Transcriptional profiling of the human liver during the reperfusion phase of transplantation

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List of abbreviations:

ANP: atrial natriuretic peptide

AP1: activator protein 1

AP2: activator protein 2

ASK1: apoptosis signal-regulating kinase-1

ATF2: activating transcription factor-2

B-cells: B-lymphocytes

Ca^{2+}: calcium

cAMP: cyclic adenosine monophosphate

CAS: CRK associated substrate

cDNA: complementary deoxyribonucleic acid

CH2A: cdc25 homology domain A

CH2B: cdc25 homology domain B

c-Myc: cellular-myelocytomatosis oncogene

N-Myc: neuroblastoma-derived myelocytomatosis oncogene

v-Myc: viral-derived myelocytomatosis oncogene

CRE: cAMP responsive element

CRK: v-crk sarcoma virus CT10 oncogene homolog (avian)

DLK: Dual Leucine zipper-bearing Kinase

DNA: deoxyribonucleic acid

DOCK180: dedicator of cytokinesis 180kDa

DRB: 6-chloro-1-β-D-ribofuranosylbenzimidazole

DSIF: DRB sensitivity-inducing factor

DUSP1: dual specificity phosphatase-1

ELK-1: Ets-like protein-1

ELMO: engulfment and cell motility protein

EPAC: exchange protein directly activated by cAMP

ERK: extracellular regulated kinase

ERP: extracellular regulated phosphatase

FAK: focal adhesion kinase

Fas: apoptosis-mediating surface antigen

FasL: fas ligand

GSH: glutathione

GSSG: glutathione disulfide

HIF-1: hypoxia inducible factor-1

H-Ras: Harvey-rat sarcoma virus oncogene

HSF-1: heat shock factor-1

HSP70: heat shock protein 70

HSP-72: heat shock protein 72

HUVEC: human umbilical-vein endothelial cells

HVH1: human vaccinia virus homologue H1

IFN-γ: interferon gamma

IL: interleukin

ILK: integrin-linked kinase

JKAP: JNK pathway-associated phosphatase

JNK: Jun N-terminal kinase

JSP: JNK stimulatory phosphatase

Kb: kilobases

K-Ras: Kristen-rat sarcoma viral oncogene

K-RasV12: constitutively active K-Ras

LD_{50}: lethal dose at which 50% of animal die

LPS: lipopolysaccharide

MAPK: mitogen activated protein kinase

MAPKK: mitogen activated protein kinase kinase

MAPKKK: mitogen activated protein kinase kinase kinase

MEFs: mouse embryonic fibroblasts

MEK: mitogen activated protein kinase kinase (MAPK)K/extracellular regulated kinase (ERK) kinase

MEKK: mitogen activated protein kinase kinase kinase (MAPK)KK/extracellular regulated kinase (ERK) kinase kinase

MKP-1: map kinase phosphatase-1

MLK: Mixed-Lineage-Kinase

mRNA: messenger ribonucleic acid

MTK-1: Map Three Kinase-1

NF1: neurofibromin 1

NF-κB: nuclear factor kappa-B

NGF: nerve growth factor

NO: nitric oxide

·NO: nitric oxide radical

Nrf2: nuclear factor E2 p45-related factor

O_2: molecular oxygen

$O_2^{\bullet -}$: superoxide

PIP_3: phosphatidylinositol-3,4,5,-triphosphate

PKA: protein kinase A

PKB/AKT: protein kinase B/v-akt murine thymoma viral oncogene homologue 1

PKC: protein kinase CP-TEFb: positive transcription elongation factor b

PRPP: 5-phosphoribosyl-1-pyrophosphate

Raf-1: v-raf-1 murine leukemia viral oncogene homologue 1

RNA pol II: ribonucleic acid polymerase II

ROS: reactive oxygen species

SAPK: stress activated protein kinase

SCF: Skp1/Cul1/F-box

Ser: serine

siRNA: short inhibitory RNA

Skp2: S-phase kinase-associated protein-2

SOS: Son Of Sevenless

Sp1: specificity protein 1/trans-acting transcription factor 1

Src: rous sarcoma oncogene

SRE: serum response element

STAT: signal transduction and activator of transcription

TAB: TAK-1 Binding protein: transforming growth factor-β-activated protein kinase binding protein

TAK-1: Transforming growth factor-β-Activated kinase-1

T-cells: T lymphocytes

TNF-α: tumour necrosis factor alpha

TPA/PMA: 12-O-Tetradecanoylphorbol-13-acetate/phorbol 12-myristate 13-acetate

Ub: ubiquitin

UV: ultraviolet light

UW: University of Wisconsin perfusion and storage solution

VSMC: vascular smooth muscle cell

WT: wild-type

Abstract

Liver transplantation continues to be the only remedy for end-stage liver disease. Moreover, the number of recipients far exceeds the number of donors and patients die on waiting lists. Unfortunately, not all grafts survive the process of transplantation and marginal livers are discarded, as they would not tolerate the stresses of ischemia-reperfusion and therefore would not survive the process of transplantation. If we are to resolve these problems, and decrease the chasm between the donor and recipient numbers, we need to characterize how a normal liver survives the process of transplantation.

We have developed a protocol that allows us to characterize the normal liver's response to transplantation. Indeed, a liver that endures the process of transplantation must be able to limit the amount of damage caused by the various stresses related to cold ischemia and warm oxygenated reperfusion. Unfortunately, the process of transplantation is not easily amenable to the reduction much less the elimination of these stresses. Therefore, a means of investigating what happens to the liver during the process of transplantation was needed that would take into account the global effect of these variables on the graft's survival.

The underlying hypothesis of my thesis is that the surviving liver invokes protective mechanisms to moderate the damage that could occur as a result of transplantation in part by regulating the level and type of expressed genes. Using microarray technology, we determined the identity of the mRNAs that revealed a degree of regulation, either up- or down-regulation during the reperfusion phase of transplantation. Furthermore, because ischemia precedes reperfusion, the process of reperfusion per se includes all of the stresses associated with ischemia, e.g. all reperfused livers were ischemia preconditioned. Thus, to conduct our analysis biopsy specimens were taken at three time-points during the peri-reperfusion phase of the operation. Our methodology not only permitted us to identify regulated genes, it also allowed us to control for recipient blood-borne contribution of messenger RNA. Because the last biopsy specimen was taken 1h post-reperfusion, our list was comprised of immediate early genes.

Of the other immediate-early genes that were on our list, we found an up-regulated gene that coded for map kinase phosphatase-1 (MKP-1). Immunohistochemistry preformed on frozen human liver sections revealed expression of MKP-1 in hepatocytes. MKP-1 is a phosphatase is best known for JNK-1, p38MAPK and

ERK1/2/5 dephosphorylation. Using transplantation relevant stresses in vitro, and HepG2 as a cellular model for hepatocytes, we characterized mkp-1 mRNA regulation. Furthermore, using the same protocol and MKP-1 shRNA expressed in HepG2, we found that a lack of MKP-1 protein expression increased apoptosis. The second gene, als2, we investigated was slightly down-regulated and coded for a protein called alsin. The latter is a RhoGEF, a guanine nucleotide exchange factor that activates Rac1, Rab5 and Ran. We proceeded to characterize alsin mRNA regulation in vitro using ischemia-reperfusion relevant stresses, as we did for MKP-1. Finally, we are seeking to determine if or how the Rho proteins (Rac1, Rab5 or Ran) are implicated during the “reperfusion” phase of the operation. All in all, our results indicate a hepatic coping mechanism invoked for the purpose of reducing the damage caused by the trauma of ischemia and reperfusion.

Résumé

La transplantation du foie demeure le seul remède pour les maladies hépatiques mortelles. Malheureusement le nombre de donneurs d'organe est inférieur au nombre de patients inscrits sur la liste d'attente. Cette situation s'aggrave lorsque l'on considère qu'un certain nombre de foies transplantés ne survivent pas et que ce ne sont pas tous les foies qui s'avèrent utilisables pour la transplantation. En effet, ces foies de « qualité inférieure » ne survivraient pas au traumatismes de l'ischémie et de la réperfusion donc ils sont refusés. Actuellement il n'existe pas de solution pour pallier ce nombre de foies à transplanter.

Nous avons développé une approche permettant de caractériser les mécanismes par lesquels un foie normal survit au processus de la transplantation. Un tel foie doit en effet être en mesure de diminuer ou du moins de contrôler les effets toxiques associés aux stress d'ischémie froide et de la réperfusion tiède oxygénée. Malheureusement, le processus de transplantation ne peut être manipulé facilement de façon à réduire ou à éliminer les variables qui lui sont associées. Compte tenu ces observations, une analyse détaillée de ce phénomène doit tenir compte de l'effet global de ces variables sur la survie de la greffe.

Une hypothèse fondamentale de mon travail de thèse présuppose qu'un foie qui survit au processus de la transplantation doit être en mesure de contrôler les effets toxiques des stress associés à ce processus. Cette régulation peut être accomplie en partie par des mécanismes de contrôle de l'expression des gènes. En utilisant des biopuces à ADN, nous avons déterminé l'identité des ARN messagers régulés positivement ou négativement au cours de la phase de réperfusion de la transplantation. Cette phase de la transplantation est ciblée pour notre étude, puisque que le processus d'ischémie précède la réperfusion, le processus de réperfusion englobe tous les stress associés à l'ischémie. Afin d'accomplir notre analyse, des biopsies hépatiques ont été prélevées à trois intervalles de temps différents. Notre protocole expérimental nous a permis non seulement d'établir une liste de gènes qui étaient régulés à la hausse ou à la baisse, une heure après le ré-établissement du flot sanguin, mais aussi nous a permis d'éliminer les ARN messagers provenant du sang du receveur. Étant donné que la dernière biopsie prélevée était à une heure après la réperfusion du foie par la veine porte hépatique, notre liste de gènes fut composé de gènes immédiats précoces.

Parmi les gènes identifiés, le messager codant pour la map kinase phosphatase-1 (MKP-1), était régulé à la hausse. En utilisant des coupes de foie humain,

l'immunohistochimie a révélé l'expression hépatocytaire de la protéine MKP-1. Cette protéine déphosphoryle les map kinases JNK-1, p38MAPK et ERK1/2/5. En simulant les stress d'ischémie et de réperfusion sur des cellules d'origine hépatomique, HepG2, nous avons caractérisé la régulation de l'ARN messager de MKP-1. De plus, en utilisant le même protocole nous avons déterminé les conséquences d'une pénurie de la protéine MKP-1 pour les cellules HepG2. Pour ce, nous avons exprimé du « shRNA » dirigé contre l'ARN de MKP-1, dans ces cellules, afin de diminuer l'expression de MKP-1. Ceci a causé une augmentation d'apoptose. Le second gène à l'étude était celui d'als2 qui code pour la protéine alsine, dont l'ARN messager était légèrement diminué en quantité lors de son évaluation par biopuces. L'alsine est une « RhoGEF », une protéine échangeuse de nucléotides, qui active les RhoGTPases Rac1, Rab5 et Ran. Nous avons procédé à la caractérisation de ce gène, en utilisant les conditions expérimentales préétablies avec MKP-1. La dernière étape se résume à déceler si ou comment les protéines RhoGTPases soient Rac1, Rab5 ou Ran, sont impliquées dans la phase de réperfusion du foie. En conclusion, nos résultats indiquent que le foie transplanté utilise différents mécanismes pour contrecarrer les effets des stress d'ischémie et de réperfusion.

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Contribution of PhD candidate (Tarek Boutros):

- For manuscript entitled: **The MAP Kinase phosphatase-1 MKP-1/DUSP1 is a regulator of human liver response to transplantation**

Designed, carried out experiments and interpreted results. Answered some of the reviewer comments. Carried out microarray experiments and contributed figures 2, 3, 4, 5 and 6 of chapter 4.

- For manuscript entitled: **Alsin the guanine nucleotide exchange factor and its involvement in human liver transplantation**

Designed, carried out experiments and interpreted results. Contributed figures 3, 4, 5 and 6 of chapter 5.

- For the review entitled: **MAP Kinase/MAPK phosphatase regulation: roles in cell growth, death and cancer**

Wrote, edited, submitted the manuscript and answered all the reviewer comments and the comments of the copy editor.

I. Chapter 1: The purpose of this study

The purpose of this study

Liver transplantation continues to be the only remedy for end-stage liver disease. As the number of recipients far exceeds the number of donors, which translates to people dying on the waiting list, some means of closing the gap between these numbers needs to be found (Clavien et al., 1992). Adding to this problem are some observations: 1) not all usable grafts survive the process of transplantation; and 2) marginal livers are discarded, as they cannot tolerate the stresses of ischemia-reperfusion and therefore would not survive the process of transplantation. Therefore one way to decrease the chasm between donor and recipient numbers is to improve survival of the usable livers and make the marginal livers amenable to transplantation. If we are to resolve these problems, we need to characterize how a normal liver survives the process of transplantation, which invokes a great number of variables that occur in part as a result of ischemic cold storage and the reperfusion with the warm recipient blood supply. These stresses have the potential to affect, directly or indirectly, the outcome of hepatic transplantation. A liver that survives the process of transplantation must be able to limit the amount of damage afforded by the various stresses related to ischemia-reperfusion. Unfortunately, the process of transplantation is not easily amenable to manipulation of these stresses, much less their elimination or their reduction in number. Therefore, a means of investigating what happens to the liver during the process of transplantation was needed that would take into account the global effect of these variables on the organ as an entity.

A reasonable assumption is that the surviving liver invokes protective mechanisms to moderate the damage that could occur as a result of transplantation in part by regulating the level and type of proteins expressed. This can be accomplished to some extent via de novo translation of messenger RNAs (mRNAs). But which ones do we look at? We investigate the mRNAs that reveal a degree of regulation, either up- or down-regulation. By what means do we detect these mRNAs? We wanted to proceed with as little bias as technology would allow, hence our use of microarrays. This technology allows as “unbiased” a sampling of mRNA regulation as is presently available. But what time during the operation do we take biopsies to evaluate mRNA regulation? Because ischemia precedes reperfusion, the process of reperfusion per se includes all of the stresses associated with ischemia, e.g. all reperfused livers are ischemia preconditioned. Thus, to characterize how a normal liver deals with the process of transplantation, biopsy specimens were taken at three time-points during the peri-

reperfusion phase of the operation. The first time-point (biopsy) was taken prior to the recipient blood reflow through the hepatic-portal vein and then two other time-points (biopsy specimens), one at ten minutes and the other at 60 minutes post-hepatic portal vein reperfusion were excised. Thus the first biopsy collected (time-point) serves as a negative control. The second of these time-points was a control for recipient blood-borne contribution of messenger RNA, when compared to the negative control. Finally, the third time-point accounts for the liver-specific contribution of mRNAs, which in principal represent the RNAs the liver requires or silences for survival, as these mRNAs would be used for the purposes of translation into protein.

The systematic identification of these RNAs and the cellular localization of their protein product would allow, theoretically, the reconstruction of part of the "reperfusion story". The second part would be to determine the stresses responsible for this regulation. The third part would deal with the consequences of the lack of regulation of each mRNA/protein during the relevant stress. This would eventually reveal how the different cell-types in the liver cope with the stress of transplantation. Once we understand how a normal liver survives the process of transplantation, we can use this profile to determine why some normal livers do not survive transplantation and perhaps establish a protocol to make marginal livers amenable to transplantation. Before we can understand how a normal liver survives the process of transplantation we need to define what a liver is as an organ and what variables and stresses exist during the process of transplantation.

In chapter 2, a review of the literature will afford a look at the nature of the variables involved in transplantation, how confident the current dogma is of the importance of these variables in transplantation outcome and where our misconceptions lie, e.g. where our knowledge fails because we lack a proper understanding of the importance some of the variables vis-à-vis transplantation. In addition, the divergence of results of animal models and humans will also be undertaken, when possible. This is an essential step not only to introduce the reader to the topic, but also serves to put our methodology, and later on our results, into perspective and revels why it was necessary to define for ourselves the problem and not simply rely on the literature and plan experiments based on other people's findings.

II. Chapter 2: A comprehensive review of the literature

Review of the literature

A. The liver

This section focuses on human liver anatomy and physiology. Some mention of species differences between human and rat or mouse liver are emphasized, when needed. More detailed analyses of the morphological differences between the livers in a great many species of mammals, birds, reptiles and amphibians have been documented (Beresford and Henninger, 1986) but will not be discussed any further in this thesis.

1. Gross anatomy: physical and functional

a. The physical anatomy. The liver is the largest solid organ in the human body comprising approximately 2-3% of the weight of an adult. The anterior view of the liver reveals that the translucent falciform ligament separates the liver into the left and the right lobes; the latter being six times larger than the former. The inferior surface of the liver reveals the quadrate lobe lodged between the gall bladder and the round ligament (a remnant of the foetal umbilical vein). The posterior view of the liver reveals the caudate lobe lying in front of the hepatic porta, posteriorly to the vena cava, separated from the quadrate lobe by the hepatic portal vein. Finally, a structure call the hilum, located in the inferior surface of the liver unites the proper hepatic artery, the hepatic portal vein, the common hepatic duct, the lymph vessels and the hepatic nerve plexus within a fibrous capsule (Kuntz, 2008b).

b. The functional anatomy. The functional anatomy is based on physiological boundaries that are considered from the distribution pattern of the hepatic portal vein, the hepatic artery, and the bile ducts, whereupon the liver is divided into 3 principal segments subdivided into 4 subsegments each, for a total of 12 segments or lobules. Conversely, the physiological boundaries can also be considered from the context of the origin of the three large ramifications of the hepatic portal vein, which divide the liver into 9 segments according to their sub-ramifications (Kuntz, 2008b; Netter, 2006).

2. The hepatic vasculature, innervation and the stroma

To best appreciate the macroanatomy of the liver as it relates to the vasculature of the liver this section will be separated according to the different type of vessels (see Figure 1) (Roberts et al., 1997). Due to the complexity of the subject, a detailed analysis cannot be given here, instead an overview highlighting some of the important points and species differences are given. Interestingly, the oxygen is supplied by the arterial system

20 % O_2 by volume and the hepatic portal venous system 15-17% by volume (Kuntz, 2008b; Tortora and Derrickson, 2009).

a. The portal venous system. The portal venous blood flowing through the hepatic portal vein derives from many sources, in essence from all the visceral organs (stomach, pancreas, spleen, small and large intestine) (Netter, 2006). At the porta hepatis, the hepatic portal vein ramifies into the left and right branches. As the portal vein branches extend through the liver they continue to ramify and the diameter of their lumen decreases until they become portal tracts. Thus the portal blood enters the sinusoids via the periportal limiting plate of the hepatocytes; a limiting plate is defined as a continuous line of hepatocytes bordering the portal tract (Kuntz, 2008b). In addition, the portal vasculature per se can be divided into conducting and distributing vasculature (Saxena et al., 1999). The conducting vasculature extends throughout the liver and assures that the blood reaches the entire breadth and depth of the organ. It is characterized by an array of ramifications the length, branching order and number of which revealing no consistent pattern. The function of the distributing vasculature is to ensure that the blood supply interacts with the different cells of the liver. It is characterized by an orderly array of ramifications guaranteeing that every cellular aspect that requires contact with blood is irrigated, though not in direct contact. In fact, the terminal branches of the conducting vasculature give rise to the first-order branches of the distributing system, whose directionality seems random (Saxena et al., 1999). Nevertheless, this pseudo-randomness ensures that each of the first-order branches of the distributing vasculature irrigates a volume 1.54 mm^3 (1.6mm x 1.2mm x 0.8 mm) of human liver parenchyma. The second- and third-order vasculatures arise from the first-order distributing system. It is the third-order ramifications that constitute the portal venules seen in the interlobular vascular septa. In humans the terminal portal veins supply the sinusoids, however in rats, the larger portal venous branches also supply blood to the sinusoids (Saxena et al., 1999).

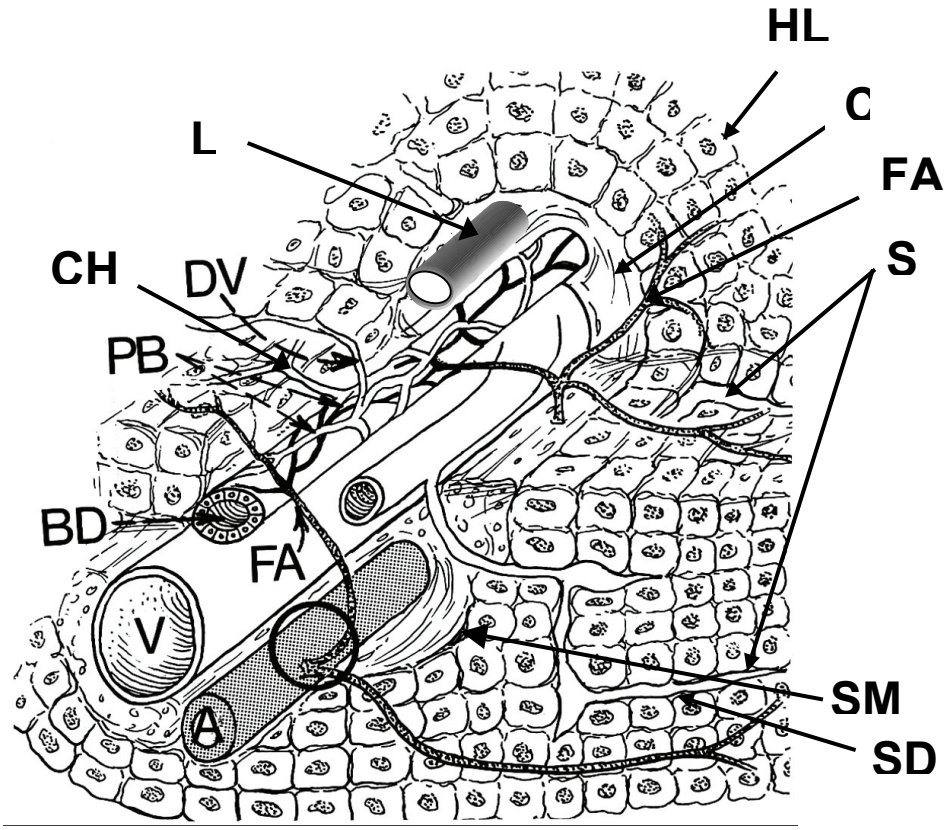

Figure 1. A cross section of the liver. FA, feeder artery for peribiliary vascular plexus (PBVP); PB, portal branch; V: branch of the hepatic portal vein; A: branch of the hepatic artery; BD: bile duct; DV: drainage vein for PBVP; C: connective tissue; L: lymphatic duct; CH: canal of Hering; S: sinusoids; HL: hepatic lobule; SM: space of Mall; SD space of Disse (Roberts et al., 1997).

b. The arterial system. The arterial vasculature provides 25% of the hepatic blood flow (Saxena et al., 1999). The abdominal aorta branches into the celiac trunk, which divides into the common hepatic artery, the left gastric artery and the splenic artery (Netter, 2006). The common hepatic artery gives rise to the three braches: the gastroduodenal artery, the right gastric artery and the proper hepatic artery (Kuntz, 2008b; Netter, 2006). The latter artery ramifies into the right, intermediate and left branches. At this point, and prior to other subdivisions, there is an arterial sphincter. As the arteries continue to subdivide, the interlobular arteries give rise to intralobular arterioles (Kuntz, 2008b). Some of the discrepancies centre on the hepatic arterioles and how they deliver blood to the hepatic parenchyma. More specifically, do the hepatic arterioles deliver blood to the sinusoidal vasculature or delivers blood to a terminal portal vein? In addition, there are variations as to how far some of the arterioles travel before

they connect to the sinusoidal vasculature in terms of providing the pericentral third of the lobule with oxygen. Interestingly, there are differences in terms of the presence of arterio-portal anastomoses, humans lack such structures, while rats have them in the terminal portal tracts (Saxena et al., 1999).

c. The venous system. Because the arterial blood eventually reaches and mixes with the hepatoportal blood, the blood flowing out from the sinusoids is composed of 75% hepatoportal and 25% arterial blood (Kuntz, 2008b). The relative difference in blood pressure between the portal system and the venous system, not their absolute numbers, is key in assuring that the perfusion of the portal tract occurs in the appropriate direction. Thus the venous vasculature drains blood form the portal sinusoids and the arterial vasculature into the central hepatic vein of the lobule (Kuntz, 2008b). More specifically, there are 6 branches of the portal system for one central vein in the classical hexagonally shaped hepatic lobule (Saxena et al., 1999). It should be noted that in humans livers, among mammals, the sinusoids drain directly in the central vein. In rat livers sinusoids drain into the venous system at all levels including the sublobular and collecting veins (Saxena et al., 1999). The central hepatic veins funnel to the sublobular veins, which merge into collecting veins (Kuntz, 2008b). The latter veins merge into five trunk veins: right and left superior hepatic vein, the right, the intermediate and the left hepatic veins; in most cases (60-70%) the intermediate and left hepatic vein form a single structure (Kuntz, 2008b). These trunk veins connect to the inferior vena cava at the posterior surface of the liver (Kuntz, 2008b).

d. The biliary system. The vessels of this system start out as an extracellular space localized between the apical surfaces of rows of hepatocytes facing one another. At this level, the canaliculi are 0.5-1.0 μm in diameter, do not have walls per se, however the apical membranes of the hepatocytes form a zone called the “pericanalicular ectoplasm” (Kuntz, 2008b). Furthermore the apical membrane is characterized by microvilli, which increases the surface area. The canaliculi form extensive networks in the shape of a polygon (Kuntz, 2008b). In an attempt to unify and characterize the finer biliary structures in the human liver, a recent review on nomenclature of the small branches of the biliary tree was undertaken (Roskams et al., 2004). Thus, the canaliculi between hepatocytes throughout the lobule lead to the canals of Hering. A canal of Hering, by definition is bordered on one side by hepatocytes and on the other side by

cholangiocytes. It forms an anatomical and physiological link between the canaliculi and the biliary tree. It is noteworthy that a canal of Hering does not necessarily stop at the limiting plate of the portal tract, but extends through it. Therefore the hepatocyte canalicular system links to the canals of Hering within the lobule in the periportal region forming star-like projections from the canals of Hering (Roskams et al., 2004). The bile moves along the canals of Hering until it reaches the bile ductules entirely formed by cholangiocytes (Roskams et al., 2004; Saxena et al., 1999). In turn the ductules may have an intralobular segment in addition to their intraportal section. The ductules will link to larger interlobular bile ducts (Roskams et al., 2004). These latter ducts are located in the connective tissue of the portal tracts and eventually empty their content into progressively larger bile ducts (Kuntz, 2008b; Ludwig et al., 1998), called septal, area or segmental ducts depending on their diameter (Marzioni et al., 2002). The bile ducts continue to merge until they form the right and left hepatic ducts which unit to form the common hepatic duct at the porta hepatis (Kuntz, 2008b) and merges with the cystic duct of the gallbladder as it exits the parenchyma of the liver. The common bile duct merges with the pancreatic duct, just prior to emptying its contents into the descending aspect of the duodenum (Netter, 2006).

e. The lymphatic system. The lymph derived from the liver accounts for 25-50% of the lymph flowing through the thoracic duct. In the liver, 80% of the lymph is formed in the portal system, while the sublobular and the capsular lymphatic vessels form the rest of the hepatic lymph (Ohtani and Ohtani, 2008). The portal lymph is formed as the blood passes through the sinusoids and the plasma is filtered within the space of Disse (Saxena et al., 1999), which is the space that is formed by the basolateral aspect of the hepatocytes, i.e. the limiting plate, and the membrane of the sinusoidal endothelial cells, which lack a basal lamina (Trutmann and Sasse, 1994). As the hepatic artery (25%) and the hepatic portal vein (75%) contribute blood to the sinusoids, the lymph also has a similar composition although it has 80% of the plasma proteins concentration (Ohtani and Ohtani, 2008). In fact, fluid, particles and proteins up to 100nm can enter the space of Disse through pores (Kuntz, 2008b). The space of Disse accounts for 33% of the intrahepatic extracellular space, which is 0.2-1.0μm wide and known to increase during hypoxia, accounts for 5-7% of the hepatic volume, and is the most important contributor of lymph (Kuntz, 2008b). The lymph flows through the collagen “honeycomb” within the space of Disse (Ohtani and Ohtani, 2008). The space of Disse is continuous

with the interstitial space of the portal tract at the origin of the sinusoids (the inlet venules) (Ohtani and Ohtani, 2008; Saxena et al., 1999). From the space of Disse, the lymph flows to the space of Mall, which is in continuity with the space of Disse. The space of Mall is a perilobular lymph space confined by the limiting plate of the hepatocytes and the periportal connective tissue (Trutmann and Sasse, 1994). The lymph continues to the portal tract to reach the lymphatic vessels (Trutmann and Sasse, 1994). The capillaries of the lymphatic system begin at the adventitia of the sublobular veins, and follow the veins until they reach the paracaval lymph nodes. In addition, the valves present in this vasculature assure the unidirectional flow of the lymph (Kuntz, 2008b). Then the lymph flows through the sublobular lymphatic vessels, the lymphatic vessels and then the inferior vena cava (Ohtani and Ohtani, 2008).

f. The hepatic innervation. This topic is covered in chapter 2 sectionC1c.

g. The stroma of the liver. Stroma refers to the connective tissue of the intersticium and includes: the capsule of the liver, the connective tissue that envelops the vasculature, Glisson's portal tract and the reticular network of the space of Disse. The capsule of the liver is essentially composed of endothelial cells, some innervation, branches of the phrenic nerve, blood, lymph and rudimentary bile ducts in a network of collagenous and elastic fibres. The perivascular connective tissue serves a structural function, in that it prevents a collapse of the venous system as a result of the negative pressure imposed by the process of respiration, which actually causes displacement of the liver by 3cm (Kuntz, 2008b); The reticular network of the space of Disse (covered in the previous sections and will be touched upon in the upcoming section on hepatic stellate cells). The Glisson's portal tract is defined as the perivascular connective tissue ensheathing the portal vein, the hepatic arterioles, bile ducts, lymph vessels and nerve fibres.

3. Microanatomy and major functions associated with the liver

a. The functional unit. Perhaps as good a place to start this section as any is to define a functional unit of the liver. A functional unit is by definition the smallest structure within an organ capable of performing all the function of the entire organ. In the kidney, the functional unit is the nephron (Saxena et al., 1999). For the liver, a functional unit that can account for the perfusion of the liver by the hepatic artery and hepatic portal vein and the efflux of the bile and the venous blood has remained elusive. Nevertheless

there have been models such as the classic lobule, the portal vein lobule and the acinus, none of which successfully explain the all the physiology and pathology observed. The acinus is the smallest functional unit of the classic lobule. It should be noted that the classic hexagonal shape of the classic lobule is seen in pigs, camels, polar bears and seals but not in human livers (Jungermann and Katz, 1989; Kuntz, 2008b). The classic lobule model explains well many aspects of the liver function including vascular and biliary flow, structural and metabolic porto-central gradients but fails to account for the lateral, porto-portal, gradients between adjacent portal tracts. Thus a functional unit of the liver is still wanting (Saxena et al., 1999).

The acinus is separated into three zones (Kuntz, 2008b). The first zone, zone 1, has the highest oxygen content and substrate concentration, where toxins have the most noxious effect and is nearest to the portal system. Zone 2 is an intermediate region with corresponding levels of oxygen and nutrients, but it lacks functional boundaries of its own. Zone 3 is closest to the central vein, and is therefore most depleted in oxygen content and nutrients. Interestingly, because it has the lowest oxygen levels it also has the lowest resistance to oxygen deficiency (Kuntz, 2008b). Zone 3 also has the lowest capacity for regeneration, upon liver damage. Superimposed on the zone 1,2, and 3 nomenclature are the zones A, B, and C which form concentric circles around the periportal field, with ever decreasing blood supply as the circles grow (see Figure 2). These zones correspond to metabolic and enzymatic zonation of hepatocytes, also called metabolic heterogeneity (Jungermann and Katz, 1989; Kuntz, 2008a). There is also ultrastructural heterogeneity, a case in point, in zone 1 Küpffer cells are more numerous than in zone 3. In addition, zone 1 is comparatively richer in microvilli, mitochondria, lysosomes and Golgi structures than in zone 3. The lumen of bile capillaries is also larger in zone 1 than zone 3. Conversely, there is less smooth endoplasmic reticulum (ER) in hepatocytes of zone 1 (15 700 μm^3) compared with the smooth ER of zone 3 (21 600 μm^3). The fenestrations of the sinusoidal endothelium also increase from zone 1 to zone 3 (Kuntz, 2008b).

The last model is the portal vein lobule, which has the periportal field in the centre, and central vein in the periphery. Therefore, based on this model the blood flows from the centre to the periphery. Conversely, the bile flows from the periphery to the centre. The main tenet of the portal vein lobule model is that the liver is glandular in nature (Kuntz, 2008b).

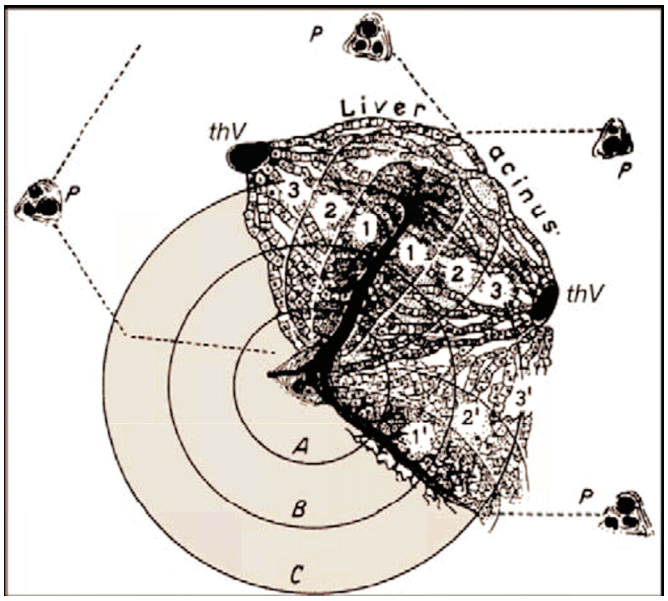

Figure 2. The functional microcirculatory liver unit revealing the acinus. Terminal hepatic vein (thV), periportal field (P). Zones of different blood supply A, B, C and 1, 2, 3. The direction of blood flow is from 1 to 3 and from A to C. Zone 1 (afferent zone): zone richest in O2, nutrients and hormones. Zone 3 (efferent zone): zone poorest in O2, nutrients and hormones, but enriched with CO2 and metabolites from zones 1 and 2 (Kuntz, 2008b).

b. The major cells types that constitute the liver and their function

This section reviews some of the basic characteristics of the different cell types and their abundance within the liver.

i. **Hepatocytes**: The hepatocytes are the major parenchymal cell-types of the liver. They are physically characterized as polygonal epithelial cells. Although the actual life span is unknown for human hepatocytes, as it involves genetic and environmental factors. The life span is approximately 191-453 days for rat hepatocytes, at which time they die by apoptosis (Kuntz, 2008b). In humans, hepatocytes comprise 60-65% of the liver cell number and 80% of liver volume with a total hepatocyte number of $300x10^9$, and a mitotic rate of 1 cell per 10000-20000 hepatocytes (Kuntz, 2008b). Interestingly, hepatocyte proliferate in the periportal space migrate toward the central vein, at a rate of 1.44μm/day. Thus their metabolic heterogeneity seems to be acquired as a function of their position within the acinus (Kuntz, 2008b). The hepatocyte

membrane is divided into three compartments via polarization. The first of these is the sinusoidal membrane, also called the basolateral membrane, which accounts for approximately 35% of the total membrane surface. As the sinusoidal membrane, which faces the space of Disse, is implicated in absorptive and secretory functions, it is covered in microvilli to increase its effective surface by six fold. Some of these microvilli not only protrude into the space of Disse, they also protrude though it and the fenestrae into the luminal side of the sinusoid, coming into direct contact with the blood; recall that the space of Disse varies between 0.2-1.0μm. The apical surface, also named (bile) canalicular surface, accounts for 15% of the hepatocyte membrane and is considered the secretory pole of the cell (Kuntz, 2008b).

The final 50% of the hepatocyte membrane is found at the intercellular fissure that is connected to the space of Disse. Separating the intercellular fissure form the apical membrane is the tight junctions, also called the zonula occludens, which nevertheless allows water and cations to be exchanged between these two compartments (Kuntz, 2008b). Other junctions increase the adherence of hepatocytes to one another, such as the intermediate junctions, or zonula adhaerens, and the desmosomes also called macula adhaerens. The desmosomes contain gap junctions (macula communicante), allowing intercellular communication between neighbouring hepatocytes. Upon cell death of one hepatocyte, the gap junction close to prevent transmission of cell death signals from one cell to another (Kuntz, 2008b). Furthermore, hepatocyte are non-terminally differentiated cells and do not normally proliferate. However, hepatocytes proceed from G_0 to G_1 upon injury to the liver or as a result of hepatectomy (Michalopoulos and DeFrances, 1997; Taub, 2004). Finally, the hepatocytes are a heterogeneous population of cells. The function of these cells will be covered in the section c on the major functions associated with the liver.

ii. **Cholangiocytes**: Cholangiocytes, also called the bile duct cells, form the larger bile ductules and ducts. As bile ducts become larger, the cholangiocytes also increase in size and become more columnar in shape (Marzioni et al., 2002). Cholangiocytes modify the bile content/composition before it reaches the gallbladder. This modification is controlled by different mechanisms, including: 1) gastrointestinal hormones, among which are vasoactive intestinal peptide, somatostatin, bombesin, gastrin and secretin; 2) peptide such as endothelin; 3) bile salts themselves and 4) the parasympathetic nerves (Marzioni et al., 2002). Secretin binding to its receptor at the

basolateral aspect of the cholangiocyte membrane increases ductal secretion of bicarbonate via a cAMP induction of protein kinase A activity, leading to opening of the cystic fibrosis transmembrane regulator (cAMP-dependent Cl^- channels) and the activation of the Cl^-/HCO_3^- exchanger at the apical membrane and the consequent secretion of bicarbonate. In addition, some of the previously mentioned hormones and peptides can antagonize each other's function. A case in point, somatostatin and gastrin inhibit the effects of secretin by reducing cAMP levels (Marzioni et al., 2002).

In the normal adult, cholangiocytes do not proliferate, however it is well recognized that the human cholangiocytes can proliferate within the context of the liver under various pathological conditions: 1) primary sclerosing cholangitis; 2) primary biliary cirrhosis; 3) liver allograft rejection; 4) graft-versus-host disease (graft is rejecting host); 5) alcoholism; and 6) extrahepatic obstruction among other conditions (Marzioni et al., 2002). It should be emphasized that the susceptibility of cholangiocytes to disease, be it acquired or congenital, can thus far be categorized according to the size of the duct. As an example, liver allograft rejection, infections and idiopathic adult ductopenia target small ducts, while biliary atresia and cholangiocarcinomas target large ducts. It may therefore not be surprising that the small, medium and large ducts are also known to express different intracellular and cell surface proteins (Marzioni et al., 2002).

iii. **Mast cells:** In normal human livers mast cells are found with an average density of 3.9 ± 3.3 cells/mm^2, with a distribution favouring the portal tract, while only 5-10% are present in the sinusoids (Farrell et al., 1995). More specifically, the mast cells are found preferentially in the periphery of the portal spaces while some are scattered in the connective tissue of the portal tract and few are located to the walls of the branches of the portal vein. The sinusoidal mast cells are in the acinar zone 2. Finally, some mast cells are also found in the connective tissue associated with Glisson's capsule (Farrell et al., 1995). Mast cells synthesize an array of mediators including: a dozen members of the interleukin family (IL-3 to IL-6 inclusive, IL-9 to IL-13 inclusive, IL-15, IL-16, IL-18 and IL-25), TNF-α, TGF-β, nerve growth factor, VEGF, chemokines, granulocyte macrophage colony stimulating factor, tryptase, chymase, metalloproteinases, peroxidase, caboxypeptidase, heparin, histamine, cathepsin, chondroitin sulfates, among other factors (Franceschini et al., 2006). The exact combination of mediators varies with the biological site from which mast cells derive and the biological process they are taking part in. As the mast cells are found in all major organs of the human body and at sites

that are in contact with the environment, such as the digestive tract, they are believed to be one of the first responders to foreign agents (Franceschini et al., 2006). Mast cells are known to be involved in liver fibrosis, following chemical, viral or autoimmune mediated damage to the liver, presumably and in part via tryptase action on hepatic stellate cells, inducing type I collagen synthesis as they do for fibroblasts (Franceschini et al., 2006). Finally, although mast cells may also be implicated in paediatric liver transplant rejection, more so in chronic than in acute rejection (Arikan et al., 2008), the correlation between adult liver and rejection has as yet to be made.

iv. **Oval cells**: These cells are believed to be the precursors of the liver epithelial cells, which are the hepatocytes and cholangiocytes (Cantz et al., 2008; Oh et al., 2002). Morphologically, they are small oval cells with ovoid nuclei, and a large nucleus to cytoplasm ratio. Under normal circumstances, these cells reside in the canals of Herring (canals formed on one side by hepatocytes and on the other by cholangiocytes) or derive from bone marrow and do not proliferate; this bone marrow derived progenitor cell may be more rare that the hepatic endogenous oval cells (Cantz et al., 2008; Oh et al., 2002). The pre-existence of oval cells in the adult tissue has also been a matter of debate as these cells may also develop from other cell types such as bile-duct or cholangiocytes (Cantz et al., 2008). It is believed that oval cells only proliferate if the injury is of such magnitude that it reaches the canals of Herring or when the hepatocytes are incapable of proliferating. In animal models, these cells are induced to proliferate upon inhibition of hepatocyte proliferation, via carbon tetrachloride treatment or other chemical treatment prior to injury. The combination of chemical insult and partial hepatectomy triggers proliferation in oval cells (Cantz et al., 2008; Oh et al., 2002). Following proliferation, oval cells can differentiate into hepatocytes or cholangiocytes (Oh et al., 2002).

v. **Sinusoidal cells**: This term groups different cells that are found in and around the sinusoids of the liver, including hepatic stellate cells, Küpffer cells, sinusoidal endothelial cells and Pit cells, which collectively comprise ~6% of the liver volume but 30-40% of total cell number (Kuntz, 2008b).

v.1. <u>Hepatic stellate cells</u>: These cells, also called Ito cells or fat storing cells, account for 3-8% of the hepatic cell population and 1.4% of the hepatic volume and are found more abundantly in zone III of the acinus. These cells are found in the space

of Disse, and regulate the width of the sinusoids. Hepatic stellate cells are the major synthesizers of the extracellular matrix (Kuntz, 2008b). A case in point the half-life of collagen, secreted by the hepatic stellate cells, is 30 days as these cells also secrete matrix-metalloproteinases to degrade it. Proteoglycans are also secreted by hepatic stellate cells, and are considered an important component of the extracellular matrix with hydrophilic and cation binding properties. There are many extracellular matrix proteins, which fulfil various functions, such as the adhesion of cells be it hepatocytes and non-parenchymal cells to the extracellular matrix such as fibronectin, laminin, nidogen, tenascin and indulin. The extracellular protein elastin imparts, as the name suggests, elastic properties to the hepatic structures (Kuntz, 2008b). In addition, under normal circumstances hepatic stellate cells store vitamin A as retinol esters in lipid globules located within their cytosol. Retinol esters found in the chylomychrons pass to the hepatocytes and are hydrolysed to retinol, which can pass into the blood or get stored in hepatic stellate cells. When injury to the liver is such that it leads to fibrosis, hepatic stellate cells lose their lipid globules, and by extension vitamin A, and greatly increase the synthesis of extracellular matrix and acquire a myofibroblastic phenotype (Kuntz, 2008b).

v.2. Küpffer cells: The liver's resident macrophages reside in the luminal surface of the capillary beds. Küpffer cells account for 25 % of the sinusoidal cells or 8-12% of the total liver cell population. Although they appear to be randomly distributed throughout the sinusoids there is a three to four fold greater number of Küpffer cells in the periportal versus the perivenous zones, although Küpffer cells may be occasionally flushed out of the sinusoids with the portal blood (Kuntz, 2008b). Küpffer cells may be the most abundant of the tissue macrophages (Crofton et al., 1978), and are characterized by the second highest phagocytic activity, after the macrophages at the vascular bed of the lungs. Küpffer cells not only secrete cytokines and growth factors, but also function to remove toxins, antigen-antibody complexes, antigens and purines (Kuntz, 2008b), such as adenine and guanine (Stryer, 1988). Finally, although usually on the luminal side of the sinusoids, Küpffer cells can also traverse the sinusoids and contact the hepatocytes directly (Crispe, 2003). While Küpffer can present antigen and cause T-cell proliferation and increase their cytokine synthesis (Crispe, 2003), they can also induce tolerance of a hepatic graft, in a rat model of spontaneous allograft acceptance post-hepatic transplantation, by a FasL-mediated cytotoxicity to T-cells

(Chen et al., 2008). The topic of Küpffer cells is also covered in chapter 2 sections B4c in the context of University of Wisconsin solution and B5 in relation to endotoxemia.

v.3. Sinusoidal endothelial cells: The endothelial cells represent ~3% of the liver volume but account for 15-20% of the hepatic cell number. As previously mentioned, the sinusoidal endothelia cells have pores that allow 100nm sized particles and proteins to pass through. However there are other pores or fenestrae with 500nm diameter, and intercellular spaces with a range of 100-500nm in size. All these "holes" serve to sieve the blood, allow for the passage of lipids, cholesterol and vitamin A (Kuntz, 2008b). Thus, these endothelial cells form fenestrated capillary beds that allow the transit of molecules without hindrance (Alrefai and Gill, 2007; Kuntz, 2008b). The sinusoidal cells can secrete inflammatory cytokines (TNF-α), growth factors (HGF, IGF, FGF), and vasoactive substances such as nitric oxide and endothelin (Kuntz, 2008b). Interestingly, these endothelial cells are also known to present antigen to T-cells of the liver (Crispe, 2003; Knechtle and Kwun, 2009), such as CD4+ and CD8+, in the context of MHC class II which seems to induce tolerance or rejection of antigen in mice (Knechtle and Kwun, 2009). Interestingly, human sinusoidal endothelial cells also express CD4+ and CD8+, ICAM-1 and VCAM-1 among other molecules in common with HUVECs, but sinusoidal endothelial cells do not express CD62E, CD31 or factor VIII related antigen that are present on HUVECs. Murine liver sinusoidal endothelial cells express all the cell surface markers expressed by their human counterpart, but also express CD80 and CD86. More importantly, murine sinusoidal endothelial cells can function as professional antigen presenting cells, while this function has not been verified for their human counterpart (Crispe, 2003). Thus not only is there a species difference in cell surface markers, there is also a difference based on the tissue from which the endothelial cells are extracted (Knechtle and Kwun, 2009). Interestingly, while antibodies to sinusoidal endothelial cells facilitate cellular rejection of human livers, rat sinusoidal endothelial cells were important in the spontaneous allograft acceptance, in a model of hepatic transplantation (Knechtle and Kwun, 2009).

v.4. Lymphocytes: The liver possesses a resident lymphocyte population. Among these are CD8+ cells, which outnumber the CD4+ lymphocytes (Crispe, 2003) and other lymphocytic populations including the natural killer (NK) cells and the natural killer T-cells (NTK); both these latter cells are referred to as Pit cells (Crispe, 2003; Kuntz, 2008b). When normal human and brain dead donor livers were

compared for their lymphocytic population, it was found that they had similar numbers of lymphocytes with an enrichment of CD8+ compared to CD4+ cells (Pruvot et al., 1995). The normal hepatic population in this scenario refers to patients undergoing laparotomy for extrahepatic disease, cholecystectomy or esophageal reflux, while blood was gotten from the periphery. Adding to the previously mentioned results, the total lymphocyte count within the liver was 2-9X10^9 cells or 0.5-3X10^6/g of hepatic tissue. These numbers are comparable to the number of lymphocytes in the peripheral blood of normal subjects (Pruvot et al., 1995). More interestingly however, both normal and donor livers revealed cell surface markers of activation on their lymphocytes, such as CD8DR, CD69 when compared to their respective peripheral blood derived lymphocytes. CD56, an NK-cell marker revealed a two fold enrichment in normal and donor livers when compared to their peripheral blood (Pruvot et al., 1995). The lymphocytes and NK-cells were predominantly found in the portal tract, while perisinusoidal space revealed fewer cells and the centro-lobular zone was all but devoid of lymphocytes (Pruvot et al., 1995). More specifically, in normal livers, CD25+ (IL-2 receptor α chain) and CD69+ lymphocytes were found in portal tract only, while in donor livers CD25+ and CD69+ cells were also present in the perisinusoidal space. No such staining was found in the centro-lobular zone. CD56+ (NK cells) were mostly found in the portal tract of the normal livers, while the donor livers revealed the presence of CD56+ cells in the perisinusoidal area (Pruvot et al., 1995). This difference in lymphocyte distribution within the hepatic parenchyma, between normal and brain dead liver donors, was attributed to the process of organ procurement, that may damage sinusoidal endothelial cells, hepatocytes or other cell types causing recruitment of T-cells (Pruvot et al., 1995). Some of these findings were echoed by another study demonstrating that the liver from brain dead donors, after transplantation and reperfusion by the recipient blood, revealed a subpopulation of lymphocytes that was different from that of peripheral blood obtained from the brain dead donors and healthy volunteers (Norris et al., 1998). Although the latter group hypothesized that the hepatic lymphocytic population could be involved in mediating the non-immune response to food based antigens that the liver receives from other organs, the function of these subpopulations of lymphocytes in humans is unknown (Norris et al., 1998). Although animal models targeting subpopulations of lymphocytes have been reported (Crispe, 2003), species differences where the immune system is concerned (Haley, 2003) warrant extreme caution when attempting to extrapolate such results to humans.

c. Major functions associated with the liver.

i. **Overall functions**. Using a panoply of different biochemical mechanisms, the liver carries out over 70 partial functions that can be distributed among eleven or so metabolic categories. These categories include: 1) amino acid and protein metabolism; 2) carbohydrate metabolism; 3) lipid and lipoprotein metabolism; 4) bile acid metabolism; 5) bilirubin metabolism; 6) hormone metabolism; 7) porphyrin metabolism; 8) acid-base balance; 9) biotransformation and detoxification; 10) vitamin metabolism, e.g. use as cofactors in enzymatic reactions and storage; and 11) homeostasis of trace elements such as iron, manganese, selenium, zinc and copper among others (Kuntz, 2008a). The liver achieves these functions by utilizing four levels of regulation. The first of these is the inter-organ communication (Kuntz, 2008a), which allows metabolites such as lactate to be transported from the muscle to the liver for gluconeogenesis, removing some of the burden from active muscle to liver (Stryer, 1988). Another example of inter-organ communication lies in the synthesis of bile acid metabolism and recycling. Bile acids are synthesized by hepatocytes, which are secreted into the small intestine, the ileum, and thereafter 95% of the bile is recycled back to the liver while 5% of bile is discharged via feces (Russell, 1999). The enterohepatic circulation delivers the complex made of albumin-bound conjugated bile acids to the space of Disse, through the fenestrations of the sinusoidal endothelial cells, and whereupon the bile acids are taken-up by the basolateral aspect of the hepatocytes (Alrefai and Gill, 2007). The second level of regulation is achieved via zonation of function. More specifically, different hepatocyte populations perform different functions depending on where they are in the acinus (this topic will be elaborated on in the upcoming section c.ii). Some functions are distributed between the various organelles within the cell, such as protein synthesis is achieved via the rough endoplasmic reticulum, while degradation is accomplished by proteasome (Lorentzen and Conti, 2006; Nandi et al., 2006; von Mikecz, 2006) and lysosome (Kuntz, 2008a). Fatty acids are synthesized in the smooth endoplasmic reticulum and degraded in the mitochondria (Kuntz, 2008a). Finally, the molecular level of regulation entails, among other mechanisms, regulation through feedback inhibition, e.g. the end product of the enzymatic process inhibits the enzyme that synthesized it such as 5-phosphoribosyl-1-pyrophosphate synthetase which transforms ribose 5-phosphate into 5-phosphorybosyl-1-pyrophosphate (PRPP) is inhibited by AMP, GMP and IMP (Stryer, 1988).

ii. **Detailed considerations of liver function**. Because the liver must perform so many functions it needs an effective method to distribute the work as to successfully perform all tasks with as little waste in energy as possible. The periportal zone receives blood from the portal vein and the hepatic artery, is rich in oxygen and metabolites (Kuntz, 2008a). The metabolic pathways this region utilizes include: 1) gluconeogenesis; 2) β-oxidation of fatty acids; 3) urea synthesis from amino acids; 4) amino acid degradation (including glutamine hydrolysis); 5) glycogen synthesis from lactate or amino acids; 6) glycogen degradation; 7) cholesterol synthesis; 8) citrate cycle and respiratory chain reactions; and 9) oxygen uptake. For its part, the perivenous zone accomplishes the following tasks: 1) glycolysis; 2) liponeogeneisis; 3) glutamine synthesis; 4) glutamate transport; 5) glycogen synthesis from glucose; 6) glycogen degradation to lactate; 7) biotransformation; and 8) ketogenesis. Although these represent some of the more important functions that the liver performs in a seemingly zonal fashion, it should be emphasized that the boundaries of these zones are not absolute barriers and there is overlap between the zones (Kuntz, 2008a). Such lack of boundaries may be very important when the liver regenerates after partial hepatectomy and must keep all its functions active (Michalopoulos and DeFrances, 1997; Taub, 2004). Now that we have taken a look at the liver anatomy and have gained an appreciation of hepatic functions, it is easy to understand why it must be replaced when this organ becomes ill beyond its ability to effect self-repair. The next section will cover the donors, the recipients, the storage solution and endotoxemia.

B. Hepatic transplantation

1. Why transplantation?

In the past, 50% of patients requiring liver transplantation were cancer related. However, the number of transplants relating to cancer currently stands at approximately 15%. The most prominent group is comprised of patients suffering from cirrhosis of the liver (58%), which can be caused by alcoholism (18%) or hepatitis C (15%). Metabolic diseases such as Wilson's disease, familial amyloidotic polyneuropathy and α-1 antitrypsin deficiency are also indications for transplantation not to mention chronic hepatitis caused by autoimmune disease or hepatitis B. Finally, cholestatic liver diseases such as primary biliary cirrhosis and sclerosing cholangitis, and acute hepatic failure which can be caused by acute viral hepatitis or drug related hepatitis (usually acetaminophen overdose) comprise the most important reasons for liver transplantation be it in Europe (Adam and Hoti, 2009) or the United States (UNOS, http://www.optn.org/organDatasource/about.asp?display=Liver)(Adam and Hoti, 2009). How the recipient's health and donor related variables affect the decision to proceed with, or influence the outcome of, liver transplantation will be dealt with in the next subsections. Some of these factors are more generally recognized than others, as having a hand in the demise of the graft. More importantly, however, these factors can combine to worsen the prognosis in terms of graft and overall patient outcome.

2. Donors

There are some liver donor variables that are responsible for a decrease in the immediate hepatic graft function upon transplantation. Of these, the degree of steatosis (fattiness of the liver), the age of the donor, the duration of both the cold and warm ischemia are known to reduce the viability of the liver after transplantation (Brokelman et al., 1999; Kamiike et al., 1988; Platz et al., 1997; Totsuka et al., 2004). Although the age, the preservation time, the effect of brain death on organ viability, among other factors related to ischemia-reperfusion will be developed to some extent in subsection C, these factors are complex and it is likely that each contributes multiple variables aiding in the liver's demise. For now it will suffice to mention that warm ischemia is associated with reduced ATP level in human livers, and as such is suspected to be an important factor in determining the graft's ability to recover (Kamiike et al., 1988). Interestingly, gender also seems to affect the outcome of liver transplantation. More specifically, female donor-

male recipient have the lowest survival rate when compared to female donor-female recipient, male donor-male recipient and male donor-female recipient (Brooks et al., 1996). The reason for the gender bias remains unclear (Csete, 2008), however gender bias and disease is a recognized phenomena (Lockshin, 2001). In addition, there seems to be a geographical bias, with North American centres reporting the female donor–male recipient problem in survival rate while Western European revealed no difference in this category of donor-recipient group (Zeier et al., 2002). Although epigenetics may have an effect on select populations (Tang and Ho, 2007), more studies need to be done to corroborate the different transplantation results before this North American/European dichotomy can be ascribed to epigenetics. It should be noted that sex differences are also noted in the rodent model, revealing estrogen as having beneficial effects on hepatic ischemia/reperfusion injury model via increased expression of hepatic endothelial nitric oxide synthase, up-regulation of superoxide dismutase, inhibition of xanthine oxidase activity, among other beneficial effects (Yokoyama et al., 2005). Because these studies were conducted on small for size animal model, these results would need to be verified for the cadaveric donor and the hormonal status of the recipient would need to be ascertained (pre- versus post-menopausal recipients).

3. Recipients

The donor is not the only source of variables capable of influencing liver transplant outcome. Some recipient health-related considerations have been noted to warrant “special considerations” (Adam and Hoti, 2009), which include: 1) alcoholic liver disease, as recidivism would damage the new liver (Adam and Hoti, 2009); and 2) diseases that reoccur such as hepatitis C and hepatocellular carcinoma among others (Kotlyar et al., 2006). Other recipient health issues have been labelled as contraindications to liver transplantation. Among these factors: 1) age of recipient, i.e. older than 65 years; 2) HIV, as most of these patients have co-infections with hepatitis B or C or both; 3) cholangiocarcinomas; and 4) technical problems such as portal vein thrombosis. Although these problems have been considered absolute contraindication in the past, with improved surgical technique, better anti-retroviral therapies and improved chemotherapy used in combination with radiotherapy, liver transplantation is also being performed on some of these patients (Adam and Hoti, 2009).

Although liver transplantation is though of as a remedy for end-stage liver disease, the following are conditions that can develop post-operatively in some patients

which, as a result, would require monitoring: 1) obesity and hyperlipidemia; 2) cardiovascular disease; 3) renal insufficiency; 4) osteoporosis; and 5) secondary malignancies (Adam and Hoti, 2009). As the hepatic transplantation process requires the use of immunosuppressive agents, and these have been implicated in a slew of diseases in the recipient (Liu and Schiano, 2007), it is at present difficult to ascertain the extent of the disease as caused by the new liver per se and the contribution of the immunosuppressive agent to the "new pathology".

4. The University of Wisconsin solution

a. Creation of the university of Wisconsin solution. The University of Wisconsin solution was created because the Collins and its derivative, the EuroCollins solutions (Collins solution modified by the European transplantation group) which were adequate for the preservation of kidneys during transplantation, were inadequate for the preservation of any other organ, such as heart, lung, liver, pancreas and intestines. Thus the University of Wisconsin solution was created based on the reasoning that a perfusion and storage solution should have the following characteristics: 1) minimize cell swelling during cold storage; 2) diminish intracellular acidosis; 3) maintain integrity of interstitial space during flush-out; 4) minimize reperfusion-related oxygen radicals; and 5) provide substrates to regenerate ATP during the reperfusion phase (Belzer and Southard, 1988). However, an "ideal" preservation solution should also have the following properties: 1) cost-effectiveness; 2) a solution able to be used for preservation of all organs (the heart and lungs are still wanting of a preservation solution that is as effective as UW is for liver and kidney); and 3) allows organ transplantation to take into account some real problems where the time-line is concerned, such as the preservation of the organ for as long as needed until it can be shipped anywhere around the world and the patient located and prepped for the operation. In order to determine the composition of the preservation solution that would best fit the previous criteria, an analysis of the contribution of the ingredients of the UW solution, which has been universally used for organ transplantation, to its effectiveness as a preservation solution was undertaken.

Table 1 in section B4 (p.53-58) summarizes the components of the UW solution. This table also compares the reason why each ingredient was originally included in the UW solution to the actual biological activity that each ingredient has. As can be gleaned from Table 1, many ingredients were added for "logical" reasons, which turned out to be either useless, contraindicated or of benefit by a completely uncharacterized or

unexpected mechanism. Other ingredients such as GSH, was added as an anti-oxidative agent (Belzer and Southard, 1988). However, as it turns out the “real story” surrounding this tripeptide, composed of glutamic acid, cysteine and glycine (Ristoff and Larsson, 2007; Wright et al., 1980) is anything but straightforward. Although the topic of glutathione may be deemed beyond the parameters of the thesis per se, the take home message it affords is nevertheless important. A closer look into this molecule, as it pertains to transplantation biology, will reveal some of the unexpected complexities that can be associated with the addition of a simple physiologically relevant ingredient to a preservation solution as well as the inherent problems of determining the role it and other ingredients plays in the composition of UW or any perfusion and preservation solution.

b. Glutathione: GSH versus GSSG. As previously stated, the reduced form of glutathione, GSH, was added as an antioxidant to the UW solution (Belzer and Southard, 1988). Although the presence of glutathione was confirmed to be an essential ingredient in UW solution for the purposes of transplantation (Southard et al., 1990), it was latter found that GSH was completely oxidized to glutathione disulfide, GSSG, in the UW solution approximately 4 days after manufacture of UW solution (Astier and Paul, 1989). This oxidation eventually occurred even if the solution was kept in a sealed bag or if sealed twice i.e. “double-bagged” (Postaire et al., 1991). In addition, fresh UW solution is known to be more effective at preservation, as measured by cardiac output, than the shelf-stored (4-6 months) UW solution (Wicomb and Collins, 1989). Thus, during the organ harvesting process, it is GSSG that perfuses the liver not GSH. Further tests revealed that the presence of glutathione in the reduced GSH form, added fresh to UW solution just prior to use for perfusion, or the mere presence of oxidized GSSG as it is in the standard UW solution, were indistinguishable for the purposes of dog liver transplantation unless the liver was kept longer that 24h ex-vivo at 4ºC (Boudjema et al., 1990). Human liver transplantation also failed to reveal any difference between GSH and GSSG, using UW solution, for the cold ischemia times less than 24h (12-16h), when liver necrosis (AST, ALT) or function (bilirubin, bile output and prothrombin time) or length of patient stay in hospital were assessed (Boudjema et al., 1991; Merion et al., 1991). As both GSH and GSSG are membrane impermeable (Hahn et al., 1978) and a lag time of 24h ex-vivo at 4ºC rarely occurs, it remains unclear at present how GSSG could participate in organ preservation. The preservation time at 4ºC was kept under 12h for the biopsy specimens used in the present study, that are the crux of this thesis. The

following paragraphs will attempt to unveil how extracellular GSSG or GSH could be useful to human liver transplantation.

c. GSH and GSSG mechanism of action. Although GSSG is reduced to GSH by glutathione reductase, within the cell (Go and Jones, 2008; Han et al., 2006a; Hansen et al., 2006; Hill and Bhatnagar, 2007; Kehrer and Lund, 1994), a membrane bound glutathione reductase capable of reducing extracellular GSSG (Ottaviano et al., 2008) within the human or animal liver sinusoids or hepatic parenchyma awaits characterization. Alternatively, GSSG may participate in mixed disulfide exchange and S-glutathionylation (also called glutathiolation) of extracellular proteins, as it would in the intracellular milieu (Han et al., 2006a) if its concentration were not so low (Hill and Bhatnagar, 2007). However, as the concentration of GSSG is 3mM, in the context of UW solution (see Table 1, section B4 p.53-58), this type of reaction could conceivably occur. The biological relevance of extracellular GSSG is buttressed by the finding that enzymes that catalyze the reduction of protein-S-S-protein disulfide bonds and protein-S-GSSG, such as thioredoxin and glutaredoxin can be found in human plasma (Lillig and Holmgren, 2007; Lundberg et al., 2004; Ottaviano et al., 2008) although extracellular glutathiolated proteins and GSH account for only 1% of the amount found in blood, with 99% of glutathiolated proteins and GSH are found in the red blood cells of healthy human (Kleinman et al., 2003). Nevertheless, local concentrations of GSSG may glutathiolate proteins and protect them against ROS and irreversible modification, inactivation or damage (Hill and Bhatnagar, 2007). Conversely, GSSG can mediate either a cytotoxic effect in promonocytic cell lines (Filomeni et al., 2003) or a slight reduction in proliferation in neuroblastoma cell lines (Filomeni et al., 2005). In light of the cytotoxicity of GSSG to promonocytes (Filomeni et al., 2003), and the results demonstrating that gadolinium chloride, which is toxic to Küpffer cells (hepatic macrophages), is hepatoprotective in rat (Giakoustidis et al., 2006; Kukan et al., 1997) and pig models (Jahnke et al., 2006; von Frankenberg et al., 2003) of liver transplantation, it would be interesting to determine if GSSG is cytotoxic to the Küpffer cells, improves hepatobiliary function, improves microcirculation or reduces oxidative stress as gadolinium chloride does (Cutrin et al., 1998; Fukuda et al., 2004; Giakoustidis et al., 2006; Jahnke et al., 2006; Kukan et al., 1997; Schauer et al., 2001; von Frankenberg et al., 2003). Conversely, Küpffer induce tolerance of a hepatic graft, in a rat model of spontaneous allograft acceptance post liver transplantation, by increased

apoptosis in T-cells in vivo (Chen et al., 2008). This was confirmed in vitro, revealing increased expression of FasL on Küpffer cells caused T-cell apoptosis. However, if gadolinium chloride is used prior to transplantation, there is less T-cell death and the liver is rejected (Chen et al., 2008). This line of reasoning is all the more important when we consider that Küpffer cells, in mice: 1) derive from bone marrow promonocytes; 2) do not have mitotic activity in the liver; and 3) that the total Küpffer cell population is replaced once every 21 days (Crofton et al., 1978). Human Küpffer cells have a much longer turnover, on the order of months (Clouston et al., 2002). Therefore, Küpffer cells seem to mediate rejection and tolerance. Distinguishing between both functions of Küpffer cells may be an essential component of human liver transplantation. Otherwise, inhibiting their function during transplantation, except perhaps for phagocytosis, which would be needed to clear debris, may be beneficial.

Interestingly, GSH is a much more potent inhibitor of recombinant MMP9 than recombinant MMP2 and GSSG inhibits MMP9 to about 45% efficacy while not inhibiting MMP2. University of Wisconsin solution inhibits both MMPs, while other ingredients of UW solution also inhibit these MMPs (Upadhya and Strasberg, 2000) (also see Table 1, p.53-58). The effect of glutathione is all the more important when we consider that the human liver effluents contain matrix metalloproteinases (MMP2 and MMP9) and that these enzymes are active at 4ºC, especially MMP9 (Upadhya and Strasberg, 2000). Thus an experimental paradigm using UW solution, with or without GSSG, and determining Küpffer cell number, function and activation status is well within reach. As any Küpffer cells that dies will eventually be replaced, it is possible to determine to what extent Küpffer cells are involved in mediating liver damage in the process of transplantation. Another aspect that could be considered, using the same paradigm with or without GSSG in UW solution, is the amount of membrane-bound extracellular proteins that were glutathiolated. Finally, the activation status of matrix metalloproteinases can be monitored as a function of cold storage time and determine if there is a correlation between MMP activity and liver survival.

d. GSH and GSSG degradation. An alternate mechanism for extracellular GSH/GSSG relates to the degradation of glutathione. Analyses of GSH and GSSG half-lives were performed in rat model using radio labelled glycine, which was incorporated into GSH and GSSG (Hahn et al., 1978). In brief, the results revealed delayed kinetics of rat renal clearance of GSSG in comparison to GSH, as the renal γ-glutamyltranspeptidase, the enzyme responsible for the cleaving of GSH into a dipeptide,

cysteinyl-glycine, was also believed to be responsible for the cleavage of GSSG into cystinyl-bis-glycine (Hahn et al., 1978). However, it should be noted that human γ-glutamyltranspeptidase localized to the hepatic sinusoidal endothelial cells (Busachi et al., 1981) while little was found in the kidney, which was the opposite of the rat (Rollins et al., 1981). Presumably, this would allow for possible cleavage of GSH in the context of human liver but not rat liver (Hahn et al., 1978). However, while in the rat 85% of the plasma glutathione is derived from the liver and can increase in response to oxidative stress (Adams et al., 1983), and over 84% is degraded and removed from plasma by the rat kidney (Hahn et al., 1978; Purucker and Wernze, 1990)), while in humans red blood cells provide plasma glutathione along with liver and muscle (Giustarini et al., 2008) and only ~49% is removed by the human kidney (Purucker and Wernze, 1990). Interestingly, in the human hepatoma cell line HepG2, extrusion of GSH precedes apoptosis (Ghibelli et al., 1998). These species differences in glutathione synthesis and extraction from plasma may have direct correlation with the interpretation of UW efficacy in liver transplantation, at least where the glutathione component is concerned.

The cysteinyl-glycine dipeptide, formed as a result of the function of γ-glutamyltranspeptidase on GSH, has the unexpected propensity of being pro-oxidative in the presence of Fe^{3+}. More specifically, cysteinyl-glycine reduces Fe^{3+} to Fe^{2+} with concomitant formation of superoxide, from molecular oxygen, which is reduced to hydrogen peroxide in the presence of water, also called a Fenton or Fenton-like reaction (Dominici et al., 1999; Paolicchi et al., 2002). Conversely, superoxide anion derived from human neutrophils, treated with lipopolysaccharide (LPS), can cause the release of iron from transferrin (Brieland and Fantone, 1991). Further degradation of glutathione occurs by dipeptidase-1 (dehydropeptidase-I) or aminopeptidase, both of which are found in rat kidney and cleave either cystinyl-bis-glycine or cysteinyl-glycine, respectively, into single amino acids (Kozak and Tate, 1982). However, in the context of humans, it is the liver that expresses these enzymes (Nakanishi et al., 1989; Tsuji et al., 1990), thus these dipeptides may be cleaved in liver and perhaps the kidney. But how is this relevant to GSH/GSSH and liver transplantation? In an attempt to understand how glutathione reduces ischemia/reperfusion related stress during the storage phase of human liver transplantation, studies on both animal models and human liver transplantation used cysteine and glycine, to determine if the effect of glutathione could be partially or totally reproduced by its constituent amino acids. Although glutamate is also a constituent of GSH, for reasons that will become clear, it has not been used in liver transplantation.

Moreover, to appreciate the possible role of each amino acid in the present scenario, we will overview some of their functions as they pertain to liver transplantation.

i. L-Glutamate. It is perhaps ironic that one of the best-characterized excitatory neurotransmitters, in terms of function, subtypes of receptors it binds to and the localization of these receptors within the central (Lisman et al., 2007; McAllister, 2007; Raiteri, 2006) and peripheral nervous system (Sawynok, 2003), L-glutamate is almost uncharacterized outside neural tissue (Gill and Pulido, 2001; Nedergaard et al., 2002). Therefore, what exists of the data pertaining to L-glutamate in the liver will be unceremoniously presented with no attempt to "guess" at glutamate's function in this organ considering the paucity of information. L-glutamate can be taken-up by the livers of rats (Haussinger and Gerok, 1983; Haussinger et al., 1989; Stoll et al., 1991), sheep and cows (Howell et al., 2001) and humans (Treyer et al., 2008). In the rat, L-glutamate is taken-up at the periveinular region and more specifically the subpopulation of hepatocytes that express glutamine synthase. In addition, using radiolabelled L-glutamate, which was taken-up by the liver, it was observed that non-labelled L-glutamate was released from the liver (Haussinger et al., 1989). As cystine, the dimer of cysteine, is transported into cells in exchange for a glutamate, via a cystine/glutamate antiporter that can be found in rat hepatocytes (McBean and Flynn, 2001), among other transport systems (Lo et al., 2008; McBean and Flynn, 2001), it is possible that the activity of the antiporter could account for the exchange between radiolabelled L-glutamate and non-labelled glutamate. However, until all the glutamate receptors, the transport systems and their function are characterized in human liver, or animal models, it will be difficult to draw any conclusions regarding the biological activity of L-glutamate in the liver. It is perhaps the nature of glutamate as an excitatory neurotransmitter and the involvement of its receptors in the sensation of pain (Sawynok, 2003), coupled with an almost complete lack of data concerning the biological effects of glutamate in the liver that explain why glutamate has not been used in liver transplantation.

ii. L-Cysteine/N-acetyl-L-cysteine. Cysteine, a non-essential amino acid is highly unstable in reduced form and therefore the N-acetylated variety, N-acetylcysteine, was developed for pharmacological purposes (Glantzounis et al., 2006). It can enter the cell and be hydrolysed by acylase I to release cysteine (Uttamsingh et al., 1998; Yamauchi et al., 2002). Although most of the activity is found in the kidney, some acylase activity is found in the liver (Yamauchi et al., 2002). Of the clinical trial using N-acetylcysteine, we focus our attention on ischemia/reperfusion relevant trials and more

specifically, those implicating human liver transplantation (Glantzounis et al., 2006). There have been many ischemia/reperfusion related studies, using small cohorts, assessing the efficacy of N-acetylcysteine during the process of transplantation, with conflicting results. In brief, the results varied from: 1) one study revealed that N-acetylcysteine had no effect on liver function, patient morbidity or mortality despite improved oxygen delivery and consumption; 2) two studies failed to reveal any beneficial effect on liver after transplantation; and 3) two studies showed that N-acetylcysteine was cytoprotective. In one study hepatocytes were protected from damage and the incidence of primary liver dysfunction decreased and in the other study N-acetylcysteine decreased levels of circulating VCAM-1 and ICAM-1. Overall the diverging results of these clinical trials were attributed to differences in time and duration of the N-acetylcysteine treatment as well as the primary end points (what was considered the most important readout for each clinical trial) (Glantzounis et al., 2006). The primary end point is all the more important as other confounding factors such as the multiplicity of function of N-acetylcysteine are considered. Among these, and perhaps the most obvious is increased glutathione synthesis as cysteine is a constituent amino acid. The other functions of N-acetylcysteine not related to GSH were discovered upon inhibition of glutathione synthesis or the more direct means using the D-stereoisomer N-acetyl-D-cysteine, as it cannot be converted to GSH. Among these functions: 1) ROS scavenging; 2) anti-apoptosis; 3) anti-mitotic and 4) direct reduction of thiols at the cell surface (Athuraliya and Jones, 2009; Galhardo et al., 2007; Glantzounis et al., 2006).

iii. Glycine. In brief, depending on the animal model used, glycine revealed a beneficial effect in rats by reducing the deleterious effect of manipulation induced stress on liver (Schemmer et al., 1999), and could substitute for glutathione in UW solution (den Butter et al., 1993), while glycine could not substitute for glutathione in either dogs (den Butter et al., 1993), or rabbits (den Butter et al., 1994) liver transplantation when added to UW solution. In a non-heart beating pig liver transplantation model, pigs receiving livers that had been treated with glycine during normothermic perfusion revealed a 20% increase in survival compared to controls receiving the same volume of saline solution (Barros-Schelotto et al., 2002). When glycine was administered in a clinical setting, it was given to recipients prior to transplantation and daily for the first week after transplantation, reducing plasma levels liver enzymes, AST and ALT, in the very limited number of patients tested, n=7, when compared to “historical controls” e.g. values gotten from past transplantations

(Schemmer et al., 2001b; Schemmer et al., 2002). A double blind study protocol examining the effect of glycine in a larger group of patients had been published (Luntz et al., 2005), but the findings of this study have not appeared as yet. Unfortunately, lack of data of the double blind study coupled with a different paradigm of human versus the animal studies makes it extremely difficult, if not impossible, to understand the mechanism by which glycine mediates its effect.

Although glycine is an inhibitory neurotransmitter of the central nervous system (Zafra et al., 1995), what will retain our attention at present is the localization of the glycine receptors, which are glycine-gated chloride channels, to various hepatic cell types including: 1) Küpffer cells (Froh et al., 2002; Ikejima et al., 1997); 2) hepatocytes (Qu et al., 2002); 3) endothelial cells (Yamashina et al., 2001; Zhang et al., 2000b); 4) T lymphocytes (Stachlewitz et al., 2000); and 5) neutrophils (Froh et al., 2002; Wheeler et al., 2000) among other cell types (Habib et al., 2006). More notably is the effect of glycine on these cells, among which: 1) decreasing TNF-α production when cultures of Küpffer cells were treated with LPS. This effect was mediated via increase chloride influx, hyperpolarizing the cells, and decreased calcium influx (Ikejima et al., 1997); 2) decreasing calcium influx in neutrophils, due their hyperpolarization upon glycine treatment, resulting in a decreased superoxide production (Wheeler et al., 2000); 3) decreasing T-cell proliferation also via hyperpolarization of cell membrane (Stachlewitz et al., 2000); 4) preventing growth factor deprivation mediated-apoptosis of sinusoidal endothelial cells (Zhang et al., 2000b); 5) inhibited non-lysosomal, calcium-dependent proteolysis, in an anoxia model using rat hepatocytes (Nichols et al., 1994); and 6) interfering with lipopolysaccharide signalling, by down-regulating interleukin-1 receptor associated kinase-4, and improving survival rate in the rats liver transplant models. More specifically, rats that received livers from rats infused with glycine 1h prior to organ harvest, showed enhanced survival compared to livers from control rats infused with saline 1h before harvest of organ (Liu et al., 2006). The significance of the latter finding will be elaborated on in the upcoming subsection dealing with endotoxemia. Finally, it is interesting to note that glycine protects renal cells from damage caused by ATP depletion (Pan et al., 2005). As can be seen from this enumeration, there is no shortage of possible effects of glycine on the liver. Thus, until otherwise demonstrated, it is logical to suppose that the glycine effect on liver survival during the process of transplantation has the potential to occur at multiple levels.

e. What have we learned? Although some UW "ingredients" have a more complex biology than others (or at least, our understanding of their biology is more complete for some of them than for others), this in-depth look at glutathione hopefully puts to rest the age-old question, "couldn't we just do this simple experiment to figure out what glutathione (or any other molecule) contributes in terms of improved preservation of the liver? " Having overviewed some of the intricacies associated with glutathione oxidation and catabolism, it is understandable why determining the exact contribution of an ingredient to the overall success of a preservation solution such as UW has been an elusive proposition. Although the last pages have touched upon the biology of exogenously added GSH, in an upcoming section (section 5 oxidative stress), the endogenous GSH/GSSG system will be covered as it pertains to the understanding of a more global picture of what happens to the liver during the process of transplantation. Furthermore, while adenosine is an ingredient of the UW and can be legitimately covered in this section, intracellular adenosine will be touched upon in the upcoming section as an end product of ATP degradation (section 2 pH and preservation), while extracellular adenosine will be overviewed in section 6a ii in the topic of ischemic preconditioning.

The other ingredients of UW solution will not be discussed in detail, but are summarized in Table 1 (p.53-58). This table underscores the time and effort that has been and continues to be invested, in an attempt to determine what components of UW are essential and which are expendable. In two decades of work, there are still unanswered questions concerning the basic composition of a perfusion and storage solution required for organ transplantation. Perhaps the answer lies in developing a solution that caters to the most sensitive organs to ischemia/reperfusion related damage, such as the heart and lungs, and determine if this composition is deleterious to the less sensitive organs such as liver, pancreas, kidney, or successful in their preservation. Thereafter, the composition of such a solution could be adjusted for use with marginal organs. However, as long as the contribution of each ingredient remains a mystery, to the overall success of the perfusion and storage process, fine-tuning the solution in question will be a haphazard proposition at best.

Finally, the use of animal models has not proven to be a great benefit in the case of glutathione, or perhaps animal models are chosen for practical purposes such as cost and characterization of their genome, and not their degree of similarity to human physiology. A case in point was the use of rats as a liver transplantation model, as previously mentioned. Other upcoming sections and subsections will highlight the

shortcoming of animal models as needed. Although financially costly and time-consuming, it may be advisable to characterize systematically an animal used as a model for normal or disease states or surgical procedures, to establish the whereabouts, expression levels and function of molecules that are directly involved in the system under study. This may seem obvious to the point of being trivial, it is nevertheless a point that has been overlooked time and again with non-trivial consequences to conclusions that have been drawn in different studies.

Table 1. The components of the UW perfusion and preservation solution

Components:	concentration	**Presumed function** of components in UW	References:	**Actual function** of components in UW	References:
Potassium lactobionate	100 mmol/L	Oxidized lactose impermeable osmotic agent, prevents cell swelling	(Belzer and Southard, 1988; Southard and Belzer, 1993)	• Intramolecular coordination of ions such as: Cu^{2+}, Mn^{2+} and Fe^{3+} and Fe^{2+} (may also coordinate to Mg^{2+} although not demonstrated). • Intermolecular coordination (2 lactobionate molecules) of ions such as: Ca^{2+} • reducing possible toxicity upon reperfusion due to hydrogen peroxide, Fenton reaction (Fe^{3+}) • reducing calcium influx into cells and preventing apoptosis • reducing hydroxyl radicals by their being compexed to Fe^{2+} • Inhibiting MMP2 and MMP9 (matrix metalloproteinases)	(Charloux et al., 1995; Shepherd et al., 1993; Southard and Belzer, 1995) see section: see chapter 2 section B4d

Table 1. The components of the UW perfusion and preservation solution (continued)

Components:	concentration	**Presumed function** of components in UW	References:	**Actual function** of components in UW	References:
KH_2PO_4	25 mmol/L	Buffer HPO_4^{2-} and prevents cell oedema	(Belzer and Southard, 1988; El-Wahsh, 2007; Fuller, 1987; Southard and Belzer, 1993)	Acts as a simple buffer, HPO_4^{2-} may coordinate to Mg^{2+}	(Stryer, 1988) (Walser, 1961)
$MgSO_4$	5 mmol/L	Electrolyte, membrane stabilization, prevents Mg flux out of cells SO_4^{2-} prevents cell oedema	(Belzer and Southard, 1988; El-Wahsh, 2007; Fuller, 1987; Southard and Belzer, 1993)	See chapter 3 section B4	
Raffinose	30 mmol/L	impermeable osmotic agent, additional osmotic support, prevents cell swelling	(Belzer and Southard, 1988; Southard and Belzer, 1993)	See chapter 3 section B4	

Table 1. The components of the UW perfusion and preservation solution (continued)

Components:	concentration	**Presumed function** of components in UW	References:	**Actual function** of components in UW	References:
Allopurinol	1 mmol/L	Inhibits xanthine oxidase and the generation of O_2 during reperfusion	(Belzer and Southard, 1988; Southard and Belzer, 1993)	See section C3a, this chapter	(Stryer, 1988)
glutathione	3 mmol/L	Intracellular GSH is depleted during cold ischemia, theoretically extracellular GSH may help reduce oxidative stress during reperfusion	(Belzer and Southard, 1988; Southard and Belzer, 1993)	The presence of glutathione is important for liver transplantation via unknown mechanisms.	For exogenous glutathione see general introduction. For endogenous glutathione section C4 this chapter.
adenosine	5 mmol/L	Cell permeable precursor of ATP synthesis	(Belzer and Southard, 1988; Southard and Belzer, 1993)	Adenosine in the rinse solution (post- UW storage and prior to graft implantation in recipient) does not lead to improved graft or patient survival at the 6 month or 5year post-transplantation time-points.	(Fisher et al., 1999)

Table 1. The components of the UW perfusion and preservation solution (continued)

Components:	concentration	**Presumed function** of components in UW	References:	**Actual function** of components in UW	References:
Hydroxyethyl starch (HES) pentafraction	50 g/L	Non-toxic colloid to prevent expansion of extracellular space via interstitial oedema during the initial flushout.	(Belzer and Southard, 1988; Southard and Belzer, 1993)	• Causes erythrocyte cell to aggregate *in vitro*, • Reduces erythrocyte deformability *in vitro* • may cause blockage of sinusoids *in vivo*.	(Chmiel and Cierpka, 2003; Mosbah et al., 2006; Panzera et al., 2005) see section III D.2 cold stress, warm reperfusion and liver histopathology
Penicillin	200 000 U/L	"prevents infection" during storage	(Belzer and Southard, 1988; Southard and Belzer, 1993)	There does not seem to be a difference between UW solution with or without penicillin, from a contamination standpoint, for human kidney transplantation using ex-vivo reperfusion pump.	(Gage et al., 1997)

Table 1. The components of the UW perfusion and preservation solution (continued)

Components:	concentration	**Presumed function** of components in UW	References:	**Actual function** of components in UW	References:
Insulin	40 U/L	Not mentioned	(Belzer and Southard, 1988; Southard and Belzer, 1993; Southard and Belzer, 1995)	Increases ischemia/ reperfusion injury to liver by increasing metabolism in the rat model.	(Li et al., 2003b; Li et al., 2004)
Dexamethasone	16 mg/L	Studies indicated that dexamethasone suppresses lysosomal enzymes during ischemia and stabilized membranes, although may not be essential for the purposes of preservation of organs.	(Belzer and Southard, 1988; Southard and Belzer, 1993)	If dexamethasone is injected pre-operatively for purposes of immunosuppression: • Increases rejection rates in cardiac transplant patients; • Inhibits early regenerative response of rat livers if livers require significant regeneration after ~16h cold storage.	(Cochrane et al., 2008; Debonera et al., 2003)

Table 1. The components of the UW perfusion and preservation solution (continued)

Components:	concentration	**Presumed function** of components in UW	References:	**Actual function** of components in UW	References:
Na^+	25 mmol/L	Na^+ and K^+: electrolytes. The Na^+ and K^+ concentrations mimic the intracellular milieu to prevent Na^+ and K^+ from moving down their respective concentration gradients.	(Belzer and Southard, 1988; Southard and Belzer, 1993)	According to experimental findings, there does not seem to be a difference to liver preservation if extracellular milieu is high K^+ and low Na^+ versus low K^+ and high Na^+.	(Southard and Belzer, 1993)
K^+	125 mmol/L				
Characteristics:			(Belzer and Southard, 1988; Southard and Belzer, 1993)		
pH	7.4				
Osmolality	320 mOsm/L				

5. Endotoxemia

Although low levels of lipopolysaccharide (LPS) is a normal constituent of hepatic-portal vein blood in humans (Jacob et al., 1977), and can increase in human plasma (4ng/ml to 600ng/ml) for a variety of causes both septic and non-septic (Caridis et al., 1972), levels greater than 12pg/ml in donor plasma lead to loss of liver function after human liver transplantation in a small cohort of patients (Zipfel et al., 2000). Although in a rat model of liver transplantation, reduced circulating TNF-α improves liver viability and decreases primary hepatic non-function post-transplantation (Goto et al., 1992), the situation in human is far more obscure. Part of the interest in LPS and inflammatory cytokines stems from observations relating LPS with human systemic inflammation (Andreasen et al., 2008). The transplantation process per se has been associated with the presence of inflammatory cytokines (Bumgardner and Orosz, 1999) as a result of at least two factors: 1) the introduction of a foreign tissue (Bumgardner and Orosz, 1999); and 2) ischemia/reperfusion is considered a non-antigen related inflammatory process (Ke et al., 2006). Although based on the previously mentioned observations, one could tentatively link the presence of endotoxin, inflammatory cytokines and liver non-function or rejection in a cause and effect dynamics, such as: endotoxin leading to increased inflammatory cytokines, which accelerate or worsen liver non-function or rejection. This scenario may not represent what is happening, however due to the low number of patients per study, it would be more accurate to speak of trends then actual cause and effect in the following examples. Endotoxemia was not correlated to graft function (Fugger et al., 1991; Maring et al., 2002), while higher post-operative levels of cytokines such as TNF-α and IL-6 were thought to predict graft rejection and infection, respectively (Fugger et al., 1991; Hoffmann et al., 1993; Imagawa et al., 1990). Another study found measurements of cytokine levels to be of limited value when predicting rejection or post-operative infection (Maring et al., 2000). In addition, there is a lack of consensus as to the benefit of gastrointestinal decontamination in surgical patients (Maring et al., 2002; Nathens and Marshall, 1999). In theory this decontamination would diminish the pathogenic microorganisms that colonize the pharyngeal and gastrointestinal tract, thereby decreasing infection, endotoxemia and inflammatory cytokines post-transplantation (Nathens and Marshall, 1999). As this treatment is directed to aerobic gram-negative bacteria and fungi (Nathens and Marshall, 1999), one study found that the patients did indeed benefit from the decontamination as aerobic gram-negative bacteria, and fungal infection were eliminated, only to be replaced

by pathogenic gram-positive bacterial infection (Zwaveling et al., 2002). Finally, this type of decontamination “does not affect” in principal obligatory anaerobic bacteria (Nathens and Marshall, 1999).

The data from patients is not as “clean” as the data from the animal models, where LPS, TNF-α and IL-6 versus prediction of hepatic graft survival are concerned. Part of the problem when dealing with humans is sheer number of variables, ranging from the length of the anhepatic phase, varying MHC disparities, variable length of time of organ conservation (Hamilton et al., 1993), and differences ABO compatibilities between donor-recipient, to case histories of the donors and recipients, among other variables (Gonzalez et al., 1994). It is therefore not surprising that the sum of these variables can be prohibitive when trying to ascertain whether or not there is a cause and effect relationship between LPS, inflammatory cytokines and prediction of graft survival. Adding to these problems, most studies use a relatively small number of patients and therefore lack the adequate power to resolve a given question that is obstructed by so many variables.

As this and the up-coming subsections have and will continue to reveal, although there is an incredible amount of data deriving from cell culture, animal models and clinical information, the data is far from pointing to a coherent much less single direction. What has been missing thus far is an answer to a simple question. Indeed for all the studies that have been published in the literature, very few queried the liver to determine what was going on globally with it during the process of transplantation. More importantly, none have focused their attention on the reperfusion phase of the operation, which already includes all the stresses of the ischemic phase, both cold and warm, just prior to the reestablishment of the recipient blood supply. For such a want of data was the impetus for our study of the liver during the reperfusion phase of the operation, the results of which are covered in chapter 3 sections B and C and chapters 4 and 5. However, all is not lost and to do justice to the relevant data that exists in the literature, concerning liver transplantation, we continue to broaden and deepen our knowledge. The upcoming subsections will review other aspects of ischemia reperfusion related damage as we cover various aspects including brain death, the pH, and cold stress-heat shock among other noxious stimuli.

C. Stresses associated with ischemia and reperfusion

Ischemia is defined as cessation of blood flow to a region and reperfusion is associated with the reestablishment of blood flow to the region that was ischemic (Fuller, 1987). There are many causes and consequences associated with ischemia and reperfusion that are not only mentioned in this section, but will also be mentioned throughout the thesis as the need arises. This section will reveal the bulk of what is known about various stresses that are part of ischemia and reperfusion mediated damage, how they have been studies and what is missing from the data to complete our knowledge. Furthermore, when possible, differences between animal and human data will be presented. Because ischemia occurs before reperfusion, and brain death is the first instigator of ischemia, that we know of, this section will herald the topic of ischemia with the demise of the brain.

1. The consequences of brain death

a. The brain death component of ischemia. How do we know that a person is brain dead? A patient is considered brain-dead when: 1) conditions that could mimic brain death such as hypothermia, acid-base, electrolyte or endocrine imbalance, drugs that can depress the activity of the nervous system, lesions to the spinal cord, etc. are eliminated; 2) if there is no cortical (e.g. motor cortex) function, as tested via noxious stimuli (pressure) to nail bed of a finger or pressure to the temporomandibular joint, this serves to assess the depth of the coma; and 3) there is brainstem death as reflected by a lack of brainstem related reflexes, via cotton swab applied to the cornea, cough response associated with bronchial suctioning, among other tests (Braunfeld, 2004; Wijdicks, 2001). After the initial finding of brain death, the examinations are repeated again, 6h to 24h later, to confirm the diagnosis. Interestingly, the interval of time between the physiologic brain death and actual declaration of brain death may be unknown (Wood and McCartney, 2007).

Brainstem death is associated with the process of herniation of the brainstem, which proceeds from rostral-to-caudal affecting the mesencephalon, the ponds and finally the medulla. As the medulla becomes ischemic, the loss of vagal nerve activity results in unopposed activity from the sympathetic nervous system. Furthermore, as the body attempts to preserve cerebral perfusion via increased levels of circulating

epinephrine and norepinephrine, together with increased cardiac output due to the stimulation by the sympathetic nervous system result in systemic hypertension. In addition circulating catecholamines can create cardiac arrhythmia with consequent myocardial ischemia. Peripheral vasoconstriction may induce pulmonary oedema, via increased tension in veins returning blood to the heart and decreasing blood-flow from the heart to systemic circulation. All these phenomena combine to create an “autonomic storm”. Interestingly, in animal models, it was found that the severity of the “autonomic storm” lasted 5-15min, and by 60min, the hemodynamic parameters returned back to normal or below normal levels. It is noteworthy that the intensity of the resolution of the storm is proportional to the intensity of the storm itself (Braunfeld, 2004). However, after this storm, a 50% drop in arterial blood pressure has been detected in dogs, pigs, baboons (Endo et al., 2000; Novitzky et al., 1988; Washida et al., 1992) and humans (Novitzky and Cooper, 1988; Novitzky et al., 1987) leading to hypoperfusion and ischemia and increase anaerobic metabolism. Thus ischemia-reperfusion injury starts with the brain death of the donor, with possibly compromised microcirculation (Braunfeld, 2004). Nevertheless hypoperfusion requires pharmacological therapy, such as dopamine, catecholamine among hormone replacement therapies, to keep the donor organs viable for organ transplantation (Braunfeld, 2004; Novitzky and Cooper, 1988; Washida et al., 1992). Adding to these observations brain death is associated with cytokine and hormonal modifications in the donor (Amado et al., 1995). Conversely, the usual organ donor suffers from traumatic brain injury is not only associated with increased intracranial pressure as mentioned above (Braunfeld, 2004), there is also increased vagal nerve activity, decreased innate immune response and increased susceptibility to infection (Kox et al., 2008). The next section will present the link between the nervous system and the immune system.

b. Neuroimmunomodulatory axis during inflammation and ischemia/reperfusion. The importance of the nervous system, both sympathetic and parasympathetic, is also highlighted in the various functions associated to these branches of the nervous systems. More specifically, vagotomy (cutting of the vagus nerve which is part of the parasympathetic nervous system) is associated with increased export of immature double positive $CD4^+$ $CD8^+$ and double negative $CD4^-CD8^-$ from the thymus of 4-6 week old mice, with a concomitant increase of these lymphocytes in both spleen and lymph nodes, while the single positive ($CD4^-CD8^+$ and $CD4^+CD8^-$) mature

lymphocytes decreased from spleen and lymph nodes in mice (Antonica et al., 1996). Conversely, vagal stimulation releases lymphocytes from rat thymus (Antonica et al., 1994). This rat vagal innervation of the thymus is thought to occur at the level of thymic stromal cells such the epithelial reticular cells in the perivascular spaces and the endothelial of post-capillary veinules and the lymphatic system, while the thymocytes are not considered as candidates for innervation based on the presence of the acetylcholine receptor on the thymic stromal cells that seems to be absent from thymocytes (Dovas et al., 1998).

In addition to the cellular effects of the vagus nerve on the lymphocytic population, this nerve also has anti-inflammatory effects. Consequently, the efferent signalling pathway of the vagus nerve has been dubbed the cholinergic anti-inflammatory pathway, as acetylcholine is a major neurotransmitter of the vagus nerve (Tracey, 2007), and one of the points of control of inflammation (Nathan, 2002). More specifically, it is known that stimulation of the vagus nerve attenuates the systemic inflammatory response to endotoxin (Borovikova et al., 2000) and improves survival in an animal model of sepsis (Wang et al., 2004). Such anti-inflammatory action has been attributed to the vagus nerve-derived acetylcholine binding to the nicotinic acetylcholine receptor, more specifically the homopentamers formed by the α7 subunit, localized on macrophages (de Jonge et al., 2005; Ulloa, 2005; Wang et al., 2003).

These immunomodulatory findings are important within the context of ischemia-reperfusion mediated injury, as vagus nerve stimulation or the use of cholinergic agonists has been linked to: 1) delayed blood reflow within the liver in the context of warm ischemia reperfusion (Nishida et al., 2000); 2) reduced circulating TNF-α in serum, liver, heart and maintaining the mean arterial blood pressure after aortic clamping (Bernik et al., 2002); 3) cholinergic agonist pretreatment reduces circulating TNF-α, IL-6, and MIP-2, 3h after partial hepatic warm ischemia (90 min) and reperfusion is applied, but this pretreatment has no appreciable effect on neutrophil infiltration or histopathology of the liver at the 24h post-reperfusion time-point (Crockett et al., 2006); 4) cholinergic agonist pretreatment reduces renal tubular necrosis and neutrophil infiltration in ischemia/reperfusion mediated injury as assessed after bilateral 45 min of ischemia was applied and 24h of reperfusion (Yeboah et al., 2008); and 5) recently a hypothesis on the reduction of ischemia-reperfusion mediated cardiac damage, as a result of using an α7 nicotinic acetylcholine receptor agonist, has been put forward (Xiong et al., 2009). All these findings and hypotheses, speak of a vagal-mediated immunosuppression.

However, as the adage would have it, “too much of a good thing is not necessarily a good thing”. A case in point, traumatic brain injury resulting in increased vagal nerve activity can be associated with decreased innate immune response and increased susceptibility to infection (Kox et al., 2008). Therefore, using the vagus nerve to moderate immune response would have to be done under strict censure. Unfortunately, many of the previously mentioned studies were done on animals the species difference in the immune system in terms of abundance and localization of neutrophils and lymphocytes in the blood, type and quantity of immunoglobulin production among other differences (Haley, 2003). In addition, a closer look at the species differences regarding hepatic innervation will shed light on how reliable the information is regarding interspecies extrapolation of data.

c. Species differences in hepatic innervation. A comparative immunohistochemical study revealed the similarities and differences in hepatic innervation, between 5 species including humans, rats, hamsters, guinea pigs and dogs (Akiyoshi et al., 1998). The livers of all these animals were innervated by the parasympathetic and sympathetic nervous system. First, a summary of the extra-hepatic innervation; where the hepatic innervation stems from. In brief, the celiac and superior mesenteric ganglia give rise to splanchnic nerves, which form the post-ganglionic aspect of the sympathetic nervous system that innervates the liver. The pre-ganglionic aspect of the parasympathetic nervous system is constituted from branches of the vagus nerve. One branch forms an anterior plexus around the hepatic artery and a posterior plexus around the portal vein and the common bile duct (Akiyoshi et al., 1998). The intra-hepatic innervation is species specific and the density of nerve fibres vary between species and also according to the structure innervated, although there are some similarities.

The portal region (including hepatic artery, portal vein, bile duct and border of the periportal region):

- What is **common for all 5 species** (humans, rats, hamsters, guinea pigs and dogs) revealed aminergic nerves (tyrosine hydroxylase positive fibres) and cholinergic nerve fibres (acetylcholine positive). These are the only types of innervation common to all 5 species.
- In guinea pigs, dogs and humans, the hepatic artery, the portal vein and the border of the periportal region are neuropeptide Y and substance P positive. In addition, the bile duct is innervated by neuropeptide Y and substance P

positive nerves in guinea pig and dog but not in human livers. Neuropeptide Y and substance P positive fibres (peptidergic nerves) innervate hepatic artery in rats and hamsters, while substance P also innervates hamster portal vein.

- Vasoactive intestinal peptide (VIP) positive nerves innervate hepatic artery, portal vein and bile duct for human, dog, guinea pigs, and hamster. Guinea pigs are also VIP positive border of the periportal region. Rats are only VIP positive in nerve fibres that innervate hepatic artery and portal vein.
- Calcitonin gene-related peptide. All structures investigated revealed positive nerves fibres for hamsters and guinea pigs. Rats and dogs are positive throughout except for the border of the periportal region. Humans are only positive for hepatic artery and bile ducts (Akiyoshi et al., 1998).

<u>The intralobular innervation (including parenchyma and central vein)</u>

- Humans, dogs and guinea pigs have innervation that is positive for tyrosine hydroxylase, neuropeptide Y and substance P but negative for the other types of nerves mentioned above, in terms of intralobular innervation. Hamsters are positive only for substance P in the innervation of the central vein, and negative elsewhere. Rats are negative for intralobular innervation throughout for all types of nerve fibres examined (Akiyoshi et al., 1998).

This in depth look at innervation of the liver is important when we consider the numerous hepatic studies using animal models, too numerous to mention here, in the following order of popularity: rat> mouse> guinea pig> rabbit> hamster> dog> cat> pig> monkey (Akiyoshi et al., 1998). In summary, the differences are in terms of structures innervated, type and density of innervation and also depth, e.g. parenchymal innervation versus the space of Disse (space between the basolateral aspect of hepatocytes and the sinusoidal endothelial cells) (Akiyoshi et al., 1998), and more specifically where the human liver is concerned, the innervation was localized within recesses between hepatocytes, hepatic stellate cells and sinusoidal endothelial cells, while Küpffer cell innervation was more contentious (Tiniakos et al., 1996). Given the differences of the previous study (Akiyoshi et al., 1998), the order of preference when studying liver disease should be dog ≈ guinea pig » hamster ≈ rat. Unfortunately this has not been the case and rats have been used as animal models of brain dead liver donors (Van der Hoeven et al., 2001; Van Der Hoeven et al., 2003) and as models for orthotopic liver

transplantation for over 35 years (Spiegel and Palmes, 1998). Therefore, given these results, the salient points are: 1) choose the animal model that best fits the human condition prior to experimentation; and 2) caution must be exercised when interpreting results until the animal model is characterized vis-à-vis the organ or system being studied.

d. Hepatic reinnervation after transplantation. After transplantation does the nervous system reinnervate the liver? The sympathetic nervous does not reinnervate even after 1 year post-transplantation. This could explain the increase in blood-flow to the liver due to lack of vasomuscular tone. The abnormal splanchnic heamodynamics that were present in the patient prior to liver transplantation, due to liver pathology, remain up to 1 year post-transplantation (Colle et al., 2004). Lack of vagal innervation reduces stimulation of hepatic progenitor cell (oval cell), found in the canals of Hering, reduces oval cell differentiation into hepatocytes and cholangiocytes (Cassiman et al., 2002). This could have deleterious effects in conditions where hepatocytes are unable to replace their numbers, although in living transplant recipients the liver regenerates normally (Colle et al., 2004). Transplant patients had postprandial hyperglycemia, by oral glucose tolerance test, which eventually returned to normal. Patients were also hyperfagic with a higher fat and lower carbohydrate diet coupled with decrease in resting energy expenditure. Transplant patients do not feel pain after liver biopsy as opposed to patients with normal innervation to their livers, which feel shoulder pain after liver biopsy. There is the development of amputation neuromas associated with failed nerve regeneration (Colle et al., 2004). Despite all these observations liver metabolism seems “normal” overall, in terms of production of proteins and coagulatory factors, detoxification and bile production. The long-term physiological consequences of hepatic denervation are as yet unknown (Colle et al., 2004). Although we have already revised what is known about hepatic denervation and nerve regeneration after transplantation, as a logical extension of brain death and the possible biological consequences associated with denervation. However once the liver is taken out of the donor, other problems arise which are associated with storage. We need to consider what happens to the liver during continued cold ischemia and more specifically how the liver and the University of Wisconsin solution affect one another in terms of acid-base composition.

2. pH and preservation

A means of measuring tissue pH is via ^{31}P-magnetic resonance spectroscopy. This method measures the chemical shift of inorganic phosphates (Pi) relative to the nicotine adenine dinucleotide peak, and uses this value to calculate pH. This technique allows the discrimination between the hepatic Pi and that of Pi contributed by UW storage solution and furthermore, the liver can remain on ice in its Styrofoam container during the process (Wolf et al., 1996). Using this method, some interesting correlations were found in relation to lower tissue pH in human livers, including older donors, longer donor hospitalization, long cold storage times (over 10h) (Wolf et al., 1996). Interestingly, there was no correlation between hepatocellular graft damage and pH. It should be noted that normal hepatic tissue pH is 7.36 (Wolf et al., 1996) while intracellular pH values vary between 6.4 and 7.4 (Chang, 1981). Although this method of examining tissue pH is non-invasive, limits contamination, can be done on the entire liver, is relatively fast (30min), it distinguishes between hepatic Pi and that of Pi contributed by UW storage solution, it does not distinguish between intracellular and interstitial pH. Combining this method with one that measures interstitial pH could solve this problem, as will be seen in the upcoming paragraph. The lack of a correlation between hepatocellular damage and pH may be related to the attempt to correlate damage within localized regions with whole liver pH estimation. Nevertheless, during ischemia intracellular pH is reduced due to hydrolysis of ATP (Fuller, 1987), as this high-energy nucleotide accounts for 3-10mM in the cytosol (Schwiebert and Zsembery, 2003) and contributes protons as it degrades into ADP and AMP, such that: $ATP^{4-} + H_2O \rightarrow ADP^{3-} + HPO_4^{2-} + H^+$; $ADP^{3-} + H_2O \rightarrow AMP^{2-} + HPO_4^{2-} + H^+$ and $AMP^{2-} + H_2O \rightarrow$ adenosine + HPO_4^{2-}. Thus degradation of ATP to AMP may contribute millimolars of protons (Chang, 1981); (the consequences of ATP degradation will be taken up in the section 3a continued ischemia, cold stress and energy status). In addition, lactate accumulates and contributes to acidosis, due to glycolysis, by a net accumulation of protons ((Fuller, 1987) (Figure 3). Under normal circumstances ~80% of the lactate in resting humans is disposed of through oxidative metabolism, while the rest ~20% is funnelled through the hepatic synthesis of glucose and glycogen (Brooks, 1998). It should be noted that glycolysis provides approximately 3% of the available chemical energy in glucose, while the oxidative metabolism, citric acid cycle and the mitochondrial respiratory chain provide ~50% of the available energy in glucose (Fuller, 1987).

Extracellular lactate, pyruvate, glucose and other metabolic measurements were done shortly after reperfusion of 18 human livers, using a microdialysis catheter, with physiological saline flowing into the liver though the outer core of the catheter and fluid flowing out from the liver into the catheter to a microvial at a rate of 0.3ul/min for 48h. This catheter had a membrane with a 20kDa cutoff, thus molecules grater than 20kDa would not pass through. The vials were kept stored and analysed at 1h (18µl) intervals. Lactate rose from 1.8mM to 5.5mM for the first three hours, then declined over the next 7 hours and stabilized at 15h after the start of measurement. Pyruvate revealed a similar time course as lactate, although the peak of pyruvate was 320µM. Throughout the 48h period of measurement there was a 25fold ratio lactate/pyruvate at the first hour of measurement (Silva et al., 2005). This was interpreted as modest ischemia during the immediate post-reperfusion phase. Glucose rose from 3mM to 10mM during the first three hours of measurements and stabilized from the third to the forty-eighth hour of measurement. As all the patients survived without any primary hepatic dysfunction or non-function, the authors concluded that these represented normal values (Silva et al., 2005). Interestingly, among the metabolites, glutamate levels were also evaluated at different intervals; they steadily declined from 70µM (2-6h post-implant) to 20 µM (43-48h post-implant). Is glutathione being degraded by γ-glutamyltranspeptidase, at the sinusoidal endothelial cells, into glutamate and cysteinylglycine (see section B4a)? Is glutamate being exported out of the cell, to import cystine (see section B4d)? So what's missing from this study? First, there was an unreported time lag between the reperfusion of the liver and the insertion and collection of the first measurement. Second, the cold ischemic time and the warm ischemic time of the liver were unreported. Third, there was no pH measurement of the fluid coming out of the liver (perhaps owing to the small volume ~18µl). Fourth, there were no biopsy specimens to reveal the histology of the liver. Fifth, there were no fluid specimens taken prior to reperfusion. It would have been interesting to determine the pH, the levels of lactate and glutamate just prior to reperfusion or during cold ischemia.

Answers to some of the previous questions came with the follow-up paper, by the same group, on lactic acidosis and liver transplantation (Silva et al., 2006). In this study, the average length of the recipient operation was 6h (compared to about 2h with the Royal Victoria Hospital transplantation group), an average cold ischemia time ~9h (same as RVH transplantation) and an average warm ischemia time ~45min (same as RVH transplantation). During the period of preparing the liver for transplantation (called the

backtable time, ~50min, not kept on ice) and the period that the recipient was prepared for receiving the new liver, a total of ~4h elapsed during which interstitial hepatic lactic acidosis rose to 12mM (Silva et al., 2006), compared to 5.5mM value during reperfusion (Silva et al., 2005). Lactic acid also increases in rat livers stored at 2ºC (Churchill et al., 1994). The 12mM of lactic acid is noteworthy as the buffering capacity of UW solution is 25mM of KH_2PO_4. In addition, reduced glutathione (GSH) can contribute 2 protons per oxidized glutathione GSSG. Thus if 3mM of GSH were initially used to manufacture UW solution, up to 3mM of protons can be released to make 1.5mM GSSG. Thus the buffering power of UW solution may not be sufficient as the length of storage time increases. Interestingly, allopurinol inhibits xanthine oxidase by acting as a substrate becoming alloxanthine, which remains bound to the active site of xanthine oxidase. This causes an increase of hypoxanthine and xanthine and a decrease of urate, which can contribute a proton per molecule when uric acid is deprotonated and becomes urate (Stryer, 1988).

Finally, when comparing patients with primary dysfunction (the liver is not functioning as expected, but is not devoid of function) to patients with good initial graft function, the levels of lactic acidosis reached over 6mM during the post-transplant phase and diminished to normal levels after 18h in the former group, while the latter group ~4mM of lactate were measured during the post-reperfusion phase of the operation and diminished to normal levels by 8h (Silva et al., 2006). This delayed clearance of lactate after reperfusion may correspond to greater mitochondrial damage, as a result of ischemia-reperfusion, in one group versus the other, although the status of the mitochondria was mot ascertained in this paper (Silva et al., 2006). The information that is lacking includes 1) evaluating the pH of the collected samples; 2) mentioning the type of preservation solution used in this study such as University of Wisconsin or other; 3) does glycerol generate glucose and is this a happenstance of available glycerol, as a result of dying or dead cells or is it a mechanism designed to use glycerol as a source of energy. As pyruvate does not increase is glycolysis active? The authors thought that the glucokinase, the first step in glycolysis, was inactive at 4ºC (Silva et al., 2006). So why bother generating glucose? Is it a failed attempt to generate energy? The answer to this question remains open.

In their third paper, this group revealed that the degree of lactic acidosis correlated with hepatocyte necrosis in zone III and complement component C4d staining (the topic of the complement component system is covered in chapter 3 section B5). This

intrahepatic lactic acidosis also correlated with sinusoidal cell injury after reperfusion. There was no correlation between glucose, pyruvate or glycerol levels and hepatic histopathology (Silva et al., 2008). One of the most elementary questions that have not been answered, as yet, is the following: are cells dying because of acidosis from extracellular or intracellular sources or both? However important the pH is during the storage of an organ, it is but one facet of the stresses that afflict the liver prior to re-implantation into the recipient. Over the next sections we will cover some of the other stresses associated with the process of transplantation and endeavour to establish their "rightful" place in the cascade of stresses.

3. Energy status and preservation

a. Sources of carbon. During the 4h period when the recipient was being prepared to receive the new liver, human hepatic glucose levels rose from 5mM to 25mM while pyruvate was undetectable in most livers (Silva et al., 2006). These findings were similar to rat livers which also revealed an increase in glucose during cold storage (Churchill et al., 1994). Glycerol measurements were taken, as an indicator of membrane damage because glycerol phosphatides (e.g., phospholipids), is the major lipid constituent of mammalian membranes (Fuller, 1991) and upon loss of energy, influx of calcium leads to activation of phospholipases which cleave glycerol from phospholipids (Silva et al., 2006; Silva et al., 2005) during this same 4h period (Silva et al., 2006). However, there is another explanation for elevated glycerol levels. In the absence of glucose, the liver may search for alternative carbon sources of energy. Funnelling glycerol into the glycolitic pathway or gluconeogenic pathway. However, as glucose concentration is increasing, and because there is a difference in temperature sensitivity of some of the enzymes implicated in glycolysis, gluconeogenesis and the pentose phosphate pathways, especially to the prolonged 4ºC incubation, some pathways, gluconeogenesis and the pentose phosphate (Figure 3), may be favoured over another (Silva et al., 2006). Another possibility also lies in the use glycerol as an alternative source of energy. NGFI-B, a transcription factor, transduced mice by tail vein injection (Pei et al., 2006) had elevated fasting, at 12h and 24h, glucose levels compared to control mice, but also an increased ability (~50%) to use glycerol as an alternative source of carbon. In human liver transplantation NGFI-B mRNA is increased at the 1h reperfusion time-point (Boutros et al., 2008b), which could be related to the liver attempting to find alternative sources of carbon as energy (the topic of NGFI-B is

covered in chapter 3 section B11). At present it remains unknown if the only way for glycerol to be used as an energy source is the funnelling through glycolysis. Thus the measure of glycerol, in of its own does not necessitate damaged membranes. Alternatively, the release of glycerol upon cell damage provides a carbon source for the surviving cells as it traverses cell membranes (Wright and Diamond, 1969).

b. ATP and the liver. Perhaps a very revealing finding, using pig liver that were machine perfused at 4ºC, is that even at 4ºC the oxygen consumption was 30% of normal. Thus even at this low temperature the liver requires oxygen for its metabolism (Vekemans et al., 2008). This continued metabolism during preservation is echoed by rat livers which loose 85% of their ATP levels by 2 h of preservation at 2ºC and 93% by 4h of preservation (Churchill et al., 1994). During this same timeline ADP levels also dropped by 54% at 4h and 67% at 8h (Churchill et al., 1994). Does human liver mirror such results? The ATP, ADP and AMP levels were indeed measured in human livers, this time as a function of warm ischemia. Levels of ATP were reduced by ~85% after 15min of warm ischemia, by ~93% after 30min, and 99% after 60 min of warm ischemia (Kamiike et al., 1988). ADP followed a similar reduction, although much less pronounced. AMP levels increased during the first 15min and remained constant until the 60min time-point, thereafter decreasing at the 2h time-point. During this timeline hypoxanthine and xanthine, the degradation products of inosine (Figure 3), increased from 15min to 2 hours, without showing signs of plateau, during warm ischemia. Inosine and urate (the deprotonated form of uric acid), the end product of inosine degradation, increased for the first 15min thereupon stabilizing for the 2h duration of warm ischemia (Kamiike et al., 1988). These observations may be due to the effects of allopurinol as the inhibition of xanthine oxidase increases hypoxanthine and xanthine and decreases urate levels (Stryer, 1988). More importantly, there was a correlation between levels of total adenine nucleotides (ATP+ ADP+ AMP) and initial graft function. More specifically, greater total nucleotide conservation, e.g. not degraded to hypoxanthine and xanthine, correlated to good initial graft function (Kamiike et al., 1988). It is important to underline that the recovery of ATP levels never exceeded the levels of total adenine nucleotide (ATP + ADP + AMP). This finding should be verified, as it implies that the purine nucleotide salvage pathway is not invoked (Figure 3), at least in the timeline used: 38-166min after reperfusion (93 ± 31 min). As the purine salvage pathway exists in rat liver (Kim et al., 1992) it is likely to be a matter of de novo transcription, rather than a species-specific mechanism, as rat livers preserved for 6h recovered 92% of their ATP levels and

livers preserved for 24h only recovered 76% of their ATP levels (Quintana et al., 2005). An answer to the question" Why is the purine salvage pathway not invoked?" will be given in the next section.

c. allopurinol and nucleotide synthesis. Allopurinol is known to react with PRPP (5-phosphorybosyl-1-pyrophosphate) (Figure 3 in yellow background) to form allopurinol ribonucleotide. PRPP is a key substrate in the biosynthesis of purines, the purine salvage pathway, and the synthesis of uridylate (UMP) from the pyrimidine synthesis pathway (Stryer, 1988). In addition, allopurinol ribonucleotide inhibits the conversion of PRPP into phosphoribosylamine by amidophosphoribosyltransferase, also called phosphoribosylpyrophosphate amidotransferase (Stryer, 1988) (Figure 3 enzyme in red bold). Furthermore, the results of the human liver transplantation are important in that they indicate that the immediate fate (the first 2h 46min post-reperfusion) of the liver lies not in the purine salvage pathway but in the total amount of adenine nucleotides (ATP + ADP + AMP) already available (Kamiike et al., 1988). Conversely, as it pertains to ATP levels, there was neither a correlation between the levels of ATP and the duration of liver cold storage, nor a correlation between ATP levels and good initial liver function or primary graft dysfunction (poor initial function) (Kamiike et al., 1988). Thus, considering these results, the noxious effects of allopurinol may outweigh its benefits. As ATP is the molecular currency of life, limiting its availability at the time of reperfusion when the cells need to effect repairs and synthesize molecules such as mRNA and proteins, at the onset of reperfusion, could prove to be a limiting aspect of liver well being. Furthermore, although there are many pathways to compensate for the reduced ATP, such as glycolysis (Stryer, 1988), purine de novo synthesis (Adam, 2005) among others (Figure 3), some pathways are more "costly" than others; the salvage pathway is less costly than the de novo synthesis pathway in terms of energy (Stryer, 1988). But there is more to cold storage than reduced ATP levels. How the duration of cold storage correlates with graft well-being will now be considered.

4. Ischemic cold stress and warm reperfusion (heat shock)

a. Length of cold stress: is it important? A retrospective analysis, in point of fact a meta-analysis, on the effect of prolonged human hepatic cold ischemia time on primary graft non-function and overall graft and patient survival reveals that there is no linear relationship between length of cold ischemia and primary non-function and overall organ and patient survival. Patients receiving grafts that had cold ischemia times over

12h and those receiving livers with cold ischemia times less than 5 hours had a worst primary non-function and organ and patient survival than those patients receiving livers with intermediate cold ischemia times (Stahl et al., 2008). The cause of this dichotomy was not clear; the authors hypothesized that this discrepancy may have been due to "urgent matches" between the donor and recipient due to recipients that were more ill than average or more difficult patients to match with donors. Another hypothesis is that this discrepancy is due to regional differences where the organs were procured and transplanted, although the authors did not have any proof to substantiate this claim (Stahl et al., 2008). A third possibility that was not mentioned by the authors was that the groups of patients in the cold ischemia times over 12h may have had worse primary non-function, organ and patient survival because of the length of cold ischemia time, where the endothelial cell viability is compromised as it occurs in rats and pigs (Huet et al., 2004; McKeown et al., 1988; Schon et al., 1998; Schutz et al., 1997; Stolz et al., 2007). The patients in the cold ischemia times less than 5h may have had worse primary non-function, organ and patient survival due to improper cooling of the organ and longer warm ischemia time, which can be deleterious to rat and pig hepatocytes (Brockmann et al., 2005; Duval et al., 2006; Huet et al., 2004; Schon et al., 1998) and biliary epithelial cells in rats and humans (Feng et al., 2009; Noack et al., 1993; Silva et al., 2008). Finally, there seems to be an inherent toxicity to preservation solutions, including UW as a function of time and temperature, 4h (37ºC) > 5 or 10h (21ºC) >>18h (4ºC) as evaluated in cultured rat hepatocytes (Rauen and de Groot, 2008). Distinguishing between ischemia-reperfusion related injury versus perfusion solution related injury may be difficult in the context of human liver, unless a non-invasive imaging test can be devised.

Another aspect that should be mentioned is that, glycogen depletion was found to be based on lobular zonation, with 48% in the periportal and 74% in the pericentrilobular zones during ischemia. Reperfusion was accompanied by further loss of glycogen in these regions to a total of 64% and 95% in the periportal and the pericentrilobular zones respectively (Cherid et al., 2003). The duration of the cold ischemia time, 64% glycogen depletion for less than 10h and 82% depletion for over 10h cold ischemia were also revealed (Cherid et al., 2003). Unfortunately, although hepatic glycogen stores are depleted during the process of transplantation, especially during cold storage, no definitive link between glycogen depletion and prediction of graft primary non-function has been established to date (Quintana et al., 2005). There is some work that needs to

be done to determine how glycogen depletion affects the liver. More specifically, what hepatic physiological aspect, say acidosis, glycogen depletion correlates with.

b. Cold stress, warm reperfusion and liver histopathology. Interestingly, cold preservation seems to be more noxious for sinusoidal endothelial cells than warm ischemia. During the period of preservation, enlargement of the fenestrae, membrane blebbing, a hallmark of apoptosis and an increased number of vacuoles of the endothelial cell cytoplasm round out the histological description of rat and pig sinusoidal endothelial cells (Huet et al., 2004; McKeown et al., 1988; Schon et al., 1998; Schutz et al., 1997; Stolz et al., 2007). Warm reperfusion of sinusoidal endothelial cells heralds apoptosis, adherence/activation of neutrophils, platelets and coagulation system in rats (Arai et al., 1996; Cywes et al., 1993; Gao et al., 1998). It should also be mentioned, that hydroxyethyl starch, a component of University of Wisconsin solution, contributes to human red blood cell aggregation in vitro (Mosbah et al., 2006). The effects of hydroxyethyl starch on red blood cells have not been demonstrated in the context of human or animal livers. Warm ischemia is more detrimental to hepatocytes than cold ischemia (Brockmann et al., 2005; Duval et al., 2006; Huet et al., 2004; Schon et al., 1998). Overall, the process of cold-ischemic storage and warm reperfusion with recipient blood supply affects hepatocytes, as seen at the histological level, hepatocyte ballooning, cholestasis, aggregates of polymorphonuclear leukocytes (Abraham and Furth, 1996; D'Alessandro et al., 1991; Gaffey et al., 1997; Kakizoe et al., 1990; Silva et al., 2008). Warm reperfusion injury damages the biliary epithelial cells than does ischemic preservation per se (Noack et al., 1993; Silva et al., 2008). Adding to these observations was the finding that gentle in situ manipulation of rat liver activated Küpffer cells (Schemmer et al., 2001a). Unfortunately, manipulation of the liver is unavoidable during the process, it is nevertheless important to take this into account as a means (pharmacological treatment) may be found to inhibit Küpffer cell activation. Finally, caution should be exercised when evaluating protein synthesis in hepatocytes, after rewarming at 37ºC for different lengths of time, as cells in suspension reveal a 30% decrease, while whole liver, reveals a 70% decrease in protein synthesis when either preparation is at 4ºC in UW (Vreugdenhil et al., 1999).

c. Cold stress and immune-related problems. Interestingly, the length of cold ischemia hepatic preservation time ~15h versus 6:30h mean times had no effect on plasma levels of C3a or C5b-9 (membrane attack complex, MAC), at 2h post-reperfusion

(the topic of complement component, C5, is covered in detail in chapter 3 section B5). However when both groups were compared to their respective controls (blood gotten 1min before reperfusion from arterial blood), there was a two-fold increase in C3a and a four-fold increase in C5b-9. Although the authors believed that the complement activation may not be a causative factor in the poor outcome of grafts due to prolonged ischemia time, their small sampling which consisted of 11 patients in ~15h ischemia group and 7 patients in the 6:30h ischemia group (Schmidt et al., 2004) may mask an effect or the effect may consist of dysfunction of the graft at a later time. Another group found no correlation between cold ischemia time of human liver and MAC deposition on hepatocytes, as hepatocytes that were positive for MAC staining and those that were negative derived from grafts that had nearly identical cold ischemic times (approximately 8:30h) (Scoazec et al., 1997). Interestingly, no correlation between the percentage of hepatocyte staining for MAC and warm ischemia was seen in their human liver biopsy specimens either (Scoazec et al., 1997).

There were differences between plasma levels of IL-8 at the 2h post-reperfusion time-point, 2 fold increase, when comparing the ~15 hours to the 6:30 hours of cold ischemic preservation groups. However there was a substantial 8-10 fold increase in IL-8 when 2 hours post-reperfusion, when each group was compared to their respective 1 min pre-reperfusion time-point (Schmidt et al., 2004). A less dramatic 2-fold IL-6 increase was noted between the cold ischemic preservation groups and their pre-reperfusion control groups. The conclusion was that IL-8 might be a causative factor in the poor outcome of grafts caused by prolonged cold ischemia time (Schmidt et al., 2004).

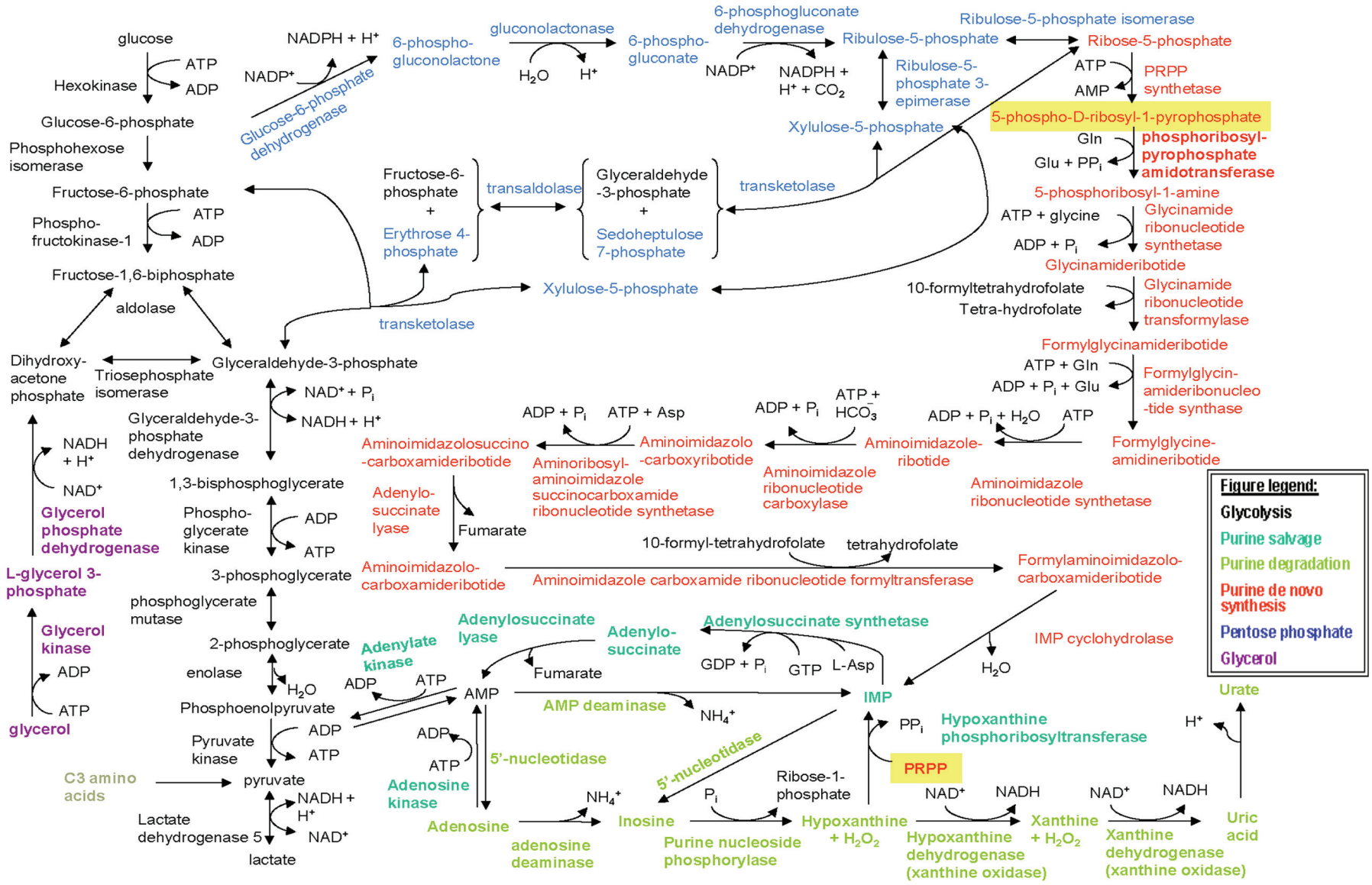
glucose
ATP
ADP
Hexokinase
Glucose-6-phosphate
Phosphohexose isomerase
Fructose-6-phosphate
Phospho-fructokinase-1
ATP
ADP
Fructose-1,6-biphosphate
aldolase
Dihydroxy-acetone phosphate
Triosephosphate isomerase
Glyceraldehyde-3-phosphate
NADPH + H+
NADP+
Glucose-6-phosphate dehydrogenase
6-phospho-gluconolactone
gluconolactonase
H2O
H+
6-phospho-gluconate
6-phosphogluconate dehydrogenase
NADP+
NADPH + H+ + CO2
Ribulose-5-phosphate
Ribulose-5-phosphate isomerase
Ribose-5-phosphate
Ribulose-5-phosphate 3-epimerase
Xylulose-5-phosphate
ATP
AMP
PRPP synthetase
5-phospho-D-ribosyl-1-pyrophosphate
Gln
Glu + PPi
phosphoribosyl-pyrophosphate amidotransferase
5-phosphoribosyl-1-amine
ATP + glycine
ADP + Pi
Glycinamide ribonucleotide synthetase
Glycinamideribotide
10-formyltetrahydrofolate
Tetra-hydrofolate
Glycinamide ribonucleotide transformylase
Formylglycinamideribotide
ATP + Gln
ADP + Pi + Glu
Formylglycin-amideribonucleo-tide synthase
Formylglycine-amidineribotide
ADP + Pi + H2O
ATP
Aminoimidazole-ribotide
ADP + Pi
ATP + HCO3−
Aminoimidazolo-carboxyribotide
Aminoimidazole ribonucleotide carboxylase
ADP + Pi
ATP + Asp
Aminoimidazolosuccino-carboxamideribotide
Aminoribosyl-aminoimidazole succinocarboxamide ribonucleotide synthetase
Aminoimidazole ribonucleotide synthetase
Fructose-6-phosphate
+
Erythrose 4-phosphate
transaldolase
Glyceraldehyde-3-phosphate
+
Sedoheptulose 7-phosphate
transketolase
transketolase
Xylulose-5-phosphate
Glyceraldehyde-3-phosphate dehydrogenase
NAD+ + Pi
NADH + H+
1,3-bisphosphoglycerate
Phospho-glycerate kinase
ADP
ATP
3-phosphoglycerate
phosphoglycerate mutase
2-phosphoglycerate
enolase
H2O
Phosphoenolpyruvate
Pyruvate kinase
ADP
ATP
pyruvate
C3 amino acids
Lactate dehydrogenase 5
NADH + H+
NAD+
lactate
NADH + H+
NAD+
Glycerol phosphate dehydrogenase
L-glycerol 3-phosphate
Glycerol kinase
ADP
ATP
glycerol
Adenylo-succinate lyase
Fumarate
Aminoimidazolo-carboxamideribotide
10-formyl-tetrahydrofolate
tetrahydrofolate
Aminoimidazole carboxamide ribonucleotide formyltransferase
Formylaminoimidazolo-carboxamideribotide
IMP cyclohydrolase
H2O
Adenylate kinase
ADP
ATP
Adenylosuccinate lyase
Adenylo-succinate
Fumarate
Adenylosuccinate synthetase
GDP + Pi
GTP
L-Asp
AMP
AMP deaminase
NH4+
IMP
PPi
Hypoxanthine phosphoribosyltransferase
PRPP
ADP
ATP
5'-nucleotidase
Adenosine kinase
Adenosine
NH4+
adenosine deaminase
Inosine
5'-nucleotidase
Pi
Ribose-1-phosphate
Purine nucleoside phosphorylase
Hypoxanthine + H2O2
NAD+
NADH
Hypoxanthine dehydrogenase (xanthine oxidase)
Xanthine + H2O2
NAD+
NADH
Xanthine dehydrogenase (xanthine oxidase)
Uric acid
H+
Urate
Figure legend:
Glycolysis
Purine salvage
Purine degradation
Purine de novo synthesis
Pentose phosphate
Glycerol

Figure 3. Schematic representation of the various pathways. Most of the pathways are referred to in the text, such as the glycolytic, the pentose phosphate and the purine salvage pathways. Gycerol funnels into the glycolysis/gluconeogenic pathway via conversion to dihydoxyacetone phosphate. The purine de novo synthesis pathway has been added, along with the other pathways in order to approximate how each pathway "fits in" in relation to the other pathways. Perhaps the most important point of this figure is the amount of energy each pathway "costs", e.g., how much ATP or GTP needs to be invested, and how much is gained. It should be noted that uric acid has a pKa of 5.4 and is deprotonated to urate (Stryer, 1988). Of course, there are other sources of energy such as amino acids (alanine, serine, cysteine) that funnel into pyruvate, while threonine can also be converted into pyruvate via aminoacetone. Alanine, serine, cysteine amino acids are referred to as C3 amino acids as they have 3 carbon atoms, while threonine (4 carbon molecule) and tryptophan (11 carbon molecule) are converted into a 3 carbon atom molecule, aminoacetone and then pyruvate. Glycine (2 carbon molecule) is converted to serine before it can be converted to pyruvate. The conversion of these amino acids to pyruvate can generate NH_4 and protons (Stryer, 1988). Ammonia is eliminated through the urea cycle ($CO_2 + NH_4^+ + 3\ ATP^{4-} + aspartate + 2H_2O \rightarrow urea + 2\ ADP^{3-} + 2\ HPO_4^{2-} + AMP^{2-} + PP_i + fumarate$). Although the formation of adenylate (adenosine monophosphate) from IMP is shown in the figure, inosine monophosphate (IMP) can also lead to the formation of GMP via xanthylate (Devlin, 2002; Murray et al., 2009; Stryer, 1988). PP_i: pyrophosphate; P_i: othophosphate; IMP: inosinate; PRPP: 5-phosphoribosyl-1-pyrophosphate.

5. oxidative stress

a. Cellular glutathione and redox potential. It had been assumed that an accurate way of measuring oxidative stress to a cell was the measure of GSH/GSSG ratios, because of the rational that one cannot measure every type of molecule or redox couple (in the present example GSH/GSSH constitute a redox couple). Thus one chooses a representative redox couple and the more abundant the redox couple is in the cell, the more representative of the redox state of the cell it will be (Schafer and Buettner, 2001) (Banhegyi et al., 2007; Han et al., 2006a). More precisely the GSH/GSSG redox couple is approximately 100-10 000x more abundant than other redox active compounds (Han et al., 2006a). Indeed, glutathione is found in the range of 1-11mM in the cell, depending on cell type (Hansen et al., 2006; Shackelford et al., 2005) and species (Igarashi et al., 1983), with a cytosolic ratio of GSH to GSSG that has been estimated at 30:1 (Banhegyi et al., 2007), 100:1 (Han et al., 2006a) or ~400:1 (Hill and Bhatnagar, 2007) depending on the cell type. Conclusions were drawn as to the oxidative status of the cell under examination, with possible biological consequences to oxidized proteins, DNA, lipids (Cesaratto et al., 2004).

The first misconception is that the GSH/GSSG ratio gives an accurate reading of the redox potential within a cell. Because two molecules of GSH form one molecule of GSSG, the reaction for GSH is second order and therefore the concentrations squared of GSH, $[GSH]^2$, more accurately reflects the reductive-oxidative equilibrium found within the cell (Han et al., 2006a). In addition, a cell with an absolute change in [GSH], without changing the GSH/GSSG ratio, would have a change in redox potential (Han et al., 2006a). Thus to determine the cellular redox potential, for a redox couple, the Nernst equation for an electrochemical cell is used: $\Delta E = \Delta E_o - [(RT/nF)\ln Q]$, and can be rewritten to accommodate a biological cell and more specifically the redox potential of a given redox couple, in the present case GSH/GSSG, for a specific compartment. ΔE_o is the electromotive force under standard conditions, R is the gas constant (8.314 $JK^{-1}mol^{-1}$), T is the temperature in Kelvin, n is the number of electrons exchanged in the chemical process, F is the Faraday constant (9.6485×10^4 Coulombs mol^{-1}), Q is the mass action expression ($Q = [GSH]^2/[GSSG]$, derived from 2GSH $\rightleftharpoons$ GSSG) (Schafer and Buettner, 2001). The RTlnQ term is a correction factor that accounts for non-standard conditions (Schafer and Buettner, 2001). Of course other considerations must be taken into account such as the pH of the intracellular environment, which can have an effect on the redox potential of a redox couple (Schafer and Buettner, 2001). The new equation

taking the effect of pH into account becomes $E_{pH} = E^{o\prime} + ((pH - 7.0) X (\Delta E / \Delta pH))$ mV, where $E^{o\prime} = E_{pH=7} = -240$mV for the GSSG/2GSH couple at pH 7.0, pH is the pH being considered, the value of $\Delta E / \Delta pH$ is dependent on the number of protons and electrons involved in the redox process (Schafer and Buettner, 2001).

Although these equations seem complicated, there are some salient points we need to consider: 1) the equation of E_{pH} is a far cry from a simple ratio GSH/GSSG; 2) if the pH shifts then a given cell would need to shift its ratio of the concentration of redox couples (the pH-dependent redox couples) to maintain the redox potential at a given level; or 3) an alternative to shifting the ratio of the concentration of the pH-sensitive redox couples, in response to a decrease in pH, may be compensated for by decreasing [lactate]/[pyruvate] ratio, which would preserve the ratios of redox couples such as $[NAD^+]$/[NADH]. However, it should be noted that redox couples exist in different ratios, $[NAD^+]$/[NADH] from 10:1 to 1000:1 and $[NADP^+]$/[NADPH] at about 1:100 (Schafer and Buettner, 2001). These become interesting considerations during the cold preservation of the liver where the intracellular ATP levels, estimated at 3-10mM decreases and protons increase anywhere from 1-19mM as 93% of ATP can be degraded during cold storage and each ATP molecule can contribute 2 protons. Furthermore, while lactate accumulates, xanthine oxidase is inhibited by an allopurinol derivative resulting in the accumulation of hypoxanthine and xanthine. Moreover, the purine de novo synthesis and salvage pathways may be hindered from functioning due to the formation of allopurinol ribonucleotide from allopurinol and PRPP (see section 2 pH and preservation and section 3a continued ischemia, cold stress and energy status). Taking these considerations into account, it may not be possible for a given cell to compensate for a decrease in intracellular pH by shifting ratios of metabolites, [lactate]/[pyruvate]. Upon reperfusion, oxidative stress could damage an already weakened system that must deal with an acidic milieu, depleted of ATP and nucleotides with little or no energy to power the proton pumps. Adding to all this, glutathione levels are not optimal as will be seen in the next subsection. Finally, is calculating E_{pH} all there is to determining the cellular redox potential and how feasible is it in the scenario of hepatic cold storage and reperfusion?

The second misconception was revealed when it was determined that the mitochondrial redox potential more accurately reflected the cellular redox potential. The mitochondrial is the most reductive environment thus far identified within the cell (Hansen et al., 2006) with a GSH concentration in the range of 5-11 mM (Shackelford et

al., 2005). Furthermore, while mitochondrial GSH derives from the cytosolic pool of GSH, as mitochondria do not have their own GSH synthetic enzymes (glutamate-cysteine ligase and GSH synthase) (Han et al., 2006a; Hansen et al., 2006), the pool of cytosolic GSH and mitochondrial GSH are kept separate; one can deplete one and not affect the other (Hansen et al., 2006). In addition, GSSG is membrane impermeable, thus once formed it resides in the mitochondria. These characteristics make for more accurate GSH and GSSG determinations, and as the greatest source of ROS in the cell is the mitochondria, it is more relevant to measure mitochondrial redox potential that it is to measure cytosolic redox potential (Han et al., 2006a; Hansen et al., 2006).

b. Glutathione depletion. In human liver transplantation GSH depletion does not correlate with hepatic graft viability (Ardite et al., 1999; Grezzana Filho Tde et al., 2006). Interestingly however, the GSH level which decreases during the cold ischemic storage period, decreases further in the first 2h of reperfusion, perhaps as a result of oxidative stress (Grezzana Filho Tde et al., 2006). It should be noted, however, that total liver biopsy GSH and GSSG were assessed in these studies (Ardite et al., 1999; Grezzana Filho Tde et al., 2006). It remains possible that graft viability correlates with mitochondrial redox potential and not with total tissue GSH/GSSG ratios. While real-time estimation of subcellular glutathione redox potential is now feasible in cell culture (Gutscher et al., 2008), a non-toxic means of estimating mitochondrial glutathione redox potential within tissue awaits the light of day. As both GSH and GSSG are cell-impermeable and the cytosolic pool of glutathione is kept separate from the mitochondrial pool, it may be possible to estimate the mitochondrial pool using a mitochondrial-specific marker used on tissue-sections. Of course, such a method would need to be controlled by more direct measurements such as those that were done in cell culture (Gutscher et al., 2008), to determine the overall effect of tissue preparation on the reduced and oxidized glutathione levels.

c. Consequences of glutathione depletion. Indeed, when mitochondrial GSH is depleted this could allow ROS to diffuse from the mitochondria to the cytosol with the possible oxidation of proteins such as phosphatases, transcription factors and caspases, among other proteins, which have a cysteine in the active site. Such a cysteine could be oxidized to sulfenic, sulfinic or sulfonic acids (Han et al., 2006a; Reddie and Carroll, 2008; Winterbourn and Hampton, 2008). It should be noted that “sulfinic” is spelled alternatively as sulfinic (Woo et al., 2003) or sulphinic (Biteau et al., 2003). Although cells are equipped with redox molecules and enzymes capable of reversing such

modifications, at least for sulfenic and sulfinic modifications (Biteau et al., 2003; Woo et al., 2003), at present, a means of reducing sulfonic modifications has as yet to be described.

Other modifications such as nitrotyrosine formation have also been noted in mice (Moon et al., 2008), rats (Skinner et al., 1997), pigs (Isobe et al., 1999; Meguro et al., 2002) and humans (Varadarajan et al., 2004). Although the previously cited studies used paradigms that vary between warm ischemia-warm reperfusion for mice and pigs (Isobe et al., 1999; Meguro et al., 2002; Moon et al., 2008) to cold ischemia-warm reperfusion in rats and humans (Skinner et al., 1997; Varadarajan et al., 2004), results with mice and pigs suggest that inducible nitric oxide synthase (iNOS) lead to production of NO that in turn had deleterious effects, while results from humans reveal that NO derived from endothelial nitric oxide synthase (eNOS) may be essential to liver survival (Varadarajan et al., 2004). This dichotomy may relate to the potential of NO or peroxynitrite ($ONOO^-$), the product of NO and superoxide ($O_2^{\bullet-}$), both of which have been implicated in nitrotyrosine formation, and either increased MAPK phosphorylation (Schieke et al., 1999; Zhang et al., 2000a) or inactivation of mitochondrial proteins (Moon et al., 2008).

An overly simplified scenario can be summarized as follows. The oxidative stress increases moderately, within the cell, inactivates a MAPK phosphatases (Kamata et al., 2005; Salmeen et al., 2003; van Montfort et al., 2003) such as MKP-1 and MKP-3 (Kamata et al., 2005) thereby allowing increased MAPK signalling (Schieke et al., 1999; Zhang et al., 2000a) until γ-glutamylcysteine synthase subunits to be transcribed via Nrf2 and JunD (Zipper and Mulcahy, 2000), among other cellular events (Shackelford et al., 2005), leading to increased GSH synthesis and increased reducing potential (Zipper and Mulcahy, 2000). However, if too much oxidative stress occurs in a short space of time, then transcription factors and other proteins can be inactivated (Han et al., 2006a; Reddie and Carroll, 2008; Winterbourn and Hampton, 2008), and this could lead to apoptosis (Han et al., 2006a). Finally if the oxidative stress is such that the caspases have their catalytic cysteine inactivated via oxidation to sulfenic or sulfinic acids, and the process is not reversed, this may lead to incapacitation of the apoptotic pathway and necrosis could ensue (Han et al., 2006a). Of course determining if such an overall scenario “plays out” in living cells, one would need to monitor the levels, location and type of reactive oxygen species that are produced, or at least determine the level and type of ROS produced versus the identity of proteins that have been oxidized and their subcellular location. In addition, there is the issue of specificity of the reaction, for

instance hydrogen peroxide may react more selectively with peroxiredoxins, than GSH or a phosphatase such as protein tyrosine phosphatase (Winterbourn and Hampton, 2008). Therefore identifying the reactive oxidative species generated, its subcellular localization, the "natural" target of each type of ROS and which targets are oxidized when the "natural" target is saturated remains an unachieved goal (Winterbourn and Hampton, 2008).

d. Redox and MAPK signal transduction. Intraperitoneal treatment of mice with L-buthionine-*S*-*R*-sulfoximine (Limon-Pacheco et al., 2007), which targets all organs and partially depletes both cytosolic and mitochondrial GSH (Hentze et al., 2003), caused an increase in p38MAPK, c-Jun and ATF-2 phosphorylation, and NF-κB translocation to the nucleus in the context of the murine liver (Limon-Pacheco et al., 2007). Adding to these observations, the MAPK pathway regulated one of the GSH synthetic enzymes. More specifically, both the catalytic and regulatory subunits of the γ-glutamylcysteine synthase, the enzyme that catalyses the first and rate-limiting step in glutathione synthesis, were regulated by ERK and p38MAPK (Zipper and Mulcahy, 2000). Moreover a chemically induced oxidative stress (menandione), via production of reactive oxygen species and glutathione depletion, inhibits MEKK1 via glutathiolation can activate ASK1, a JNK MAPKKK (Boutros et al., 2008a; Cross and Templeton, 2004). Conversely, dominant active form of H-Ras (H-RasV12) in NIH 3T3 fibroblasts can increase superoxide production and cell cycle progression in an using ERK and JNK-independent pathways (Irani et al., 1997). Other redox sensitive molecules are also implicated in the MAPK pathway. A case in point, glutaredoxin and thioredoxin are first and foremost implicated in the reduction of protein-S-GSSG and protein-S-S-protein disulfide bonds in many subcellular locals (Lillig and Holmgren, 2007). However, glucose deprivation induced metabolic stress caused a GSH-dependent release of ASK1 from glutaredoxin or a GSH-independent release of ASK1 from thioredoxin and consequent activation of ASK1 in a human cell line (Song and Lee, 2003). This release of ASK1-thioredoxin couple and activation of ASK1, as a result of toxicity to the liver was confirmed in mice (Nakagawa et al., 2008), human hepatocytes (Lim et al., 2008) and TNF-α treated human kidney cell line (Liu et al., 2000b). Regenerating the active (reduced form) of glutaredoxin and thioredoxin entails that thioredoxin reductase and NADPH reduce the oxidized thioredoxin, while two molecules of GSH reduce glutaredoxin, forming one molecule of GSSG in the process. GSSG is reduced by glutathione reductase using NADPH (Lillig and Holmgren, 2007). Adding to the previous observations, ligands such

as TNF-α, IFN-γ, among other cytokines can produce reactive oxygen species in an array of cell types, including hepatocytes (Thannickal and Fanburg, 2000). Although the participation of inflammatory mediators may be an important considered during the hepatic reperfusion phase of the operation (Jaeschke, 2003), there is virtually no data on the role physiological levels of reactive oxygen species in the normal function of the liver. Finally, an intuitive means of curtailing the damage caused by ischemia-reperfusion mediated stress is to habituate the liver to the conditions of ischemia and/or reperfusion. One of the ways of achieving this is through ischemic preconditioning and ischemic postconditioning.

6. Ischemic preconditioning/postconditioning

a. Ischemic preconditioning

i. Advantages and pitfalls of preconditioning. Ischemic preconditioning consists of the practice of exposing tissue to one or more brief periods of ischemia followed by reperfusion (blood reflow for 10-15 min) to habituate the tissue to ischemia-reperfusion relevant stresses prior to long periods of ischemia followed by reperfusion, which could potentially be lethal. The protective effect of ischemic preconditioning appears in two distinct phases. The immediate effect occurs after transient ischemia and lasts for 2-3hours while the late phase begins 12-24hours after transient ischemia and lasts for 3-4 days (Carini and Albano, 2003). Unfortunately, the therapeutic window of transient ischemia represents a challenge, as it must be confined to not less than 5 min and not more than 15 min of transient ischemia for the procedure to be successful (Carini and Albano, 2003). Alternatively, chemically induced (doxorubicin) oxidative stress also lessened reperfusion related injury to the liver (Carini and Albano, 2003). Another preconditioning paradigm, used three courses of thermal preconditioning of rat skin flap, innervated by the right seventh intercostals territory, increased hepatic HSP70 expression and decreased ischemia-reperfusion mediated damage as measured by ALT and AST (liver enzymes in the blood) (Lin et al., 2001). Supporting the previous findings is that the double knockout HSP70.1 and HSP70.3 mice, used in an in vivo model of cardiac warm ischemia-reperfusion were afforded early but not late protection by ischemic preconditioning, while their wild-type littermates had both early and late protection from ischemic-reperfusion induced damage when they received ischemic preconditioning. Thus ischemic preconditioning relies on HSP70.1 and HSP70.3 for late protection but not early protection, from ischemia-reperfusion mediated damage

(Hampton et al., 2003). Because the late effects of preconditioning, start at 12-24hours after transient ischemia it would indicate a transcriptional component as opposed to the early effects of preconditioning, which may be due to post-translational modifications.

In pigs, ischemic preconditioning, followed by 2h of cold ischemia, prevented reperfusion-related hepatic injury (Ricciardi et al., 2001). The opposite results were revealed in dog livers that were subjected to 48 cold-ischemia, where ischemic preconditioning (10min of ischemia and 10min reperfusion) had no protective effect, while ischemic preconditioning protected against warm ischemia-reperfusion by reducing AST and ALT release (less necrosis) from 1-24 hour post-reperfusion. Ischemic preconditioning also preserved ATP levels in the liver, lessened myeloperoxidase activity, a marker of neutrophil infiltration and increased bile flow at the 1-hour post-reperfusion time-point in canine livers (Compagnon et al., 2005).

Such a narrow therapeutic window makes transient ischemia not only difficult to control in real time, in the context of human surgery, but also represents a real problem as the quality of human livers may vary from one donor to the other, especially where marginal livers are concerned. This makes the application of transient ischemia a chancy proposition at best. Perhaps this is a major reason accounting for the lack of trials implicating human hepatic ischemic preconditioning in brain-dead donors. Nevertheless, a retrospective study comparing brief donor cardiopulmonary arrest (same as the length of stay in intensive care unit) versus no donor cardiopulmonary arrest (brain trauma) revealed an effect that the authors interpreted as simulating ischemic preconditioning. Interestingly, AST and ALT levels in donors with cardiopulmonary arrest were higher than brain trauma prior to transplantation, however both AST and ALT levels were significantly lower post-transplantation (Totsuka et al., 2000).

ii. Mechanism of preconditioning. Part of the benefit of hepatic ischemic preconditioning lies in the improved blood-flow through the liver and more specifically, the enhanced red blood cell flow through the sinusoids in the capillary system and post sinusoidal venules, although the volume of blood per second was not changed in partially hepatiectomized rats regardless of whether the livers were preconditioned or not (Eipel et al., 2005). Interestingly, ischemic preconditioning delayed hepatocyte proliferation, at the 24h-time-point, after partial hepatectomy although the liver mass was eventually regained with similar time-course as the partial hepatectomy without preconditioning at the 7-day time-point (Eipel et al., 2005). In addition, although cardiac ischemic preconditioning has been associated with the adenosine A_1 activation, that of

the liver requires the adenosine A_{2A} type receptor signalling (Carini and Albano, 2003). The latter receptor funnels through PKC-δ and PKC-ε and p38MAPK in the rat (Carini et al., 2001a). Mice exposed to transient hepatic ischemia for less than 5 min or more than 10min reveal neither p38MAPK activation nor ischemic preconditioning (Carini and Albano, 2003). Some of the early mechanisms invoked by hepatic preconditioning include a preconditioning-mediated reduction of intracellular acidosis in hepatocytes by a PKC and p38MAPK-mediated mechanism, however lactate production was not modified (Carini et al., 2001b). Furthermore, in a rat model of 60min warm ischemia, adenosine and adenosine A_{2A} receptor agonists decreased ALT and AST release, improved the rate of blood flow compared to untreated controls and decreased hepatic TNF-α and IL-8 and decreased neutrophil infiltration at the 6h post-reperfusion time-point (Harada et al., 2000), possibly by decreasing neutrophil-hepatic endothelial cell interaction (Howell et al., 2000). Similar results were found in vitro using freshly isolated human cells. The adenosine or A_{2A} type receptor agonist decreases activated human neutrophil elastase release in vitro and decreased LPS-stimulated human monocytes synthesis and release of TNF-α also in vitro (Harada et al., 2000). Other early mechanisms include the increase in reduced glutathione (GSH) levels in the liver and diminished Küpffer cell activation. Furthermore, early preconditioning effects also include the increased AKT/PKB activity in hepatocytes, which may block proapoptotic signals (Carini and Albano, 2003). The late effects of preconditioning, starting 24h and lasting for 2-4 days, also include improved bile production and decreased leukocyte infiltration, enhanced blood flow through the sinusoids and lessened AST and ALT release (Carini and Albano, 2003). However in addition to these effects, the late effects of preconditioning include the increased expression of heat shock proteins (HSP70, HSP27), which can prevent caspase activation via interaction with proapoptotic molecules such cytochrome c, apoptosis inducing factor and apoptotic protease activation factor-1. Thus heat shock proteins seem to be important in late phase of ischemic preconditioning. Other hepatoprotective mechanisms also include decreased TNF-α during reperfusion (Carini and Albano, 2003). Although ischemic preconditioning is used as a means of habituating the liver to the upcoming ischemia and subsequent anoxia, an analogous treatment dubbed ischemic postconditioning is used as a means of accustoming the liver to oxidative stress. This is the topic of the next section.

b. Ischemic postconditioning

An interesting study compared the efficacy of ischemic preconditioning to ischemic postconditioning using either warm hepatic ischemia or liver transplantation (cold-ischemia warm reperfusion) in the rat (Wang et al., 2008a). The ischemic preconditioning was done twice (2 treatments), 5 min ischemia/ 5min reperfusion per treatment. The ischemic postconditioning was done 3 times, for each time 30 seconds of reperfusion and 30 seconds of ischemia (reocclusion). Warm ischemia lasted for 30minutes by clamping on the hepatic hile. Cold ischemia lasted for 2h at 4ºC (Wang et al., 2008a). Hepatic enzyme levels AST and ALT, and Fas expression were decreased by preconditioning or postconditioning, to the same degree, regardless of the warm ischemia or transplantation paradigms used, when compared to their respective controls (warm ischemia or transplantation no treatment); sham controls were not presented (Wang et al., 2008a). Apoptosis was decreased to the same extent by preconditioning or postconditioning, in the warm ischemia model, however in the transplantation model, ischemic postconditioning revealed greater reduction in apoptosis than ischemic preconditioning. Rat survival rate was similar in the ischemic preconditioning and the ischemic postconditioning using either the warm hepatic ischemia or the orthotopic liver transplantation (Wang et al., 2008a). Using ischemic postconditioning (5 periods of 5 seconds of reperfusion and 5 seconds of clamping per period) in a rat model of partial hepatic ischemia, revealed reduced lipid peroxidation in the post-conditioned liver compared to the ischemic liver without any postconditioning (Teixeira et al., 2009). In addition, both endothelial nitric oxide synthase and inducible nitric oxide synthase were up-regulated by ischemic postconditioning, which may be hepatoprotective against reperfusion injury (Carini and Albano, 2003; Wang et al., 2009).

The review of the literature speaks of a scenario that is anything but simple for human liver transplantation. The number of variables associated with the physical and physiological stresses to the liver during the process of transplantation, the cell types within this organ and the possible cross-talk that result from their coping with these stresses, the donor and recipient-related variables, the ingredients of the perfusion and preservation solution, that have never been properly characterized, are among the litany of variables that are part of the process of transplantation. Furthermore, because the process of transplantation is not easily amenable to manipulation of these stresses, much less their elimination, a means of addressing what happens to liver during the

process of transplantation was needed that would take into account the global effect of these variables on the organ as an entity. Thus as the liver transits from brain dead donor to cold ischemic storage in synthetic buffer (UW solution) and finally to the warm reflow of the recipient blood, it must cope with the previously mentioned stresses, if it is to survive the process of transplantation. A means of doing so lies with the de novo translation of proteins from mRNAs. Thus we used human liver biopsy specimens to determine which liver-specific mRNAs were regulated during the reperfusion phase of the operation. In chapter 3, we will review the technology used, microarray, to query the biopsy specimens as well as what transcripts we identified and what possible role, if any, their protein product could have during the process of human liver transplantation.

III. Chapter 3: Microarray technology and the human liver genes we identified

Microarray technology and the human liver genes we identified

Given the number of variables afforded by the process of transplantation and that the reperfusion phase of the operation harbours all the stresses associated with the ischemic phase of transplantation, we focused our attention on gathering biopsy specimens during the reperfusion phase of the operation. The first biopsy collected at the R0 time-point permits us to control for blood reflow (negative control). The second of these time-points, R10, was a control for recipient blood-borne contribution of messenger RNA. Finally, the third time-point, R60, accounts for the liver-specific contribution of mRNAs, which in principal represent the RNAs the liver modulates in response to transplantation. Furthermore, we desired to conduct an analysis with as little bias as possible we therefore chose to use microarray technology as a means of querying the liver's response to transplantation. Armed with the question: How does a normal liver survive the process of transplantation? We were ready to determine, by transcriptional profiling using the three time-point biopsy specimens, how the liver moderates its response to the cascade of stresses associated with transplantation.

A. Microarray technology

At present we will review what is known about microarray technology, its strengths and weaknesses and the logistics associated with using human biopsy specimens from a healthy organ. As previously stated, we used microarray technology as it afforded the least biased approach to querying the human liver's response to transplantation. Furthermore, because we are dealing with human biopsy specimens from transplanted livers, this implies many logistic and biological limitations. The first limitation is consent, as the patients or their family must consent to the biopsies. The second limitation is the liver, as only livers that are fit for transplantation can be used for the study. The third, is that the size of the specimen, which is small at the outset as the liver needs to be viable for transplantation, is further divided into three sections, one for immunohistochemistry, one for total RNA extraction and the third piece for protein extraction. The fourth limitation lies with the process of transplantation, as it is a random process, because the death of a donor is of course a random event. The fifth limitation is that the transplantation procedure must be carried out at the Royal Victoria Hospital (RVH), which implies that the patient at the RVH must be at the top of the list of

recipients. Indeed, if a donor dies in Montreal but the recipient is out of province or in another hospital, then no sample can be collected as this study, i.e. biopsy collection cannot be imposed on a transplant group from another hospital.

Furthermore, the transplantation process carries with it a procession of variables, none of which can be easily eliminated. Among the variables are, 1) the different surgeons (from the same transplant group) are performing transplantation; 2) the lengths of time the donor has been brain dead; 3) the donor livers; 4) the diverse lengths of cold preservation time; 5) the varying lengths of warm ischemia time; 6) the different recipients, although the blood derived mRNA has been taken into account, because of the R60-10 versus R60-0 comparisons, the blood borne factors such as hormones, cytokines, endotoxin, viruses etc may modulate mRNA even at the 1h post-reperfusion; 7) the blood pressure variations from one donor to another and one recipient to another; and 8) the degree of match during tissue typing, realizing that the more desperate the situation is to save a patient's life, the less exact the match, although this variable may affect later outcomes such as organ rejection. Another limitation is financial. As our laboratory is not equipped to spot our own microarrays, we had to acquire them from another lab, which imposed a limit on the number of samples that could be processed. In conclusion, given the number of variables and the limited number of samples, a rigorous statistical analysis of the microarray data was not possible. Nevertheless, this study gave us a starting point, a list of genes that we could investigate and determine what role, if any, the protein products of these genes had during the process of transplantation.

In the absence of statistics, we were nevertheless able to diminish the impact of artefactual results through a variety of means. First, we used chips that had duplicate spots of the same EST (expressed sequence tag, which is defined as part of an expressed gene, in this case part of an mRNA including the 3' untranslated region). The length of the EST is determined by the reverse-transcriptase. The 3' UTR is present but there can also be coding sequences. Length of the cDNA was between 300 and 2000 bp. Double spotting is useful in revealing problem areas and problem spots were eliminated during the quantification process. Furthermore, when both spots were good, the average of duplicate spots was used as a single measurement during the quantification process. We also carry out dye swaps, which is a form of technical replicate (Nantel, 2006), as Cy5-labelled R60 (R=reperfusion time point 60 min) or R10 and Cy3-labelled R0 were labelled in another experiment Cy3-labelled R60 or R10 and Cy5-labelled R0 (Boutros et al., 2008b). Due to the limitations of our samples technical

replicates, in the form of repeating the entire process for each sample, including dye-swaps, could not be done. Pooling RNA was not done as it limited the detection of mRNA species that were inherently more variable than others (Nantel, 2006). As we do not know a priori what to expect from human liver transcriptional profiling, given the number of variables, we preferred to allow each sample “to tell its own story”. Biological replicates were done as follows, recalling that each liver had 3 biopsies taken: 7 different livers were used with 7 Cy5-labelled R60 hybridized concurrently with 7 Cy3-labelled R0 and their corresponding dye swap 7 Cy3-labelled R60 and 7 Cy5-labelled R0. Of the 7 different livers, we had enough total RNA to do 4 other labellings such as 4 Cy5-labelled R60 and 4 Cy3-labelled R10 with the dye swap 4 Cy3-labelled R60 and 4 Cy5-labelled R10 (Table 1).

Table 1. Summary of microarray analyses carried out in our study. Samples compared are indicated. Cy3 and Cy5 labelling are indicated in green and red respectively. L: liver; R: reperfusion; times 0, 10min and 60 min post hepatic portal vein reperfusion.

	L6R0	L6R10	L6R60	L7R0	L7R10	L7R60	L11R0	L11R10	L11R60	L26R0	L26R10	L26R60	L27R0	L27R10	L27R60	L28R0	L28R10	L28R60	L30R0	L30R10	L30R60
L6R0																					
L6R10																					
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L30R60																					

Comparing the list of genes generated by the R60-R10 normalization with the one generated by the R60-R0 normalization allowed us to eliminate the globin genes as being contributed by the recipient blood; R60-R10 revealed no globin gene regulation and R60-R0 revealed up-regulation of globin genes. We started by validating some of the genes that were most strongly and reproducibly regulated in terms of the number of ESTs that were identified for these genes and across patients.

It should be cautioned that this method of data transformation tends to underestimate fold change in the abundance of a transcript. While independent validation may be required on a per gene basis (Nantel, 2006) other methods used to validate our list of genes include finding patterns of expression via gene ontology-based clustering and STRING network representation which can reveal what category of function or response each gene relates to within the context of a cell (or extracellular) and how the identified genes relate to each other respectively. But this is not the end of the analysis it is only the beginning.

Given our experimental paradigm, microarray analyses afford an opportunity to identify “new” target genes and perhaps their eventual protein products out of the already existing panoply of proteins expressed within the cell, especially those that are related to the management of stress. Once we have our list of genes and determine how they may relate to one another using annotation-based analysis, identifying the possible role a gene or its protein product plays within the context of liver transplantation is perhaps the ultimate validation. One way of doing this, prior to actual experimentation, is via an investigation of the literature to determine what is known about the gene and protein product in general and in the context of ischemia-reperfusion related stresses. The next section affords a detailed look at some of the most-interesting genes identified in the microarray study, whether these were up-regulated (Boutros et al., 2008b) or down-regulated (Boutros et al. unpublished observations). The depth of detail is, of course, proportional to what is known about each gene and its protein product. One final note about a frequent misconception concerning transcriptional profiling experiments, microarrays do not directly measure gene expression and cannot distinguish between the diverse mechanisms that could modulate transcript levels, as the section on mkp-1 will reveal, there is no want of mechanisms to regulate the levels of expression of its mRNA. Such diversity assures that mRNA expression is tightly regulated in terms of time and species being regulated. Such control explains, in part, why we should not expect a linear correlation between mRNA and protein levels. Of course protein production has its

own mechanisms of regulation, some of which have been mentioned in the context of MKP-1 protein and other proteins throughout sections B and C. It is therefore easy to understand, given the possible combinations of control at the mRNA and protein levels, that linear relationships between mRNA and protein level of expression may be few and far between.

B. Genes identified through microarray analyses that were previously reported to be involved during ischemia-reperfusion

By far and large, the genes identified through our microarray investigation (Boutros et al., 2008b) have been previously implicated in ischemia-reperfusion related stresses in either human studies or animal models. As such, an attempt to relate these proteins to the outcome of human liver transplantation has been attempted in this section. As the first original contribution concerns the map kinase phosphatase-1 (MKP1), and we had exhaustively reviewed the literature available about this phosphatase, it is presented first in some detail. It should be mentioned, however, that the length and breadth of each section depends on the availability of the overall knowledge for each protein, and how this knowledge relates to ischemia-reperfusion related stresses.

1. Map kinase phosphatase-1 (MKP-1)

The following section, which covers general information about Map kinase phosphatase-1, is an abbreviated although updated excerpt of the review entitled “Mitogen Activated Protein (MAP) Kinase/MAPK Phosphatase Regulation: Roles in Cell Growth Death and Cancer” (Boutros et al., 2008a) found in the appendix section, subsection C. For a more complete discussion on MKP-1, the reader is referred to the review.

MAP kinase phosphatase-1 (MKP-1) is one of many phosphatases coded by the mammalian genome (Alonso et al., 2004; Arena et al., 2005; Keyse, 2000; Tonks, 2005) and a member of a sub-family of phosphatases known as the dual-specificity phosphatases (Camps et al., 2000; Dickinson and Keyse, 2006; Farooq and Zhou, 2004; Theodosiou and Ashworth, 2002). Initially identified as one of a set of genes that are expressed in cultured murine cells during G0/G1 transition (Lau and Nathans, 1985), it is part of a family, most of which demonstrate a wide tissue distribution (Camps et al.,

2000). As their name implies, the dual specificity phosphatases can dephosphorylate 2 types of residues, threonine and tyrosine. The function of these phosphatases is to dephosphorylate, and therefore inactivate, the MAP kinases (Liu et al., 2005; Slack et al., 2001), ERK's (Duff et al., 1995; Sarkozi et al., 2007; Sun et al., 1993), p38MAPKs (Kaiser et al., 2004), and JNKs (Sanchez-Perez et al., 1998). This dephosphorylation activity has been shown to be context dependent; not all three types of MAP kinases are targeted for dephosphorylation in a given situation, at least in the case of MKP-1 (Wu and Bennett, 2005; Wu et al., 2005b).

As the archetypal member of its family, MKP-1 continues to be the most exhaustively studied. Although the three dimensional structural analysis remains to be determined, much of the function and domains of MKP-1 protein is known and the gene and its regulation is also characterized. As proteins are the products of genes, the first issue to be addressed will be the mkp-1 gene.

a. mkp-1 gene, structure, promoter and enhancer

The human mkp-1 gene contains 4 exons and 3 introns coding for an inducible mRNA that is approximately 2.4kb long (Kwak et al., 1994). The promoter/enhancer region of this gene contains multiple AP2, Sp1, CRE sites while only one site for AP1, NF1 and TATA-box (Kwak et al., 1994; Pursiheimo et al., 2002) (Figure 1). Other binding motifs, such as an E-box and three GC boxes are localized between positions –110 and –30 (Ryser et al., 2004). Finally, a possible binding site for p53 protein is found in the second intron (Li et al., 2003a). These binding sites may explain the numerous factors that can transactivate the mkp-1 gene (see next section). Interestingly, while there is an E-box consensus sequence, as previously mentioned, in both the murine and human mkp-1 promoter, the transcription factor dimer c-Myc/Max is unable regulate mkp-1 expression due to unfavourable flanking regions (Sommer et al., 2000). Thus, the presence of a transcription factor's consensus sequence does not predict modulation via its sequence and the presence of appropriate flanking regions can determine the final outcome as to gene transcription. Finally, the transcription factor E2F1 is necessary to ensure responsiveness, of the mkp-1 gene, to hydrogen peroxide (Wang et al., 2007).

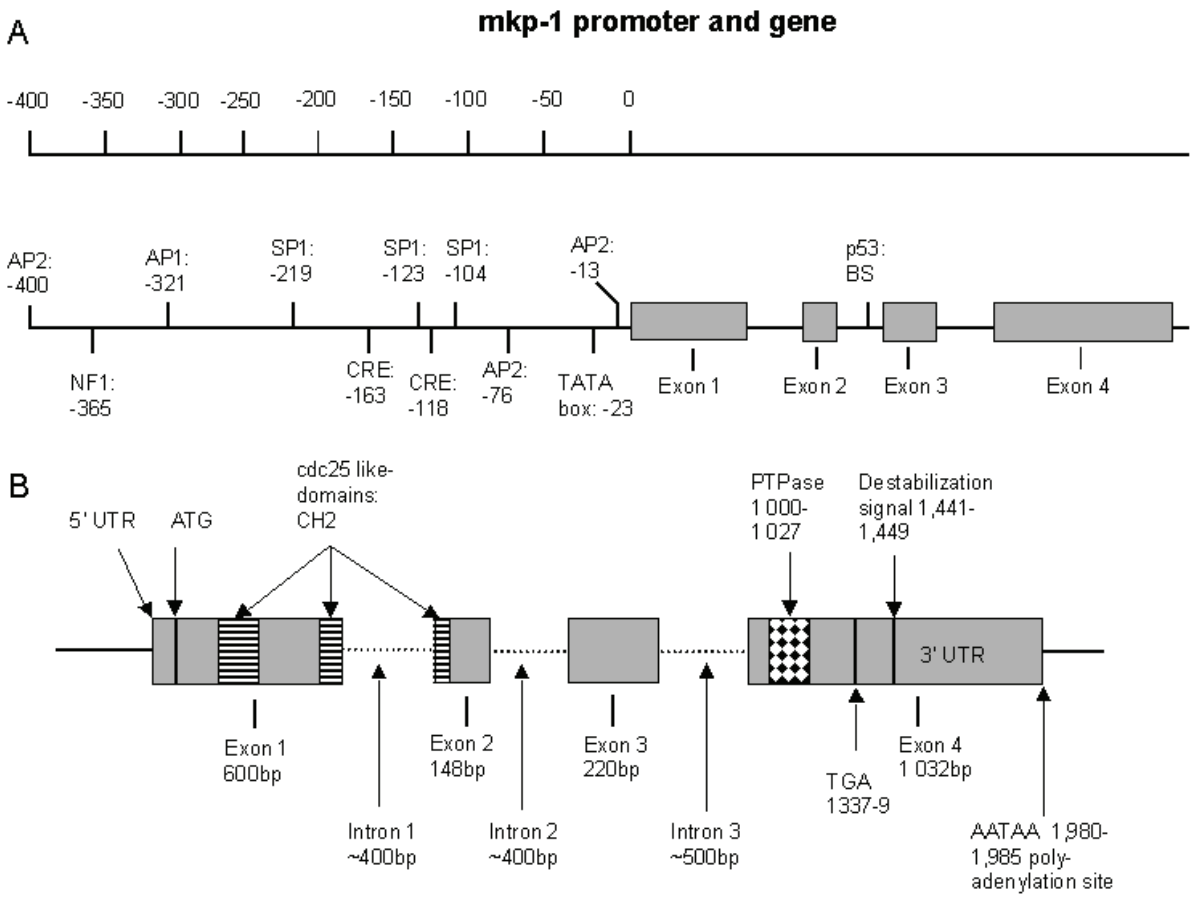


Figure 1. MKP-1 promoter and gene. A) Although the MKP-1 promoter demonstrates the potential for binding many transcription factors, caution should be used when inferring the binding of a transcription factor even when its consensus sequence is present in the promoter region, because flanking regions may influence binding (see text). B) A more detailed look at the MKP-1 gene reveals that the exons 1 and 4 bear the crux of the important domains of gene product, while the CH2B domain is coded by exons 1 and 2. Interestingly, there is no known domain coded by exon 3 discovered thus far. Reproduced with kind permission form the American Society of Pharmacology and Experimental Therapeutics. All rights reserved.

b. mkp-1 mRNA

i. Regulation of mkp-1 mRNA expression levels. As with many immediate-early genes coding for regulatory proteins, an abundance of factors up-regulate mkp-1 mRNA levels in different cell types. For example: serum in mouse and rat fibroblasts (Bokemeyer et al., 1996), dexamethasone, a glucocorticoid analogue, in human mammary epithelial cells MCF10A-Myc (Wu et al., 2004; Wu et al., 2005b), glucagon in rat hepatocytes (Schliess et al., 2000), insulin in rat hepatoma cell line H4IIE-C3 (Lornejad-Schafer et al., 2003), and atrial natriuretic peptide (ANP) in human umbilical-vein endothelial cells (HUVEC) (Furst et al., 2005). Other factors such as arachidonic acid, increases mkp-1 mRNA expression in cultures of rat aortic vascular smooth muscle cells (Metzler et al., 1998). Stressful conditions, such as heat shock in human skin fibroblasts (Keyse and Emslie, 1992), and in murine macrophages (Wong et al., 2005); osmotic shock in H4IIE-C3 rat hepatoma cell line (Lornejad-Schafer et al., 2003; Schliess et al., 1998); hypoxia in neonatal rat tissues (Bernaudin et al., 2002), hypoxia in PC12, Hep3B (Seta et al., 2001) and HepG2 cells (Liu et al., 2005; Liu et al., 2003; Seta et al., 2001), cobalt chloride, a hypoxia mimic, in HepG2 (Liu et al., 2003) and ischemia in rat forebrain (Takano et al., 1995; Wiessner et al., 1995) also modulate mkp-1 transcript. In addition, DNA damaging agents such as hydrogen peroxide in human skin fibroblasts (Keyse and Emslie, 1992) and vascular smooth muscle cells (Metzler et al., 1998) and other DNA damaging agents (Keyse and Emslie, 1992) produce similar effects. In contrast, no mkp-1 transcript was detected in four different unstimulated hepatoma cell lines (Kwak and Dixon, 1995). It is not known whether the reported increases in mRNA correspond to an increased stability or de novo synthesis of the mRNA.

ii. mRNA stability. The mkp-1 mRNA has a half-life of 1-2h, which varies according to the stimulant (Lau and Nathans, 1985). This variation in half-life may stem from the different mechanisms that determine the amount of mkp-1 mRNA that accumulates. In addition, a given stimulus may lead to the activation of multiple effectors, only some of which would be relevant to the query of interest. A case in point, heat shock up-regulates p38MAPK activity, and inhibition of this MAPK reduces the heat shock-mediated increase in mkp-1 mRNA levels (Wong et al., 2005). Conversely, stabilization of the mkp-1 transcript via heat shock in RAW 264.7 murine macrophages may not depend on heat shock factor-1 (HSF-1). A luciferase assay, using WT mkp-1 murine promoter upstream of the luciferase gene, demonstrated luciferase activity in HSF-1 -/-

fibroblasts (Wong et al., 2005). Other mechanisms exist to modulate mRNA stability. Although tristetraprolin (TTP) affects mkp-1 mRNA, this topic will be covered later in this chapter as TTP is one of the genes we identified at the 1h time-point during the reperfusion phase of the liver transplantation (Boutros et al., 2008b). In another study, treating HeLa cells with 1mM of H_2O_2 increased mkp-1 mRNA from 2-8h and its stability from 40min half-life to well over 2h half-life (Kuwano et al., 2008). Interestingly, of the RNA-binding proteins that were tested, HuR, NF90 and TIAR bound the 3' untranslated region of mkp-1 mRNA, while neither TIA-1 nor AUF1 bound to this region. After H_2O_2 treatment, the amount of mkp-1 mRNA bound to either HuR or NF90 increased, which stabilized mkp-1 mRNA, while the amount of mkp-1 mRNA bound to the translational repressors TIA-1 or TIAR decreased following the same treatment. Using these conditions HuR and NF90 seem to function independently of one another, and HuR binding to mkp-1 mRNA lasted for 8h, while NF90 binding to mkp-1 mRNA lasted 4h (Kuwano et al., 2008). This difference in mRNA binding may be related to translation, a topic that will be covered in the upcoming section, protein stability and de novo protein synthesis.

Modulating the mkp-1 mRNA stability is not presently considered for therapy, while modulating the stability of other mRNAs is being considered (Eberhardt et al., 2007). As the MKP-1 protein has a short-half life (see protein stability), targeting the mRNA expression could represent a therapeutic target. The potential of using mkp-1 mRNA stability, as a means of control, is further highlighted by observations demonstrating the involvement of MKP-1 substrates, p38MAPK, JNK1 and ERK1/2 in the signal transduction cascade relating to the stabilization of mRNAs (Eberhardt et al., 2007). But transplantation has a great many variables, and mkp-1 is only the proverbial drop in the bucket. Is the modulation of mkp-1 mRNA stability worth the effort? Before we can answer this question, we need to understand all the mechanisms that affect mkp-1 mRNA and protein levels and where mkp-1 fits into the array of transplantation-related variables.

iii. De novo transcription. Although previously mentioned studies using either hypoxia or ischemia did not reveal the mechanism of mkp-1 mRNA regulation, in human cervical carcinoma cell line SiHa, hypoxia up-regulates mkp-1 mRNA expression without stabilization; this mechanism is thought to protect hypoxic cells against apoptosis (Laderoute et al., 1999). Treatment of mesangial cells with hydrogen peroxide induced

transcription of mkp-1 messenger, in an AP-1 dependent manner, without affecting stability (Xu et al., 2004). Of note, while the ectopic expression of MKP-1 induced apoptosis in untreated mesangial cells, it also reduced hydrogen peroxide mediated apoptosis. Conversely, catalytically inactive MKP-1 neither induced apoptosis in untreated cells nor protected cells from apoptosis upon hydrogen peroxide treatment. Thus, although MKP-1 reduced oxidative-stress mediated apoptosis, inactivation of basal levels of ERK1/2 activity may account for the apoptosis induced by ectopic expression of MKP-1 in untreated cells (Xu et al., 2004).

iv. mRNA elongation. Under basal conditions in the rat neuroendocrine cells, GH4C1, a complex made from DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) inhibited RNA polymerase II (pol II) at the promoter proximal region of the MKP-1 gene (Fujita et al., 2007), and not within the first exon as previously reported (Ryser et al., 2001). Upon stimulation with thyrotropin-releasing hormone, the positive transcription elongation factor b (P-TEFb) was recruited and phosphorylated both the c-terminal repeats of Spt5, one of the subunits of DSIF (Yamaguchi et al., 1998), and Ser-2 within the c-terminal domain of pol II. These modifications allowed NELF to detach from pol II, which continued elongation with P-TEFb and DSIF complexed to it (Fujita et al., 2007).

As we have seen in this section, the many factors and mechanisms regulating the level of mkp-1 mRNA underscore the importance of MKP-1's function. These multiple levels of control of the mkp-1 mRNA speak of an expression that is precisely and exquisitely controlled by stimulants in space and time. Furthermore, each one of the mechanisms represents a level of control with a potential for therapeutic intervention. However, this is only the control for the mkp-1 mRNA expression levels. The next section will reveal that the MKP-1 protein also boasts its modulators.

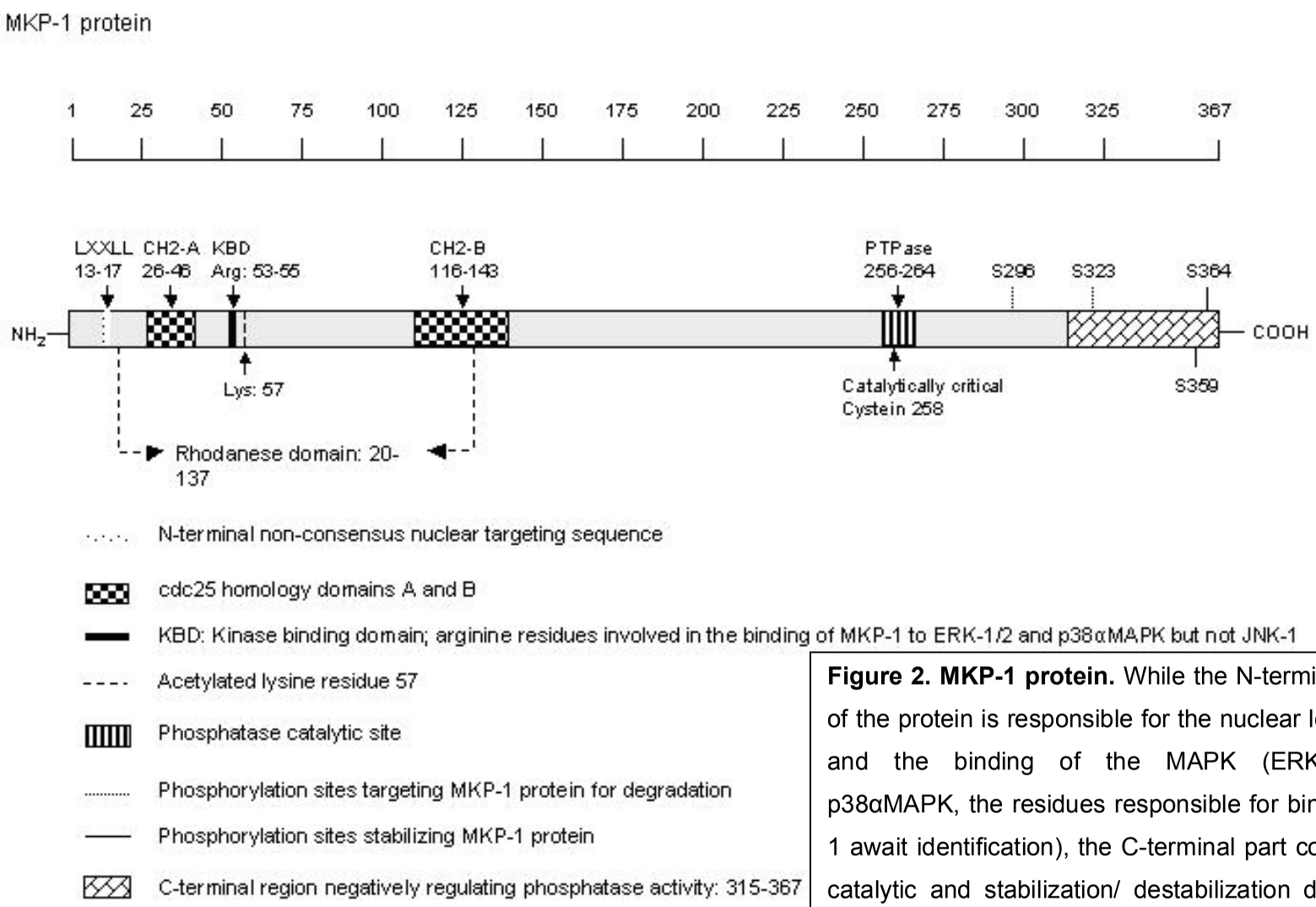


Figure 2. MKP-1 protein. While the N-terminal aspect of the protein is responsible for the nuclear localization and the binding of the MAPK (ERK-1/2 and p38αMAPK, the residues responsible for binding JNK-1 await identification), the C-terminal part contains the catalytic and stabilization/ destabilization domains of the protein. Reproduced with kind permission form the American Society of Pharmacology and Experimental Therapeutics. All rights reserved.

c. MKP-1 protein function and affinity

MKP-1 is a nuclear phosphatase. This phosphatase dephosphorylates proteins of the MAPK family, in the following order of affinity: p38MAPK ≥ JNK » ERK1/2 (Camps et al., 2000; Farooq and Zhou, 2004; Franklin and Kraft, 1997), and within these, ERK2 > ERK1 (Slack et al., 2001). MKP-1 also dephosphorylates ERK5 though it can bind to ERK5 in both stimulated and unstimulated cells (Sarkozi et al., 2007). However, MAPK bind to different sites within MKP-1. Indeed, while MKP-1 binding of ERK2, ERK1 and p38αMAPK depends on the same arginine residues (arginine 53-55) (Figure 2) that of JNK1 depends on as yet unidentified residues, although within the first N-terminal-188 residues of MKP-1 (Slack et al., 2001). While ERK5 binding to MKP-1 may be similar to ERK1/2, this awaits experimental verification. When the arginine 53-55 residues are mutated, MKP-1 retains its phosphatase activity toward JNK1 but not toward ERK2 or p38α (Slack et al., 2001). Identification of the MKP-1 residues that bind JNK1 will allow a more precise control for eventual therapeutic purposes. Although the order of MAPKs dephosphorylation is commonly accepted (Camps et al., 2000; Farooq and Zhou, 2004), that of ERK1/2 has been debated. Indeed, MKP-1 does not appear to dephosphorylate ERK1/2, but competes with ERK effectors and prevents their binding and phosphorylation-mediated activation (Wu et al., 2005a). Based on this model, it is suggested that the binding of MKP-1 to ERK1/2 prevents the ERK1/2-ELK-1 interaction, and therefore phosphorylation and activation of ELK-1 in the nucleus. In turn, this would prevent the activation of the serum response element (SRE) by ELK-1 (Wu et al., 2005a). Elucidating the manner, in which MKP-1 binds to the various MAPKs, would be a first step in determining why a difference exists in the affinity between MKP-1 and the different MAPKs. Some advances have been made as will be presented shortly. Another possibility lies in the observation that phosphorylated ERK2 can homodimerize (Khokhlatchev et al., 1998) and thereafter actively translocated to the nucleus, while monomeric phospho-ERK can enter the nucleus by passive diffusion (Adachi et al., 1999). ERK dimers may simply be unable to interact with MKP-1.

Other results reveal that MAPKs are not the only type of substrate for MKP-1. more specifically, a myc-tagged MKP-1 mutant protein, with a cysteine 258 to serine point mutation at the catalytic site, was able to bind histones H3, H2B, H2A and H4 in a pull-down assay from COS-1 cells (Kinney et al., 2009). In addition, purified wild-type but not mutant MKP-1 protein from COS-1 cells, was able to dephosphorylate purified histone H3 at Ser-10 purified from HUVECs (Kinney et al., 2009). Phosphorylation (Ser-

10) and acetylation (Lys-14) of histone H3 had been noted in response to EGF treatment in a mouse fibroblast cell line (C3H 10T1/2), and shear stress in HUVECs (Illi et al., 2003). These histone posttranslational modifications lead to increased c-fos, c-jun in both cell types, while genotoxic stress, but not heat shock (42ºC) or H_2O_2, increased mkp-1 mRNA levels in C3H 10T1/2 (Clayton et al., 2000; Li et al., 2001). In this way, MKP-1 may participate in the silencing of gene transcription via histone dephosphorylation. To what extent this mechanism is important still remains to be resolved.

Interestingly, a recent study found that MKP-1 protein was acetylated via the acetyltransferase p300, at Lys-57, in murine RAW macrophage cell line when treated with lipopolysaccharide (LPS) and trichostatin A (TCA), a histone deacetylase inhibitor but MKP-1 acetylation was not found in control cells (Cao et al., 2008). Acetylation of MKP-1 at Lys-57 increases its interaction with ERK1 and p38MAPK and enhances their dephosphorylation compared to non-acetylated MKP-1. This acetylation-mediated increase in dephosphorylation was estimated at 25% for p38MAPK; no estimation was given for ERK1. In wild-type mice given TCA treatment, 2 days before and 3 days after LPS treatment, there was 100% survival for 8 days post-LPS treatment, while 100% of the mice given LPS only treatment died after 3 days; both TCA and LPS were given intraperitoneally (Cao et al., 2008). TCA decreased leukocyte infiltration in the liver, oedema and necrosis. More importantly serum levels of TNF-α and IL-1β increased more dramatically in mkp-1 null mice in response to LPS, when compared to wild-type mice (Boutros et al., 2008a; Cao et al., 2008), and TCA did not decrease TNF-α and IL-1β to the same extent in mkp-1 null mice as it did in wild-type mice; there is approximately a 50% difference in the TCA mediated inhibition of TNF-α and IL-1β in the serum of wild-type mice and freshly isolated macrophages when compared to the serum of mkp-1 null mice and their freshly isolated macrophages (Cao et al., 2008). Although these data indicate that MKP-1 mediates the anti-inflammatory activity of histone deacetylase, the more salient point for our concerns regarding human liver transplantation is that an exogenous factor used in cancer treatment can reduce inflammation and leukocyte infiltration of the liver and increase anti-inflammatory process. It would be worthwhile to determine if this treatment, which seems to target MKP-1, can be used in the context of liver transplantation to decrease the inflammatory process, incurred as a result of the operation per se and possibly endotoxemia (see chapter 2 section B5) and limit the damage that the immune cells can mediate. In line with this concept dexamethasone

(3mg/kg or 30mg/kg) administered to mice wild-type versus mkp-1 null 30min prior to LPS treatment increased survival (100%) after 7 days post-LPS, while mkp-1 null mice treated with 30mg/mg of dexamethasone died (100%) after 36h post-LPS. Serum levels of inflammatory cytokines, and ex-vivo cytokine production of macrophages, were also decreased in dexamethasone treated wild-type mice compared to mkp-1 null mice (Wang et al., 2008b). As dexamethasone injected pre-operatively for purposes of immunosuppression in human transplant recipients increased cardiac rejection rates and inhibited early regenerative response of rat livers if livers required significant regeneration after ~16h cold storage (see Table 1 chapter 2 section B4, p.53-58), timing and caution are obviously warranted as species and exact paradigm may greatly influence the outcome.

Finally, MKP-1's dephosphorylating activity is restricted to the nucleus (Camps et al., 2000; Wu et al., 2005a). The targeting of MKP-1 to the nucleus is ascribed to a LXXLL motif located in the NH_2-terminus proximal to the cdc25 homology domains A (CH2A) (Wu et al., 2005a) (Figure 2). Although this sequence is not considered to be a consensus nuclear targeting sequence (Cartier and Reszka, 2002; Christophe et al., 2000), it acts as a nuclear targeting sequence for MKP-1 (Wu et al., 2005a). MKP-1 also contains a rhodanese domain located within residues 20-137 (UniProtKB/Swiss-Prot), known to catalyze a sulphur transfer reaction. Interestingly, MKP-1 lacks a critical cysteine residue within the rhodanese domain that would allow it to be enzymatically active. The function of this catalytically inactive rhodanese domain is currently unknown (Bordo and Bork, 2002). Understanding how MKP-1 is targeted to the nucleus, on its own or via binding to another molecule, can add another level of control over this phosphatase.

i. Regulation of MKP-1 protein expression levels. In addition to the transcriptional regulation, MKP-1 protein expression is increased in various cell types by different factors. For example: in insulin treated rat smooth muscle cells (Takehara et al., 2000) and rat hepatoma cell (Lornejad-Schafer et al., 2003), glucagon treated rat hepatocytes (Schliess et al., 2000), dexamethasone, treated human breast epithelial cell lines MCF-7 and MDA-MB-231 (Wu et al., 2004; Wu et al., 2005b), epidermal growth factor treated mouse embryonic fibroblasts (Wu and Bennett, 2005); ANP treated HUVEC and rat lung (Furst et al., 2005); overexpression of cytochrome P450 2C9 or treatment with 11,12-epoxyeicosatrienoic acid on HUVECs (Potente et al., 2002);

arachidonic acid treated rat aortic vascular smooth muscle cell (VSMC) cultures (Metzler et al., 1998); hypoxia treated neurons of newborne piglets (Mishra and Delivoria-Papadopoulos, 2004), hypoxia treated PC12 (Seta et al., 2001) and HepG2 cells (Liu et al., 2005); serum treated mouse fibroblasts (Sun et al., 1993); peroxide treated VSMC (Metzler et al., 1998) and HUVECs (Furst et al., 2005). As noted for mRNA levels: 1) it is unknown whether the increase in protein levels is due to de novo translation or increased stabilization; and 2) none of these factors or treatments are specific for MKP-1 protein regulation as they target many effectors, while others are adaptation to changes in the environment. Although at first glance there seems to be fewer agents and conditions that up-regulate MKP-1 protein versus the litany of agents and conditions that control its mRNA, this is due to the lack of investigation of MKP-1 protein regulation when its mRNA was studied.

ii. Protein stability and de novo protein synthesis. The MKP-1 protein half-life varies from 40 min (Charles et al., 1992) to 2 hour (Noguchi et al., 1993). This difference in half-life can be accounted for by the various mechanisms available to the cell for the modulation of MKP-1 protein stability, as will be presently seen. Interestingly, ERK1/2 reduce MKP-1 protein degradation by phosphorylating the ^{359}Ser and ^{364}Ser residues (Brondello et al., 1999), while glucocorticoids also decrease MKP-1 degradation albeit by an unknown mechanism (Kassel et al., 2001). On the other hand, ERK1/2 is also responsible for degradation of MKP-1 (Lin et al., 2003), via phosphorylation of ^{296}S and ^{323}S residues (Lin and Yang, 2006) (Figure 2). Once phosphorylated, Skp2 (also called, SCF^{Skp2}, Skp1/Cul1/F-box protein Skp2), targets MKP-1 for degradation via the ubiquitin proteasomal pathway (Lin et al., 2003). This degradation also involves PKCδ through an unknown mechanism (Choi et al., 2006). Although ERK-2 is a better substrate for MKP-1 than is ERK-1, as determined by yeast two-hybrid system (Slack et al., 2001), ERK1 can phosphorylate MKP-1 in vitro (Brondello et al., 1999). Furthermore, as ERK-1 and ERK-2 share 83% identity in protein sequence (Boulton et al., 1990), it remains possible that one is responsible for phosphorylation-mediated stabilization of MKP-1 while the other targets this phosphatase for proteasomal degradation via phosphorylation on other residues. Otherwise, ERK1 and ERK2 may serve redundant functions. Finally, the p38MAPK may also be a stabilizing agent, or have a role in translational events, since SB203580 an inhibitor that preferentially targets p38α/p38βMAPK, partially blocks the ERK-dependent cis-diaminedichloroplatinum II (cisplatin)-mediated accumulation of MKP-1 protein without affecting mRNA levels (Wang et al., 2006).

Finally, in contrast to what happens in IMR-90 cells, human lung fibroblasts, which constitutively express MKP-1, COS-7 cells induced de novo MKP-1 synthesis during the recovery phase after heat shock (Yaglom et al., 2003), while hyperosmolarity delays insulin induced MKP-1 protein expression (Lornejad-Schafer et al., 2003). Finally, the mRNA binding protein HuR also participates in enhancing MKP-1 de novo protein synthesis, as more mkp-1 mRNA is associated with polysomes after treating HeLa cells with 1mM of H_2O_2 when compared to control cultures (Kuwano et al., 2008). In this paradigm, although the general translation of proteins was reduced, translation of mkp-1 mRNA specifically increased. Although NF90 also bound mkp-1 mRNA (also see p.100), this protein acted as a translational repressor in HeLa cells treated with 1mM of H_2O_2 versus untreated control cells. Using these conditions HuR and NF90 seem to function independently of one another. Greatly reduced MKP-1 protein levels and a corresponding increase in phospho-ERK, phospho-JNK1 and phospho-p38MAPK accompanied the silencing of HuR (Kuwano et al., 2008). The silencing of NF90, was accompanied by a modest decrease in MKP-1 protein, and a modest increase in phospho-JNK1 and phospho-p38MAPK, while no effect on phospho-ERK was seen (Kuwano et al., 2008). The silencing of MKP-1 did not have an effect on cell viability in this paradigm (Kuwano et al., 2008), perhaps owing to the multiplicity of dual specificity phosphatases (Boutros et al., 2008a). Another possibility, concerns the phosphorylated MAPKs that were detected in this study (Kuwano et al., 2008), were derived from whole cell lysates and thus it was possible that there were different levels of cytosolic versus nuclear phospho-MAPKs, which would explain the lack of an effect on cell viability seen when silencing MKP-1 (Kuwano et al., 2008). Finally, later on in the HSP70 section, we will consider how heat shock protein-70 (HSP-70) interacts with MKP-1 protein to stabilize and conserve its activity.

iii. Protein activity. A mechanism that limits MKP-1 protein activity lies within the C-terminal region of MKP-1 (Figure 2), which auto-inhibits the phosphatase activity. In the same line of thought, the C-terminal truncated form of MKP-1 has higher phosphatase activity without modifying its substrate specificity (Hutter et al., 2002). Thus far, there is an overall consensus on the increased phosphatase activity of MKP-1 upon binding to one of a number of active MAPKs such as ERK1/2, JNK1 or p38MAPK (Slack et al., 2001), and in turn, MKP-1 dephosphorylates the MAPK it was bound to (Farooq and Zhou, 2004; Hutter et al., 2000). However, considering that the C-terminal portion of

MKP-1 can inhibit its phosphatase activity, determining if the C-terminal fragment, in of its own, can bind to other proteins may reveal another level of control for this phosphatase. Although previous binding assays failed to reveal interaction of MKP-1 with other proteins, other than the usual MAPKs, these assays relied on the phosphatase activity of MKP-1 for a readout. Some chemicals such as benzofuran and sanguinarine, an alkaloid plant poison (Garcia et al., 2006), also block MKP-1 activity (Lazo et al., 2006; Vogt et al., 2005).

These past sections have revealed a level of control over the MKP-1 protein that mirrors the many facets of control over its mRNA. Clearly the amount of MKP-1 protein is under strict censure in space and time. As is the case with the mRNA, each level of control over the MKP-1 protein is a potential therapeutic target. Since MKP-1 inactivates JNK1, p38MAPK and ERK1/2, not surprisingly stimulants capable of increasing MAPK activity also augment MKP-1 protein levels. The elegance of MAPKs up-regulating MKP-1 protein levels assures that under normal circumstances, the MAPKs limit the duration of their activity in space and time. Thus a circuit comprised of stimulant/MAPK pathway/MKP-1 protein is established, after a discrete period of time assuring the proper response for each cell type. However, the relevance of all this MKP-1 mRNA and protein regulation and the consequences of down-regulating MAPK pathways is best appreciated in light of an overview of MAPK signal transduction pathways in a linear (Figure 3) and MAPK cross-talk model (Figure 4). These figures give a synopsis of the consequences of MAPK function and how these MAPK interact with one another. Due to the complex nature of the signal transduction pathways, these figures present concepts that are beyond the scope of this thesis but are included in the figure to make a more complete image.

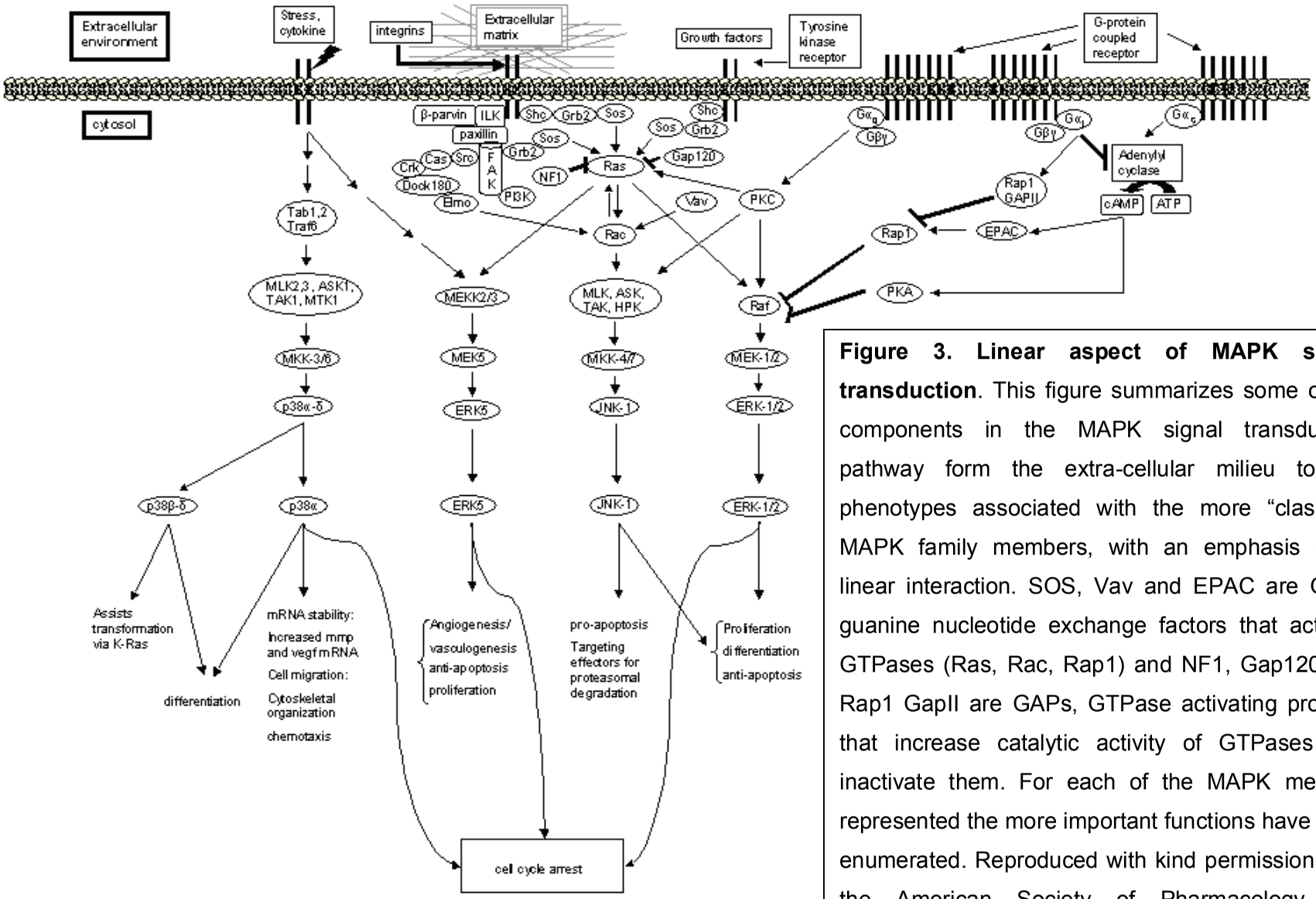


Figure 3. Linear aspect of MAPK signal transduction. This figure summarizes some of the components in the MAPK signal transduction pathway form the extra-cellular milieu to the phenotypes associated with the more "classical" MAPK family members, with an emphasis on a linear interaction. SOS, Vav and EPAC are GEFs guanine nucleotide exchange factors that activate GTPases (Ras, Rac, Rap1) and NF1, Gap120 and Rap1 GapII are GAPs, GTPase activating proteins that increase catalytic activity of GTPases and inactivate them. For each of the MAPK member represented the more important functions have been enumerated. Reproduced with kind permission form the American Society of Pharmacology and Experimental Therapeutics. All rights reserved.

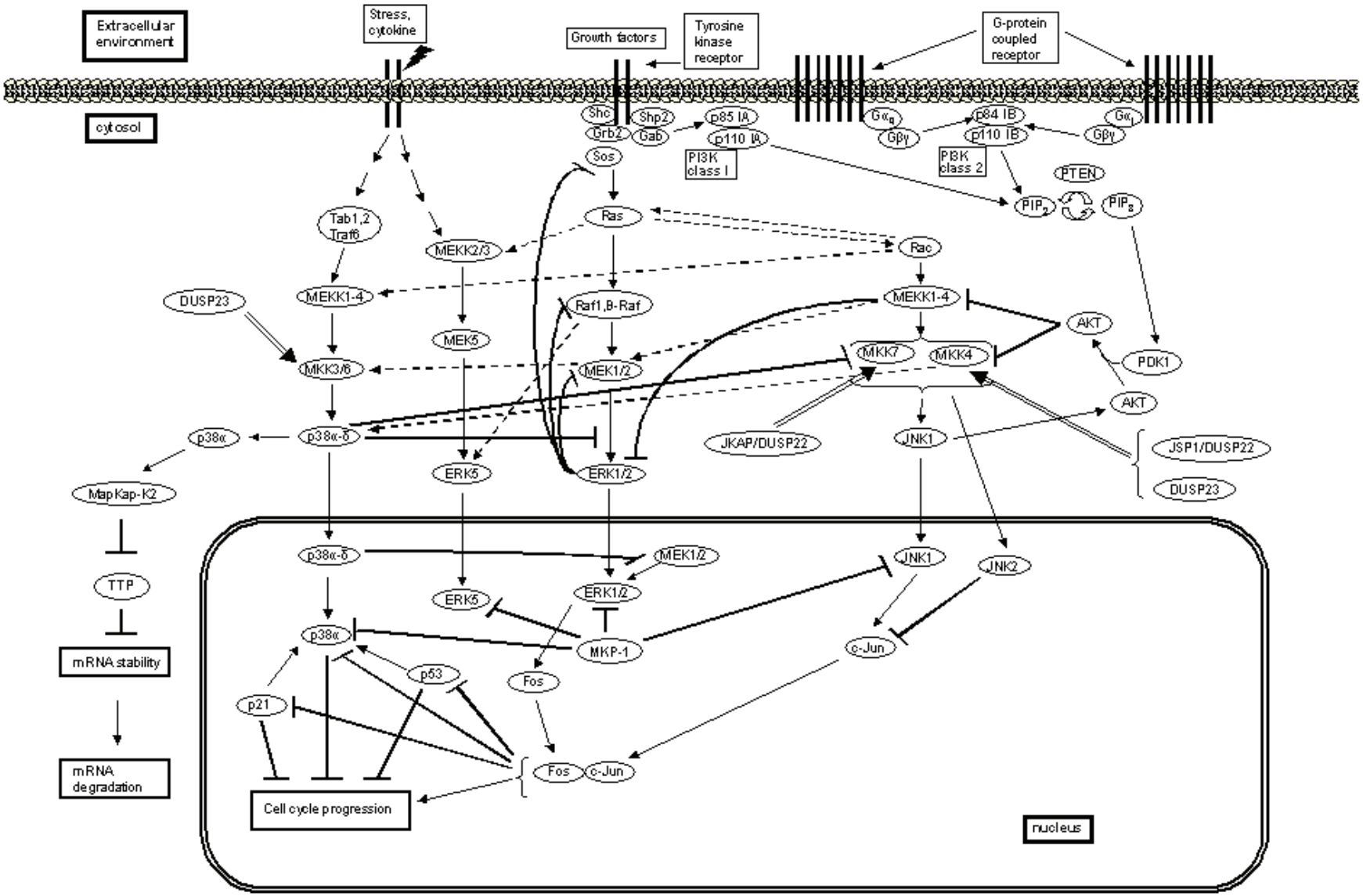

Extracellular environment
Stress, cytokine
Growth factors
Tyrosine kinase receptor
G-protein coupled receptor
cytosol
Shc
Grb2
Shp2
Gab
Sos
p85 IA
p110 IA
PI3K class I
Gαq
Gβγ
p84 IB
p110 IB
PI3K class 2
Gαi
Gβγ
PTEN
PIP2
PIP3
Tab1,2 Traf6
MEKK2/3
Ras
Rac
DUSP23
MEKK1-4
Raf1,B-Raf
MEKK1-4
AKT
MEK5
MKK7
MKK4
PDK1
MKK3/6
MEK1/2
AKT
p38α
p38α-δ
JKAP/DUSP22
JNK1
JSP1/DUSP22
ERK5
ERK1/2
DUSP23
MapKap-K2
p38α-δ
MEK1/2
JNK1
JNK2
TTP
ERK5
ERK1/2
p38α
MKP-1
c-Jun
mRNA stability
p53
Fos
p21
Fos
c-Jun
mRNA degradation
Cell cycle progression
nucleus

Figure 4. Crosstalk of the MAPK signal transduction pathway. Summary of the crosstalk and feedback inhibition implicating the MAPK signal transduction pathway. Solid single arrows indicate activating signal transduction within a module (except JNK→ Akt which is a priming phosphorylation). Hatched arrows indicate activating crosstalk between signalling modules. Solid double arrows indicate activating dephosphorylation by phosphatases (dual specificity phosphatases 22 and DUSP23). Solid lines-bars are inhibitory phosphorylation or dephosphorylation. Open arrows indicate cycling between two forms of PIP. Reproduced with kind permission form the American Society of Pharmacology and Experimental Therapeutics.

The mkp-1 mRNA expression is regulated by different stresses and given the number and different sources of stresses activated during the process of transplantation, the study of mkp-1 expression and function represents an interesting starting point. This is all the more true as MKP-1 protein targets JNK-1 and p38MAPK for dephosphorylation in preference to the ERKs. This aspect will be elaborated on in the first original contribution, which presents our findings on mkp-1 mRNA regulation and protein function. Our attention will now focus of heat hock proteins, as they have also been identified by microarray analysis as being up-regulated. In addition, we will also consider how HSP70 interacts with MKP-1.

2. Heat shock proteins 70 and 40 (HSP70 and HSP40)

a. HSP70 animal models: an overview. The chaperone function of the heat shock protein (HSP) family is well documented. This includes the control of other protein activity and their folding, among other functions (Bukau et al., 2006). Nevertheless, to get a better appreciation of HSP70 and its function, we will consider the information derived from knockout mice. There are three HSP70 genes with the following correlative match between species (rat HSP70.1 = mouse HSP70.1 = human HSP70.2; rat HSP70.2 = mouse HSP70.3 = human HSP70.1; and rat HSP70.3 = human HSP70-hom = mHSP70t) (Lee et al., 2001; Walter et al., 1994). Most HSP70.1 knockout mice are viable with no obvious phenotypic abnormalities during development, but are sensitized to osmotic stress when 3% NaCl is added to their drinking water, while the renal medulla reveals increased apoptosis (Shim et al., 2002). Another group also characterized HSP70.1-/- or HSP70.3-/- mice as viable and fertile with no obvious morphological abnormalities (Huang et al., 2001). A third group generated HSP70.1 and HSP70.3 double nulls were fertile and had normal development but a 12% decrease in weight when compared to wild-type littermates (Hunt et al., 2004). While HSP70 may not be absolutely required during development, conditions of stress are another matter.

There are many models that reveal the importance of inducible HSP70s (e.g. HSP70.1/HSP70.2/HSP70.3 depending on the species) in the mitigation of ischemia-reperfusion mediated damage. A case in point, HSP70.1-/- mice showed a significantly greater infarction volume and DNA fragmentation in their cortex when compared to WT-mice at 24h after 120 minutes of warm focal cerebral ischemia. Therefore, HSP70s (Giffard and Yenari, 2004) and more specifically HSP70.1 plays an important role in the early protection of the brain, at least after acute focal cerebral ischemia in mice (Lee et al., 2001; Lee et al., 2004). An HSP70 co-factor, HSP40 (Bukau et al., 2006) was also know to be up-regulated as a result of cerebral ischemia in mice, gerbils (Tanaka et al., 2002) and rats (Tamura et al., 2003).

b. HSP70 and surgery. It should be noted that HSP70s have been considered important in hepatic surgery (Yamamoto et al., 1998). In a retrospective analysis, low levels of heat shock protein 70 mRNA from human liver biopsy specimens and cells that slough off in the organ perfusate (perfusion solution used to equilibrate the Na^+ and K^+ prior to organ implantation into recipient) had been considered as an early marker of acute liver rejection after cold ischemic preservation. Furthermore, it was assumed that the higher levels of hsp70 mRNA, prior to reperfusion, were due to the

brain-dead donor prior to organ harvesting (Flohe et al., 1998). The HSP70 chaperone function could explain the clinical findings that human HSP70 increased expression reduced hepatic plasma transaminase (AST, ALT) levels of patients after warm ischemia reperfusion undergoing partial hepatectomy for localized lesions (Boeri et al., 2003). Conversely, plasma concentrations of HSP70 and amount of blood loss were considered prognostic markers of postoperative liver dysfunction after liver resection (Kimura et al., 2004).

c. Lack of HSP70 and DNA. Although the value of HSP70s has been revealed, thus far, to be important in different animal models and human ischemia-reperfusion related injuries, a final animal model will be detailed. As previous mentioned, HSP70.1/3 double null mice were fertile and had no overt phenotype save 12% reduction in weight when newborne (Hunt et al., 2004). However, a detailed investigation revealed interesting differences (Hunt et al., 2004): 1) MEFs isolated from HSP70.1/3 double null mice had longer doubling times than wild-type littermates; this difference may be caused by genomic instability; 2) a three-fold increase in the “spontaneous” chromosome and chromatid gaps and breaks and also during whole body heat shock, when age and sex-matched mice wild-type and HSP70.1/3 double null mice were compared; 3) 2.5-fold decrease in telomerase activity perhaps because of decreased telomerase stability (this is based on the observation that HSP70 normally binds to the catalytic subunit of telomerase in the absence of the template RNA component (Forsythe et al., 2001)); 4) increase end-to-end chromosome association in double knockouts when compared to wild-type; 5) decreased inhibition of DNA synthesis associated with radiation exposure; and 6) double null MEFs had decreased DNA repair capabilities in S phase when heat shock was followed by irradiation, when compared to wild-type MEFs. Finally, perhaps an important point for the present discussion is the observation that while HSP70.1 and HSP70.3 are expressed in many organs, the mouse liver only expresses HSP70.1 (Hunt et al., 2004). This aspect may be relevant in a liver that endeavours to replace damaged tissue due to ischemia and reperfusion, via cell proliferation.

d. HSP70 and MKP-1. Another aspect of HSP70 function is its transient binding to proteins involved in cell cycle such as: p53, Cdc4, p27/Kip1, cMyc among others (Hunt et al., 2004) including proteins not directly related to the cell cycle such as MKP-1. Within the context of MKP-1, it should be noted that a single heat shock treatment of IMR-90 human lung fibroblasts causes aggregation of MKP-1 protein in an HSP-72-dependent manner, preventing MKP-1 from denaturation and preserving its

function; this aggregation is partially reversed in the recovery phase from the heat shock treatment (Yaglom et al., 2003). A double heat shock treatment increases MKP-1 serine phosphorylation and activity via interaction with HSP70 in human bronchial epithelial cells (Lee et al., 2005). Considering that MKP-1 is being stabilized in its phosphorylated state, this stabilization may be the consequence of general chaperone function e.g., preventing MKP-1 from unfolding, or part of a mechanism designed to limit the deleterious effects of fever. In the context of ischemia-reperfusion, HSP70 could stabilize proteins such as MKP-1 that inactivate part of the MAPK-axis that concerns the stress response.

e. HSP70 and HSP40. Although not mentioned until now, HSP70 substrate binding ability is dependent on the presence of ATP, HSP40 and target substrate availability. It is the hydrolysis of ATP that drives the conformational change in the c-terminal part of HSP70 necessary to generate a substrate-binding domain. HSP40 increases the catalytic activity of the NH_2-terminal ATPase domain of HSP70 (Bukau and Horwich, 1998). The lack of ATP during stress may hinder substrate binding and therefore increase protein aggregation during stress. Taking some of these observations into account, lack of HSP70 expression not only explains the absence of late protection otherwise afforded by ischemic preconditioning (Hampton et al., 2003), as mentioned in chapter 2 section C5, but considering the aforementioned findings concerning HSP70.1/3 double null mice (Hunt et al., 2004), lack of HSP70 may also explain the hepatic dysfunction seen in patients, which may lead to dysfunction in 1-12 months or longer, the aetiologies of which are numerous (Hubscher, 2009). Animal models to be assayed are liver regeneration and transplantation in single HSP70.1 null and HSP70.1/3 double null mice; such a study, which would help determine the role of HSP70 in the regenerating liver, has not appeared as yet. Interestingly, our results reveal that both HSP70 and HSP40 increase during reperfusion.

3. The other heat shock proteins (HSPA1L, HSPH1, HSPCA, HSPA8 and HSP70B')

a. Overview. These heat shock proteins have been grouped together as there is very little relevant information available. A host of heat shock proteins have been previously identified in relation to gene expression mediated by hypoxia in HepG2 cells (Sonna et al., 2003). The latter study used cultured HepG2 cells in 1%O_2-5%CO_2,

hypoxia at 37ºC, for 24h and compared this to normal "room oxygen" (~21%O_2)-CO_2 at 37ºC (Sonna et al., 2003). Of the heat shock proteins identified in this study (Sonna et al., 2003), we identified HSPA1L (HSP70-like), HSPH1 (heat shock 105kDa/110kDa protein1; Entrez-Gene (http://www.ncbi.nlm.nih.gov/sites/entrez) nomenclature), HSPCA (HSP90 protein 1 alpha-like 3) and HSPA8 (HSP70 protein 8) as being up-regulated (Boutros et al., 2008b). However, HSPA1L was not regulated, while HSPA8 and HSPCA were down-regulated (Sonna et al., 2003). Although our results indicate that HSPH1 is up-regulated (Boutros et al., 2008b), the hypoxia study subdivided heat shock 105kDa and 110kDa protein1 into two categories; the first not significantly regulated and the latter significantly down-regulated (Sonna et al., 2003). For the rest of this section, the Entrez-Gene nomenclature will be used. The difference in our study and the hypoxia study are numerous, including whole organ versus cell line, reperfusion with all its variables such as, cold storage, heat shock, whole blood, blood pressure and manipulation stress, which come to mind. Nevertheless, the obvious absence of heat shock in the hypoxia paradigm may be a sufficient explanation to account for the differences between our results and those of Sonna et al. (2003).

b. Interaction between some of the HSPs. All is not so linear, however, as the interaction of HSP90 with HIF-1α (Minet et al., 1999) results in the stabilization of the latter protein via the PAS domain independently of oxygen tension, prolylhydoxylase action, the von Hippel-Lindau/ Elongin C/ Elongin B E3 ubiquitin ligase complex and the proteasome (Liu et al., 2007). This may account for the differences in basal levels of HIF-1α among different cell types (Liu et al., 2007). HIF-1α is one of many HSP90 "client" proteins, which includes transcription factors, kinases and cytoskeletal proteins, among others (Wegele et al., 2004). In addition, although chaperone machinery can function in parallel, observations suggest that they also function sequentially or coordinately, for example cellular complexes made of HSP70-Hop (Hsp organizing protein) -HSP90 are found, in the absence of client protein. Hop is one of the co-chaperones implicated in preparing steroid hormone receptors for bind to their ligands, which in the present example form HSP70-steroid receptor-Hop-HSP90 complex (Wegele et al., 2004). Binding of the steroid to the steroid hormone receptor, causes the latter to dimerize and become less amenable to bind HSP90. This is an oversimplification of the steroid hormone receptor cycle, which requires the presence of other proteins for activation (Wegele et al., 2004). This glimpse may represent a fraction of the potential list of client

proteins that require the formation of a complex to activate them, upon reperfusion of the human liver.

c. HSPs versus ATP and ADP. The HSPH1 (heat shock 105kDa/110kDa protein1) was relatively recently identified in humans (Ishihara et al., 1999) and was known to confer thermal resistance (Oh et al., 1997) analogously to other members of the HSP family. However, HSP105 is capable of preventing protein aggregation due to heat shock in the presence of ADP in contrast to ATP as required by HSP70 (Yamagishi et al., 2003). Although overexpression of HSP70 or stressful preconditioning can protect cells against ATP depletion, no mention of the contribution of HSP105 was made (Kabakov et al., 2002). This may be useful in the case of human liver transplantation, as ATP is depleted during the cold storage (~10h) and may not be completely replenished in the early phase of reperfusion. In a cell free assay or using rabbit reticulocyte lysates, HSP105 prevented heat (42ºC) mediated aggregation of luciferase but did not help the folding of protein and prevented HSC70/HSP40 mediated renaturation of luciferase (Yamagishi et al., 2000). Further results demonstrated that either wild-type HSC70 or HSC70K71A, incapable of binding ATP, was able to increase the ATPase activity of HSP105. This is all the more interesting as HSP105 alone has no ATPase activity and HSP105 decreases HSP70 ATPase activity, in the presence of potassium and without HSP40 (Yamagishi et al., 2004). As the molar ratio of HSP105 to HSP70 in the cell is approximately 1:10, and given the previously mentioned observations and the paucity of data concerning HSP105, it is difficult to interpret the role of this heat shock protein in human liver transplantation beyond what has already been mentioned.

d. HSPs and specific stress response. HSP70B' was originally identified, in 1990, as a stress-inducible novel member of the HSP70 family (Leung et al., 1990). HSP70B' is a more basic protein than the HSP70 and HSC70 family members. Interestingly, although human fibroblasts up-regulate HSP70 mRNA upon 42ºC, HSP70B' mRNA was not up-regulated at this temperature, but required 44-45ºC heat-shock treatment (Leung et al., 1990). If HSP70 expression is down-regulated (siRNA) prior to heat shock, HSP70B' protein is up-regulated in human colon carcinomas and primary human non-transformed colonocytes, while the complementary experiment siRNA to HSP70B' had no effect on levels of HSP70 (Noonan et al., 2007). In addition, cadmium chloride treatment led to the up-regulation of HSP70 mRNA but not to that of HSP70B' in fibroblasts (Leung et al., 1990), while $ZnSO_4$ did not up-regulate HSP70B' mRNA homogeneously across cell types (human colon carcinoma and primary human

non-transformed colonocyte cell lines) (Noonan et al., 2007). HSP70B' mRNA is also up-regulated upon proteasomal inhibition in human colon carcinoma and primary human non-transformed colonocyte cell lines, (Noonan et al., 2007). Furthermore, it should be noted that while HSP70B' mRNA expression is found in pigs, cows and humans, a rodent ortholog has not been found (Noonan et al., 2007), perhaps due to lack of annotation. Nevertheless, lack of HSP70B' should be taken into account as many models of ischemia-reperfusion and transplantation use rats and mice. Finally, 24h of hypoxia (1% O_2) treated HepG2 cells, with reoxygenation kept under 2min (the time it took to lyse cells to extract total RNA) revealed no increase in HSP70B' mRNA (Sonna et al., 2003). Our results revealed an up-regulation of the expression of this gene along with the other HSPs. This HSP may be modulated by different stimuli than the other members of its family. This point could be painstakingly verified using a slew of transplantation-relevant stimuli and determining which ones regulate this gene and protein.

4. Plasminogen activator inhibitor type-1 (PAI-1)

a. Overview. Plasminogen activator inhibitor type-1 is a serine proteinase inhibitor and targets both tissue-type and urokinase-type plasminogen activators, t-PA and u-PA respectively; both t-PA and u-PA cleave plasminogen into plasmin (Lijnen, 2005). In turn, the most important function of plasmin is the degradation of fibrin core of the thrombus, e.g. the intravascular thrombolysis (Novokhatny, 2008). Other functions of plasmin have included tissue remodelling, cell migration and inflammation (Novokhatny, 2008). Interestingly, in support of the fibrin degradation function of plasmin, human with PAI-1 deficiency have been identified. A case in point, the extended members of a family revealed 19 individuals that were heterozygous null for the PAI-1 gene without any unusual bleeding after trauma or surgery and 7 members that were homozygous PAI-1 null that had greater bleeding only after trauma or surgery (Fay et al., 1997). The authors concluded that the primary function of PAI-1 in vivo was to regulate vascular fibrinolysis (Fay et al., 1997). The lack of increased bleeding associated with decreased PAI-1 levels in patients was confirmed by others (Agren et al., 2006). Other patients with various degrees of PAI-1 deficiency have also been identified associated with varying degrees of blood clotting problems (Lee et al., 1993; Zhang et al., 2005). Nevertheless, it should be noted that some normal blood donors have low, about 30% of the normal PAI-1 activity (i.e. 70% deficient) in their plasma (Santamaria et al., 2007), while the levels of PAI-1

antigen is usually higher than the activity per se (Morimoto et al., 2004). Our results reveal an increased level of PAI-1 mRNA at the 1h post-reperfusion time-point.

b. PAI-1, transplantation and regulation. Conversely, high levels of PAI-1 can promote microthrombi, which may lead to multiorgan failure (Vergouwen et al., 2004). Although the pro- and anti-coagulatory factors return to normal after liver transplantation for the intrinsic and extrinsic coagulatory pathways (Velasco et al., 1992), as PAI-1 is partially derived from the hepatocytes, platelets and endothelial cells among other cell types (Lijnen, 2005; Tsantes et al., 2008), it remains possible that high levels of PAI-1 post-transplant could lead to microthrombi. In keeping with this line of thought, the 4G allele is associated with increased ischemia after aneurysmal subarachnoid haemorrhage (Vergouwen et al., 2004). The 4G/5G tract polymorphism (one G-C couple is deleted, transforming a 5G-C tract into a 4G-C tract) located within the pai-1 promoter causes an increase in PAI-1 activity in the plasma (Eriksson et al., 1995). Although the following topics will not be covered in detail, it is noteworthy that the 4G/4G polymorphism is associated with various diseases/conditions, such as susceptibility to sepsis (Hermans et al., 1999; Menges et al., 2001; Westendorp et al., 1999), multiorgan dysfunction due to severe trauma (Menges et al., 2001) and myocardial infarction (Eriksson et al., 1995). The exact mechanism that linked reduced fibrinolysis, due to 4G/4G polymorphism, increased inflammatory cytokines (IL-1 and TNF-α) increased susceptibility to sepsis and multiorgan dysfunction was not known (Menges et al., 2001). Notably, LPS (endotoxin) administered to normal volunteers increased expression of tissue-type plasminogen activator (t-PA) antigen from 10ng/mL to 52ng/mL in 3h and PAI-1 antigen rose from 7U/mL to 49 U/mL in 5h, while t-PA activity increased form 1.2ng/mL at baseline to 13.9ng/mL in 2h but was undetectable at 3h (Suffredini et al., 1989). It was believed that t-PA could prevent thrombosis and then t-PA would be controlled by PAI-1 (Suffredini et al., 1989). In the context of transplantation, only the human kidney transplant recipient survival was assessed. In summary, PAI-1 promoter 4G/5G polymorphism in the donor or recipient did not correlate with recipient survival. However, donor, but not recipient, PAI-1 promoter 4G/5G polymorphism influenced kidney graft survival in the patient category that suffered form graft failure due to interstitial fibrosis and tubular atrophy (Rerolle et al., 2008).

c. PAI-1 and species differences. Some of the phenotypes seen in human PAI-1 4G/5G promoter polymorphism have been replicated in mice. A brief overview of these phenotypes is now undertaken. Data obtained from PAI-1 null mice in include:

normal fertility, normal macroscopic and microscopic histology (Carmeliet et al., 1993a) and lack of spontaneous bleeding upon partial tail amputation or appendectomy (Carmeliet et al., 1993b). The murine model of hepatic regeneration after Fas-mediated apoptosis, using an agonistic anti-Fas antibody intraperitoneally injected into wild-type and PAI-1 null mice, revealed the same percentage of death in both wild-type and PAI-1 null mice. However, both hepatocyte growth factor and proliferating nuclear cell antigen appeared 24 h earlier in PAI-1 null mice compared to wild-type controls (Shimizu et al., 2001). Others have noted that the human hepatoma HepG2 cell line can express PAI-1 under 1-2% O_2 hypoxic (but not 8% O_2) conditions (Fink et al., 2002) and rat hepatocytes express PAI-1 under 8% O_2 (Kietzmann et al., 1999). In addition, the extracellular matrix of HepG2 cells, more specifically vitronectin, binds PAI-1 (Owensby et al., 1991). Although liver regeneration is especially important for either living donor liver transplantation or brain dead split-liver transplantation (as only a hepatic lobe is used), even whole liver may require some regeneration to replace dead or dying tissue. It would be valuable to determine how PAI-1 affects human liver regeneration or survival, via a 4G/5G promoter polymorphism prospective study. Would liver transplant patients benefit from a temporary decrease in PAI-1 activity?

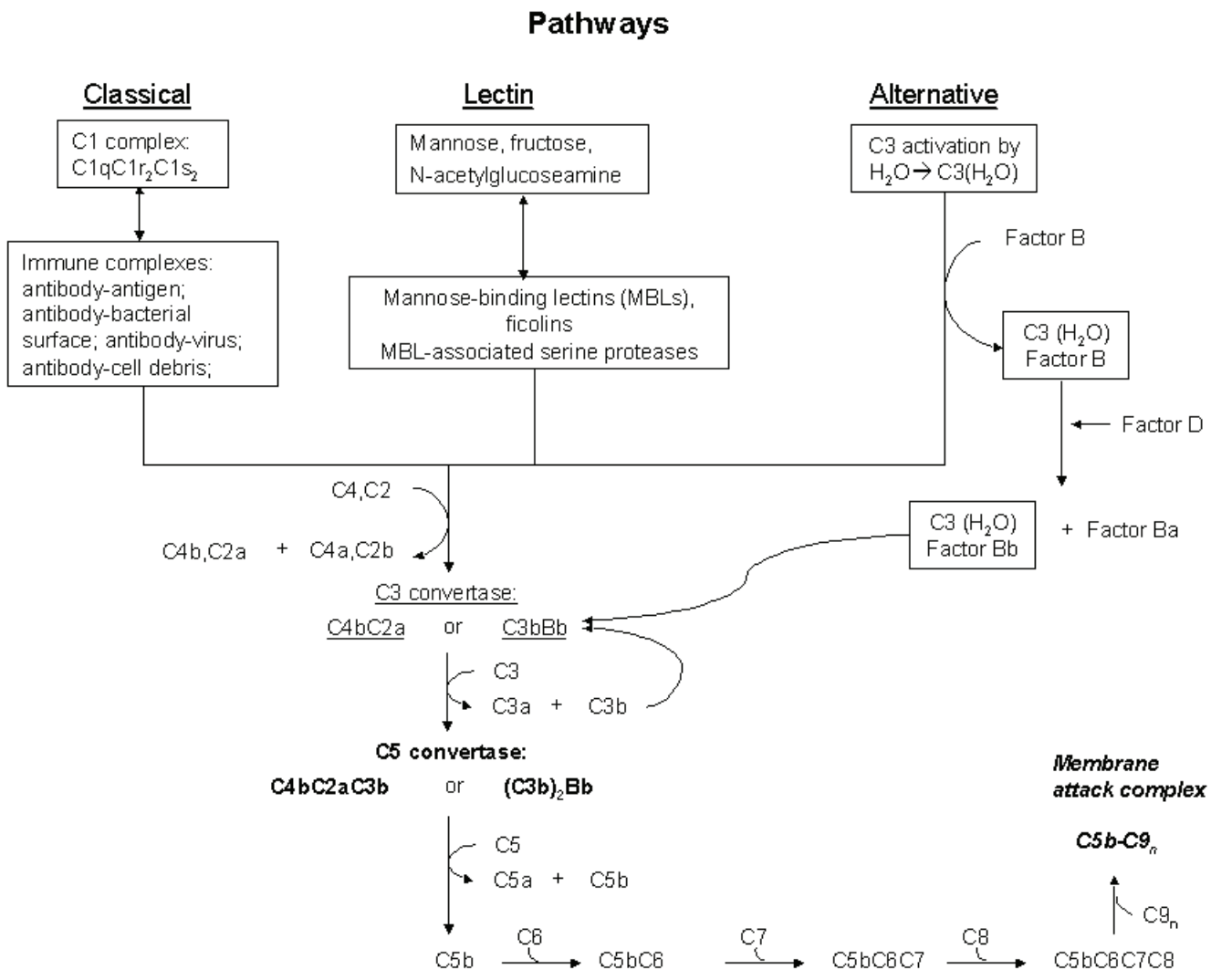
There is still much to be learned as to how PAI-1 affects liver and other organ physiology. As with other molecules, the effects of PAI-1 depend on level of expression, activity, timing, localization, and the presence of other molecules. In spite of the paucity of information where human organ transplantation is concerned, it is easy to understand that blood clotting at the proper time and place is essential during the process of transplantation, while excess PAI-1 can result in microthrombi which can be detrimental to blood circulation in the capillary system within organs such as the brain and liver among others.

5. Complement component 5 (C5)

a. A brief overview of the complement component system. There are three pathways that can activate the complement component system. The first two will be immediately enumerated: 1) the classical pathway characterized by the activation of complement component 1 complex, more specifically initiated by the recognition of antigen-antibody on cell surfaces or antibody and another surface such as, bacterial surfaces, viruses, C-reactive protein and cell debris (Fosbrink et al., 2005) by the Cq1 component of the Cq1-$C1r_2$-$C1s_2$ complex; and 2) the lectin pathway initiated by

microbial sugars such as mannose, fructose and N-acetylglucosamine, which bind either mannose binding lectins (MBL), especially MBL2 the others have not been proven to be involved, or ficolins, which in turn bind one of mannose binding lectins (MBL)-associated serine proteases (MASP) (Figure 5). Both the classical and the lectin pathways cleave complement component 4 (C4) into the anaphylatoxin C4a and the major fragment C4b. This latter fragment binds to C2 and one of three complexes, C1, MBL-MASP or ficolins-MASP cleave C2 when it is bound to C4b into C2a and C2b in a magnesium dependent manner. C4bC2a remain attached to one another and form the C3 convertase cleaving C3 into C3a and C3b. The latter fragment binds to the C3 convertase, forming a C4bC2aC3b complex known as a C5 convertase. The cycle of cleavage repeats with this latter convertase proteolytically activating C5 into C5a and C5b. The complement component C5b binds to C6-C8 and up to 16 copies of C9 to form a pore, the membrane attack complex (MAP), which causes the lysis of cells. The third pathway termed "alternative" proceeds in a similar manner except that C3 is not cleaved into C3a and C3b, rather C3 changes its conformation irreversibly, at a very low rate, to C3(H_2O). Both C3b and C3(H_2O) can bind to factor B. In the context of the C3(H_2O)factor B complex, factor D can cleave factor B (into Bb and Ba fragments) and create a C3bBb; the C3 convertase. Another C3b fragment can bind to C3 convertase to generate a $(C3b)_2Bb$ complex, a C5 convertase which proceeds as previously described to form the membrane attack complex (Gros et al., 2008).

Figure 5. Schematic representation of the complement component cascade. The activation cascade reveals not only the classical, the lectin and the alternative pathways, but also the points in the cascade where they overlap. Notice that there are two C3 convertases and C5 convertases. $C9_n$ is the complement component 9, where n is any number from 1-16. See text for complete detail.


Pathways
Classical
Lectin
Alternative
C1 complex: C1qC1r2C1s2
Mannose, fructose, N-acetylglucoseamine
C3 activation by H2O→ C3(H2O)
Immune complexes: antibody-antigen; antibody-bacterial surface; antibody-virus; antibody-cell debris;
Mannose-binding lectins (MBLs), ficolins
MBL-associated serine proteases
Factor B
C3 (H2O) Factor B
Factor D
C4,C2
C4b,C2a + C4a,C2b
C3 (H2O) Factor Bb
+ Factor Ba
C3 convertase:
C4bC2a or C3bBb
C3
C3a + C3b
C5 convertase:
C4bC2aC3b or (C3b)2Bb
C5
C5a + C5b
Membrane attack complex
C5b-C9n
C9n
C5b
C6
C5bC6
C7
C5bC6C7
C8
C5bC6C7C8

b. The complement system and the University of Wisconsin solution. At present, it is important to mention how the previous cascade of proteins fits into the context of transplantation. The perfusion and preservation solution UW contains among its components $MgSO_4$ (5mM) used as an electrolyte and membrane preservation and raffinose (30mM), an impermeable osmotic agent, to provide additional osmotic support and prevent cell swelling (see Table 1 chapter 2 section B4, p.53-58). The first observation is related to the complexes, C1, MBL-MASP or ficolins-MASP requiring magnesium to cleave C2, when bound to C4b, into C2a and C2b. The second observation lies in the composition of raffinose. This trisaccharide is composed of D-galactose, D-glucose and D-fructose (Streitwieser A, 1985). The third is lactobionic acid (oxidized lactose), which is composed of galactose and gluconic acid (Tomlinson et al., 1978) and a component of UW solution at a concentration of 100mM (see Table 1 chapter 2 section B4, p.53-58). Three comments should be mentioned about these monosaccharides: 1) fructose has the potential to bind lectins/ficolins, which in turn bind MASP as part of the lectin pathway of complement activation, see above text; 2) galactose-terminating glycoproteins can bind to receptors on hepatocytes and be endocytosed (Lodish, 1991; Stockert and Morell, 1983) for example, asialoglycoprotein receptor can bind single galactose residue with low affinity ~1mM (Lee et al., 1983; Lodish, 1991) found on HepG2 cells (Bischoff and Lodish, 1987); 3) serum ficolins are synthesized by the liver and cell-associated ficolins are predominantly expressed on phagocytic cells (such as Küpffer cells) (Garred et al., 2009; Runza et al., 2008); 4) mannose-binding lectin 2 is synthesized in the liver (Garred et al., 2009); and 5) the mannose binding lectin staining was associated with ischemia-reperfusion related damage in human kidney transplantation and primary non-function of the graft. This study used a machine perfusion as a method of preservation; unfortunately the identity of the perfusion solution was not mentioned (de Vries et al., 2004).

c. C5 Complement component. As the function of C5 lies in its component parts, namely C5a and C5b, their function will be overviewed. The C5a receptor is expressed in many tissues including hepatocytes, hepatic stellate cells, Küpffer cells, the HepG2 cell line, T-cells, B-cells, mast cells, smooth muscle cells and endothelial cells among other (Monk et al., 2007) and human liver (Haviland et al., 1995). As such C5a component has been implicated in smooth muscle contraction, enhanced vascular permeability, increased expression of adhesion molecules, increased chemotaxis of inflammatory cells, increased oxidative burst in neutrophils (Guo and Ward, 2005; Monk

et al., 2007), and decreased production of inflammatory cytokines (IL-12, IL-23, IL-27) (Hawlisch et al., 2005). Although the mRNA for subunits specific to IL-12 and IL-23 were up-regulated during ischemia and reperfusion of the mouse liver, only IL-23 induced Küpffer production of TNF-α (Husted et al., 2006). Unfortunately, cytokines as many other proteins have pleiotropic effects, and IL-12, IL-23 and IL-27 are no exception. These cytokines have been implicated in animal models of immunological acceptance and rejection (Goriely and Goldman, 2007; 2008), while IL-27 exacerbates a mouse model of sepsis (Neurath, 2007; Wirtz et al., 2006).

All the previously mentioned functions of C5a reveal an immunological axis to this component. However, C5a is also involved in liver regeneration, as revealed when C5 deficient mice are reconstituted with C5a or C5 in a chemical injury model (Mastellos et al., 2001) or when mice are reconstituted with C5a in a partial hepatectomy injury model (Strey et al., 2003). Conversely, rat and mouse models of warm ischemia-reperfusion of the kidney, liver, intestine, heart and limb results in C3a, C5a activation and formation of the membrane attack complex, C5b-9_n where n is any number (1-16) of copies of the complement component 9. These models benefit from anti-C5aR and inhibition of C5b-9_n complex formation via antibody and mice that are deletion mutants in the one of the components of the components of the complement components also revealed reduced injury (Arumugam et al., 2004a; Arumugam et al., 2004b; Austen et al., 1999; De Vries et al., 2003; Guo and Ward, 2005; Heijnen et al., 2006; Kyriakides et al., 1999; Vakeva et al., 1998; Wada et al., 2001; Zhang et al., 2007; Zhou et al., 2000). In a rat model of liver transplantation using cold ischemia-warm reperfusion, the membrane attack complex was causative in inducing liver ischemia-reperfusion related injury (Fondevila et al., 2008).

d. C5, the membrane attack complex and liver transplantation. In the next paragraphs an overview of the involvement of the complement component system, with a special emphasis on complement components, C5 and the C5b-9_n, membrane attack complex (MAC), in patients during the process of transplantation. Patients that are deficient in C5 to varying degrees will also be addressed. The efficacy of the C5b-9_n complex to initiate lysis depends on many factors, including cell surface inhibitors, and the nature of the target membrane, such as bacteria, virus envelops, anucleated cells (platelets, red blood cells) and nucleated cells (Niculescu and Rus, 2001). The distinction between such categories comes into its own when we consider that nucleated cells can

escape the lytic function of a limited number of MAC on the cell surface by endocytosis and exocytosis of this complex (Niculescu and Rus, 2001).

The membrane attack complex, C5b-9_n, increased by 2-fold in blood samples obtained from patients at 5 minutes post-hepatic reperfusion as compared to 5 minutes pre-reperfusion but returned to normal at 1h post-reperfusion (Bellamy et al., 2004). Another study found a four fold increase of MAC in patients at 2h post-hepatic reperfusion (the results of this study were detailed in the cold stress and heat shock, chapter 2 section C3 (Schmidt et al., 2004). Perhaps the discrepancy is due to different time-points, 2h versus 1h post-reperfusion, giving the liver a chance to synthesize more proteins of the complement component system. However, it is very unlikely that the increase in MAC at the 5 min time-point (Bellamy et al., 2004) is the result of new protein synthesis, but rather the release of pre-existing protein from the graft or possibly a systemic reaction from the recipient to the new liver caused by the simultaneous hemodynamic changes (a reduced systemic vascular resistance and an increase in cardiac output), which occur simultaneously in patients (Bellamy et al., 2004).

In regards to the first possibility, the MAC complex had been localized to extracellular deposits, in the connective tissue of the portal tracts (the connective tissue that groups the hepatic portal vein, hepatic artery and the bile duct) (Polihronis et al., 1993; Scoazec et al., 1997), along with soluble inhibitors clusterin and vitronectin in preoperative and control human liver biopsy specimens (Scoazec et al., 1997). All post-operative staining was done on biopsy specimens obtained immediately after reperfusion. The percentage of hepatocytes staining for MAC deposition correlated positively with neutrophil and platelet accumulation in the liver and levels of AST in serum, an indicator of necrotic damage, but negatively with serum levels of the coagulation factor V (accelerin), an indicator of de novo protein synthesis (Scoazec et al., 1997). In line with these results MAC deposition had been correlated with hepatocyte necrosis in hepatitis patients (Pham et al., 1995). While plasma levels of C5b-9_n (MAC) complex continues to rise in the blood (measured in plasma) for the first hour post-reperfusion, which was the last time-point (Tomasdottir et al., 1993) and decreased 6-12h after reperfusion, although remained higher than preoperative values (Ronholm et al., 1994). Interestingly, levels of serum C5a did not fluctuate between preoperative and post-operative values (Ronholm et al., 1994; Tomasdottir et al., 1993). This may be due to binding of C5a to leukocytes (Ronholm et al., 1994). As to how systemic changes result in the activation of complement component proteins by the recipient, as opposed

to the activation by the grafted liver per se, this may be due to clamping of the portal and inferior caval veins, during the anhepatic phase of the operation (old liver gone, new liver not yet connected in the recipient), resulting in hypoperfusion of the gut and translocation of bacterial endotoxin, generation of reactive oxygen species or formation of immune complexes and injured endothelial cells (Ronholm et al., 1994).

e. C5, the membrane attack complex and signal transduction. All the previously mentioned studies speak of an involvement of the complement component C5b in the formation of the membrane attack complex C5b-9_n, which is presumed to lead to cell lysis. Although this complex does indeed form a pore that ultimately results in cellular destruction, the C5b-9_n complex also functions in signal transduction (Fosbrink et al., 2005). To understand the involvement of C5b-9_n complex, it is important to revise the process of C5b-9_n assembly. Once C5 is cleaved into C5a and C5b, this latter fragment binds to a C6 molecule, allowing reversible binding of C5b-6 to the plasma membrane. C7 binds to C5 and creates C5b6-7 (C5b-7) complex allowing interaction with the phospholipids of the plasma membrane. At this stage of complex formation, C5b-7 monomer or its dimer (DiScipio et al., 1988) can stimulate the formation of cAMP and other second messengers diacylglycerol and ceramide (Niculescu and Rus, 2001) but neither form pores nor do they have cytolytic activity (Fosbrink et al., 2005). C8 and C9 bind to C5b-7 monomer or dimer complex (DiScipio et al., 1988), increasing the amount of secondary messengers (Niculescu and Rus, 2001). Up to 16 C9 monomers can bind C5b-8, which forms a 0.4-3nm pore and lyse cells when found in large number on the plasma membrane even in the absence of C9 (Fosbrink et al., 2005). The C9 component binds to C5b-8 forming a C5b-9_n pore complex, that has a channel diameter of 1-11nm depending on the number of C9 molecules in the C5b-9_n complex (Niculescu and Rus, 2001).

The C5b-8 complex can activate cAMP, while C5b-9_n activates PKC. Interestingly, C5b-8 is less effective than the MAC complex at activating PKC, and C5b-7 cannot mediate such an activation (Carney et al., 1990). C5b-9_n also releases growth factors, such as basic fibroblast growth factor and platelet derived growth factor (Niculescu and Rus, 2001) and C5b-9_n induces release IGF-I from smooth muscle cells (Fosbrink et al., 2005). C5b-9_n, but not C5b-7, increases DNA synthesis (Niculescu et al., 1997), and also increases proliferation and migration in human aortic endothelial cells via an AKT-mediated mechanism (Fosbrink et al., 2009; Fosbrink et al., 2006). C5b-7 and

C5b-9_n increase ERK1 activation with different kinetics, 10min and 2 min respectively, while C5b-6 does not mediate this activation in a human B lymphoblastic cell line (Niculescu et al., 1997). In addition, C5b-9_n activates JNK1 and p38MAPK in human aortic smooth muscle cells (Fosbrink et al., 2005). Human aortic smooth muscle cells proliferate via Raf1-ERK1, in response to C5b-9_n (Niculescu et al., 1999).

f. Complement C5 deficiency in human. Several C5-deficient families have been reported (Aguilar-Ramirez et al., 2009; Delgado-Cervino et al., 2005; Peter et al., 1981; Rosenfeld et al., 1976a; Rosenfeld et al., 1976b; Sanal et al., 1992; Snyderman et al., 1979; Wang et al., 1995). In general, sera from homozygous C5-deficient patients have been noted with the following characteristics: 1) lack of neutrophil chemotactic activity (attributed to C5a) in the presence of immune complex, aggregated IgG, or LPS; 2) lack of microbial cytolytic activity, which is attributed to C5b, as part of the C5b-9_n complex, see above text (Rosenfeld et al., 1976a; Rosenfeld et al., 1976b; Snyderman et al., 1979). Interestingly, there is no direct correlation between C5 deficiency and symptoms. A patient lacking detectible C5 activity (<0.01%) suffered from systemic lupus erythematosus, while her half-sister (1-2% of normal C5 activity) was reported to be healthy (Rosenfeld et al., 1976a; Rosenfeld et al., 1976b). In an unrelated family, a patient that lacked C5 activity in her serum (less that 0.5% of normal) had chronic disseminated gonococcal infection (Neisseria gonorrhoeae), while her twin enjoyed “normal health” with the same level of C5 activity in her serum (Snyderman et al., 1979). Although sera from both twins can lyse bacteria, they could not cause chemotactic activity for normal human leukocytes, via classical or alternative complement pathways using IgG or cobra venom respectively. Conversely, it should be noted that in vivo, leukocytes eventually accumulated at sites of inflammation in the “diseased” sibling (Snyderman et al., 1979).

Since one can have a “normal phenotype” and have low levels of C5, and as this complement component has all the previously mentioned functions, and the complement system can have noxious effects, it may be eventually possible to monitor the levels of C5b-9_n complex and deplete the complement component system (Trendelenburg, 2007), until innocuous or even beneficial levels are attained especially during the inflammatory period that accompanies major surgery resolves. Indeed, our results demonstrate that the C5 mRNA is slightly down-regulated upon reperfusion (Boutros et al. unpublished

observations), which seem to go hand in hand with the premise that “a little C5 goes a long way”.

Thus far we have seen proteins of many different families phosphatases, chaperones, coagulatory and innate immune system with the potential for interaction at multiple levels. However, not all are so easily slotted. So without further ado, the Ras homologue gene family, RhoB is presented.

6. Ras homolog gene family, member B (RhoB)

a. RhoB function. As the name implies, this protein is a member of the Ras superfamily and Rho subfamily of GTPases (Wennerberg et al., 2005). RhoB mRNA is ubiquitously expressed and is regulated as a function of age in skeletal muscle and lung tissue, but remains unchanged in liver, brain and kidney as mice age. This age-related modification is based on accumulation of histone modification, more specifically histone methylation, which down-regulates gene expression (Yoon et al., 2007). Interestingly, RhoB can regulate its own expression at the transcriptional level (Fritz and Kaina, 1997). The RhoB protein has been implicated in many other functions, among which: 1) protecting cells from genotoxic stress (Fritz and Kaina, 2001; Fritz et al., 1995; Westmark et al., 2005); 2) regulating epidermal growth factor receptor trafficking (Gampel et al., 1999); 3) recruiting the AKT kinase PDK1, which is downstream of PI3K, to endosomes (Flynn et al., 2000); 4) recruiting Dia 1 (Diaphanous related forming) to endocytic vesicles (Fernandez-Borja et al., 2005); 5) trafficking of PDGFR-β to recycling endosomes and late endosomes in smooth muscle cells (Huang et al., 2007); 6) targeting of AKT and Src to the nucleus and activating ERK pathway (Huang et al., 2007); 7) In freshly plated MEFs that were RhoB null, targeting Src to the periphery and activation of Src (adhesion induced activation) was detected in 20% of cells as compared to 80% of MEFs expressing wild-type RhoB. Src is recruited to RhoB containing late-endosomes via a Rab11 dependent mechanism requiring polymerized actin (Sandilands et al., 2004); 8) controlling levels of c-Myc expression, by targeting GSK-3β to the nucleus, which would otherwise phosphorylate c-Myc and target it for polyubiquitination and proteasomal degradation (Huang et al., 2006); and 9) RhoB null bone marrow macrophages have reduced expression of integrins αVβ2 and αVβ3 at their cell surface, reduced attachment to ICAM-1 and vitronectin respectively, and reduced cell spreading on artificial substrata (Wheeler and Ridley, 2007). Our results reveal an increased expression of RhoB mRNA.

b. RhoB regulation. Based on these findings, RhoB is clearly involved in the targeting of proteins involved in signal transduction, cell survival and proliferation. In the event of cell damage or in assuring cell survival during the process of transplantation, RhoB can have a multitude of positive functions on a host of cells. The function of RhoB can be modulated by post-translational modifications. Some of these modifications impact on RhoB function in the following way: 1) RhoB is prenylated, which decreases its stability (Stamatakis et al., 2002); 2) of the two forms of prenylation, farnesylated RhoB is associated with antiapoptotic functions, prevents cell cycle arrest and actin cytoskeletal disruption (Allal et al., 2002; Prendergast, 2001), while geranylgeranylated RhoB suppresses Ras-mediated transformation and is proapoptotic (Du et al., 1999; Liu et al., 2000a; Liu and Prendergast, 2000; Mazieres et al., 2005; Prendergast, 2001); and 3) palmytoylation of RhoB is also required for is anti-transforming and proapoptotic functions (Wang and Sebti, 2005).

c. RhoB and stress. Surprisingly, there is comparatively little that is known about the role of RhoB in transplantation. In cultures of human glioblastoma cell line U87, ROS derived from NADPH oxidase increased level of GTP-RhoB under hypoxic conditions (1% O_2) and this increase was reversed by an NADPH oxidase inhibitor diphenyllene iodonium. In turn, GTP-RhoB increased active AKT leading to increased Ser-9 phosphorylation of GSK-3β, inactivating the latter. These hypoxic conditions lead to decreasing Tyr216 phosphorylation of GSK-3β, which is an activating phosphorylation. Ultimately, inactivation of GSK-3β leads to stabilization of HIF-1α (Skuli et al., 2006). In a human glioblastoma xenograft experiment, dominant negative conditional expression of RhoB (RhoBN19) caused cells to migrate along blood vessels. In this model, not only did RhoBN19 decrease the size and density of the blood vessels in the tumour, but also decreased MMP2 activity, while MMP9 was not mentioned (Ader et al., 2003).

In deceased patients that suffered from stroke, whose clinical history was known, a significant lesional up-regulation of RhoA and RhoB was observed beginning 2 to 10 days after ischemia and continuing for 4 to 38 months after focal cerebral infarction when compared to control brains. The cellular sources of both molecules included polymorphonuclear granulocytes, monocytes/macrophages and reactive astrocytes (Brabeck et al., 2003). The authors believed that the expression of these RhoGTPases prevented neuronal survival, increased production of inflammatory cytokines, TNF-α, mediated monocytes/macrophages recruitment among other negative effects based on scientific literature of RhoA and RhoB implicating these RhoGTPases in model systems

used to study these events and not to direct experimental evidence (Brabeck et al., 2003). In a mouse model of cerebral infarction, transient ischemia for 1h, followed by reperfusion for up to 24h, heralded the expression of RhoB mRNA and protein and preceded DNA single-strand breaks in neurons. More specifically from 1h to 24h post-reperfusion, RhoB protein was seen in the region that suffered ischemia; these regions had actin cytoskeletal remodelling (Trapp et al., 2001). What was missing form the data in human and mouse model was cause and effect, which may be more amenable in animal models than in human.

Although transcriptional regulators are important, other proteins are required to re-establish hepatic homeostasis. In the following section, we will overview one such molecule.

7. Insulin-like growth factor binding protein 1 (IGFBP1)

a. Overview. The human IGFBP1 cDNA was first characterized in 1988 (Brinkman et al., 1988) and since then five other IGFBPs have been identified, IGFBP2-6. IGFBPs bind to IGF1 and IGF2 (insulin-like growth factors) to mediate their bioavailability (Rajpathak et al., 2009). The focus will be on IGFBP1, however, which is mostly synthesized by the human liver and kidney (Brinkman et al., 1988), and more specifically found in human hepatocytes (Arany et al., 1994; Scharf et al., 1995) and the human hepatoma cell line, HepG2 (Ren et al., 1992) and binds both IGF1 and IGF2 with nearly equal affinity (Clemmons and Underwood, 1991). Despite the observation that IGFs have pleiotropic effects during development and after birth (Butler and LeRoith, 2001) IGF receptors are almost absent from the liver (Dupont and LeRoith, 2001). Therefore if the effect of IGFBP1 on the liver is partially mediated through the IGFs it is unlikely through the binding of IGF1 or IGF2 to IGF receptors even though the liver is the most important source of IGFs (O'Dell and Day, 1998). Interestingly, IGFBP1 can bind to $\alpha_5\beta_1$ integrin receptor increasing migration of Chinese hamster ovary cells (Jones et al., 1993) and $\alpha_5\beta_1$ integrin receptor is found on: 1) hepatocytes and linked to directional motility in vitro (Biname et al., 2008); and 2) hepatic stellate cells (HSC) and related to the collagen type I production in early passage-freshly isolated HSC but not in fully activated later passage HSC (Dodig et al., 2007). Although the liver is known to regulate the adhesion molecules and their receptors during pathogenesis (Jaeschke, 1997), how $\alpha_5\beta_1$ integrin receptor may be relevant to transplantation biology needs to be elucidated.

b. IGFBP1, IGF1 and insulin. While IGFBP1 only treatment increases blood glucose levels, IGF1 decreases blood glucose levels in the rat and if a combination of both IGF1 and IGFBP1 is used, IGFBP1 prevents IGF1-mediated decrease of blood glucose levels (Lewitt et al., 1991). Indeed, endogenous levels IGFBP1 are inversely proportional to IGF1 and insulin levels (Rajpathak et al., 2009). Insulin also increases hepatic-derived, and perhaps adipocyte-derived, IGF1 levels in the blood. Given this relationship of IGFBP1, IGF1 and insulin, IGFBP1, was believed to be implicated in adult obesity, type II diabetes and insulin resistance, although no clear picture has as yet emerged as insufficient data prevent the establishment between cause and effect between levels of IGFBP1 and risk to type II diabetes (Rajpathak et al., 2009). Indeed, IGFBP1 had been known to regulate the efflux of IGF1 from the vascular space to the tissue, in a rat cardiac perfusion model, in an insulin-dependent fashion (Bar et al., 1990). As previously stated, insulin decreased IGFBP1 mRNA expression levels, while other factors increased IGFBP1 levels such as glucocorticoids (Kelley et al., 1996), fasting or protein free diet (e.g. starch feeding) in hepatic parenchymal and non-parenchymal cells (Takenaka et al., 1993). IGFBP1 levels also fluctuated according to circadian rhythm (Clemmons and Underwood, 1991). The identity of the non-parenchymal cells was neither identified nor was it a pure population (Takenaka et al., 1993), rather it was a mixed population based on a previous extraction method (van Berkel and van Tol, 1978).

c. IGFBP1 and relevance to transplantation. In regards to the process of transplantation, IGFBP1 is up-regulated during the reperfusion phase of the operation in rats, especially when insulin is omitted from UW solution (Li et al., 2004) and the UW used for human liver transplantation is also devoid of insulin (Boutros et al., 2008b). In addition, hypoxia (1% or 2% O_2) (Seferovic et al., 2009; Sugawara et al., 2000) or leucine deprivation (Seferovic et al., 2009), an essential amino acid, also increased the phosphorylation status of IGFBP1 at multiple sites in HepG2 cells. Interestingly, the highly phosphorylated forms of IGFBP1, at least two, had greater binding affinity to IGF1, 100-1000 fold, and resulted in decreased proliferation of HepG2 cells in vitro in the presence of IGF1 (Seferovic et al., 2009). In patients with liver disease, serum levels of IGFBP1 and insulin were increased prior to transplantation and these levels returned to normal after transplantation. Insulin resistance may have played a role in increased serum levels of IGFBP1 (Weber et al., 2002). In addition to these findings, ER-stress was not only associated with human liver transplantation, our laboratory's results

(Emadali et al., 2005), but also responsible for up-regulation of IGFBP1 at the mRNA, and protein level, via the transcription factor ATF4 in primary human hepatocytes and HepG2 cells (Marchand et al., 2006). Interestingly, both intracellular and extracellular (conditioned media) levels of IGFBP1 protein were increased by chemically induced ER-stressors (Marchand et al., 2006). ATF4 is selectively transcribed and translated as a result of signalling via double-stranded RNA-activated protein kinase-like ER kinase (PERK)/eukaryotic initiation factor-2 α-subunit (eIF2α) mediated mechanism. Indeed, cell stress causes PERK activation, which phosphorylates eIF2α and increases the translation of ATF4 among other factors (Dever, 2002). Notably, ATF4 null mouse embryonic fibroblasts require non-essential amino acid supplementation as they are deficient in an array of genes involved in amino acid transporter and metabolism and these cells also have a paucity of reducing equivalents and also require cysteine, N-acetylcysteine, or glutathione supplementation (β-mercaptoethanol and DTT also rescue such cells) or iron chelation (Harding et al., 2003). Interestingly, IGFBP2 but not IGFBP1 was identified as a gene that was down-regulated in ATF4 null mouse embryonic fibroblasts (Harding et al., 2003). Perhaps the differences in ATF4 involvement in IGFBP1 regulation comparing (Marchand et al., 2006) and (Harding et al., 2003) may be due to cell-type and/or species differences as both studies use tunicamycin as an ER-relevant stressor. Although mechanisms involving ER stress are conserved (Dever, 2002), the results using mouse embryonic fibroblasts should formally be reproduced in human hepatocytes or HepG2 cell lines, as such results could shed light on the mechanisms during the process of transplantation, and explain the relevance of the composition of University of Wisconsin solution in ER-stress occurring during the reperfusion phase of the operation, e.g. iron chelation or the importance of glutathione in UW solution (see chapter 2 section B4) as a means of reducing ER-stress.

d. IGFBP1 and intracellular function. Finally, IGFBP1 also has an intracellular function in an anti-apoptotic mechanism that involves its binding to BAK at the mitochondria, thus preventing p53-mediated apoptosis in HepG2 cells. Interestingly, this mechanism is dependent on a p53-mediated increase of IGFBP1 mRNA and protein levels, either as a direct or indirect target of p53 action on IGFBP1 (Leu and George, 2007). In mice, IGFBP1 protein was localized to mitochondrial fraction and in serum in response to 24h fasting, or 90min post-non-lethal treatment of cisplatin, a platinum based compound that forms DNA adducts (Boutros et al., 2008a), anti-Fas agonist antibody or epidermal growth factor (Leu and George, 2007). Cisplatin dramatically

increased both mitochondrial and serum levels of IGFBP1, while fasting and EGF increased serum levels of IGFBP1 more than mitochondrial fraction of this protein (Leu and George, 2007). Finally the anti-Fas treatment of mice increased IGFBP1 at the mitochondrial fraction in liver samples, while nearly undetectable levels in the serum were found. In all cases, IGFBP1 coimmunoprecipitated with BAK, found at the mitochondria, from murine hepatic extracts (Leu and George, 2007). It is an interesting finding that should be reproduced, as the presence of intracellular IGFBP1 may counteract the potentially toxic substances the liver is exposed to under normal circumstances and may also explain why the liver is resistant to p53-mediated apoptosis (Leu and George, 2007), and that although IGFBP1 null mice have a normal phenotype, they have a reduced hepatic regenerative capacity after partial hepatectomy (Leu et al., 2003).

As we have seen in this section, part of the extracellular function of IGFBP1 is related to the energy status of a cell. However it is not the only factor that responds to the energy status of the cell. In the up-coming section, we will review another player that participates in the cell's attempt to re-establish its normal homeostasis, in terms of energy.

8. Phosphoenolpyruvate carboxykinase 1 (soluble), (PEPCK1/PCK1)

a. Overview. The human phosphoenolpyruvate carboxykinase-1 PCK1 (also called PEPCK1, PEPCK-C, PCK-C, as it is cytosolic), gene was first described in 1993 (Ting et al., 1993). This enzyme catalyses a critical step in gluconeogenesis as it converts oxaloacetate to phosphoenolpyruvate, using GTP to catalyze the reaction (Hanson and Reshef, 1997). Expression of PCK1 has since been found in different organs such as liver (Yu et al., 1993), kidney, white and brown adipose tissue, small intestine among other organs (Hanson and Reshef, 1997). PCK1 in mammalian liver is up-regulated by starvation mediated by increased cAMP, and down-regulated by high-carbohydrate diet. In fact, refeeding rats after a 24h starvation period caused a decrease in pck1 mRNA by 50% after 45min of refeeding, and 90% decrease in pck1 mRNA after 3h of refeeding as compared to starvation levels (Kioussis et al., 1978). Similar results had been gotten with glucose refeeding of rats, where mRNA and PCK1 protein decreased post-refeeding, with a half-life of 30-45min and 45-60min respectively (Kioussis et al., 1978; Tilghman et al., 1974). Some hormones up-regulated PCK1,

including glucagon, and thyroid hormone, while insulin decreased pck1 mRNA expression (Hanson and Reshef, 1997). Glucocorticoids can either increase PCK1 protein by acting directly on hepatocytes, in the absence of insulin, or decrease PCK1 protein in the fed or starved (overnight) rat livers, when the glucocorticoid analogue triamcinolone is injected intraperitoneally. The glucocorticoid dichotomy is most likely caused by glucocorticoid-mediated systemic insulin release, as judged by increased insulin levels in the hepatic portal vein, with a time-course that precedes decreased PCK1 protein levels. Insulin decreases levels of cAMP (Czech, 1977), which is known to increase PCK1 protein levels (Gunn et al., 1975). In support of this hypothesis chemically (alloxan) induced diabetic rats revealed increase in hepatic PCK1 protein synthesis when injected with the glucocorticoid analogue triamcinolone (Gunn et al., 1975). Our results reveal increased PCK1 mRNA levels.

b. PCK1 and oxygen-hypoxia. In addition, oxygen levels are also known to mediate glucagon regulation of the PCK1 gene and its corresponding protein, as arterial O_2 (16% tension) levels up-regulate PCK1 expression levels, post-glucagon treatment, to a greater extent than venous O_2 (8% tension) in primary rat hepatocytes (Hanson and Reshef, 1997; Hellkamp et al., 1991; Kietzmann et al., 1992). These results mirror the hepatic gradient in oxygen tension with greater levels of oxygen in the periportal than in the perivenous area (Jungermann and Katz, 1989) and the predominant localization of pck1 mRNA and protein expression in the periportal hepatocytes (Hellkamp et al., 1991). Conversely cobalt chloride ($CoCl_2$) the prolylhydroxylase inhibitor used to chemically simulate hypoxia (Boutros et al., 2008b), inhibited glucagon-mediated increase in pck1 mRNA and protein expression (Kietzmann et al., 1992). Primary hepatocytes also reduced their maximal glucagon-mediated induction pck1 mRNA levels and protein activity even though the culture media containing the $CoCl_2$ (pretreatement) had been replaced with fresh culture media (Kietzmann et al., 1992). Within the rat pck1 gene promoter, the cyclic response element 1 was responsible for glucagon and the cyclic response element 2 was responsible for the oxygen-meditated modulation respectively (Bratke et al., 1999); these are only two of the many cis-regulatory sites in the promoter of this gene that account for modulation of pck1 by many different factors/stimuli in a tissue-specific manner. The human gene is controlled by similar factors, as the rat and mouse gene, although the promoter has some differences in its cis-regulatory elements (Hanson and Reshef, 1997). In a pulse chase experiment performed on rat hepatoma cell lines, dibutyryl cyclic AMP increased pck1 mRNA half-life form 40min to 240min (Hod

and Hanson, 1988), presumably by an mRNA binding protein with an approximate mass of 100kDa (Nachaliel et al., 1993), although this protein was never identified.

c. PCK1 and warm ischemia. After 1h warm hepatic lobular ischemia in a rat model (2 out of 4 lobes gene subjected to ischemia), pck1 mRNA expression continued to decrease up to 1h after reperfusion, thereafter levels returned to normal at the 48h time-point. The control hepatic lobes of the same rat, not subjected to ischemia, decreased the expression of pck1 mRNA expression at the 4h "post-reperfusion" time-point and pck1 mRNA expression was re-established at the 24h time-point (Gingalewski et al., 1996). Unfortunately, taking the non-ischemic lobes from the same rat as a control has the unfortunate problem of introducing variables associated with the occlusion of half the liver. Once ischemia is introduced in two liver lobes, the total amount of blood would be funnelled though the non-clamped branches of the artery and hepatic portal vein into two of the four lobes of the liver. The stretch and blood pressure associated with the same amount of blood funnelling through half the hepatic volume, the non-ischemic lobes, greater amount of blood borne hormones per hepatocyte will be detected in the non-ischemic lobes, among other variables are introduced into the system. This is all the more important as the ischemic lobes were the left and median, while the smaller right and caudate lobes were non-ischemic, thus more that 50% of the liver was occluded in the experiment (Gingalewski et al., 1996).

Thus far, we have attended to modulation of stress, via HSPs and MKP-1, attended to the energy needs of the cell via IGFBP1 and PCK1. But what of the function of detoxification? Does the liver need to reduce the bile and bilirubin levels while it is coping with the process of transplantation? The up-coming section will answer this question.

9. ABCC3 (MRP3)

ATP-binding cassette, sub-family C (CFTR/MRP) member 3, ABCC3 also called MRP3, is an export protein for anionic conjugates normally expressed at the basolateral membrane of hepatocytes and cholangiocytes (Kullak-Ublick et al., 2004; Suzuki and Sugiyama, 2000), whose expression is up-regulated in liver diseases (Roberts et al., 2002; Suzuki and Sugiyama, 2000), such as primary biliary cirrhosis, hepatitis C and also up-regulated near to necrotic regions in human livers (Ros et al., 2003). In a murine model, after two-thirds partial hepatectomy, up-regulation of mrp3 mRNA and protein at 24h-48h is seen at the basolateral membrane in hepatocytes surrounding the central and

portal veins (Csanaky et al., 2009). Although under normal circumstance MRP3 is expressed at low levels, the increased expression after partial hepatectomy is thought to eliminate bile acids, conjugated and unconjugated bilirubin from the liver to the blood to limit toxicity to the regenerating liver (Csanaky et al., 2009). Increased MRP3 also occurs after 90% partial hepatectomy in rats (Chang et al., 2004). Although disease and liver regeneration up-regulated MRP3 expression in humans and rats (Chang et al., 2004; Csanaky et al., 2009; Geier et al., 2007; Roberts et al., 2002; Ros et al., 2003; Suzuki and Sugiyama, 2000), no MRP3 regulation was detected in a rat model of partial (lobar) warm hepatic ischemia, for an hour, followed by 3, 6, 24 or 48h of reperfusion (Tanaka et al., 2008). Interestingly a slight reduction in expression was seen in human biopsy specimens at the 1h post-reperfusion time-point (Boutros et al. unpublished results). If we take the previously mentioned results concerning pathology and hepatic regeneration into account, this want of an expression during human liver transplantation, or rat lobar warm ischemia, may be cautiously interpreted as an indicative that the liver is not undergoing a massive life-saving effort. Alternatively, it may indicate that the 1h time-point may be too early for the expression of this gene in humans, although none was seen for the rat at any of the time-points of reperfusion (Tanaka et al., 2008).

Although we have covered modulation of stress via HSPs and MKP-1, energy status via PCK1 and IGFBP1 all these and other genes require transcriptional regulation. In addition, a given cell may increase its level of transcription factors in order to meet its needs. In the next sections we will cover some of these transcription factors.

10. FosB, JunB and v-Jun (c-Jun)

a. Overview. FosB, JunB and c-Jun are members of the Fos and Jun inducible transcription factor families (Herdegen and Leah, 1998). Fos and Jun family members are known to mix and match to form an activator protein-1 (AP-1) transcription factor dimer, although other transcription factor families such as ATF and Maf can also form AP-1 in the presence of the two previous families. All these factors are composed of basic region-leucine zipper containing proteins, creating an array of complexes capable of binding DNA with different affinities at different binding sites (Herdegen and Leah, 1998; Shaulian and Karin, 2002). Furthermore, depending on the identity of the proteins composing the dimer, AP-1 has been involved in the modulation of proliferation, pro- and anti-apoptosis, differentiation, and angiogenesis among other functions (Milde-Langosch, 2005; Shaulian and Karin, 2002).

b. Fos, Jun and ischemia reperfusion. Although FosB was identified as one of the genes regulated by murine hippocampal ischemia-reperfusion, no defining role was cited (Nagata et al., 2004). However, in cardiac ischemia-reperfusion the AP-1 dimer composed of FosB and JunB lead to increased MMP2 synthesis in a ROS dependent mechanism (Alfonso-Jaume et al., 2006). This could have implications for hepatocytes, as liver effluents contain matrix metalloproteinases, MMP2 and MMP9 (Upadhya and Strasberg, 2000). Interestingly, decreased JunB but not Fos (presumably c-Fos, although it was not mentioned) mRNA was evident when liver was stored at 37ºC for up to 4 hours. Fos mRNA increased from time zero to the 30 minutes of hepatic incubation at 37ºC, thereupon decreasing (Almeida et al., 2004). The data thus far implicate FosB and JunB in warm ischemia-reperfusion injury, although in different model systems, but with very little information regarding the consequences attached to such regulation. However other problems surface when we consider the possible function of FosB and JunB: 1) the paucity of data, especially cold ischemia-warm reperfusion; 2) the number of combinations of dimers that Fos, Jun, ATF and Maf can form leading to different AP-1 complexes (Herdegen and Leah, 1998; Milde-Langosch, 2005; Shaulian and Karin, 2002); 3) the multiplicity of functions that AP-1 has been implicated in as a result of these different complexes (Herdegen and Leah, 1998; Milde-Langosch, 2005; Shaulian and Karin, 2002); and 4) the virtual absence of data relating the expression of the FosB and JunB, or other members of the transcription factor families, much less their mRNA, with direct evidence for the formation of such dimers at the promoters of genes. For example we could assay for the mRNA regulation of fosB and junB after cold ischemia-warm reperfusion, then also assay in the same cells for FosB/JunB dimer binding to the promoter of a target gene, using a CHIP on Chip assay for the latter part (Sikder and Kodadek, 2005). All these factors easily explain why it is difficult to predict the function of FosB and JunB in human liver transplantation.

Nevertheless some information does exist for c-jun mRNA up-regulation, 1h post-reperfusion in porcine liver, and was believed to be due to the length of cold storage and oxidative stress, although the storage solution was Histidine-Tryptophan-Ketoglutarate not University of Wisconsin solution and the perfusion was done ex-vivo using artificially reconstituted blood, using porcine plasma reconstituted with porcine erythrocytes, platelets, leukocytes and albumin (Wieland et al., 2000). Compare this paradigm as opposed to human liver transplantation, UW storage solution and in vivo reperfusion where junB, c-jun and fosB mRNA are up-regulated (Boutros et al., 2008b). Notably, in

the former study, both c-fos and c-jun mRNA were co-expressed (Wieland et al., 2000). In an earlier study, using lobar murine hepatic ischemia-reperfusion, c-fos and c-jun mRNA were co-expressed (Schlossberg et al., 1996). At the 1-3 hours post-reperfusion time-point, c-fos and c-jun mRNA expression were believed to be associated with tissue repair, while the 6-20h post-reperfusion time-point revealed decreased c-fos mRNA expression and sustained c-jun mRNA expression was associated with regions of hepatic lobe necrosis and apoptosis (Schlossberg et al., 1996). More recently, a 60min warm total hepatic ischemia followed by 2h of reperfusion in rats was associated with nuclear c-Jun protein, inactive cytosolic NF-κB and caspase-3 activation in hepatocytes, increased liver enzymes in serum (AST, ALT) used as a mark of necrosis, when compared to sham control livers (Giakoustidis et al., 2008).

Given these results and our lack of detection of c-fos mRNA it is possible that either FosB expression counteracts c-Jun expression at the protein levels or there may be deleterious effects associated with the expression of c-jun assuming that the same cells express c-Jun, FosB and JunB. Although noxious effects may not appear in the early 1h post-reperfusion time-point (Boutros et al., 2008b).

11. nuclear receptor subfamily 4, group A, member 1, transcript variant 1 (NGFI-B, Nur77, TR3)

a. Overview of general function and localization. NGFI-B (nerve growth factor-induced clone B (Li et al., 2000)) is a nuclear hormone receptor transcription factor that does not appear to require the presence of ligand to bind DNA. The DNA binding domain of NGFI-B recognizes the NGFI-B response element within the promoter of a target gene and binds to this element as a monomer or NGFI-B can bind to a Nur response element as a homodimer (Philips et al., 1997). NGFI-B is expressed in many tissues including the liver. Not only can serum induce the mRNA of NGFI-B, as in the case of other proteins, disparate consequences are linked to different durations of NGFI-B protein activation. Long-term activation may lead to apoptosis, while transient activation does not. In addition, to monomers and homodimers, NGFI-B can form heterodimers with nuclear hormone receptor transcription factors that can bind ligands; these include the retenoic acid receptors, retinoid X receptor and peroxisome proliferator activated receptors. MAPKs and AKT can phosphorylate NGFI-B, causing its inactivation and retention in the cytoplasm (Hsu et al., 2004). More specifically, AKT phosphorylates NGFI-B at residue Ser-350 in its DNA binding domain and reduces DNA binding activity,

however the phosphorylation of Ser-350 (Pekarsky et al., 2001) (Ser-351 in human NGFI-B (Han et al., 2006b)) also allows 14-3-3ζ protein to dock; the docking of 14-3-3ζ and the reduced DNA binding activity may have a cause and effect relationship or they can be complementary; this is as yet undetermined. The result of the AKT-mediated phosphorylation of NGFI-B and its sequestration by 14-3-3ζ, result in decreased apoptosis in fibroblasts and T-cell hybridomas (Masuyama et al., 2001). Further evidence of the vital role of MAPK signal transduction derives from evidence that JNK-mediated phosphorylation of NGFI-B may inhibit the latter's nuclear accumulation, however inhibition of AKT activity is necessary to allow translocation of NGFI-B from nucleus to cytosol, as phosphorylation of Ser-351 inhibits export of human NGFI-B from the nucleus to the cytosol (Han et al., 2006b). In addition, ERK-mediated phosphorylation of NGFI-B at Ser-105 in rat (Katagiri et al., 2000), or Ser-140 in human NGFI-B (Han et al., 2006b), was necessary for NGFI-B export from nucleus.

Once in the cytosol NGFI-B can interact with mitochondrial Bcl-2 irrespective of its JNK-related phosphorylation status (Han et al., 2006b). More importantly, the DNA binding domain of NGFI-B is not required for its proapoptotic activity (Li et al., 2000). More specifically, NGFI-B/ΔDBD (NGFI-B that lacks DNA binding domain and localizes to the cytosol; no need to treat cells with proapoptotic agents) (Li et al., 2000; Lin et al., 2004) binding to Bcl-2 is mediated via the loop region in the N-terminal (1-80 aa) of Bcl-2 and the DC1 (467-536 aa, and more specifically Leu-487) region of NGFI-B (Lin et al., 2004). Coexpression of NGFI-B/ΔDBD and Bcl-2 caused cytochrome c release from mitochondria and apoptosis in HEK293T cells, whereas coexpression of Bax and Bcl-2, prevented Bax mediated mitochondrial-mediated apoptosis in the same cell line. Furthermore the hydrophobic groove in Bcl-2 is not required for its proapoptotic but is essential for its antiapoptotic activity. The NGFI-B/ΔDBD interaction with Bcl-2 causes the latter to expose its BH3 domain, allowing enhanced interaction of Bcl-2 with Bcl-X_L and Bak using primary peripheral blood lymphocytes (Lin et al., 2004). Similar results had been found with endogenous NGFI-B. Interestingly, the proapoptotic molecules A23187, VP-16, TPA and the retinoids MM11453 and MM11384, cause the translocation of wild-type NGFI-B from the nucleus to the mitochondrial surface not the matrix (Li et al., 2000). Thus apoptotic signals can cause the translocation of NGFI-B from the nucleus to the cytosol, changing its function.

In addition to these studies, another finding using AHPN treated human neuroblastoma and esophageal squamous carcinoma cell lines revealed NGFI-B

translocation not only to mitochondria, with eventual caspase-9 activation, but also to the endoplasmic reticulum, with caspase-4 activation, in response to this proapoptotic stimulus (Liang et al., 2007). As our laboratory has shown that ER-stress occurs during the reperfusion phase of the operation (Emadali et al., 2005), it would be interesting to determine if this is accompanied with NGFI-B translocation to the ER and caspase-4 activation. Our results also reveal an increase in NGFI-B mRNA.

b. NGFI-B and alternative sources of energy. Using adenoviral NGFI-B transduced mice (by tail vein injection), these mice revealed elevated fasting glucose levels, at 12h and 24h, compared to control mice, but also an increased ability (~50%) to use an alternative source of carbon such as glycerol. Their ability to use pyruvate was only marginally enhanced. Given that PEPCK, a rate-limiting enzyme, is required to synthesize glucose from pyruvate, but is not required for glucose synthesis from glycerol, and that NGFI-B expression does not increase PEPCK levels, the in vivo findings support the in vitro experiments (Pei et al., 2006). Although hepatic glycogen stores are depleted during transplantation, especially during cold storage, no definitive link between glycogen depletion and prediction of graft primary non-function has been established to date (Quintana et al., 2005). NGFI-B up-regulation (Boutros et al., 2008b) may be the livers' attempt to find an auxiliary source of energy for glucose synthesis. The University of Wisconsin solution contains sugars in the form of raffinose and oxidized lactose (lactobionic acid), it does not contain glucose (see Table 1 chapter 2 section B4, p.53-58). It is not clear at present which aspect of transplantation regulates NGFI-B (see chapter 2 section C3a for gluconeogenesis).

c. NGFI-B and graft acceptance. Other results had implicated NGFI-B expression in long-term graft acceptance, in a murine cardiac transplantation model (Tao and Hancock, 2008). Wild-type C57BL/6 recipient mice rejected BALB/C hearts in ~ 8 days, while C57BL/6 T-lymphocyte-specific NGFI-B-transgenic mice rejected the BALB/C hearts after 100 days. Based on their results, the authors concluded that the allograft acceptance was due to both the increased number of T-regulatory cells and an increased activation-induced T-effector cell apoptosis (Tao and Hancock, 2008). Transcriptional profiling of BALB/C grafted hearts, 7 days post-transplantation into WT C57BL/6 recipients or into C57BL/6 T-lymphocyte-specific NGFI-B-transgenic mice, revealed greater pro-inflammatory mediators, and more leukocyte infiltration, in the BALB/C hearts grafted in WT mice than in hearts grafted in C57BL/6 T-lymphocyte-specific NGFI-B-transgenic mice. The preceding results speak of a scenario that could

involve NGFI-B mediated/reducing expression of pro-inflammatory regulators. If the results in a murine cardiac transplantation model (Tao and Hancock, 2008) were extended to human liver transplantation, they may prove valuable as increased ischemia-reperfusion related damage might lead to increased inflammatory response, greater leukocyte infiltration, which may accelerate the process of rejection of the graft. Greater NGFI-B activity in T-cells may prove useful for reducing immunologically mediated damage. Up until now, there has been a review of transcription factors, we identified by microarray (Boutros et al., 2008b), which positively modulate gene regulation.

12. Inhibitor of DNA binding-2 (ID2) and v-Myc (N-Myc)

a. ID2 an overview. The inhibitor of DNA binding-2 protein is a member of the helix-loop-helix transcription factor family, with inhibitory functions toward DNA binding and cell differentiation, hence its name when the first family member was originally identified in 1990 now called ID1 (Benezra et al., 1990). The helix-loop-helix domain is required for homo- or heterodimerization and the basic region, which is absent in the ID subfamily, is responsible for DNA binding (Norton et al., 1998). Because ID proteins lack the DNA binding region, but still have a dimerization domain, they act as negative regulators of the helix-loop-helix transcription factor family. ID2 was originally cloned 1991 (Sun et al., 1991), and a few years later, the ID2 null mice were created (Yokota et al., 1999). Although these mice have a normal phenotype at birth, they lack lymph nodes and Peyer's patches (secondary lymphoid organs seen as nodes on the antimesenteric side of the intestine from the duodenum to the rectum in mice) and a paucity of NK cell population (reduced to 10% of normal numbers) due to a defect in the precursor population, the lymphotoxin producing cells, although lymphatic vessels appear normal. However, one quarter of the ID2 null mice died during the neonatal period and eventually their phenotype revealed retarded growth, without further explanation as to the cause (Yokota et al., 1999). A viable explanation concerning the smaller size of these mice could be attributed to the decreased clonal expansion coupled with early differentiation of cell populations.

b. ID2 function. In keeping with some of the variables associated with transplantation, ID2 mRNA was induced in response to growth factor stimulation in early and late G_1 (Hara et al., 1994; Norton et al., 1998), which occurs upon the reestablishment of blood flow to the liver, after a ~10h storage in UW at 4ºC.

Interestingly, human ID2 mRNA is expressed in both foetal and adult livers (Boutros et al., 2008b; Terai et al., 2000). Overexpressing ID2 in rat hepatic stellate cells reduces smooth muscle actin and collagen-1 messenger, hallmarks of activated hepatic stellate cells e.g. acquiring a myofibroblastic phenotype, as well as a slight reduction in MMP2 and MMP9 mRNA (Tajima et al., 2007). Another transplantation-relevant stressor such as hypoxia can stimulate expression of ID2 via HIF-1 in a neuroblastoma cell line (Lofstedt et al., 2004) and conversely ID2 reduces VEGF secretion and HIF-1α protein levels, in a hepatocellular carcinoma cell line (Tsunedomi et al., 2008). Interestingly, ID2 binds retinoblastoma (Rb) protein at the beginning of S-phase, and prevent the binding of Rb to either N-Myc (also called v-Myc) or c-Myc allowing cell cycle progression (Lasorella et al., 2000). In addition, either N-Myc or c-Myc could cause the up-regulation of ID2 at the mRNA and protein levels. ID2 null MEFs are incapable of entering into cell cycle after serum stimulation, and when both ID2 and c-Myc are present in the cell, they are coordinately regulated; no mention was made of N-myc (Lasorella et al., 2000). In the context of the liver, the upregulation of ID2 and N-Myc (Boutros et al., 2008b) could be a prelude to hepatocyte proliferation for the purposes of regeneration/repair mechanism post-transplantation. In support of these ideas, ID2 was identified as a candidate gene for cell-cycle progress to the S-phase after murine partial hepatectomy (Togo et al., 2004). Other studies have linked the dissociation of the ID2 protein from the c-Myc promoter and the "priming" of hepatocytes for proliferation, exit of G_0 and entry into G_1, in response to partial hepatectomy in rats (Rodriguez et al., 2006) or chemical depletion of GSH (reduced form of glutathione) within the rat liver (Torres et al., 2009). Thus ID2 is involved in alleviating transcriptional repression at the myc promoter and then permitting Myc protein to initiate transcription at promoters, when ID2 binds to transcriptional repressors such as Rb proteins (Lasorella et al., 2000).

c. N-Myc. At this point it is important to mention that N-Myc null mice were deficient in organogenesis, which included the lung, the stomach, the liver, the heart and the central and peripheral nervous systems (Sawai et al., 1993). More specifically, extensive apoptosis in hepatocytes in N-Myc null mice was observed in contrast to their wild-type counterpart livers (Giroux and Charron, 1998). Although N-Myc was thought to be essential during organogenesis, while c-Myc was believed to be required throughout the life of the animal (Sawai et al., 1993), N-Myc was also implicated in proliferation, differentiation and apoptosis (Lee and Reddy, 1999) depending on the environment, especially where nutrition was concerned (Rossler et al., 2001). Conversely, N-myc was

also implicated in VEGF secretion irrespective of serum conditions (Kang et al., 2008). In the context of human liver transplantation, with its constellation of potentially noxious stimuli, N-Myc may have an anti-apoptotic activity and prepare the liver for repair. A conditional knockout of the N-Myc gene would not only bypass the developmental issues with organogenesis associated with N-Myc null mice, it would permit the assaying the N-Myc involvement in the liver while taking into account the transplantation-relevant stresses. Given all these data, it would be interesting to determine how ID2 transcription and protein function relate to that of N-Myc transcription and protein activity during liver transplantation.

All this transcription must be modulated at some point to eliminated unwanted mRNA and fine tune the levels of mRNA that are to be translated. Tristetraprolin is one such protein. The next section will investigate how this protein participates in the regulation of mRNAs.

13. Tristetraprolin (TTP)

a. Overview. Tristetraprolin (TTP) binds to AU-rich elements (AREs) on the 3'-untranslated region of mRNAs and destabilizes them (Dean et al., 2004). However it does not necessitate the presence of the poly(A) tail of the mRNA to do so (Lai and Blackshear, 2001). TTP binds to and destabilizes mkp-1 mRNA via an unknown mechanism (Lin et al., 2008), although TTP which also destabilizes TNF-α mRNA (Dean et al., 2004) does so in a microRNA (miRNA) dependent manner (Jing et al., 2005). Interestingly, TTP protein can regulate its own mRNA stability, which allows the TTP protein to regulate the levels of its own expression (Brooks et al., 2004; Tchen et al., 2004), as well as the mRNA of inflammatory cytokines and vegf mRNA (Essafi-Benkhadir et al., 2007), among other mRNAs (Lai et al., 2006b). Nevertheless, although borne without apparent phenotype, TTP null mice revealed an up-regulation of TNF-α mRNA and protein with symptoms such as erosive arthritis, dermatitis, conjunctivitis, among others as well as anti-DNA and anti-nuclear antibodies found in serum as the mice age, resulting in 34% death by 7 months of age (Taylor et al., 1996). Furthermore, TTP is involved in mRNA decapping (Fenger-Gron et al., 2005; Simon et al., 2006), which is an important step for mRNA degradation (Chan and Slack, 2006; Simon et al., 2006), and the recruiting of the Xrn1 5'→3' exonuclease and a component of the exosome, scl75 (Hau et al., 2007).

b. Regulation of TTP function. First, ERK1/2 are implicated in the export of ttp mRNA from the nucleus into the cytosol, in a tlp2-protooncogene-MEK1/2-ERK1/2 dependent mechanism, in murine macrophages stimulated with LPS (Dumitru et al., 2000). Furthermore, ERK and p38MAPK are required for tnf-α stabilization in the cytosol (Rutault et al., 2001), via TTP protein inhibition (Deleault et al., 2008), while JNK activation enhances translation of tnf-α mRNA (Swantek et al., 1997) in part by interfering with the ERK/p38MAPK effect (Deleault et al., 2008). In addition, proteasome inhibition also stabilizes TTP protein (Deleault et al., 2008). Other results had implicated both p38MAPK-MK2 and ERK in the increased mRNA and protein levels of TTP (Hitti et al., 2006; Taylor et al., 1995; Zhu et al., 2001) as well as in the stabilization of TTP via phosphorylation at Ser-52 and Ser-178, two of many phosphorylation site identified by mass spectrometry (Cao et al., 2006). The lack of Ser-52 and Ser-178 phosphorylation leads to nuclear accumulation and degradation of TTP in murine macrophage cell line (Brook et al., 2006). Although TTP becomes stable as a result of phosphorylation, it does not bind to ARE sequences in mRNAs (Brook et al., 2006; Hitti et al., 2006; Zhu et al., 2001). Indeed there are many proteins that regulate the phosphorylation status of TTP (Cao et al., 2007) and interact to regulate TTP protein activity toward the destabilization of mRNA (Sandler and Stoecklin, 2008).

c. TTP and ischemia-reperfusion. Following rat whole forebrain 10 min ischemia and up to 72h of reperfusion, cytoplasmic poly(A) mRNAs initially relocalized to granules that were positive for the translation initiation factor eIF4G and the poly(A) binding protein (PABP). These granules were negative for S6 (protein of the small ribosomal S40 subunit), TIA-1 (T-cell internal antigen-1) and TTP, indicating that they were not part of the ribosomes, stress granules or processing bodies. Other results reveal that TTP is up-regulated in cultured HepG2 cells in 1%O_2-5%CO_2, hypoxia, for 24h when compared to normal “room oxygen” (~21%O_2)-CO_2 at 37ºC (Sonna et al., 2003). In addition, TTP was identified as one of the genes that was up-regulated at the 16h reperfusion time-point, that followed 5min of warm ischemia of the canine left coronary artery occlusion perfusing the anterior wall of the heart, as a model of late ischemic preconditioning of the myocardium, believed to have a protective effect as part of an anti-inflammatory response (Zubakov et al., 2003). Finally we also detected up-regulation of ttp mRNA at the 1h post-reperfusion time-point, during the human liver transplantation (Boutros et al., 2008b). Needless to say that there is still much work that needs to be done to determine the subcellular localization of TTP protein, its

phosphorylation status, the mRNAs targeted by TTP and its overall involvement during ischemia reperfusion. Perhaps down-regulating TTP expression prior to hepatic transplantation could give us a clue as to how the liver uses TTP during this multi-stress process. The regulation of mRNA levels is fine and well, but proteins must also be dealt with, in terms of their degradation and regulating their signal transduction capabilities when necessary.

14. Ubiquitin B (UbB)

a. Overview. Ubiquitin is part of a gene family that comprises UbA, UbB and UbC (Wiborg et al., 1985). Interestingly, the ubb gene codes for three mature ubiquitin proteins coded for in tandem (Baker and Board, 1987; Wiborg et al., 1985), while uba gene codes for 1 ubiquitin protein and ubc gene codes for 9 mature ubiquitin proteins. The relative abundance of each mRNA seems similar in different tissues of the same species, while different species have varying expression of UbA, UbB and UbC (Wiborg et al., 1985). It should be noted however that ubiquitin is conserved among different eukaryotes (Catic and Ploegh, 2005). Enzymes categorized as activating enzyme (E1), conjugating enzyme (E2) and ligase (E3) are responsible for activating and transferring ubiquitin to proteins (Hochstrasser, 2006; Jentsch and Pyrowolakis, 2000). The consequences of protein ubiquitination are numerous and will not be discussed in detail in this thesis. Instead, an overview of the consequences of ubiquitination and how it relates to human liver transplantation will be more informative. Perhaps the most documented of these consequences is proteasomal degradation in cytosol (Pines and Lindon, 2005) and nucleus (von Mikecz, 2006). The proteasome is a multiunit complex responsible for degrading proteins into peptides to recycle misfolded, damaged, or normal proteins and as part of the control for cell cycling (Cheng, 2009).

b. Functions, sites and consequences of ubiquitination. A ubiquitin chain, with a minimum of 4 ubiquitins (Thrower et al., 2000), that is linked via its Lys-48 residue to a Lys residue in an acceptor protein, targets proteins for degradation or can result in the regulation of transcription (Chau et al., 1989; Flick et al., 2004; Hochstrasser, 2009), while a ubiquitin chain that is linked via its Lys-63 residue to a Lys residue in an acceptor protein, targets proteins for DNA repair (Hofmann and Pickart, 1999; Spence et al., 1995), cell cycle regulation (Spence et al., 2000) and signal transduction (Wang et al., 2001). Monoubiquitination of histones controls gene expression or endocytosis of ion channels and transmembrane receptors and directs

these to lysosomes (Hicke, 2001). However, the process of ubiquitination is not confined strictly to Lys-48, Lys-63, polyubiquitination or monoubiquitination within the cell at any given time. Rather ubiquitination, as any other cellular process occurs for other reasons and in a spectrum of possibilities within the cell. For instance, the ubiquitin involvement in complex circumstances as signal transduction, occurs from growth factor and cytokine receptors all through the kinases involved in the signal transduction pathway, up to and including transcription factors (Karin and Gallagher, 2009; Laine and Ronai, 2005; Mukhopadhyay and Riezman, 2007; Sun and Chen, 2004) and also occurs in the process of autophagy (Kirkin et al., 2009). Finally, ubiquitination is reversible and the length of the ubiquitin chain can be edited (Hicke, 2001; Mukhopadhyay and Riezman, 2007).

c. Ubiquitination and ischemia. Considering all the potential involvement of ubiquitin in cellular processes, it is perhaps not surprising that there are reports of ubiquitin association with animal models of ischemia. A case in point, ubb gene transcript was slightly up-regulated, as evaluated by in situ hybridization, in rat following increasing times of focal-warm-cerebral ischemia, up to 24h of ischemia, with no reperfusion (Noga et al., 1997). In contrast to these previous results, 20min of warm-forebrain ischemia followed by 30min of reperfusion decreases levels of ubb mRNA, but these levels increase dramatically after 4-6h of reperfusion in the cortex and the hippocampus. Levels returned back to normal from 24h after reperfusion (Noga and Hayashi, 1996). This increase in mRNA is preceded by increased ubiquitin conjugates in the mitochondrial fraction during the early reperfusion phase (30min-4h) that followed transient (5min) total cerebral ischemia in gerbils (Hayashi et al., 1992). The amount of mitochondrial ubiquitin insoluble conjugates increased with the length of ischemic time, 2-10min, and 1h of reperfusion (Hayashi et al., 1993). The observations relating to ubiquitin and ubb gene expression can be unified if we consider that ischemia depletes free ubiquitin in gerbil hippocampus (Morimoto et al., 1996), and therefore more would need to be synthesized if the system were overwhelmed. Furthermore, the proteasome activity was reduced in mouse brains, in tissue homogenates, using an artificial substrate to assay proteasome activity, after 10min of focal cerebral ischemia and up to 24h of reperfusion (Keller et al., 2000).

It should be noted that protein aggregation itself could lead to an impairment of the proteasome. This is exemplified an in vitro model using HEK293 human embryonic kidney cells, expressing proteins that are prone to aggregation such as huntingtin

fragment and cystic fibrosis transmembrane conductance regulator, which are implicated in neurodegenerative disorder (Bence et al., 2001). Finally a single or dinucleotide deletion or single nucleotide insertion of ubiquitin B transcript called molecular misreading (van Den Hurk et al., 2001), was first noticed in sporadic Alzheimer and Down syndrome (van Leeuwen et al., 1998) leading to the production of UBB^{+1} protein product, has also been noted in after transient global cerebral ischemia in 8-9 weeks old gerbils (Yamashiro et al., 2007). This mutation causes the expression of 16 extra amino acids at the C-terminal of ubiquitin and was believed to block the ubiquitin-proteasomal degradation system in neuronal cells, when UBB^{+1} protein product is ubiquitinated at Lys-29 and Lys-48 but not if ubiquitinated at Lys-48 only (Lindsten et al., 2002). Interestingly, $ubiquitin^{+1}$ protein was found in the hepatocytes, within cytoplasmic inclusions called Mallory bodies, named after Dr Mallory who first described them (Zatloukal et al., 2007), in patients with steatohepatitis, a disease with unknown aetiology (McPhaul et al., 2002). How ubiquitination affects human liver transplantation should be determined.

Section B of this chapter has overviewed the proteins coded by mRNAs identified by our microarray results (Boutros et al., 2008b) that had been identified in previous studies as being modulated by aspects of ischemia and reperfusion. In the up-coming section, genes not previously identified by such stresses are presented.

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C. Genes unknown in the field of ischemia-reperfusion and identified by our microarray results

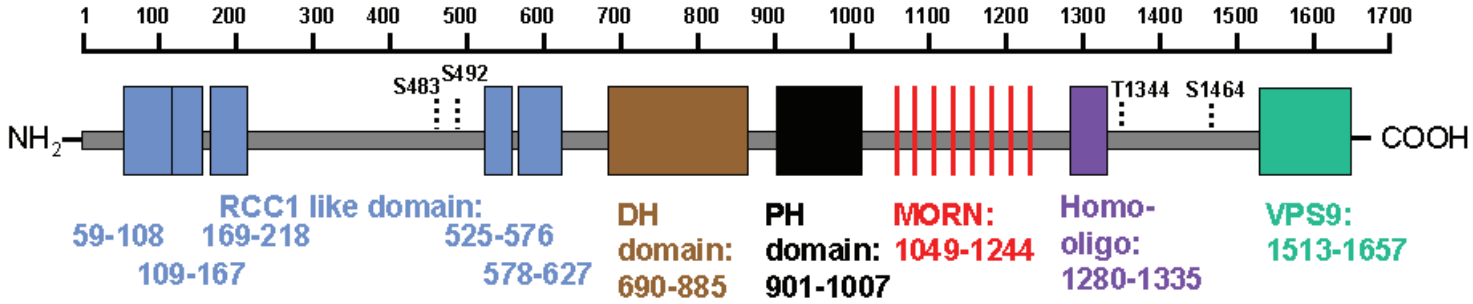
The genes that have been identified by microarray that have not been implicated in ischemia-reperfusion are presented in this section. A brief explanation as to how they could contribute to the survival of the graft is also given.

1. Alsin (ALS2)

a. Overview. ALS2 was originally identified as a locus linked to an autosomal recessive form of amyotrophic lateral sclerosis with early onset and slower clinical progress, compared to the other forms of ALS (Hentati et al., 1994). Mutations in the ALS2 gene coding for the alsin protein were identified a few years latter (Hadano et al., 2001; Yang et al., 2001). Alsin is known in the fields of juvenile onset of ALS which causes upper and lower motor neurons degeneration (Chandran et al., 2007), primary lateral sclerosis upper motor neuron degeneration (Panzeri et al., 2006; Rowland, 2005) and infantile ascending hereditary spastic paraplegia which affects primarily the lower motor neurons, although it is heterogeneous (Eymard-Pierre et al., 2002), however it is all but completely uncharacterized outside the field of neurodegeneration.

A RhoGEF is a guanine nucleotide exchange factor, exchanging GDP for GTP thereby activating members of the Ras superfamily (Quilliam et al., 1995; Rossman et al., 2005). Therefore as a RhoGEF alsin is an activator of Rac1 and Rab5 (Topp et al., 2005; Topp et al., 2004) and presumably Ran (Ras-related nuclear) (Eymard-Pierre et al., 2006), or at least alsin can act as a weak RanGEF in a cell free system (Otomo et al., 2003) (Figure 6).

Alsin protein structure



RCC1 like domain: **R**egulator of **C**hromosome **C**ondensation. Activates RAN or mediates protein-protein interaction

DH domain: **D**iffuse **B** cell **L**ymphoma (**DBL**) **H**omology domain, part of a RhoGEF for Rac1 together with the pleckstrin homology (PH) domain.

PH domain: **P**leckstrin **H**omology domain binds to 1) β/γ subunit of heterotrimeric G proteins; 2) phosphatidylinositol-4,5-bisphosphate; and 3) phospho-Ser/Thr residues.

MORN: **M**embrane **O**ccupation and **R**ecognition **N**exus motif, possible linker allowing membrane-protein interaction.

Alsin Homo-oligomerization domain

VPS9: **V**acuolar **P**rotein **S**orting **9** domain catalyzes nucleotide exchange on Rab5.

Serine and threonine phosphorylation sites with as yet unknown consequences

Figure 6. The alsin protein. Although RCC1, DH/PH and VPS9 represent the Ran, Rac1 and Rab5 RhoGEF domains respectively, the domains that actually function as RhoGEF in the in vivo situation is different (see text). Homo-oligomerization is achieved through the 1280-1335 amino acid residues between MORN and VSP9 regions, which are required for Rab5 RhoGEF function. The function of phosphorylation has not been determined.

b. Consequence of Alsin function. As an activator of Rac1, Rab5 and Ran, alsin has the potential to be involved in multiple functions, among which are: 1) cytoskeletal rearrangement and oxidative stress via Rac1 as this Ras family member is known to be involved in formation of lamellipodia, generation of reactive oxygen species (as Rac1 is a subunit of NADPH oxidase in human cells), endocytosis and apoptosis, among other Rac1 functions (Bosco et al., 2009); 2) increased endosomal trafficking via activation of Rab5, which is best known for its involvement in early endosomal trafficking (Gorvel et al., 1991), but like Rac1 it is also involved in actin cytoskeleton remodelling among other functions (Lanzetti et al., 2001); and 3) although not demonstrated to be a RanGEF in vivo, alsin mediated activation of Ran could lead to diverse functions ranging from the shuttling of proteins across the nuclear pore complex, via importins and exportins, apoptosis, microtubule reorganization among many functions of Ran which vary according to its effectors (Rensen et al., 2008). Among the molecules that are shuttled by Ran, to and from the nucleus, we can enumerate: 1) p53, the retinoblastoma protein (Rb) and the von Hippel-Lindau (VHL) tumour suppressors; 2) E2F4 and NFκB transcription factors; and 3) miRNA export via exportin 5 (Rensen et al., 2008). The miRNA export may function in conjunction with tristetraprolin, as TNF-α mRNA uses TTP and miRNA for its degradation (see TTP section in section B above); this possibility needs to be verified in terms of the cell-types that expresses TTP, alsin and determine whether or not miRNA export from the nucleus is relevant to transplantation relevant stress. In addition, both Ran (Rensen et al., 2008) and alsin (Millecamps et al., 2005) are found at the centrosome, which is described as a semi-conservative non-membrane bound organelle (Schatten, 2008). The centrosome per se is implicated in multiple functions including: 1) cell motility; 2) cell polarity; 3) maintaining the cell shape; 4) vesicular transport; and 5) the cell cycle (Schatten, 2008). Ran is also implicated in many aspects of cell division as such: 1) Ran is localized at the centrosomes throughout the cell cycle; 2) Ran is implicated in centrosome duplication; 3) spindle pole formation; and 4) microtubule dynamics, among other aspects (Schatten, 2008).

When overexpressed Alsin was found at centrosomes in human SW13 cells, an adrenal cortex carcinoma cell line, and in cholinergic LA-N-2 and a catecholaminergic SK-N-SH neuronal cell lines while the endogenous alsin was undetectable. The function of alsin at the centrosome was unknown with only indirect speculation as to its role in intracellular transport (Millecamps et al., 2005). A more severe phenotype was found in COS-7 monkey kidney cells when alsin was overexpressed including disorganization of

tubulin aster, Golgi fragmentation and mitochondrial redistribution. The organelle disruption may have been due to loss of microtubule foci (Millecamps et al., 2005). Species and cell-type specific differences are only one aspect of the variables associated with alsin. Alsin protein has two different forms.

c. Splice forms of alsin. Alsin expression in human adult tissue (heart, skeletal muscle, kidney, liver and brain especially the cerebellum) was detected as two transcripts one 6.5kb corresponding to the full-length 185kDa protein 1-1657 amino acids, and a smaller transcript at 2.6kb corresponding to 1-396 amino acids of the alsin protein (Hadano et al., 2001). It should be noted that although the liver revealed a greater expression of the 2.6kb than 6.5kb, the sample also revealed signs of degradation (Hadano et al., 2001). The naturally occurring short form is produced by alternate splicing at the 5' donor site after exon 4 resulting in a stop codon after the first 25 amino acids of intron 4 (Hadano et al., 2001). The adult mouse also revealed expression of full-length alsin in cerebellum and the liver (Devon et al., 2005). The als2 gene has binding sites for various transcription factors including (N-Myc, c-Fos, NF-κB among others) (Devon et al., 2005).

d. Domains of the alsin protein. While the alsin protein is a RhoGEF for Rab5, as mentioned above, alsin requires homo-oligomerization for its Rab5-RhoGEF function but not its binding to Rab5 (Kunita et al., 2004). Alsin homo-oligomerization is achieved through the 1280-1335 amino acid residues, however the structure of the alsin homo-oligomers is unknown (Kunita et al., 2004). Although the 660-1657 amino acid residues of alsin gives full Rab5 RhoGEF activity, a point mutation in VSP9 domain Pro-1603-Ala targets alsin to early endosome, without creating the enlargement of endosomes seen in the alsin 660-1657 protein in neuronal cells but not COS-7 or HeLa cells. A point mutation at Leu-1617-Ala abolishes both the enlarged endosome phenotype and the alsin 660-1657 protein targeting to early endosome (Otomo et al., 2003). Interestingly, the RCC1-like domain (Figure 6) of the full-length alsin protein seems to inhibit the recruitment of alsin to the endosomes, perhaps by the interaction of RCC1-like domain with MORN-VSP9 region, accounting for the diffuse cytoplasmic localization of full-length alsin (Otomo et al., 2003).

Similarly, while alsin residues 685-1026 are sufficient for Rac1 interaction, they are insufficient for Rac1 activation (Topp et al., 2005; Topp et al., 2004). The minimal alsin residues conferring Rac1 activation has not been published as yet. Moreover alsin may require some post-translational modification, such as phosphorylation or a co-factor,

as it is incapable of acting as RhoGEF in a cell free assay (Topp et al., 2005; Topp et al., 2004), but does act as a Rac1 RhoGEF within cells (Topp et al., 2005; Topp et al., 2004). Furthermore, within the context of a cell, alsin was localized to dendrites, axon and cell body, in neurons and also in membrane ruffles and lamellipodia in NIH3T3 cells (Topp et al., 2004). In addition, it is not known if alsin homo-oligomerization is required for its function as a RhoGEF for Rac1 or Ran. Alsin is also phosphorylated at Ser-483 and Ser-492 during G1 and M phases of the cell cycle in HeLa cells (Daub et al., 2008; Dephoure et al., 2008), while EGF treatment of HeLa cells lead to Ser-1464 phosphorylation (Cantin et al., 2008). Alsin is also phosphorylated on threonine-1344 in response to genotoxic stress, such as ionizing radiation of human embryonic kidney 293T cells; DNA damage is believed to regulate this latter phosphorylation (Matsuoka et al., 2007). At present it is difficult to ascertain the functional relevance of the alsin phosphorylations as the information regarding these phosphorylations is located in the supplementary information without any mention of alsin in the main the text of the previously cited original contributions.

e. The homologous protein ALS2CL. Recently another protein, the product of als2 homologous gene als2cl (als2 c-terminal like), similar to the C-terminal region of alsin has been characterized ALS2CL with one major splice variant 5kb coding for a 108kDa protein and 17 minor spice variants of unknown function (Hadano et al., 2004). Interestingly, in human tissue heart and kidney revealed by far the most expression, a very intense smear from 10kb-4kb, while other tissues such as skeletal muscle, lung, spleen and liver revealed a fraction of the expression ~1% of the heart and kidney. Mouse tissue revealed the most abundant expression in liver of the 5kb with other organs such as kidney, lung and heart revealing ~5% of the expression in the liver (Hadano et al., 2004). The relevance of this protein becomes clear when we consider that ALS2CL protein can interact with the alsin protein to reduce alsin function. More specifically ectopic expression of full-length alsin has a diffuse cytoplasmic distribution, while the co-expression of alsin and ALS2CL protein relocalized alsin to punctate structures within the cytoplasm (Suzuki-Utsunomiya et al., 2007). Furthermore, while a truncated alsin construct 695-1657 caused an enlargement of endosome phenotype, coexpression with ALS2CL protein relocalized alsin 695-1657 to microtubules but not actin. The amino acid residues 329-651 of ALS2CL interact with the 1351-1657 amino acid residues of alsin affects localization but not alsin RhoGEF activity toward Rab5 (Suzuki-Utsunomiya et al., 2007). It should be noted that ALS2CL can homo-dimerize via

329-582 and 652-953 amino acid residues, but very little homo-oligomerization is detected (Suzuki-Utsunomiya et al., 2007). There is still more work to be done to determine under what circumstances the alsin-ALS2CL interaction takes place and what the consequences are of this interaction when endogenous levels of both proteins are expressed. Again the species-specific difference, in the expression of the ALS2CL protein warrants caution should a transplantation model be conducted in mice.

f. Alsin null mice. Although there are four research groups that have generated als2 null mice with some similarities and differences between the groups, these mice concern the modeling of the juvenile onset of ALS pathology, which is well beyond the scope of human liver transplantation. However some of the phenotypes of these mice may concern liver transplantation, as such although a brief look at their phenotype will be undertaken, some of the more salient point will be detailed. Generally, all agree that the mice had no overt phenotype at birth. The mice were also fertile and had a life span similar to their wild-type littermates, suggesting that alsin had no role, or a redundant role with other RhoGEFs during development. The differences between the mice generated by the various laboratories are believed to be due in part to the use of different targeting vectors and different embryonic stem cell lines. Perhaps worse, these mice, which were generated as models of human neuropathologies involving alsin, failed to reveal phenotypes that were “on the menu”. A case in point, the mice had no upper or lower motor neuron deficiencies, which is a hallmark of ALS2, although a reduced number of motor neuron axons was evident in one of the studies (Chandran et al., 2007). The reason for the differences observed between murine and human phenotypes is currently unknown, but could have been due to species differences or to gain of function of the residual alsin in the context of human pathologies.

A more detailed look at the phenotypes afforded by the als2 null mice reveals an altered neuronal endocytosis in some als2 null mice (Devon et al., 2006; Hadano et al., 2006). Interestingly, two research groups reported on the altered endocytotic phenotype concerning EGFR in als2 null fibroblasts (Hadano et al., 2006) or IGF1R in cerebellar granule neurons and BDNF (brain derived neurotrophic factor) in cortical neurons but no altered phenotype of transferrin in cortical neurons, presumably via the transferrin receptor (Devon et al., 2006). These results suggest that alsin could be involved in receptor endocytosis in hepatocytes or other cell types within the context of human liver transplantation. The third group implicated alsin deficiency with the redistribution of glutamate receptor interacting protein 1 (GRIP1), as alsin interacts, via its RCC1-like

domain, with the PDZ domain of GRIP1. The absence of this interaction, brought about a reduction of glutamate receptor-2 (GluR2) subunit at the synaptic surface of neurons. GluR2 is one of four receptor subunits, labelled GluR1-4, implicated in the formation of the tetrameric AMPA-type glutamate receptor. The absence of GluR2 from the AMPA-type glutamate receptor tetramer, increases Ca^{2+}-mediated neuronal excitotoxicity. Indeed, the presence of GluR2 as a subunit renders AMPA-type glutamate receptor impermeable to calcium (Lai et al., 2006a). Although human livers express metabotropic receptor GluR5 (Treyer et al., 2008) the role of glutamate in the liver (see chapter 2 section B4d.i.) is grainy at best. Adding to the previously mentioned results, the absence of alsin increased neuronal susceptibility to paraquat-induced oxidative stress, in vivo, via intraperitoneal injection, and in neuronal cultures (Cai et al., 2005). The greater sensitivity of cells to oxidative stress is of obvious importance in human liver transplantation, however these results, as those concerning receptor endocytosis, would need to be confirmed in the liver and in some of the more prominent cell types, such as hepatocytes, Küpffer cells, hepatic stellate cells, sinusoidal endothelial cells, among other cell types.

In summary, considering that alsin can activate Rac1, Rab5 and potentially Ran, and the multiplicity of functions that each of these RhoGTPase has, the potential list of alsin-mediated functions may be very long. Although our microarray results revealed a slight decrease in alsin mRNA (Boutros et al. unpublished results), this could be explained that such a multiplicity of function requires a more stable environment or that the appropriate time for alsin expression had not arrived. Nevertheless, to confirm or dismiss these possibilities, the function of alsin needs to be investigated during the process of human liver transplantation, or at least in an in vitro model using ischemia-reperfusion relevant stresses. Indeed although some data does exist for alsin, there is virtually nothing outside of the previously mentioned pathologies, and no data exists concerning alsin mRNA regulation. What of the alsin phosphorylation? The role of this phosphorylation has as yet to be determined. Does the alsin protein always homo-oligomerize? Although the data suggests that this oligomerization is vital to the Rab5 RhoGEF activity (see above text), nothing is known for the oligomerization of alsin versus the RhoGEF function toward Rac1 and Ran. Are there any other posttranslational modification for the alsin protein? None reported as yet. Of course, as there are four different ALS null mice, stemming from four research laboratories, using these mice in a model of transplantation could yield interesting results. For all these reasons we

considered characterizing alsin involvement in transplantation relevant stresses, this is the topic of chapter 5.

The RhoGEF alsin, along with its effectors Rac1, Rab5 and Ran, have been covered in the previous pages and we have mentioned RhoB in section B of this chapter. In the next section, another member of the Rho family will be presented and how it differs from the other members of this family will be addressed.

2. RND1 (Rho6)

a. Overview. RND1 is one of three RND proteins, a sub-group of the Rho family of GTPases. However contrary to the other members of the RhoGTPases, RNDs are constitutively bound to GTP (Nobes et al., 1998). RhoGEFs do not mediate the exchange of GDP for GTP for RNDs, as they do for other GTPases and GTPase activating proteins (GAPs) do not increase the inherent GTPase activity of RNDs, as they do for other members of GTPases thereby returning the GTPases to the GDP-bound inactive form; the lack of GAP activity on RNDs is due to the observation that neither RND1 nor RND3 have intrinsic GTPase activity. Finally, guanine dissociation inhibitors (GDIs, also called RhoGDIs), which bind to the GTPases, in their GDP-bound form, and interact with geranylgeranyl moiety of the GTPases, keeping them in the cytosol in the resting state, do not regulate RND proteins. This can be explained by the observation that RND1, for example does not bind GDP. More specifically the affinity for GTP is 100-fold that of GDP. As a 10:1 intracellular ratio exists between GTP and GDP, this reduces the likelihood of GDP interacting with RND (Nobes et al., 1998).

b. Regulation and function. This sub-group of the RhoGTPases is controlled at the level of transcription/translation, subcellular localization and phosphorylation. RND interacts with the plasma membrane via the positively charged amino acids in the N-terminal aspect of the protein (Chardin, 2006; Nobes et al., 1998). RND1 mRNA is predominantly expressed in the adult human liver and brain. In the rat, hepatocyte expression of RND1 protein accounts for most if not all the RND1 hepatic expression. In addition, RND1 expression localized to adherens junctions, in quiescent, confluent fibroblast cultures (Nobes et al., 1998). Fibroblast overexpressing RND1, via vector microinjection, rounded up (hence the name RND1 or round1), and resisted actin cytoskeletal rearrangement upon using stimulants such as platelet derived growth factor. In addition, RND1 expression in rat embryonic fibroblasts lead to the loss of cell-matrix adhesion, focal adhesions, and basal stress fibres; focal adhesions serve to anchor

stress fibres to the extracellular matrix (Chardin, 2006; Nobes et al., 1998). Interestingly, when epithelial cells such as Madin-Darby canine kidney cells are microinjected with RND1 expressing vector, actin stress fibres juxtaposed to basal membrane are eliminated, however the cells did not round up or dissociate. However, actin filaments associated with adherens junctions in the lateral membrane remained intact even though RND1 expression was associated with adherens junctions (Nobes et al., 1998).

Although RhoGAP does not regulate RND1 per se, as mentioned above, RND1 can nevertheless recruit p190 RhoGAP to inactivate RhoGTPases such as RhoA by hydrolysis of GTP into GDP. In neurons RND1 interacts with plexin-B1, which is a receptor for sema-4D, resulting in loss of adhesion followed by neurite retraction. Plexin-B1 expression in COS-7 cells leads to cell rounding. This reveals a cell-type difference in RND1 function. RND1 also interacts with non-phosphorylated FRS2β in unstimulated cells. Upon FGF (fibroblast growth factor) stimulation, FGFR phosphorylates FRS2β at multiple sites leading to RND1 release and the possible interaction with p190 RhoGAP leading to Rho inhibition. Lack of RND1 in PC12 cells leads to lack of neurite outgrowth upon FGF treatment (Chardin, 2006).

The interactions between RND1 and RhoA have also been implicated in smooth muscle contraction of the rat intestine; longitudinal strips of rat ileum were dissected and one end was tied to a force transducer. RDN1 prevents RhoA mediated increase in contraction at constant intracellular calcium levels, also called RhoA mediated calcium sensitization. This RhoA induced contraction is thought to proceed via Rho-kinase phosphorylation of myosin light-chain phosphatase thereby inhibiting the phosphatase and also via direct Rho-kinase mediated phosphorylation of myosin light-chain. However the effect of RND1 on RhoA depends on the latter not having started its calcium mediated sensitization, otherwise RND1 becomes ineffective (Loirand et al., 1999). Human adult dermis microvascular endothelial cells also reveal up-regulation in RND1 in response to the inflammatory cytokine IL-1β, but the physiological function of this up-regulation was undetermined (Warton et al., 2004).

The information of RND1 is still quite sparse. The paucity of information in the liver is surprising given the abundance of adult human hepatic mRNA (Nobes et al., 1998). How this Rho member is involved in human liver transplantation is not at all clear. Having said this, as RND1 is implicated in the elimination of stress fibres, this may be contraindicated for cell survival during the process of transplantation; such a process includes thermal stress, mechanical stress as a result of manipulation of the liver, UW

solution and blood-induced sheer stress, as these fluids pass through the sinusoids of the liver, among other stresses. RhoGEFs, RhoGAPs and RhoGDIs do not regulate RND1, but this Rho member is transcriptionally regulated (see above text). Delaying increased RND1 expression upon reperfusion could allow the liver to return to more normal conditions, prior to removing stress fibres. Our results reveal slight down-regulation of RND1 1h post-reperfusion time point (Boutros et al. unpublished results). While our laboratory's previous results demonstrated that there was actin cytoskeletal remodelling in hepatocytes, it was not determined if RND1 was implicated in the re-establishment of "normal" actin cytoskeletal rearrangement in the HepG2 cell used as an in vitro model of ischemia reperfusion (Emadali et al., 2006).

In the next three sections, the three other proteins, whose transcripts were identified with our microarray analysis (Boutros et al., 2008b)(Boutros et al. unpublished results), will be presented in the form of an overview. As these are apparently disparate proteins, or at least they appear so due to the paucity of information relating to them, no attempt to artificially link these proteins will be done.

3. Small nuclear RNA-activating protein 50 (SNAP50)

SNAP50 transcription factor was first cloned in 1996 as a subunit of the SNAP50 complex ($SNAP_c$) (Henry et al., 1996), which includes SNAP190, SNAP50, SNAP45, SNAP43 and SNAP19 (Henry et al., 1995; Ma and Hernandez, 2001). In addition, SNAP50 was found to bind to SNAP43 and DNA (Henry et al., 1996). $SNAP_c$ binds to the proximal sequence element found in human small nuclear RNA (snRNA) gene promoters and with other transcription factors such as Oct-1, recruits TATA-box binding protein to TATA-box, which allows RNA polymerase II and III to initiate transcription at snRNA promoters (Hanzlowsky et al., 2006; Jawdekar and Henry, 2008). This is a very superficial view of the transcriptional machinery that is involved in the transcription of snRNA, which include TATA-binding and TATA-less promoters (Jawdekar and Henry, 2008). In addition, each subunit of $SNAP_c$ is capable of interacting with other proteins, such as the interaction of SNAP190 with Oct-1 or that of SNAP50 with retinoblastoma protein, among other interactions. Such interactions are thought to regulate snRNA transcription depending on the environmental conditions (Jawdekar and Henry, 2008). Interestingly, RNA polymerase III is active during late G_1, S and G2 phases of the cell cycle while during the M, the G_0 and the early G_1 phases of the cell cycle it is repressed (Jawdekar and Henry, 2008) and hepatocytes in the adult liver are in G_0 phase (Haber et

al., 1993; Michalopoulos and DeFrances, 1997). In addition, genotoxic stress (in the form of UV light) down-regulated many snRNA genes transcribed by RNA polymerase II and III. The cause of this down-regulation was unknown (Jawdekar and Henry, 2008). Small nuclear RNA are implicated in a multitude of cellular functions, including mRNA splicing, rRNA processing, stimulation of RNA polymerase II elongation (Jawdekar and Henry, 2008). Although our results reveal slight down-regulation of SNAP50 mRNA (Boutros et al. unpublished results), at present it is difficult to determine the reason for this down-regulation. It may be the result of the G_0 phase of the hepatocytes or the result of cell stress. As the SNAP50 is one of five subunits of $SNAP_c$ required, along with SNAP190, for the binding of DNA, regulating SNAP50 could be one way of controlling the $SNAP_c$ activity as promoters that bind $SNAP_c$ are considered strong promoters and therefore moderation of $SNAP_c$ may be required (Jawdekar and Henry, 2008). Further testing is required to understand the involvement of SNAP50 during the process of human liver transplantation.

4. Delta-sleep-inducing peptide immunoreactor (DSIPI)

This protein is named DSIPI (Delta-sleep-inducing peptide immunoreactor, because it shares immunoreactivity with the sequence unrelated delta-sleep-inducing peptide, which is a nonapeptide) also called TSC22D3-1 (Transforming growth factor β-stimulated clone 22 domain family member 3 splice form 1), or TSC22-related inducible leucine zipper-3c or GILZ2 (glucocorticoid-induced leucine zipper 2). GILZ (GLIZ1) was first identified, in 1997, as a dexamethasone (glucocorticoid) induced gene in T-lymphocytes (D'Adamio et al., 1997). However, it should be noted that only recently were the various splice forms, different products of the same gene, identified and named GILZ1-4 (Soundararajan et al., 2007). The increased mRNA expression identified by our microarray results (Boutros et al., 2008b) corresponded to murine GILZ2 (Soundararajan et al., 2007). GILZ2 protein is expressed in many adult mouse and adult rat tissues including the liver (Soundararajan et al., 2007). In normal humans GILZ expression was localized to Küpffer cells, with no mention as to the splice form that it corresponded to (Hamdi et al., 2007). Aldosterone is a major corticosteroid involved in regulating electrolyte and fluid homeostasis in vertebrates and it up-regulates both gilz1 and gilz2 mRNAs in murine inner medullary collecting duct cells (mIMCD3) (Fiol et al., 2007). No explanation was offered as to the possible implication of such regulation in the former study. Interestingly, expressing GILZ2, in a highly differentiated mouse kidney cortical

collecting duct cell line (mpkCCD$_{C14}$), lead to cell surface expression of an epithelial sodium channel (ENaC), but had no effect on sodium current. GILZ2 slightly counteracted the EGF-mediated inhibition of the cell surface expression of this sodium channel in human HEK293 embryonic epithelial kidney cells (Soundararajan et al., 2007). In addition, GILZ2 had no effect on HEK293 proliferation upon serum stimulation and very modest reduction of phospho-ERK1/2 levels and very little interaction with c-Raf (Soundararajan et al., 2007). GILZ2 also had no effect on NFκB transcriptional activity, using a reporter gene, in mouse kidney epithelial cells (CV1-b) (Soundararajan et al., 2007).

The promoter of the human GILZ gene reveals a host of binding sites for various transcription factors including glucocorticoid responsive element (GRE), signal transducer and activator of transcription-6 (STAT-6), nuclear factor of activated T-cell (NFAT), c-Myc among others (Asselin-Labat et al., 2004). Although a review on GILZ was recently published (Ayroldi and Riccardi, 2009), it concerns GILZ1 not GILZ2 and the function of these splice forms are non-overlapping (Soundararajan et al., 2007). At present there is not enough information specifically on GILZ2 function to hypothesize on the role of GILZ2 during transplantation. The final gene that will be presented also codes for a protein that has not been characterized to any great extent, nor is it known to what family, if one exists, it belongs to.

5. Decidual protein induced by progesterone (DEPP)

Decidual protein induced by progesterone (DEPP) is also called fasting-inducing gene (FIG) or fat-specific expressed gene (FSEG). Decidual protein induced by progesterone is a protein that is specifically expressed in arteries with almost no expressed in veins (Shin and Anderson, 2005). DEPP is expressed during mouse development in a subset of endothelial cells of the dorsal aorta, as evaluated by in situ hybridization, at stage E8.75 but diminishes and disappears by stages E10.5 and E13.5, respectively. Homozygous null mice had a normal phenotype where the development and patterning of the blood vessels were concerned in both the embryos and the yolk sacs at E9.5 or E15.5. The mice were borne with a normal phenotype and were fertile be it on a 129/C57Bl6 mixed or a 129 pure background (Shin and Anderson, 2005).

In the adult mouse, endothelial cells of many tissues were also heterogeneously DEPP positive such as small diameter blood vessels in the fat around the dorsal aorta, the heart (in a subset of myocardial cells of the atrio-ventricular and bulbo-ventricular

canals but not the endothelial cells of the atria or ventricles), the kidney (glomeruli) and the liver. DEPP expression also appeared in epithelial cells of the lung, pancreatic islet cells and hepatocytes. DEPP was also expressed heterogeneously in the uterine epithelial cells of pregnant female mice, but uterine epithelial cells of non-pregnant female mice revealed no DEPP expression (Shin and Anderson, 2005). In the context adult neovascularization and more specifically, tumour and wounded skin angiogenesis, DEPP was also expressed heterogeneously, although it was not known if the endothelial cells that were DEPP positive during development were recruited for angiogenesis or if the endothelial cells that were recruited for angiogenesis up-regulated DEPP protein expression. Furthermore, there was no difference in angiogenesis profile between the DEPP null, DEPP heterozygous or the DEPP wild-type mice (Shin and Anderson, 2005).

The biological significance of this heterogeneity is not known, and the biological role of DEPP has as yet to be established, but it does not seem to involve proliferation or differentiation or apoptosis of endothelial cells (Shin and Anderson, 2005). Furthermore, as it is a cytosolic protein with several putative serine, threonine and tyrosine phosphorylation sites, it is thought to function in a signal transduction pathway (Shin and Anderson, 2005). Another report suggests that HEK293 cells, exogenously expressing DEPP protein, slightly increases phospho-ERK levels and increases Elk1 transcriptional activity as judged by a reporter gene assay (Watanabe et al., 2005). It remains possible that the function of the DEPP protein is related to stress. In line with this reasoning hypoxia (1-2% O_2) for 24h, in a chamber, up-regulates DEPP mRNA expression in malignant glioma U251 cell line, and is thought to be a prelude to angiogenesis, although this hypothesis (Ragel et al., 2007) is based on an interpretation of the results presented in the DEPP null mouse paper (Shin and Anderson, 2005) that were the antithesis of the conclusions of the authors. Our results reveal that depp mRNA increased at the 1h post-reperfusion time-point (Boutros et al., 2008b). Although hypoxia could conceivably up-regulate the expression of depp mRNA and protein, in hepatocytes and the arterial aspect of the sinusoidal endothelial cells, there is much to do before a solid hypothesis could be built around the expression of this gene and the role of this protein during human liver transplantation.

The past two sections have presented the genes that were identified by microarray during the 1h post-reperfusion time-point. As could be gleaned by their respective summaries, the genes with the best characterized proteins in the literature, lent themselves more easily to speculations as to their involvement in the process of

human liver transplantation than the others with a more limited description. Of course this does not imply that the description of their involvement during liver transplantation is necessarily correct; this holds true for the genes whose expression and protein function have been modeled in animals. The hypotheses, offered on a per gene basis, are only a starting point a means of trying to fathom a list of immediate-early genes vis-à-vis the multitude of stresses associated with the process of transplantation. This is necessary if we are to understand how gene expression and the protein products relate, not only to the stresses associated with transplantation, but also to each other. Although literature does give some information, it does not give detail in terms of mechanisms and subcellular function the way a detailed analysis does.

The next chapter will present an original contribution, which describes a transcript identified by microarray analysis, that of Map Kinase Phosphatase-1 (MKP-1). Chapter 4 details the stresses associated with transplantation, and how these are relevant to mkp-1 mRNA regulation as well as the consequences, or lack thereof, of the MKP-1 protein function in an in vitro model of cell survival. Thus without further ado, the first original contribution on MKP-1 is presented in the following chapter.

IV. Chapter 4

The MAP Kinase phosphatase-1 MKP-1/DUSP1 is a regulator of human liver response to transplantation

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A. Abstract

Orthotopic liver transplantation continues to be the only remedy for end-stage liver disease. In an attempt to decrease the ever-widening gap between organ donor and recipient numbers, and ultimately make more livers amenable to transplantation, we characterized the healthy human liver's response to ischemia and reperfusion-induced injury during transplantation. This was carried out by transcriptional profiling using cDNA microarray to identify genes whose expression was modulated at the 1-hour post-reperfusion time-point. We observed that the map kinase phosphatase-1/dual specificity phosphatase-1 (MKP-1/DUSP1) mRNA was strongly and significantly up-regulated. Validation of this observation was carried out using RT-PCR, immunoblotting and immunohistochemistry. In addition, we characterized the signalling pathways regulating MKP-1 expression using the human hepatoma cell line HepG2. Finally, by combining MKP-1 silencing with reperfusion-associated stresses, we revealed the preferential role of this protein in attenuating the activity of the JNK and p38MAPK pathways, and the resulting apoptosis, making MKP-1 a potential target for therapeutic intervention.

B. Introduction

Orthotopic liver transplantation (OLT) remains the only therapeutic option for end-stage liver diseases. Unfortunately, an increasing number of patients requiring transplantation and a widening lag time to organ availability reduce the number of required transplantations. Strategies can be devised to alleviate this problem, such as: i) including marginal livers in donor pools and ii) reducing the incidence of graft failure. Ischemia Reperfusion Injury (IRI) is one of the major contributors to post-transplant graft non-function and dysfunction (Clavien et al., 1992). During the reperfusion phase of the operation, blood-derived heat-shock and oxygen, and liver-derived reactive oxygen species produced by Küpffer cells and hepatocytes can damage the graft (Massip-Salcedo et al., 2007). Such oxidative stress promotes hepatic cell apoptosis, necrosis, activation of transcription regulators, such as NF-κB or AP-1 (Luedde and Trautwein, 2006) and induces the expression of pro-inflammatory factors such as interferon-gamma and tumour necrosis factor-α (TNF-α) (Le Moine et al., 2000). Further damage occurs by the activation of neutrophils, macrophages and lymphocytes, aggravating the reperfusion injury (Casillas-Ramirez et al., 2006). Events occurring during ischemia and within the first few hours post-reperfusion largely determine the graft's ability to tolerate IRI and recover. Unfortunately, our understanding of the mechanisms involved in the re-establishment of cellular and organ homeostasis during the reperfusion phase remains incomplete. Therefore, the identification of key molecules involved in the re-establishment of liver homeostasis represents an important avenue of investigation.

Transcriptome analysis carried out on human liver transplantation biopsies revealed IRI-mediated parallel activation of pathways which can lead to oxidative/inflammatory stress, apoptosis or cell proliferation (Conti et al., 2007; Defamie et al., 2008). Although some characterization exists, networks controlling transcriptional activation, specific to the early reperfusion phase of human liver transplantation, remain poorly characterized. In an attempt to conduct an unbiased analysis and identify the genes regulated during the reperfusion phase of liver transplantation, we performed a transcriptional profiling analysis on human liver biopsies collected prior to, 10min and 60 min after reperfusion. As a result, we identified approximately 30 genes whose expression level was significantly modulated during the first hour of reperfusion. Among these, we observed the induction of mkp-1 mRNA expression, the transcript coding for the dual-specificity phosphatase, MKP-1, during the reperfusion phase. Consequently,

we investigated MKP-1's involvement during this phase of liver transplantation and demonstrated that its regulation was part of a specific transcriptional program.

C. Materials and Methods

1. Materials – Hydrogen peroxide, $CoCl_2$, BAPTA-AM, Antimycin A were purchased from Sigma. DTT was from Bioshop. Trizol, DMEM and Rnase H were from Invitrogen. Tunicamycin was from Calbiochem. Fetal Bovine Serum (FBS) was purchased from Hyclone. Anti-calnexin antibody was from Stressgen. All the oligonucleotide primers used in this study were purchased from AlphaDNA (Montreal, Qc, Canada). Thermoscript RT-PCR System, Superscript II and Superscript III RT RNAse H- (Invitrogen). PD98059 (Cell Signaling), roscovitin, chelerythrine and DRB (CalBiochem), genistein (Sigma).

2. Patients and tissue collection - 0.5 cm^3 biopsies were surgically removed in the course of 8 liver transplantation procedures performed within the McGill University Health Centre with approval of the institution's ethics committee (ERB 05-003) as previously described (Emadali et al., 2007; Emadali et al., 2006; Emadali et al., 2005) (Figure 1A). Briefly, liver biopsies were collected during re-implantation of the organ in the recipient, just prior to clamp removal from the hepatic portal vein (R0), and 10 minutes (R10) and 60 minutes (R60) after the blood flow had been re-established through the portal vein. The 10 min time-point biopsy specimen accounts for any mRNA contributed by the recipient blood when performing the microarray analysis (Figure 1B). The average cold ischemia phase was 8h30 ± 2h15 whereas the average warm ischemia time was 48 min ± 26min. For every time-point, each biopsy was divided into three pieces. Two pieces were snap- frozen and stored in liquid nitrogen until RNA or protein extraction. The third piece was embedded in OCT prior to being snap-frozen in liquid nitrogen.

3. RNA isolation - Total RNA was extracted using Trizol following the manufacturer's instructions. Frozen tissue samples were put in Trizol prior to homogenization. The quantity of RNA was evaluated by either a RiboGreen kit (Molecular Probes) using CytoFluor 2300 (Millipore) to evaluate fluorescence intensity (for microarrays) or spectrophotometry at 260 nm (for RT-PCR). The quality of the RNA was determined by non-denaturing agarose gels. The relative purity was determined by $A_{260/280}$ ratio by spectrophotometry.

4. RNA labeling – We used a slightly modified version of a previously described method (Nantel et al., 2002). Briefly, cyanine 5-dCTP and cyanine 3-dCTP were used to label the cDNA, prepared from total RNA deriving from: control, 10min and 60 min reperfusion time-points. The reaction was stopped and the RNA degraded by incubating

with RNAse-H and RNAse-A for 15 min at 37ºC. RNA was purified using Qiagen columns.

5. Hybridization - The method is essentially described previously (Nantel et al., 2002), with the following modifications. Each probe on the Human 19k micro-arrays were spotted in duplicate and distributed between two slides (A and B). As a complete set of printed 19k ESTs is comprised of a slide A and a slide B, hybridization was carried out with both slides face to face, instead of having a coverslip over each slide. This "sandwiching" of slides A and B allowed for a more homogeneous and accurate hybridization within a complete set of 19k genes, than independent hybridizations of slides A and B would have afforded. Hybridizations were done for the 0-60min and 10-60min sets, with corresponding dye-swaps for each set.

6. Microarray analyses – For microarray analyses, 7 livers were biopsied at R0, R10 and R60 (Reperfusion time 0, 10 and 60 min). For all 7 patients, we used DNA microarray to compare RNA from the R60 samples to their respective R0 samples. However, of the 7 biopsy sets (R0, R10, R60) (see Table 1, chapter 3 section A, p91), three R10 biopsies did not contain enough tissue to extract sufficient mRNA for microarray analysis, consequently we could only compare 4 of the R60-R10 analyses. For each of the biopsy time-points dye-swaps were done, thus using 2 microarrays per biopsy-time-point for a total of 22 microarrays.

The microarrays were scanned using a ScanArray 5000 scanner (Perkin-Elmer-Cetus) at a 10μm resolution. The resulting TIFF files were quantified with QuantArray software (Perkin-Elmer-Cetus). Each spot of the array had to satisfy three criteria of quality control for inclusion into normalization and analysis: 1) the signal intensity had to be greater than the surrounding background; 2) the raw intensities of duplicate spots, per gene, had to be within 50% of each other; 3) the signal intensity for each spot had to be within the dynamic range of the photomultiplier tube. This was determined by the user with the help of a scatter plot of the $\log_{10}$ of background-subtracted intensities (Nantel et al., 2002).

7. Data analyses - Normalization and analysis were conducted using GeneSpring v5.0 (Agilent Technologies). The ratio of intensity of the two channels was normalized by fitting a Lowess curve to the log-intensity versus log-ratio plot for the entire array. We selected a list of significantly-modulated genes with an average change in transcript abundance of at least 2-fold in either the R60R0 or the R60R10 comparisons. Genes whose expression was significantly regulated were then annotated

using the g:Profiler program (http://bitt.cs.ut.ee/gprofiler/index.cgi) and clustered based on their GO annotation (http://www.geneontology.org/) using the PermutMatrix program (Caraux and Pinloche, 2005). Functional network analysis was carried out using the STRING suite (Snel et al., 2000; von Mering et al., 2007). The resulting scale-free functional interaction network was then annotated using the Medusa program (Hooper and Bork, 2005).

8. RT-PCR analyses - HSP70 (forward: 5'-ATG GCC AAA GCC GCG GCG-3', reverse: 5'-CTT GTC TCC GTC GTT GAT CA-3'); MKP-1 (forward: 5'-AAC AGT CGA CCC CAT GGG G-3', reverse: 5'-ACC AGG AGC TGA TGT CTG CC-3'); and S16 (Emadali et al., 2005) primers were designed as follows. Semi-quantitative RT-PCR analysis was performed as previously described (Nguyen et al., 2004).

9. Cell culture and treatments - HepG2 cells were cultured in DMEM with 10% foetal bovine serum (FBS) until the experiments. To evaluate MKP-1/DUSP1 mRNA expression in response to various environmental stresses, HepG2 cells were plated at 2.0 x 10^6 cells per 60mm plate in DMEM with 10% FBS. After 2 days, the growth medium was replaced with 2 ml of DMEM supplemented with 10% FBS and containing one of the various stressors (see below) or the vehicle control. To mimic the cold stress/heat shock aspect of transplantation, HepG2 cells were plated as above. Two days later, the cells were washed twice with either 4ºC PBS or 37ºC PBS and afterwards incubated with DMEM or DMEM with 10% FBS or University of Wisconsin (UW) Beltzer solution, at 4ºC or 37ºC for 5 hours. As the cultures incubated at 4ºC were outside the CO_2 incubator, both DMEM and DMEM with 10% FBS used for incubation at 4ºC or 37ºC, had 20 mM HEPES buffer added. After this incubation period, the various media were removed and replaced by 37ºC DMEM with 10% FBS, no HEPES, and incubated for various time-points at 37ºC. To determine the signalling pathways used by the various stressors, kinase inhibitors were used. More specifically, HepG2 were plated at a density of 2 x 10^6 cells per 60mm plate and cultured until ~80% confluent. All kinase inhibitors were used overnight at the final concentrations reported in figure 6. The next day, either antimycin A, DTT or heat shock was used as stressors. For the inhibitor assay, the cells were neither washed with PBS nor was the medium (DMEM with 10% FBS) replaced prior to the addition of stimulator. MKP-1 silencing was carried out using the previously reported plasmids (Wu et al., 2004) and using 5 and 10ug of plasmid DNA. The transfection protocol was previously described (Emadali et al., 2006).

10. Kinase assays – Kinase assays were performed as previously described (Chevet et al., 1999). Briefly, cell lysates were collected at different times post-reperfusion and JNK-1, $p38^{MAPK}$ and ERK-1 were immunoprecipitated using specific antibodies (SantaCruz Biotechnologies). Immunoprecipitates were incubated in the presence of $^{32}P\gamma$-ATP (100 µM) and 2 µg of the respective kinase substrates Myelin Basic Protein (for ERK-1), GST-Jun (for JNK-1) and GST-ATF2 (for $p38^{MAPK}$) at 30ºC for 15 min. The supernatants were then collected, TCA precipitated and the resulting pellets counted on a beta scintillation counter.

11. Immunobloting and immunochemistry - Immunoblot analyses were carried out as described previously (Chevet et al., 1999). Liver tissue sections (12 µm thick) on glass slides were fixed in 3.7% formaldehyde. Tissue sections were immunostained using an anti-MKP-1 antibody (Santa Cruz Biotechnologies) using the Envision+® rabbit kit (Dako, Mississauga, ON) according to the manufacturer's instructions. Sections were then counterstained using hematoxylin (Vector, Burlington, ON) then dehydrated and mounted using Permount (Fisher, Nepean, ON).

12. Apoptosis measurement – This was carried out as previously described (7). Briefly, HepG2 cells were subjected or not to MKP-1 silencing and to a 2h in vitro cold ischemia as described above. Twenty-four hours later the percentage of apoptotic cells was determined. Apoptosis was measured by Annexin V staining using the manufacturer's recommendations (BD Biosciences) and quantified using FACS analysis.

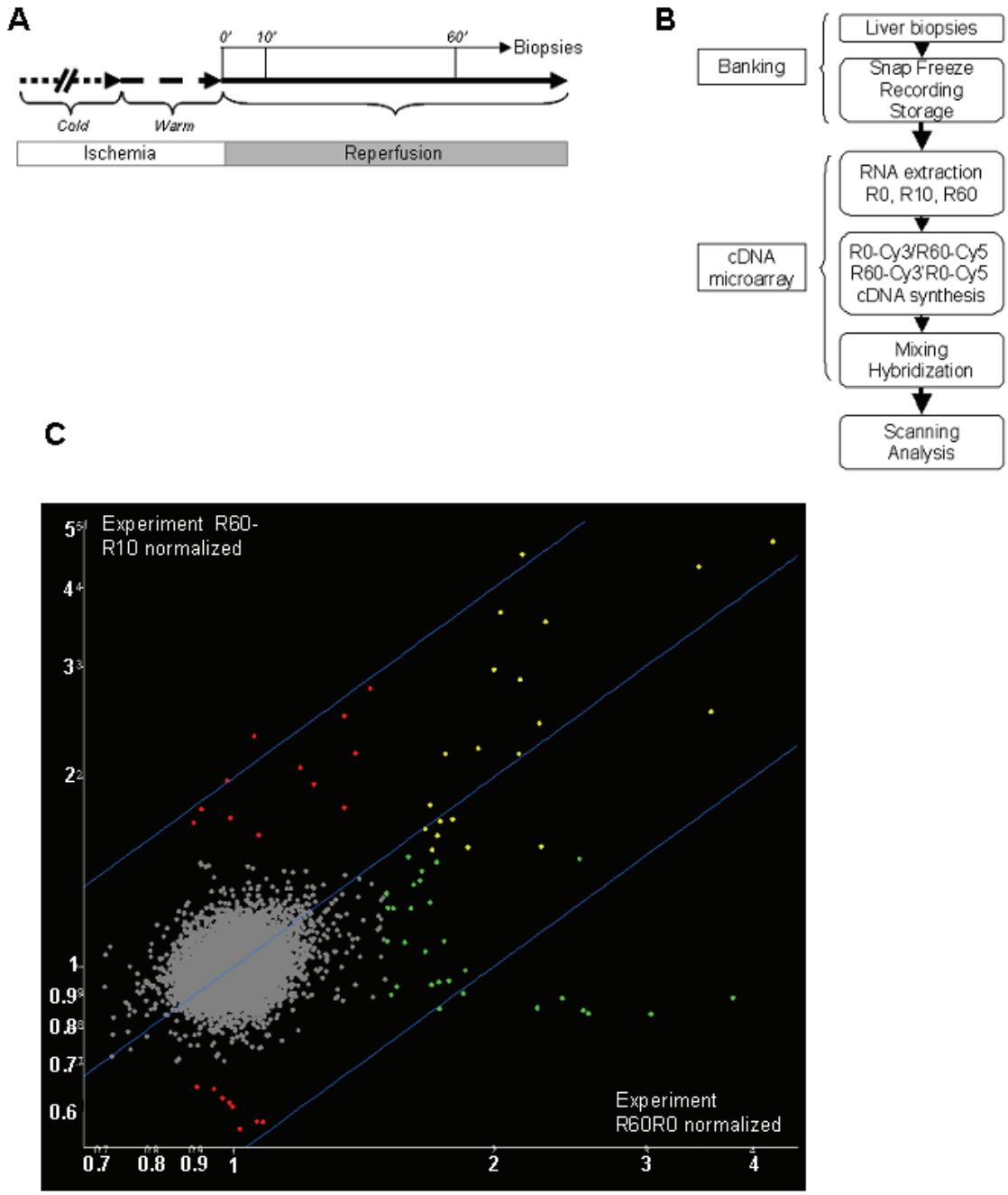
D. Results

1. Reperfusion-mediated up-regulation of MKP-1 mRNA

Using three time-point biopsy-specimens (see Materials and Methods), we elaborated and compared two normalized lists of genes: first list 60 min vs 10 min (R60R10) and second list 60 min vs 0 min (R60R0). Comparing these two lists allowed us to eliminate any mRNA whose apparent increase in expression was the result of the recipient blood, during the reperfusion phase of the operation. For instance, the 60 vs 0 min normalization revealed a 3-fold increase in globin mRNA levels while the 60 min vs 10 min normalization did not (Table 2 p. 179, 180 and Supplementary tables 1 and 2 see http://www3.interscience.wiley.com/journal/121511581/suppinfo). This observation suggests that the increasing amount of globin mRNA is most likely contributed by the recipient blood, rather than an increased expression in liver tissue. The globin mRNAs were therefore eliminated from further analyses, as they were not significantly regulated. A more complete analysis of gene expression profiling is given in Table 2 (p. 179, 180). Based on the comparison of both lists of genes, R60R10 and R60R0, we generated a scatter plot (Figure 1C), which led to a preliminary list of genes devoid of recipient blood-derived contributions. After subtracting the false positives, we kept only the transcripts that showed at least a 1.5-fold change in abundance during the first hour of reperfusion. Although fold variation was important, consistency in the results across patients and "multiple hits" with different ESTs (expressed sequence tags) within the same array had higher priority (Enjalbert et al., 2003; Nantel et al., 2002). These results were recapitulated using a scatter plot of the fluorescence intensities on the Experiment and Control channels (Supplementary Figure 1). The functional distribution of the 23 non-redundant genes, with a significant change in expression upon reperfusion, was established using a Gene Ontology (GO) – based analysis with the g:Profiler program. The GO annotation revealed that the up-regulated genes collectively belonged to 10 functional groups which where partitioned into 2 major clusters (Figure 1D). The first cluster was mainly associated with functions relating to the biological processes associated with responses to stress, chemical and biotic stimuli (GO:0006950; GO:0042221; GO:0009607) and the second was associated with the regulation of biological, cellular and developmental processes (GO:0048519; GO:0048523; GO:0050793). Not surprisingly, our list is comprised of immediate-early genes due to the 60 min time-point. To evaluate potential functional interactions existing between these 23 genes, we used the STRING suite as described in Materials and Methods. Interestingly,

out of the 23 genes selected above, 21 were found to belong to a highly connected functional network of 23 nodes and 80 edges based on experiment, database and literature-related information (Figure 1E). Moreover, the gene network built from our transcriptional profiling revealed the expression of mRNA coding for proteins implicated in signalling (purple), transcription (orange), chaperone (blue) and metabolic (green) functions during the reperfusion phase of the operation.

Our microarray results were confirmed, using RT-PCR, for samples previously used for microarray experiments as well as independent samples. RT-PCR revealed a 9-fold and 4-fold increase for Hsp70 and MKP-1 mRNAs, respectively, in the 60 min time-point biopsies compared to the 0 time-point biopsies (Figure 2A). Immunoblotting analysis of MKP-1 protein, using an anti-MKP-1 antibody on liver biopsy extracts, revealed a 1.5 fold increase following normalization to the endoplasmic reticulum (ER) membrane-resident protein calnexin (Figure 2B). Finally, immunohistochemistry performed on frozen human liver sections revealed both cytoplasmic and nuclear staining for MKP-1 (Figure 2D) when compared to a control antibody (Figure 2C). Collectively, these data indicate that the ischemia-conditioned liver responds to reperfusion-related stresses, in part, by up-regulating MKP-1 mRNA and protein in hepatocytes.


A
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Snap Freeze
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Storage
cDNA
microarray
RNA extraction
R0, R10, R60
R0-Cy3/R60-Cy5
R60-Cy3'R0-Cy5
cDNA synthesis
Mixing
Hybridization
Scanning
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Experiment
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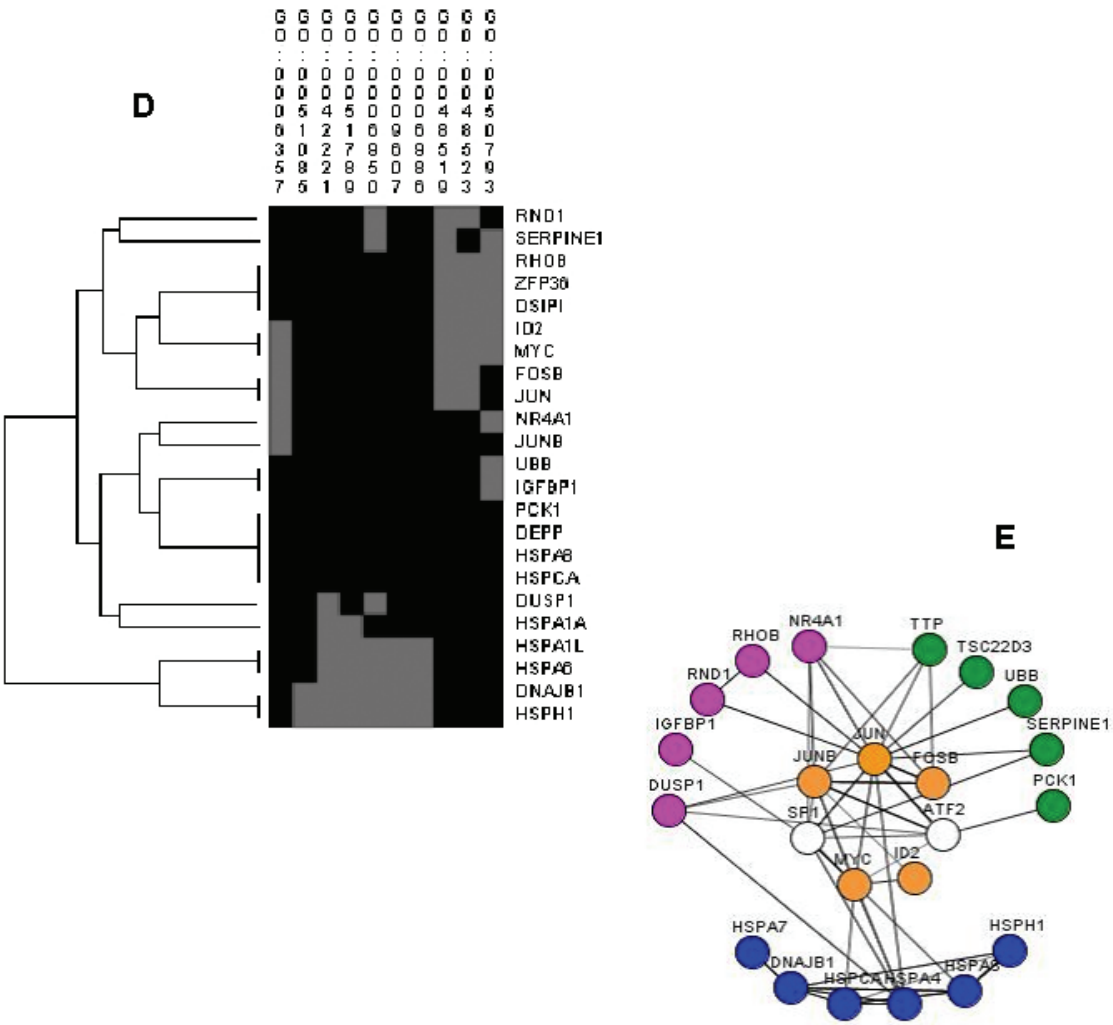
Figure 1. A-C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RHOB
ZFP36
DSIPI
ID2
MYC
FOSB
JUN
NR4A1
JUNB
UBB
IGFBP1
PCK1
DEPP
HSPA8
HSPCA
DUSP1
HSPA1A
HSPA1L
HSPA6
DNAJB1
HSPH1
E
RHOB
NR4A1
TTP
TSC22D3
RND1
UBB
IGFBP1
JUN
SERPINE1
JUNB
FOSB
DUSP1
PCK1
SP1
ATF2
MYC
ID2
HSPA7
HSPH1
DNAJB1
HSPCA
HSPA4
HSPA8

Figure 1. D, E

Figure 1 - Microarray analysis of human liver biopsies collected during the reperfusion phase of transplantation. A. Time-line representation of the biopsy collection protocol followed in our study. **B**. Flow-chart representation of the experimental procedure used in our microarray analysis. **C**. Scatter-plot representation of the gene distribution on a per normalization basis. The genes from the R60R10 group are shown in red, the genes form the R60R0 group (including globin genes) are shown in green, and the genes that have been modulated in both groups (R60R10 and R60R0) are shown in yellow. . **D**. Gene Ontology-based clustering of the known up-regulated genes during human liver reperfusion. (GO:0006357 = regulation of transcription from RNA polymerase II promoter; GO:0051085 = chaperone co-factor dependent protein folding; GO:0048519 = negative regulation of biological process; GO:0050793 = regulation of developmental process; GO:0048523 = negative regulation of cellular process; GO:0009607 = response to biotic stimulus; GO:0051789 = response to protein stimulus; GO:0006986 = response to unfolded proteins; GO:0009607 = response to biotic stimulus; GO:0006950 = response to stress; GO:0042221 = response to chemical stimulus). **E.** STRING network representation of 21 out of the 23 genes identified in Table 2 (p.179, 180). The nodes represent genes whose protein products have been categorized according to the following colour code: blue nodes for chaperone functions; purple nodes, signalling; green nodes, metabolism (RNA and proteins); orange nodes, transcription. The edges are representative of the various interaction types available through STRING suite based on experimental, databases and text mining.

Table 1: Clinical characteristics of the transplanted patients

Liver biopsies (R0, R10, R60) were collected from patients (#6, 7, 11, 26, 27, 28, 30). This table reports the status of the patient (a, b, c indicate the cause of death as a: recurrence of viral hepatitis C; b: cerebrovascular accident; c: myocardial ischemia and infarction, respectively) and of the graft as well as blood concentrations at 22/29h and 5/6d post- reperfusion of Aspartate aminotransferase (AST; norm 6-35 units/L); Alanine aminotransferase (ALT, norm 6-45 units/L); Total bilirubin (TB; norm 1.7-18.9 umol/L); Alkaline phosphatase (ALP; norm 25-115 units/L). International Normalization Ratio (INR: norm 0.88-1.13). Shaded areas indicate values in the normal range. N = No, Y = Yes; ND = not determined.

	L6	**L7**	**L11**	**L26**	**L27**	**L28**	**L30**
Patient death	N	N	Y	Y	Y	N	N
Reason of death	-	-	a	b	c	-	-
Graft death	N	N	-	-	-	N	N
AST (units/L) 22/29h	804	2066	720	1089	179	2692	ND
ALT (units/L) 22/29h	894	1921	378	1212	144	1032	1349
TB (umol/L) 22/29h	24	83	34	212	88	66	40
ALP (units/L) 22/29h	83	107	70	61	130	50	85
INR 22/29h	2.11	1.64	1.45	2.56	1.49	5.86	1.78
AST (units/L) 5/6d	48	62	62	ND	80	45	33
ALT (units/L) 5/6d	277	496	101	ND	104	233	215
TB (umol/L) 5/6d	27	85	127	ND	170	265	38
ALP (units/L) 5/6d	202	152	95	ND	117	52	67
INR 5/6d	1.16	1.16	1.27	ND	1.29	2.57	1.18

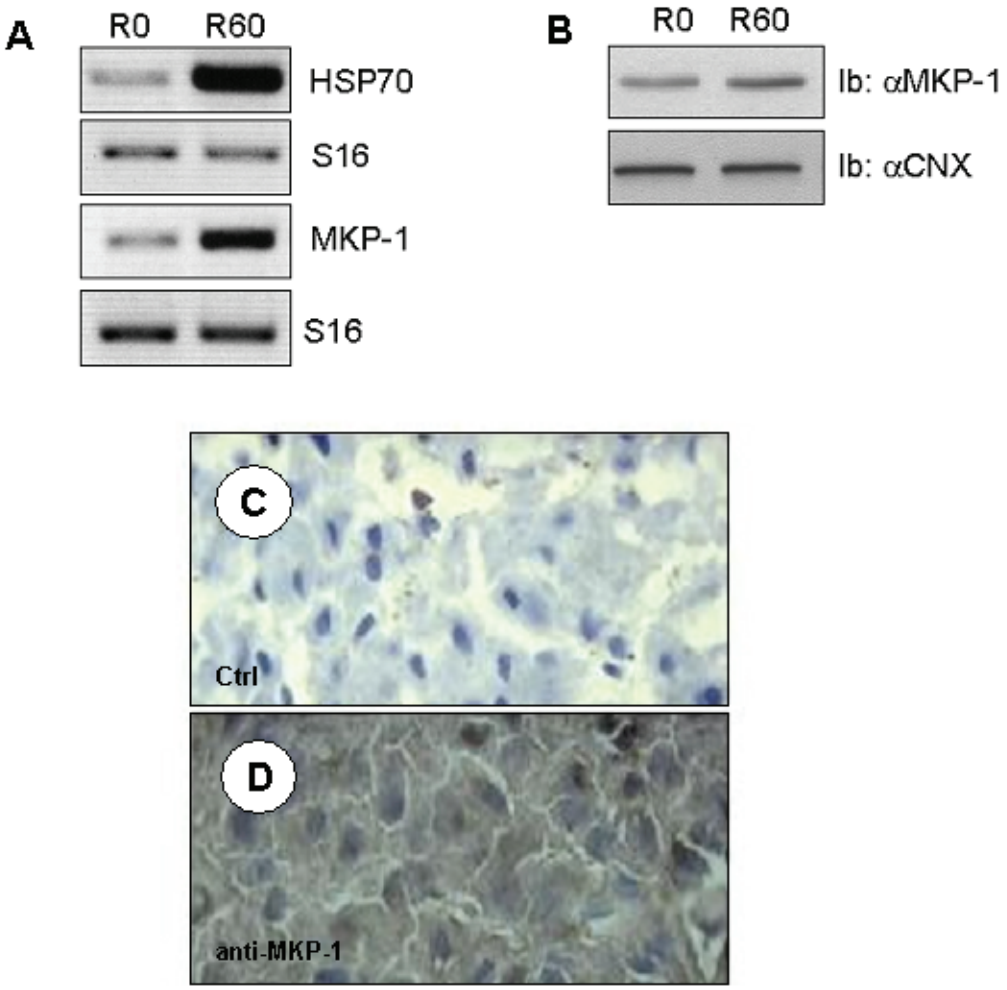

Figure 2 –Validation of microarray results. **A**. Validation by RT-PCR. Two biopsies collected at 0 and 60 min reperfusion time-points were chosen from three different donor patients. For each biopsy, total RNA was used for RT-PCR. HSP70 or MKP-1 messenger was amplified from the zero and 60min reperfusion time-points using the same number of cycles for both time-points. S16, a gene coding for a ribosomal protein, was used as a control (30 cycles). This is a representative gel of 3 experiments **B**. Total protein extracted from human liver biopsies collected at zero and 60min time-points were used for Western blot. Anti-MKP-1 antibody was used to reveal MKP-1, while an anti-calnexin antibody was used as a loading control. Representative immunoblot, 1 of 3 different experiments. Immunohistochemistry: 12µm thick frozen human liver sections. Images taken at 100X magnification. Hematoxilin staining reveals the nuclei in blue. **C.** R0 secondary antibody only. **D**. R60 rabbit anti-MKP-1, 1/250 dilution. The staining appears to be both nuclear and cytosolic in hepatocytes.

Table 2: List of all the up-regulated genes in R60 samples vs. R10 samples presenting at least two values with fold change > 1.5 in the sample set. This list was generated following the removal of genes whose expression was found up-regulated at R10 (as part of the blood contribution to this transcriptome analysis). Complementary DNA probe, Genbank accession, entry description, gene names are indicated as well as the average fold increase in 7 samples and the p value observed for each probe. Finally, the number of samples where the fold increase was found to be > 1.5 is also indicated.

cDNA probes	Swissprot/TrEMBL Acc	Description	Gene name	Average Fold increase	Number of cases with Fold Increase >1.5
H04421	P28562	Dual specificity phosphatase 1	DUSP1	3.71	7
H29136	P28562	Dual specificity phosphatase 1	DUSP1	4.01	7
R79387	P28562	Dual specificity phosphatase 1	DUSP1	3.14	4
H17836	P34931	ym41b04.r1 Soares infant brain 1NIB Homo sapiens cDNA clone IMAGE:50615	HSPA1L	2.45	4
W63752	P25685	DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1	2.32	2
W40235	Q92598	Heat shock 105kDa/110kDa protein 1	HSPH1	2.12	3
BI715153	P17066	Heat shock 70kDa protein 6 (HSP70B')	HSPA6	2.14	4
N27681	P08107	Heat shock 70kDa protein 1A	HSPA1A	6.48	7
T74240	P08107	Heat shock 70kDa protein 1A	HSPA1A	2.91	4
BM450631	Q14568	Heat shock 90kDa protein 1, alpha-like 3	HSPCA	1.87	5
H62639	P11142	Heat shock 70kDa protein 8	HSPA8	1.88	4
BM920804	Q92730	Rho family GTPase 1	RND1	2.82	3
BI761144	Q92730	Rho family GTPase 1	RND1	1.65	3
W67471	P62745	Ras homolog gene family, member B	RHOB	1.21	1
R32051	P62745	yh63a08.r1 Soares placenta Nb2HP Homo sapiens cDNA clone IMAGE:134390	ARHB/ RHOB	2.53	4

Table 2 (continued). List of all the up-regulated genes in R60 samples vs. R10 samples presenting at least two values with fold change > 1.5 in the sample set. This list was generated following the removal of genes whose expression was found up-regulated at R10 (as part of the blood contribution to this transcriptome analysis). Complementary DNA probe, Genbank accession, entry description, gene names are indicated as well as the average fold increase in 7 samples and the p value observed for each probe. Finally, the number of samples where the fold increase was found to be > 1.5 is also indicated.

cDNA probes	Swissprot/TrEMBL Acc	Description	Gene name	Average Fold increase	Number of cases with Fold Increase >1.5
AA046598	P08833	Insulin-like growth factor binding protein 1	IGFBP1	1.80	4
AL575338	P08833	Insulin-like growth factor binding protein 1	IGFBP1	1.34	2
AA043477	P26651	Zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	2.39	6
BQ066997	P26651	Zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	1.73	4
W38673	Q02363	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	ID2	1.75	6
R83815	Q02363	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	ID2	1.51	3
BM782284	P62988	Ubiquitin B	UBB	2.44	3
BM999610	P05412	V-jun sarcoma virus 17 oncogene homolog (avian)	JUN	2.77	5
BQ048940	P17275	Jun B proto-oncogene	JUNB	2.12	3
H14887	P53539	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	2.64	5
H43827	A2A2C9	V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1.60	2
W87741	A2A2C9	V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1.54	3
AA115157	Q99576	TSC22 domain family, member 3	DSIPI	1.92	5
R01835	Q9NTK1	ye84g08.r1 Soares fetal liver spleen 1NFLS Homo sapiens cDNA clone IMAGE:124478.	DEPP	1.80	4
R86197	P35558	Phosphoenolpyruvate carboxykinase 1 (soluble)	PCK1	1.47	3
R21221	P05121	Serine (or cysteine) proteinase inhibitor, clade E member 1	SERPINE1	1.30	3
N92185	P22736	Nuclear receptor subfamily 4, group A, member 1	NR4A1	1.84	5

2. IRI-related stresses leading to the up-regulation of MKP-1 mRNA

In an attempt to determine which of the stresses, implicated in the process of human liver transplantation, was involved in the up-regulation of MKP-1 mRNA and protein expression, we used the well characterized HepG2 cultures as a model (Emadali et al., 2006). The ischemia and reperfusion-associated stresses were divided into four broad categories, namely thermal, oxidative and metabolic stresses, as well as chemically induced hypoxia-reperfusion. Each category was, in turn, subdivided into components (Figures 3-5). First, we assessed the effects of a 4-37ºC thermal stress versus normothermia using three different media to account for the influence of thermal stress and storage solutions on the expression of the MKP-1 transcript. More specifically, HepG2 cells were incubated with either DMEM + 10% FBS, DMEM only or University of Wisconsin (UW) solution at 4°C (Figure 3A) or 37°C (Figure 3B), for 5h. Thereafter, all cultures (Figure 3A, B) were incubated with DMEM + 10% FBS at 37°C for the indicated times. The heat shock effect on MKP-1 mRNA expression was most noticeable (Figure 3A) as compared to its normothermic counterpart (Figure 3B). However, the use of UW solution delayed the induction of MKP-1 mRNA expression in the heat shock model (Figure 3A). Finally, the presence of 10% FBS slightly increased the expression of MKP-1 mRNA in the normothermic paradigm (Figure 3B), but only when cultures were previously cultured in DMEM and not in UW solution.

The next transplantation-relevant stress modeled was the reducing-oxidative (redox) stress, using DTT or H_2O_2 as a reducing or oxidizing agent respectively. DTT induced both a time and dose-dependent expression of MKP-1 mRNA, with the 30mM concentration being more effective than the 100mM at all times tested (Figure 4B). In contrast, hydrogen peroxide which had previously been reported to increase MKP-1 mRNA expression (Xu et al., 2004), had no effect on this messenger using the present doses, times and cellular model (Figure 4A). Thus, in our model reducing, not oxidative, stress was a more potent inducer of MKP-1 mRNA expression. The facet of metabolic stress relating to ischemia-reperfusion was subdivided into three components (Figure 5A-C). Antimycin-A, a known mitochondrial complex III inhibitor, was one of the strongest MKP-1 mRNA inducers in our experimental model (Figure 5A). The next metabolic stressor to be tested alone or in conjunction with antimycin A was tunicamycin, the N-glycosyl transferase inhibitor was used to simulate the Unfolded Protein Response (UPR) (Figure 5B). As the UPR was activated during both phases of liver transplantation (Emadali et al., 2005), MKP-1 mRNA expression might represent a potential target of this

pathway. When tunicamycin was used alone, there was a slight induction of the MKP-1 transcript while the combination of both stressors seemed to have an anergistic effect (Figure 5B) in comparison to antimycin-A only treatment. In contrast, calcium depletion related stress had no effect on MKP-1 mRNA expression level, as seen with the membrane permeable calcium chelator BAPTA-AM (Figure 5C) irrespective of the concentrations or times tested.

Finally, as both ischemia and hypoxia have been implicated in the regulation of MKP-1 mRNA (see discussion), we used cobalt chloride ($CoCl_2$), a prolyl hydroxylase inhibitor, to stabilize HIF-1α, and simulate hypoxia. Using a 100μM dose of $CoCl_2$ and various time-points, we did not observe any modulation of the MKP-1 mRNA levels in either “hypoxic” or “reperfusion” phases (data not shown).

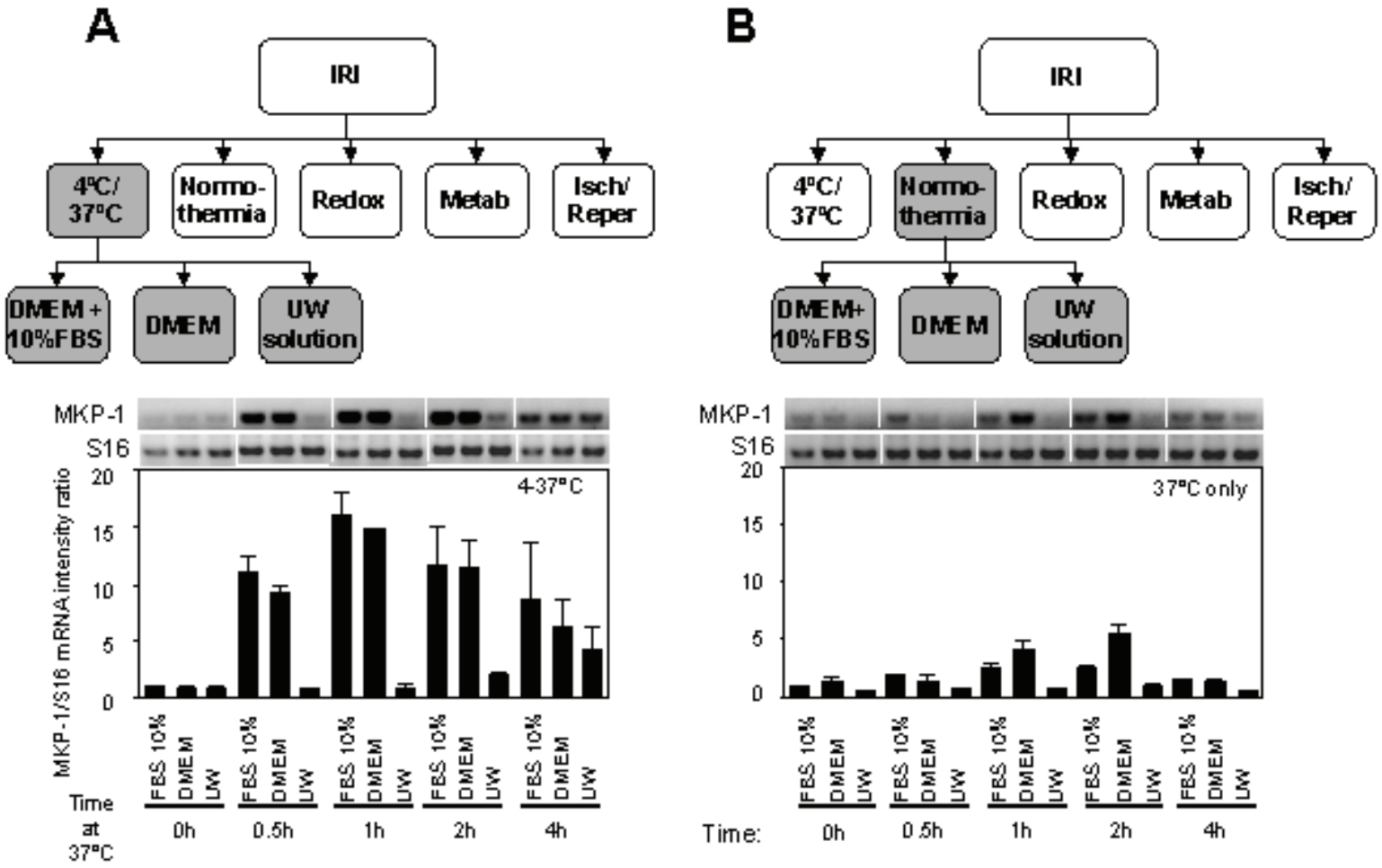
A
IRI
4ºC/ 37ºC
Normo-thermia
Redox
Metab
Isch/ Reper
DMEM + 10%FBS
DMEM
UW solution
MKP-1
S16
MKP-1/S16 mRNA intensity ratio
4-37ºC
FBS 10%
DMEM
UW
Time at 37ºC
0h
0.5h
1h
2h
4h
B
IRI
4ºC/ 37ºC
Normo-thermia
Redox
Metab
Isch/ Reper
DMEM+ 10%FBS
DMEM
UW solution
MKP-1
S16
37ºC only
Time:
0h
0.5h
1h
2h
4h

Figure 3

Figure 3 – Thermal stress mediated expression of MKP-1/DUSP1 mRNA in HepG2 cultures. **A**. The effect of cold stress-warm reperfusion (4-37ºC) in three different media (DMEM + 10% FBS, DMEM only and UW) on MKP-1/DUSP1 mRNA expression by RT-PCR. Normalization was carried out using S16 mRNA. **B**. Same media as A, but under normothermic conditions (no cold stress or heat shock). Quantification for A and B was performed on 2 independent experiments and is represented +/- SEM.

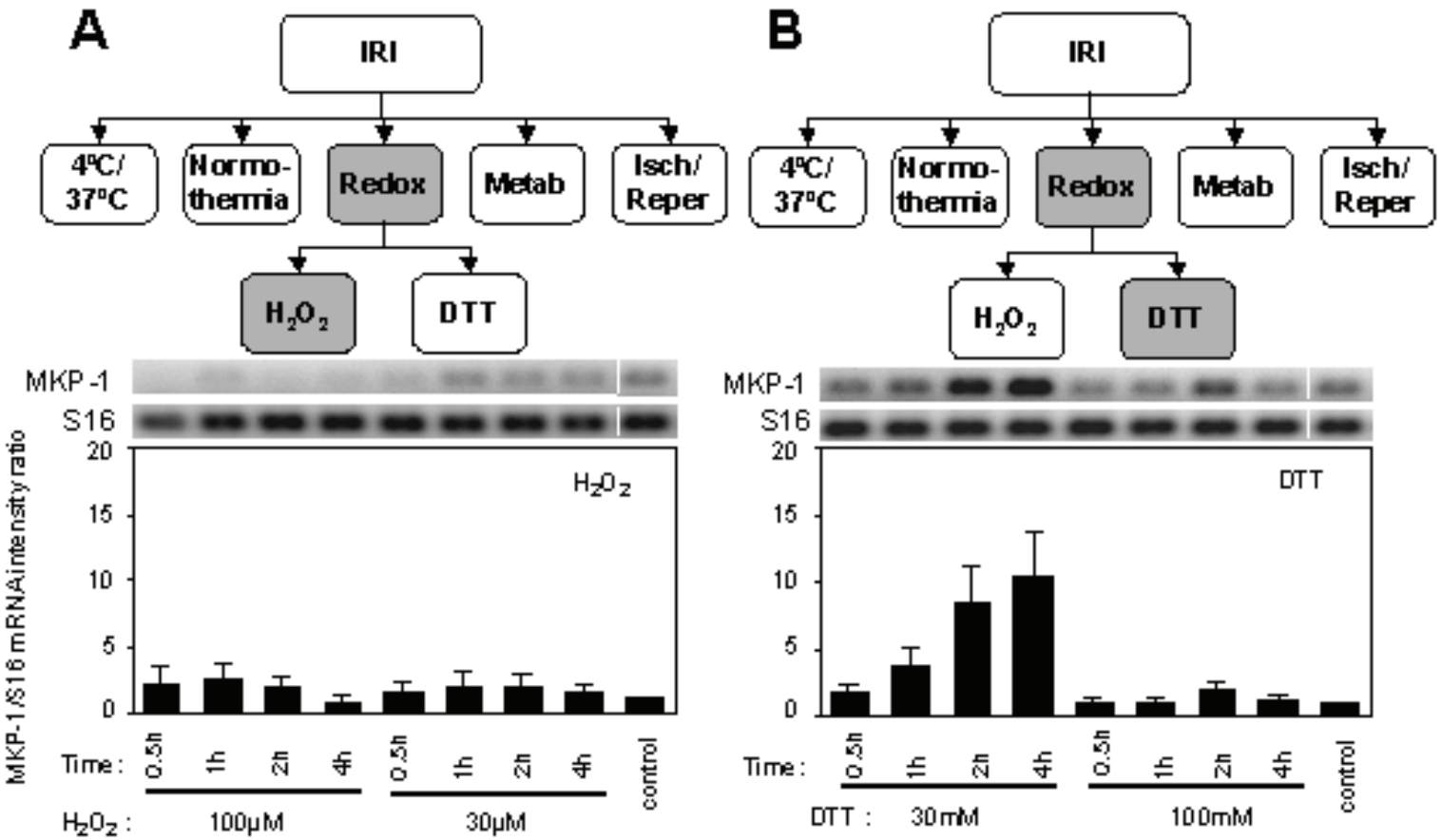
A
IRI
4ºC/ 37ºC
Normo-thermia
Redox
Metab
Isch/ Reper
H2O2
DTT
MKP-1
S16
MKP-1/S16 mRNA intensity ratio
20
15
10
5
0
H2O2
Time:
0.5h
1h
2h
4h
0.5h
1h
2h
4h
control
H2O2 :
100µM
30µM
B
IRI
4ºC/ 37ºC
Normo-thermia
Redox
Metab
Isch/ Reper
H2O2
DTT
MKP-1
S16
20
15
10
5
0
DTT
Time:
0.5h
1h
2h
4h
0.5h
1h
2h
4h
control
DTT :
30mM
100mM

Figure 4

Figure 4 – Oxidative stress mediated expression of MKP-1/DUSP1 mRNA in HepG2 cultures. A. The expression of MKP-1/DUSP1 mRNA was evaluated by RT-PCR in HepG2 cells treated with either 100µM or 30 µM H_2O_2 for 0.5, 1, 2 and 4h. **B.** The same experiments were carried out using DTT instead of H_2O_2. Normalization was carried out using S16 mRNA. Quantification was performed on 3 independent experiments and is represented +/- SEM.

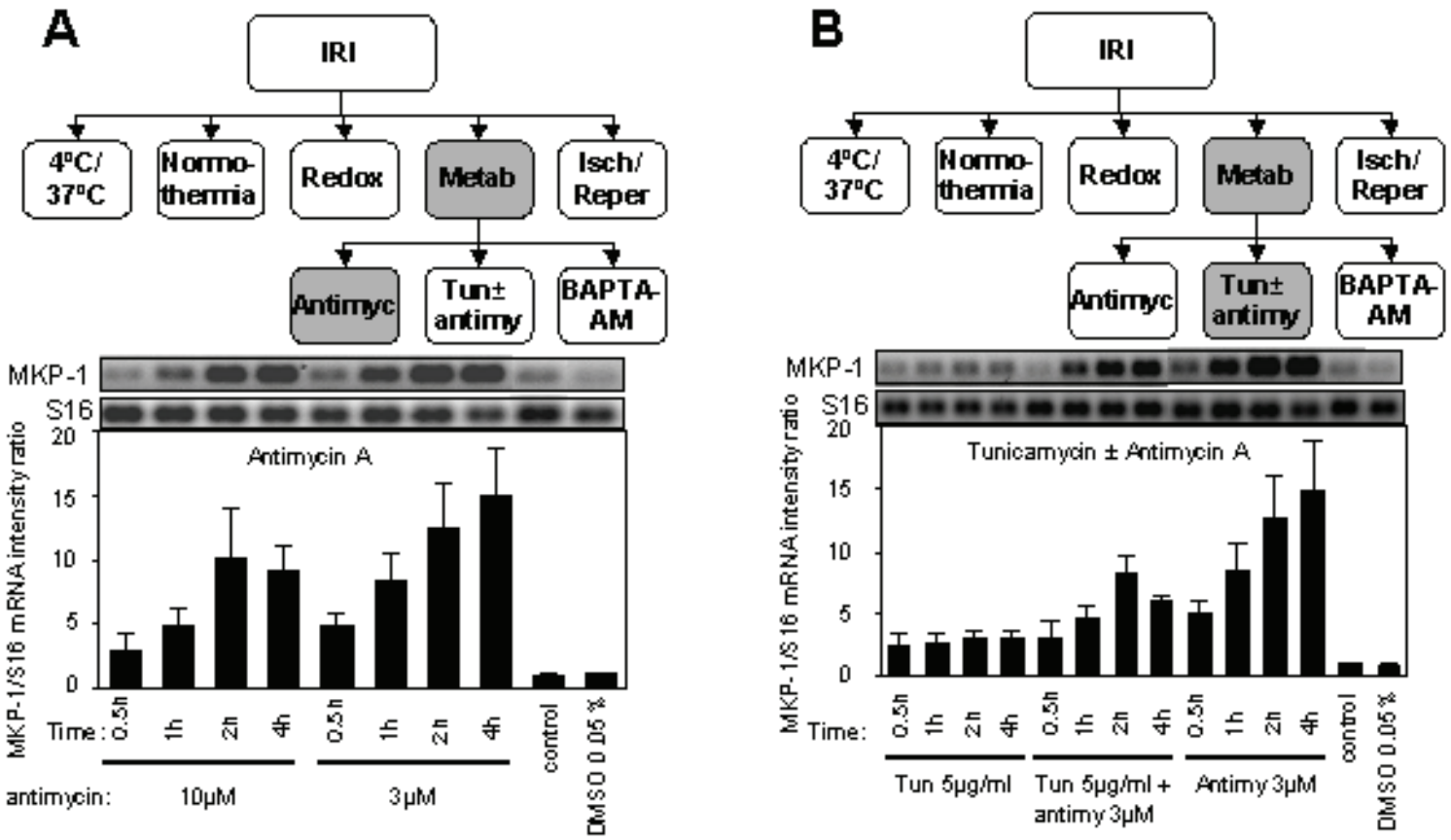
A
IRI
4°C/ 37°C
Normo-thermia
Redox
Metab
Isch/ Reper
Antimyc
Tun± antimy
BAPTA-AM
MKP-1
S16
Antimycin A
MKP-1/S16 mRNA intensity ratio
20
15
10
5
0
Time:
0.5h
1h
2h
4h
0.5h
1h
2h
4h
control
DMSO 0.05 %
antimycin:
10µM
3µM
B
IRI
4°C/ 37°C
Normo-thermia
Redox
Metab
Isch/ Reper
Antimyc
Tun± antimy
BAPTA-AM
MKP-1
S16
Tunicamycin ± Antimycin A
MKP-1/S16 mRNA intensity ratio
20
15
10
5
0
Time:
0.5h
1h
2h
4h
0.5h
1h
2h
4h
0.5h
1h
2h
4h
control
DMSO 0.05 %
Tun 5µg/ml
Tun 5µg/ml + antimy 3µM
Antimy 3µM

Figure 5

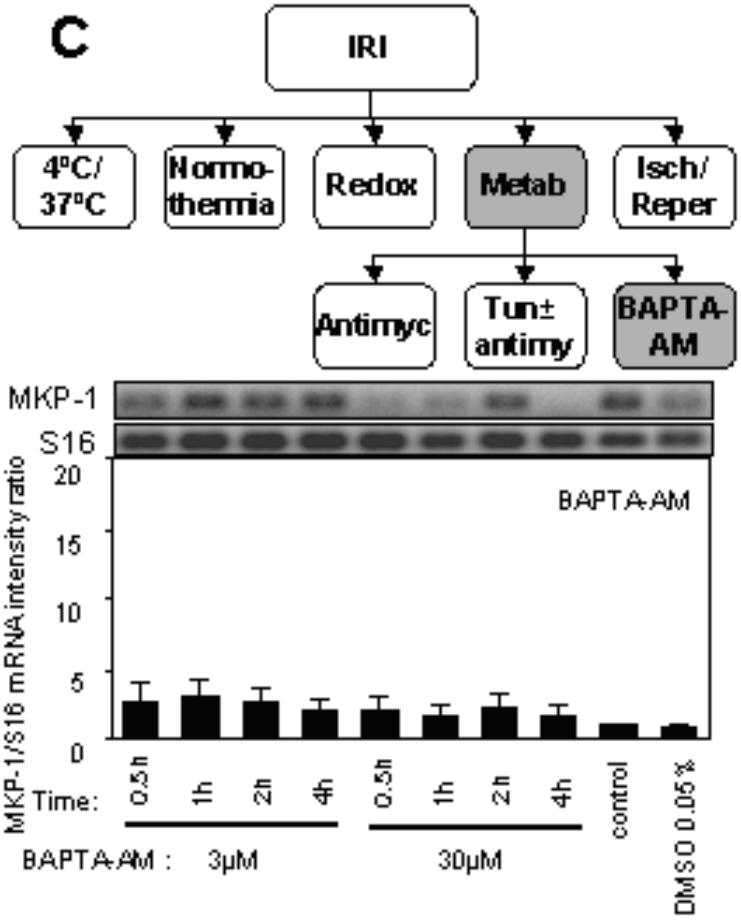

Figure 5 – Metabolic stress mediated expression of MKP-1/DUPS1 in culture. A. HepG2 cells were treated with 10 or 3 µM antimycin A for 0.5, 1, 2 or 4 h and MKP-1/DUSP1 mRNA expression was evaluated by RT-PCR. **B.** The combination of antimycin A (3 µM) and tunicamycin (5µg/ml) has an intermediate effect on the up-regulation of MKP-1 mRNA in relation to the modest effect of tunicamycin only and the potent antimycin A only regulation of the transcript, at all the times tested. **C.** Similar experiments were carried out using 3 and 30 µM BAPTA-AM as stressor. Normalization was carried out using S16 mRNA. Quantification was performed on 3 independent experiments and is represented +/- SEM.

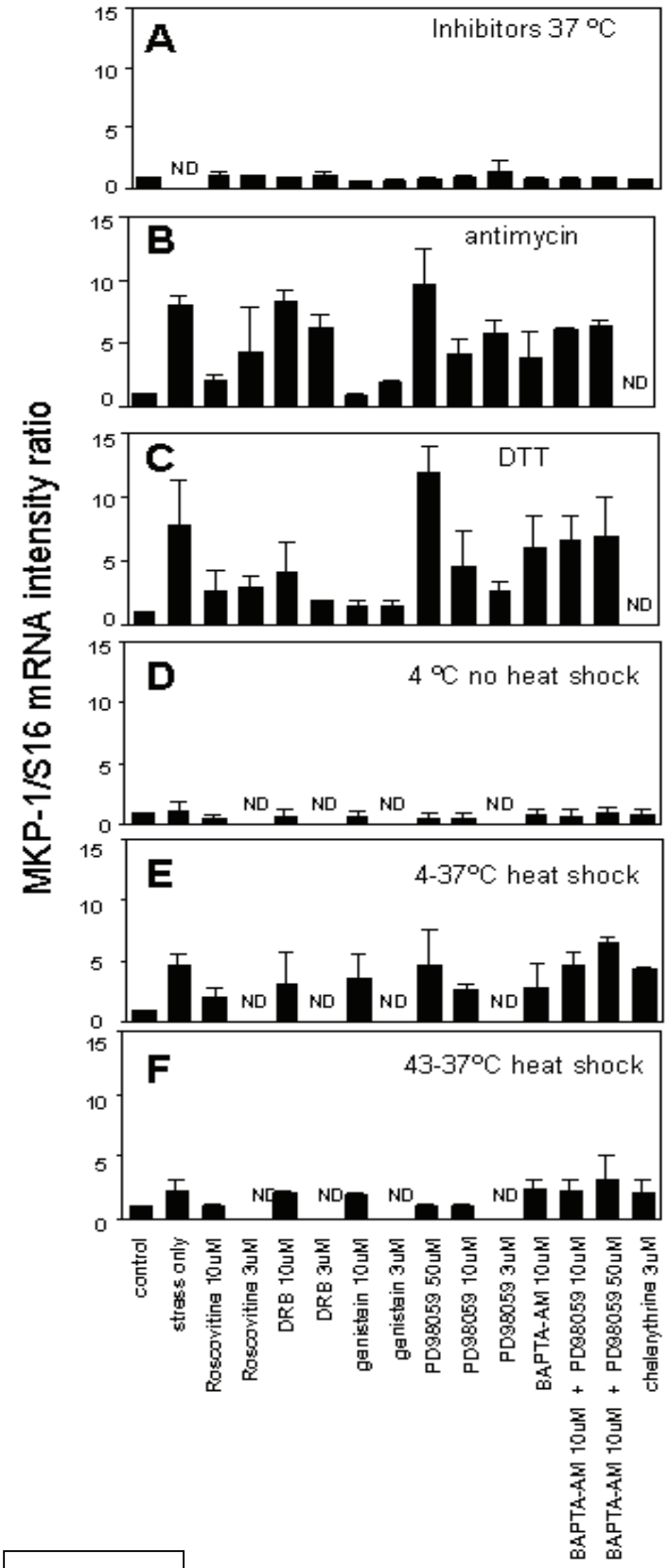
MKP-1/S16 mRNA intensity ratio
A
Inhibitors 37 °C
B
antimycin
C
DTT
D
4 °C no heat shock
E
4-37°C heat shock
F
43-37°C heat shock
ND
control
stress only
Roscovitine 10uM
Roscovitine 3uM
DRB 10uM
DRB 3uM
genistein 10uM
genistein 3uM
PD98059 50uM
PD98059 10uM
PD98059 3uM
BAPTA-AM 10uM
BAPTA-AM 10uM + PD98059 10uM
BAPTA-AM 10uM + PD98059 50uM
chelerythrine 3uM

Figure 6

Figure 6 – Impact of various kinase inhibitors on stress-mediated MKP-1/DUSP1 mRNA expression. The effect of 5 kinase inhibitors and a calcium chelator, thereafter collectively referred to as inhibitors in this figure legend, on stress-mediated MKP-1/DUSP1 expression in HepG2 cells was evaluated by RT-PCR. **A.** Inhibitors only, at 37°C. **B.** Antimycin A ± inhibitors at 37°C. **C.** DTT ± inhibitors at 37°C. **D.** Cold stress (4°C) ± inhibitors. **E.** 4-37°C: heat shock ± inhibitors. **F.** 43-37°C: heat shock only (no cold stress) ± inhibitors. All inhibitors were left overnight. Normalization was carried out using S16 mRNA and quantification is represented by +/- SEM.

3. Signalling specificity of stress-induced MKP-1 mRNA expression

As modulation of MKP-1 mRNA has been associated with many signal transduction pathways, including MAPKs and calcium, we used kinase inhibitors in conjunction with DTT, antimycin A or heat shock to characterize the upstream signalling pathways leading to MKP-1 regulation (Figure 6). Because these inhibitors were reported to either decrease IR mediated damage or abolish the beneficial effects of ischemic preconditioning (see discussion), we determined their ability to modulate MKP-1 mRNA in the absence of stressors (Figure 6A), or in conjunction with stress-mediated induction of the transcript (Figure 6B-F). Compared to the untreated control, none of the inhibitors had any significant effect on the expression of the transcript at 37°C (Figure 6A) or at 4°C (Figure 6D). Upon use of either antimycin A (Figure 6B) or DTT (Figure 6C), the most effective inhibitor of MKP-1 mRNA up-regulation was the tyrosine kinase inhibitor genistein (3 or 10μM). The casein kinase-2 (CK2) inhibitor DRB (3μM) was very effective at inhibiting DTT- but not antimycin A-mediated up-regulation of the messenger, thus implicating a CK2-dependent pathway in DTT-induced stress. Moreover, roscovitine 10μM (a cyclin-dependent kinase inhibitor) and PD98059 10μM (a MEK-1 inhibitor) were effective at preventing MKP-1 mRNA up-regulation upon DTT, antimycin A or heat shock treatment (Figure 6B, C, E and F). These results delineate major signalling pathways responsible for the up-regulation of MKP-1 mRNA, and suggest that the activation of the MEK/ERK pathway may be an important axis in curtailing IRI via MKP-1. Although non-redundant signalling pathways can be activated, the various stresses may lead to MKP-1 mRNA up-regulation as a common denominator, whether or not they funnel through common effectors to achieve this regulation.

4. Targeting MKP-1 expression alters SAPK activation upon IRI-related stress

We sought to prevent stress-induced MKP-1 mRNA expression in our cellular model in order to determine the impact of MKP-1 silencing on stress-mediated MAPK/SAPK activation. This line of investigation revealed that: i) MKP-1 mRNA was up-regulated during the reperfusion phase of liver transplantation; ii) all three MAPK pathways were activated upon liver transplantation (Emadali et al., 2005); iii) MKP-1 attenuated IRI-mediated damage in cardiomyocytes (Kaiser et al., 2004) and iv) the reduction of ischemia-mediated cardiac infarct size in MKP-1 cardiac transgenic mice (Kaiser et al., 2004). Thus, we silenced the expression of MKP-1 using an ShRNA (Sh)

based strategy as previously reported (Wu et al., 2004). MKP-1 protein expression was determined at both 24 and 48h post-transfection (Sh) by immunoblotting using an anti-MKP-1 antibody and compared these results to cells treated with the transfection reagent alone (Ctrl) or a scrambled ScrRNA sequence (Scr) (Wu et al., 2004). The expression of MKP-1 was reduced by 70-80% at 48h post-transfection of Sh compared to Scr and Ctrl. The 48h time-point was used for the subsequent experiment (Figure 7A).

To evaluate the impact of MKP-1 silencing on the activation status of MAPK/SAPK pathways, confluent HepG2 cultures were placed in the UW solution at 4°C in the presence of antimycin-A for 1h, which is used as a model for ATP-depletion during the ischemic phase (Emadali et al., 2006). Following this treatment, the medium was replaced by DMEM + 10% FBS at 37°C, to mimic the “reperfusion” step. Cells were collected at 60, 120, 240 and 300 min post “reperfusion” and lysates were evaluated for ERK-1, p38MAPK and JNK-1 activity (Figure 7B). In ScrRNA transfected cells, both p38MAPK and ERK followed a similar pattern of activation, reaching a peak at 60 min post-reperfusion, then decreasing to reach basal levels of activity at 240 min. JNK activation also reached a peak at 60 min post-reperfusion, but this activity was sustained and did not return to basal levels even after 300 min. Interestingly, in MKP-1 ShRNA transfected cells, ERK activity remained similar to that observed in ScrRNA transfected cells. However, using the same concept, both p38MAPK and JNK displayed a delayed inactivation with a sustained activity (≥ 2 fold above basal level) when MKP-1 ShRNA versus ScrRNA were used. These results suggest that MKP-1 may selectively participate in the inactivation of both JNK and p38MAPK during the reperfusion phase and consequently participate in cell survival. This is reflected by the number of apoptotic cells at the 24h time-point following MKP1 ShRNA transfection versus ScrRNA control (Figure 7c). These results suggest that a reduction in MKP-1 expression allows for longer JNK activity leading to increased apoptosis as previously suggested in other experimental paradigms (Ventura et al., 2006).

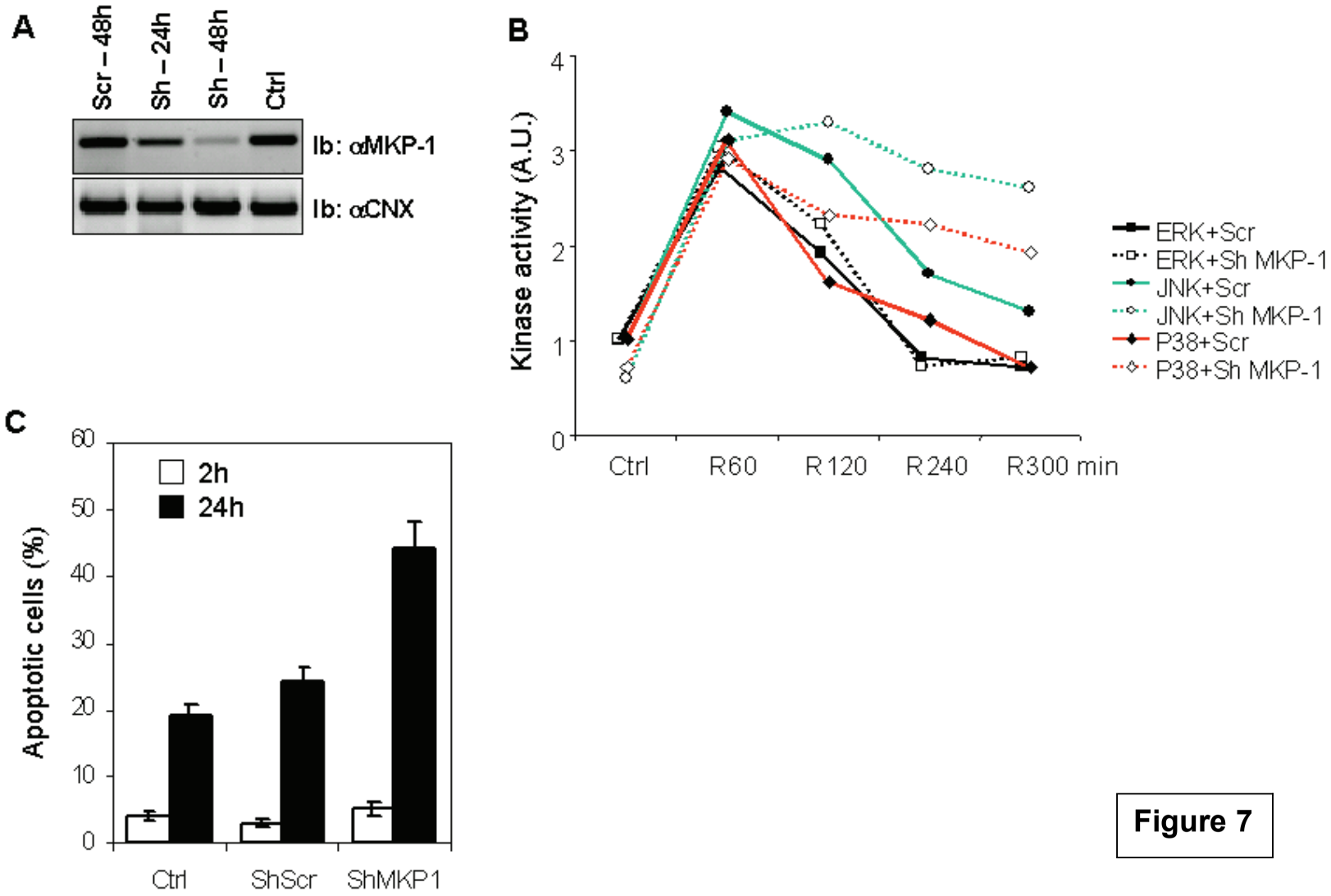
A
Scr – 48h
Sh – 24h
Sh – 48h
Ctrl
Ib: αMKP-1
Ib: αCNX
B
Kinase activity (A.U.)
0
1
2
3
4
Ctrl
R60
R120
R240
R300 min
ERK+Scr
ERK+Sh MKP-1
JNK+Scr
JNK+Sh MKP-1
P38+Scr
P38+Sh MKP-1
C
Apoptotic cells (%)
0
10
20
30
40
50
60
2h
24h
Ctrl
ShScr
ShMKP1

Figure 7

Figure 7 – MKP-1/DUSP1 silencing affects SAPK/MAPK activation upon IR-related stress in HepG2 cells. **A.** MKP-1/DUSP1 silencing was carried out using an ShRNA-based approach. Twenty-four and 48h post-transfection, MKP-1/DUSP1 protein expression was evaluated by immunoblotting. Standardization was carried out using an antibody against the endoplasmic reticulum resident protein calnexin. **B.** The activation of p38MAPK, JNK-1 and ERK-1 was measured in HepG2 cells subjected to IR-related stress following transfection with an ShRNA targeting MKP-1 (open symbols) or a scrambled sequence (Scr; closed symbols). **C**. HepG2 cells were transfected or not with a plasmid containing a scrambled ShRNA or MKP-1 targeting ShRNA. Thirty-six hours post transfection, cells were subjected to a 2h cold ischemia, then placed in standard culture medium and 22h later, the percentage of apoptotic cells determined (white bars indicate the percentage of apoptotic cells after 2h cold ischemia whereas the black bars report the same information but after 24h).

E. Discussion

To our knowledge, the present study represents the first to evaluate mRNA expression profiles specifically during the reperfusion phase of human liver transplantation, taking the recipient blood-borne mRNA into account. Although reports have already described mRNA expression profiles in human liver transplantation (Conti et al., 2007; Defamie et al., 2008), these studies did not dissociate the ischemic phase from the reperfusion phase of transplantation. Our analysis consequently provides a clear picture of early regulatory events during the reperfusion phase of the operation. RNA expression profiling during the reperfusion phase of transplantation has led to the identification of 23 non-redundant regulated genes. Most of the up-regulated genes belonged to regulatory and stress-related pathways. This indicated that besides the expected up-regulation of known immediate early genes (jun, fos), a number of genes involved in signalling were also identified. Finally, using a STRING-based analysis, supported by experiment, database and literature-related information, we found that 21 of the immediate early genes identified in this study belonged to a functional network. This combination of mRNA expression may represent a hepatic immediate early gene response as a consequence of ischemia-conditioned reperfusion-related stress. This is one of the avenues currently under investigation.

Interestingly, our study differed from other reports on hepatic transcriptional profiling. More precisely, we found a low number of identical entries between our study and that of Conti et al. (Conti et al., 2007) or that of Defamie et al. (Defamie et al., 2008) in the up-regulated gene categories. The low correspondence of identical entries representing regulated genes, between our study and the others, may be attributed to i) our study comparing the 60min reperfusion time-point to a pre-reperfusion time-point (zero time-point) during the re-implantation phase of the operation (the reperfusion phase is separated from the ischemic phase); ii) the zero time-point, 10 min and 60 min time-points derive from the same organ, for a given set; iii) a difference in the time-point of samples analyzed (long (2-3h) reperfusion time in the Conti study vs. a 1h reperfusion time-point in our work); iv) their lists of up-regulated genes were mostly comprised of larger (~ 270 down to 8) fold increases for Conti et al. than ours, ~3.5 down to 1.5-fold. The genes with the lower fold increase were not listed (Conti et al., 2007); and v) although there is a methodological difference, our study, the Conti study and the Defamie study use microarray chips from different sources, this may not account for difference in results (Guo et al., 2006; Shi et al., 2006).

The dual specificity MKP-1 has a role in the regulation of ischemia/reperfusion related injury. Cardiac transgenic mice over-expressing MKP-1 were protected from cardiac IRI whereas knockout mice were more sensitive (Kaiser et al., 2004). Although the transgenic and knockout mice were on different genetic backgrounds, a rat model of global cerebral ischemia also confirmed these findings (Kawahara et al., 2004). Taken together, these observations led us to investigate the potential role of MKP-1 in liver cells upon IRI. Others and we previously demonstrated that the three axes of the MAPK pathways (p38MAPK, JNK and ERK) were activated upon liver reperfusion (Emadali et al., 2005; Uehara et al., 2005). As such, an increase in MKP-1 expression may be part of a regulatory mechanism to moderate this activation and allow the liver to return to its basal state following transplantation. We propose a model in which, following transplantation, an initial phase that is dependent on the MAPK pathway (ERK, p38MAPK or JNK) would increase the expression of MKP-1. This increase would promote mainly the dephosphorylation and therefore inactivation of JNK and p38MAPK, thus allowing hepatocytes to survive IRI during human liver transplantation.

Many of the inhibitors used in this study were previously reported to influence the extent of ischemia/reperfusion-mediated damage. Interestingly, none of them led to increased MKP-1 mRNA levels, at 37ºC or 4ºC, when used overnight. As MKP-1 is an immediate-early gene, it remains possible that the inhibitors stimulate MKP-1 expression at an earlier time-point. The experiments carried out at 37ºC or 4ºC were used as controls for the thermal or chemical stress-inducing agents. Thus, roscovitine and genistein inhibited both antimycin A and DTT mediated increase in MKP-1 mRNA, while roscovitine also inhibited heat-shock mediated increase in this transcript. BAPTA-AM had no appreciable effect on MKP-1 mRNA levels. As the process of organ transplantation invokes multiple stressors, it remains possible that the signal transduction inhibitors moderate the deleterious effects of ischemia/reperfusion in vivo while allowing the increased expression of endogenous regulators such as MKP-1 at the gene or protein level. Otherwise, their mechanism of action may not rely on MKP-1. These alternative possibilities remain to be tested in vivo.

Other inhibitors have a deleterious effect, when administered prior to ischemic preconditioning, a method used to reduce liver injury (Carini and Albano, 2003). This is confirmed for genistein and chelerythrine as they eliminate the beneficial effects of ischemic preconditioning (Ricciardi et al., 2001). Our results show that some of the inhibitors tested such as genistein or PD98059, did reduce stress-mediated up-regulation

of MKP-1 mRNA, caused by DTT and antimycin A, while PD98059 also inhibits heat-shock induced MKP-1 transcript. Interestingly, our results also demonstrate that DRB was most effective against DTT induced MKP-1 mRNA. DTT induces ER-stress and is used to model a facet of UPR. It is possible that lack of cold preservation, heat-shock, or blood-supply reduces ER-stress, when the tissue is reperfused ex-vivo with a 37ºC buffer solution (Krebs-Henseleit) (Kim et al., 1999).

The evaluation of reperfusion-induced immediate early gene response, during human liver transplantation, has provided us with a number of novel and potentially therapeutic targets. The liver's adaptive response to transplantation represents an effective means of understanding and improving the marginal livers response to transplantation. This is a necessary step if a protocol is to be created that would allow these livers to be successfully transplanted. In so doing, the chasm between donor and recipient numbers would dwindle.

The MKP-1 saga has shed light on how a liver-specific cell-type, hepatocytes, may survive the stresses associated with ischemia-reperfusion, by negatively regulating the function of stress-associated kinases JNK-1 and p38MAPK, and allowing ERK1 signalling to proceed. However, as previously mentioned, there are many types of stresses associated with the process of liver transplantation. Therefore it is logical to assume that there will be more than one type of molecule used by the cells in the liver to curtail the effects of stress. In the next chapter, chapter 5, we will examine the need of liver cells to down-regulate, or at least prevent the up-regulation of, another transcript identified by microarray. Chapter 5 will reprise the in vitro modeling of the stresses associated with transplantation, found in chapter 4, however this time the focus is the alsin (als2) mRNA and determine what the consequences of the alsin protein function is vis-à-vis the process of transplantation, more specifically the stresses associated with this process (manuscript in preparation).

V. Chapter 5: alsin

Alsin the guanine nucleotide exchange factor and its involvement in human liver transplantation

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Manuscript in preparation

A. Abstract

Orthotopic liver transplantation continues to be the only remedy for end-stage liver disease. Although marginal livers could increase the number of organs available for transplantation, they are not amenable to ischemia-reperfusion-mediated injury (IRI). We recently transcriptionally profiled the normal human liver's response to IRI, during the reperfusion phase of the operation, as a prelude to understanding the lack of viability of marginal livers after transplantation. Of the immediate-early genes that were identified at the 1h-post reperfusion time-point, the als2 gene coding for the alsin protein, demonstrated a slight decrease in the level of its transcript. Alsin is a protein that is associated with juvenile form of amyotrophic lateral sclerosis (ALS), among other juvenile neurodegenerative diseases. Although alsin is a known RhoGEF, for Rac1, Rab5 and possibly Ran, there is no data extant on the modulators of the als2 transcript, much less information about the consequences of the alsin protein function outside the field of neurodegeneration. Having validated the results of our transcriptional profiling, using RT-PCR, immunoblotting and immunohistochemistry, we used the human hepatoma cell line HepG2 as an in vitro model, and determined the profile of reperfusion-related stresses that modulate this transcript. We also determined some of the signalling pathways that regulate this transcript. Finally, the combination of Alsin silencing coupled with stresses associated with reperfusion, and the use of alsin constructs to determine which aspect of alsin function is crucial during the reperfusion phase of the operation. This is a necessary step if possible avenues of intervention are to be found.

B. Introduction

Briefly, orthotopic liver transplantation (OLT) remains the only therapeutic option for end-stage liver diseases. Unfortunately, an increasing number of patients requiring transplantation coupled to an organ shortage compound the problem of organ procurement for patients. Including marginal livers in the donor pools and reducing the incidence of graft failure are viable alternatives to remedy the situation. As a first step in understanding the previously stated problems, we transcriptionally profiled the human liver during the reperfusion phase of the operation in order to define the normal livers response to transplantation. We identified a set of genes that were regulated at the 1-hour post-reperfusion time point (Boutros et al., 2008b). Given that our understanding of the mechanisms involved in the reestablishment of cellular and organ homeostasis remain incomplete, especially during the first hour post-reperfusion, we investigated key molecules that might be involved in the re-establishment of homeostasis. To this end, we previously identified the dual specificity phosphatase MKP-1 as a transcript that was up-regulated during the reperfusion phase of the operation and used an in vitro model to determine the consequences of MKP-1 function vis-à-vis HepG2 survival during ischemia-reperfusion related stresses. Our results revealed that MKP-1 down-regulated activated JNK-1 and p38MAPK, but not ERK-1 in HepG2 cells. Furthermore, we found that MKP-1 expression during ischemia-reperfusion related stresses lead to apoptosis. This may represent one axis of the liver's attempt to reestablish homeostasis after transplantation (Boutros et al., 2008b).

To further characterize the mechanisms responsible for the reestablishment of homeostasis, we selected another gene identified by transcriptional profiling, which is the als2 mRNA coding for a protein named alsin. As no published data exists concerning the regulation of the als2 transcript, we characterized the regulation of this transcript, using the same ischemia-reperfusion-relevant stresses, we previously used (Boutros et al., 2008b). To determine the consequences of alsin function, as it relates to ischemia-reperfusion related injury, we are identifying the cell-type expressing alsin, using an antibody we are developing. In addition, alsin is a guanine nucleotide exchange factor and an activator of Rac1, Rab5 and possibly Ran, Rho GTPases. Furthermore, these RhoGTPases are involved in an array of biological functions including: 1) Rac1 involvement in actin cytoskeleton remodelling, which occurs in the reperfusion phase of the operation (Emadali et al., 2006), NADPH oxidase function, lamellipodia formation and apoptosis (Bosco et al., 2009); 2) Rab5 mediating early vesicle transit and

modulation of vesicle bound receptor signalling and involved in actin cytoskeleton remodelling (Gorvel et al., 1991; Lanzetti et al., 2001); and 3) Ran assures the shuttling of proteins across the nuclear pore complex, via importins and exportins, apoptosis, microtubule reorganization among many functions of Ran which vary according to its effectors (Rensen et al., 2008). Given the previous observations, we decided to design various constructs that would express various domains of alsin to determine how these RhoGTPase, were implicated in the process of transplantation, in terms of aiding or hindering the reestablishment of homeostasis.

C. Materials and Methods

1. Materials – Hydrogen peroxide, $CoCl_2$, BAPTA-AM, Antimycin A were purchased from Sigma. DTT was from Bioshop. Trizol, DMEM and Rnase H were from Invitrogen. Tunicamycin was from Calbiochem. Fetal Bovine Serum (FBS) was purchased from Hyclone. Anti-calnexin antibody was from Stressgen. All the oligonucleotide primers used in this study were purchased from AlphaDNA (Montreal, Qc, Canada). Thermoscript RT-PCR System, Superscript II and Superscript III RT RNAse H- (Invitrogen). PD98059 (Cell Signaling), roscovitin, chelerythrine and DRB (CalBiochem), genistein (Sigma).

2. Patients and tissue collection - 0.5 cm^3 biopsies were surgically removed in the course of 8 liver transplantation procedures performed within the McGill University Health Centre with approval of the institution's ethics committee (ERB 05-003) as previously described (Emadali et al., 2007; Emadali et al., 2006; Emadali et al., 2005) and as schematically represented in Figure 1A. Briefly, biopsies from reperfused livers were collected during re-implantation of the organ in the recipient, just prior to clamp removal from the hepatic portal vein (R0), and 10 minutes (R10) and 60 minutes (R60) after the blood flow has been re-established through the portal vein. The average cold ischemia phase was 8h30 ± 2h15 whereas the average warm ischemia time was 48 min ± 26min. For every time-point, each biopsy was divided into three pieces, snap frozen and stored in liquid nitrogen until RNA or protein extraction. The third piece was embedded in OCT prior to being snap frozen in liquid nitrogen.

3. RNA isolation - Total RNA was extracted using Trizol following the manufacturer's instructions. Frozen tissue samples were put in Trizol before homogenization. The quantity of RNA was evaluated by spectrophotometry at 260 nm for RT-PCR. The quality of the RNA was determined by non-denaturing agarose gels. The relative purity was determined by $A_{260/280}$ ratio by spectrophotometry.

4. Cell culture and treatments - HepG2 cells were cultured in DMEM with 10% foetal bovine serum (FBS) until the experiments. To evaluate MKP-1/DUSP1 mRNA expression in response to various environmental stresses, HepG2 cells were plated at 2.0×10^6 cells per 60mm plate in DMEM with 10% FBS. After 2 days, the growth medium was replaced with 2 ml of DMEM supplemented with 10% FBS and containing one of the various stressors (see below) or the vehicle control. To mimic the cold stress/heat shock aspect of transplantation, HepG2 cells were plated as above. Two days later, the cells were washed twice with either 4ºC PBS or 37ºC PBS and afterwards incubated with

DMEM or DMEM with 10% FBS or University of Wisconsin (UW) Beltzer solution, at 4ºC or 37ºC for 5 hours. As the cultures incubated at 4ºC were outside the CO_2 incubator, both DMEM and DMEM with 10% FBS used for incubation at 4ºC or 37ºC, had 20 mM HEPES buffer added. After this incubation period, the various media were removed and replaced by 37ºC DMEM with 10% FBS, no HEPES, and incubated for various time-points at 37ºC. To determine the signalling pathways used by the various stressors, kinase inhibitors were used. More specifically, HepG2 were plated at a density of 2×10^6 cells per 60mm plate and cultured until ~80% confluent. All kinase inhibitors were used overnight at the final concentrations reported in figure 6. The next day, either antimycin A, DTT or heat shock was used as stressors. For the inhibitor assay, the cells were neither washed with PBS nor was the medium (DMEM with 10% FBS) replaced prior to the addition of stimulator. MKP-1 silencing was carried out using the previously reported plasmids (Wu et al., 2004) and using 5 and 10ug of plasmid DNA. The transfection protocol was previously described (Emadali et al., 2006).

5. The anti-alsin antibody – To manufacture the anti-alsin antibody, the following alsin peptide sequence (TETLDRQEEVFENTLVANDQSVATELNAVSAQITSS DAMSSQQNVMGTTEISSARNIPSYPDTQAVNEYLRKLSDHSVREDSEHGEKPVPSQPL LEEAIPN) was used. After transforming bacteria (the rosetta strain of *E. coli*) we purified the peptide, and proceeded to inject it in chicken to generate IgY polyclonal antibodies.

6. RT-PCR analyses - alsin (forward: 5'- ATGGACTCAAAGAAGAGAAGC-3', reverse: 5'-ACATGTACGCGACACCATTG-3') and S16 (Emadali et al., 2005) primers were designed. Semi-quantitative RT-PCR analysis was performed as previously described (Nguyen et al., 2004).

D. Results

Human liver biopsies were collected during the reperfusion phase of the operation at three different time-points and snap frozen. The zero time-point corresponds to the period prior to the reestablishment of the recipient's blood flow through the hepatic portal-vein while the 10 min and 60 min time-points correspond to the length of time following blood flow restoration through the graft (Figure 1A). The 10 min time-point biopsy specimen accounts for any mRNA contributed by the recipient blood when performing the microarray analysis.

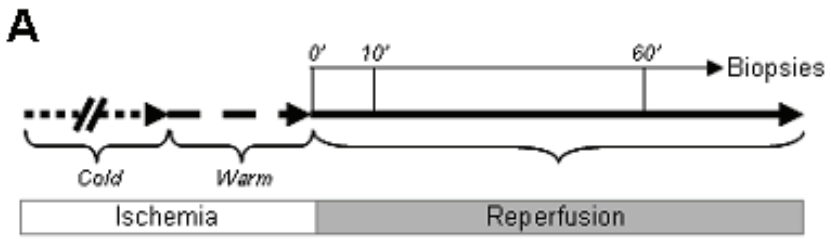


Figure 1 – Time-line of human liver biopsies collected during the reperfusion phase of transplantation. A. Time-line representation of the biopsy collection protocol followed in our study. Work in progress.

Brief overview of the transcriptional profiling analysis

Briefly, each spot in the microarray had to pass three criteria of quality control for inclusion into the process of normalization and analysis: 1) the signal intensity had to be greater than the surrounding background; 2) the raw intensities of duplicate spots, per gene, had to be within 50% of each other; and 3) the signal intensity for each spot had to be within the dynamic range of the photomultiplier tube. Not surprisingly, our list is comprised of immediate-early genes due to the early 60 min reperfusion time-point (Boutros et al., 2008b). Using these three time-point biopsy-specimens, we elaborated and compared two normalized lists of genes, the 60 min vs 10 min and the 60 min vs 0 min, which allowed us to eliminate any mRNA whose apparent increase in expression was the result of the recipient blood, during the reperfusion phase of the operation. We

obtained the final list of genes comprising 31 unique mRNAs that passed all our filters (Boutros et al., 2008b).

als2 mRNA and protein expression in human liver biopsy specimens

Work in progress. See section F “What needs to be done” (this section would account for Figures 2A-E).

Breakdown of IRI-related stresses surprisingly leads to the up-regulation of als2 mRNA

In an attempt to determine which of the stresses implicated in the process of human liver transplantation was involved in the regulation of als2 mRNA and alsin protein levels, we used a well characterized human hepatoblastoma cell line, HepG2, as an *in vitro* model (Emadali et al., 2006). In addition, both the ischemia and reperfusion-associated stresses were divided into four broad categories, namely thermal stress, oxidative stress, metabolic stress, and chemically mimicked hypoxia-reperfusion. Each category was, in turn, subdivided into components (Figures 3 - 5). We first assessed the effects of a 4-37ºC thermal stress versus normothermia using three different media to account for the influence of thermal stress and storage solutions on the expression of the als2 transcript. More specifically, in both Figures 3A and 3B, HepG2 cells were incubated with either DMEM + 10% FBS, DMEM only or University of Wisconsin (UW) solution at 4°C (Figure 3A) or 37°C (Figure 3B), for 5h. Thereafter, all cultures were incubated with DMEM + 10% FBS at 37°C for various times. Interestingly, contrary to our results with mkp-1 mRNA (Boutros et al., 2008b), heat shock delayed expression of als2 mRNA (Figure 3A) as compared to its normothermic counterpart when 10% FBS is always present (Figure 3B). However, if the cells are incubated in serum free conditions at 4°C (Figure 3A) or 37°C (Figure 3B), for 5h, heat shock has no effect in contrast to normothermic conditions. Finally, although the UW solution delayed and decreased the induction of als2 mRNA expression in the heat shock model (Figure 3A), the UW solution only appears to decrease the als2 mRNA expression without delaying its onset in the normothermic model (Figure 3B).

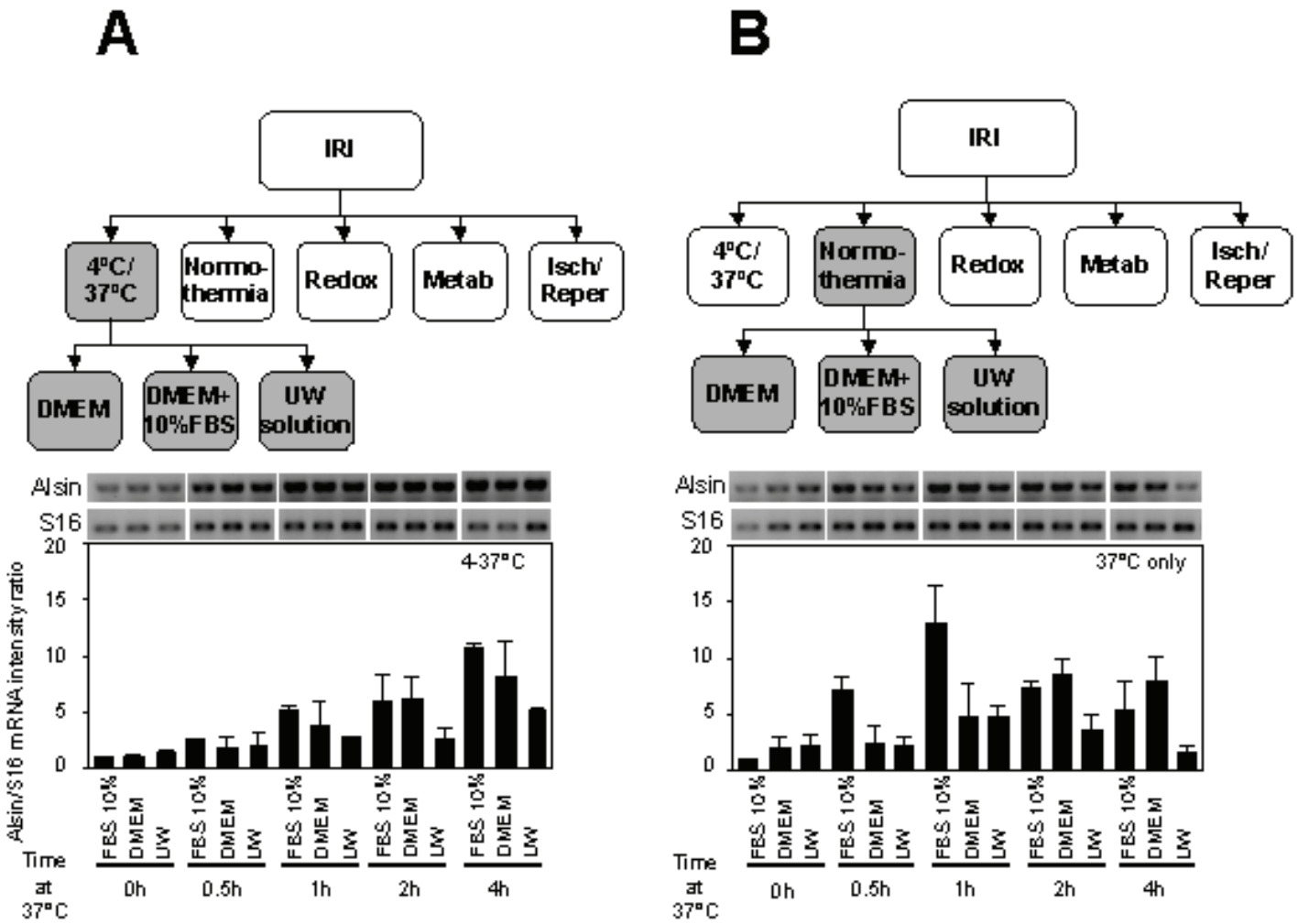

A
IRI
4°C/ 37°C
Normo-thermia
Redox
Metab
Isch/ Reper
DMEM
DMEM+ 10%FBS
UW solution
Alsin
S16
4-37°C
Alsin/S16 mRNA intensity ratio
20
15
10
5
0
FBS 10%
DMEM
UW
Time at 37°C
0h
0.5h
1h
2h
4h
B
IRI
4°C/ 37°C
Normo-thermia
Redox
Metab
Isch/ Reper
DMEM
DMEM+ 10%FBS
UW solution
Alsin
S16
37°C only
20
15
10
5
0
FBS 10%
DMEM
UW
Time at 37°C
0h
0.5h
1h
2h
4h

Figure 3

Figure 3 – Temperature stress mediated expression of alsin/als2 mRNA in HepG2 cultures. **A**. The effect of cold stress warm reperfusion (4-37ºC) in three different media (DMEM + 10% FBS, DMEM only and UW) on alsin/als2 mRNA expression by RT-PCR. Normalization was carried out using S16 mRNA. **B**. Same media as A, but under normothermic conditions (no cold stress or heat shock). Quantification for A and B was performed on 2 independent experiments and is represented +/- SEM.

The next transplantation-relevant stress to be modeled was the reducing/oxidative (redox) stress. To this end, we used the reducing agent DTT or oxidizing agent H_2O_2 to model the redox stress. DTT showed both a time and dose-dependent effect on als2 mRNA expression, with the 30mM concentration being more effective than the 100mM at all times tested (Figure 4A). In contrast, hydrogen peroxide had either no effect on this messenger or a slight inhibitory effect at the 30min time-point and 100μM dosage (Figure 4B). These results suggest that, in our cellular model, reducing stress up-regulates als2 mRNA expression, while oxidative stress has an inhibitory function.

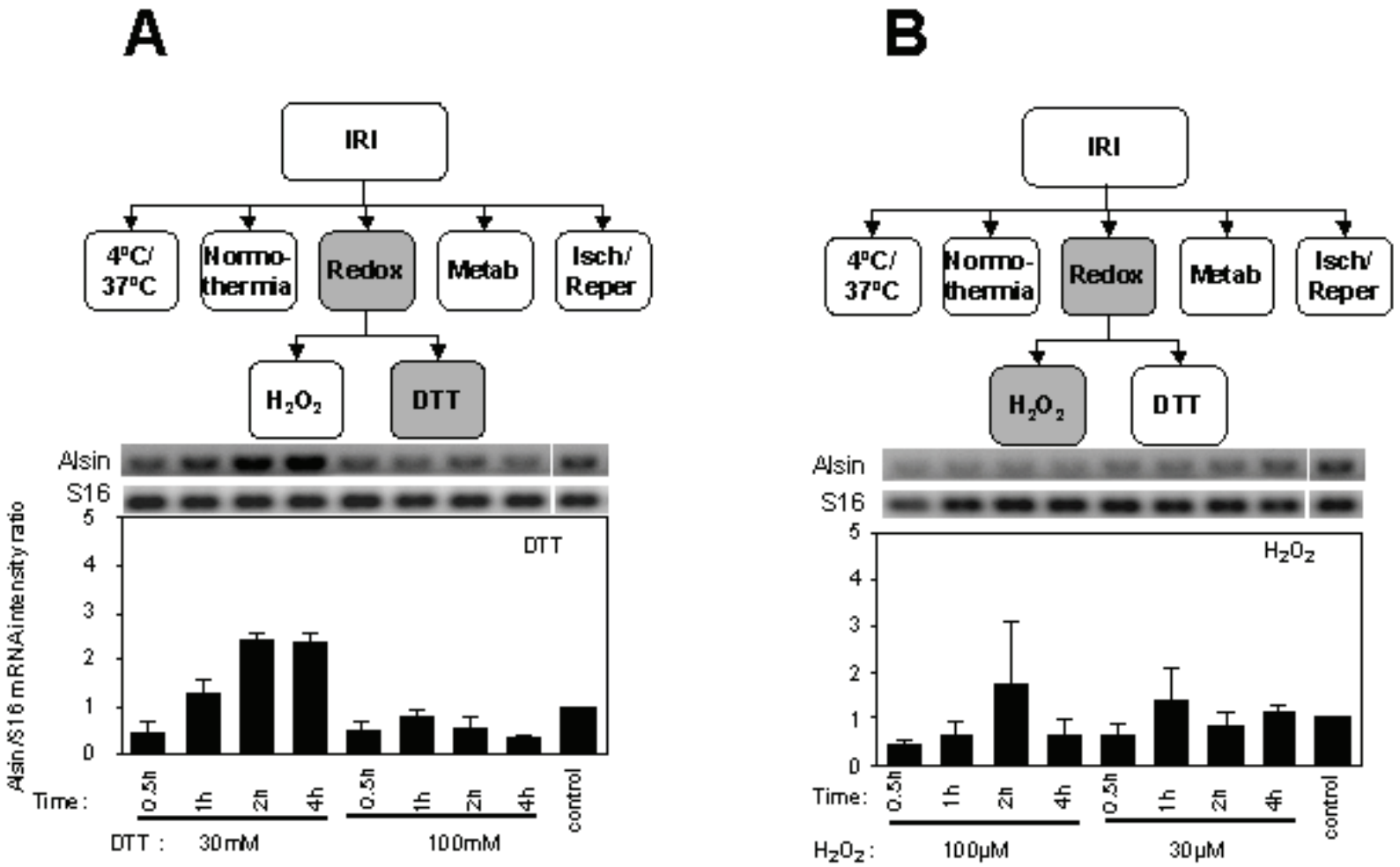

A
IRI
4°C/ 37°C
Normo-thermia
Redox
Metab
Isch/ Reper
H2O2
DTT
Alsin
S16
Alsin/S16 mRNA intensity ratio
DTT
Time:
0.5h
1h
2h
4h
control
DTT :
30mM
100mM
B
IRI
4°C/ 37°C
Normo-thermia
Redox
Metab
Isch/ Reper
H2O2
DTT
Alsin
S16
H2O2
Time:
H2O2 :
100μM
30μM

Figure 4

Figure 4 – Oxidative stress mediated expression of alsin/als2 mRNA in HepG2 cultures. A. The expression of alsin/als2 mRNA was evaluated by RT-PCR in HepG2 cells treated with either 100µM or 30 µM H_2O_2 for 0.5, 1, 2 and 4h. **B.** The same experiments were carried out using DTT instead of H_2O_2. Normalization was carried out using S16 mRNA. Quantification was performed on 3 independent experiments and is represented +/- SEM.

The metabolic stress facet of ischemia-reperfusion (Figure 5A-C) is subdivided into three components. Antimycin A only, a known mitochondrial complex III inhibitor, induced als2 mRNA (Figure 5A). To determine the effects of different metabolic stresses, antimycin A is used in conjunction with tunicamycin, the N-glycosyl transferase inhibitor, that is used to chemically simulate the Unfolded Protein Response (UPR) facet of endoplasmic reticulum stress (Figure 5B). Indeed, as the UPR is activated during both phases of liver transplantation (Emadali et al., 2005), als2 mRNA expression may consequently represent a potential target of this stress-related pathway. When tunicamycin is used alone, there is no effect on the als2 transcript and the combination of both stressors had no effect (Figure 5B) in comparison to antimycin A only treatment. In addition, calcium depletion related stress did not have any effect on als2 mRNA expression level, as seen with the membrane permeable calcium chelator BAPTA-AM (Figure 5C) irrespective of the concentrations or times tested.

Finally, as both ischemia and its in vitro counterpart hypoxia have been implicated in the regulation of MKP-1 mRNA (see discussion), we used cobalt chloride ($CoCl_2$), a prolyl hydroxylase inhibitor known to stabilize HIF-1α and chemically simulate hypoxia. Using a 100μM dose of $CoCl_2$ and various time-points, we did not observe any variation of als2 mRNA levels either in the “hypoxic” or the “reperfusion” phase of the experiment (data not shown).

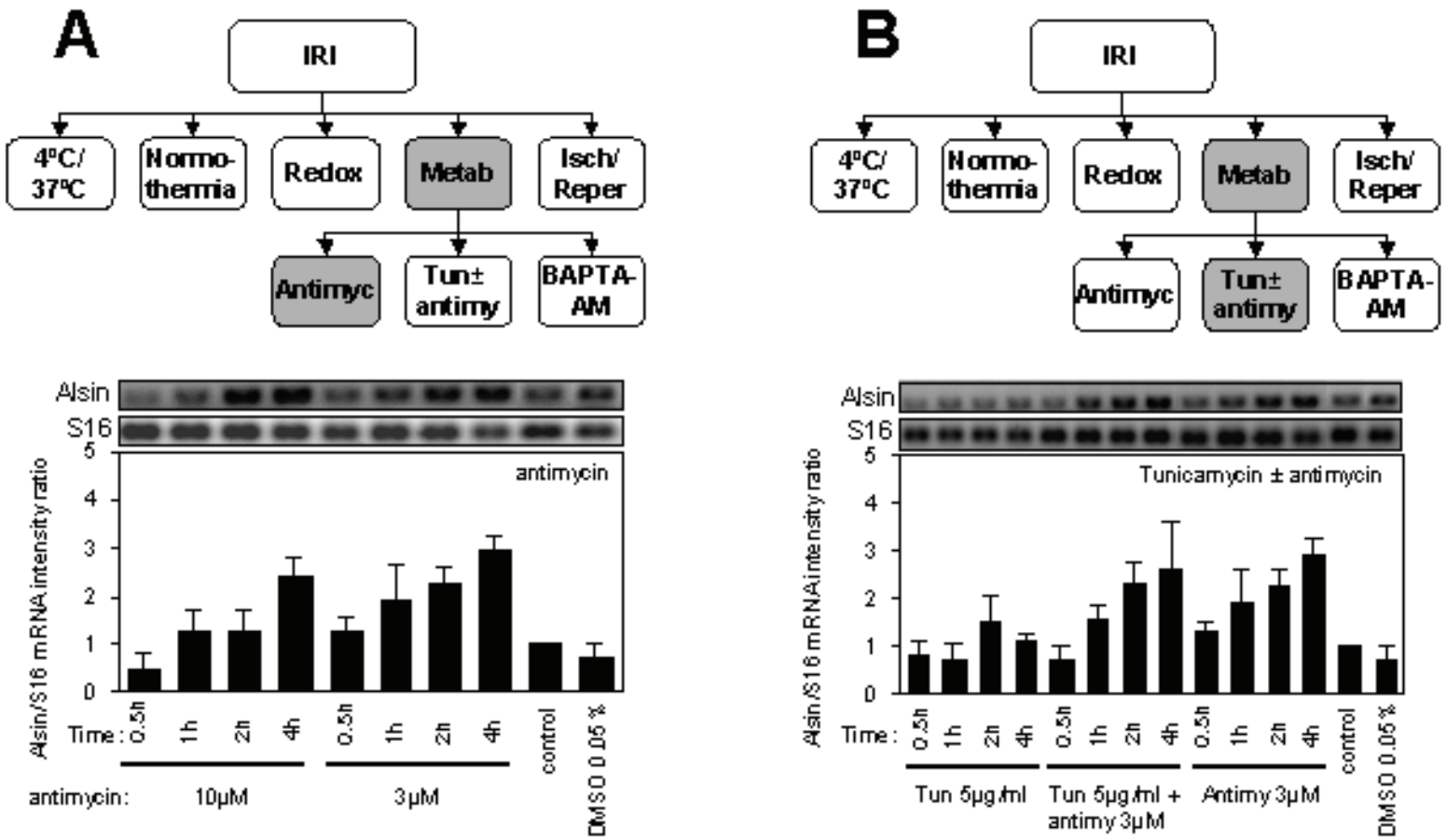

A
IRI
4ºC/ 37ºC
Normo-thermia
Redox
Metab
Isch/ Reper
Antimyc
Tun± antimy
BAPTA-AM
Alsin
S16
antimycin
Alsin/S16 mRNA intensity ratio
Time:
0.5h
1h
2h
4h
control
DMSO 0.05 %
antimycin:
10µM
3µM
B
IRI
4ºC/ 37ºC
Normo-thermia
Redox
Metab
Isch/ Reper
Antimyc
Tun± antimy
BAPTA-AM
Alsin
S16
Tunicamycin ± antimycin
Alsin/S16 mRNA intensity ratio
Time:
Tun 5µg/ml
Tun 5µg/ml + antimy 3µM
Antimy 3µM

Figure 5 A-B

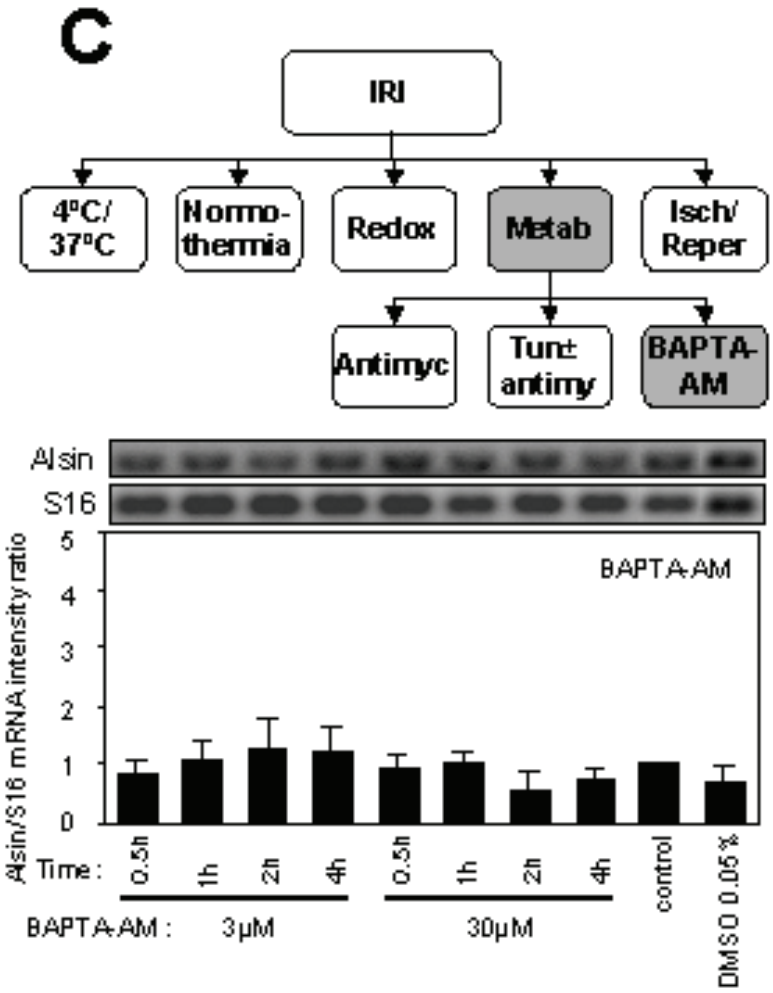


Figure 5 – Metabolic stress mediated expression of alsin/als2 in culture. A. HepG2 cells were treated with 10 or 3 µM antimycin A for 0.5, 1, 2 or 4 h and alsin/als2 mRNA expression was evaluated by RT-PCR. **B.** The antimycin A (3 µM) treatment up-regulates the als2 transcript, while the combination of antimycin A and tunicamycin (5µg/ml) shows no difference in relation to the antimycin A only and tunicamycin only has no effect on this transcript. **C.** Similar experiments were carried out using 3 and 30 µM BAPTA-AM as stressor. Normalization was carried out using S16 mRNA. Quantification was performed on 3 independent experiments and is represented +/- SEM.

Signalling specificity of stress-induced alsin/als2 mRNA expression

As modulation of als2 mRNA has never been described, we used various kinase inhibitors in conjunction with DTT, antimycin A or heat shock to characterize the upstream signalling pathways leading to als2 regulation in our model (Figure 6). As these inhibitors have been documented to either decrease ischemia-reperfusion mediated damage or abolish the beneficial effects of ischemic preconditioning (see discussion), we determined their ability to modulate als2 mRNA either in the absence of stressors (Figure 6A), or in conjunction with stress-mediated induction of als2 mRNA (Figure 6B-F). As the various inhibitors were tested, we found that, compared to the untreated control, only the tyrosine kinase inhibitor genistein 3μM had an inhibitory effect on the expression of the transcript at 37°C (Figure 6A), while the MEK inhibitor, PD98059 50μM reduced the expression of als2 transcript at 4°C (Figure 6D). Upon use of either antimycin A (Figure 6B) or DTT (Figure 6C), the most effective inhibitors of als2 mRNA up-regulation were either genistein (3 or 10μM). The MEK inhibitor PD98059 3μM was very effective at inhibiting DTT- but not antimycin A-mediated up-regulation of the als2 messenger, thus suggesting the involvement of an ERK dependent pathway in DTT-induced stress. Our results delineate major signalling pathways responsible for the up-regulation of als2 mRNA and indicate that the activation of the tyrosine kinase and MEK/ERK pathways are axes that may be of importance in this modulation upon IRI. In addition, although non-redundant signalling pathways can be activated, the signalling specificity achieved by the various stresses may still lead to als2 mRNA up-regulation as a common denominator. Alternatively, the non-redundant signalling pathways funnel through common effectors leading to als2 transcript regulation.

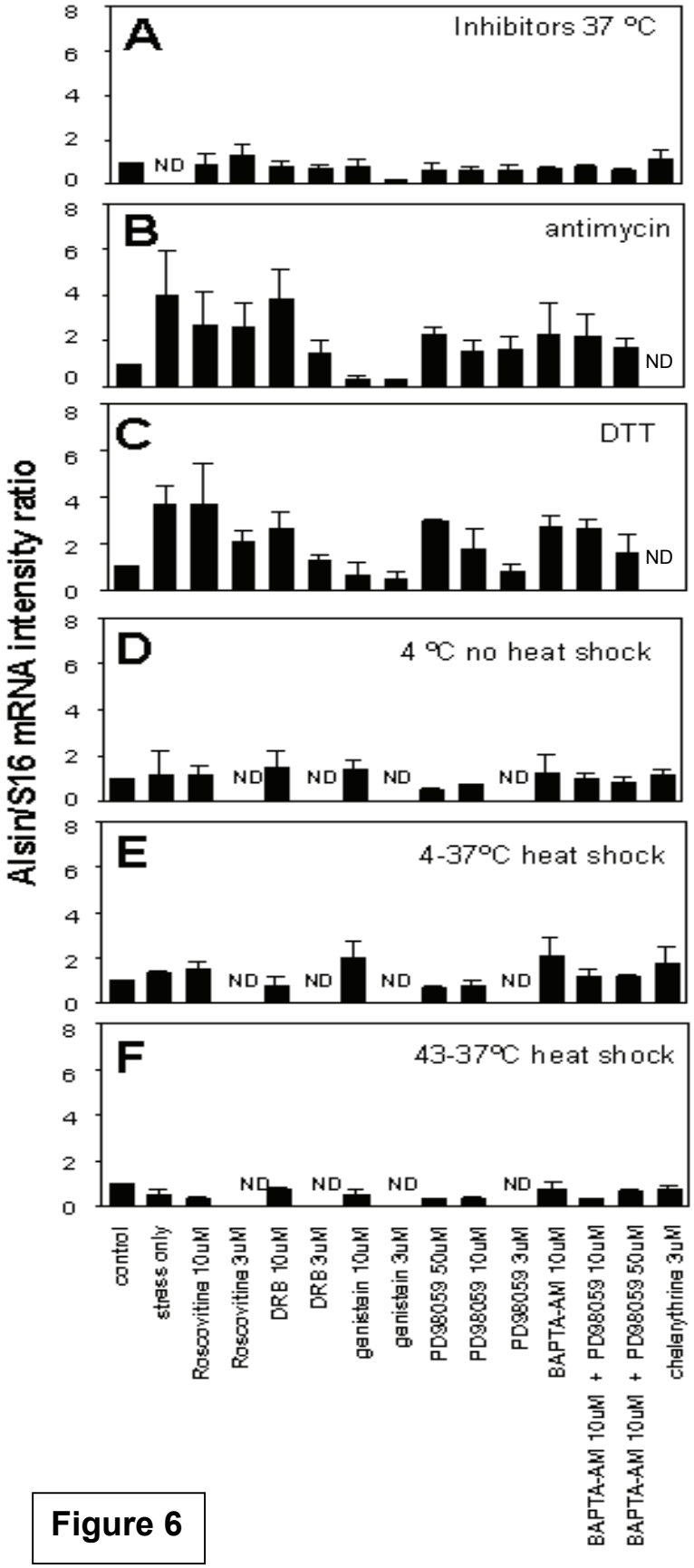

A
Inhibitors 37 °C
B
antimycin
C
DTT
D
4 °C no heat shock
E
4-37°C heat shock
F
43-37°C heat shock
ND
Alsin/S16 mRNA intensity ratio
0
2
4
6
8
control
stress only
Roscovitine 10uM
Roscovitine 3uM
DRB 10uM
DRB 3uM
genistein 10uM
genistein 3uM
PD98059 50uM
PD98059 10uM
PD98059 3uM
BAPTA-AM 10uM
BAPTA-AM 10uM + PD98059 10uM
BAPTA-AM 10uM + PD98059 50uM
chelerythrine 3uM

Figure 6

Figure 6 – Impact of various kinase inhibitors on stress-mediated alsin/als2 mRNA expression. The effect of 5 kinase inhibitors and a calcium chelator, thereafter referred to as inhibitors in this figure legend, on stress-mediated als2 expression in HepG2 cells was evaluated by RT-PCR. **A.** Inhibitors only, at 37°C. **B.** Antimycin A ± inhibitors at 37°C. **C.** DTT ± inhibitors at 37°C. **D.** Cold stress (4°C) ± inhibitors. **E.** 4-37°C: heat shock ± inhibitors. **F.** 43-37°C: heat shock only (no cold stress) ± inhibitors. All inhibitors were left overnight. Normalization was carried out using S16 mRNA and quantification is represented by +/- SEM.

E. Discussion

Stressors that modulate als2 mRNA. Our results reveal that heat shock (Figure 3A) delays the up-regulation of als2 mRNA caused by serum (Figure 3B) in our HepG2 in vitro model. This in vitro result mirrors our in vivo microarray findings, which reveal a slight down-regulation of als2 mRNA. The difference between our in vitro up-regulation and our in vivo down-regulation, may lie in part in the comparison of tissue culture cell line to an organ biopsy specimen. The length of time 5h cold ischemia, versus 10h for the liver could also contribute to the differences, especially where ATP levels are concerned (see chapter 2 section C2). There is also the possibility of the influence of pH levels. The UW solution pH 7.4 has a buffering capacity of 25mM KH_2PO_4, which may not be adequate. Given 12mM lactic acid during storage of the liver (Silva et al., 2006), 3mM protons from GSSG (if 3mM of GSH were initially used to manufacture UW solution, up to 3mM of protons can be released to make 1.5mM GSSG). However the cultures that were put in the cold 4ºC were supplemented with 20 mM HEPES buffer because they were outside the CO_2 incubator, as were their sister cultures at 37ºC inside the incubator to control for the effect of HEPES. The UW stored liver is devoid of HEPES. The effect of HEPES on als2 gene expression has not been examined, and may be causative in the difference in als2 mRNA expression. However intracellular pH levels are known to decrease upon increasing degradation of ATP (Fuller, 1987), which causes a decrease in pH (Chang, 1981) (also see chapter 2 section C2). Thus there could be multiple effects.

Although bovine serum is used in vitro and human blood is used in vivo, it is unlikely that the direct contact of serum with the cell line versus human plasma in the case of the liver as the sinusoidal endothelial fenestrations, pores and intercellular space, account for 100-500nm (Kuntz, 2008b). One possibility is that the recipient blood may have disease-related blood borne factors that inhibit als2 mRNA expression, versus healthy bovine serum. Alternatively, there could be a species-specific effect, bovine serum versus human blood. This latter possibility could be tested using human serum in vitro, although the price and the possibility of disease make such use both costly and risky.

Adding to the previously mentioned possibilities, was the finding that reducing stress in the form of DTT increased als2 mRNA (Figure 4A) while oxidative stress in the form of 100μM of H_2O_2 caused a slight decrease in this messenger (Figure 4B). This is in line with alsin being a RhoGEF, an activator, of Rac1, which is a subunit of NADPH

oxidase (Bosco et al., 2009). Thus the modulation of alsin may be necessary to equilibrate the redox status in the cell. Of the metabolic stressors, only the mitochondrial complex III inhibitor antimycin A increased als2 mRNA (Figure 5A). Neither tunicamycin, the N-glycosyl transferase inhibitor used to simulate ER-stress, nor the membrane permeable calcium chelator BAPTA-AM had an effect. Finally, we used cobalt chloride ($CoCl_2$) to chemically simulate hypoxia and found no modulation at the "hypoxic" or the "reperfusion" phase of the experiment (data not shown). Thus both heat shock, oxidative stress and pH of the UW solution storing the liver may have an additive or synergistic effect in the inhibition of the als2 mRNA expression in vivo, where liver biopsy specimens are concerned.

Pathways used to modulate alsin/als2 mRNA. Many of the inhibitors used in this study were previously reported to influence the extent of ischemia/reperfusion-mediated damage. Of the inhibitors that are protective against ischemia/reperfusion-related damage, roscovitine protects both liver and kidney from ischemia-reperfusion induced injury (Aydemir et al., 2002; Topaloglu et al., 2003), while genistein improves the survival of mice subjected to total liver ischemia (Yamamoto et al., 1996). Finally, BAPTA-AM reduces the rat cortical infarct volume after focal cortical ischemia (Tymianski et al., 1993). Of these inhibitors that were protective against ischemia-reperfusion related injury, only genistein was inhibitory to als2 mRNA regulation even basal levels when no stressor was used (Figure 6A).

Other inhibitors have a deleterious effect, when administered prior to ischemic preconditioning, a method used to reduce liver injury (Carini and Albano, 2003). This is confirmed for genistein and chelerythrine as they eliminate the beneficial effects of ischemic preconditioning (Ricciardi et al., 2001). An in vitro model of ischemic preconditioning, also confirm the previous results (Carini et al., 2000; Carini et al., 2001a). The MEK-1/2 inhibitor PD98059 increases apoptosis in cardiomyocytes upon ischemia/reoxygenation, while isolated rat hearts demonstrates slower functional recovery when treated with PD98059 prior to global ischemia/reoxygenation (Yue et al., 2000). Others demonstrated that intra-myocardial infusion of PD98059 in porcine heart abolished the protective effects of ischemic preconditioning (Strohm et al., 2000). Our results show that some of the inhibitors tested such as genistein or PD98059, did reduce stress-mediated up-regulation of als2 mRNA, caused by antimycin A (Figure 6B) and DTT (Figure 6C). Finally, while ischemic preconditioning rabbit hearts activates casein

kinase-2, DRB fails to abolish the positive effects of preconditioning (Kim et al., 1999). Interestingly, our results demonstrate that DRB was most effective against DTT induced als2 mRNA. DTT induces ER-stress and is used to model a facet of UPR. It is possible that lack of cold preservation, heat-shock, or blood-supply reduces ER-stress, when the tissue is reperfused ex-vivo with a 37ºC buffer solution (Krebs-Henseleit) (Kim et al., 1999). Genistein, which had abolished the positive effect of ischemic pretreatment also inhibited als2 up-regulation by DTT, while PD98059 pretreatment also worsened the ischemia-reperfusion induced injury and inhibited DTT but not antimycin A mediated als2 mRNA up-regulation. Thus an aspect of preconditioning may involve redox-regulation. Whether alsin is involved in other aspects such as endocytosis, early endosomal trafficking and nuclear shuttling remains to be determined.

F. What needs to be done

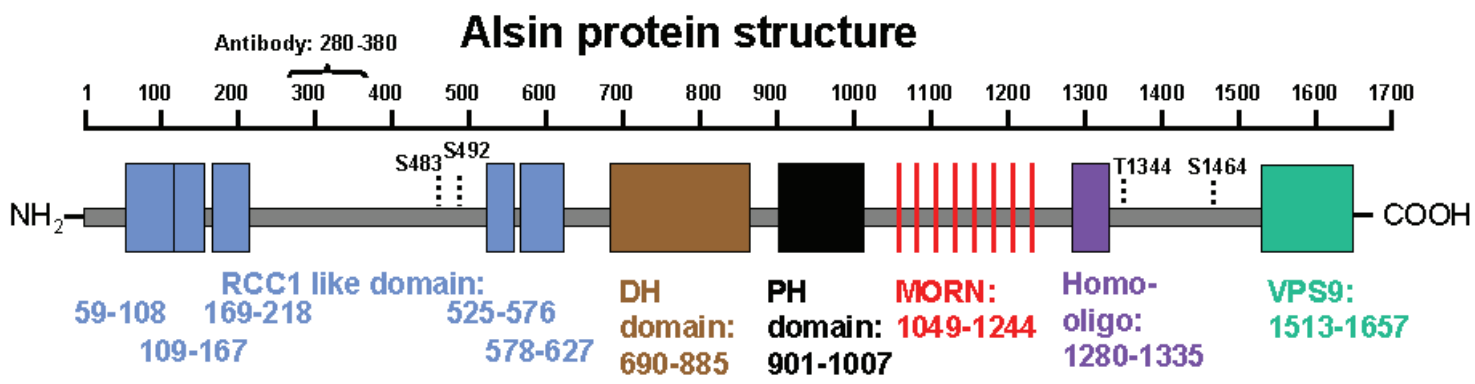
als2 mRNA and protein expression in human liver biopsy specimens

Following the elaboration of the list of genes identified by microarray analysis, a validation step using semi-quantitative RT-PCR is being carried out for als2. To this end, samples used previously for the microarray experiments, as well as independent samples would be analysed. RT-PCR to reveal the als2 expression levels 60 min time-point biopsies compared to the 0 time-point biopsies (Figure 2A). Similarly using the anti-alsin antibody that we are characterizing (Figure 2B), immunoblotting analysis of alsin on liver biopsy extracts to reveal the level of expression (Figure 2C) following normalization to the endoplasmic reticulum membrane-resident-protein calnexin. Finally, immunohistochemistry on frozen human liver sections using the anti-alsin antibody to reveal the localization of alsin to (Figure 2E) when compared to a control (Figure 2D).

The role of alsin in IRI-related stress

Moreover, based on alsin protein (Figure 7) we are currently making 7 alsin constructs (Figure 8), each of which will activate specifically Rac1, Rab5 or Ran in the chosen cell line. The constructs with an asterisk have already been made and sequenced. Two constructs are left to design. This will help us to determine the consequences of Ras-related effector activation during *in vitro* modeling of transplant-relevant stresses (heat-shock, metabolic and reducing-oxidizing stresses). Thereafter various outcomes will be measured: 1) the actin cytoskeletal rearrangement will be evaluated microscopically, as our laboratory has previously done (Emadali et al., 2006);

2) the NADPH oxidase function will be assessed by measuring intracellular ROS production via 2′,7′-Dichlorofluorescein diacetate in intact cells (Vejrazka et al., 2005); 3) the Rab5 endocytotic function will be evaluated using the non-toxic component of the cholera toxin, cholera toxin subunit b (Hagiwara et al., 2009); 4) the cell proliferation will be evaluated by counting; and 5) the previously identified cell line transfected by alsin construct will be used to activated Ran. Subcellular fractionation and Western blot techniques will be used to identify down-stream Ran effectors that translocate to the nucleus or cytosol. Cell proliferation and apoptosis will also be assayed.



RCC1 like domain: **R**egulator of **C**hromosome **C**ondensation. Activates RAN or mediates protein-protein interaction

DH domain: **D**iffuse **B** cell **L**ymphoma (**DBL**) **H**omology domain, part of a RhoGEF for Rac1 together with the pleckstrin homology (PH) domain.

PH domain: **P**leckstrin **H**omology domain binds to 1) β/γ subunit of heterotrimeric G proteins; 2) phosphatidylinositol-4,5-bisphosphate; and 3) phospho-Ser/Thr residues.

MORN: **M**embrane **O**ccupation and **R**ecognition **N**exus motif, possible linker allowing membrane-protein interaction.

Alsin Homo-oligomerization domain

VPS9: **V**acuolar **P**rotein **S**orting **9** domain catalyzes nucleotide exchange on Rab5.

Serine and threonine phosphorylation sites with as yet unknown consequences

Figure 7. The Alsin protein. Although RCC1, DH/PH and VPS9 represent the Ran, Rac1 and Rab5 RhoGEF domains respectively, the domains that actually function as RhoGEF in the in vivo situation is different (see text). Homo-oligomerization is achieved through the 1280-1335 amino acid residues between MORN and VSP9 regions, which are required for Rab5 RhoGEF function. The function of phosphorylation has not been determined. The antibody corresponds to 280-380 amino acid sequence.

Alsin constructs

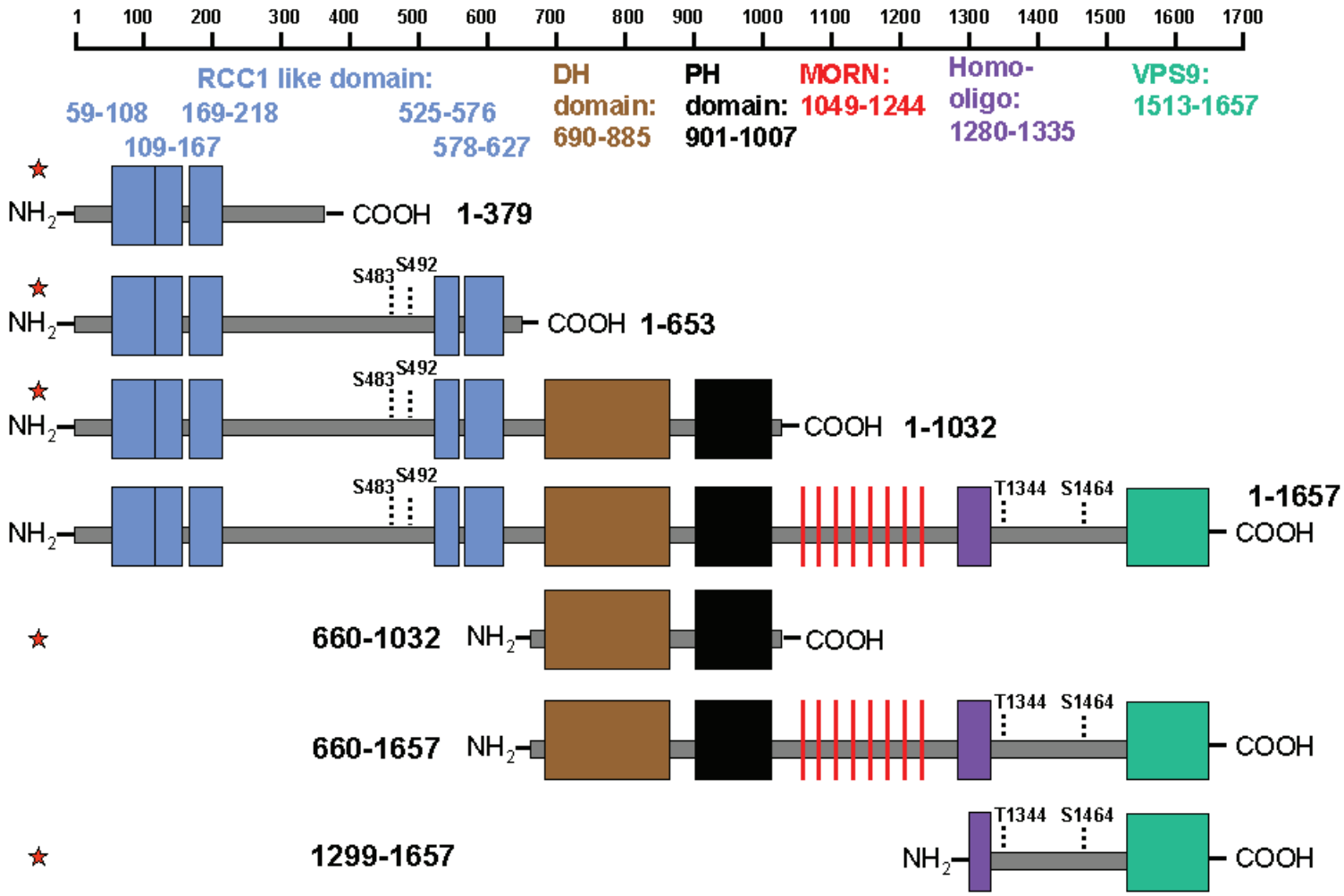


Figure 8. Alsin constructs. Alsin constructs revealing various deletion mutants designed to activate selectively one RhoGTPase. Although RCC1, DH/PH and VPS9 represent the Ran, Rac1 and Rab5 RhoGEF domains respectively, the domains that actually function as RhoGEF in the in vivo situation is different. For Rac1 DH/PH domain is sufficient for binding but not activation, while the minimal alsin design for activation is not known. The 1299-1657 would be sufficient to bind to Rab5, but optimal activation is reported to be done by the 660-1657 construct. For the Ran construct, although RCC1 bind to Ran and full length alsin gives a modest activation of Ran in a cell free system, Ran activation in the context of a cell was not reported (see chapter 3 section C1).

While the MKP-1 story has shed light on the negative regulation of the stress-associated kinases JNK-1 and p38MAPK, and the alsin chronicle awaits full revelation, we can nevertheless say something about alsin. According to our results, alsin seems to be involved in redox regulation. In the general discussion, I will endeavour to bring everything together. The ultimate purpose is to create a more complete view of the process of liver transplantation.

Chapter 6: General discussion

A. What have we learned from our results?

1. Overview. The transcriptional profiling of the normal human liver during the reperfusion phase of transplantation is an important first step, in the characterization of the normal liver's response to this "artificial" process that is plagued by multiple variables, and potentially multiple noxious stimuli. The reason we chose to focus our attention on the reperfusion aspect of the human liver transplant operation is that the cold ischemic phase only represents a partial number of the total stresses that an organ is subjected to during the process of transplantation. Cold ischemic period does not include: 1) mechanical stress associated with blood flow and surgical manipulation of the organ. Although the organ is flushed with UW solution, it is not flushed directly but within the context of the body; in situ, therefore the sinusoids may not be exposed to the same amount of sheer stress; 2) the warm ischemic phase related to the re-implantation of the organ prior to blood-flow; and 3) oxidative stress due to blood flow among other plasma-related factors and stresses. Thus the reperfusion phase affords a more complete gamut of stresses and in consequence, a more global response of the liver to the ischemia-conditioned reperfusion-related stresses. Accordingly, our transcriptional profiling analysis has allowed us to query the tissue on a global scale and determine how the liver is dealing with the sum of the stresses, at the 1h post-reperfusion time-point. Our profiling revealed a list of immediate-early genes that have been presented in chapter 3 sections B and C. Of the identified genes, we selected to focus our attention on MAP kinase phosphatase-1 and alsin. The reasons for our choices are presented in the following sections.

2. MKP-1. Of the immediate early genes that may be implicated in damage control, we identified the dual specificity phosphatase MKP-1, which was found to be up-regulated during the reperfusion phase of the operation. We believe that the up-regulation of MKP-1 serves to limit the signal transduction of the various members of the MAPK family, more specifically JNK and p38MAPK, and reduce the possibility of MAPK-triggered apoptosis, based on the following observations: 1) our localization of MKP-1 to hepatocytes by immunohistochemistry (Boutros et al., 2008b); 2) our laboratory's previous findings of phosphorylated members of MAPK family (ERK, JNK and p38MAPK) during the reperfusion phase of the operation (Emadali et al., 2005); 3) our *in vitro* HepG2 experiments indicating that MKP-1 can reduce JNK-1 and p38MAPK-

phosphorylation levels, but not ERK1 phosphorylation levels (Boutros et al., 2008b); 4) our findings that MKP-1 silencing, via shRNA in HepG2 cells, increases apoptosis when these cells are exposed to ischemia-reperfusion relevant stresses in vitro (Boutros et al., 2008b); and 5) previously published animal models of cardiac ischemia/reperfusion mediated injury demonstrating that the presence of MKP-1 was instrumental in reducing infarct the size (Kaiser et al., 2004). This would appear to be one of the mechanisms invoked by the hepatocytes to mitigate damage by the MAPKs. As MKP-1 is a ubiquitously expressed stress-responsive gene (Boutros et al., 2008a), it would be interesting to determine if the other cell types within the liver also control MAPKs via up-regulation of MKP-1, or if they use phosphatases to target MAPKs (Boutros et al., 2008a). Using MKP-1 expression to inactivate the MAPK member that is most expressed in the cell is an interesting prospect. However, once the levels of the targeted MAPK become controlled, or the stressor is inactivated, MKP-1 may target other members of the MAPK family members if it continues to be expressed. This should be considered in the event of therapeutic targeting of MKP-1. In any case MKP-1 expression may not be the determining factor in liver well-being post transplantation. Other variables, such as the presence of other genes or the UW perfusion solution must also be taken into consideration as will be revealed shortly.

3. Alsin. Our preliminary results with alsin reveal that its corresponding mRNA is regulated by the redox status. More specifically the reducing agent DTT up-regulated als2 mRNA, while hydrogen peroxide (H_2O_2), an oxidative stressor slightly down-regulated this messenger. A plausible interpretation of these results is that alsin is activating Rac1, a subunit of NADPH oxidase (Bosco et al., 2009). Of course this would need to be verified by assaying the amount of reactive oxygen species (chapter 5 section E). Other functions of Rac1, such as apoptosis and actin cytoskeletal rearrangement would also need to be assayed to determine which of the Rac1 functions alsin is targeting. The other RhoGTPases, Rab5 and Ran cannot be discounted. As such vesicle size, early endosomal trafficking and alsin colocalization with Rab5 can be assayed (Gorvel et al., 1991; Lanzetti et al., 2001; Otomo et al., 2003), while microtubule dynamics and the shuttling of proteins can be determined in both the cytosolic and the nuclear compartments to verify the effect of alsin on Ran activity (Schatten, 2008) (also chapter 3 section C1 and chapter 5 section E). This would give a more complete picture in regards to the implications of alsin function during the cell's attempt to deal with ischemia-reperfusion relevant stresses (Boutros et al. manuscript in preparation).

4. Unpublished results. Our microarray study revealed a number of genes that have either been previously implicated in organ- or cell-related ischemia reperfusion or completely uncharacterized vis-à-vis this paradigm; these have been dealt with in chapter 3 sections B and C. At present, a reiteration of what has been mentioned in these sections will not be done. Instead some of the more salient points summarizing their expression, or a lack thereof, will be given in an attempt to construct a clearer picture of the liver's response to transplantation. Although the heat shock protein family, is well documented for their folding of proteins (Bukau et al., 2006), they are also implicated in apoptosis at many levels (Beere, 2005; Yenari et al., 2005). Thus their presence could serve for both the prevention of protein aggregation, caused by different types of noxious stimuli and hence accounting for the presence of the different heat shock protein family member expression (Chapter 3 section B2), they could also help cell survival by interfering with apoptosis (Beere, 2005; Yenari et al., 2005). The transcription factors, c-Jun, JunB, FosB and N-Myc would be needed to transcribe the appropriate genes the cell needs to survive (chapter 3 sections B10 and B12). NGFB-I could be the liver's attempt to use sources of energy other than glucose, such as glycerol (chapter 3 section B11). ID2 the inhibitor of DNA binding 2 would be needed to inhibit the transcription of genes that are not immediately needed or even deleterious to the survival of the cell at that point in time (chapter 3 section B12). Round1 (RND1) could restructure the actin cytoskeleton to normal after ischemia-reperfusion imposed remodelling of the actin cytoskeleton, perhaps to ensure cell survival (Emadali et al., 2006). RhoB may target pro-survival molecules Akt and Src to appropriate subcellular compartments (chapter 3 section B6).

The liver's attempt to re-equilibrate its metabolism may be partially reflected by the presence of insulin-like growth factor binding protein-1 and phosphoenolpyruvate carboxykinase-1 or may have intracellular implications (chapter 3 sections B7 and B8). PAI-1 up-regulation may be the result of blood loss, and the liver's attempt to increase coagulation, thereby decrease exsanguination (chapter 3 section B4). Although MRP3 exports bile acids, as well as conjugated and unconjugated bilirubin from the liver to the blood to limit toxicity to the regenerating liver (Csanaky et al., 2009), this mechanism may not be relevant during transplantation, or may not be on the list of priorities for hepatic survival (chapter 3 section B9). Alternatively, this transcript should be up-regulated to limit toxicity to the liver. SNAP50 is down-regulated (Boutros et al, unpublished results), as a subunit of the transcription factor $SNAP_C$, one of its functions

is to transcribe snRNA which are implicated in the maturation of mRNA (chapter 3 section C3). One possibility is that the immediate early genes we identified do not require maturation by snRNAs, or that the function of $SNAP_C$, and by extension SNAP50, may not be immediately required or that there are sufficient snRNAs to accomplish the task. Two of the transcripts, DSIPI and DEPP, are not sufficiently characterized and their role in liver transplantation remains nebulous (chapter 3 sections C4 and C5). Finally, all this transcription and translation require control, at the transcriptional level by tristetraprolin which destabilizes mRNA and ubiquitin B which can tag, polyubiquitinate, proteins and target them for proteasomal degradation (chapter 3 sections 13 and 14). This assures that the immediate early genes that are transcribed and their protein products do not linger in the cell past the point of their use. Overall the liver seems to be setting into motion mechanisms to assure its survival.

B. Where do we go from here?

1. Our results. Needless to say the first step is to verify the identity of the cell types that expresses each of the identified genes. Thereupon an appropriate protocol could be set-up to determine the involvement of the protein product of each gene vis-à-vis cell survival, and overall hepatic well-being, in relation to ischemia and reperfusion related stresses and determine if the previously mentioned hypotheses hold way. Of course we would also need to establish how these genes fit in relation to each other. Once this is done, we would have a better image of what happens to the human liver, from an in vitro model standpoint, at the 1h post-reperfusion time point. To test theses in vitro finding, a judiciously selected animal model, coupled to adenoviral-mediated gene expression (constitutively active or dominant negative proteins or shRNA), could be used to confirm or eliminate the hypotheses based on in vitro results. This would allow each gene to be assayed on a cell-type specific basis. However, we should not forget some of the species differences exist in terms of vascular anatomy (chapter 2 section A2a,b,c) and hepatic innervation (chapter 2 section C1c) and the requirement of glutathione, because glycine as a substitute would be insufficient in the University of Wisconsin solution (chapter 2 section B4d.iii). Thus results from animal models of ischemia/reperfusion cannot be used to infer the behaviour of human liver during the process of transplantation. Rather data from multiple species should be used as guidelines and whatever known species differences exist should be taken into account when interpreting the data.

2. The University of Wisconsin solution. This thesis has endeavoured to present and dissect the constituents of the University of Wisconsin solution and determine how each ingredient contributes to the well being or the demise of the liver during storage and after reperfusion. First and foremost is the lack of buffering power of the UW solution as 12mM of lactic acid (Silva et al., 2006) can be generated by the liver during hepatic storage, 3mM protons from GSH to GSSG oxidation (chapter 2 sections C2 and C3). Such a lack of buffering capacity may have an effect on hepatic viability, especially for marginal livers. Another obvious shortcoming is that the components of the UW solution have not been rigorously tested in terms of their actual mechanisms of action and toxicity (Table 1 chapter 2 section B4, p.53-58). Of course we have just overviewed the toxicity via contribution of protons. Other components also need to be tested.

Raffinose was added to UW solution as an impermeable osmotic agent, additional osmotic support, prevent cell swelling and lactobionate was used also used as an impermeable osmotic agent, prevents cell swelling and later found to coordinate Cu^{2+}, Mn^{2+} and Fe^{3+} and Fe^{2+} among other ions (Table 1 chapter 2 section B4, p.53-58). However, raffinose is composed of galactose, glucose and fructose (Streitwieser A, 1985), and lactobionate is composed of galactose and gluconic acid (Tomlinson et al., 1978). Given their composition, can fructose and galactose be recognized by lectins/ficolins and asialoglycoprotein receptors, respectively, when they are in the context of raffinose? Could raffinose and lactobionate be considered as foreign sugars by the liver as if they were derived from a microorganism? Although the University of Wisconsin solution has been tried and tested (El-Wahsh, 2007; Southard and Belzer, 1995), given that 30mM of raffinose and 100mM of lactobionate are used in UW solution, it may be advisable to conduct an experiment with radiolabelled raffinose or lactobionate (one of the monosaccharides can be labelled) and determine if such interactions between galactose or fructose and their potential partner proteins do occur, and verify if raffinose or lactobionate can activate the complement component system (chapter 3 section B5a). In this event other impermeable osmotic agents may be used to replace these sugars. This may be advisable when dealing with marginal livers, and may explain why they are less tolerant toward ischemia reperfusion injury that normal livers.

3. Other considerations. The complement component system (chapter 3 section B5) is not properly characterized where the membrane attack complex function is concerned. A case in point, a detailed analyses of the function of each of the complexes,

C5b-7, C5b-8 and C5b-9_n in terms of C5b-7 monomer and dimer, C5b-8 function versus C5b-9_n, and the various forms of C5b-9_n as this complex changes form having 1 C9 up to 16 C9 molecules incorporated in C5b-9_n. This analyses should be carried out on a cell-type, developmental stage, differentiated versus undifferentiated cell basis. One can also include normal versus pathological conditions in the analyses. In addition there are plasma membrane bound inhibitors such as: 1) decay accelerating factor, that inhibits the formation of C3 convertase, which converts C3 into C3a and C3b (C3 convertase is part of the C5 convertase, which is formed by C3convertase-C3b); 2) CD59 inhibits C9 binding to C5b-9_n; and 3) C8-binding protein/homologous restriction factor sequesters C8 and inhibits C5b-8 formation which prevents C5b-9_n formation (Fosbrink et al., 2005) (chapter 3 section B5, figure 5). In short, there is a long road before we understand this how the C5b-7, C5b-8 and C5b-9_n complexes function and the consequence of their regulation.

What do we know about the active components of C5, C5a and C5b as part of the complement component system thus far? While both have pro-inflammatory effects, can lead to attraction of immune cells and lysis of both foreign micro-organisms and endogenous cells that need to be eliminated, to much or inappropriate activities of these and other members of the complement component cascade can lead to diseases. Conversely the absence of C5 also leads to disease. However, in small quantities, C5a leads to liver regeneration, in animal models, the human counterpart has not been demonstrated as yet. C5b as part of an immunological complex can lead to signal transduction and cell survival, proliferation and other biological responses depending on the identity of the complex, C5b-7, C5b-8 or C5b-9_n, and the number of components monomers/dimers and C9 multimers. There are many unanswered questions. How much C5 in the plasma does one need to be healthy? Based on what has been previously mentioned, some people seem healthy with very low C5 activity while their identical siblings have diseases.

These considerations are nothing but a sampling of the questions that need to be answered. As there are many pieces missing from the picture, there is still much information that is unknown or experiments that have not been done in humans, which causes the picture to be very grainy. Even if all these answers come to light, there are still many more considerations, many more variables associated with the process of transplantation. For instance, although our laboratory has previously published results concerning the hepatic phosphoproteomics, during the reperfusion phase of the

operation (Emadali et al., 2006), phosphorylation per se is known to have at least five different consequences for proteins: activation, inactivation, stabilization, destabilization and priming (Boutros et al., 2008a). Therefore, there is still much work to be done where appreciating the consequences of phosphorylation is concerned in terms of protein function. Is this all there is to liver transplantation, genes, phosphorylation and a good storage solution? What if we master transplantation tomorrow?

C. Reality sets-in

Although the purpose of our study is clear, by defining what constitutes a normal response to transplantation, we will be in a better position to understand why marginal livers fail, let us not oversimplify the situation. Marginal livers may fall into different categories, such as age-related, steatotic(fatty)-related, fibrotic-related among other subdivisions of marginality, which may not have a common resolution. In addition, the profile of a normal liver's response to transplantation is only the first step. There are other considerations at hand. The first of these is the prevention of immunological rejection with the requisite treatment of the patient with immunosuppressants. If one considers the plethora of syndromes that result from their use (Liu and Schiano, 2007), it is worthwhile if not necessary to find a means of inducing immunotolerance between the host and graft. While both of the previous goals are laudable, they offer little solace to patients with a recurrent disease. As the name implies such a disease would eventually destroy the graft. Therefore even if both of the first two hurdles are overcome the liver would eventually fail. So why bother establishing the normal liver's response to transplantation? As cures are hard to come by, determining the normal liver's response to transplantation is a legitimate course of action if we are to use marginal livers and increase the number of recipients. In addition, resolving the problem of immunotolerance would also reduce the immunosupressant load, and the syndromes associated with them, and may help in mounting an immune related defence against some of the recurrent diseases. Thus, as a journey of a thousand miles begins with a single step, we have taken our first stride.

VII. References:

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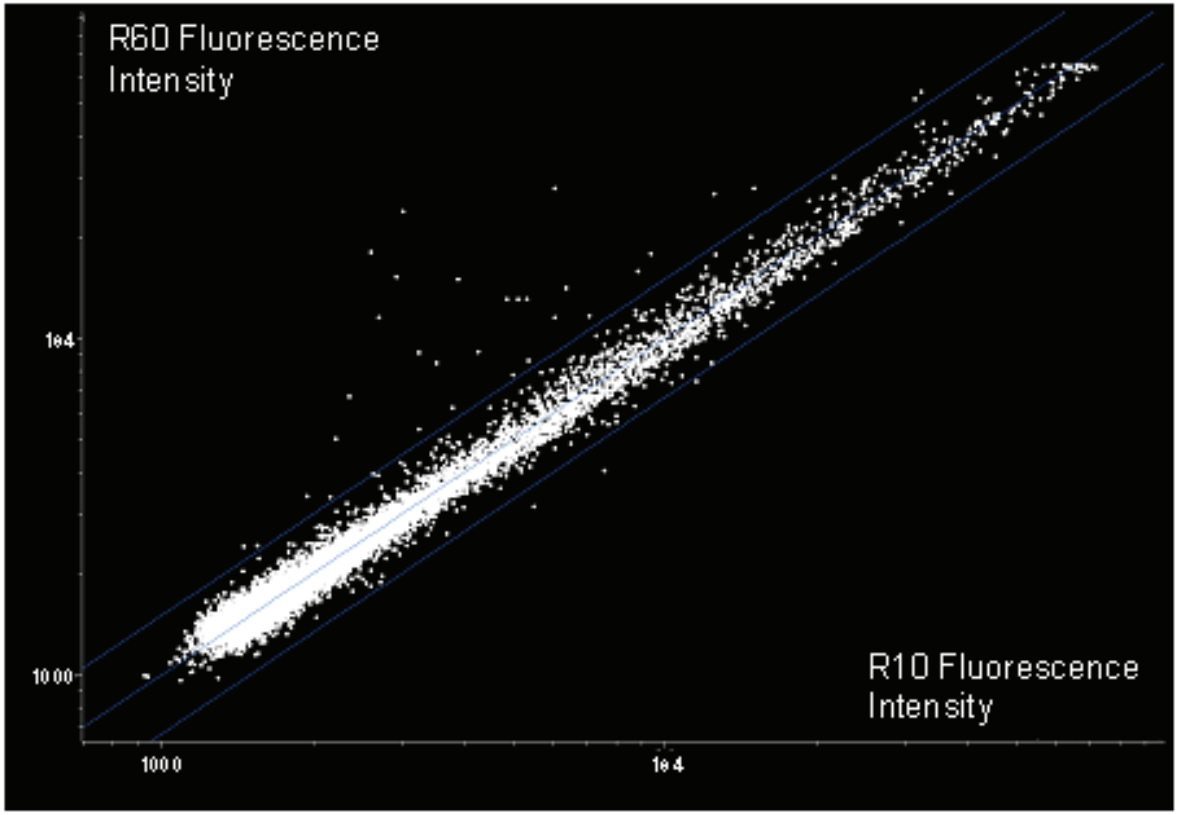
VIII. Appendices

A. Supplementary information for chapter 4



Supplementary Figure 1. Scatter plot of the fluorescence intensities on the Experiment and Control channels. The spots with an average change in fluorescence ratio of more than 1.5-fold (diagonal blue lines) that show acceptable fluorescence intensity and low-intensity spots tended to have a fluorescence ratio that would approach 1.

B. Published review

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Mitogen-Activated Protein (MAP) Kinase/MAP Kinase Phosphatase Regulation: Roles in Cell Growth, Death, and Cancer

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***Abstract*——Mitogen-activated protein kinase dual-specificity phosphatase-1 (also called MKP-1, DUSP1, ERP, CL100, HVH1, PTPN10, and 3CH134) is a member of the threonine-tyrosine dual-specificity phosphatases, one of more than 100 protein tyrosine phosphatases. It was first identified approximately 20 years ago, and since that time extensive investigations into both mkp-1 mRNA and protein regulation and function in different cells, tissues, and organs have been conducted. However, no general review on the topic of MKP-1 exists. As the subject matter pertaining to MKP-1 encompasses many branches of the biomedical field, we focus on the role of this protein in cancer development and progression, highlighting the potential role of the mitogen-activated protein kinase (MAPK) family. Section II of this article elucidates the MAPK family cross-talk. Section III reviews the structure of the *mkp-1* encoding gene, and the known mechanisms regulating the expression and activity of the protein. Section IV is an overview of the MAPK-specific dual-specificity phosphatases and their role in cancer. In sections V and VI, mkp-1 mRNA and protein are examined in relation to cancer biology, therapeutics, and clinical studies, including a discussion of the potential role of the MAPK family. We conclude by proposing an integrated scheme for MKP-1 and MAPK in cancer.**

I. Introduction

MAP[1] kinase phosphatase-1 (MKP-1) is one of many phosphatases coded by the mammalian genome (Keyse, 2000; Alonso et al., 2004c; Arena et al., 2005; Tonks, 2005) and is a member of a subfamily of phosphatases

[1] Abbreviations: MAP, mitogen-activated protein; MKP-1, MAP kinase phosphatase-1; ERK, extracellular regulated kinase; MAPK, mitogen activated protein kinase; JNK, Jun N-terminal kinase; MEK, mitogen activated protein kinase kinase (MAPK)/extracellular regulated kinase (ERK) kinase; H-Ras, Harvey-rat sarcoma virus oncogene; K-Ras, Kristen-rat sarcoma viral oncogene; N-Ras, neuroblastoma ras oncogene; Raf-1, v-raf-1 murine leukemia viral oncogene homologue 1; c-Myc, cellular-myelocytomatosis oncogene; CDK, cyclin-dependent kinase; AP, activator protein; PAK, p21-activated protein kinase; FAK, focal adhesion kinase; Src, rous sarcoma oncogene; MAPKKK, mitogen activated protein kinase kinase kinase; MKK, mitogen-activated protein kinase kinase; MEKK, mitogen activated protein kinase kinase kinase (MAPK)/extracellular regulated kinase (ERK) kinase kinase; MLK, mixed-lineage kinase; TAK-1, transforming growth factor-β-activated kinase-1; ASK, apoptosis signal-regulating kinase; JIP, JNK-interacting proteins; IκBα, inhibitor of nuclear factor-κBα; KO, knockout; TAB, TAK-1 binding protein, transforming growth factor-β-activated protein kinase binding protein; MAPKAP-K, MAPK-activated protein kinase; TTP, tris-tetraprolin; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; HSP, heat shock protein; MAPKK, mitogen activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; SOS, son of sevenless; HUVEC, human umbilical vein endothelial cell; ER, estrogen receptor; HSF-1, heat shock factor-1; WT, wild-type; TNF-α, tumor necrosis factor-α; miRNA, microRNA; EGF, epidermal growth factor; DSIF, 6-chloro-1-β-D-ribofuranosylbenzimidazole sensitivity-inducing factor; ELK-1, Ets-like protein-1; Skp2, S-phase kinase-associated protein-2; PK, protein kinase; SCF, Skp1/Cul1/F-box; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; cisplatin, (*cis*-diaminedichloroplatinum II); LPS, lipopolysaccharide; Ro 31-8220, 3-[1-3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl) maleimide (bisindolylmaleimide IX); VHR, vaccinia virus

known as the dual-specificity phosphatases (Camps et al., 2000; Theodosiou and Ashworth, 2002; Farooq and Zhou, 2004; Dickinson and Keyse, 2006). Initially identified as one of a set of genes that are expressed in cultured murine cells during the G_0/G_1 transition (Lau and Nathans, 1985), it is part of a family, most of which demonstrate a wide tissue distribution (Camps et al., 2000). As their name implies, the dual-specificity phosphatases can dephosphorylate two types of residues, threonine and tyrosine. The function of these phosphatases is to dephosphorylate and therefore inactivate the MAP kinases (Slack et al., 2001; Liu et al., 2005a), ERKs (Sun et al., 1993; Duff et al., 1995; Sarközi et al., 2007), p38MAPKs (Kaiser et al., 2004), and JNKs (Sánchez-Pérez et al., 1998). This dephosphorylation activity has been shown to be context-dependent; not all three types of MAP kinases are targeted for dephosphorylation in a given situation, at least in the case of MKP-1 (Wu and Bennett, 2005; Wu et al., 2005b).

As the archetypal member of its family, MKP-1 continues to be the most exhaustively studied. Thus, much of the function and domains of MKP-1 is known; unfortunately, the three-dimensional structural analysis remains to be determined. Although the MKP-1 protein function is characterized in many cells and tissues in both animal models and humans, in this review we will focus on the relationship between MKP-1 and the axes of the MAPK family, emphasizing the involvement of both MKP-1 and the family of MAPK in cancer. As MKP-1 is one of the MAPK-specific dual-specificity phosphatases, which include other MKPs and atypical dual-specificity phosphatases, we also summarize the substrate specificity, the expression pattern, and the involvement of these phosphatases in cancer. Finally, we also review the expression of MKP-1 and chemotherapeutic agents, their mechanism of action, and relevant clinical trials.

homolog 1-related; DUSP1, dual specificity phosphatase-1; HVH, human vaccinia virus homolog; PAC-1, phosphatase in activated T-cells; Pyst, phosphorylates tyrosine serine threonine; JKAP, JNK pathway-associated phosphatase; JSP, JNK stimulatory phosphatase; LMW-DSP, low-molecular-weight dual-specificity phosphatase; AKT, v-akt murine thymoma viral oncogene homolog 1; STYX, phospho-serine/threonine/tyrosine interacting like-1; MDSP, muscle-specific dual specificity phosphatase; TMDP, testis and skeletal muscle dual-specificity phosphatase; IL-6, interleukin-6; IL-10, interleukin-10; IL-12p70, interleukin-12 protein 70 kDa; GSK glycogen synthase kinase; MEFs, mouse embryonic fibroblasts; shRNA, short hairpin RNA; PDK, phosphoinositide-dependent kinase-1; γ2A/AT_1, angiotensin type 1 receptor only; Jak, Janus tyrosine kinase; siRNA, short inhibitory RNA; transplatin, *trans*-diaminedichloroplatinum II; SP600125, anthra[1,9-*cd*]pyrazol-6 (2*H*)-one; Fas, apoptosis-mediating surface antigen; FasL, Fas ligand; BBI, Bowman-Birk inhibitor; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; DETA-NONOate, (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate; NO, nitric oxide; PARP, poly(ADP-ribose) polymerase; NF-κB, nuclear factor κB; HIF-1, hypoxia inducible factor-1; CBP, cyclic AMP response element-binding protein; APC, adenomatous polyposis coli; Wnt, wingless; NSCLC, non–small-cell lung cancer.

II. Mitogen-Activated Protein Kinase Family: An Overview

To appreciate the possible consequences of phosphatase function, a thorough overview of the axes of MAPK will be undertaken. Although for simplicity, a linear presentation is given for each of the three branches of the MAPK cascade (Cuevas et al., 2007) (Fig. 1), the MAPKs interact with one another, either directly or indirectly, which will be addressed in section II.E (Fig. 2). An overview of the contribution of MAPK modules to the tumorigenic phenotype will be presented in sections V.C on MAPK and cancer and also in sections V.D and VI.

A. Ras-Raf-Mitogen-Activated Protein Kinase Kinase/Extracellular-Regulated Kinase Kinase Kinase-Extracellular-Regulated Kinase Interactions

A thorough review of this branch of MAPK signal transduction must start with the monomeric GTPase Ras, which is associated with this archetype-signaling module Raf-MEK-ERK. Ras has three isoforms: H-Ras, N-Ras, and K-Ras; the latter isoform has two splice forms that result from the use of an alternative C terminus, leading to the products K-Ras4A and K-Ras4B (Schubbert et al., 2007). Furthermore, the signaling of these isoforms is partially controlled on the basis of Ras subcellular distribution, in addition to regional distribution across the plasma membrane (Hancock, 2003; Mor and Philips, 2006) that results from post-translational modifications (Konstantinopoulos et al., 2007). Adding to this, there are three Raf isoforms: Raf1 (also called C-Raf), A-Raf, and B-Raf (Wellbrock et al., 2004) that serve nonredundant functions based on the phenotypes of knockout mice (Gerits et al., 2007). The Ras isoforms have different binding affinities to the Raf proteins (Wellbrock et al., 2004). In the context of cells, B-Raf seems to be the dominant form that activates MEK1/2, whereas the isoforms Raf1 and A-Raf may regulate duration of signaling of MEK-ERK among other functions (Wellbrock et al., 2004).

Another means of defining signal specificity is by controlling subcellular localization, a key element of signal transduction, which can be mediated via scaffolding protein. Scaffolds such as MORG1, KSR, and paxillin, in conjunction with other proteins (MP1, p14, and 14-3-3) that act as scaffolds in their own right, target the Raf1-MEK-ERK module to late endosome, plasma membrane, and focal adhesions, respectively (Kolch, 2005). Although MEK can interact with ERK in their unphosphorylated forms with or without scaffolding proteins (Yoon and Seger, 2006), the activation of ERK by MEK forces them apart because of structural changes in phosphorylated ERK. Once activated, ERK1 and ERK2 catalytic activities increase (170,000–250,000 times over basal activity) and have at least 160 potential effectors in the cytosol and nucleus (Yoon and Seger, 2006).

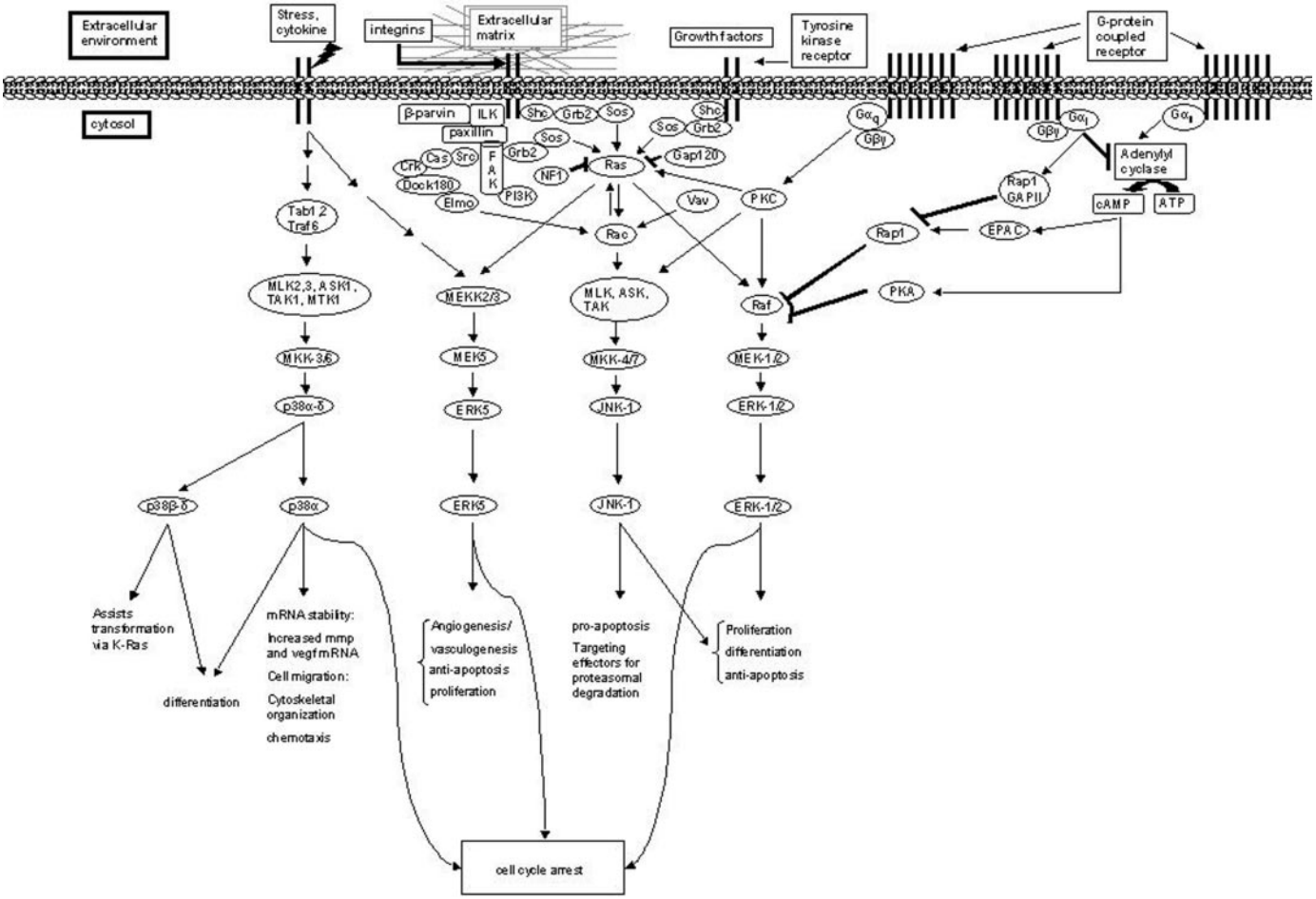

FIG. 1. Linear aspect of MAPK signal transduction. This figure summarizes some of the components in the MAPK signal transduction pathway from the extracellular milieu to the phenotypes associated with the more "classic" MAPK family members, with an emphasis on a linear interaction. SOS, Vav, and exchange protein directly activated by cAMP (EPAC) are guanine nucleotide exchange factors that activate GTPases (Ras, Rac, and Rap1) and neurofibromin 1 (NF1). Gap120 and Rap1 GapII are GTPase-activating proteins (GAPs) that increase catalytic activity of GTPases and inactivate them. For each of the MAPK members represented, the more important functions have been enumerated. See sections II.A–D and V.D. ILK, integrin-linked kinase; Cas, CRK-associated substrate; Elmo, engulfment and cell motility protein.

Among these are transcription factors, kinases, phosphatases, cytoskeletal proteins, and apoptosis-related proteins (Yoon and Seger, 2006).

B. Extracellular-Regulated Kinases

The oldest known and best characterized members of the MAPK family, the ERKs boast at last count six isoform family members (ERK1–5 and ERK7/8) (Bogoyevitch and Court, 2004). By far the most studied are ERK1/2 and ERK-5. ERK1/2 were the first members to be characterized (Bogoyevitch and Court, 2004). The signaling events activating ERK1/2 are usually initiated at the plasma membrane via receptor tyrosine kinases (McKay and Morrison, 2007) and funnel, in part, through the Raf-MEK1/2-ERK module, the outcome of which depends on cell surface receptor density, the amount of ligand, the duration of signaling dictated in part by the rate of internalization and inactivation of receptor, and the cell type under consideration (Marshall, 1995; Murphy and Blenis, 2006). More importantly, duration and strength of signaling can be uncoupled. Two signals may last for an equal amount of time but have different strengths and, conversely, signaling strength can be equal but last for various lengths of time. A combination of strength and duration dictates different outcomes for a given cell-type in response to stimuli (Murphy and Blenis, 2006), with possible outcomes ranging from sustained high activation corresponding to senescence, apoptosis, and differentiation, whereas sustained lower levels of activation correlate with cell proliferation (Agell et al., 2002). The opposite situations in which sustained or transient ERK activation leading to cell proliferation can also be found, depending on cell type, receptor number, and other factors (Marshall, 1995). Tyrosine kinase receptor-mediated activation of ERK1/2 proceeds via a Raf-MEK-ERK signaling module and the activation of ERK1/2 requires threonine and tyrosine dual phosphorylation in a threonine-glutamic acid-tyrosine (T-E-Y) motif (Turjanski et al., 2007). Other cell surface receptors, such as G protein-coupled receptors, e.g., the integrins, among others, can also transmit their signal via MAPK modules or modu-

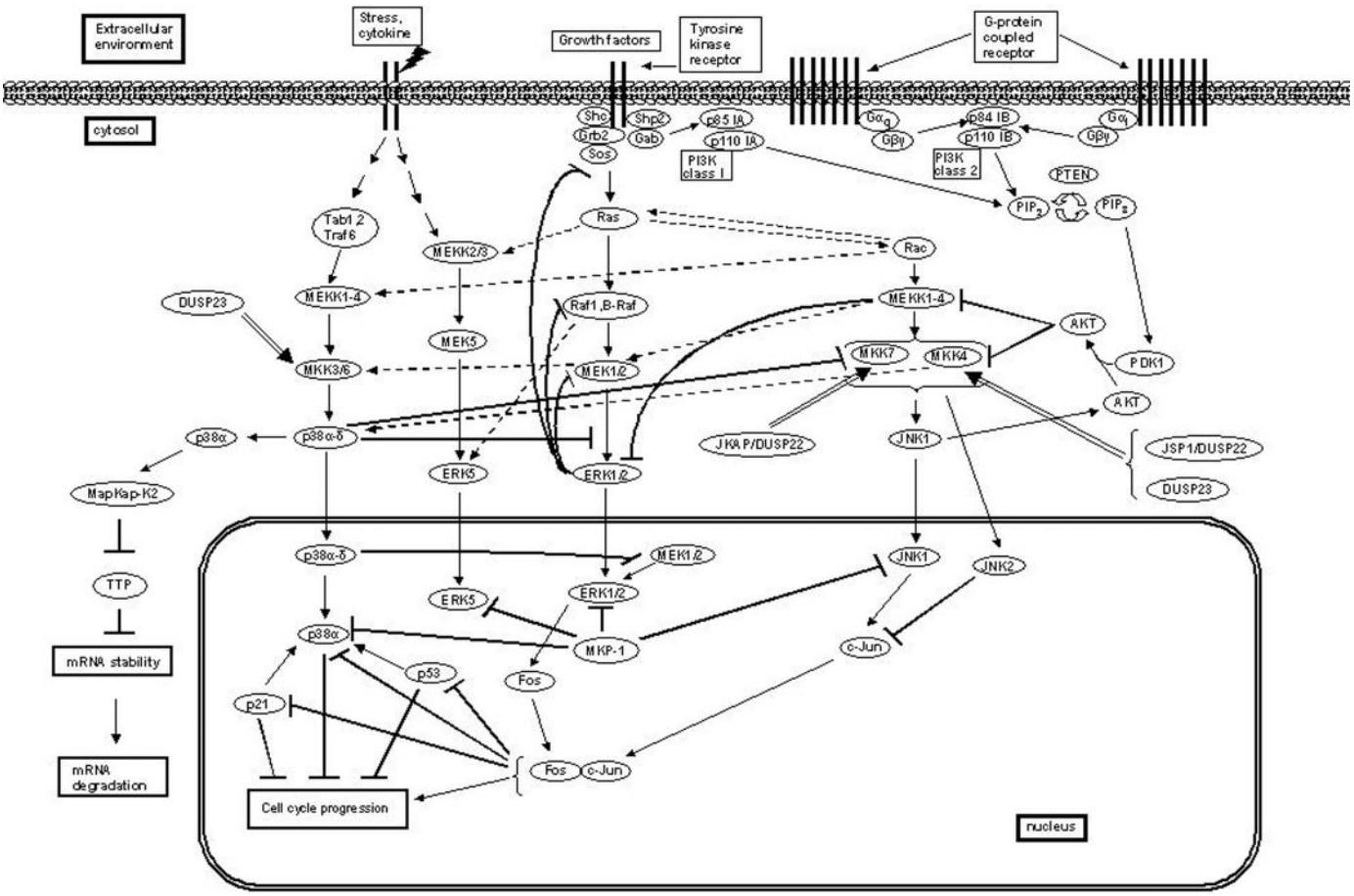

FIG. 2. Cross-talk of the MAPK signal transduction pathway. Summary of the cross-talk and feedback inhibition implicating the MAPK signal transduction pathway. Solid single arrows indicate activating signal transduction within a module (except JNK → Akt which is a priming phosphorylation). Hatched arrows indicate activating cross-talk between signaling modules. Solid double arrows indicate activating dephosphorylation by phosphatases. Solid lines-bars are inhibitory phosphorylation or dephosphorylation. Open arrows indicate cycling between two forms of phosphatidylinositol phosphate (PIP). For more details about these interactions, see section II.E. on MAPK cross-talk and section IV, an overview of the dual-specificity phosphatase family. PTEN, phosphatase with tensin homology.

late the tyrosine kinase receptor signaling (Martin and Vuori, 2004; Olson and Hallahan, 2004; Hannigan et al., 2005; McLean et al., 2005; Cully et al., 2006; Engelman et al., 2006; Holz et al., 2006; Kohno and Pouyssegur, 2006; Goldsmith and Dhanasekaran, 2007; Hehlgans et al., 2007; Mayor et al., 2007; Schubbert et al., 2007).

ERK1/2 activity or a lack thereof has many consequences that will be considered in the context of their functions. Much is known about the involvement of these kinases in various biological processes; however, as ERK1 (p44MAPK) and ERK2 (p42MAPK) share 83% identity and are regulated by similar factors and conditions, distinguishing between the contribution of either kinase to ERK signaling has been challenging. As a detailed review of ERK1/2 in cell cycle progression has been undertaken elsewhere (Chambard et al., 2007; Meloche and Pouyssegur, 2007), we will provide an overview of some of the functions reported for ERK1/2 at different phases of the cell cycle as follows:

- **Preparation for the cell cycle:** ERK1/2 prepare the cell for the cell cycle by 1) synthesis of ribosomal RNA, 2) contribution to chromatin remodeling, 3) increased pyrimidine nucleotide synthesis (Chambard et al., 2007), 4) increased protein synthesis (Meloche and Pouyssegur, 2007), and 5) participation in protein translation (Meloche and Pouyssegur, 2007).
- **G_0-G_1 transition:** ERK1/2 are also implicated in the stabilization of c-Myc, which together with Max forms a heterodimeric transcription factor, one of the essential steps for cells to proceed from G_0 to late G_1 (Jones and Kazlauskas, 2001).
- **G_1-S transition:** 1) Both transient and sustained activation of ERK1/2 can phosphorylate Elk and induce transcription of the c-*fos* gene; however, only sustained ERK1/2 nuclear activity can stabilize c-Fos protein within the nucleus, which leads to increased fra-1 transcription and decreased cyclin D1 transcription, among other transcriptional activities. The sustained ERK1/2 nuclear activity and c-Fos stability dwindle, and Fra-1 and other transcription factors initiate transcription of cyclin D1. 2) ERK1/2 can also up-regulate cyclin D1 via increased stability of c-Myc transcription factor

(Chambard et al., 2007). 3) ERK1/2 phosphorylation of CDK family members, which in turn phosphorylate the retinoblastoma family, is a necessary step for retinoblastoma family members to dissociate from the E2F family members and initiate transcription of genes coding for proteins involved in DNA replication (Meloche and Pouyssegur, 2007). 4) ERK1/2 mediate inhibition of antiproliferative gene transcription throughout the G_1 phase by an AP-1-dependent mechanism (Yamamoto et al., 2006).

- **G_2-M transition:** ERK1/2 activity is required during the early G_2 phase and DNA damage checkpoints during G_2 (Meloche and Pouyssegur, 2007) but is dispensable from this point on for the rest of mitosis (Shinohara et al., 2006). ERK1/2 are also involved in cell cycle arrest, a topic that will be discussed in section V.C on MAPK and cancer.

Although the facet of ERK1/2 function in the control of cytoskeletal remodeling is reviewed in Pullikuth and Catling (2007), we summarize some of the more important points for this review. MP1-p14-PAK signaling has been implicated in PAK-mediated MEK1-ERK activation involving the remodeling of focal adhesion and actin filaments during cell spreading (Pullikuth and Catling, 2007). ERK1/2 also increase tubulin polymerization via inhibitory phosphorylation of a tubulin-sequestering protein, stathmin/Op18, which is also targeted by PAK and CDK5 for inactivating phosphorylation. Although ERK1/2 phosphorylate and activate the calcium-dependent protease calpain, which is implicated in focal adhesion assembly and disassembly, the relationship of calcium, calmodulin, and MAPK signal transduction is a complex network of interactions that goes beyond the scope of this review (Agell et al., 2002; Cook and Lockyer, 2006). However, we will briefly summarize the interaction of some of the previously mentioned players. FAK directs calpain to focal adhesions and is itself degraded by calpain during focal adhesion turnover. Furthermore, FAK may serve as scaffold for ERK1/2 and calpain, allowing these two proteins to interact. These findings are further supported by evidence that *Fak*-null cells have decreased ERK1/2 signaling, decreased calpain activity, and decreased microtubule stability, leading to a reduction in focal adhesion disassembly and reduced cell migration. Such results are explained in light of other data implicating FAK as a necessary component of focal adhesion disassembly but not in focal adhesion formation. Moreover, growing microtubules can target focal adhesions for disassembly. In this way, ERK-mediated tubulin polymerization targets focal adhesion disassembly and participates in the remodeling of the cytoskeleton, focal adhesion turnover, and cell shape and motility (Pullikuth and Catling, 2007).

ERK1/2 also participate in cell differentiation by modulating the stability, activity, and protein-protein interaction of various proteins in a cell type-specific manner (Yoon and Seger, 2006). ERK1/2-mediated degradation of BCL6 allows B-cell differentiation and participates in antibody production. ERK1/2 phosphorylation of the Ets transcription factor ERF, results in a decrease in its repression of gene transcription. Another ERK effector, GATA1, increases its protein-protein interaction after phosphorylation. This modification is implicated in both the development and differentiation of erythroid cells. Stathmin-related functions in the cell cycle and differentiation are also regulated by ERK-mediated phosphorylation (Yoon and Seger, 2006).

The previously mentioned studies were complemented by work on ERK1 and ERK2 knockout mice in an attempt to distinguish between the functions of these isoforms. Some *Erk1*-null mice have no overt phenotype, develop normally, and are fertile, whereas others reveal increased locomotor activity (Gerits et al., 2007) and decreased adiposity due to impaired adipocyte differentiation (Aouadi et al., 2006), among other phenotypes (Gerits et al., 2007). *Erk2*-null mice die in utero from embryonic day 6.5 from lack of mesoderm differentiation to embryonic day 8.5 from lack of proper placental development, depending on the murine background (Gerits et al., 2007). Although ERK1 and ERK2 are frequently thought of as being simultaneously regulated, these results demonstrate that they have nonoverlapping functions under certain conditions. Systematically identifying effectors that recognize one or the other kinase would go a long way to determine the nonoverlapping functions of each kinase.

ERK5, the next best characterized member of the family was cloned in 1995 (Wang and Tournier, 2006) and is also called big MAPK1 (BMK1) owing to its molecular mass of 98 kDa. In mice, this MAPK has three splice forms: ERK5a, ERK5b, and ERK5c. ERK5a is a catalytically active kinase, whereas the shorter N-terminal-truncated forms, ERK5b and ERK5c, are kinase-dead (Yan et al., 2001). The latter two kinases can interfere with ERK5a binding to its upstream kinase MEK5. The N-terminal domain of ERK5a contains three domains: 1) cytoplasmic targeting, 2) MEK5-binding domain, and 3) oligomerization domain (Yan et al., 2001). MEK5 is expressed as two splice forms, MEK5α and MEKβ. The former activates ERK5, whereas the latter acts as a dominant-negative regulator of ERK5 activation (Cameron et al., 2004). In addition, The ERK5 C-terminal kinase contains both a nuclear localization signal and nuclear export signal and an autoinhibitory domain. Upon activation, ERK5 can phosphorylate its C terminus, thereby exposing its nuclear localization sequence (Buschbeck and Ullrich, 2005). A C-terminal truncated splice form, termed ERK5-T, is unable to translocate to the nucleus although it can bind to and sequester ERK5 in the cytosol (McCaw et al., 2005). In nonstimulated cells, ERK5 is either nuclear or diffuse throughout the cell, depending on cell type. Activation of

ERK5 requires a T-E-Y motif (Mody et al., 2003) and can result from mitogenic factors or chemical or physical stresses.

ERK3 and ERK4 are from the same subfamily and are covered together here because there is very little published information on ERK4. ERK3, first identified in 1991 (Boulton et al., 1991), is believed to be a protein of approximately 100 kDa. A 63-kDa form, once believed to be a form of ERK3, has been renamed ERK4 (Coulombe and Meloche, 2007). This ERK4 is not to be confused with the presumed 45-kDa ERK1b splice form, which shares immunoreactivity with ERK1 and is also named ERK4. The mRNA of both *erk3* and *erk4* can be detected in multiple tissues although ERK4 has a more restricted expression pattern (Coulombe and Meloche, 2007). The common ERK1/2 and ERK5 activation motif T-E-Y is replaced by S-E-G in ERK3 and ERK4. Thus, on the basis of current evidence, it is believed that neither of these kinases is dually phosphorylated. However, B-Raf and MEK1/2 have been implicated in the increase of ERK3 protein levels (Hoeflich et al., 2006). ERK4 has thus far not been characterized. Although the function of ERK3 remains unknown, the subcellular distribution of ERK3 is both cytosolic and nuclear and is not affected by mitogens or chemical stress; it is targeted to the nucleus in response to thermal stress. Furthermore, the change in distribution does not require ERK3 to be enzymatically active or its activation motif to be phosphorylated (Coulombe and Meloche, 2007).

As ERK6 is also known as p38γMAPK, this protein is discussed in section II.D on p38MAPKs.

ERK7 was identified in 1999 (Abe et al., 1999), and ERK8 was initially identified as a novel MAPK (Abe et al., 2002). ERK8 is now considered to be the human ortholog of the rodent ERK7; thus, the ERK7 designation will be used for ERK7 and ERK8 (Saelzler et al., 2006; Coulombe and Meloche, 2007). ERK7 mRNA is ubiquitously expressed in human adult tissues. Although the protein has an activation loop with a T-E-Y motif similar to that of ERK1/2 and ERK5, it is constitutively phosphorylated, via autophosphorylation, whereas its half-life is determined by polyubiquitination and proteasomal degradation, similar to ERK3 (Coulombe and Meloche, 2007). The C-terminal portion of ERK7 also determines full kinase activity. Stress, mitogens, and kinases, such as Src and Ret, can induce phosphorylation and activation of ERK7 (Coulombe and Meloche, 2007).

C. c-Jun NH_2-Terminal Kinases

This kinase family is stimulated by stress, cytokines, and growth factors (Roberts and Der, 2007). These stimuli activate the signaling module comprising of MAPKKK-MKK4/7-JNK (Bogoyevitch and Kobe, 2006; Raman et al., 2007; Roberts and Der, 2007). More specifically, JNK-related MAPKKKs are composed of MEKK1, MEKK4, dual leucine zipper-bearing kinase, MLK1–4, leucine zipper-bearing kinase, TAK-1, ASK1, and zipper sterile-α-motif kinase. In turn, these funnel through MKK4 and MKK7, activating the JNKs (Raman et al., 2007; Roberts and Der, 2007). The c-Jun NH_2-terminal kinase family is composed of three isoforms, JNK1, JNK2, and JNK3, which are divided into splice forms. The four splice forms of JNK1 are arranged as follows: α1 and β1 splice forms (p46) and α2 and β2-splice forms (p54). The α1 and β1 splice forms (p46) differ from each other by alternative exon usage, leading to substitution between kinase domains IX and X, which determine substrate specificity. The same exon usage characterizes the difference between the α2 and β2 splice forms. The p46 differ from the p54 splice forms by the C-terminal region that is alternatively spliced. The function associated with the longer C-terminal region found in p54 and lacking in p46 remains unclear. JNK2 also has splice forms (α1, α2, β1, and β2) and molecular masses analogous to that of JNK1, whereas JNK3 has only the α1 (p46) and α2 (p54) splice forms (Waetzig and Herdegen, 2005). There are very few data comparing the effects of all 10 splice forms in a single biological system (Gupta et al., 1996; Yang et al., 1998; Tsuiki et al., 2003). Both JNK1 and JNK2 are ubiquitously expressed, and JNK3 is more specific to the brain and heart and testis. The activation of JNKs requires threonine and tyrosine dual phosphorylation in a threonine-proline-tyrosine (T-P-Y) motif. MKK7 preferentially targets threonine 183 and MKK4 phosphorylates tyrosine 185 (Bode and Dong, 2007).

Underlying all these splice forms are differently localized effectors, cytosol versus nucleus (Bogoyevitch and Kobe, 2006), scaffolding proteins such as JNK-interacting proteins (JIPs), among others, that sequester and determine subcellular localization, and functions of JNKs (Raman et al., 2007), which include cell migration activity (Huang et al., 2003., 2004; Bogoyevitch and Kobe, 2006) and proapoptotic and antiapoptotic activities (Liu and Lin, 2005) relating to the duration of JNK signaling (Ventura et al., 2006), among others (Bogoyevitch and Kobe, 2006). More than 1 h of signaling by JNK is associated with proapoptotic activity, whereas shorter durations are linked to antiapoptotic activities (Ventura et al., 2006). Adding to these observations is the seemingly contradictory JNK-mediated inhibition of the apoptotic pathway via phosphorylation (Yu et al., 2004) and stimulation of the prodeath pathway also via JNK activity (Maundrell et al., 1997; Fuchs et al., 1998b). JNK also participates in cell cycle progression via the c-Jun component of the AP-1 transcription factor (Mikhailov et al., 2005; Bogoyevitch and Kobe, 2006; Heasley and Han, 2006; Perdiguero et al., 2007).

JNK is also implicated in cell migration, as the chemical inhibition or the dominant-negative form of JNK can impair cell migration of human, rodent, and bovine cells (Huang et al., 2004). JNK phosphorylates paxillin on Ser-178, and a point mutation Ser-178→Ala preventing

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TABLE 1

Summary of the MAP kinase dual-specificity phosphatases and their involvement in cancer

This table summarizes the findings concerning the MKPs that target MAP kinases as substrates. For the purposes of clarity, the first name of each phosphatase corresponds to the official symbol from the National Center for Biotechnology Information GENE database (http://www.ncbi.nlm.nih.gov/sites/entrez). Other names that vary with species are also given and can be found on other databases (PubMed, part of NCBI, or at http://harvester.fzk.de/harvester) or other review articles (Alonso et al., 2004). The expression level and function of each phosphatase is given in relation to the process of tumorigenesis, when available. The occasional contradiction in tissue distribution is caused by differences in detection methods (Northern blot versus reverse transcriptase-polymerase chain reaction). Finally, although for each phosphatase MAPK substrate specificity is given, depending on cell type and paradigm, the reported specificity can vary.

MKP Family	Primary Subcellular Localization	MAPK Substrate Specificity	Normal Tissue Expression and Distribution of the mRNA or Protein	Expression in Tumors relative to Normal Tissue, Consequences of Atypical Expression to Tumor Cell Lines and in Vivo Tumors	Knockout Mice Phenotype	References
DUSP1, *MKP-1*, *erp*, *CL100*, *HVH1*, *PTPN10*, *Ptpn16*, *3CH134*	Nuclear	p38 ~ JNK ≫ ERK	Inducible, ubiquitous	Increase or decrease, depending on tumor (see text)	Develop normally: immune- and diet-related phenotype revealed (see text)	See text
DUSP2, *PAC-1*	Nuclear	ERK ≫ p38 ~ JNK Pac-1 variant without intrinsic phosphatase activity increases MKP-1 phosphatase activity	Inducible in hematopoietic tissue Some expression in thymus, spleen, kidney. and lung Pac-1 variant has no phosphatase activity; found in large granular lymphocyte leukemia and pancreatic islet cells	Decreased in acute leukemia Increased in ovarian carcinoma Increased in breast cancer cell line: increased apoptosis Overexpressed in large granular lymphocyte leukemia No direct correlation with non-Hodgkin's lymphoma subtype, may be involved in lymphomagenesis	Develop normally: *Dusp2*$^{-/-}$ mice protected from arthritis; increased JNK1 activity with decreased ERK and p38 activities	Rohan et al., 1993; Ward et al., 1994; Chu et al., 1996; Kim et al., 1999; Kothapalli et al., 2003; Givant-Horwitz et al., 2004; Jeffrey et al., 2006; Cerhan et al., 2007; Wu et al., 2007
DUSP4, *MKP-2*, *HVH2*, *TYP*	Nuclear	ERK ~ JNK > p38 C-terminal domain of MKP-2 reduces its phosphatase activity	Ubiquitous, inducible	Increased MKP-2 protein if Raf-1/MEK is active Exocrine pancreatic tumor cell lines inverse correlation between survival of rats and *mkp-2* mRNA expression in ascites hepatomas Overexpressed in human breast cancers Early onset of high-grade breast cancer associated with loss of chromosome arm 8p, loss of genes including MKP-2 6 weeks after s.c. injection, in athymic female nude mice mammary pads, fewer mice had tumors when MKP-2-transfected MCF-7 cells were injected compared with MCF-7 containing empty vector or MCF-7 parental lines Ovarian serous borderline tumors express higher levels of MKP-4 than serous carcinomas Difference in phenotype may be due in part to MKP-4 expression MKP-2 siRNA protects MEFs from hydrogen peroxide-induced apoptosis MKP-2 can induce apoptosis in hydrogen peroxide-treated-*p53*$^{-/-}$ MEFs		Guan and Butch, 1995; King et al., 1995; Chu et al., 1996; Yokoyama et al., 1997; Yip-Schneider et al., 2001; Hutter et al., 2002; Wang et al., 2003, 2007a; Armes et al., 2004; Sieben et al., 2005; Shen et al., 2006
DUSP5, *HVH3*, *Cpg21*, *Gm337*	Nuclear	ERK ≫ JNK ~ p38 ERK-2 ~ ERK1 ≫ ERK3 ~ ERK5 ~ ERK7 DUSP5 transports and sequesters unphosphorylated ERK2 to the nucleus Phosphatase-dead DUSP5 translocates ERK2 to nucleus and MEK can activate ERK2 in nucleus Crystal structure of DUSP5 catalytic domain revels head-to-tail dimers	Inducible Bone marrow, tonsillar B-cells, brain, lung, and eosinophile	Overexpression in lung and colon cancer cell lines, causes decreased colony formation Granta 519 cells (used as an in-vitro model for mantle cell lymphoma) reduce their proliferation by ~50%, via an unknown mechanism, when DUSP5 siRNA is ectopically expressed		Temple et al., 2001; Ueda et al., 2003; Mandl et al., 2005; Jeong et al., 2007; Ortega-Paino et al., 2008

TABLE 1—*Continued.*

MKP Family	Primary Subcellular Localization	MAPK Substrate Specificity	Normal Tissue Expression and Distribution of the mRNA or Protein	Expression in Tumors relative to Normal Tissue, Consequences of Atypical Expression to Tumor Cell Lines and in Vivo Tumors	Knockout Mice Phenotype	References
DUSP6, *MKP-3*, *PYST1*, *rVH6*	Cytosolic	ERK ≫ JNK ~ p38 ERK1/2 ≫ ERK5 Monomer ERK binds to monomer MKP-3 ERK2 binding to MKP-3 → rearrangement MKP-3 catalytic site MKP-3 (C^{293}→S) causes cytoplasmic retention of ERK; this retention allows MEK to phosphorylate ERK ERK1/2 phosphorylates MKP-3 serine 159 and/or serine 197 and mTOR can phosphorylate MKP-3 at serine 159, leading to MKP-3 proteasomal degradation ERK5, p38, and JNK do not phosphorylate MKP-3	Constitutive Mkp-3 mRNA expression in mouse embryo in centers associated with cell proliferation and patterning: presegmental paraxial mesoderm, limb bud, brachial arch mesenchyme, midbrain/hindbrain isthmus, nasal, dental, hair, and mammary placodes	Expression of DUSP6 gene product is reduced by hypermethylation in human pancreatic cell lines and pancreatic tumors Decrease associated with invasive carcinoma phenotype of pancreatic cancer Tamoxifen treatment in conjunction with MKP-3 overexpression in MCF-7 cells increases 1) MCF-7 colony formation in soft agar, 2) growth rate in athymic ovariectomized nude mice, and 3) levels of phospho-ERK and reduces 4) MKP-3 phosphatase activity Inactivation of MKP-3 protein was associated with progression from high-grade dysplasia/pancreatic intraepithelial neoplasia (grade 3) to invasive carcinoma → lack of DUSP6 may be associated with invasive phenotype Ovarian cancer cell lines OVCAR3, OVCA420, OVCA429, OVCA433, DOV13–5, SKOV3, and A2780cp, reveal lower MKP-3 protein expression, compared with immortalized human ovarian surface epithelium; the lower expression was accounted for by inhibiting proteasomal degradation of MKP-2 40% of human ovarian cancer samples revealed reduced MKP-3 protein expression compared with normal ovarian samples Ectopic expression of MKP-3 in A2780cp cells 1) reduces cell proliferation and anchorage-independent growth in vitro, 2) reduces growth rate in vivo when injected into nude mice, and 3) increases cell sensitivity to cisplatin-mediated death in vitro and in nude mice (reduced tumor size)	1) At embryonic days 8.5–17.5 F_1 heterozygous offspring of germline chimeras: *DUSP6* (*MKP-3*)-null mice have normal mendelian frequency with no obvious phenotype 2) Postnatal development: *DUSP6* (*MKP-3*)-null mice reveal postnatal lethality depending on genetic background 3) Null mice that survived to weaning: can have skeletal dwarfism, craniosynostosis, developmental delays, and/or malformations of the middle ear component caused by negative regulation of FGF signaling	Groom et al., 1996; Muda et al., 1996; Wiland et al., 1996; Camps et al., 1998; Brunet et al., 1999; Stewart et al., 1999; Dickinson et al., 2002a; Furukawa et al., 2003, 2005; Kim et al., 2003b; Karlsson et al., 2004; Marchetti et al., 2005; Xu et al., 2005; Cui et al., 2006; Li et al., 2007; Arkell et al., 2008; Bermudez et al., 2008; Chan et al., 2008
DUSP7, *PYST2*, *MKPX*, *B59*	Cytosolic	ERK > p38 ≫ JNK PYST2-L (long) has phosphatase activity and PYST2-S (short) has no catalytic activity.	Constitutive Very little expression in peripheral blood mononuclear cells Humans: skeletal muscle, brain, heart, kidney, pancreas, placenta, little expression in lung and liver	Ectopic B59 expression reduces the number of foci per plate when v-raf or H-ras-transformed NIH 3T3 are cultured PYST2-S and PYST2-L are coded by different open reading frames of the same gene PYST2-L is coded by exons 1, 2, and 3b and PYST2-S is coded by exons 3a and 3b; the catalytic domain is coded by exon 2; PYST2-S may compete with PYST2-L for common factors at the transcription, translation, or regulatory levels Increased PYST2-L mRNA in leukocytes of patients with acute myelogenous leukemia than in with normal leukocytes; leukemic cell lines also reveal an increase that is not related to an amplification of the gene PYST2-L mRNA is also increased in solid tumors derived from different tissues PYST-2 is expressed 5-fold higher in leukemic leukocytes (before chemotherapy) when compared with leukocytes from the same patient after remission		Shin et al., 1997; Dowd et al., 1998; Levy-Nissenbaum et al., 2003a,b, 2004
DUSP8, *HVH-5*, *HVH8* *Nttp1*, *M3/6*, *HB5*	Nuclear and cytosolic	JNK ~ p38 ≫ ERK M3/6 binds to JIP1 to dephosphorylate JNK M3/6 can also bind to JIP2 JNK phosphorylates M3/6 → does not affect stability	Constitutive Adult mouse tissue: eye, brain, lung, and heart Fetal human tissue: brain and lung Human: skeletal muscle, heart, and different structures of the brain may be involved in olfactory processing			Martell et al., 1995; Muda et al., 1996; Theodosiou et al., 1996; Nesbit et al., 1997; Bernabeu et al., 2000; Johnson et al., 2000; Willoughby et al., 2003

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TABLE 1—*Continued.*

MKP Family	Primary Subcellular Localization	MAPK Substrate Specificity	Normal Tissue Expression and Distribution of the mRNA or Protein	Expression in Tumors relative to Normal Tissue, Consequences of Atypical Expression to Tumor Cell Lines and in Vivo Tumors	Knockout Mice Phenotype	References
DUSP9, MKP-4, Pyst3	Cytosolic Dependent on nuclear export signal and nuclear export protein CRM1	ERK ≫ p38 > JNK1 ERK2 > ERK1 ≫ ERK3, ERK5, ERK7 p38α ≫ p38γ, p38δ	Human placenta, kidney Mice: placenta, kidney, testis (protein)	Decrease of MKP-4 in squamous cell carcinoma leads to increased invasion and metastasis In developing tumors: squamous cell carcinoma-producing lineages coexpressing MKP-4 and GFP injected into neonatal BALB/C had smaller tumors that did not express GFP → conclusion: tumors derived from nontransduced cell population; in the same experiment, control cells transduced with GFP only developed into a tumor that was GFP-positive In established tumors: 35 days after inoculation of H1299 cells, with either MKP-4-Tet or control vector, into nude female mice, tumors formed and mice were fed tetracycline Tetracycline-responsive MKP-4-expressing H1299 cells formed smaller tumors than H1299 control cells; cells in MKP-4-expressing tumors were larger and had decondensed nuclei compared with control cells In vitro: MKP-4 expression caused cell enlargement and cell death in tumorigenic cells but not in the nontumorigenic parental cell line In vitro: overexpression of MKP-4 leads to cell death caused by cell cycle arrest at G_2-M and microtubule disruption	Embryonically lethal owing to lack of labyrinth development (placental defect) When placental defect rescued: not required for embryonic development Males$^{-/y}$ are born healthy and fertile males$^{-/y}$ derive from females$^{-/+}$ × males$^{-/y}$	Muda et al., 1997; Dickinson et al., 2002b; Christie et al., 2005; Liu et al., 2007
DUSP10, MKP-5	Nuclear and cytosolic	p38 > JNK ≫ ERK p38α,β ≫ p38γ, δ	Inducible Some expression in human liver and skeletal muscle; mouse heart, lung, liver, skeletal muscle, and kidney.	Decreased up-regulation of *mkp-5* mRNA in response to 1,25-D in cell lines from human prostate cancer and squamous cell carcinoma; low 1,25-D is associated with increased risk of prostate cancer in older men Overexpression of *mkp-5* reduces apoptosis in nonstressed *p53*-null cells expressing WT p53, constitutively active JNKK, and *mkp-5* compared with cells only expressing WT p53 and constitutively active JNKK	Develop normally: Protects against excessive T- cell cytokine production in response to viral infection.	Tanoue et al., 1999, 2001; Theodosiou et al., 1999; Buschmann et al., 2001; Zhang et al., 2004; Nonn et al., 2006
DUSP16, MKP-7, Mkpm	Cytosolic	JNK ~ p38 ≫ ERK p38α,β ≫ p38γ,δ MKP-7 binds to JIP1 to dephosphorylate JNK2 MKP-7 can also bind to JIP2 MKP-7 binds to β- arrestin 2 to dephosphorylate JNK3 MKP-7 binds to JNK via C-terminal (371–665) and Leu-166, Leu-168 in catalytic domain of MKP-7 MKP-7 binds to p38 via: Arg-56, Arg-57 in rhodanese domain and Leu-166, Leu-168 of MKP-7 MKP-7 binds to ERK via C-terminal (371–665) and Arg-56, Arg-57 of MKP-7 Dephosphorylation: Leu-166, Leu-168 of MKP-7 are important in MAPK inactivation Residues 569–604 of MKP-7 are involved in its degradation ERK phosphorylates MKP-7 (Ser-446), increasing its stability	Inducible Some expression in mouse heart, testis, and kidney and to lesser extent in brain and liver	Hemizygosity has no direct linkage to leukemia Overexpression reduces BCR-ABL-induced transformation in rat-1 fibroblasts in unstimulated cells MKP-7 binds to β-arrestin 2 in the cytoplasm Upon stimulation, β-arrestin 2 translocates to the receptor (angiotensin type 1a receptor) where MKP-7 dissociates from it; β-arrestin 2 can bind to ASK1 and indirectly to MKK4 to stimulate JNK3 activation; active JNK3 can cause MKP-7 to dissociate from β-arrestin 2; 30–60 min after stimulation, the MKP-7 reassociates with β-arrestin 2 to inactivate JNK3		McDonald et al., 2000; Matsuguchi et al., 2001; Tanoue et al., 2001; Hoornaert et al., 2003; Masuda et al., 2003; Willoughby et al., 2003; Katagiri et al., 2005; Willoughby and Collins, 2005
STYXL1, DUSP24, MK-STYX		Presumed catalytically inactive Cys→Ser substitution at catalytic site		Expression is increased in Ewing's sarcoma family of tumors		Wishart and Dixon, 1998; Siligan et al., 2005

mTOR, mammalian target of rapamycin; FGF, fibroblast growth factor; 1,25-D, 1,25-dihydroxyvitamin-D_3; JNKK, Jun N-terminal kinase kinase.

this phosphorylation causes a slowing of cell movement in a variety of cell types (Bogoyevitch and Kobe, 2006). JNK associates with focal adhesions, in a JIP-dependent manner, and MEKK1 localized with FAK and α-actinin (Huang et al., 2004; Bogoyevitch and Kobe, 2006). JNK has also been found to localize to membrane ruffles and along microtubules. JNK phosphorylates Tau, preventing it from promoting microtubule assembly or binding to microtubules if hyperphosphorylated (Goedert et al., 1997; Bogoyevitch and Kobe, 2006).

Active JNK mediates its function by regulating the activity and stability of some effectors via phosphorylation. However, active JNK can also mediate degradation of its effectors, such as c-Jun (Gao et al., 2004) and inhibitory molecules such as IκBα (Ki et al., 2007) by phosphorylating and targeting them for polyubiquitination and proteasomal degradation and also by increasing E3 ligase activity (Gao et al., 2004; Chang et al., 2006). Conversely, nonphosphorylated JNK can also bind to these same effectors, such as c-Jun, p53, activating transcription factor-2, and JUNB, among others, in nonstimulated cells, and target them for degradation via polyubiquitination and the proteasomal pathway, possibly by acting as an adaptor protein. In addition, decreasing the levels of JNK can increase the protein levels of JNK effectors (Fuchs et al., 1996, 1997, 1998a; Bode and Dong, 2007). Although the exact mechanisms governing these observations remain to be determined, overall it seems that JNK controls the levels of its effector proteins in both stimulated and nonstimulated conditions. Furthermore, as dephosphorylated JNK, via MKP-1 or other phosphatase (Tables 1 and 2), can target JNK effectors for degradation, inactivation of JNK would require monitoring for possible consequences, as this protein is not simply "turned off."

An array of JNK knockout mouse work has been done for JNK1, JNK2, and JNK3. JNK1 KO phenotypes vary from the normal phenotype to one with increased Th2 cytokine production and improved cardiac function under acute pressure overload. Similar variations can be seen for JNK2 KO mice, with phenotypes ranging from normal, prone to obesity, increased Th2 cytokine production, and so on. There are fewer results for JNK3 (fewer KO mice generated), which reveal either a normal phenotype or resistance to kainic acid treatment (Gerits et al., 2007). Furthermore, whereas JNK1/JNK3 and JNK2/JNK3 double knockouts develop normally, JNK1/JNK2 double knockouts die because of a lack of developmentally controlled apoptosis (Kuan et al., 1999; Gerits et al., 2007). Taken together, the JNK1/JNK2 double KO suggests that JNK1 and JNK2 can compensate for each other during development. In addition, the different phenotypes associated with JNK1 and JNK2 single KO mice may be associated with the multiplicity of the JNK1 and JNK2 splice forms or genetic background, as the severity of the phenotype associated with knockouts can be mouse strain-specific (Linder, 2006; Yoshiki and Moriwaki, 2006). Although the background of the mice was not always mentioned in the work (Kuan et al., 1999; Gerits et al., 2007), knocking in one splice form at a time, across genetic backgrounds, would uncover the contribution of the murine strain to the differences in phenotypes observed, whereas knocking them in, within the same genetic background, would reveal the functional differences between the various splice forms.

D. p38 Mitogen-Activated Protein Kinases

This signal transduction pathway is also stimulated by stress, cytokines, and growth factors (Roberts and Der, 2007). These stimuli mediate their effect by activating a signaling module that is composed of MAPKKK-MKK3/4/6-p38MAPK (Han and Sun, 2007). The MAPKKKs are composed of MLK2 and MLK3, dual leucine zipper-bearing kinase, ASK1, map three kinase-1, and TAK-1. These kinases, in turn, activate MKK3/4/6, which activate p38MAPK (Brancho et al., 2003; Han and Sun, 2007; Raman et al., 2007; Roberts and Der, 2007). Of note, the MKK-independent means of p38αMAPK activation/repression rely on TAK-1 binding protein (TAB1, TAB2, and TAB3 or T-cell receptor). These activators are more cell type and stimulus-restricted. How they differ in terms of the consequences of downstream signaling, in relation to the usual MKK-dependent activation or phosphatase-mediated repression is still being determined (Cuenda and Rousseau, 2007). The JNK scaffolding proteins JIPs, among other scaffolding proteins, can also bind to p38MAPKs and affect their subcellular localization (Raman et al., 2007).

The p38MAPK family, which is also part of the stress-activated MAPKs (Han and Sun, 2007), has four isoforms (α, β, γ, and δ) (Han and Sun, 2007; Raman et al., 2007). Expression patterns vary according to the isoform. p38αMAPK, which is the most extensively characterized of its family members, has a wide expression pattern and so does p38βMAPK (Cuenda and Rousseau, 2007; Mayor et al., 2007). p38γMAPK is expressed in skeletal muscle, whereas p38δMAPK is detected in small intestine, pancreas, testis, and kidney. Based on sequence identity and substrate specificity, these isoforms can be subdivided as α, β and γ, δ. A potential means of distinguishing these isoforms relies on the use of ATP competitive inhibitors as the ATP-binding pocket for the α, β isoforms is different in amino acid composition from the corresponding ATP-binding pocket in γ, δ. Furthermore, the level of expression of these isoforms as well as their upstream activators, MKK3 and MKK6, vary across stimuli and cell types. The latter kinases target a threonine-glycine-tyrosine (T-G-Y) motif on their p38MAPK substrates (Cuenda and Rousseau, 2007).

Some of the functions of the p38αMAPK isoform involve increasing mRNA stability (Dean et al., 2004) via MAPKAP-K2, which phosphorylates the RNA-binding

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TABLE 2

Summary of the MAP kinase atypical dual-specificity phosphatases and their involvement in cancer

This table summarizes the findings concerning MKPs and atypical dual-specificity phosphatases that target MAP kinases as substrates. For the purposes of clarity, the first name of each phosphatase corresponds to the official symbol from the National Center for Biotechnology Information GENE database (http://www.ncbi.nlm.nih.gov/sites/entrez). Other names that vary with species are also given and can be found on other databases (PubMed, part of NCBI, or at http://harvester.fzk.de/harvester) or other review articles (Alonso et al., 2004). The expression level and function of each phosphatase is given in relation to the process of tumorigenesis, when available. The occasional contradiction in tissue distribution is caused by differences in detection methods (Northern blot versus reverse transcriptase-polymerase chain reaction). Finally, although for each phosphatase MAPK substrate specificity is given, depending on cell type and paradigm, the reported specificity can vary.

Atypical Phosphatase	Primary Subcellular Localization	MAPK Substrate Specificity	Normal Tissue Expression and Distribution of the mRNA or Protein	Expression in Tumors Relative to Normal Tissue, Consequences of Atypical Expression to Tumor Cell Lines and in Vivo Tumors	Knockout Mice Phenotype	References
DUSP3, *VHR*, *T-DSP11*	Nuclear	ERK ~ JNK ≫ p38 STAT5 dephosphorylation VHR (Y^{138}) may create binding for STAT5 SH2 domain VHR (C^{124}→S); VHR (Y^{138}→F); either mutation → dominant negative for STAT5 dephosphorylation VHR (Y^{138}) autodephosphorylation intra- or intermolecular	Constitutive Human: heart, brain, skeletal muscle, breast, ovary	VHR siRNA-mediated decrease induced cell-cycle arrest in HeLa cells VHR highly expressed in HeLa, SiHa, CaSki, C33, and HT3 cervical cancer cell lines with nuclear localization compared with HT3 primary keratinocytes (from hysterectomies) that have no VHR nuclear staining High-grade squamous intraepithelial lesions and squamous cell carcinomas are associated with strong nuclear and cytoplasmic VHR protein Primary cervical adenocarcinomas and adenocarcinoma in situ are also associated with high VHR staining in cytoplasm but no nuclear immunoreactivity VHR may be a marker for cervix carcinoma		Kamb et al., 1994; Todd et al., 1999, 2002; Rahmouni et al., 2006; Hoyt et al., 2007; Henkens et al., 2008
DUSP12, *GKAP*, *HYVH-1*, *LMW-DSP4*, *T-DSP4*, *VH1*, *mVH1*	Cytosolic	No specificity toward MAPK. May increase level of phospho-ERK1 Dephosphorylates pNPP and a phosphotyrosine -containing peptide "RAYTIDE" but not a phosphoserine-containing peptide Dephosphorylates and accelerates glucokinase activity	Inducible: expression follows cyclin D1 accumulating during G_1/S phase in NIH 3T3 Some expression in mouse kidney, liver, lung, heart thymus, spleen, brain, and skeletal muscle	Increased expression owing to increased gene copy number in LS3x, LS21, LS43, and MS8x sarcomas		Munoz-Alonso et al., 2000; Zhang et al., 2000; Aoki et al., 2001; Kresse et al., 2005
DUSP13A, *MDSP*, *BEDP*	Cytosolic	No specificity toward MAPK MDSP has phosphatase activity toward P-Tyr and P-Thr Unknown substrate	Skeletal muscle (protein) Highest levels in adult mouse			Chen et al., 2004
DUSP13B, *TMDP*, *TS-DSP6*		No MAPK specificity Dephosphorylates P-Tyr (MBP) and P-Thr (MBP) with similar efficiency Possibly active before substrate binding	Testis: highest levels in adult mouse (protein)			Nakamura et al., 1999; Chen et al., 2004; Kim et al., 2007
DUSP14, *MKP6*, *MKP-L*	At the plasma membrane	JNK > ERK ~ p38	Ubiquitous	HCT-116 cell lines transfected with Kruppel-type zinc-finger protein (ZBP-89) up-regulates JNK1/2 phosphorylation by down-regulating MKP-6 (DUSP14) expression ,leading to enhanced apoptosis		Marti et al., 2001; Bai et al., 2004
DUSP15, *VHY*, *LMW-DSP10*, *T-DSP10*	Cytoplasmic face of plasma Membrane (myristolated)		mRNA Testis protein (pachytene spermatocytes)			Alonso et al., 2004b
DUSP18, *DUSP20*, *LMWDSP20*	Cytosolic and nuclear	JNK ≫ p38 ~ ERK	Adult: ubiquitous Fetal: all tissues tested Interaction between C-terminal domain of DUSP18 and its catalytic domain may enhance stabilization of catalytic domain and account for its thermal stability; the optimum activity for DUSP18 is at 55°C and 80% of WT activity is retained at 70°C	All carcinoma cell lines and cell "normal" lines tested		Wu et al., 2003, 2006b; Jeong et al., 2006

TABLE 2—*Continued.*

Atypical Phosphatase	Primary Subcellular Localization	MAPK Substrate Specificity	Normal Tissue Expression and Distribution of the mRNA or Protein	Expression in Tumors Relative to Normal Tissue, Consequences of Atypical Expression to Tumor Cell Lines and in Vivo Tumors	Knockout Mice Phenotype	References
DUSP19, *LDP-2*, *SKRP1*, *TS-DSP1*, *DUSP17*, *LMW-DSP3*	Cytosolic	JNK ≫ ERK ~ p38 SKRP1 also interacts directly with MKK7 and ASK1	Mouse: ubiquitous Human adult: heart, lung, liver, pancreas			Nakamura et al., 2002; Zama et al., 2002a,b; Cheng et al., 2003
DUSP21, *LMWDSP21*	Cytosolic and nuclear		Testis			Hood et al., 2002
DUSP22, *LMW-DSP2*, *TS-DSP-2*, *VHX*, *JSP1*, *JKAP*, *MKPX*	Cytosolic and nuclear (myristolated)	p38 ≫ JNK > ERK VHX inactivates ERK-2 JKAP associates with MKK7 *JKAP*-null cells have reduced JNK activation in response to cytokines JSP1 activates MKK4 LMW-DSP2 dephosphorylates STAT3 DUSP22 dephosphorylates ERα at Ser-118	Inducible Lymphoid cells adult murine tissue: heart, brain, liver, kidney and testis	LMW-DSP2 interacts directly and dephosphorylates STAT3 in murine testicular GC-1 cells; LMW-DSP2 also prevents STAT3 translocation to the nucleus DUSP22 mRNA increases in response to estrogen in ERα-positive human breast cancer cell lines MCF-7 and T47D Luciferase reporter assay in MCF-7 or HeLa cells: 1) DUSP22 siRNA enhanced estrogen/ERα-induced luciferase activation (HeLa cells were ERα-transfected, MCF-7 endogenous ERα); 2) DUSP22 siRNA reduced dihydrotestosterone/androgen receptor-induced luciferase activation (in androgen receptor HeLa cells); 3) DUSP22 siRNA had no effect on dexamethasone-induced luciferase activation in HeLa cells Pulldown assays indicate that DUSP22 interacts directly with ERα	JKAP-null mice are borne with predicted mendelian ratios and are healthy throughout adult life	Aoyama et al., 2001; Shen et al., 2001; Alonso et al., 2002; Chen et al., 2002; Sekine et al., 2006, 2007
DUSP23, *MOSP*, *VHZ*, *LDP-3*, *DUSP25*, *FLJ20442*	Cytosolic: apical submembrane area and nuclear: nucleolus	ERK ≫ p38 ~ JNK Enhances activation of p38 and JNK during osmotic stress via MKK4 and MKK6 activation	Human fetal tissue: ubiquitous except spleen; human adult: colon, testis, pancreas, liver heart, lung Mouse adult: ubiquitously expressed LDP-3			Alonso et al., 2004a; Takagaki et al., 2004, 2007; Wu et al., 2004a; Agarwal et al., 2008
DUSP26, *MKP8*, *NATA1*, *SKRP3*, *LDP-4*	Nuclear and Golgi apparatus	p38 ≫ JNK ~ ERK	Mouse: skeletal muscle and brain, cerebellum Mouse LDP-4 expressed in brain except hippocampus	Increased copy number of gene in anaplastic thyroid tumors and cell lines; DUSP26 inhibits caspase-3 activity in anaplastic thyroid cell lines Expression is also increased in retinoblastoma, neuroblastoma, and epithelioblastoma		Vasudevan et al., 2005; Hu and Mivechi, 2006; Takagaki et al., 2007; Yu et al., 2007
DUPD1, *DUSP27*, *FMDSP*	Cytosolic		Skeletal muscle, liver and adipose tissue			Friedberg et al., 2007
DUSP28, *VHP*						Strausberg et al., 2002
PTPMT1, *MOSP*, *PLIP*, *PNAS-129*	Mitochondrial: anchored to the matrix face of the inner mitochondrial membrane	Currently unknown Knockdown of *PTPMT1* changes P-Ser/Thr and P-Tyr profiles in mitochondrial proteins	Rat: testes, liver, kidney, and endocrine cells of the pancreas Rat islets and insulinoma cell line INS-1 832/13 express PTPMT1 but not the exocrine-derived cell line PANC-1	Knockdown of *PTPMT1* expression in the pancreatic insulinoma cell line INS-1 832/13 increases 1) ATP production by ~80%, 2) ATP/ADP ratio as ADP levels remain unchanged, and 3) glucose-stimulated insulin secretion		Pagliarini et al., 2005
STYX, *hStyxb*		Inactive phosphatase owing to Cys→Gly substitution in catalytic site	Ubiquitous		Null males are infertile because of abnormal sperm production. Female offspring of null animals are fertile	Wishart et al., 1995; Wishart and Dixon, 2002

STAT, signal transduction and activator of transcription; pNPP, *p*-nitrophenyl phosphate; P, phospho; MBP, myelin basic protein; ERα, estrogen receptor-α; VHP, VH1-like phosphatase1.

effectors, such as tristetraprolin (TTP) (Dean et al., 2004), heterogeneous nuclear ribonucleoprotein A0 (Rousseau et al., 2002), and poly(A)-binding protein (Bollig et al., 2003), thereby modulating their mRNA binding capacity. TTP and other AU-rich element-binding proteins are involved in reducing the stability of mkp-1 mRNA (Lin et al., 2008), VEGF mRNA (Claffey et al., 1998), and MMP mRNA (Akool et al., 2003; Huwiler et al., 2003), among other transcripts (Eberhardt et al., 2007). In addition, p38αMAPK and p38βMAPK phosphorylate KH-type splicing regulatory protein, an mRNA-binding protein, and inhibit its binding and destabilization of mRNAs coding for proteins involved in the differentiation of myoblasts into myotubes (Briata et al., 2005). In addition, p38δ is associated with keratinocyte maturation (Efimova et al., 2003; Cuenda and Rousseau, 2007). In the same line of thought, p38αMAPK also participates in inhibition of cellular proliferation (Mikhailov et al., 2005; Heasley and Han, 2006; Perdiguero et al., 2007).

p38αMAPK can modulate the actin cytoskeleton via MAPKAP-K2 and HSP27, a required step for migration (Rousseau et al., 2000, 2006). The unphosphorylated form of HSP27 binds to actin, capping it and blocking polymerization. Other proteins downstream of MAPKAP-K2, such as LIM kinase 1, induce actin polymerization via inhibitory phosphorylation of cofilin, an actin-depolymerizing protein and CapZ-interacting protein-CapZ also affect actin polymerization (Cuenda and Rousseau, 2007). These are only some of the proteins involved in actin polymerization, one of the actin remodeling events (Disanza et al., 2005). A complete picture of how p38MAPK modulates cell migration is still wanting.

Knockout mice for each of the p38MAPKs have been generated. Overall, mice null for the isoforms p38β, p38γ, or p38δMAPK reveal no overt phenotype and develop normally. Null mice for p38αMAPK are embryonically lethal because of either placental defects or erythroid differentiation, depending on the genetic background of mice (Gerits et al., 2007). None of the p38MAPK isoforms is able to substitute for the lack of p38αMAPK. Results from a recent study revealed that p38α embryo-specific knockout mice [placentas are *p38α*(+/+)] develop into pups; however, most die at the 4-day postnatal time point (Hui et al., 2007), whereas another study used mice with an inducible deletion of the *p38α* gene, which allows the mice to develop to adulthood (Ventura et al., 2007). The phenotypes of these mice will be discussed in light of MAPK involvement in cancer (see section V.C on MAPK and cancer).

E. Mitogen-Activated Protein Kinase Cross-Talk

Until now, for reasons of simplicity and clarity, the axes of the MAPKs (Raf-MEK1/2-ERK, MEKK-MKK4/7-JNK, and MEKK-MKK3/4/6-p38MAPK) have been discussed independently of one another regarding their families, functions, and signal transduction pathways as a MAPKKK → MAPKK → MAPK model. However, evidence reveals this is not the case in cell lines or primary cells. Relationships between the members of a given module are not always linear, and both stimulatory and inhibitory interactions exist within and across MAPK modules (Fig. 2).

The cross-talk between the modules occurs at every level. For example, Ras activates Rac independently of PI3K (Lambert et al., 2002) or via PI3K (Bar-Sagi and Hall, 2000), leading to cytoskeletal remodeling (Innocenti et al., 2003), cell migration (Holly et al., 2005; Shin et al., 2005), and directional movement (Sasaki et al., 2004; Sasaki and Firtel, 2006). However, the Ras-PI3K interaction is not always required (Lim and Counter, 2005) and can be inhibited when the MAPK pathway is constitutively active, leading to cell cycle arrest (Menges and McCance, 2008). Less is known about Rac-mediated activation of Ras (Zugaza et al., 2004). Other members of the MAPK signal transduction pathway also interact with both stimulatory and inhibitory consequences. Some of the functional consequences of p38α- and p38βMAPK-mediated inhibition of JNK signal transduction include decreased in vitro cell transformation and cell proliferation (Wada et al., 2008). In addition, the JNK effector c-Jun is also implicated in reduced liver regeneration, after partial hepatectomy, as seen in c-*jun*-null mice (Hilberg et al., 1993; Eferl et al., 1999) or conditional c-*jun*-null mice (Behrens et al., 2002). This phenotype was rescued in mice carrying a conditional deletion of c-*jun* and a complete *p53*-null mutation [c-*jun*(−/−)*p53*(−/−)] or a conditional c-*jun* and complete *p21*-null mutation [c-*jun*(−/−)*p21*(−/−)] (Stepniak et al., 2006). Interestingly, there is no overt effect of the *p53*-null mutation on the capacity of the liver to regenerate after partial hepatectomy (Stepniak et al., 2006). Adding to the previous observations, mice carrying c-*jun*(−/−)*p53*(−/−) or c-*jun*(−/−)*p21*(−/−) reveal lower levels of phosphorylated p38MAPK than conditional c-*jun*(−/−) mice do (Stepniak et al., 2006). Mice bearing both conditional null mutations for c-*jun* and *p38αMAPK* genes revealed normal liver regeneration (Stepniak et al., 2006; Wada et al., 2008). Together p53 and p21 increase phosphorylation levels of p38α, and all three inhibit cell cycle progression, whereas c-Jun, the downstream effector of JNK, inhibits p38α, p53, and p21. In the absence of c-Jun, all three are active and the cell cycle is inhibited, unless one of the three proteins is inhibited, allowing the cell cycle to progress. Although this relationship is seen in the regenerating liver (Stepniak et al., 2006), such interactions need to be established for other systems, especially when normal regulatory systems are overridden. Another study showed that p38α antagonizes the JNK-c-Jun pathway via inhibition of MKK7 (Hui et al., 2007). The regulatory loop between p38MAPK and JNK can be extended, as both p38α and the MKK4/7-JNK-c-Jun pathway inhibit ERK1/2 activation and function in cell lines (Zhang et al., 2001; Shen et al., 2003). More specifically, c-Jun

transcriptional activity is required for ERK inhibition (Shen et al., 2003), whereas JNK inhibits ERK1/2 activation in primary cells and possibly p38MAPK (Jeffrey et al., 2006) although the mechanism was not known for either study (Shen et al., 2003; Jeffrey et al., 2006).

MAPK family members can also modulate their own signal transduction pathway. This cross-talk occurs at many levels (Cuevas et al., 2007; Han and Sun, 2007; Schwacke and Voit, 2007); there is also feedback inhibition at the mRNA (Ambrosino et al., 2003) and protein levels (Dhillon et al., 2007). For example, 1) p38α reduces the stability of *mkk6* mRNA (Ambrosino et al., 2003), which is the transcript for one of the p38MAPK kinases, 2) ERK can directly phosphorylate and inhibit Raf1 and son of sevenless (SOS) (a RasGEF and activator of Ras) or indirectly phosphorylate and inhibit SOS via activation of ribosomal s6 kinase 2 (Dhillon et al., 2007), 3) ERK can also inhibit B-Raf and MEK via phosphorylation (Yoon and Seger, 2006), and 4) JNK2 reduces JNK1-mediated phosphorylation of c-Jun (Hochedlinger et al., 2002). Other regulatory mechanisms exist in the form of the plant homeodomain of MEKK1, one of the MKK4 kinases, ubiquitinating ERK1/2 and targeting them for proteasomal degradation (Lu et al., 2002). In addition, Raf1 can bind to ERK5 and enhance its phosphorylation in a MEK5-independent, Ras-mediated mechanism (English et al., 1999). Adding to this complex scenario, members of a signaling module, such as the Raf kinases, may have functions outside these modules (Hindley and Kolch, 2002), although the physiological relevance of Raf function outside of the MAPK module is still in debate.

Even as the consequences of the interactions between members of the MAPK family are increasingly being recognized (Hui et al., 2007; Ventura et al., 2007), we have a long way to go before we understand how, when, where, and what happens as a consequence of the interactions between the various isoforms and splice forms of the MAPK family, in addition to the contribution of their and other signaling modules. Knockout mice are available for the various members of the MAPK modules (Gerits et al., 2007), MEKK1–4 connection maps are available (Cuevas et al., 2007), and models of MAPK, MAPKK, and MAPKKK interactions have been designed, revealing the possible level of complexity (Schwacke and Voit, 2007). What we lack is detailed information, a connection map based on subcellular localization, MAPK splice form, cell, development, and stimulus-specific identification of the players involved in signal transduction. However, other variables also come into play. Among these are the consequences of phosphorylation, such as priming, inhibition, stabilizing, activating, and targeting of proteins for polyubiquitination and degradation. The consequences attributed to phosphorylation is a topic that is covered throughout the review. Furthermore, the function associated with nonphosphorylated JNK and possibly other MAPK members also needs to be considered. The influence of scaffolding proteins, which group different players and determine their localization and availability for interaction, also influence the outcome of signal transduction. When taken into account, all of these factors will modify the interaction maps and models. However, there is one facet that remains to be explored: the time line. Chronology is a dimension that can be used to hone in these models (Kholodenko, 2006) and determine when a specific interaction is occurring. All of these potential interactions need to be verified experimentally to distinguish factual from potential interactions.

The preceding sections have dealt with activation and inactivation of kinases by other kinases. However, cells have access to a host of phosphatases that modulate MAPK activity directly or by modulating activity of upstream kinases. Indeed, although our main focus is MKP-1, adding to all this complexity is an appreciable list of dual-specificity phosphatases, the MKPs and the atypical phosphatases that target MAPKs and their kinases with varying degrees of specificity in the cytosol or the nucleus (Tables 1 and 2; Fig. 2) (see also section IV on the dual-specificity phosphatase family). As MKP-1 is one of many phosphatases located at the MAPK nexus, understanding how all the players interact to modulate signal transduction pathways is a necessary task if we are to complete the picture vis-à-vis cell biology. To this end, we review what is known about MKP-1 regulation at the mRNA and protein levels, and we endeavor to determine how this phosphatase interacts with the MAPKs in the normal and pathological state.

III. Regulation of Mitogen-Activated Protein Kinase Phosphatase-1 Expression and Activity

A. mkp-1 *Gene Structure, Promoter, and Enhancer*

The human *mkp-1* gene contains four exons and three introns coding for an inducible mRNA that is approximately 2.4 kilobases long (Kwak et al., 1994). The promoter/enhancer region of this gene contains multiple AP-2, *trans*-acting transcription factor 1, and cAMP-responsive element sites but only one site for AP-1, neurofibromin 1, and TATA box (Kwak et al., 1994; Pursiheimo et al., 2002) (Fig. 3). Other binding motifs, such as an E box and three GC boxes are localized between positions –110 and –30 (Ryser et al., 2004). Finally, a possible binding site for p53 protein is found in the second intron (Li et al., 2003). These binding sites may explain the numerous factors that can transactivate the *mkp-1* gene (see section III.B). Interestingly, although there is an E box consensus sequence, as previously mentioned, in both the murine and human *mkp-1* promoter, the transcription factor dimer c-Myc/Max is unable to regulate *mkp-1* expression because of unfavorable flanking regions (Sommer et al., 2000). Thus, the presence of the consensus sequence of a transcription factor does not predict modulation via its sequence, and the presence of appropriate flanking regions

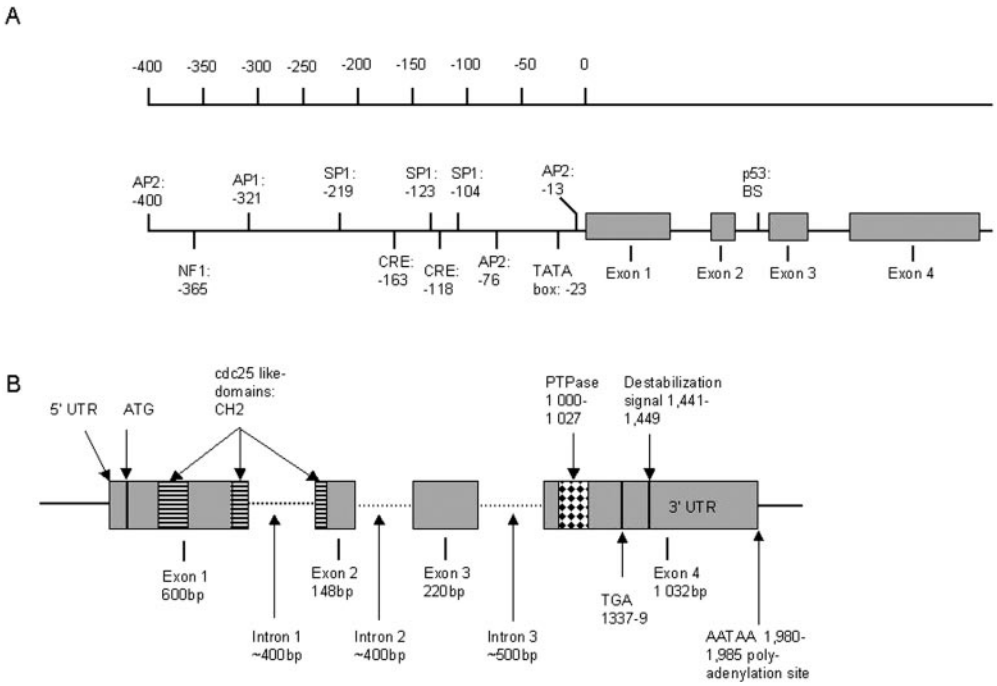

FIG. 3. MKP-1 promoter and gene. A, although the MKP-1 promoter demonstrates the potential for binding many transcription factors, caution should be used when inferring the binding of a transcription factor even when its consensus sequence is present in the promoter region, because flanking regions may influence binding (see text). B, a more detailed look at the *mkp-1* gene reveals that exons 1 and 4 bear the crux of the important domains of gene product, whereas the CH2B domain is coded by exons 1 and 2. Interestingly, at this time no known domain coded by exon 3 has been discovered. NF1, neurofibromin 1; Sp1, *trans*-acting transcription factor 1; CRE, cAMP-responsive element; UTR, untranslated region; PTPase, phosphoprotein tyrosine phosphatase; bp, base pairs.

can determine the final outcome as to gene transcription. Finally, the transcription factor E2F1 is necessary to ensure responsiveness of the *mkp-1* gene to hydrogen peroxide (Wang et al., 2007b).

Despite its high degree of conservation, there is a single nucleotide polymorphism reported in the *mkp-1* gene, although it occurs in the first intron and is not believed to influence transcription or translation efficiency. However the latter conclusion awaits formal experimental validation (Suzuki et al., 2001).

B. mkp-1 mRNA

1. Regulation of mkp-1 mRNA Expression Levels. As with many immediate-early genes coding for regulatory proteins, an abundance of factors up-regulate mkp-1 mRNA levels in different cell types, for example, serum in mouse and rat fibroblasts (Bokemeyer et al., 1996), dexamethasone, a glucocorticoid analog in human mammary epithelial cells MCF10A-Myc (Wu et al., 2004b, 2005b), glucagon in rat hepatocytes (Schliess et al., 2000), insulin in rat hepatoma cell line H4IIE-C3 (Lornejad-Schafer et al., 2003), and atrial natriuretic peptide in human umbilical-vein endothelial cells (HUVECs) (Furst et al., 2005). Other factors such as arachidonic acid increase mkp-1 mRNA expression in cultures of rat aortic vascular smooth muscle cells (Metzler et al., 1998). Stressful conditions, such as heat shock in human skin fibroblasts (Keyse and Emslie, 1992) and in murine macrophages (Wong et al., 2005), osmotic shock in rat hepatoma cell line H4IIE-C3 (Schliess et al., 1998; Lornejad-Schafer et al., 2003), hypoxia in neonatal rat tissues (Bernaudin et al., 2002), hypoxia in PC12, Hep3B (Seta et al., 2001), and HepG2 cells (Seta et al., 2001; Liu et al., 2003, 2005a,b), cobalt chloride, a hypoxia mimic, in HepG2 cells (Liu et al., 2003), and ischemia in rat forebrain (Takano et al., 1995; Wiessner et al., 1995) also modulate the *mkp-1* transcript. In addition, DNA-damaging agents such as hydrogen peroxide in human skin fibroblasts (Keyse and Emslie, 1992) and vascular smooth muscle cells (Metzler et al., 1998) and other DNA-damaging agents (Keyse and Emslie, 1992) produce similar effects. In contrast, no *mkp-1* transcript was detected in four different unstimulated hepatoma cell lines (Kwak and Dixon, 1995). It is not known whether the reported increases in mRNA correspond to increased stability or de novo synthesis of the mRNA.

There is no shortage of factors and conditions that regulate mkp-1 mRNA. These represent a potential arsenal from which to choose, corresponding to nearly every contingency that could arise should increased levels of mkp-1 mRNA be required. However, at what point, if any, do these factors use the same signal transduction mechanisms to generate their effect? Heat shock and hypoxia/ischemia-reperfusion, for example, generate reactive oxygen species and protein misfolding affecting the endoplasmic reticulum (ER) (Görlach et al., 2006), the mitochondria (Benedetti et al., 2006), the cytosol (Hartl and Hayer-Hartl, 2002), and the nucleus (Ham-

mond et al., 2007). In turn, these compartments communicate with each other. The ER and mitochondria interact with each other via calcium and reactive oxygen species (Görlach et al., 2006), and, for example, oxidative stress can cause calcium influx from both the extracellular milieu and ER or sarcoplasmic reticulum (Ermak and Davies, 2002). Furthermore, the ER-unfolded protein response, calcium flux, and reactive oxygen species also affect the nuclear compartment via transcriptional changes (Alonso et al., 2006; Görlach et al., 2006) or DNA damage (Hammond et al., 2007). Although this is a simplified overview, it reveals that using some of the previously mentioned stimuli for the purposes of up-regulating mkp-1 mRNA may not be straightforward. As the responses to different stresses are adaptations that allow the cell to react to its internal and external environment, these stresses target a host of functions within the cell. Thus, although there are many factors that can up-regulate mkp-1 mRNA, finding a treatment that specifically targets *mkp-1* would be a first step.

2. mRNA Stability. The mkp-1 mRNA has a half-life of 1 to 2 h, which varies according to the stimulant (Lau and Nathans, 1985). This variation in half-life may stem from the different mechanisms that determine the amount of mkp-1 mRNA that accumulates. In addition, a given stimulus may lead to the activation of multiple effectors, only some of which would be relevant to the query of interest. For example, heat shock up-regulates p38MAPK activity, and inhibition of this MAPK reduces the heat shock-mediated increase in mkp-1 mRNA levels (Wong et al., 2005). Conversely, stabilization of the *mkp-1* transcript via heat shock in RAW 264.7 murine macrophages may not depend on HSF-1. A luciferase assay, using a WT *mkp-1* murine promoter upstream of the luciferase gene, demonstrated luciferase activity in *HSF-1*(−/−) fibroblasts (Wong et al., 2005).

Other mechanisms exist to modulate mRNA stability. TTP binds to AU-rich elements on the 3′-untranslated region of mRNAs and destabilizes them (Dean et al., 2004). TTP binds to and destabilizes mkp-1 mRNA via an unknown mechanism (Lin et al., 2008), although TTP, which also destabilizes TNF-α mRNA (Dean et al., 2004), does so in a microRNA (miRNA)-dependent manner (Jing et al., 2005). Furthermore, TTP is involved in mRNA decapping (Fenger-Gron et al., 2005; Simon et al., 2006), which is an important step for mRNA degradation (Chan and Slack, 2006; Simon et al., 2006). At present neither the decapping mechanism nor the involvement of miRNA have been confirmed as mechanisms for mkp-1 mRNA degradation. Modulating the mkp-1 mRNA is not presently considered for therapy, whereas modulating the stability of other mRNAs is being considered (Eberhardt et al., 2007). As the MKP-1 protein has a short-half-life (see section III.C.2 on protein stability), targeting the mRNA expression could represent a therapeutic target. The potential of using mkp-1 mRNA stability as a means of control is further highlighted by observations demonstrating the involvement of MKP-1 substrates, p38MAPK, JNK1, and ERK1/2, in the signal transduction cascade relating to the stabilization of mRNAs (Eberhardt et al., 2007).

3. De Novo Transcription. Although previously mentioned studies using either hypoxia or ischemia did not reveal the mechanism of mkp-1 mRNA regulation, in the human cervical carcinoma cell line SiHa, hypoxia up-regulates mkp-1 mRNA expression without stabilization; this mechanism is thought to protect hypoxic cells against apoptosis (Laderoute et al., 1999). Treatment of mesangial cells with hydrogen peroxide induced transcription of *mkp-1* messenger, in an AP-1-dependent manner, without affecting stability (Xu et al., 2004). Of note, although the ectopic expression of MKP-1 induced apoptosis in untreated mesangial cells, it also reduced hydrogen peroxide-mediated apoptosis. Conversely, catalytically inactive MKP-1 neither induced apoptosis in untreated cells nor protected cells from apoptosis upon hydrogen peroxide treatment. Thus, although MKP-1 reduced oxidative stress-mediated apoptosis, inactivation of basal levels of ERK1/2 activity may account for the apoptosis induced by ectopic expression of MKP-1 in untreated cells (Xu et al., 2004). Using MKP-1 expression to inactivate the MAPK member that is most expressed in the cell is an interesting prospect. However, once the levels of the targeted MAPK become controlled or the stressor is inactivated, MKP-1 may target other members of the MAPK family if it continues to be expressed. This should be considered in the event of therapeutic targeting of MKP-1. A possible resolution to this problem may lie in the flexibility of the system. The ability to up-regulate mRNA without its concomitant stabilization not only allows greater flexibility in manipulating the system but also permits the assaying of the gene where transcription is concerned. Lack of efficient transcription can be distinguished from lack of stabilization of the mRNA; therefore, a more precise therapeutic intervention can be envisaged should the need arise.

4. mRNA Elongation. The calcium-mediated regulation of gene transcription is quite complex, as it can depend on the frequency of Ca^{2+} oscillation (Dolmetsch et al., 1998), the subcellular localization (Hardingham et al., 1997; Hardingham and Bading, 1999), and the amplitude and duration (Dolmetsch et al., 1997). In line with the previously mentioned complexity, a prolonged Ca^{2+} signal induces a much greater MKP-1 up-regulation compared with a transient Ca^{2+} signal in the neuronal-like CA-77 cell line (Durham and Russo, 2000). Although the importance of calcium-mediated *mkp-1* gene regulation in Rat-1 fibroblasts (Cook et al., 1997; Scimeca et al., 1997) is known, the mechanism by which Ca^{2+} mediates this regulation remained elusive.

A possible mechanism by which Ca^{2+} up-regulates mkp-1 mRNA is described for the rat neuroendocrine cell line GH4C1 (Ryser et al., 2001). When the GH4C1 cell line is treated with thyrotropin-releasing hormone, a

calcium-mediated up-regulation of *mkp-1* is observed, in contrast to the calcium-independent up-regulation of the same gene via EGF (Ryser et al., 2001). In the former case, a calcium-sensitive block in elongation located within the first exon, 300 base pairs downstream of the transcriptional initiation start site, of the rat *mkp-1* gene was thought to be the cause. In the latter case, EGF-mediated enhanced initiation and elongation were calcium-independent (Ryser et al., 2001). Recently, this group revisited the mechanism of inhibition and reported that under basal conditions in the GH4C1 cell line, a complex made from 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole sensitivity-inducing factor (DSIF) and negative elongation factor-inhibited RNA polymerase II at the promoter proximal region of the *mkp-1* gene and not within the first exon (Fujita et al., 2007). Upon stimulation with thyrotropin-releasing hormone, the positive transcription elongation factor b was recruited and phosphorylated both the C-terminal repeats of Spt5, one of the subunits of DSIF (Yamaguchi et al., 1998), and Ser-2 within the C-terminal domain of polymerase II. These modifications allowed negative elongation factor to detach from polymerase II, which continued elongation with positive transcription elongation factor and DSIF complexed to it (Fujita et al., 2007).

This mechanism raises some questions. Is this calcium-sensitive block in elongation strictly part of "normal" calcium-mediated signaling or can it be the result of stress-related mechanisms? Many proteins are sensitive to intracellular calcium proteins, acting as sensors and calcium-binding proteins (Agell et al., 2002; Haeseleer et al., 2002; Cook and Lockyer, 2006). Determining to what extent this mechanism applies to other mRNAs and cell types would be the next step. As the main cellular calcium storehouse is the endoplasmic reticulum (Görlach et al., 2006; Høyer-Hansen and Jäättelä, 2007), could the calcium-sensitive block in elongation also be part of an ER stress response (Görlach et al., 2006)? If not, could the calcium be derived from perinuclear or nuclear stores (Alonso et al., 2006)? The provenance of calcium would be a clue as to the mechanisms that govern this block.

As we have seen in this section, the many factors and mechanisms regulating the level of mkp-1 mRNA underscore the importance of the function of MKP-1. These multiple levels of control of the mkp-1 mRNA speak of an expression that is precisely and exquisitely controlled by stimulants in space and time. Furthermore, each one of the mechanisms represents a level of control with a potential for therapeutic intervention. However, this is only the control for mkp-1 mRNA expression levels. The next section will reveal that the MKP-1 protein also boasts its modulators.

C. Mitogen-Activated Protein Kinase Phosphatase-1 Protein Function and Affinity

This section presents an overview of MKP-1 affinity and function and the mechanisms that allow cells to modulate the function of this protein. The consequences of this modulation will be addressed in light of chemotherapeutic agents and cellular and animal models in sections V and VI.

MKP-1 is a nuclear phosphatase. This phosphatase dephosphorylates proteins of the MAPK family, in the following order of affinity: p38MAPK ≥ JNK ≫ ERK1/2 (Franklin and Kraft, 1997; Camps et al., 2000; Farooq and Zhou, 2004) and within these ERK2 > ERK1 (Slack et al., 2001). MKP-1 also dephosphorylates ERK5 although it can bind to ERK5 in both stimulated and unstimulated cells (Sarközi et al., 2007). However, MAPK binds to different sites within MKP-1; indeed, whereas MKP-1 binding of ERK2, ERK1, and p38αMAPK depends on the same arginine residues (arginine 53–55) (Fig. 4), that of JNK1 depends on as yet unidentified residues, although within the first N-terminal 188 residues of MKP-1 (Slack et al., 2001). Although ERK5 binding to MKP-1 may be similar to that of ERK1/2, this theory awaits experimental verification. When the arginine 53 to 55 residues are mutated, MKP-1 retains its phosphatase activity toward JNK1 but not toward ERK2 or p38α (Slack et al., 2001). Identification of the MKP-1 residues that bind JNK1 will allow more precise control for eventual therapeutic purposes. The ability either to modify the binding domains of MKP-1 (ERK/p38MAPK versus JNK1) independently of one another or to hinder the binding of the MKP-1 domains to effector molecules, via small competitive inhibitors, represents a promising avenue of endeavor. Determining the three-dimensional structure of MKP-1 would be necessary to distinguish between ERK and p38MAPK, which bind to the same site. More subtle target mutations within MKP-1 itself may be needed for the purposes of limiting MKP-1 interaction with either ERK or p38MAPK, while allowing binding to the other. With the advent of high-throughput assays for dual-specificity phosphatases (Tierno et al., 2007), inhibitors that distinguish between MKP-1 binding to ERK1/2, JNK1, and p38αMAPK may be identified soon.

Although the order of MAPK dephosphorylation is commonly accepted (Camps et al., 2000; Farooq and Zhou, 2004), with an occasional exception as seen in rat pinealocytes (Price et al., 2007), that of ERK1/2 has been debated. Indeed, MKP-1 does not seem to dephosphorylate ERK1/2 but competes with ERK effectors and prevents their binding and phosphorylation-mediated activation (Wu et al., 2005a). Based on this model, it is suggested that the binding of MKP-1 to ERK1/2 prevents the ERK1/2-ELK-1 interaction and therefore phosphorylation and activation of ELK-1 in the nucleus. In turn, this would prevent the activation of the serum response element by ELK-1 (Wu et al., 2005a). Elucidating the manner in which MKP-1 binds to the various MAPKs would be a first step in determining why a difference exists in the affinity between MKP-1 and the different MAPKs. Another possibility lies in the obser-

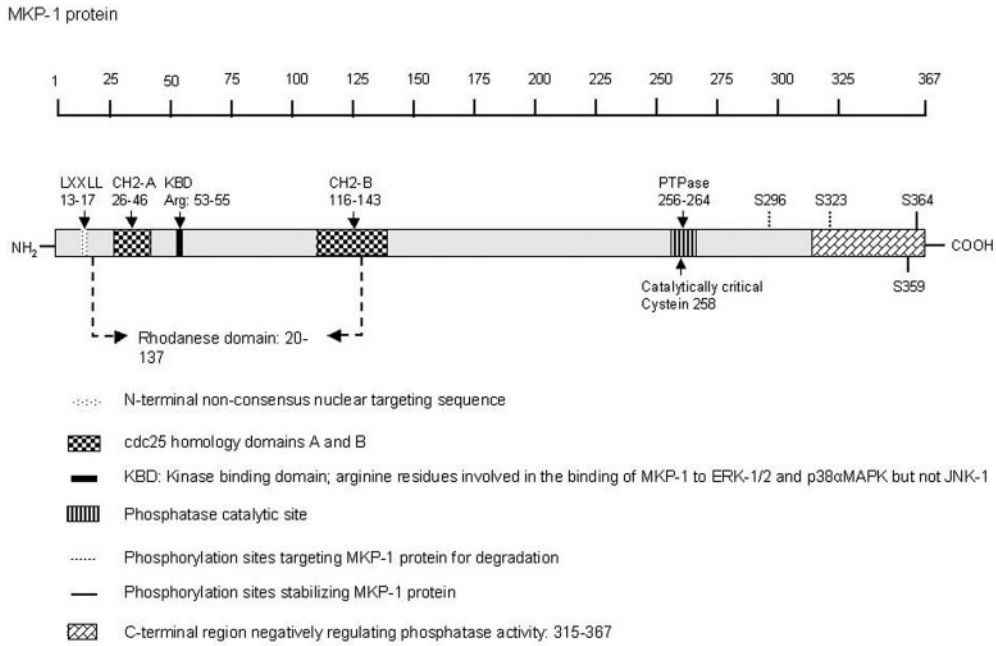

FIG. 4. MKP-1 protein. Although the N-terminal aspect of the protein is responsible for the nuclear localization and the binding of the MAPK (ERK1/2 and p38αMAPK, the residues responsible for binding JNK-1 await identification), the C-terminal part contains the catalytic and stabilization/destabilization domains of the protein. PTPase, phosphoprotein tyrosine phosphatase.

vation that phosphorylated ERK2 can homodimerize (Khokhlatchev et al., 1998) and thereafter actively translocate to the nucleus, whereas monomeric phospho-ERK can enter the nucleus by passive diffusion (Adachi et al., 1999). ERK dimers may simply be unable to interact with MKP-1.

The dephosphorylating activity of MKP-1 is restricted to the nucleus (Camps et al., 2000; Wu et al., 2005a). The targeting of MKP-1 to the nucleus is ascribed to a LXXLL motif located in the $NH_{2\ terminus}$ proximal to the cdc25 homology domains A (Wu et al., 2005a) (Fig. 4). Although this sequence is not considered to be a consensus nuclear targeting sequence (Christophe et al., 2000; Cartier and Reszka, 2002), it acts as a nuclear targeting sequence for MKP-1 (Wu et al., 2005a). MKP-1 also contains a rhodanese domain located within residues 20 to 137 (UniProtKB/Swiss-Prot), known to catalyze a sulfur transfer reaction. Interestingly, MKP-1 lacks a critical cysteine residue within the rhodanese domain that would allow it to be enzymatically active. The function of this catalytically inactive rhodanese domain is currently unknown (Bordo and Bork, 2002). Understanding how MKP-1 is targeted to the nucleus, on its own or via binding to another molecule, can add another level of control over this phosphatase. The possibility of sequestering MKP-1 in the cytosol, as a means of limiting its phosphatase activity in the nucleus, carries the underlying assumption that MKP-1 could not dephosphorylate its effector MAPKs while sequestered in the cytosol; otherwise one set of variables would be exchanged for another.

To summarize, MKP-1 is currently known as a nuclear phosphatase. Despite this, there is a report of a non-nuclear localization for this protein. Treating a human lymphoblastic cell line with nerve growth factor increases mkp-1 mRNA synthesis and protein stability (up to 6 h) with a corresponding increase in translocation of the MKP-1 protein to the mitochondrial compartment (Rosini et al., 2004). The significance of this finding awaits further validation.

1. Regulation of Mitogen-Activated Protein Kinase Phosphatase-1 Protein Expression Levels. In addition to the transcriptional regulation, MKP-1 protein expression is increased in various cell types by different factors as exemplified by the following: insulin-treated rat smooth muscle cells (Takehara et al., 2000) and rat hepatoma cells (Lornejad-Schafer et al., 2003), glucagon-treated rat hepatocytes (Schliess et al., 2000), dexamethasone-treated human breast epithelial cell lines MCF-7 and MDA-MB-231 (Wu et al., 2004b, 2005b), epidermal growth factor-treated mouse embryonic fibroblasts (Wu and Bennett, 2005), atrial natriuretic peptide-treated HUVECs and rat lung cells (Furst et al., 2005), overexpression of cytochrome P450 2C9 on treatment with 11,12-epoxyeicosatrienoic acid in HUVECs (Potente et al., 2002), arachidonic acid-treated rat aortic vascular smooth muscle cell cultures (Metzler et al., 1998), hypoxia-treated neurons of newborn piglets (Mishra and Delivoria-Papadopoulos, 2004), hypoxia-treated PC12 (Seta et al., 2001) and HepG2 cells (Liu et al., 2005a, 2005b), serum-treated mouse fibroblasts (Sun et al., 1993), and peroxide-treated vascular smooth muscle cells (Metzler et al., 1998) and HUVECs (Furst et al., 2005). As noted for mRNA levels, 1) it is unknown whether the increase in protein levels is due to de novo translation or increased stabilization, and 2) none of

these factors or treatments are specific for MKP-1 protein regulation as they target many effectors, whereas others are adaptations to changes in the environment. Although at first glance there seem to be fewer agents and conditions that up-regulate MKP-1 protein compared with the litany of agents and conditions that control its mRNA, this situation is due to the lack of investigation of MKP-1 protein regulation when its mRNA was studied.

2. Protein Stability and De Novo Protein Synthesis. The MKP-1 protein half-life varies between 40 min (Charles et al., 1992) and 2 h (Noguchi et al., 1993). This difference in half-life can be accounted for by the various mechanisms available to the cell for the modulation of MKP-1 protein stability, as will be seen presently. A single heat shock treatment of IMR-90 human lung fibroblasts causes aggregation of MKP-1 protein in an HSP72-dependent manner, preventing MKP-1 from denaturation and preserving its function; this aggregation is partially reversed in the recovery phase from the heat shock treatment (Yaglom et al., 2003). Moreover, ERK1/2 reduce MKP-1 protein degradation by phosphorylating the ^{359}Ser and ^{364}Ser residues (Brondello et al., 1999), whereas glucocorticoids also decrease MKP-1 degradation, albeit by an unknown mechanism (Kassel et al., 2001). On the other hand, ERK1/2 are also responsible for degradation of MKP-1 (Lin et al., 2003) via phosphorylation of ^{296}Ser and ^{323}Ser residues (Lin and Yang, 2006) (Fig. 4). Once phosphorylated, Skp2 (also called SCF^{Skp2} of Skp1/Cul1/F-box protein Skp2), targets MKP-1 for degradation via the ubiquitin proteasomal pathway (Lin et al., 2003). This degradation also involves PKCδ through an unknown mechanism (Choi et al., 2006). Although ERK-2 is a better substrate for MKP-1 than is ERK1, as determined by the yeast two-hybrid system (Slack et al., 2001), ERK1 can phosphorylate MKP-1 in vitro (Brondello et al., 1999). Furthermore, as ERK1 and ERK2 share 83% identity in protein sequence (Boulton et al., 1990), it remains possible that one is responsible for phosphorylation-mediated stabilization of MKP-1, whereas the other targets this phosphatase for proteasomal degradation via phosphorylation on other residues. Otherwise, ERK1 and ERK2 may serve redundant functions. Finally, the p38MAPK may also be a stabilizing agent or have a role in translational events, because SB203580, an inhibitor that preferentially targets p38α/p38βMAPK, partially blocks ERK-dependent *cis*-diaminedichloroplatinum II (cisplatin)-mediated accumulation of MKP-1 protein without affecting mRNA levels (Wang et al., 2006). Finally, in contrast to what happens in IMR-90 cells, which constitutively express MKP-1, COS-7 cells induced de novo MKP-1 synthesis during the recovery phase after heat shock (Yaglom et al., 2003), whereas hyperosmolarity delayed insulin-induced MKP-1 protein expression (Lornejad-Schafer et al., 2003).

3. Protein Activity. Dipyridamole, an inhibitor of phosphodiesterases and nucleoside transport, stimulates tyrosine phosphorylation of MKP-1 in the murine macrophage cell-line RAW 264.7 by as yet an unknown mechanism. Dipyridamole also blocks LPS-mediated up-regulation of cyclooxygenase-2 protein levels via inhibition of p38MAPK phosphorylation (Chen et al., 2006). In addition, a double heat shock treatment increases MKP-1 phosphorylation and activity via interaction with HSP70 in human bronchial epithelial cells (Lee et al., 2005). The chaperone functions of the HSP family, which include the control of activities of other proteins and their refolding, among other functions, are well documented (Bukau et al., 2006). In consideration of the fact that MKP-1 is being stabilized in its phosphorylated state, this stabilization may be the consequence of general chaperone functions, e.g., preventing MKP-1 from unfolding or part of a mechanism designed to limit the deleterious effects of fever. How MKP-1, fever, and cancer are related to each other is a topic that will be discussed in more detail in section V.

Another mechanism that limits MKP-1 protein activity lies within the C-terminal region of MKP-1 (Fig. 4), which autoinhibits the phosphatase activity. In the same line of thought, the C-terminal truncated form of MKP-1 has higher phosphatase activity without modifying its substrate specificity (Hutter et al., 2002). Thus far, there is overall consensus on the increased phosphatase activity of MKP-1 upon binding to one of a number of active MAPKs such as ERK1/2, JNK1, or p38MAPK (Slack et al., 2001), and, in turn, MKP-1 dephosphorylates the MAPK it was bound to (Hutter et al., 2000; Farooq and Zhou, 2004). However, if we consider that the C-terminal portion of MKP-1 can inhibit its phosphatase activity, determining whether the C-terminal fragment on its own can bind to other proteins may reveal another level of control for this phosphatase. Although previous binding assays failed to reveal interactions of MKP-1 with other proteins, other than the usual MAPKs, these assays relied on the phosphatase activity of MKP-1 for a readout.

Unfortunately, the three-dimensional structure for the MKP-1 protein is still unknown and thus "tailor-made" inhibitors are not available. However, compounds such as benzofuran block MKP-1 protein function (Lazo et al., 2006), whereas others, such as Ro 31-8220, inhibit kinase activity (McKenna and Hanson, 1993; Hers et al., 1999; Chepurny et al., 2002; Hofmann, 2004) and also prevent MKP-1 protein expression (Beltman et al., 1996; Zhang et al., 2003). Sanguinarine, an alkaloid plant poison (Garcia et al., 2006) known for its antibacterial activity (Vollmer, 2006), has also been characterized to inhibit MKP-1 activity and, as a result, increases levels of phospho-ERK and phospho-JNK after a 30-min treatment in the human pancreatic cancer cell line PANC1 (Vogt et al., 2005). Nevertheless, a direct link associating sanguinarine-induced cell death (Ding et al., 2002) and reduced MKP-1 activity is still wanting.

The preceding sections have revealed a level of control over the MKP-1 protein that mirrors the many facets of control over its mRNA. Clearly the amount of MKP-1 protein is under strict censure in space and time. As is the case with the mRNA, each level of control over the MKP-1 protein is a potential therapeutic target. Because MKP-1 inactivates JNK1, p38MAPK, and ERK1/2, it is not surprising that stimulants capable of increasing MAPK activity also augment MKP-1 protein levels. The elegance of the up-regulation of MKP-1 protein levels by MAPK assures that under normal circumstances the MAPKs limit the duration of their activity in space and time. Thus, a circuit comprising a stimulant/MAPK pathway/MKP-1 protein is established after a discrete period of time, assuring the proper response for each cell type.

IV. Overview of the Dual-Specificity Phosphatase Family

The human genome codes for many types of phosphatases, among which are more than 100 protein tyrosine (Alonso et al., 2004c), lipid (Suzuki et al., 2008), carbohydrate (Worby et al., 2006; Vilchez et al., 2007), and RNA (Deshpande et al., 1999) phosphatases. The protein tyrosine phosphatases can be categorized according to sequence similarity, substrate specificity, subcellular localization, and other factors. Depending on how the tyrosine phosphatases are categorized into subgroups, the exact number and identity of the members vary within each subgroup (Alonso et al., 2004c; Farooq and Zhou, 2004). Reviewing all of the members of the protein tyrosine phosphatase family is beyond the scope of this review. However, here we summarize the MKP family and the atypical dual-specificity phosphatases, which have MAPKs as substrates, in terms of function, tissue distribution, and role in cancer (Tables 1 and 2). The atypical dual-specificity phosphatases differ from the MKPs in that the members of the latter subgroup have N-terminal domains that determine substrate specificity, whereas the former lack this domain (Alonso et al., 2004c). In general, phosphatases also differ by whether they are in an active conformation before ligand binding (e.g., VHR/DUSP3, HVH3/DUSP5, and MKP-5/DUSP10) or whether ligand binding mediates a change in conformation of the phosphatase, which enables the formation of a functional catalytic site (e.g., MKP-1/DUSP1, MKP-3/DUSP6, MKP-4/DUSP9, and PAC-1/DUSP2) (Camps et al., 1998; Alonso et al., 2004c; Farooq and Zhou, 2004; Owens and Keyse, 2007).

Kinetic studies revealed that the MKP-mediated dephosphorylation of MAPKs, which are dually phosphorylated at the T-X-Y motif (where X is Glu for ERK1/2/5, Gly for p38MAPKs, and Pro for JNKs), proceeds through a two-step dephosphorylation process: 1) binding of MAPK to MKP, targeting phospho-tyrosine, as this residue is first to be dephosphorylated by the catalytic site, and then release of the MKP-MAPK complex; and 2) binding of MAPK to MKP, dephosphorylation of the phospho-threonine residue, and then release of both proteins (Alonso et al., 2004c; Farooq and Zhou, 2004).

Although substrate specificity varies depending on the phosphatase, there is obvious overlap between the specificities. This overlap can be explained in terms of gene duplication and evolutionary divergence (Alonso et al., 2004c). However, these overlaps may be reduced if we consider subcellular localization, tissue distribution, or other factors. Unfortunately, most of the reports on the tissue expression concern only mRNA and not the protein product of the phosphatases. When substrate specificity was investigated, only one representative member of the most common MAPKs were used, for obvious practical considerations, leaving open the possibility that the other isoforms and splice forms of MAPKs might interact differently with each phosphatase. Other observations suggested that a given phosphatase was not a simple "off switch" for its kinase substrate. This was exemplified by the following observations, which will be developed shortly: 1) a DUSP2 (PAC-1) variant, without intrinsic phosphatase activity, enhanced MKP-1 phosphatase activity; 2) absence of DUSP2 increased JNK activity and decreased ERK and p38MAPK activities; 3) DUSP5 transported and sequestered unphosphorylated ERK2 to the nucleus; 4) the DUSP7 (Pyst2) catalytically inactive short form (Pyst2-S) can regulate the function of the long form (Pyst2-L); 5) DUSP22 activated the JNK pathway; and 6) DUSP23 enhanced p38MAPK and JNK activities (Tables 1 and 2; Fig. 2).

Although the exact mechanisms by which the previously mentioned observations occurred remain to be determined, there were some concepts relating to each that had been unveiled. The first of these was that absence of DUSP2 increased JNK activity leading to decreased ERK and p38MAPK activities. Whereas the increased JNK activity in the absence of phosphatase activity was a straightforward concept, decreased kinase activities of ERK and p38MAPK were thought to be a JNK-mediated effect on the latter MAPKs (Jeffrey et al., 2006). Indeed, JNK and its signaling module are believed to exert both positive and negative control over the other MAPKs (Fig. 2) (see also section II.E. on MAPK cross-talk). Therefore, when a phosphatase is overexpressed in an attempt to inactivate its preferred MAPK substrate, it would be necessary to determine 1) the activity of other members of the MAPK family, given the cross-talk between the MAPK family and their signaling modules and 2) the possibility that the phosphatase can activate upstream kinases. Of note, JKAP (DUSP22) activated the JNK pathway via activation of MKK7 (but not MKK4) (Chen et al., 2002) and JSP1 (DUSP22) activated MKK4 (not MKK7) (Shen et al., 2001). Conversely, LMW-DSP2 (DUSP22) had been implicated in inactivation of JNK and p38MAPK through an indirect mecha-

nism (Aoyama et al., 2001) (Fig. 2). As the activation status of the upstream kinases was not investigated in the latter study, the difference between these results (Aoyama et al., 2001) and the results of the two former studies (Shen et al., 2001; Chen et al., 2002) remained unclear. Human JKAP differs in its C-terminal sequence, which is slightly longer than that of JSP1 or LMW-DSP2. These three phosphatases are most likely splice forms of the *dusp22* gene (Chen et al., 2002), which could account for their different affinities for MKK7 and MKK4 in different cell types. Similar to DUSP22 protein, DUSP23 protein could activate MKK4 (upstream kinase of p38MAPK and JNK) and MKK6 (upstream kinase of p38MAPK) (Takagaki et al., 2004). In summary, DUSP22 (JKAP, JSP1) and DUSP23 dephosphorylated and activated MKK4, MKK6, and MKK7 (Shen et al., 2001; Chen et al., 2002; Takagaki et al., 2004). Conversely, AKT phosphorylated and inactivated MLK3, ASK, and MEKK4 (Bogoyevitch and Kobe, 2006). How cells manipulate the activity of these phosphatases and kinases in response to specific stimuli in health and disease may be determined by the level of expression of a given phosphatase, which seems to vary according to tumor type, such as MKP-1 (see sections V and VI), and other phosphatases (Tables 1 and 2).

Adding to these observations were the findings that inactive phosphatases were not merely dominant-negative proteins. Whereas Pyst2-S regulates the function and activity of Pyst2-L by competing for effectors and transcription and/or translation factors, among other possible mechanisms (Levy-Nissenbaum et al., 2003a,b, 2004), a PAC-1 inactive variant enhanced the function of MKP-1 (Kothapalli et al., 2003) (Table 1). Moreover, there were at least two phosphatases within the group we are considering in this review, MK-STYX (STYXL1) and STYX, with inactive catalytic sites due to a naturally occurring substitution, Cys→Ser and Cys→Gly, respectively (Wishart and Dixon, 1998). MK-STYX is classified as a member of the MKP subfamily, and STYX is considered an atypical dual-specificity phosphatase, as it had no N-terminal substrate-binding motif. Although the substrates of these phosphatase-dead proteins have as yet to be determined, the consequences of lacking an inactive phosphatase were revealed from results with STYX male null mice (Table 2). The male mice were infertile, whereas their female null counterparts remained fertile, although both seemed to have a "normal" overall phenotype (Wishart and Dixon, 2002). This subtle difference in fertility for a catalytically inactive phosphatase that had a ubiquitously expressed mRNA warrants further investigation. Until the proteomic expression profile of STYX and its function and substrate specificity are known, it will be difficult to determine the reason for this gender-specific infertility. There may be other gender-specific differences in the STYX-null mice. The function of MK-STYX remains unclear although it has been implicated in cancer (Table 1). One role these phosphatase-dead proteins could have is to sequester their MAPK substrates and "preserve" them in active form, protected from dephosphorylation. Alternatively, catalytically inactive "phosphatases" could maintain the unphosphorylated MAPK sequestered in their subcellular localization ready for activation by their upstream kinases. Such an effect was seen with the inactive form of DUSP5, due to an experimentally induced point mutation. Whereas both the active and inactive forms of DUSP5 transported unphosphorylated ERK2 to the nucleus, the inactive form of DUSP5 allowed activation of ERK2 by MEK1/2 in the nucleus in response to mitogen (Mandl et al., 2005).

Another twist in this story lies in the rare event in mammalian cells of two different proteins of the same family coded by the same gene as a result of different open reading frames, as is the case for DUSP13A (MDSP) and DUSP13B (TMDP) (Chen et al., 2004) (Table 2). These two proteins share 42% sequence identity. Interestingly, this locus has an ortholog in mouse and a similar arrangement of phosphatases in puffer fish (*Fugu rubripes*), indicating evolutionary conservation and perhaps importance (Chen et al., 2004). MDSP and TMDP display phosphatase activity toward phosphotyrosine and phosphothreonine when tested against artificial substrates. However, the naturally occurring substrates and the consequence of their function await identification (Nakamura et al., 1999; Chen et al., 2004). Other phosphatases such as M3/6 (DUSP8) and MKP-7 (DUSP16), can bind to the scaffolding protein JIP1, and MKP-7 can decrease MLK3-JNK-JIP1-mediated phosphorylation of c-Jun (Willoughby et al., 2003). As Tables 1 and 2 reveal, there are many phosphatases that await characterization for function, distribution, and involvement in cancer or other diseases. When more phosphatases are characterized, in terms of binding to scaffolding proteins, expression pattern, and involvement during normal and disease processes, a more comprehensive picture will emerge as to how kinases and phosphatases interact to generate cell phenotypes.

In sections V and VI, we will focus our attention on MKP-1 and its role in cancer, both its pro- and anticancer activities (Ducruet et al., 2005), vis-à-vis the MAPK family, cell and animal models, and MKP-1 expression in cancer patients. Finally, chemotherapeutic agents used for the treatment of various tumors in animal models or patients are discussed when they pertain to the expression of MKP-1 or to its function. A recurring theme is that MKP-1 can sometimes be directly linked to tumor phenotypes, whereas at other times it is more of a bystander. Perhaps this is not only a consequence of the complexities associated with MAPK signal transduction but also has a basis in the multiplicity of nuclear and cytosolic phosphatases with overlapping MAPK substrate specificity.

V. Mitogen-Activated Protein Kinase Phosphatase-1, Animal Models, and Cancer

Considering the level of control of the mkp-1 mRNA and protein (see section III.B–C), it is not surprising to find many agents that modulate their expression levels. Indeed, each naturally occurring point of control over the mRNA or protein represents a potential therapeutic target. These agents could modulate MKP-1 enzymatic activity, the rate of protein/mRNA synthesis, the rate of protein/mRNA degradation, unblocking elongation of the mRNA, and the methylation/demethylation of the *mkp-1* gene, among other levels of control. As the pathology of cancer entails a lack of or inappropriate restraint at one or more of these levels, chemotherapeutic agents that target a specific facet of control have been and continue to be sought after in an attempt to exert exogenous management when endogenous mechanism fails. However, as a considerable number of genes and proteins are controlled by a given mechanism, each chemotherapeutic agent, although specific for a certain regulatory mechanism, may target many or all genes and proteins controlled by that mechanism.

Having outlined the mechanisms that control mkp-1 mRNA and protein expression and stability, we will review what is known about *mkp-1* organ-specific transgenic and *mkp-1*-null mice and how this modulation of *mkp-1* affects normal development and adult mouse phenotypes when exogenous stressors are applied.

A. mkp-1 *Transgenic Mice*

mkp-1 constitutive cardiac transgenic mice expressing high levels of MKP-1 (3.7 times normal levels) die between postnatal days 7 and 15 because of a lack of developmental cardiac hypertrophy, whereas mice expressing moderate levels of MKP-1 (1.8 times normal levels) survive to adulthood although with abnormal cardiac morphology as determined with more detailed histological analysis and visualization of function by echocardiography. The founder mice are able to survive because of the mosaic distribution of the transgene (Bueno et al., 2001).

B. mkp-1 *Knockout Mice*

1. Phenotype. *mkp-1* knockout mice have no overt phenotype from histology to behavior. The genotype is expressed in a predictable mendelian distribution. Interestingly, *mkp-1*-null mice do not demonstrate increased ERK1/2 phosphorylation (Dorfman et al., 1996). Moreover, there are no differences between *mkp-1*(−/−) and *mkp-1*(+/+) littermates in the developmental phase or in various organ systems, such as the neurological, cardiac, hematological, and endocrinological systems (Salojin et al., 2006).

2. Immunological Consequences. Although *mkp-1*(−/−) mice seem to be normal overall, the null mice have an exaggerated innate immune response to LPS and increase their serum levels for various cytokines (TNF-α, IL-6, interferon-γ, and IL-10) (Salojin et al., 2006; Zhao et al., 2006) as well as IL-12p70 and monocyte chemotactic protein-1, compared with their *mkp-1*(+/+) littermates (Salojin et al., 2006). This difference in serum cytokine levels underscores the survival of both sets of mice after LPS challenge, with all of the *mkp-1*(−/−) mice dying within 48 h and all the wild-type mice surviving after 72 h after LPS injection. Furthermore, *mkp-1*(−/−) mice demonstrate a greater severity and incidence of arthritis in a chicken type II collagen-mediated model of rheumatoid arthritis (Salojin et al., 2006). Other studies showed similar survival kinetics for LPS-mediated endotoxic shock in *mkp-1*(−/−) mice versus their *mkp-1*(+/+) littermates (Hammer et al., 2006; Zhao et al., 2006).

LPS challenge also causes renal, hepatic, and pulmonary damage (Zhao et al., 2006). This effect was assessed by measuring blood urea nitrogen and blood alanine aminotransferase activity and histological analysis, respectively. The lungs revealed thickening of the alveolar septa, edema, and infiltration by leukocytes in the interstitial space. In LPS-treated *mkp-1*(−/−) mice blood nitrate levels were elevated compared with those in their *mkp-1*(+/+) littermates. This finding was paralleled by reduced blood pressure and hypotension in *mkp-1*(−/−) mice compared with their *mkp-1*(+/+) littermates (Zhao et al., 2006).

Among the genes up-regulated as a result of LPS challenge in *mkp-1*-null versus WT littermates are the chemokines CCL3, CCL4, and CXCL2, also called macrophage inflammatory proteins 1α, 1β, and 2, respectively, at the mRNA and protein levels. As these chemokines have the potential to recruit leukocytes, they may contribute to damage, leading to lethality. In addition, IL-6 and IL-10 were both up-regulated as a result of LPS in *mkp-1*(−/−) mice, leading to SOC3, NFIL3, Ndr1, and Gadd45γ up-regulation (Hammer et al., 2006). MKP-1 may therefore be required to down-regulate LPS- and IL-10-regulated genes (Hammer et al., 2006).

In vitro, bone marrow-derived dendritic cells and peritoneal macrophages stimulated with LPS show differences in levels of secreted cytokines (TNF-α, IL-6, IL-10, and IL-12p70) compared with *mkp-1*(−/−) with *mkp-1*(+/+) mice (Zhao et al., 2006). Peritoneal macrophages from *mkp-1*-null mice have prolonged phosphorylated p38MAPK and JNK in response to LPS compared with wild-type mice (Zhao et al., 2006). Cytokine production is altered in *mkp-1*-null versus wild-type mice upon LPS treatment whether or not they have been primed with interferon-γ or thioglycollate (Zhao et al., 2006). Others found that bone marrow-derived macrophages increased their p38MAPK phosphorylation in response to LPS in *mkp-1*-null mice compared with their wild-type counterparts (Salojin et al., 2006). In both primary and immortalized murine alveolar macrophages (MH-S), LPS induces both mkp-1 mRNA and protein synthesis (Zhao et

al., 2005). This MKP-1 induction correlates with down-regulation of ERK, JNK, and p38MAPK phosphorylation status in primary murine alveolar macrophages as demonstrated when WT and *mkp-1*-null cells are treated with LPS (Zhao et al., 2005). This group also used MH-S cells to evaluate the effect of some steroids on the induction of mkp-1 mRNA versus their anti-inflammatory activity. They found a positive correlation between the ability of a given steroid to induce mkp-1 mRNA and its anti-inflammatory activity (Zhao et al., 2005).

So what has all this to do with cancer? Within the confines of infection/MKP-1 there is a cancer connection. At present there are five observations that merit our attention: 1) the increase in inflammatory cytokine response to an LPS challenge, when *mkp-1*(−/−) mice are compared with their *mkp-1*(+/+) littermates (see section V.B.2); 2) chronic inflammatory processes, caused by either bacteria or viruses, leading to cancer (Karin et al., 2006); 3) the use of fever induced by bacterial infection (Busch-Coley treatment) to induce remission in some cancers (Hobohm, 2001); 4) fever caused by chemotherapeutic agents (Davis and Raebel, 1998); and 5) the number of immunomodulatory-related phosphatases (Tables 1 and 2). Furthermore, retrospective analysis revealed that the success of the Busch-Coley treatment was enhanced with the following four criteria: 1) the cancer was of mesodermal embryonic origin; 2) treatment was given three times per week for at least 6 months; 3) the patient achieved a fever of approximately 39°C for 12 to 24 h after each injection; and 4) the patient has an otherwise healthy immune system that has not been weakened by radiotherapy or chemotherapy (Hobohm, 2001). Taken together, these observations raise a few interesting questions: 1) If bacterial and viral infection induce some cancers, can these tumors be targeted by a modernized version of the Busch-Coley treatment, the mixed bacterial vaccine? 2) If lack of MKP-1 expression increases the production of inflammatory cytokines to the point of death in *mkp-1*-null mice, can judicious targeting of this or another phosphatase involved in reducing inflammation be used to increase efficiency of such a vaccine? 3) More importantly, what are the phosphatase and MAPK profiles for chronic inflammation that does not lead to cancer versus the profiles that lead to chronic inflammation-mediated tumorigenesis or inflammation-induced tumor regression for a given tissue? Understanding these differences will be a first step in 1) dealing with chronic inflammation-induced cancer, 2) increasing the efficacy of a mixed bacterial vaccine targeting cancer of mesodermal origin, 3) manipulating MAPKs and phosphatases to enhance the efficacy of vaccine, and 4) extending these observations outside the confines of cancer from mesodermal origin to tissues deriving from other embryological origins.

3. Diet-Induced Obesity. *mkp-1*-null mice are lean and resistant to diet-induced obesity. Activated p38MAPK, JNK1, and ERK levels are elevated in skeletal muscle and white adipose tissue, compared with levels in similarly treated wild-type mice, and the liver also demonstrates elevated p38MAPK and JNK1 activity whereas ERK activity remains unchanged. Some researchers believed that inactivation of the *mkp-1* gene may be an important element in uncoupling diet-induced obesity from glucose intolerance(Wu et al., 2006a). However, how the activities of p38MAPK, JNK1, and ERK relate to this phenotype await elucidation.

One of the most unexpected findings for a phosphatase that controls p38MAPK, JNK1, ERK1/2, and ERK5 activities is a lack of obvious phenotype in *mkp-1*-null mice. Normal development coupled with normal fertility in null mice is difficult to reconcile considering the ubiquitous expression of the mkp-1 mRNA and protein in virtually all tissues examined. One key piece of information that we lack to resolve this conundrum is which phosphatases compensate for the lack of MKP-1 in null mice during development? More importantly, why are these phosphatase(s) failing to compensate upon stress?

C. Mitogen-Activated Protein Kinase and Cancer: An Overview

MKP-1 and other dual-specificity phosphatases target members of the MAP kinases and inactivate their function in both the cytosol and the nucleus (Tables 1 and 2). To appreciate the possible involvement of members of the MAPK family in cancer, we will present an overview of what is known about their contribution to the process or inhibition of oncogenesis and how this relates to cell and animal models as well as to human tumors in this and the following section.

1. Ras-Raf Cancer Connection. Both Ras, the upstream activator of the Raf-MEK-ERK module, and Raf have been implicated in cancer (Schubbert et al., 2007; Barault et al., 2008). More specifically, the Ras isoforms have been associated with different cancers when they have an activating mutation or developmental diseases when the proteins that control Ras activity are mutated (Roberts and Der, 2007; Schubbert et al., 2007). Of the Rafs, B-Raf has the highest incidence of activating mutations, whereas Raf1 activating mutations are rare and A-Raf activating mutations have not been found (Emuss et al., 2005). Reasons for the discrepancy in frequency of association with cancer include the following: 1) the constitutive phosphorylation of B-Raf at S445 gives it a higher basal activity than Raf1 basal activity (Mason et al., 1999); and 2) the negative charge at the N terminus of B-Raf is due to an aspartic acid residue instead of the tyrosine found at the homologous site in Raf1; the tyrosine requires phosphorylation for activation. Thus, the constitutive phosphorylation combined with the N terminus negative charge is believed to contribute to B-Raf being more easily activated by a point mutation. Thus, the B-Raf mutation is frequently associated with cancers (Schubbert et al., 2007). In contrast, a single point mutation is rarely sufficient to activate A-Raf or Raf1 (Well-

brock et al., 2004). In addition, B-Raf activation by Ras is more direct than that of A-Raf and Raf1. The two latter isoforms require other kinases, scaffolding proteins, or phosphatases for activation (Wellbrock et al., 2004). Finally, it should be noted that Raf is only one of many effectors of Ras, which include: the catalytic subunit of class I PI3K (p110α–δ) (Martin and Vuori, 2004; Olson and Hallahan, 2004; Hannigan et al., 2005; McLean et al., 2005; Cully et al., 2006; Engelman et al., 2006; Holz et al., 2006; Kohno and Pouyssegur, 2006; Goldsmith and Dhanasekaran, 2007; Hehlgans et al., 2007; Mayor et al., 2007; Roberts and Der, 2007; Schubbert et al., 2007), phospholipase Cε, and some RhoGEFs of Rac and Ral that have been implicated in Ras-mediated oncogenesis (Roberts and Der, 2007).

2. Extracellular-Regulated Kinases. Although ERK1/2 functions permeate cell cycle regulation on their own, their functions are not sufficient to enter into the cell cycle. This fact is underscored by the finding that Ras overactivation on its own is insufficient for cell cycle entry and would result in growth arrest (Hirakawa and Ruley, 1988), whereas other results reveal that overexpression of growth factor receptors in PC12 cells leads to differentiation (Marshall, 1995) and sustained ERK activation in fibroblasts leads to proliferation (Marshall, 1995). Thus, the level within the signal transduction pathway, which is responsible for overstimulation, along with cell type may influence phenotypic outcome (see section II.B on ERKs). Furthermore, ERK also increases CDK inhibitor proteins p21, p27, and p53, among others, which results in cell cycle arrest (Smalley, 2003; Dhillon et al., 2007) and phosphorylation of CDC25c, leading to its ubiquitination and proteasomal degradation (Eymin et al., 2006), whereas Raf can also induce cell cycle arrest via p21 (Smalley, 2003). Thus, tumors must counteract this effect and can do so via AKT and RhoGTPase proteins (Dhillon et al., 2007). AKT inhibits the CDK inhibitor proteins p21 and p27 and the cyclin D1 inhibitor glycogen synthase kinase (GSK3β) (LoPiccolo et al., 2007; Manning and Cantley, 2007). However, greater levels of ERK mRNA, protein, and phosphotyrosine ERK1/2 were found in primary breast cancer cells compared with surrounding normal tissue (Sivaraman et al., 1997). Considering the level of involvement of ERK1/2 in the cell cycle, a phosphatase capable of inactivating ERK1/2 can have any number of effects, depending on when the phosphatase is expressed and to what extent its effects can be bypassed, assisted, or inhibited via other members of the MAPK family.

Interestingly, ERK3 expression has been associated with inhibition of proliferation and the induction of cell differentiation. Overexpression of ERK3 or ERK7 can inhibit the cell cycle in the S phase (Coulombe and Meloche, 2007). It remains to be determined whether this effect is part of the "normal" function of ERK3 or ERK7 or is due to overexpression. ERK5 has been implicated in cell cycle progression, although the extent of this involvement, the cell type, and the circumstance await further clarification, as *erk5*-null MEFs cycle through the S phase, and the increase in cyclin D1 can be uncoupled from ERK5 activation status, although mitogen signaling through ERK5 and cyclin D1 transcription can be regulated by ERK5. Because ERK1/2 are implicated in many aspects of the cell cycle, ERK5 may serve redundant functions with its more famous family members or it may have a more restricted role. For instance, ERK5 is implicated in neuronal and endothelial cell survival (Wang and Tournier, 2006), whereas leukemia cells increase levels of ERK5 via stabilization of the protein in an Abl kinase-dependent manner, leading to transformation (Buschbeck et al., 2005). Overexpression of shRNA for ERK5 in the EL-4 T-lymphoma cell line failed to develop tumors in mice when injected subcutaneously compared with mice with vector control-treated cells, which did develop tumors (Garaude et al., 2006). Recently, down-regulation of miRNA 143, which targets ERK5 expression levels, has been implicated in human cancers (Akao et al., 2006, 2007). Homeobox gene (*HOXB9*) expression, identified in Hodgkin's lymphoma, corresponds to active ERK5 (Nagel et al., 2007). Conversely, medulloblastoma cell lines increase apoptosis upon MEK5/ERK5 exogenous expression in a MEF2-dependent manner (Sturla et al., 2005). Finally, ERK5 also has a role in angiogenesis and prostate cancer (see section V.D.8 on angiogenesis and vasculogenesis and section VI.E.3 on prostate cancer).

3. c-Jun NH_2-Terminal Kinases. As previously mentioned (see section II on the MAPK family), active JNKs can have both pro- and antiapoptotic activities, whereas active and unphosphorylated JNKs modulate the stability of their effector proteins via polyubiquitination and the proteasomal pathway, although via different mechanisms. This array of functions is accompanied by an equally varied response regarding JNK function and tumorigenesis as revealed in this section.

Jnk2-null mice demonstrated lower 12-*O*-tetradecanoylphorbol-13-acetate-mediated tumorigenesis compared with WT control mice (Chen et al., 2001), whereas *Jnk1*-null mice demonstrated enhanced 12-*O*-tetradecanoylphorbol-13-acetate-mediated tumorigenesis compared with their WT control mice (She et al., 2002). The JNK1/2 double knockouts affect neither ERK, p38MAPK, nor MKK4 or MKK7 protein expression levels. WT MEFs expressed high p46-JNK1 and p54-JNK2 and low levels of p54-JNK1 and p46-JNK2, and JNK3 was not detected (Tournier et al., 2000). p38MAPK, MKK4, and MKK7 kinase activities were down-regulated after stress compared with those in WT MEFs, whereas ERK kinase activity was not visibly affected upon serum stimulation. When stressed, JNK1/2 double knockout fibroblasts fail to show apoptosis in vitro (Tournier et al., 2000). Conversely, no tumors developed with either JNK1/2 double null fibroblasts or WT MEFs when injected into athymic nude mice (Kennedy et al., 2003). Ras-transformed

JNK1/2 double KO MEFs developed greater tumor mass than Ras-transformed-WT MEFs when injected in athymic nude mice, as a result of reduced apoptosis, generating both a greater number and size of nodules (Kennedy et al., 2003). In vivo JNK complementation studies support a role for JNK in interfering with Ras-mediated cell survival, contrary to in vitro results, which suggest that JNK is a positive regulator of Ras-mediated oncogenesis (Kennedy et al., 2003). A constitutively active form of Ras (RasV12) led to persistent JNK1 activity in NCl-H82 human small-cell lung cancer cells, but had a more modest effect on ERK activity. The importance of JNK1 activity was revealed when a dominant-negative form of JNK1 reduced both cell number and colony number in RasV12-transformed NCl-H82 cells in an AP-1-dependent manner (Xiao and Lang, 2000). Other systems reveal that JNK1 is essential for survival of B-lymphoblasts (Hess et al., 2002). Therefore, the involvement of JNK1 and JNK2 in cancer is not uniquely prodeath in either human cell lines or animal models (Bode and Dong, 2007). JNK3 has a more localized distribution than either JNK1 or JNK2. Lack of JNK3 has been associated with human brain tumors (Yoshida et al., 2001). In addition, activation segment mutation in JNK1 and kinase domain mutation in JNK2 have been reported (Greenman et al., 2007), although mutations in the families of Jun and Fos, which are components of the AP-1 transcription factor, are as yet unknown (Verde et al., 2007).

Despite the previously mentioned results, a clear image of exact role of JNKs in tumor development and progression is still wanting. For instance, JNK can activate or inhibit the proapoptotic pathway via phosphorylation and phosphorylate AKT to prime it for interaction with its kinase PDK1 (Bogoyevitch and Kobe, 2006). Active AKT can phosphorylate and inhibit MKK4, ASK, and MLK3, the upstream kinases of JNK (Bogoyevitch and Kobe, 2006) (Fig. 2). In addition, when looking at JNKs to find an answer, we must also consider the function of unphosphorylated JNK as it can target its effectors for degradation (Fuchs et al., 1997, 1998a; Bode and Dong, 2007). Unfortunately, when analyzing the JNKs and their function in many systems, we often relegate nonactive JNK to be part of a loading control for phosphorylated JNK rather than a molecule that has a bona fide function in its own right. Taking the latter into account, the normal function of JNKs (see section II.C on the MAPK family), their possible role in tumorigenesis (this section), the different targets of the Jun and Fos family of transcription factors (Verde et al., 2007), and the contribution of other MAPKs (MAPK cross-talk) and phosphatases in signal transduction (Fig. 2), among other factors, we find it is easy to realize why a definitive image of JNK and cancer is still wanting.

4. p38 Mitogen-Activated Protein Kinases. Increased p38MAPK signaling is associated with MMP-9 in leukemia (Ringshausen et al., 2004) and MMP-2 in prostate cancer (Xu et al., 2006), whereas production of MMP-2 (Kim et al., 2003a) and urokinase plasminogen activator was mediated by H-Ras (Behren et al., 2005). Overall, MMP production is initially associated with the creation of a tumor microenvironment for enhanced growth and later with metastasis (Overall and López-Otín, 2002). p38α is also involved in angiogenesis; this topic will be covered in section V.D.8 on angiogenesis and vasculogenesis. Although, HSP27 is implicated in p38αMAPK-mediated cell migration (Rousseau et al., 2000, 2006), collectively as a family HSPs can be indicators of poor prognosis in cancer treatment (Ciocca and Calderwood, 2005). p38α/βMAPK is also implicated in H-Ras-mediated invasion of human breast epithelial cells (Kim et al., 2003a), and contact inhibition, a characteristic of nontransformed cells (Hoff et al., 2004), is achieved by sustained p38α activity (Faust et al., 2005), whereas p38γ assists K-Ras-mediated transformation (Tang et al., 2005). p38MAPK is also involved in cell cycle arrest by directly phosphorylating CDC25A, leading to degradation of this phosphatase (Khaled et al., 2005; Kittipatarin et al., 2006) or indirectly phosphorylating CDC25B via MAPKAP-K2 (Manke et al., 2005). The CDCs are a family of phosphatases regulating the cell cycle (Rudolph, 2007a), which are overexpressed in various cancers (Boutros et al., 2007; Rudolph, 2007b) and targeted for therapeutic intervention (Boutros et al., 2007). Although p38MAPK is activated by telomere shortening associated with replicative senescence, it is also linked to replicative senescence that is unrelated to telomere length (Han and Sun, 2007).

Although p38α KO mice are embryonically lethal, as mentioned in section II on the MAPK family, p38α embryo-specific knockouts [placentas are *p38α*(+/+)] develop into pups but most die at 4 days after birth (Hui et al., 2007). Hematopoietic cells and MEFs from these mice are associated with uncontrolled proliferation in vitro (Hui et al., 2007). Another study revealed that mice with an inducible deletion of the *p38α* gene lack differentiation in murine lung progenitor cells (Ventura et al., 2007). Other animal models support a role for p38MAPK and some of its effectors in the prevention of tumorigenesis (Han and Sun, 2007). p38MAPK is also implicated in human tumors, as a lack of p38MAPK signaling is associated with a myosarcoma, a childhood solid tumor characterized by lack of differentiation of myoblasts into myotubes (Puri et al., 2000) and other human tumors are linked to defects in the p38MAPK signal transduction pathway (Han and Sun, 2007).

Collectively members of the p38MAPK family have the potential to influence tumorigenicity on many levels, notwithstanding the lack of phenotype associate with the KO mice for the β, γ, and δ isoforms of p38MAPK during development (see section II.D). This is recognized when the best-characterized isoform, p38αMAPK, is used as an example with its involvement in chemo-

taxis, MMP production, and modulation of mRNA stability, proliferation, differentiation, and so on. Thus, inappropriately expressed or inhibited expression of p38MAPK isoforms can deregulate cell function on multiple levels. Likewise, an inappropriately expressed phosphatase that is capable of dephosphorylating p38αMAPK (Tables 1 and 2), such as MKP-1, could have deleterious consequences for the phenotype of the cell. Conversely, a cell overexpressing p38MAPK or a mutated oncogene, such as H-Ras, could benefit from expressing a p38αMAPK-specific phosphatase. This benefit holds true not only for p38MAPK but also for the other axes of MAPK. Earlier studies revealed that quiescent rat embryonic fibroblast REF-52 cells cease to synthesize DNA in response to the constitutively active form of Ras when MKP-1 is coexpressed (Sun et al., 1994). Conversely, NIH 3T3 cells demonstrate an MKP-1-mediated increase in Raf1 and MEK-1/2 activities, in both a Ras-dependent and -independent manner (Shapiro and Ahn, 1998). This effect could be due to MKP-1 inactivating ERK and preventing its inhibitory phosphorylation of the upstream kinases. In addition, PKA down-regulates Raf1, which can lead to the down-regulation of ERK1/2. As this latter kinase has been known to phosphorylate and stabilize MKP-1 protein (Brondello et al., 1999), growth factor signaling that activates ERK would be counterbalanced by PKA and MKP-1 (Pursiheimo et al., 2002).

Differential ERK activity in the cytosol versus the nucleus was observed in response to a conditionally expressed Ha-Ras in NIH 3T3 cells. Cytosolic ERK2 remained active in response to Ha-Ras for much longer than nuclear ERK2, which was dephosphorylated by MKP-1 (Plows et al., 2002). Others found that when an angiotensin II signaling system is reconstituted with either angiotensin type 1 receptor only (γ2A/AT$_1$) or in combination with Jak2 (γ2A/AT$_1$/Jak2), Jak2 inactivates ERK2 in 30 min in an MKP-1-dependent manner. In the absence of Jak2, ERK2 inactivation requires 120 min (Sandberg et al., 2004). As cytosolic MAPK has effectors different from those of nuclear MAPK (Yoon and Seger, 2006), by selectively inactivating the latter, MKP-1 can inhibit MAPK-mediated transcription of genes while allowing MAPK to maintain signal transduction capabilities within the cytosol.

D. Cancer-Related Mechanisms

Many of the chemotherapeutic agents mentioned in this section have been approved by the Food and Drug Administration for certain types of cancers when they are used alone or in conjunction with other chemotherapeutic agents, radiotherapy, or surgical intervention. The following list serves as an example (for a more complete listing the reader is directed to the Web site http://www.cancer.gov/cancertopics/druginfo/alphalist). Among uses of the reagents, 1) bortezomib has been approved for multiple myeloma, 2) cisplatin for ovarian cancer that has metastasized and for bladder cancer, 3) decitabine for myelodysplastic syndrome, 4) doxorubicin to treat several types of cancers, and 5) paclitaxel for metastatic and recurrent breast cancer. In addition, as this section is divided into different segments according to cancer phenotype, the chemotherapeutic agents have been cross-referenced accordingly.

1. Apoptosis. Because the increase in MKP-1 expression is linked to a decrease in JNK activity and a corresponding increase in cancer cell survival via a decrease in JNK-mediated apoptosis (Mizuno et al., 2004), various molecules have been used to increase apoptosis in an attempt to thwart the growth of tumors. It should be emphasized that none of the following compounds or any stress or growth factor previously mentioned, targets *mkp-1* exclusively except *mkp-1* siRNA/shRNA. The following compounds usually possess pleiotropic effects, and MKP-1 regulation is only one facet when it occurs. How the presence of MKP-1, whether endogenous or exogenously introduced, affects the function of these compounds will be addressed.

a. Platinum. Platinum-based drugs such as cisplatin and transplatin are well established chemotherapeutic agents that act (in part) by cross-linking DNA (Pascoe and Roberts, 1974a,b; Zwelling et al., 1979; Giraud-Panis and Leng, 2000). The difference in method of cross-linking is believed to underlie the effects of the *cis*-isomer, in its ability to block transcription and DNA replication in some tumors, whereas the *trans*-isomer is ineffective (Boudvillain et al., 1995; Mymryk et al., 1995; Siddik, 2003; Wang and Lippard, 2005). The result of the interaction between cisplatin and the molecular machinery of a tumor is multifactorial and incompletely understood (Siddik, 2003; Wang and Lippard, 2005). Recently, cisplatin was found to up-regulate MKP-1 in human lung H460 and ovarian OVCAR3 cancer cell lines (Wang et al., 2006). Furthermore, *mkp-1*-null MEFs were more sensitive to cisplatin-induced apoptosis in a JNK-dependent manner, whereas the wild-type counterparts were more resistant. Indeed, the JNK inhibitor SP600125 blocked cisplatin-mediated phosphorylation of JNK and increases cell survival by decreasing cisplatin-induced apoptosis (Wang et al., 2006). In addition, cisplatin is a strong inducer of JNK1 activity but weak inducer of MKP-1 expression, whereas transplatin up-regulates MKP-1 and weakly induces JNK1 activity (Sánchez-Pérez et al., 1998, 2000). These results mirror the survival of Pam212 and 293T cells, as cisplatin induces more cell death than transplatin.

b. Deacetylation/demethylation. 5-Aza-2′-deoxycytidine (decitabine) can cause reexpression of a gene that was hypermethylated and, as a result, inactivated as long as the treatment continues (Baylin, 2005). Decitabine is a deoxycytidine analog that is capable of being incorporated into DNA after phosphorylation by deoxycytidine kinase (Oki et al., 2007). As a nitrogen atom is

in the 5′-position of the ring in the decitabine molecule (in place of a carbon atom in the cytosine ring), methylation occurs, but there is no hydrogen at position C-5 to complete the reaction and free the DNA methyltransferase; the enzyme thus becomes "trapped" (Yoo and Jones, 2006). Although the resultant hypomethylation can occur throughout the genome (Oki et al., 2007), loss of methylation in repeat elements near the centromere does not herald the loss of genomic integrity (Bird, 2002).

The prostate cancer cell line PC-3 up-regulated mkp-1 mRNA expression in response to either decitabine or a combination of trichostatin A (a histone deacetylase inhibitor) (Yoshida and Horinouchi, 1999) and decitabine treatment (Rauhala et al., 2005). Overall, this method served to detect the epigenetically down-regulated genes by suppression of subtractive hybridization and cDNA microarray analysis. The results indicate that DNA methylation may be part of a mechanism used by PC-3 cells for the down-regulation of *mkp-1* gene expression. Interestingly, whereas hormone-refractory prostate carcinomas showed lower levels of MKP-1 protein and mRNA expression levels, untreated prostate carcinomas revealed lower MKP-1 protein expression, whereas mRNA levels were no different from those of benign prostate hyperplasia (Rauhala et al., 2005). All together, the results suggest that down-regulation of MKP-1 is an early event in human prostate tumorigenesis (Rauhala et al., 2005).

c. Glucocorticoids. Although the therapeutic potential of endogenous glucocorticoids has been known for some time (Buckingham, 2006), the in vivo functions of both endogenous and exogenously administered glucocorticoids are still being discovered (Distelhorst, 2002; Hayashi et al., 2004b; Stellato, 2004; Buckingham, 2006). Some of these functions include up- or down-regulation of transcription (Hayashi et al., 2004b) and stabilization/destabilization of mRNA (Stellato, 2004) in addition to apoptosis.

Interestingly, whereas glucocorticoids induce apoptosis in lymphocytes (Distelhorst, 2002), pre-B acute lymphoblastic leukemia cell line 697 is more resistant to hydroxyurea-mediated apoptosis than to glucocorticoid-mediated apoptosis when mkp-1 is stably transfected in this cell line (Abrams et al., 2005). Other studies revealed that dexamethasone used before a chemotherapeutic agent, such as paclitaxel or doxorubicin, has an antiapoptotic effect on breast cancer cells (MCF-7 and MDA-MB-231) (Wu et al., 2004b). MDA-MB-231 cells ectopically expressing MKP-1 and treated with paclitaxel have a number of apoptotic cells similar to that of sister cultures expressing MKP-1 and treated with dexamethasone before paclitaxel treatment. Moreover, in the absence of MKP-1 ectopic expression, dexamethasone/paclitaxel-treated cultures have fewer apoptotic cells than cultures treated with paclitaxel only. Interestingly, MKP-1 and dexamethasone offer a similar level of protection to MDA-MB-231 cells against paclitaxel-induced apoptosis (Wu et al., 2004b). When MKP-1 levels are reduced using siRNA, paclitaxel induces the same level of apoptosis in MDA-MB-231 cells, whether or not dexamethasone is present. When endogenous levels of MKP-1 are present, dexamethasone pretreatment reduces the number of apoptotic cells compared with the paclitaxel treatment only paradigm. Thus, it would seem that dexamethasone affords antiapoptotic protection to MDA-MB-231 cells, in part via MKP-1 (Wu et al., 2004b). The same group found that paclitaxel-induced phosphorylation of MAPK (ERK1/2 and JNK) in MDA-MB-231 cells is decreased by pretreatment with dexamethasone; under such conditions (dexamethasone/paclitaxel treatment) MKP-1 protein expression increases. If MKP-1 siRNA is used, both ERK1/2 and JNK phosphorylation levels increase, whereas phospho-p38MAPK levels remain unaffected (Wu et al., 2005b). Although MKP-1 is implicated in the mechanism by which dexamethasone reduces apoptosis in breast cancer cell lines, the exact mechanism awaits further elucidation. As glucocorticoids have pleiotropic effects (Distelhorst, 2002; Hayashi et al., 2004b; Stellato, 2004; Buckingham, 2006), MKP-1 may be one facet by which dexamethasone rescues cells from paclitaxel-induced apoptosis. With the use of dexamethasone as an antiemetic agent or to reduce acute toxicity, its side effect of being an antiapoptotic agent for breast cancer cells in vitro needs to be confirmed in vivo.

d. Fas/Fas ligand. Tumors are known to escape the innate and acquired immune detection systems and avoid death by using an array of responses (Abrams, 2005). Among the escape mechanisms available to tumors, avoidance of Fas/FasL-mediated apoptosis by different methods has been implicated in tumorigenicity. Tumors resist Fas-mediated signaling, by means of 1) down-regulation of Fas expression, 2) synthesis of secreted forms of Fas, 3) synthesis of Fas that lacks signal transduction capabilities, and 4) mutations in downstream effectors of Fas (Abrams, 2005). In addition, mutations in Fas-mediated signaling (Shin et al., 2002) and the lack of Fas-FasL interaction (Owen-Schaub et al., 1998; Koshkina et al., 2007) have been implicated in the ability of tumors to metastasize. Another way tumors could control the immune system is by way of FasL expression on tumor cells, which may induce apoptosis in Fas-expressing immune cells, although this latter model is controversial (Abrams, 2005).

Given the possible involvement of Fas/FasL in cancer, it is not surprising that this system has become a target of study. Mouse embryonic fibroblasts BALB/3T3 stably expressing Fas, designated FH2 cells, showed improved viability when transfected with constitutively activate forms of either K-Ras [constitutively active K-Ras (K-RasV12)], Raf (Raf-CAAX), or MAPKK (SDSE-MEK) when stimulated with anti-Fas antibody, compared with control FH2 cells stimulated anti-Fas antibody only.

When mkp-1 cDNA is cotransfected, the protective effects of the constitutively active forms of K-Ras, Raf, or MEK against anti-Fas antibody are abolished (Kazama and Yonehara, 2000). Thus, sustained MAPK (ERK) activity could inhibit Fas-mediated apoptosis.

DU145 human prostate cancer cells cotransfected with androgen receptor and androgen receptor coactivator, down-regulated their 5α-dihydrotestosterone-induced chloramphenicol acetyltransferase activity (chloramphenicol acetyltransferase activity assay) upon MKP-1 ectopic expression, in the presence or absence of Her-2/Neu receptor (erbB2 receptor; v-erb-b2 erythroblastic leukemia viral oncogene homologue 2 receptor/neuro/glioblastoma derived oncogene homologue receptor) (Yeh et al., 1999). Furthermore, a conditionally expressing MKP-1 construct protected DU145 cells from FasL-induced caspase-1 and caspase-3 activation and mitochondrial membrane depolarization associated with apoptosis (Srikanth et al., 1999). In addition, JNK activation via transfection of ASK1 or a constitutively active MEKK1 into DU145 cells is also down-regulated by overexpression of MKP-1 in these cells. Thus, in the human prostate cancer cell line DU145, MKP-1 overexpression protects against many agents inducing apoptosis (Srikanth et al., 1999).

e. Mitogen-activated protein kinase phosphatase-1 and the proteasome.

i. An overview. The proteasome complex is a multisubunit 26S complex composed of a 20S protease and 19S regulatory complexes. The latter can be further subdivided into base and lid multisubunit complexes (Nandi et al., 2006). Although the proteasome is known for its protein degradation activity via the ubiquitin-proteasomal pathway (Lorentzen and Conti, 2006; Nandi et al., 2006), it is increasingly being recognized as a means of controlling transcription (Muratani and Tansey, 2003). As cancer, among other diseases, is associated with both mutations in the ubiquitin-proteasomal pathway per se and mutations in proteins degraded by this pathway, it is also being targeted for pharmacological intervention (Adams, 2004; Nalepa et al., 2006). At present, which of the proteasomes is responsible for the degradation of MKP-1, the cytosolic (Lorentzen and Conti, 2006; Nandi et al., 2006) or the nuclear proteasome (von Mikecz, 2006), remains undetermined. Conversely, as transcription factors are targeted to the nuclear proteasome (von Mikecz, 2006), MKP-1 may also be targeted by this complex.

Many proteins are degraded by the proteasomal pathway, a minute fraction of which are the kinases and phosphatases. Thus, the effect of proteasomal inhibitors is most likely the result of a complex series of interactions in space and time and not the result of increasing expression of a single protein, such as MKP-1.

ii. Bortezomib. Among the pharmacological arsenal that exists to fight cancer, bortezomib is a synthetic reversible inhibitor of the proteasome-related chymotrypsin-like activity (APEX Trial, 2003; Jagannath et al., 2007; Richardson et al., 2006). Some bortezomib-related functions that have been characterized and retain our attention include 1) induction of apoptosis in tumors (Voorhees et al., 2003), 2) increasing JNK activity in various models (Meriin et al., 1998; Hideshima et al., 2003; Yang et al., 2004), and 3) up-regulation of *mkp-1* transcription levels (Orlowski et al., 2002). In line with some previous observations, transient adenovirus-mediated expression of MKP-1 in myc-transformed human mammary epithelial cells (A1N4-myc) or stable MKP-1 overexpression in BT-474 breast carcinoma cells protected these cell lines from bortezomib-induced apoptosis (Small et al., 2004). In the complementary experiment, disruption of MKP-1 in *MKP-1*-null MEFs or use of siRNA to silence mkp-1 mRNA, in conjunction with proteasome inhibitors, increased the susceptibility of mammary epithelial and breast carcinoma cells to apoptosis (Small et al., 2004). Anthracyclines were used as MKP-1 inhibitors (Small et al., 2003) in conjunction with proteasome inhibitors to augment apoptosis in vitro and in xenograft model (see section V.D.4) (Small et al., 2004).

iii. Bowman-Birk inhibitor. The Bowman-Birk inhibitor (BBI), an 8-kDa soybean-derived serine protease inhibitor, has been used in a phase IIa clinical trial (Armstrong et al., 2000; Meyskens, 2001). This serine protease inhibitor is known to inhibit trypsin from one of the domains in its double-headed structure and chymotrypsin from the other inhibitory domain (Birk, 1985). To better characterize the anticancer activity of the BBI, Chen et al. (2005) treated MCF-7 cells with this inhibitor and found that the antichymotrypsin activity of the BBI blocked the chymotrypsin-like activity of the proteasome. Among the proteins that accumulated in MCF-7 cells as a result of proteasomal inhibition, MKP-1 levels correlated with ERK1/2 inactivation and cell growth inhibition (Chen et al., 2005).

iv. MG132. With use of a hyperosmotic dehydration paradigm on rat hepatoma cell line H4IIE-C3 in conjunction with the proteasome inhibitor MG132, MKP-1 protein stabilization and increased mRNA levels were observed (Lornejad-Schafer et al., 2005). However, it is noteworthy that injection of MG-132 into the substantia nigra compacta of C57 black mice causes apoptosis to dopaminergic neurons (Sun et al., 2006). Such toxicity warrants further testing, because cancer treatment is not usually a one-time event.

f. (Z)-1-[N-(2-Aminoethyl)-N-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate. Use of DETA-NONOate, a nitric oxide (NO) donor with a 20-h half-life at 37°C, on the breast cancer cell lines MDA-MB-468, ZR 75–30, and MDA-MB-231 led to NO-mediated up-regulation of MKP-1, dephosphorylation of ERK1/2, and subsequent dephosphorylation of Akt (PKB) in the MDA-MB-468 and ZR 75–30 cell lines but not the MDA-MB-231 cell line. This up-regulation of MKP-1 and dephosphoryla-

tion of ERK1/2 and AKT led to apoptosis in the two former cell lines but not the latter (Pervin et al., 2003). The source of the difference in response to DETA-NONOate between these breast cancer cell lines is currently unknown. The MDA-MB-231 cell line can up-regulate MKP-1 expression (Orlowski et al., 2002). As levels of JNK1 were not assayed, it remains possible that one of the differences relating to apoptotic activities lies in levels of phosphorylated JNK1. JNK1 can phosphorylate AKT, a priming phosphorylation, leading to enhanced phosphorylation and activation of AKT by PDK1 (Bogoyevitch and Kobe, 2006) and inhibition of upstream kinases if JNK (section V.C.3). Alternatively, the NO effectors (Bonavida et al., 2006; Hirst and Robson, 2007; Mocellin et al., 2007) may be responsive in MDA-MB-468 and ZR 75–30 cell lines but unresponsive in MDA-MB-231 cells. Although this treatment is not effective for all cell lines, it represents a nonhormonal method of modulating MKP-1 expression.

g. Radiation. In vitro experiments demonstrate that γ-radiation up-regulates mkp-1 mRNA levels (Kasid et al., 1997). More recently, both normal human skin fibroblasts and A431 cells up-regulated ataxia telangiectasia-mutated kinase activity in response to radiation, which led to dephosphorylation of ERK1/2 in an MKP-1-dependant manner. The results of ERK1/2 dephosphorylation by MKP-1 were reprised in a xenograft model using A431 cells and irradiated nude mice (Nyati et al., 2006).

Short-wavelength ultraviolet light (UVC) up-regulates ERK2 and JNK1 activities followed by an increase in MKP-1 protein levels, leading to down-regulation of JNK1 activity in HeLa cells (Liu et al., 1995). In the C3H 10T1/2 murine cell line, p38MAPK is preferentially linked to *mkp-1* gene induction (Li et al., 2001), whereas ectopic expression of MKP-1 in U937 cells protected them from UV-induced apoptotic cell death, inhibited JNK1 activity, and induced caspase-3 activation and DNA degradation (Franklin et al., 1998). Finally, low doses of UVC decrease mkp-1 mRNA in transcription-coupled repair-deficient human fibroblasts (Hamdi et al., 2005). Collectively, these findings demonstrate that MKP-1 can protect cells from chemical- as well as radiation-induced JNK activation. This could be a significant finding as ~50% of patients that receive chemotherapy are also given radiotherapy (Bentzen, 2006).

2. Antiapoptosis. MKP-1 has also been implicated in antiapoptotic effects, by the suppression of caspase-3-mediated apoptosis in MEFs in the presence of anisomycin (Wu and Bennett, 2005). Moreover, cisplatin activated poly(ADP-ribose) polymerase (PARP) and caspase-3 by inducing their cleavage, resulting in an increase in caspase-3 activity in *mkp-1*-null MEFs, whereas wild-type MEFs are less robust in their responses when treated similarly (Wang et al., 2006). Interestingly, the proteasome inhibitor MG132 increased MKP-1 in H4IIE rat hepatoma cells in a hyperosmotic environment and also activated caspase-3 (Lornejad-Schafer et al., 2005). MKP-1 expression in MDA-MB-231 cells via transfection or adenoviral transduction reduced paclitaxel (a microtubule stabilizing agent, which inhibits cell proliferation via reduced mitotic activity) (Jordan and Wilson, 2004)- or mechlorethamine (alkylating agent)-mediated increase in caspase-3/7 activity (Small et al., 2007). Caspase-3/7 can activate MAPKs, such as ERK1/2, p38MAPK, and JNK, via Mst1, an upstream kinase for MAPKKK, in an extrinsic apoptotic pathway-dependent mechanism (Song and Lee, 2008). More specifically, caspase-7 cleaves Mst1, producing a 40-kDa fragment, which activates JNK and p38MAPK, whereas caspase-3 produces a 36-kDa fragment of Mst1, which activates ERK (Song and Lee, 2008). The functional significance of the MAPK activation in this scenario awaits further investigation.

Caspases are characteristically associated with apoptosis and interference with this function is categorized as antiapoptotic (Riedl and Salvesen, 2007). However, caspases also have nonapoptotic functions, including cell maturation, induction of differentiation, and cytokine maturation, among other roles (Lamkanfi et al., 2007). In addition, although PARP activation is a hallmark of apoptosis, as it can curtail the DNA-repair mechanisms, PARP can also be implicated in the inflammatory response by modulation of NF-κB transcriptional activity (Lamkanfi et al., 2007). In turn, NF-κB can inhibit JNK-mediated apoptosis (Papa et al., 2006). However, MEKK1 activates IκBα kinase, which phosphorylates IκBα, leading to its dissociation from NF-κB and proteasomal degradation. Alternatively, JNK phosphorylates IκBα, also leading to polyubiquitination and proteasomal degradation (Ki et al., 2007). In either case, the dissociation of IκBα from NF-κB leads to the activation of NF-κB (Lee et al., 1997). Thus, MEKK1 and JNK can activate a transcription factor, NF-κB, via phosphorylation of IκBα, to potentially synergize with or antagonize JNK activity.

Adenovirus-mediated expression of MKP-1 in BT-474 or A1N4-myc cells reduced their DNA fragmentation after doxorubicin, paclitaxel, or mechlorethamine treatment. MKP-1 expression is also able to reduce DNA fragmentation with combined doxorubicin/mechlorethamine treatment in BT-474 cells. These DNA fragmentation results mirror those derived from treating wild-type MEFs and *mkp-1*-null MEFs with doxorubicin, paclitaxel, or mechlorethamine (Small et al., 2007). The presence of MKP-1 protects human mammary epithelial cells, breast carcinoma cells, and mouse embryonic fibroblasts from various agents inducing DNA fragmentation by a JNK/c-Jun-mediated mechanism (Small et al., 2007).

Considering the potential involvement of MKP-1 in protecting tumor cell lines, it may be necessary to use an antisense or siRNA strategy to down-regulate MKP-1

before use of these chemotherapeutic agents in certain cases. Another possibility is to screen drugs for their ability to induce apoptosis in tumors without inducing the expression of MKP-1.

The preceding sections on apoptosis and antiapoptosis underscore the aforementioned observation that the expression of MKP-1 protein alone cannot predict the biological outcome for a given cell type. The identity of the MAPK that is active at the time of MKP-1 enzymatic function is more predictive although not absolutely so. There are other kinases and phosphatases that may be simultaneously active at the time of MKP-1 expression and activity that will have an impact on the ultimate fate of the cell (Tables 1 and 2; Fig. 2).

3. Differentiation. Activation of the Src kinase family member $p59^{Fyn}$ via mitogen-tyrosine kinase receptor signaling increases both ERK activity and phosphorylation of the melanocyte-specific transcription factor Microphthalmia (Wellbrock et al., 2002). This results in the degradation of the melanocyte differentiation-inducing transcription factor via the proteosomal pathway as well as a decrease in mkp-1 mRNA expression. Conversely, overexpression of MKP-1 decreases Microphthalmia degradation via ERK dephosphorylation and facilitates melanocyte differentiation (Wellbrock et al., 2002). The finding that MKP-1 overexpression can, under certain circumstances, induce cells to differentiate could be interesting if proven true for tumors, as one of the therapeutic axes for the control of tumors relies on inducing them to differentiate to the cell lineage from which they originate, whether the tumors are of hematological origin (Tsiftsoglou et al., 2003) or solid tumors (Honma and Akimoto, 2007).

4. Proliferation. PKCε is implicated in the up-regulation of MKP-1 in bone macrophages when stimulated with macrophage colony-stimulating factor (Valledor et al., 1999) or lipopolysaccharide (Valledor et al., 2000). Although MKP-1 expression did not rely on ERK activation in these studies, inactivation of ERK1/2 coincides with MKP-1 protein expression. When macrophage colony-stimulating factor was used, MKP-1 up-regulation reduced macrophage proliferation without inducing apoptosis. Thus, ERK is neither part of an autoregulatory loop nor does inhibition of proliferation herald apoptosis. MKP-1 negatively regulates cell cycle transition (G_0/G_1) in the absence of DNA damage and in response to growth factors (Li et al., 2003). In addition, constitutive expression of MKP-1 blocks G_1-specific gene expression (Brondello et al., 1995). In line with this result, cyclin D1 expression was decreased in a U28 clonal cell line induced to express MKP-1 (Manzano et al., 2002). These results go hand in hand with the previous findings relating to how *mkp-1* was initially identified, as one of a set of genes that are expressed in cultured murine cells during the G_0/G_1 transition (Lau and Nathans, 1985). Considering the level of involvement of ERK1/2 in cell cycle progression (see section II on the MAPK family), MKP-1 overexpression leading to cell cycle inhibition is in line with its function as an ERK1/2 phosphatase. Expression of the inactive form of MKP-1 (C258A) acts as a dominant-negative, decreasing cell viability in response to both cisplatin and transplatin in 293T and Pam212 cells while not interfering with the ability of JNK1 to phosphorylate c-Jun (Sánchez-Pérez et al., 2000). Lack of MKP-1-phosphatase activity (C258A) may help preserve the kinase activity of JNK1 toward c-Jun, protecting JNK from dephosphorylation by other phosphatases. This protection could also be extended to ERK1/2-mediated activity. As c-Jun can be part of the AP-1 transcription factor (Ozanne et al., 2007) and ERK1/2 mediate inhibition of antiproliferative gene transcription throughout the G_1 phase by an AP-1 dependent mechanism (Yamamoto et al., 2006), among other ERK1/2-related cell cycle mechanisms (see section II on the MAPK family), dominant-negative MKP-1 may cause cells to proceed through the cell cycle via an AP-1-mediated mechanism (Verde et al., 2007). Platinum-based compounds can cross-link DNA, resulting in increased apoptosis (Pascoe and Roberts, 1974a,b; Zwelling et al., 1979; Giraud-Panis and Leng, 2000). In addition, the consequences of JNK1 activity, antiapoptotic versus proapoptotic, relies on its duration (Ventura et al., 2006), which could lead to phosphorylation of proapoptotic proteins and induction of apoptosis (Bogoyevitch and Kobe, 2006; Ventura et al., 2006). Furthermore, *mkp-1*-null MEFs have a higher incidence of apoptosis when deprived of serum for 48 h compared with WT culture and lower proliferation rates in the presence of serum compared with WT cultures (Wu and Bennett, 2005). These results suggest that, in the absence of serum, MKP-1 expression targets JNK1, whereas a lower proliferation rate for mkp-1-null MEFs, in the presence of serum, could be the consequence of overstimulation of the Ras-Raf-MEK-ERK pathway, as this has a tendency to lead to reversible or permanent cell cycle arrest (Meloche and Pouyssegur, 2007).

The previously mentioned in vitro results are reflected by animal models, as studies in nude mice revealed that intraperitoneal but not subcutaneous growth of cell lines U28 and M18, clones of UCI101 and A2780, respectively, conditionally expressing MKP-1, have reduced tumor size in comparison with the same cell lines not induced to express MKP-1 (Manzano et al., 2002). Other results using nude mice revealed delayed and attenuated tumorigenicity when MKP-1 antisense-transfected PANC-1 or T3M4 cells are injected, compared with mock or untransfected cells (Liao et al., 2003). In addition, *nu/nu* mice that developed BT-474 cell-based tumors increased their MKP-1 protein levels 24 h after bortezomib treatment and decreased MKP-1 protein expression when they were injected with doxorubicin (a repair inhibitor and redox-mediating DNA damaging agent) (Minotti et al., 2004) compared with vehicle controls as assessed in tumor tissues.

Moreover, the combination of bortezomib and doxorubicin had intermediate levels of MKP-1 protein coupled to the highest apoptosis and phospho-JNK levels compared with either treatment alone. In addition to higher phospho-JNK levels, higher ERK1/2 levels were also found in this bortezomib/doxorubicin-treated xenograft model. These findings led the authors to conclude that such a combination of treatments could be an effective antitumor therapy, if the potentially self-limiting antiapoptotic effect of ERK could be avoided or inhibited (Small et al., 2004). The experiments with nude mice highlight the behavioral difference between tumor cell lines derived from various tissues. They also reveal that a tumor behaves differently, depending on the tissue that hosts its growth. The degree to which the environment exerts an influence on tumor biology, triggering adaptation, has important consequences for metastases as will be revealed in section V.D.6.

As a nuclear dual-specificity phosphatase, MKP-1 is well poised to dephosphorylate ERK and prevent it from initiating transcriptional activation, among other functions. As indicated by the plethora of factors in previous sections, there is no shortage of reagents that up-regulate MKP-1 protein or mRNA. However, up-regulation of mkp-1 mRNA or stabilization of MKP-1 protein, without interfering with the proapoptotic activity of JNK1 while reducing JNK-mediated antiapoptotic activity would not be straightforward. Another possibility relies on the difference in the MAPK binding site within MKP-1 itself. A modified MKP-1 molecule with a mutated JNK1 binding site to prevent inactivation of this MAPK, while allowing the ERK and p38MAPK (arginine residues 53–55) (Fig. 4) to bind to MKP-1 may be an alternative. An intervention that is more amenable than gene therapy for use in patients relies on the design of a small molecule that inhibits the JNK1-binding site within MKP-1 without affecting the ERK-p38MAPK-binding site. Such a strategy would require a more precise definition of the JNK1-binding site within MKP-1 and the three-dimensional structure of this phosphatase. All of these scenarios rely on identifying not only the MAPK isoform and splice form responsible for the tumorigenic phenotype but also the exact function it is playing at a given time point as the effectors and therefore the function of the kinase can vary across space and time.

5. Anchorage-Independent Growth. Soft agar is used for assaying anchorage-independent growth (Anderson et al., 2007) and also serves as a cloning assay (Agre and Williams, 1983). In an assay for anchorage-independent growth, MKP-1 antisense reduced the ability of PANC1 and T3M4 cells to form colonies in soft agar (Liao et al., 2003). A2780 and UCI101 cells, originating from papillary serous adenocarcinomas and stably transfected with MKP-1 cDNA, gave similar results (Manzano et al., 2002). In addition, the lung tumor cell line HCC2429, showed increased mkp-1 mRNA levels when transfected with a Notch3 dominant-negative receptor upon serum stimulation, with concomitant reduction in ERK1/2 phosphorylation, a reduction of growth in soft agar, and an increase in both apoptosis and dependence on exogenous growth factors (Haruki et al., 2005).

Upon cell-matrix or cell-cell detachment, a type of apoptosis called anoikis is usually triggered, preventing normally adherent cells from adherence-independent survival (Reddig and Juliano, 2005; Eccles and Welch, 2007). The Raf-MEK-ERK pathway, activated by mitogens, can inhibit anoikis in certain transformed cells but not in primary cells or "normal" cell lines (Reddig and Juliano, 2005). In addition, ERK1/2, JNK, and p38MAPK are involved in cell motility, focal adhesion, and cell proliferation, among other functions. However, given the level of cross-talk (Fig. 2) and MKP-1 phosphorylates these MAPKs and ERK5, distinguishing between a direct effect of MKP-1 cell growth in soft agar, for instance, versus an indirect effect becomes problematic. A high-throughput soft agar assay (Anderson et al., 2007), will accelerate the resolution of this dilemma concerning information on the identity of the MAPKs involved and the timing of their involvement in anchorage-independent growth and other mechanisms.

6. Metastasis. Eighty percent of metastases derived from tumors of epithelial origin (bladder, breast, colon, and prostate) revealed little if any mkp-1 mRNA expression by in situ hybridization (Loda et al., 1996). Interestingly, the metastases that were positive for mkp-1 mRNA also overexpressed either *neu* or *egfr* mRNA (Loda et al., 1996). In accordance with the previous study, metastasis to the lymph nodes derived from prostate adenocarcinomas revealed no mkp-1 mRNA expression (Magi-Galluzzi et al., 1997). In addition, increased expression of ERK mRNA and protein was found in lymph node metastases derived from human breast cancer, compared with surrounding normal lymph node tissue (Sivaraman et al., 1997). Lymph node metastases derived from human prostate cancer revealed c-Jun and c-Fos protein expression and phosphorylated ERK (Ouyang et al., 2008). These results reflect the increasing recognition of the involvement of AP-1 transcription factor in positively modulating tumor invasion by up-regulating proinvasive genes and down-regulating anti-invasive genes (Ozanne et al., 2007).

These findings can be appreciated in light of reports on the following proteins being potential metastasis suppressors: MKK4, MKK6, and MKK7, which activate p38MAPK and JNK, p38MAPK, or JNK, respectively; and Raf kinase inhibitor protein, which is an inhibitor of Raf-mediated ERK phosphorylation. Interestingly, tumors that express metastasis suppressor genes can complete all aspects of metastasis with the exception of proliferation at the secondary site (Eccles and Welch, 2007). Thus, MKP-1 expression would be required if p38MAPK or JNK activity would be deleterious to tumor survival or proliferation at the site of metastasis.

These observations on metastasis are interesting but need to be confirmed for a greater number of cases and

across tumor types. If a lack of MKP-1 expression demonstrates a direct correlation with metastasis and not a bystander effect, then reexpression of MKP-1 under appropriate conditions may inhibit or delay proliferation at the site of metastasis. However, as ERK, p38MAPK, and JNK can participate in cell motility (see section V.C on MAPK and cancer) and motile cells are nonproliferative (Eccles and Welch, 2007), overexpression of MKP-1 may serve to curtail the motility aspect of metastasis to the secondary site. It is noteworthy that MKP-1 conditionally expressing cell line U28 decreases formation of lamellipodia and filopodia and cell motility when treated with doxycycline treatment (Manzano et al., 2002). This in vitro result should be confirmed in vivo, using cell lines conditionally expressing MKP-1 as a model of metastasis in animals, to distinguish the level of involvement of MKP-1 in metastasis versus a bystander effect. With timing of MKP-1 expression under control, differentiating variables, such as establishment of a premetastatic niche (Kaplan et al., 2005), proliferation, cell motility, inhibition of anoikis, invasion, and proliferation at the secondary site (Steeg, 2003), would be more feasible. As the microenvironment can affect tumor metastatic potential and the tumor can, in turn, affect its environment (Eccles and Welch, 2007), these studies may have to be repeated for different sites of primary tumor growth and secondary sites of metastases to account for these variables. However, this analysis is not enough. We should not forget that a metastasis is a clonal event and that each colonizing cell must survive from the primary tumor from which it detaches until it reaches the secondary site and proliferates (Eccles and Welch, 2007). Therefore, each metastasis represents a certain signature of genomic and proteomic expression profiles deriving from the primary tumor that can be modified to accommodate the site of metastasis. The use of multiple cell lines should be considered when animal models of metastasis are used to account for interclonal variability. Finally, as the current aim is to identify groups of genes involved in the process of metastasis, in addition to other aspects of tumor biology including survival and proliferation at the secondary site (Eccles and Welch, 2007), we should not be surprised that a given gene or its protein product, be it *mkp-1* or another, is not always involved in the survival, dissemination, or other aspects of metastasis or cancer biology.

7. Hypoxia. Under normoxic conditions (~21% O_2), HIF-1α is constitutively synthesized and degraded, whereas HIF-1β is constitutively synthesized and found in the nucleus and is not subject to regulation by the level of oxygen. Degradation of HIF-1α during normoxia involves a multistep process that includes prolyl hydroxylases, factor inhibiting hypoxia-inducible factor, and polyubiquitination by the von Hippel Lindau protein complex targeted for proteasomal degradation. Prolyl hydroxylation targets HIF-1α for polyubiquitination and proteasomal degradation. Factor inhibiting hypoxia-inducible factor asparagine hydroxylates HIF-1α, preventing its interaction with p300/CBP. One of the long-term consequences of HIF-1 function is angiogenesis, among others, and cancers that prevent the timely degradation of HIF-1α are highly vascularized (Kaelin, 2005).

As mentioned earlier, both hypoxia and ischemia have been associated with mkp-1 mRNA (Takano et al., 1995; Wiessner et al., 1995; Seta et al., 2001; Bernaudin et al., 2002; Liu et al., 2003, 2005a) and protein (Seta et al., 2001; Mishra and Delivoria-Papadopoulos, 2004; Liu et al., 2005a) regulation in various systems. In turn, MKP-1 can limit HIF-1 activity (Liu et al., 2003) via dephosphorylation of ERK (Liu et al., 2005a). More specifically, ERK phosphorylates p300 (Sang et al., 2003), a cofactor for HIF-1 activity (Arany et al., 1996) and HIF-1α (Richard et al., 1999), which increases HIF-1 transcriptional activity. In addition, MKP-1 expression was found to reduce the interaction of p300 with HIF-1 in HepG2 cells (Liu et al., 2005b). Conversely, ERK5 can phosphorylate HIF-1α and target it for polyubiquitination and proteasomal degradation, during hypoxia, in a prolyl hydroxylase-independent manner (Pi et al., 2005). In turn, ERK5 can be dephosphorylated and inactivated by MKP-1 and MKP-3 in an ERK1/2-mediated mechanism (Sarközi et al., 2007). Adding to these results is the observation that hypoxia activates p38α and increases VEGF synthesis, a prelude to angiogenesis (Rousseau et al., 2000). Overall, this is an elegant example of how phosphatases and kinases interact to regulate each other and a key regulator of hypoxia, namely HIF-1α. This interaction assures an appropriate level of HIF-1 activity and the consequence of its activation.

Phosphorylation is not the only post-translational modification that modulates HIF-1α function. It is noteworthy that one of the possible post-translational modifications of HIF-1α (Brahimi-Horn et al., 2005) is cysteine nitrosation of HIF-1α (Yasinska and Sumbayev, 2003), via inducible nitric-oxide synthase or a NO donor. Nitrosation of HIF-1α activates its interaction with p300, whereas HIF-1α acetylation by ARD1 leads to its destabilization (Jeong et al., 2002). However, nitric oxide has a complex relationship with the components of this pathway, as it can stabilize HIF-1α under normoxia or inhibit its induction under hypoxia while inhibiting prolyl hydroxylase function, among other functions (Kaelin, 2005). As with many components of signal transduction, duration and timing may dictate the outcome of the NO interaction with HIF-1α. These seemingly unrelated observations come into their own when we consider that both p300 and CBP possess acetyltransferase activity (Iyer et al., 2004). Both H3 and H4 histone tails are required for p300-mediated acetylation of histones H2A and H2B, within chromatin and consequent transcriptional activation (An et al., 2002). The stress-related transcriptional activation of the *mkp-1* gene has been related to phosphorylation-acetylation of

histone H3 and chromatin remodeling (Li et al., 2001). Although these results are suggestive, direct evidence of p300 involvement in *mkp-1* gene transcription via chromatin remodeling is still wanting. It should be noted that in a previous study, histone H3 was acetylated on lysine 14 (Li et al., 2001) and that p300/CBP-associated factor is known to acetylate histone H3 (Herrera et al., 2000) on this residue (Lau et al., 2000). Although histone acetylation is a recognized modification at sites of actively transcribed genes (Saunders et al., 2006), histone H3 methylation, one of several histone post-translational modifications (Margueron et al., 2005), is associated with transcriptional elongation (Vakoc et al., 2005), a mechanism that not only controls mkp-1 mRNA (see section III.B.4 on mRNA elongation) but also the elongation of other messengers (Saunders et al., 2006). How ERK1/2, ERK5, and MKP-1, among other phosphatases and kinases, interact with HIF-1α to regulate gene transcription via histone modification and other mechanisms and conversely limit the effect of HIF-1α is a picture that needs further development.

8. Angiogenesis and Vasculogenesis. Adenoviral infection of HUVECs with the constitutively activated form of MEK6, MEK6E, the p38MAPK kinase, increases MKP-1 protein expression via p38MAPK, as pretreatment of these cells with SB203580 abolishes this increase. MEK6E also induces lamellipodia formation and cell migration in HUVECs. Furthermore, dominant-negative p38αMAPK inhibits the effects of MEK6E on lamellipodia formation, cell migration, and cell proliferation. The conclusion was that MKP-1 served, in part, to shift the balance of phosphorylated ERK and p38, which may have led to altered endothelial cell function (McMullen et al., 2005). Interestingly, among the p38MAPK KO mice, p38α is associated with chemotaxis (Rousseau et al., 2006) and with VEGF-mediated endothelial cell migration (Cuenda and Rousseau, 2007). Phosphorylated JNK and low MKP-1 protein expression levels also seem to favor angiogenesis in carcinomas (Shimada et al., 2007).

As MKP-1 inactivates p38α and JNK, its involvement in angiogenesis has been attributed to negative modulation of the MAPK pathway. In contrast, treating sections of descending aorta from WT mice and *mkp-1*-null mice with VEGF-A_{165} or thrombin revealed that endothelial cell sprouting was greatly reduced in *mkp-1*-null aortic sections, compared with similarly treated sections from WT control mice (Kinney et al., 2008). How do we reconcile these contradictory findings? The MAPKs were not equally affected by the absence of MKP-1, and residual activity (3- to 5-fold) was seen 3 h after VEGF stimulation, with JNK having the highest phosphorylation. At present the identity of the MAPK responsible for the failure of endothelial cell sprouting remains unknown. As each member of the MAPK family is involved in a multiplicity of functions, lack of endothelial cell sprouting could be caused by increased apoptosis due to JNK activation for extended periods of time, disruption in actin cytoskeleton remodeling affecting cell motility and chemotaxis, and disruption in p38MAPK-mediated mRNA stability possibly decreasing protein expression, among other normal functions attributed to the MAPK family (see section II on the MAPK family).

It is noteworthy that the *mkp-1*-null mice and their *mkp-1*(+/+) littermates had no overt phenotypic difference in the developmental phase or in various organ systems, such as the neurological, cardiac, hematological, and endocrinological systems (Salojin et al., 2006). At present the difference between the previous results (Kinney et al., 2008) and the in vivo findings in *mkp-1*-null mice (Salojin et al., 2006) are unclear. Although gross anatomy was unaffected, it remains possible that the capillary beds of organs in null mice may have a different cytoarchitecture than that found in WT murine organs. This issue needs to be addressed before any firm conclusion as to the difference between both studies can be drawn. Finally, from a practical standpoint, the use of MKP-1 to inhibit angiogenesis would need to rely on preventing the tumor from initiating the signaling cascade associated with angiogenesis rather than trying to affect endothelial cell biology using an MKP-1-based strategy.

Another player in angiogenesis and vasculogenesis was identified as ERK5. The importance of ERK5 in maintaining vascular integrity was revealed when a conditionally expressed ERK5 was "turned off" in adult animals with different efficiencies in various organs. These animals hemorrhaged from organs as a result of "leaky" capillary vasculature, resulting from large fenestrations and erythrocyte extravasation (Hayashi et al., 2004a). More specifically, the endothelial cells linking the various organs changed morphology and became round and apoptotic. The reduction/absence of ERK5 revealed a dependence of endothelial cells on ERK5 for survival and prevention of apoptosis (Hayashi et al., 2004a). Global (nonconditional) ERK5- or MEK5-null mice revealed cardiovascular developmental problems (Yan et al., 2003; Wang et al., 2005) that were reproducible in endothelial cell-specific ERK5-null mice, which also demonstrated vasculogenic and angiogenic problems (Hayashi et al., 2004a). The endothelial cell-specific ERK5 knockout mice revealed the same deficiency in yolk sac vasculature development as ERK5 global knockout mice (Hayashi et al., 2004a). Conversely, cardiomyocyte-specific ERK5 knockout mice had normal heart development up to 1 year. Thus, the phenotype associated with ERK5 global knockout mice and in endothelial cell-specific ERK5 knockout mice, was due to abnormal development of the endothelial cells lining the interior of the heart and not to the cardiomyocytes per se (Hayashi et al., 2004a). Finally, ventricular hypertrophy was accompanied by increased ERK5 activation (Kacimi and Gerdes, 2003), whereas overexpression of MEK5 also leads to cardiac hypertrophy (Nicol et al., 2001).

Section V.D.7 on hypoxia revealed that ERK5 was part of a network of kinases and phosphatases that regulate HIF-1 activity. ERK5 phosphorylated HIF-1α, leading to its polyubiquitination and proteasomal degradation (Pi et al., 2005). Completing the role of ERK5 in angiogenesis and vasculogenesis are the findings of this section, which reveal that ERK5 is essential for endothelial cell survival even in adult animals (Hayashi et al., 2004a). In addition, phosphatases such as MKP-1 and MKP-3 can dephosphorylate and inactivate ERK5, by an ERK1/2-mediated mechanism in renal epithelial cells (Sarközi et al., 2007). Thus, in summary, MKP-1 has both proangiogenic, if endothelial cells express normal levels, and antiangiogenic propensities, if the tumor is expressing MKP-1. How all the players interact under normal circumstances and during tumorigenesis within a tissue to maintain, repair, and signal for neoangiogenesis still requires elucidation.

Overall it seems that, depending on the type of tissue the tumor derives from, the environment it is growing in, and the stage, grade, and more specifically the identity of the MAPK that is expressed (JNK1, ERK1/2, and ERK5), MKP-1 seems to have opposite functions depending on levels of expression and the cell type expressing it: 1) MKP-1 can help tumors to avoid apoptosis or can interfere with proliferation and tumor growth and inhibit angiogenesis; or 2) normal levels of MKP-1 are required for endothelial cell sprouting as demonstrated by the lack of MKP-1 being a detriment to endothelial cell sprouting in response to VEGF-A_{165} and thrombin. Adding to the complexity, chemotherapeutic agents can induce *mkp-1* expression. As MKP-1 targets MAPKs for dephosphorylation and given the level of cross-talk between the MAPK family member and their signaling modules (see section II), in addition to the presence of other phosphatases (Tables 1 and 2) (see also section IV), the outcome of a given therapy is anything but straightforward.

However, there is hope of untangling the MKP-1 knot. The finding that *mkp-1*-null mice are viable unless challenged by diet or LPS (see section V.B) allows the possibility of determining whether *mkp-1*(−/−) mice are more susceptible to chemically induced tumors than their *mkp-1*(+/+) littermates. Thereafter, as the tumor develops, it can be monitored in terms of proliferation, anchorage-independent growth, metastasis, apoptosis, and so on. The tumors from *mkp-1*-null mice can be compared with those of their WT littermates in terms of size, the development of capillary beds within the tumor, metastatic potential, and other variables. Such experiments can be fine tuned with conditionally expressed *mkp-1* cell lines injected into nude mice. Determining at what point in tumor biology is MKP-1 necessary and at which crossroads other phosphatases are able to bypass the need for MKP-1 is well within reach.

VI. Mitogen-Activated Protein Kinase Phosphatase, Cancer in Humans, and Relevant Models

This section is an overview of MKP-1 in relation to different tumors, but there are conflicting results regarding the importance of MKP-1 here as there have been in previous sections. These can derive from the activity or lack thereof of other phosphatases and kinases, whose presence was not determined. The absence of such information coupled with the complexities allowed by the interaction between the members of the MAPKs and their signaling modules, in addition to the phosphatases that inactivate them, easily accounts for the discrepant information.

A. Breast Cancer

Human breast cancers, ductal and lobular carcinomas, revealed constitutive levels of mkp-1 mRNA in the surrounding tissue, whereas lesions expressed higher levels and the more aggressive tumors had little to no mkp-1 mRNA expression (Loda et al., 1996). However, there is a strong correlation between the expression of the *neu* oncogene and ERK1/2 and MKP-1 protein expression (Loda et al., 1996). It is also possible that the *neu* oncogene (also called ERBB2 and HER2), by stimulating the Raf-MEK-ERK pathway (Grant et al., 2002), could protect ERK1/2 from inactivation by MKP-1 while this phosphatase inactivates JNK1 and p38MAPK, based on MKP-1 substrate specificity. In this scenario, MKP-1 expression could be deleterious. However, Ras overactivation, Raf, and ERK can induce cell cycle arrest (Hirakawa and Ruley, 1988; Smalley, 2003; Eymin et al., 2006; Dhillon et al., 2007). In this situation in which the *neu* oncogene correlates with MKP-1, phosphatase activity may reduce strong sustained ERK activity, favoring senescence or differentiation, which leads to sustained weak activity or transient ERK activity favoring proliferation (see sections II and V).

B. Gastrointestinal Cancers

1. Colon Cancer. Early lesions of human colon adenomata show robust mkp-1 mRNA expression, which is inversely proportional to tumor grade (Loda et al., 1996). This finding goes hand in hand with the concept that low-grade tumors avoid JNK1-induced death via MKP-1 up-regulation, whereas high-grade tumors down-regulate MKP-1 to allow maximal proliferation and possibly metastasize.

A recent study revealed that although both constitutively active forms of K-$Ras^{G12D/+}$ and N-$Ras^{G12V/+}$ were expressed in the murine colon epithelium, only the active form of K-$Ras^{G12D/+}$ produced hyperplasia but not neoplasia of the colon epithelium by increasing the number of progenitor cells (Haigis et al., 2008). K-$Ras^{G12D/+}$ but not N-$Ras^{G12V/+}$ signaled through the MEK-ERK pathway, whereas neither Ras isoform modulated

JNK activation. Conversely, both K-Ras$^{G12D/+}$ and N-Ras$^{G12V/+}$ decreased levels of phosphorylated AKT (Haigis et al., 2008). More importantly, K-Ras$^{G12D/+}$ activated MEK. However, although phosphorylated MEK was detected from the bottom to the top of the intestinal crypts, only the differentiated cells at the top of the crypts expressed high levels of phosphorylated ERK. This lack of ERK activation in other regions that revealed high phospho-MEK was most likely caused by up-regulation of MKP-3 (Haigis et al., 2008). As ERK can inhibit MEK and Raf (Fig. 2) (see also section II on MAPK cross-talk), increased MKP-3 can inactivate cytosolic ERK, leading to stronger MEK activation as detected (Haigis et al., 2008). MKP-3 is involved in mediating inhibition of cell proliferation and inhibition of cell differentiation, depending on the system being tested (Kim et al., 2004; Marchetti et al., 2004). Because there is hyperplasia in the colon of mice, in the study by Haigis et al. (2008), MKP-3 may have prevented cell differentiation induced by ERK. Other observations from the same study revealed that constitutively active N-Ras$^{G12V/+}$ protects murine colon epithelium from dextran sodium sulfate-induced apoptosis but not from radiation-induced apoptosis, whereas K-Ras$^{G12D/+}$ did not afford as much protection from apoptosis induced by these agents (Haigis et al., 2008). The same study also revealed that mice harboring K-Ras$^{G12D/+}$ or N-Ras$^{G12V/+}$ combined with a conditional allele of *Apc*(−/+) (the adenomatous polyposis coli gene) was sufficient to cause high-grade dysplasia and an increased number of tumors in K-Ras$^{G12D/+}$ *Apc*(−/+) but not in N-Ras$^{G12V/+}$ *Apc*(−/+) mice, compared with mice harboring wild-type K-Ras *Apc*(−/+) or N-Ras *Apc*(−/+). More importantly, MEK activation was shown to be unnecessary in K-Ras$^{G12D/+}$ *Apc*(−/+)-mediated tumorigenesis, even though Raf activity was essential for this transformation (Haigis et al., 2008).

APC is a multidomain protein involved in the development of intestinal and other cancers (Aoki and Taketo, 2007). Although the exact mechanism by which lack of APC or its C-terminal truncated form contributes to the formation of cancer is currently unknown, it is believed to involve mutations in the β-catenin-binding region. Furthermore, loss of APC leads to nuclear accumulation of β-catenin and the activation of transcription factors, part of the Wnt signaling pathway, which in the adult mice leads to cell proliferation and polyp formation (Aoki and Taketo, 2007). To put the previous observations in context, in unstimulated cells, β-catenin is found at adherence junctions, and excess cytosolic β-catenin is phosphorylated, within the context of a complex composed of APC, Axin, CK1α, GSK3β, and PP2A, targeted for ubiquitination and proteasomal degradation (Willert and Jones, 2006). In addition, APC and Axin are found in the nucleus and may regulate β-catenin in this compartment as well. Upon Wnt stimulation, APC, CK1α, andGSK3β are dissociated from their complex, leading to an accumulation of β-catenin. The β-catenin acts as a scaffolding protein for transcription factors and chromatin-remodeling complexes, among other proteins and complexes (Willert and Jones, 2006).

So how does all this tie in with the MAPKKK-MAPKK-MAPK pathway? ERK and Wnt3α/β-catenin can stimulate fibroblast proliferation, and siRNA to either ERK1/2 or β-catenin reduces fibroblast proliferation (Yun et al., 2005). Conversely, β-catenin can lead to down-regulation of activated ERK and decreased proliferation in an Axin-mediated lysosomal-dependent degradation of both WT H-Ras and H-Ras^{L61}. This effect is seen only in cells that have WT β-catenin, not in cells that have the β-catenin S33Y mutation, such as HepG2 and HCT-116 cell lines, because this is a nondegradable form of β-catenin (Jeon et al., 2007). Whereas GSK3β phosphorylates and targets β-catenin for proteasomal degradation (Liu et al., 2002), both AKT (Cross et al., 1995) and p38MAPK (Thornton et al., 2008) phosphorylate GSK3β at the N terminus and C terminus, respectively, inhibiting GSK3β function and allowing β-catenin to accumulate. Adding to these observations, Axin, as a scaffolding protein, can lead to JNK activation and apoptosis when Axin is overexpressed (Luo and Lin, 2004) and induces apoptosis by scaffolding p53, among other proteins (Lin and Li, 2007), whereas, conversely, *p53*-null mice develop spontaneous tumors (Donehower et al., 1992). A study using EB-1 human colon cancer cells revealed p53-dependent regulation of MKP-1, which was reflected by *p53*(+/+) MEFs up-regulating MKP-1 in response to stress, whereas *p53*(−/−) MEFs did not. In this context, MKP-1 overexpression induced apoptosis in colon cancer cells (Liu et al., 2008). These results are more interesting because nonphosphorylated JNK can target p53 for ubiquitination and proteasomal degradation (Fuchs et al., 1998a). Thus, MKP-1 inactivates JNK, inactive JNK targets p53 for degradation, and p53 up-regulates MKP-1 and increases apoptosis. This regulatory loop is itself modulated by other MAPKs, phosphatases, and scaffolding proteins.

Taken together, all these studies imply that the stoichiometry of scaffolding proteins, such as APC, β-catenin, and Axin contributes to the cell fate by determining subcellular localization and/or the activation status of proteins. In turn, kinases and phosphatases regulate each other via transcription factors to modulate the level of expression and activity. The consequence of kinase-phosphatase-transcription factor interaction/regulation, which may also depend on stoichiometry, contributes to the determination of cell phenotype.

2. Gastric Cancer. Human primary gastric tumors, adenocarcinomas, expressed increased MKP-1 protein levels compared with normal gastric tissues (Bang et al., 1998). An evaluation of ERK1/2 activity revealed greater levels in gastric tumors compared with patient-matched normal gastric tissue. The authors believed not only that the increase in MKP-1 expression was a consequence of

increased ERK1/2 activity but also that MKP-1 was contributing to carcinogenesis. Unfortunately, other MAPK family members (JNK1 and p38MAPK) were not evaluated. In light of the previously mentioned observations and experimental data, it is possible that the target of MKP-1 activity is JNK1 or p38MAPK and not ERK1/2. Another possibility is that MKP-1 reduces ERK1/2 activity without abolishing it, favoring proliferation over other outcomes (see section II.B on the ERKs).

3. Hepatocellular Carcinoma. Eighty percent of human hepatocellular carcinomas revealed little if any mkp-1 mRNA labeling by in situ hybridization (Loda et al., 1996). Tumor size and serum levels of α-fetoprotein were increased in MKP-1-negative tumors compared with MKP-1-positive tumors (Tsujita et al., 2005). In addition, disease and overall survival rates after hepatectomy decreased in patients whose tumors were not expressing MKP-1 compared with patients with MKP-1-positive tumors. Overall, the results suggested that MKP-1 could be used as an independent predictor of patient survival outcome after hepatectomy for patients with hepatocellular carcinomas (Tsujita et al., 2005). This is the only human-based study that cites MKP-1 as being an independent predictor of patient survival.

Two murine models of liver-specific p38αMAPK deletion, one noninducible and the other inducible, were used with the following results: 1) spontaneous tumors did not form in livers in either group of mice (noninducible or inducible), over the course of 1 year; and 2) the diethylnitrosamine-induced hepatocellular carcinoma murine model revealed that diethylnitrosamine-increased hepatocyte proliferation was mediated via the JNK/c-Jun pathway (Hui et al., 2007). Phosphatase expression (MKP1–7, Pac1, VHR, VH3, and DUSP8) revealed no difference at the mRNA level, in liver tumors or normal liver tissue of p38α conditional deletion mutant mice compared with liver tumors or normal liver tissue of their "floxed" control counterparts. There was no mention of phosphatase protein expression in this study (Hui et al., 2007). Another study used three murine models of liver specific c-Jun mutations: 1) an inducible c-Jun deletion mutant, 2) a nonconditional c-Jun deletion mutant, and 3) a JunAA mutant that cannot be phosphorylated in the N-terminal position by JNKs (Eferl et al., 2003). This study revealed that c-Jun was required for initiation and progression of chemically induced tumorigenesis, but not for the survival of the tumor in the late phases of tumor progression. It is noteworthy that whereas JunAA was sufficient for the induction of hepatic tumorigenesis (Eferl et al., 2003), N-terminal phosphorylated c-Jun was required for proliferation of adult hepatocytes and regeneration of liver mass after partial hepatectomy (Behrens et al., 2002). This study revealed different requirements for normal hepatocyte proliferation versus those for hepatocellular carcinoma. In addition, hepatocytes isolated from conditional c-*Jun*(−/−) and *p53*-null mice, more specifically c-*Jun*(−/−)*p53*(−/−) and c-*Jun*(−/−)*p53*(−/+) mice, revealed that c-*Jun*(−/−)*p53*(−/−) double null mice had reduced p53-mediated cell death upon TNF-α treatment, as measured by lactate dehydrogenase release, compared with c-*Jun*(−/−)*p53*(−/+) double mutant mice. Thus, c-Jun protects hepatocytes from p53-induced cell death, possibly by preventing transcription of the proapoptotic gene *noxa*, which is not required for tumor cell proliferation (Eferl et al., 2003). Overall, these results demonstrate that a given protein can have different functions in normal versus pathological settings. Even if a protein is not mutated, tumors may coerce proteins to function at atypical times or places. The untimely or inappropriate expression of a normal protein could give rise to gain of function in terms of protein-protein interaction, owing to the different domains, stoichiometric considerations, and subcellular distribution of the protein.

C. Lung Cancer

A comparison of all-*trans* retinoic acid-responsive and -unresponsive non–small-cell lung cancer (NSCLC) cells revealed that the early phase of *t*-RA-mediated inhibition of serum-induced JNK1 activity relied on blocking the activity of MKK4, whereas sustained inhibition of JNK1 activity relied on up-regulation of MKP-1 expression (Lee et al., 1999). Vicent et al. (2004) investigated MKP-1 expression in lung cancer and in cell lines. There was a higher MKP-1 expression in NSCLC versus small-cell lung cancer cell lines. Interestingly, there was no obvious relationship between individual clinicopathological variables or MAPK phosphorylation status, as evaluated via immunohistochemistry, and MKP-1 expression. However, in patients with NSCLC, MAPK activation was associated with stage of tumor. Nuclear JNK activation was associated with early stages of cancer, whereas activated p38MAPK was correlated with phosphorylated ERK in both the nucleus and cytoplasm. In addition, ERK was associated with advanced stages of tumors. Overall, for patients with NSCLC, there is an improved survival rate for those with >50% of tumor nuclei staining positive for MKP-1, versus those with <50% of tumor nuclei stained (Vicent et al., 2004). These findings lend support to the hypothesis that tumors survive JNK proapoptotic signaling via MKP-1 expression in early stages and down-regulate MKP-1 when ERK is expressed in late stages. The survival rates of patients expressing nuclear MKP-1 protein reflect this finding: although the overall amount of protein expression is important, the subcellular localization also needs consideration especially when one is determining whether a correlation exists between a given protein and patient survival outcome.

D. Urogenital Cancers

1. Renal Cell Carcinomas. Renal cancer cell lines Caki-1 and KU 20–01 (the latter was derived from clear

cell carcinoma) decreased MKP-1 protein expression and revealed persistent JNK1 activation and apoptosis in response to combined treatment with Ro-31-8220 and anisomycin (Mizuno et al., 2004). In contrast, renal cancer cell lines ACHN and 769P were refractory to such combined treatment, as their MKP-1 levels were not modified and JNK1 was not activated. Renal cell carcinomas that express MKP-1 may escape apoptotic cell death by limiting JNK1 activation (Mizuno et al., 2004).

2. Bladder Cancer. Although normal bladder urothelium expressed low levels of mkp-1 mRNA, the level of expression of this transcript was inversely proportional to the grade of the bladder tumor. These tumors were categorized in situ as high-grade transitional cell dysplasia and carcinomas. EGF receptor was expressed in late stages of bladder cancer, but there was no coexpression with mkp-1 mRNA (Loda et al., 1996). In bladder cancers, the down-regulation of MKP-1 may have coincided with down-regulation of JNK1, although this possibility was not verified. However, absence of MKP-1 may not be required for high ERK levels, as ERK1 activity was consistently higher in prostate, colon, and breast cancer tissues compared with levels in their normal counterparts, even in the presence of mkp-1 mRNA and protein, when detected (Loda et al., 1996). As the MKP-1 protein was not always detected, given the level of mkp-1 mRNA and protein regulation, it could be that the MKP-1 protein was phosphorylated and targeted for proteasomal degradation by ERK1/2.

Other results from human urothelial carcinoma cell lines expressing high JNK-low MKP-1 (UMUC14 cell line) and low JNK-high MKP-1 (UMUC6 cell line) levels revealed that JNK activation and low levels of MKP-1 correlated with the ability of a tumor cell line to induce angiogenesis in a chorioallantoic membrane assay (Shimada et al., 2007). This group also found that phosphorylated JNK and decreasing MKP-1 expression levels correlated with increasing grade, invasiveness, and increased amount of microvasculature in human urothelial carcinomas (Shimada et al., 2007). Thus, in this scenario, high levels of phospho-JNK corresponded to an aggressive tumor phenotype, not apoptosis. The mechanism that caused activated JNK to correlate with tumor grade and stage was unclear, and it was not known how long JNK activity lasted, as duration of JNK activity can have anti- or proapoptotic consequences (see section II.C on the JNKs). Furthermore, as ERK5 is involved in angiogenesis and vasculogenesis (see section V.D.8 on angiogenesis and vasculogenesis), it is presently unknown whether cross-talk exists between JNK and ERK5 or to what extent JNK is required for angiogenesis. In addition, some results clearly indicate that JNK can be prosurvival or prodeath: 1) experiments in nude mice revealed that Ras-transformed JNK1/2 double KO MEFs developed greater tumor mass than Ras-transformed WT MEFs in athymic nude mice as a result of reduced apoptosis generating both greater number and size of nodules (Kennedy et al., 2003); and 2) JNK1 is essential for survival of B-lymphoblasts (Hess et al., 2002).

The apparent contradictions between the previously mentioned results regarding JNK activity require the ability to distinguish between the possible outcomes of active or inactive JNK and to identify the multiple factors that correlate with advanced tumor grade and stage versus induction of apoptosis. In the present case, with JNK, some of these factors include 1) the effectors of active JNK when it is protumorigenic versus antitumorigenic (Bogoyevitch and Kobe, 2006), 2) the targeting of JNK effectors to proteasomal degradation by both unphosphorylated JNK (Fuchs et al., 1997, 1998a; Bode and Dong, 2007) and phosphorylated JNK (Gao et al., 2004; Ki et al., 2007), which regulates the availability of the effectors for cell function, 3) the active JNK-mediated increase in E3 ligase activity, part of protein ubiquitination and degradation, which will decrease effector levels degraded by this pathway (Gao et al., 2004; Chang et al., 2006), 4) the implication of AP-1, an effector of c-Fos and c-Jun, in the control of invasion (Ozanne et al., 2007), 5) the presence of other MAPKs, given the level of cross-talk between them (see section II.E on MAPK cross-talk), 6) the presence of ERK1/2, which can stabilize/destabilize MKP-1 (Brondello et al., 1999; Lin et al., 2003; Lin and Yang, 2006), 7) the presence of phosphatases, DUSP22 and DUSP23, that can activate MKK7, MKK4, and MKK6, the upstream kinases of JNK and p38MAPK (Shen et al., 2001; Chen et al., 2002; Takagaki et al., 2004); 8) scaffolding proteins such as JIP (Raman et al., 2007) that can interact with MKP7 and JNK, resulting in the inactivation of JNK (Willoughby et al., 2003), and 9) the presence of other dual-specificity phosphatases that inactivate JNK and other members of the MAPK family (Tables 1 and 2).

3. Prostate Cancer. Strong mkp-1 mRNA expression was detected in basal cells of the human prostatic acini (Loda et al., 1996), whereas MKP-1 protein localized to normal basal and secretory cells (Magi-Galluzzi et al., 1997). Overall, mkp-1 mRNA expression decreased as the prostate tumor grade advanced from the lower to higher grades (Loda et al., 1996, Magi-Galluzzi, 1997). Interestingly, all areas of preinvasive prostate lesions or intraepithelial neoplasia expressed high levels of mkp-1 mRNA and ERK1 and JNK1 proteins. A high mkp-1 mRNA level of expression was assessed as being >50% of cells that are *mkp-1*-positive. It was believed that the mechanism that accounted for transformation via the Ras pathway was also responsible for the up-regulation in MKP-1 synthesis. For patients with invasive adenocarcinomas that were not treated with androgen ablation, the less differentiated the carcinoma was (using the Gleason score system) the fewer the tumors that showed high levels of mkp-1 mRNA expression. Interestingly, when tumor-normal prostate tissue pairs were assessed for mkp-1 mRNA expression versus JNK1 and ERK1

activities, there was an inverse correlation between the levels of mkp-1 mRNA expression and JNK1 activity but not ERK1 activity (Magi-Galluzzi et al., 1997). In patients treated with androgen ablation, neoplastic tumors were found to express low to undetectable levels of mkp-1 mRNA, although JNK1 protein expression remained detectable. In addition, an inverse correlation developed between mkp-1 mRNA expression and apoptosis after androgen ablation. In general, mkp-1 mRNA expression decreased after hormonal ablation, across all tumor grades. MKP-1 protein mirrored its mRNA expression. It was believed that MKP-1 expression correlated with the ability of human prostate tumors to evade apoptosis during the early stages of tumorigenesis (Magi-Galluzzi et al., 1997). Similar to the previously mentioned studies, hormone-refractory prostate carcinomas demonstrated lower mkp-1 mRNA and protein levels compared with benign prostate hyperplasia and untreated prostate carcinomas (Rauhala et al., 2005).

Noble rats were used as a model of sex hormone-induced prostatic dysplasia and carcinoma (Leav et al., 1996). ERK protein, mkp-1 mRNA, and protein expression were detected in both the dorsal and the lateral lobes of the prostate of wild-type rats and decreased after castration. Normal patterns of expression returned, with time, after exogenous testosterone treatment for several days (Leav et al., 1996). Interestingly, wild-type rats treated with a combination of testosterone and estradiol-17β, a known inducer of dysplasia in the dorsal and lateral lobes of the prostate (Noble, 1982), showed an overall decrease of mkp-1 mRNA and protein expression within dysplastic legions, whereas ERK levels were unaffected (Leav et al., 1996). It was believed that selective up-regulation of MKP-1 could contribute to the dysplastic phenotype via ERK mitogenic activity, as MKP-1 did not target ERK and decreased JNK activity.

Recently, both MEK5 and ERK5 have been involved in some facets of cancer. MEK5 overexpression in prostate cancer correlated with increased bony metastasis and poor patient survival outcome (Mehta et al., 2003). The same group found that ERK5 nuclear localization corresponded with poor prognosis and hormone-insensitive prostate cancer in a subset of patients (McCracken et al., 2007). In vitro, MEK5 overexpression increased proliferation, motility, and metastasis of prostate cancer cells (Mehta et al., 2003). Expression levels of nuclear phosphatases were not mentioned in these studies.

Adding to the previous findings, human prostate tumors revealed higher c-Fos and c-Jun proteins in nuclei of tumor epithelial cells, which correlated with more advanced stages of the disease. Interestingly, phosphorylated ERK also correlated with the presence of c-Fos and c-Jun. Although c-Jun correlated with disease recurrence in a subset of patients, c-Jun was not an indicator of patient survival outcome (Ouyang et al., 2008). c-Fos and c-Jun can be part of AP-1, which has been associated with control of invasion via up-regulation of genes favoring invasion and down-regulating genes inhibiting invasion (Ozanne et al., 2007). AP-1 is also implicated in proliferation (Shaulian and Karin, 2001). Thus, determining the mRNA levels of AP-1 target genes as well as the levels of their protein products may yield a correlation between the levels of the effectors and patient survival outcome. However, as AP-1 can be composed of Jun family homodimers or Fos and Jun family heterodimers, lack of correlation of one member of the family does not exclude involvement of other family members.

In summary, hormone-refractory prostate carcinomas as well as hormonal ablation down-regulate mkp-1 mRNA and protein across all tumor grades. These results are echoed by observations that the expression of *mkp-1* is inversely proportional to tumor grade. Although these findings confirm the importance of hormonal treatment in *mkp-1* regulation, as revealed in section III, there is no lack of agents that regulate *mkp-1* at the mRNA and protein levels. If MKP-1 modulation can lead to the control of hormone-refractory prostate carcinomas, finding an agent that bypasses the need for hormonal treatment, without stimulating mitogenesis, may be within reach. Furthermore, as overexpression of MEK5 and nuclear localization of ERK5 are associated with poor prognosis, MKP-1 expression or another nuclear phosphatase, may serve to decrease ERK5 activity and improve prognosis. Overall, determining whether MKP-1 or another phosphatase can be used to regulate prostate cancer biology will depend on the telltale signature of prostate cancer and which molecules are absolutely required for tumor development and progression in vivo.

4. Uterine Leiomyoma Cells. Leiomyomata are benign tumors that derive from smooth muscle cells (Cramer and Patel, 1990). Uterine leiomyoma cells (GM10964) down-regulated mkp-1 mRNA and protein levels after 17β-estradiol treatment, whereas normal uterine smooth muscle cells down-regulated only mkp-1 mRNA levels (Swartz et al., 2005). This difference in MKP-1 protein levels in GM10964 cells versus normal smooth muscle cells after 17β-estradiol may underlie the difference in proliferation between both cell lines. It would be interesting to determine whether ERK1/2 were involved in MKP-1 protein down-regulation after 17β-estradiol treatment in the context of uterine leiomyoma cells (GM10964) and other tumors.

5. Ovarian Carcinomas. MKP-1 protein expression was reduced in low-grade malignant tumors compared with benign cysts and normal surface epithelium. Conversely, ovarian carcinomas reveal the full spectrum of expression, from negative to strong MKP-1 protein expression. Although there did not seem to be a correlation between MKP-1 protein expression and overall patient survival, there was a significant correlation between MKP-1 protein expression versus a shorter progression-

free survival (the time it takes to detect the disease clinically or pathologically) (Denkert et al., 2002). It would seem that MKP-1 was permissive to the survival of ovarian carcinomas, based on the shorter progression-free survival data. In contrast, the lack of correlation between overall patient survival and MKP-1 expression would argue against such a conclusion. This seeming contradiction between shorter progression-free survival and overall patient survival outcome could have stemmed from the subcellular localization of MKP-1 protein and the duration of JNK activity, which can be modulated by MKP-1, dictating the antiapoptotic versus proapoptotic outcomes. Other phosphatases and MAPKs could also have contributed to patient survival outcome.

Another study compared the mRNA expression pattern of 68 phosphatases from immortalized ovarian surface epithelium with that of cancer cell lines and found that immortalized ovarian surface epithelium had higher mRNA expression patterns in 4 of the 68 phosphatases. MKP-1 was one of the phosphatases thus identified (Manzano et al., 2002). The MKP-1 expression pattern was characterized in primary ovarian cancers versus normal ovaries, and results revealed that MKP-1 protein expression was decreased in primary ovarian cancers compared with that in normal ovaries. The expression of MKP-1 also decreased with increasing stage of the disease (Manzano et al., 2002). Nude mice injected with ovarian cancer cell lines conditionally expressing MKP-1 had reduced tumor size in comparison with the same cell lines not induced to express MKP-1 (Manzano et al., 2002).

Both the Denkert et al. (2002) and the Manzano et al. (2002) studies revealed a tendency for tumors to lose MKP-1 expression with advancing tumor stage. However, to understand the contribution of MKP-1 to the process of tumorigenesis, the first group used a human survival study, whereas the latter used a nude mouse model and injected mice with cell lines conditionally expressing MKP-1. As both studies used different paradigms, the cause of divergence between their results could be ascribed to 1) different levels of MKP-1 expression, 2) different species, 3) tumors developing at their "natural site" (Denkert et al., 2002) versus tumors developed from cell lines injected at sites determined by experimentation (Manzano et al., 2002), and 4) heterogeneity of naturally occurring tumors versus the more homogeneous clones of cell lines used to inject nude mice.

In summary, in the early phase of cancer (low grade and early lesions), the tumor evades JNK1-induced death by up-regulating MKP-1 and in the more advanced stages of tumorigenesis down-regulates MKP-1 to allow for proliferation and increased tumor mass. As metastases have little if any *mkp-1* expression, it is possible that the absence of *mkp-1* heralds the metastatic process for a given tumor. There are exceptions to this observation, and MKP-1 can be expressed in conjunction with receptors such as Neu and EGF receptor, in advanced tumor grades. However, at this point there is no definitive evidence that MKP-1 can be used as a marker for patient survival in most cancers. The reason is that the consequence of MKP-1 activity depends, to a great extent, on the function of the MAPK at the time that MKP-1 (or another phosphatase) is inactivating it. Thus, if MKP-1 reduces JNK activity, it could favor antiapoptosis or proliferation over cell death. If MKP-1 targets ERK1/2, it may lead to enhanced proliferation instead of differentiation. If ERK5 activity is reduced, it may reduce angiogenesis or endothelial cell survival. If MKP-1 targets p38MAPK, it could prevent p38MAPK-mediated cell cycle arrest. Therefore, depending on the function of a particular MAPK, in addition to the interactions of all the other players at any given time point, MKP-1 or another phosphatase may skew the balance in one direction or another. The combination of these effects, among others, will determine cell fate.

VII. Conclusion

As shown in this review, although MKP-1 has a deceptively simple function as a dual-specificity MAPK phosphatase, the array of stimulants and the level of control at the mkp-1 mRNA and protein levels reveal a scenario that is anything but simple. Adding to this complexity, the ubiquitously expressed MKP-1 does not lead to an obvious phenotype when knockout mice are generated. This observation leads to the logical conclusion that the numerous members of the dual-specificity phosphatase family can compensate for the loss of MKP-1 during the developmental stage or that it is not required during development. In contrast, cardiac transgenic mice expressing approximately 4-fold normal levels are not viable past the 15-day postnatal time point, whereas a 2-fold increase allows the cardiac transgenic mice to survive. Considering these observations and all of the reports on MKP-1 and cancer, how can we use this information to understand the role of MKP-1 in cancer? Is it even relevant for cancer biology? The lack of phenotype in *mkp-1*-null mice and the obvious phenotype in transgenic mice present an interesting dichotomy. Keeping the effect of the genetic background of the mice as a possible explanation for the difference between null and transgenic mice, can overexpressing this phosphatase be used to target tumor proliferation or other aspects of cancer biology such as metastasis, angiogenesis, or differentiation?

Before answering such questions we need to take a hard look at where we stand. First, we should recognize that reports on the importance of MKP-1 in cancer have been divergent, with some implicating MKP-1 as a major player in cancer and others demonstrating that MKP-1 is present only as a bystander. This apparent contradiction stems, in part, from the function of MAPKs, which dictate the biological outcome under normal and patho-

logical circumstances. For instance, activation of ERK1/2 leads to proliferation, differentiation, and cell motility, among other phenotypes (Meloche and Pouyssegur, 2007; Raman et al., 2007). This multiplicity of phenotypes holds true for JNK1, which leads to cell survival or apoptosis (Bogoyevitch and Kobe, 2006), and for p38MAPK, which is implicated in cell differentiation and tumor dormancy, among other phenotypes (Zarubin and Han, 2005; Ranganathan et al., 2006) (sections II and V). Depending on which MAPK is expressed and what function it is implicated in, MKP-1 or other phosphatases will seem to have divergent effects as they inactivate their target MAPK.

Other factors also come into play in the MAPK-MKP-1 saga. For example, the subcellular distribution of active versus unphosphorylated MAPK can be recognized when a specific MAPK, such as ERK, is considered. As ERK is known to stabilize or destabilize MKP-1 via phosphorylation, the net result for a given cell of the ERK-MKP-1 interaction will depend on the mechanisms that determine ERK inactivation by MKP-1 versus ERK-mediated stabilization of MKP-1 or targeting of MKP-1 for degradation via polyubiquitination and the proteasomal pathway. In general, the consequences of phosphorylation are varied and thus far include activation, inactivation, stabilization, targeting for proteasomal degradation, and priming. Therefore the context of phosphorylation is crucial to understanding its ensuing consequences. As a result, how a stimulus affects a given cell type in relation to the ERK-MKP-1 interaction will depend on which of the three outcomes results from an ERK-MKP-1 interaction. In turn, this result will also unveil the effect on JNK1, p38MAPK, and ERK5 as they are also targeted by MKP-1. At present it is unknown whether JNK1, p38MAPK, or ERK5 can stabilize or target MKP-1 for degradation via phosphorylation as ERK1/2 do.

Adding to this complexity, unphosphorylated JNK can bind to and target its effectors for polyubiquitination and proteasomal degradation (Bode and Dong, 2007) (see also section II.C on the JNKs). As JNK has different targets in the cytosol versus the nucleus (Bogoyevitch and Kobe, 2006), the outcome of the function associated with unphosphorylated JNK will depend on chronological as well as spatial distribution. It is currently unknown whether binding to the unphosphorylated form of JNK1 can target MKP-1 for polyubiquitination and consequent degradation or whether ERK and p38MAPK can target their effector when they are in an unphosphorylated state. The subcellular distribution and activation states of ERK, JNK1, and p38MAPK will contribute to activation, inactivation, or degradation of cytosolic and nuclear effectors, gene regulation, and ultimately cell fate.

The situation regarding the axes of MAPK is far from being this linear. Cross-talk exists at every level of the MAPK signal transduction cascade and across MAPK modules(Zhang et al., 2001; Shen et al., 2003; Cuevas et al., 2007) (see also section II on MAPK cross-talk). We need to improve our understanding of the role that the various axes of the MAPK family have in cancer. For instance, there are four splice forms of JNK1 and JNK2 and two of JNK3 (Waetzig and Herdegen, 2005), several forms of p38MAPK (Olson and Hallahan, 2004), and the presence of monomers and dimers of ERK (Khokhlatchev et al., 1998; Adachi et al., 1999; Zambrowicz and Sands, 2003) in addition to all the isoforms of ERK, some of which, such as ERK3/4 and ERK7, await full characterization. As more data concerning all of these factors come to light, our understanding of MKP-1 and the modulatory role of other phosphatases will increase.

We should not forget other factors and observations. The finding that MKP-1 and MKP-3 can limit HIF-1α activity may be an important observation. This result needs to be confirmed in other tumor cell types as well as in xenograft tumor animal models. If proven to be a general effect in tumors, it would suggest, for instance, that MKP-1 is hardly expressed in advanced tumor grades when ERK1/2 are expressed or in metastases. However, ERK5 is involved in tumor proliferation and phosphorylation of HIF-1α and targeting of it for proteasomal degradation. As MKP-1 inactivates ERK5, characterizing how ERK1/2, ERK5, HIF-1α, and phosphatases (MKP-1, MKP-3, or other) function in primary tumor survival and how they "cooperate" during angiogenesis may reveal the mechanisms tumors use to strike a balance between proliferation and mediating angiogenesis among other functions.

We are now in a position to devise a method of answering the questions: Is the amount of MKP-1 relevant for cancer phenotype? More importantly, can MKP-1 overexpression be used to target tumor proliferation, metastases, angiogenesis, or other cancer phenotypes? To answer the first question, malignant tissue can be compared with its normal counterpart. All malignant tissues would need to be categorized according to type, stage, grade, gender, and chemotherapeutic, radiotherapeutic, or other treatments used, as these can also affect gene and protein expression. Thereafter, screening the malignant tissues and their normal control counterparts according to the localization of the MAPK isoform and splice form would need to be accompanied by screening for MKP-1 protein with emphasis on its localization to the nucleus. When unphosphorylated forms of MAPK are localized, the expression level of their effectors should be determined. Tumor banks are well suited for this type of study. However, to determine the activation status of these MAPKs, fresh biopsy specimens of relevant tumors may need to be obtained. For tumors that do not reveal a direct correlation between various aspects of their phenotype and MKP-1 expression, screening for other members of the dual-specificity phosphatase family may be in order, considering that they

not only target the same substrates but also have positive and negative modulatory roles on their target proteins.

For tumors that do demonstrate a correlative effect between their phenotype and MKP-1 expression, animal models can be devised to distinguish between a secondary role versus cause and effect of MKP-1 expression in relation to tumor phenotype. Thereafter, the answer to the question relating to the manipulation of MKP-1 expression levels to modify tumor biology can be addressed using animal models and cells conditionally expressing MKP-1. Cells with different oncogenic phenotypes, such as uncontrolled proliferation, metastasis, and angiogenic potential, may reveal changes in phenotype when expressing MKP-1 at different time points, as they develop into tumor masses within the context of an animal.

Another facet that may yield promising results is the targeting of the immune system. The number of phosphatases involved in immunomodulation (Tables 1 and 2) could represent a potential target for immunologically based antitumor therapy such as the mixed bacterial vaccine (Busch-Coley treatment). However, before such an effort could be undertaken, a more complete identification of the phosphatases involved in immunomodulation, a characterization of their role, and the exact mechanism relating bacterial infection to the ability of the immune system to recognize cancerous cells would need to be revealed in some detail.

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The MAP Kinase Phosphatase-1 MKP-1/DUSP1 Is a Regulator of Human Liver Response to Transplantation

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Orthotopic liver transplantation (OLT) continues to be the only remedy for end-stage liver disease. In an attempt to decrease the ever-widening gap between organ donor and recipient numbers, and ultimately make more livers amenable to transplantation, we characterized the healthy human liver's response to ischemia and reperfusion-induced injury during transplantation. This was carried out by transcriptional profiling using cDNA microarray to identify genes whose expression was modulated at the 1-h postreperfusion time point. We observed that the map kinase phosphatase-1/dual-specificity phosphatase-1 (MKP-1/DUSP1) mRNA was strongly and significantly upregulated. Validation of this observation was carried out using reverse transcriptase-polymerase chain reaction (RT–PCR), immunoblotting and immunohistochemistry. In addition, we characterized the signaling pathways regulating MKP-1 expression using the human hepatoma cell line HepG2. Finally, by combining MKP-1 silencing with reperfusion-associated stresses, we reveal the preferential role of this protein in attenuating the activity of the JNK and p38^{MAPK} pathways, and the resulting apoptosis, making MKP-1 a potential target for therapeutic intervention.

Key words: Gene expression profiling, live donor transplantation, liver transplantation, living donor transplantation, MAP kinase, signaling, signal transduction, whole liver transplantation

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Introduction

Orthotopic liver transplantation (OLT) remains the only therapeutic option for end-stage liver diseases. Unfortunately, an increasing number of patients requiring transplantation and a widening lag time to organ availability reduce the number of required transplantations. Strategies can be devised to alleviate this problem, such as: (i) including marginal livers in donor pools and (ii) reducing the incidence of graft failure. Ischemia reperfusion injury (IRI) is one of the major contributors to posttransplant graft nonfunction and dysfunction (1). During the reperfusion phase of the operation, blood-derived heat- shock and oxygen, and liver-derived reactive oxygen species produced by Küpffer cells and hepatocytes can damage the graft (2). Such oxidative stress promotes hepatic cell apoptosis, necrosis, activation of transcription regulators, such as NF-κB or AP-1 (3) and induces the expression of proinflammatory factors such as interferon-gamma and tumor necrosis factor-α (TNF-α) (4). Further damage occurs by the activation of neutrophils, macrophages and lymphocytes, aggravating the reperfusion injury (5). Events occurring during ischemia and within the first few hours postreperfusion largely determine the graft's ability to tolerate IRI and recover. Unfortunately, our understanding of the mechanisms involved in the reestablishment of cellular and organ homeostasis during the reperfusion phase remains incomplete. Therefore, the identification of key molecules involved in the reestablishment of liver homeostasis represents an important avenue of investigation.

Transcriptome analyses carried out on human liver transplantation biopsies revealed IRI-mediated parallel activation of pathways that can lead to oxidative/inflammatory stress, apoptosis or cell proliferation (6,7). Although some characterization exists, networks controlling transcriptional activation, specific to the early reperfusion phase of human liver transplantation, remain poorly characterized. In an attempt to conduct an unbiased analysis and identify the genes regulated during the reperfusion phase of liver transplantation, we performed a transcriptional profiling analysis on human liver biopsies collected prior to, 10 min and 60 min after reperfusion. As a result, we identified approximately 30 genes whose expression level was significantly upregulated during the first hour of reperfusion. Among these, we observed the induction of map kinase phosphatase-1(MKP-1) mRNA expression, the transcript coding for the dual-specificity phosphatase, MKP-1,

Consequently, we investigated MKP-1's involvement during reperfusion and demonstrated that its regulation was part of a specific transcriptional program.

Materials and Methods

Patients and tissue collection

Three biopsies, 0.5 cm, were surgically removed in the course of eight liver transplantation procedures performed within the McGill University Health Centre with approval of the institution's ethics committee (ERB 05–003) as previously described (8–10) (Figure 1A). Briefly, liver biopsies were collected during reimplantation of the organ in the recipient, just prior to clamp removal from the hepatic portal vein (R0), and 10 min (R10) and 60 min (R60) after the blood flow had been reestablished through the portal vein. The 10-min time point biopsy specimen accounts for any mRNA contributed by the recipient blood when performing the microarray analysis (Figure 1B). The average cold ischemia phase was 8h30 ± 2h15 whereas the average warm ischemia time was 48 min ± 26 min. For every time point, each biopsy was divided into three pieces. Two pieces were snap-frozen and stored in liquid nitrogen until RNA or protein extraction. The third piece was embedded in OCT prior to being snap-frozen in liquid nitrogen.

RNA isolation

Total RNA was extracted using Trizol following the manufacturer's instructions. Frozen tissue samples were put in Trizol prior to homogenization. The quantity of RNA was evaluated by either a RiboGreen kit (Molecular Probes) using CytoFluor 2300 (Millipore) to evaluate fluorescence intensity (for microarrays) or spectrophotometry at 260 nm (for **reverse transcriptase-polymerase chain reaction** [RT–PCR]). The quality of the RNA was determined by nondenaturing agarose gels. The relative purity was determined by $A_{260/280}$ ratio by spectrophotometry.

Microarray analyses

For microarray analyses, seven livers were biopsied at R0, R10 and R60 (reperfusion time 0, 10 and 60 min). For all seven patients, we used DNA microarray to compare RNA from the R60 samples to their respective R0 samples. However, of the seven biopsy sets (R0, R10, R60) (see Table 1), three R10 biopsies did not contain enough tissue to extract sufficient mRNA for microarray analysis, consequently we could only compare four of the R60–R10 analyses. For each of the biopsy time points dye swaps were done, thus using two microarrays per biopsy time point for a total of 22 microarrays.

The microarrays were scanned using a ScanArray 5000 scanner (Perkin-Elmer-Cetus) at a 10 μm resolution. The resulting TIFF files were quantified with QuantArray software (Perkin-Elmer-Cetus). Each spot of the array had to satisfy three criteria of quality control for inclusion into normalization and analysis: (1) the signal intensity had to be greater than the surrounding background; (2) the raw intensities of duplicate spots, per gene, had to be within 50% of each other; and (3) the signal intensity for each spot had to be within the dynamic range of the photomultiplier tube. This was determined by the user with the help of a scatter plot of the $\log_{10}$ of background-subtracted intensities (11).

Data analyses

Normalization and analysis were conducted using GeneSpring v5.0 (Agilent Technologies). The ratio of intensity of the two channels was normalized by fitting a Lowess curve to the log-intensity versus log-ratio plot for the entire array. We selected a list of significantly modulated genes with an average change in transcript abundance of at least 2-fold in either the R60R0 or the R60R10 comparisons. Genes whose expression was significantly regulated were then annotated using the g:Profiler program (http://www.bioinf.ebc.ee/gprofiler/index.cgi) and clustered based on their GO annotation (http://www.geneontology.org/) using the PermutMatrix program (12). Functional network analysis was carried out using the STRING suite (13,14). The resulting scale-free functional interaction network was then annotated using the Medusa program (15).

Results

Reperfusion-mediated upregulation of MKP-1 mRNA

Using three time point biopsy specimens (see Materials and Methods), we elaborated and compared two normalized lists of genes: first list 60 min versus 10 min (R60R10) and second list 60 min versus 0 min (R60R0). Comparing these two lists allowed us to eliminate any mRNA whose apparent increase in expression was the result of the recipient blood, during the reperfusion phase of the operation. For instance, the 60-min versus 0-min normalization revealed a 3-fold increase in globin mRNA levels while the 60-min versus 10-min normalization did not (Table 2 and supporting tables). This observation suggests that the increasing amount of globin mRNA is most likely contributed by the recipient blood, rather than an increased expression in liver tissue. The globin mRNAs were therefore eliminated from further analyses, as they were not significantly regulated. A more complete analysis of gene expression profiling is given in supporting Tables S1, S2 and S3. Based on the comparison of both lists of genes, R60R10 and R60R0, we generated a scatter plot (Figure 1C), which led to a preliminary list of genes devoid of recipient blood-derived contributions. After subtracting the false positives, we kept only the transcripts that showed at least a 1.5-fold change in abundance during the first hour of reperfusion. Although fold variation was important, consistency in the results across patients and 'multiple hits' with different expressed sequence tags (ESTs) within the same array had higher priority (11,16). These results were recapitulated using a scatter plot of the fluorescence intensities on the Experiment and Control channels (supporting Figure S1). The functional distribution of the 23 nonredundant genes, with a significant change in expression upon reperfusion, was established using a gene ontology (GO)-based analysis with the g:Profiler program. The GO annotation revealed that the upregulated genes collectively belonged to 10 functional groups that where partitioned into two major clusters (Figure 1D). The first cluster was linked to functions relating to the biological processes associated with responses to stress, chemical and biotic stimuli (GO:0006950; GO:0042221; GO:0009607) and the second was associated with the regulation of biological, cellular and developmental processes (GO:0048519; GO:0048523; GO:0050793). To evaluate potential functional interactions existing between these 23 genes, we used the STRING suite as described in Materials and Methods. Interestingly, out of the 23 genes selected above, 21 were found to belong to a highly connected functional network of 23

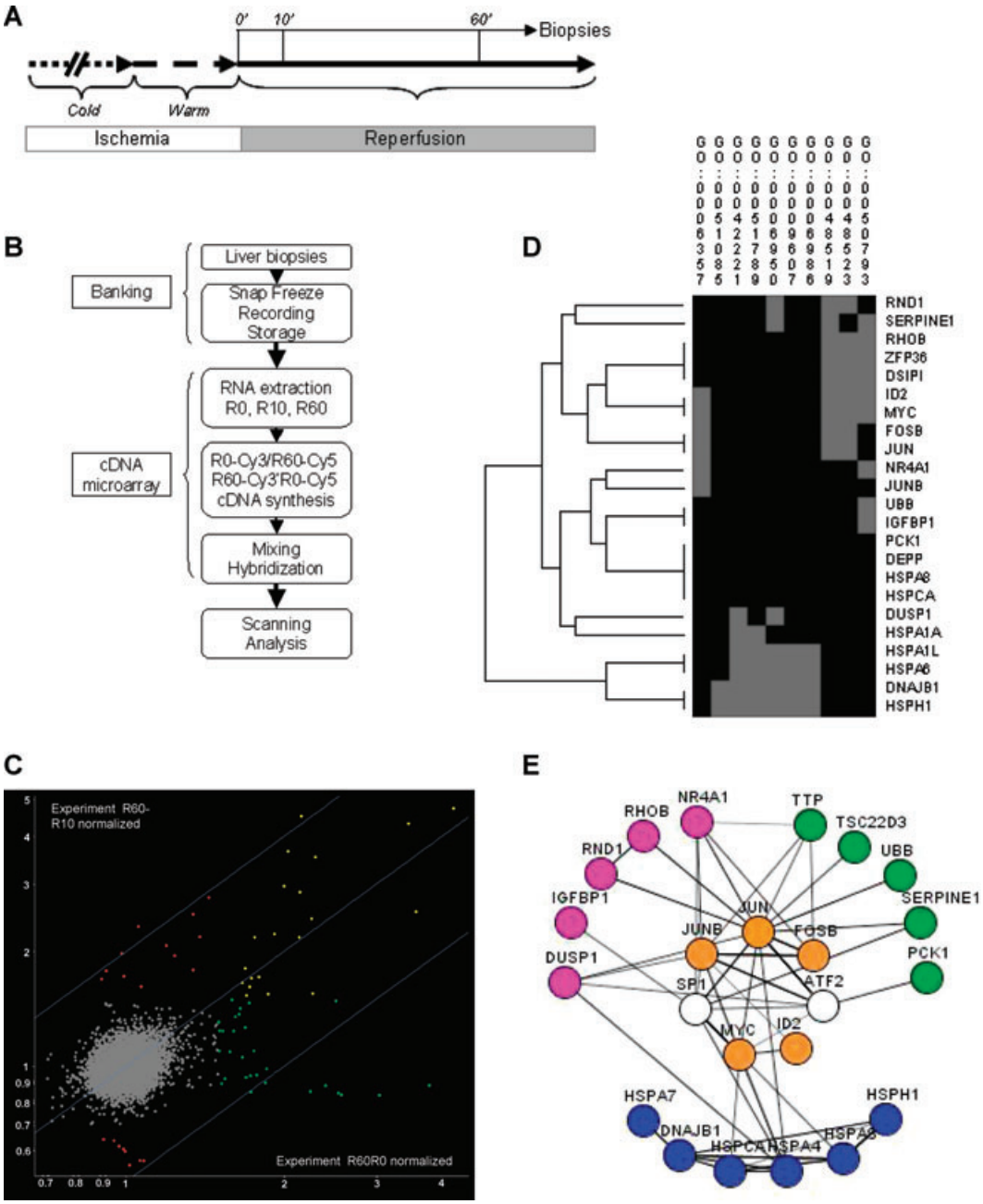

Figure 1: Microarray analysis of human liver biopsies collected during the reperfusion phase of transplantation. (A) Timeline representation of the biopsy collection protocol followed in our study. (B) Flow-chart representation of the experimental procedure used in our microarray analysis. (C) Scatter plot representation of the gene distribution on a per normalization basis. The genes from the R60R10 group are shown in red, the genes from the R60R0 group (including globin genes) are shown in green and the genes that have been modulated in both groups (R60R10 and R60R0) are shown in yellow. (D). Gene ontology-based clustering of the known upregulated genes during human liver reperfusion. (GO:0006357 = regulation of transcription from RNA polymerase II promoter; GO:0051085 = chaperone cofactor-dependent protein folding; GO:0048519 = negative regulation of biological process; GO:0050793 = regulation of developmental process; GO:0048523 = negative regulation of cellular process; GO:0009607 = response to biotic stimulus; GO:0051789 = response to protein stimulus; GO:0006986 = response to unfolded proteins; GO:0009607 = response to biotic stimulus; GO:0006950 = response to stress; GO:0042221 = response to chemical stimulus). (E) STRING network representation of 21 out of the 23 genes identified in Table 2. The nodes represent genes whose protein products have been categorized according to the following color code: blue nodes for chaperone functions, purple nodes for signaling, green nodes for metabolism (RNA and proteins) and orange nodes for transcription. The edges are representative of the various interaction types available through STRING suite based on experimental, data bases and text mining.

Table 1: Clinical characteristics of the transplanted patients

	L6	L7	L11	L26	L27	L28	L30
Patient death	N	N	Y	Y	Y	N	N
Reason of death	–	–	a	b	c	–	–
Graft death	N	N	–	–	–	N	N
AST (units/L) 22/29 h	804	2066	720	1089	179	2692	ND
ALT (units/L) 22/29 h	894	1921	378	1212	144	1032	1349
TB (umol/L) 22/29 h	24	83	34	212	88	66	40
ALP (units/L) 22/29 h	83	107	70	61	130	50	85
INR 22/29 h	2.11	1.64	1.45	2.56	1.49	5.86	1.78
AST (units/L) 5/6 day	48	62	62	ND	80	45	33
ALT (units/L) 5/6 day	277	496	101	ND	104	233	215
TB (umol/L) 5/6 day	27	85	127	ND	170	265	38
ALP (units/L) 5/6 day	202	152	95	ND	117	52	67
INR 5/6 day	1.16	1.16	1.27	ND	1.29	2.57	1.18

Liver biopsies (R0, R10, R60) were collected from patients (no. 6, 7, 11, 26, 27, 28, 30). This table reports the status of the patient (a, b, c indicate the cause of death as a: recurrence of viral hepatitis C; b: cerebrovascular accident; c: myocardial ischemia and infarction, respectively) and of the graft as well as blood concentrations at 22/29 h and 5/6 day postreperfusion of aspartate aminotransferase (AST; norm 6-35 units/L), alanine aminotransferase (ALT, norm 6–45 units/L), total bilirubin (TB; norm 1.7–18.9 umol/L) and alkaline phosphatase (ALP; norm 25–115 units/L). International normalization ratio (INR: norm 0.88–1.13). Shaded areas indicate values in the normal range. N = no; Y = yes; ND = not determined.

nodes and 80 edges based on experiment, data base and literature-related information (Figure 1E). Moreover, the gene network built from our transcriptional profiling revealed the expression of mRNA coding for proteins implicated in signaling (purple), transcription (orange), chaperone (blue) and metabolic (green) functions during the reperfusion phase of the operation.

Our microarray results were confirmed, using RT–PCR, for samples previously used for microarray experiments as well as independent samples. RT–PCR revealed a 9-fold and 4-fold increase for Hsp70 and MKP-1 mRNAs, respectively, in the 60-min time point biopsies compared to the 0 time point biopsies (Figure 2A). Immunoblotting analysis of MKP-1 protein, using an anti-MKP-1 antibody on liver biopsy extracts, revealed a 1.5-fold increase following normalization to the endoplasmic reticulum (ER) membrane-resident protein calnexin (Figure 2B). Finally, immunohistochemistry performed on frozen human liver sections revealed both cytoplasmic and nuclear staining for MKP-1 (Figure 2D) when compared to a control antibody (Figure 2C). Collectively, these data indicate that the ischemia-conditioned liver responds to reperfusion-related stresses, in part, by upregulating MKP-1 mRNA and protein in hepatocytes.

IRI-related stresses leading to the upregulation of MKP-1 mRNA

In an attempt to determine which of the stresses, implicated in the process of human liver transplantation, was involved in the upregulation of MKP-1 mRNA and protein expression, we used the well-characterized HepG2 cultures as a model (9). The ischemia and reperfusion-associated stresses were divided into four broad categories, namely thermal, oxidative and metabolic stresses, as well as chemically induced hypoxia-reperfusion. Each category was, in turn, subdivided into components (Figures 3–5). First, we assessed the effects of a 4–37°C thermal stress versus normothermia using three different media to account for the influence of thermal stress and storage solutions on the expression of the MKP-1 transcript. More specifically, HepG2 cells were incubated with either Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS), DMEM only or University of Wisconsin (UW) solution at 4°C (Figure 3A) or 37°C (Figure 3B), for 5 h. Thereafter, all cultures (Figure 3A and B) were incubated with DMEM + 10% FBS at 37°C for the indicated times. The heat-shock effect on MKP-1 mRNA expression was most noticeable (Figure 3A) as compared to its normothermic counterpart (Figure 3B). However, the use of UW solution delayed the induction of MKP-1 mRNA expression in the heat-shock model (Figure 3A). Finally, the presence of 10% FBS slightly increased the expression of MKP-1 mRNA in the normothermic paradigm (Figure 3B), but only when cultures were previously cultured in DMEM and not in UW solution.

The next transplantation-relevant stress modeled was the reducing-oxidative (redox) stress, using dithiothreitol (DTT) or H_2O_2 as a reducing or oxidizing agent, respectively. DTT induced both a time- and dose-dependent expression of MKP-1 mRNA, with the 30 mM concentration being more effective than the 100 mM at all times tested (Figure 4B). In contrast, hydrogen peroxide, which had previously been reported to increase MKP-1 mRNA expression (17), had no effect on this messenger using the present doses, times and cellular model (Figure 4A). Thus, in our model reducing, not oxidative, stress was a more potent inducer of MKP-1 mRNA expression. The facet of metabolic stress relating to ischemia reperfusion was subdivided into three components (Figure 5A–C). Antimycin A,

Table 2: List of all the upregulated genes in R60 samples versus R10 samples presenting at least two values with fold change > 1.5 in the sample set

cDNA probes	Swissprot/ TrEMBL Acc	Description	Gene name	Average fold increase	Number of cases with fold increase >1.5
H04421	P28562	Dual-specificity phosphatase 1	DUSP1	3.71	7
H29136	P28562	Dual-specificity phosphatase 1	DUSP1	4.01	7
R79387	P28562	Dual-specificity phosphatase 1	DUSP1	3.14	4
H17836	P34931	ym41b04.r1 Soares infant brain 1NIB Homo sapiens cDNA clone IMAGE:50615	HSPA1L	2.45	4
W63752	P25685	DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1	2.32	2
W40235	Q92598	Heat shock 105kDa/110kDa protein 1	HSPH1	2.12	3
BI715153	P17066	Heat shock 70kDa protein 6 (HSP70B')	HSPA6	2.14	4
N27681	P08107	Heat shock 70kDa protein 1A	HSPA1A	6.48	7
T74240	P08107	Heat shock 70kDa protein 1A	HSPA1A	2.91	4
BM450631	Q14568	Heat shock 90kDa protein 1, alpha-like 3	HSPCA	1.87	5
H62639	P11142	Heat shock 70kDa protein 8	HSPA8	1.88	4
BM920804	Q92730	Rho family GTPase 1	RND1	2.82	3
BI761144	Q92730	Rho family GTPase 1	RND1	1.65	3
W67471	P62745	Ras homolog gene family, member B	RHOB	1.21	1
R32051	P62745	yh63a08.r1 Soares placenta Nb2HP Homo sapiens cDNA clone IMAGE:134390	ARHB/ RHOB	2.53	4
R32051	P62745				
AA046598	P08833	Insulin-like growth factor binding protein 1	IGFBP1	1.80	4
AL575338	P08833	Insulin-like growth factor binding protein 1	IGFBP1	1.34	2
AA043477	P26651	Zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	2.39	6
BQ066997	P26651	Zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	1.73	4
W38673	Q02363	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	ID2	1.75	6
R83815	Q02363	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	ID2	1.51	3
BM782284	P62988	Ubiquitin B	UBB	2.44	3
BM999610	P05412	V-jun sarcoma virus 17 oncogene homolog (avian)	JUN	2.77	5
BQ048940	P17275	Jun B proto-oncogene	JUNB	2.12	3
H14887	P53539	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	2.64	5
H43827	A2A2C9	V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1.60	2
W87741	A2A2C9	V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1.54	3
AA115157	Q99576	TSC22 domain family, member 3	DSIPI	1.92	5
R01835	Q9NTK1	ye84g08.r1 Soares fetal liver spleen 1NFLS Homo sapiens cDNA clone IMAGE:124478.	DEPP	1.80	4
R86197	P35558	Phosphoenolpyruvate carboxykinase 1 (soluble)	PCK1	1.47	3
R21221	P05121	Serine (or cysteine) proteinase inhibitor, clade E member 1	SERPINE1	1.30	3
N92185	P22736	Nuclear receptor subfamily 4, group A, member 1	NR4A1	1.84	5

This list was generated following the removal of genes whose expression was found upregulated at R10 (as part of the blood contribution to this transcriptome analysis). Complementary DNA probe, Genbank accession, entry description and gene names are indicated as well as the average-fold increase in seven samples and the p-value observed for each probe. Finally, the number of samples where the fold increase was found to be >1.5 is also indicated.

a known mitochondrial complex III inhibitor, was one of the strongest MKP-1 mRNA inducers in our experimental model (Figure 5A). The next metabolic stressor to be tested alone or in conjunction with antimycin A was tunicamycin. The N-glycosylation inhibitor was used to simulate the unfolded protein response (UPR) (Figure 5B). As the UPR was activated during both phases of liver transplantation (8), MKP-1 mRNA expression might represent a potential target of this pathway. When tunicamycin was used alone, there was a slight induction of the MKP-1 transcript while the combination of both stressors seemed to have an anergistic effect (Figure 5B) in comparison to antimycin A-only treatment. In contrast, calcium depletion-related stress had no effect on MKP-1 mRNA expression level, as seen with the membrane-permeable calcium chelator 1,2-bis-(o-Aminophenoxy)-ethane-N,N,-N',

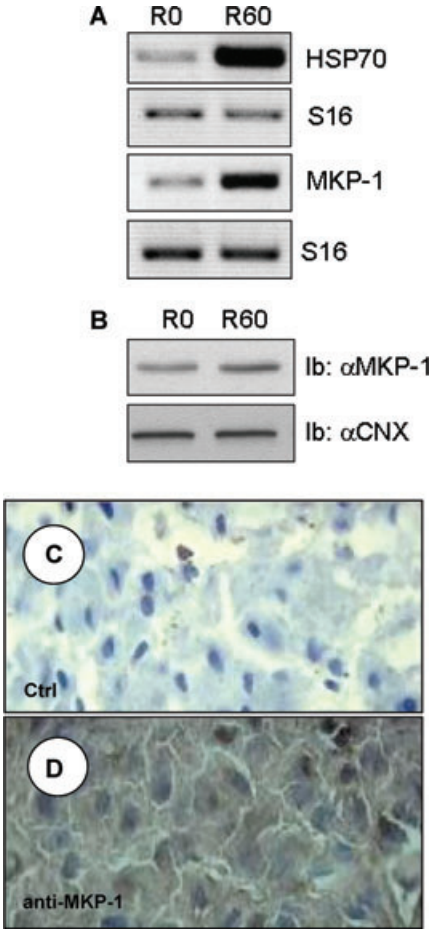

Figure 2: Validation of microarray results. (A) Validation by RT–PCR. Two biopsies collected at 0- and 60-min reperfusion time points were chosen from three different donor patients. For each biopsy, total RNA was used for RT–PCR. HSP70 or MKP-1 messenger was amplified from the 0- and 60-min reperfusion time points using the same number of cycles for both time points. S16, a gene coding for a ribosomal protein, was used as a control (30 cycles). This is a representative gel of three experiments. (B) Total protein extracted from human liver biopsies collected at 0- and 60-min time points were used for Western blot. Anti-MKP-1 antibody was used to reveal MKP-1, while an anticalnexin antibody was used as a loading control. Representative immunoblot: 1 of 3 different experiments. Immunohistochemistry: 12-μm thick frozen human liver sections. Images taken at 100× magnification. Hematoxilin staining reveals the nuclei in blue. (C) R0 secondary antibody only. (D) R60 rabbit anti-MKP-1, 1/250 dilution. The staining appears to be both nuclear and cytosolic in hepatocytes.

N′-tetraacetic acid tetra(acetoxymethyl) ester (Figure 5C) irrespective of the concentrations or times tested.

Finally, as both ischemia and hypoxia have been implicated in the regulation of MKP-1 mRNA (see Discussion), we used cobalt chloride ($CoCl_2$), a prolyl hydroxylase inhibitor, to stabilize hypoxia-inducible factor-1 alpha, and simulate hypoxia. Using a 100-μM dose of $CoCl_2$ at various time points, we did not observe any modulation of the MKP-1 mRNA levels in either 'hypoxic' or 'reperfusion' phases (data not shown).

Signaling specificity of stress-induced MKP-1 mRNA expression

As modulation of MKP-1 mRNA has been associated with many signal transduction pathways, including mitogen-activated protein kinases (MAPKs) and calcium, we used kinase inhibitors in conjunction with DTT, antimycin A or heat shock to characterize the upstream signaling pathways leading to MKP-1 regulation (Figure 6). Because these inhibitors were reported to either decrease IR-mediated damage or abolish the beneficial effects of ischemic preconditioning (see Discussion), we determined their ability to modulate MKP-1 mRNA in the absence of stressors (Figure 6A), or in conjunction with stress-mediated induction of the transcript (Figure 6B–F). Compared to the untreated control, none of the inhibitors had any significant effect on the expression of the transcript at 37°C (Figure 6A) or at 4°C (Figure 6D). Upon use of either antimycin A (Figure 6B) or DTT (Figure 6C), the most effective inhibitor of MKP-1 mRNA upregulation was the tyrosine kinase inhibitor genistein (3 or 10 μM). The casein kinase-2 (CK2) inhibitor 5,6-dichloro-1-B-D-ribofuramosylbenzinidazole (3 μM) was very effective at inhibiting DTT- but not antimycin A-mediated upregulation of the messenger, thus implicating a CK2-dependent pathway in DTT-induced stress. Moreover, roscovitine 10 μM (a cyclin-dependent kinase inhibitor) and PD98059 10 μM (a MEK-1 inhibitor) were effective at preventing MKP-1 mRNA upregulation upon DTT, antimycin A or heat-shock treatment (Figure 6 B, C, E and F). These results delineate major signaling pathways responsible for the upregulation of MKP-1 mRNA, and suggest that the activation of the MEK/ERK pathway may be an important axis in curtailing IRI via MKP-1. Although nonredundant signaling pathways can be activated, the various stresses may lead to MKP-1 mRNA upregulation as a common denominator, whether or not they funnel through common effectors to achieve this regulation.

Targeting MKP-1 expression alters stress-activated protein kinase (SAPK) activation upon IRI-related stress

We sought to prevent stress-induced MKP-1 mRNA expression in our cellular model in order to determine the impact of MKP-1 silencing on stress-mediated MAPK/SAPK activation. This line of investigation revealed that: (i) MKP-1 mRNA was upregulated during the reperfusion phase of liver transplantation; (ii) all three MAPK pathways were activated upon liver transplantation (8); (iii) MKP-1 attenuated IRI-mediated damage in cardiomyocytes (18); and (iv) the reduction of ischemia mediated cardiac infarct size in MKP-1 cardiac transgenic mice (18). Thus, we silenced the expression of MKP-1 using an ShRNA (Sh)-based strategy as previously reported (19). MKP-1 protein expression was determined at both 24- and 48-h posttransfection

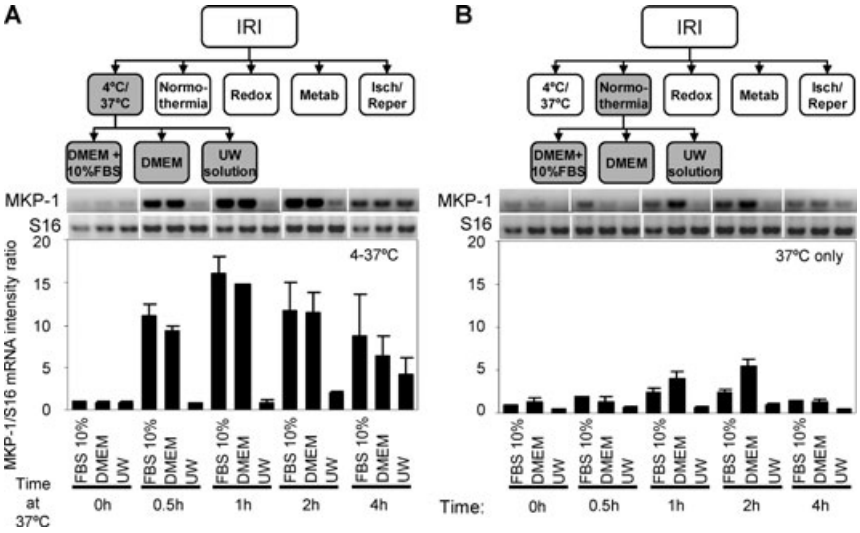

Figure 3: Themal stress mediated expression of MKP-1/DUSP1 mRNA in HepG2 cultures. (A) The effect of cold stress–warm reperfusion (4–37°C) in three media (DMEM + 10% FBS, DMEM only and UW) on MKP-1/DUSPI mRNA expression by RT—PCR. Normalization was carried out using S16 mRNA. (B) Same media as A, but under normothermic conditions (no cold stress or heat shock). Quantification for A and B was performed on two independent experiments and is represented ± SEM.

(Sh) by immunobloting using an anti-MKP-1 antibody and compared these results to cells treated with the transfection reagent alone (Ctrl) or a scrambled ScrRNA sequence (Scr) (19). The expression of MKP-1 was reduced by 70–80% at 48-h posttransfection of Sh compared to Scr and Ctrl. The 48-h time point was used for the subsequent experiments (Figure 7A).

To evaluate the impact of MKP-1 silencing on the activation status of MAPK/SAPK pathways, confluent HepG2 cultures were placed in the UW solution at 4°C in the presence of antimycin A for 1 h, which is used as a model for ATP depletion during the ischemic phase (9). Following this treatment, the medium was replaced by DMEM + 10% FBS at 37°C, to mimic the 'reperfusion' step. Cells were collected at 60, 120, 240 and 300 min post 'reperfusion' and lysates were evaluated for extracellular regulated kinase-1 (ERK-1), $p38^{MAPK}$ and Jun-N-terminal kinase-1 (JNK-1) activity (Figure 7B). In ScrRNA transfected cells, both $p38^{MAPK}$ and ERK followed a similar pattern of activation, reaching a peak at 60-min postreperfusion, then decreasing to reach basal levels of activity at 240 min. JNK activation also reached a peak at 60-min postreperfusion, but this activity was sustained and did not return to basal levels even after 300 min. Interestingly, in MKP-1 ShRNA transfected cells, ERK activity remained similar to that observed in ScrRNA-transfected cells. However, using the same concept, both $p38^{MAPK}$ and JNK displayed a delayed inactivation with a sustained activity (≥ 2-fold above basal level) when MKP-1 ShRNA versus ScrRNA were used. These results suggest that MKP-1 may selectively participate in the inactivation of both JNK and $p38^{MAPK}$ during the reperfusion phase and consequently participate in cell survival. This is reflected by the number of apoptotic cells at the 24-h time point following MKP1 ShRNA transfection versus ScrRNA control (Figure 7C). These results suggest that a reduction in

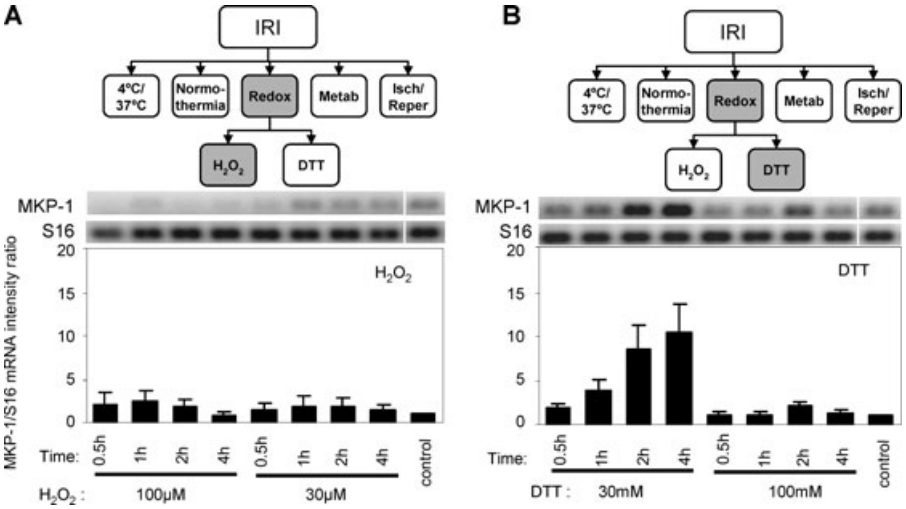

Figure 4: Oxidative stress-mediated expression of MKP-1/DUSP1 mRNA in HepG2 cultures. (A) The expression of MKP-1/DUSP1 mRNA was evaluated by RT–PCR in HepG2 cells treated with either 100 μM or 30 μM H_2O_2 for 0.5, 1, 2 and 4 h. (B) The same experiments were carried out using DTT instead of H_2O_2. Normalization was carried out using S16 mRNA. Quantification was performed on three independent experiments and is represented ± SEM.

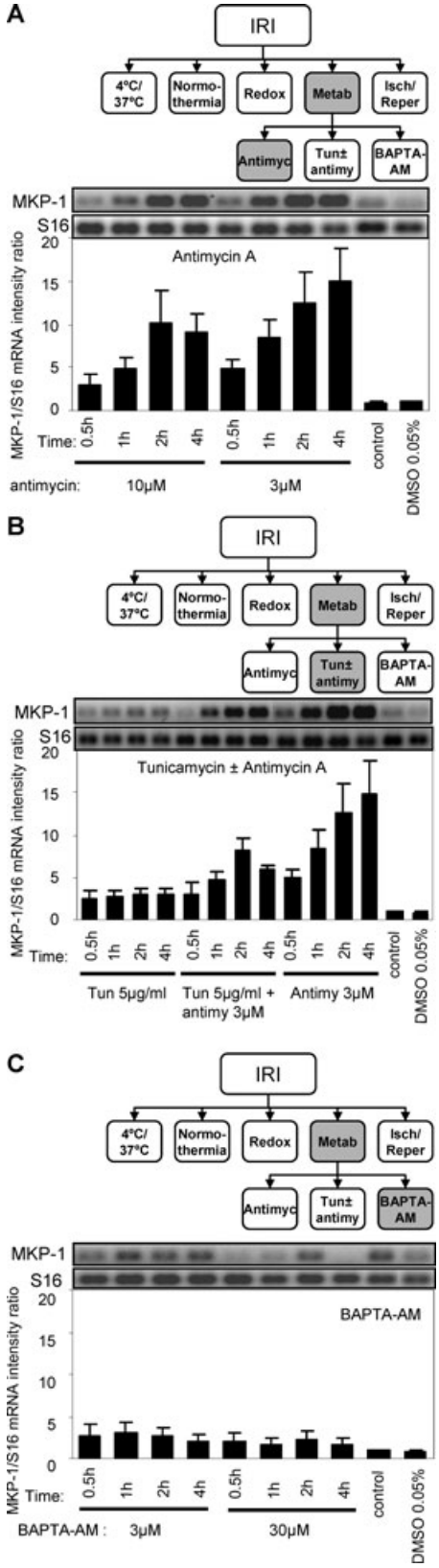

Figure 5: Continued.

MKP-1 expression allows for longer JNK activity leading to increased apoptosis as previously proposed in other experimental paradigms (20).

Discussion

To our knowledge, this study is the first to evaluate mRNA expression profiles specifically during the reperfusion phase of human liver transplantation, taking the recipient blood-born mRNA into account. Although reports have already described mRNA expression profiles in human liver transplantation (6,7), these studies did not dissociate the ischemic phase from the reperfusion phase of transplantation. Our analysis consequently provides a clear picture of early regulatory events during the reperfusion phase of the operation. RNA expression profiling during the reperfusion phase of transplantation has led to the identification of 23 nonredundant regulated genes. Most of the upregulated genes belong to regulatory and stress-related pathways. This indicated that besides the expected upregulation of known immediate-early genes (jun, fos), a number of genes involved in signaling were also modified. Finally, using a STRING-based analysis, supported by experiment, data base and literature-related information, we found that 21 of the immediate early genes identified in this study belonged to a functional network. This combination of mRNA expression may represent a hepatic immediate-early gene response as a consequence of ischemia-conditioned reperfusion-related stress. This is one of the avenues currently under investigation.

Interestingly, our study differed from other reports on hepatic transcriptional profiling. More precisely, we found a low number of identical entries between our study and that of Conti et al. (6) or that of Defamie et al. (7) in the upregulated gene categories. The low correspondence of identical entries representing regulated genes, between our study and the others, may be attributed to: (i) our study comparing the 60-min reperfusion time point to a prereperfusion time point (zero time point) during the reimplantation phase of the operation (the reperfusion phase is separated from the ischemic phase); (ii) the 0 time point, 10 min and 60 min time points derive from the same organ, for a given set;

Figure 5: Metabolic stress-mediated expression of MKP-1/DUPS1 in culture. (A) HepG2 cells were treated with 10 μM or 3 μM antimycin A for 0.5, 1, 2 or 4 h and MKP-1/DUSP1 mRNA expression was evaluated by RT–PCR. (B) The combination of antimycin A (3 μM) and tunicamycin (5 μg/mL) has an intermediate effect on the upregulation of MKP-1 mRNA in relation to the modest effect of tunicamycin only and the potent antimycin A only regulation of the transcript, at all the times tested. (C) Similar experiments were carried out using 3 μM and 30 μM BAPTA-AM as stressor. Normalization was carried out using S16 mRNA. Quantification was performed on three independent experiments and is represented ± SEM.

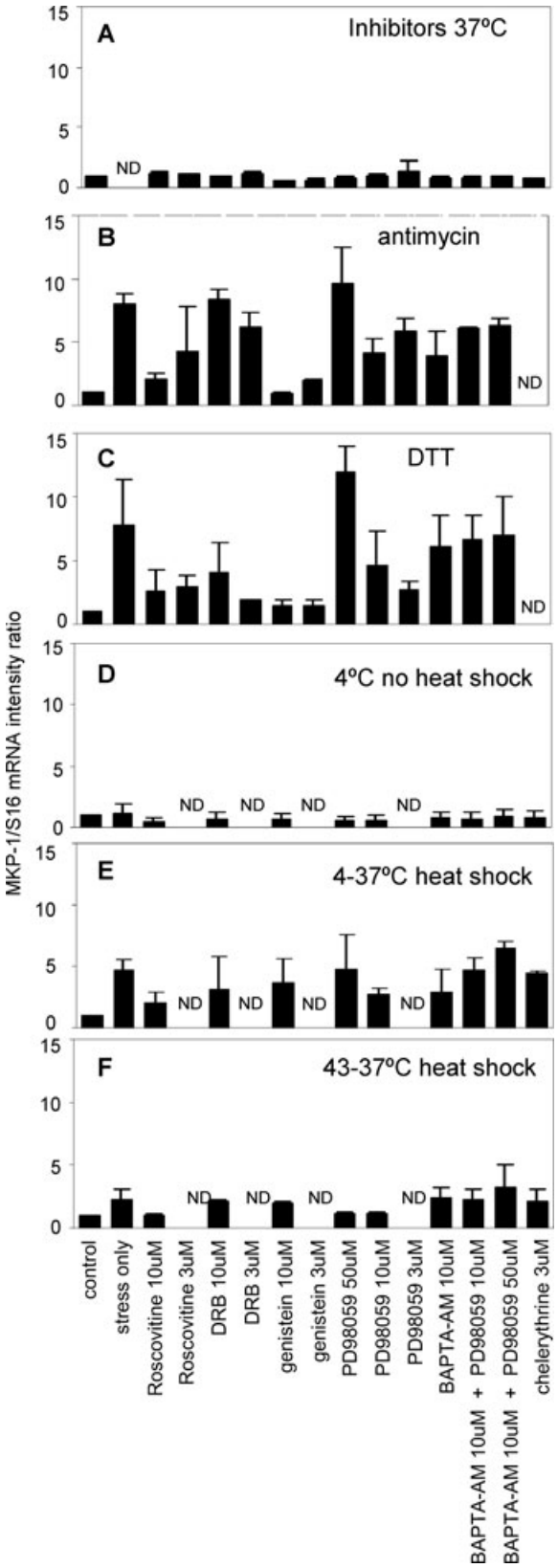

Figure 6: Continued.

(iii) a difference in the time point of samples analyzed (long [2–3 h] reperfusion time in the Conti study vs. a 1-h reperfusion time point in our work); (iv) their lists of upregulated genes mostly comprised larger (∼270-down to 8-) fold increases for Conti et al. than ours, ∼3.5-down to 1.5-fold. The genes with the lower fold increase were not listed (6); and (v) although our study and the Conti and the Defamie studies use microarray chips from different sources, this may not account for difference in results (21,22).

The dual-specificity MKP-1 has a role in the regulation of ischemia/reperfusion-related injury. Cardiac transgenic mice overexpressing MKP-1 were protected from cardiac IRI whereas knockout mice were more sensitive (18). Although the transgenic and knockout mice were on different genetic backgrounds, a rat model of global cerebral ischemia also confirmed these findings (23). Taken together, these observations led us to investigate the potential role of MKP-1 in liver cells upon IRI. Others and we previously demonstrated that the three axes of the MAPK pathways ($p38^{MAPK}$, JNK and ERK) were activated upon liver reperfusion (8,24). As such, an increase in MKP-1 expression may be part of a regulatory mechanism to moderate this activation and allow the liver to return to its basal state following transplantation. We propose a model in which, following transplantation, an initial phase that is dependent on the MAPK pathway (ERK, $p38^{MAPK}$ or JNK) would increase the expression of MKP-1. This increase would promote mainly the dephosphorylation and therefore inactivation of JNK and $p38^{MAPK}$, thus allowing hepatocytes to survive IRI during human liver transplantation.

Many of the inhibitors used in this study were previously reported to influence the extent of ischemia/reperfusion-mediated damage. Interestingly, none of them led to increased MKP-1 mRNA levels, at 37°C or 4°C, when used overnight. As MKP-1 is an immediate-early gene, it remains possible that the inhibitors stimulate MKP-1 expression at an earlier time point. The experiments carried out at 37°C or 4°C were used as controls for the thermal or chemical stress-inducing agents. Thus, roscovitine and genistein inhibited both antimycin A and DTT-mediated increase in MKP-1 mRNA, while roscovitine also inhibited heat-shock-mediated increase in this transcript. BAPTA-AM had no appreciable effect on MKP-1 mRNA levels. As the process

Figure 6: Impact of various kinase inhibitors on stress-mediated MKP-1/DUSP1 mRNA expression. The effect of five kinase inhibitors and a calcium chelator, thereafter collectively referred to as inhibitors in this figure legend, on stress-mediated MKP-1/DUSP1 expression in HepG2 cells was evaluated by RT–PCR. (A) Inhibitors only, at 37°C. (B) Antimycin A ± inhibitors at 37°C. (C) DTT ± inhibitors at 37°C. (D) Cold stress (4°C) ± inhibitors. (E) 4–37°C: heat shock ± inhibitors. (F) 43–37°C: heat shock only (no cold stress) ± inhibitors. All inhibitors were left overnight. Normalization was carried out using S16 mRNA and quantification is represented by ± SEM.

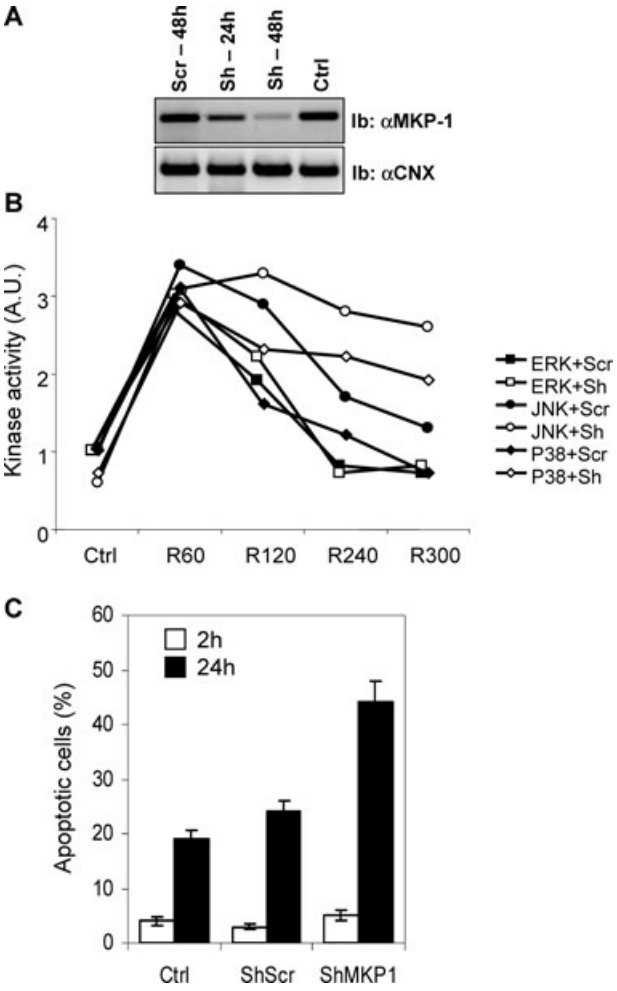

Figure 7: MKP-1/DUSP1 silencing affects SAPK/MAPK activation upon IR-related stress in HepG2 cells. (A) MKP-1/DUSP1 silencing was carried out using an ShRNA-based approach. Twenty-four and 48-h posttransfection, MKP-1/DUSP1 protein expression was evaluated by immunobloting. Standardization was carried out using an antibody against the endoplasmic reticulum resident protein calnexin. (B) The activation of $p38^{MAPK}$, JNK-1 and ERK-1 was measured in HepG2 cells subjected to IR-related stress following transfection with an ShRNA targeting MKP-1 (open symbols) or a scrambled sequence (Scr; closed symbols). (C) HepG2 cells were transfected or not with a plasmid containing a scrambled ShRNA or MKP-1 targeting ShRNA. Thirty-six hours posttransfection, cells were subjected to a 2-h cold ischemia, then placed in standard culture medium and 22 h later, the percentage of apoptotic cells determined (white bars indicate the percentage of apoptotic cells after 2-h cold ischemia whereas the black bars report the same information but after 24 h).

of organ transplantation invokes multiple stressors, it remains possible that the signal transduction inhibitors moderate the deleterious effects of ischemia/reperfusion *in vivo* while allowing the increased expression of endogenous regulators such as MKP-1 at the gene or protein level. Otherwise, their mechanism of action may not rely on MKP-1. These alternative possibilities remain to be tested *in vivo*.

Other inhibitors have a deleterious effect, when administered prior to ischemic preconditioning, a method used to reduce liver injury (25). This is confirmed for genistein and chelerythrine as they eliminate the beneficial effects of ischemic preconditioning (26). Our results show that some of the inhibitors tested such as genistein or PD98059, did reduce stress-mediated upregulation of MKP-1 mRNA, caused by DTT and antimycin A, while PD98059 also inhibits heat-shock-induced MKP-1 transcript. Interestingly, our results also demonstrate that DRB was most effective against DTT-induced MKP-1 mRNA. DTT induces ER stress and is used to model a facet of UPR. It is possible that lack of cold preservation, heat shock, or blood supply reduces ER stress, when the tissue is reperfused *ex vivo* with a 37°C buffer solution (Krebs-Henseleit) (27).

The evaluation of reperfusion-induced immediate-early gene response, during human liver transplantation, has provided us with a number of novel and potentially therapeutic targets. The liver's adaptive response to transplantation represents an effective means of understanding and improving the marginal livers response to transplantation. This is a necessary step if a protocol is to be created that would allow these livers to be successfully transplanted. In doing so, the chasm between donor and recipient numbers would dwindle.

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Supporting Information

The following supporting information is available for this article.

Figure S1. Scatter plot of the fluorescence intensities on the experiment and control channels. The spots with an average change in fluorescence ratio of more than 1.5-fold (diagonal blue lines) that show acceptable fluorescence intensity and low-intensity spots tended to have a fluorescence ratio that would approach 1.

Table S1. Summary of microarray analyses carried out in our study. Samples compared are indicated. Cy3 and Cy5 labeling are indicated in green and red respectively.

Table S2. The fluorescence intensities for the experiment (R60) and control (R0 or R10) on a per-chip and per-sample basis, as well as the fluorescence intensity for the ratios red/green and its dye swap green/red, on a per-chip basis and per-sample basis.

Table S3. The average of the fluorescence intensities ratios for the red/green and its dye swap green/red for each normalized experiment, R60R0 or R60R10.

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