Chaperonin10: an endothelial-derived, erythropoietin-dependent differentiation factor

By

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Abstract

Erythropoietin (EPO) stimulates endothelial cells to produce various factors that support the formation of erythroid cells. We identified by 2D electrophoresis/mass spectrometry one such factor as being chaperonin 10 (cpn10). Cpn10 was released in human umbilical vein endothelial cells (HUVEC) medium after EPO treatment; it decreased the proliferation of the erythroleukemic K562 cells, while stimulating differentiation in the erythroid TF-1 cells and skin fibroblasts. We analyzed the early events initiated by the addition of cpn10 in K562 and TF-1 cells and found significant changes in the phosphorylation levels of glycogen synthase kinase 3 (GSK-3) and cofilin-1. Further experiments using GSK-3 inhibitors in the presence or absence of cpn10 showed an alteration in the proliferation and differentiation patterns previously observed in TF-1 cells, suggesting a possible role for GSK-3 in cell differentiation as part of the signal transduction pathway triggered by cpn10. This is the first evidence linking cpn10 to erythropoiesis.

Abrégé

L'érythropoïétine (EPO) stimule les cellules endothéliales à produire différents facteurs qui soutiennent la formation des érythrocytes. En effectuant une électrophorèse-2D / spectrométrie de masse, on a identifié la chapéronine10 (cpn10) comme étant un de ces facteurs. Cpn10 est secrétée par les cellules endothéliales HUVEC après un ajout d'EPO; elle diminue la prolifération des cellules érythroleucémiques K562 et elle stimule la différentiation des érythrocytes TF-1 et des fibroblastes. On a observé qu'une des actions immédiates initiées par cpn10 dans les cellules K562 et TF-1 était de changer significativement la phosphorylation de GSK-3 (glycogen synthase kinase 3) et cofilin-1. Des inhibiteurs de GSK-3 utilisés en présence de cpn10 ou seuls ont altéré le processus de prolifération et différentiation observés auparavant avec les cellules TF-1, en suggérant ainsi que GSK-3 puisse jouer un rôle dans la différentiation cellulaire déclenchée par cpn10. C'est la première fois qu'un lien est décrit entre cpn10 et l'érythropoïèse.

Table of contents

List of figures

List of abbreviations

ADP:	adenosine diphosphate
Akt:	protein kinase B
Apaf-1:	apoptotic protease-activating factor 1
ARNT:	aryl hydrocarbon receptor translator
ATP:	adenosine triphosphate
AZT:	azidothymidine
Bad:	Bcl-2-antagonist of cell death
Bcl-2:	B-cell lymphoma protein 2 alpha
BDNF:	brain-derived neurotrophic factor
BFU-E:	burst-forming unit erythroid
BMEC:	bone marrow endothelial cells
BSA:	bovine serum albumin
C21:	C-terminal peptide of TSP-4, last 21 amino acids
Cdc42:	Cell division control protein 42 homolog
CFU-E:	colony-forming unit erythroid
C.P.M.:	counts per minute
cpn10:	chaperonin 10
DMEM:	Dulbecco's Modified Eagle Media
DTT:	dithiothreitol
EAE:	experimental autoimmune encephalomyelitis
EBM-2:	Endothelial Cell Basal Medium-2

EDTA: ethylene diamine tetraacetic acid

EGTA: ethylene glycol tetraacetic acid

ELISA: Enzyme-Linked ImmunoSorbent Assay

EPF: early pregnancy factor

EPO: erythropoietin

EPO-R: erythropoietin receptor

ER: endoplasmic reticulum

ERK: extracellular signal - regulated kinase

FBS: fetal bovine serum

FGF: fibroblast growth factor

GEMM: the common precursor for Granulocytes, Erythrocytes, Monocytes and Megakaryocytes

GDP: guanosine diphosphate

GM: granulocytes and macrophages

GM-CSF: granulocyte-macrophage colony-stimulating factor

GSK-3: glycogen synthase kinase 3

Grb2-SOS: growth factor receptor binding 2 - SOS

GTP: guanosine triphosphate

HAF: hypoxia-associated factor

HIF-1α/ β: hypoxia-inducible transcription factor 1 alpha/beta

HIF-PHD: hypoxia-inducible transcription factor 1 prolyl-4-hydroxylases

HIV: human immunodeficiency virus

HNF-4: hepatocyte nuclear factor 4

HRE: hypoxia-response element

Hsp: heat-shock protein

HUVEC: human umbilical vein endothelial cells

IGF-1: insulin-like growth factor-1

IGFBP-3: insulin-like growth factor binding protein 3

IL: interleukin

INF-γ: interferon gamma

Jak2: Janus kinase 2

kDa: kilo Daltons

KIE: kidney inducible element

LIE: liver-inducible element

LIMK1: LIM domain kinase 1

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinase

MEK: mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase

MEP: megakaryocytes and erythroid cells

MOI: multiplicity of infection

MS: multiple sclerosis

NF-κB: nuclear factor κB

NK: natural killer

NRE: negative regulatory element

NRLE: negative regulatory liver element

O-DDD: oxygen-dependent degradation motif

p300/CBP: p300 / cAMP response element binding protein (CREB) - binding protein

p90RSK: p90Ribosomal kinase

Par6/PKC: Par6-atypical protein kinase C

PBS: phosphate-buffered saline

PI-3K: phosphatidylinositol 3-kinase

PKC: protein kinase C

rhEPO: recombinant human erythropoietin

RANTES: regulated upon activation, normal T cell expressed and secreted

SCF: stem cell factor

SDS-PAGE: sodium dodecyl sulfate - polyacrylamide gel electrophoresis

Shc: Src (steroid receptor coactivator) homologue and collagen homologue

STAT5: signal transducer and activator of transcription 5

TBST: Tween-Tris-buffered saline

Th1/Th2: type 1/type 2 T helper cell

TLR: toll-like receptor

TNF-α: tumor necrosis factor alpha

TPO: thrombopoietin

TSP: thrombospondin

VEGF: vascular endothelial growth factor

VHL: von Hippel-Lindau tumour suppressor protein

Foreword

The present thesis is not manuscript-based; however, the results presented herein are also the subject of an article already accepted for publication in the *Journal of Cellular Physiology*. Proof of acceptance together with the complete title of the article and authors' names are attached as appendixes. As first author, I have made the greatest contribution to this investigation. Dr. Gulzhakhan Sadvakassova, a former research assistant in our laboratory, started this research project with the 2D electrophoresis experiment that lead to the discovery of cpn10 as an endothelial-derived EPO-dependent factor, which constitutes the main subject of this thesis. Dr. Sadvakassova also helped me in setting up and successfully achieving certain experiments, such as the proliferation assays, the determinations of differentiation factors in TF-1 cells and the productive Lentiviral transduction of fibroblasts. The entire research was conducted with the guidance and incommensurable support of my supervisor, Dr. Luis Fernando Congote. Without their help, the completion of this project would not have been possible.

I. Erythropoietin and its role in erythropoiesis

1. General facts about erythropoietin

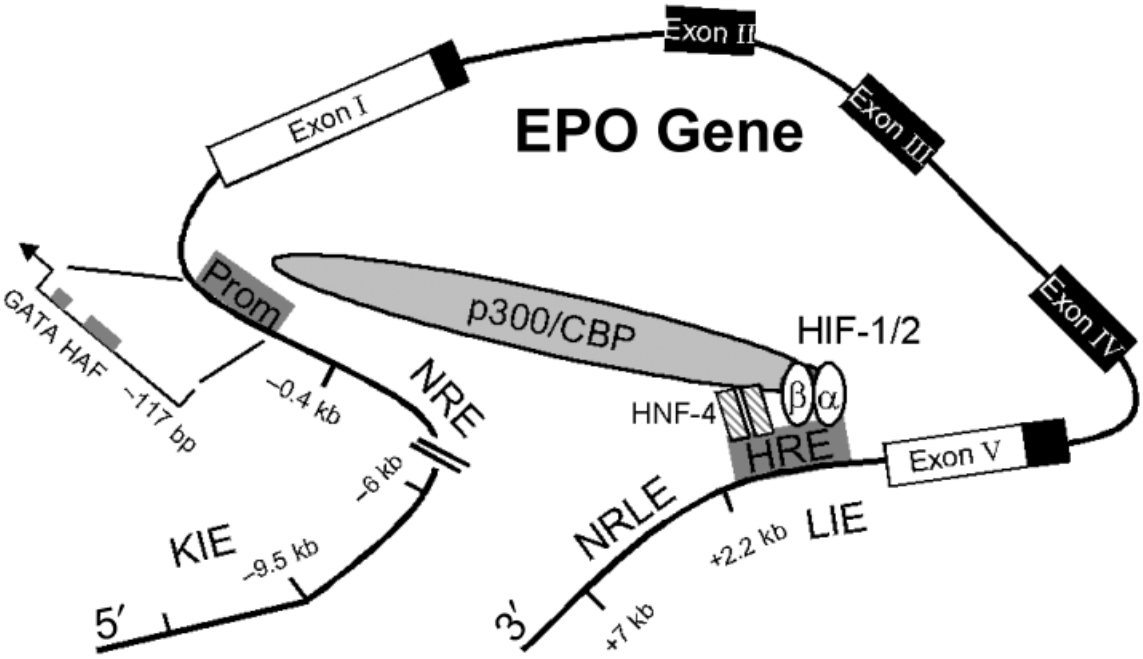
Although the role of red blood cells in oxygen transport and tissue distribution was known since the second half of the nineteenth century, it was only in 1906 that Carnot and Deflandre discovered that a humoral factor was responsible for the increase of erythrocyte production in rabbits. They called this substance “hemopoietine”, but this name was changed in 1948 to the one still used today, “erythropoietin” [1]. The first role attributed to erythropoietin (EPO) as a hormone was the increase of red blood cell production by promoting the proliferation and differentiation of erythroid precursors in the bone marrow [2] in response to hypoxia [3]. Its main sites of production are the hepatocytes in the fetal liver and the fibroblast-like peritubular interstitial cells in the adult kidney [4, 5]. However, recent evidence exposits secondary sites of EPO production in adults, including the brain, vascular system, heart and bone marrow [6].

2. Hypoxic regulation of the EPO gene

In spite of what one would expect, a reduced number of red blood cells does not stimulate the production of EPO, whose secretion is mainly regulated by the tissues’ oxygen availability. In response to anemia or hypoxia, there is an increase in transcription of the EPO gene. This gene contains the hypoxia-response element (HRE) within its 3’ enhancer

region, which binds to the hypoxia-inducible transcription factor 1 (HIF-1α/β). HIF-1β is insensitive to oxygen levels and is constitutively expressed, whereas HIF-1α expression is tightly regulated by hypoxia. The C-terminal of HIF-1α contains an oxygen-dependent degradation motif (O-DDD) with conserved proline residues at positions 402 and 564. In the presence of oxygen, iron and 2-oxoglutarate, these proline residues are hydroxylated by specific prolyl-4-hydroxylases (HIF-PHDs), reaction that enables HIF-1α to bind to the von Hippel-Lindau tumour suppressor protein (VHL). The complex formed by HIF-1α and VHL is polyubiquitinated by E3 ligase and targeted for degradation [7]. Therefore, HIF-PHDs are responsible for impeding the HIF-1α translocation to the nucleus and the subsequent EPO gene transcription under normal oxygen conditions. Normally, in conditions of low oxygen, the hydroxylation by HIF-PHD does not occur; HIF-1α is stabilized and can translocate to the nucleus. There it will form a dimer with HIF-1β (also known as the aryl hydrocarbon receptor translator, ARNT) and bind to the HRE in the 3' enhancer of the EPO gene. Moreover, the HIF-1α contains N- and C-terminal activation domains that bind to coactivators such as p300/cAMP response element binding protein (CREB)-binding protein (CBP). The constitutive hepatocyte nuclear factor 4 (HNF-4), expressed in liver, renal cortex and certain intestinal cells, also binds to the HRE as well as to p300/CBP; the latter is considered a "bridging" coactivator because it binds simultaneously to the EPO gene promoter, causing a loop in the

Fig. 1. The EPO gene and its oxygen-dependent regulation. In the presence of normal oxygen levels, proline resides within the hypoxia-inducible transcription factor 1 alpha (HIF-1α) get hydroxylated, which allows it to interact with the von Hippel-Lindau tumour suppressor protein (VHL) and targets it for degradation. During hypoxia, this hydroxylation doesn't occur. HIF-1α is stabilized, it can translocate to the nucleus and form a dimer with the hypoxia-inducible transcription factor 1 beta (HIF-1β). This dimer can bind to the hypoxia-response element (HRE) within the enhancer, a region also bound by the hepatocyte nuclear factor 4 (HNF-4). Both HIF-1α and HNF-4 bind the "bridging" coactivator p300/cAMP response element binding protein (CREB)-binding protein (CBP), required for the full expression of the EPO gene. Additional gene regulatory elements include the kidney inducible element (KIE), the negative regulatory element (NRE), the liver-inducible element (LIE) and the negative regulatory liver element (NRLE). The minimal promoter consists of the GATA-binding site and the hypoxia-associated factor (HAF). This schematic representation of the gene is not to scale. (Reproduced from [7] with the permission of the journal *Clinical and Experimental Pharmacology and Physiology*).


EPO Gene
Exon I
Exon II
Exon III
Exon IV
Exon V
Prom
p300/CBP
HIF-1/2
HNF-4
β
α
HRE
NRE
GATA HAF
−117 bp
−0.4 kb
−6 kb
KIE
−9.5 kb
5′
NRLE
+2.2 kb
LIE
3′
+7 kb

DNA [7]. A schematic representation of the EPO gene and the various factors involved in its oxygen-dependent regulation is depicted in **Fig. 1**. This cooperative binding stimulates the transcription of EPO and other hypoxia-responsive genes. The entire EPO system is highly sensitive, allowing the hemoglobin levels to be relatively constant under normal conditions and rapidly adapt according to oxygen availability.

3. Role of EPO in red blood cell formation

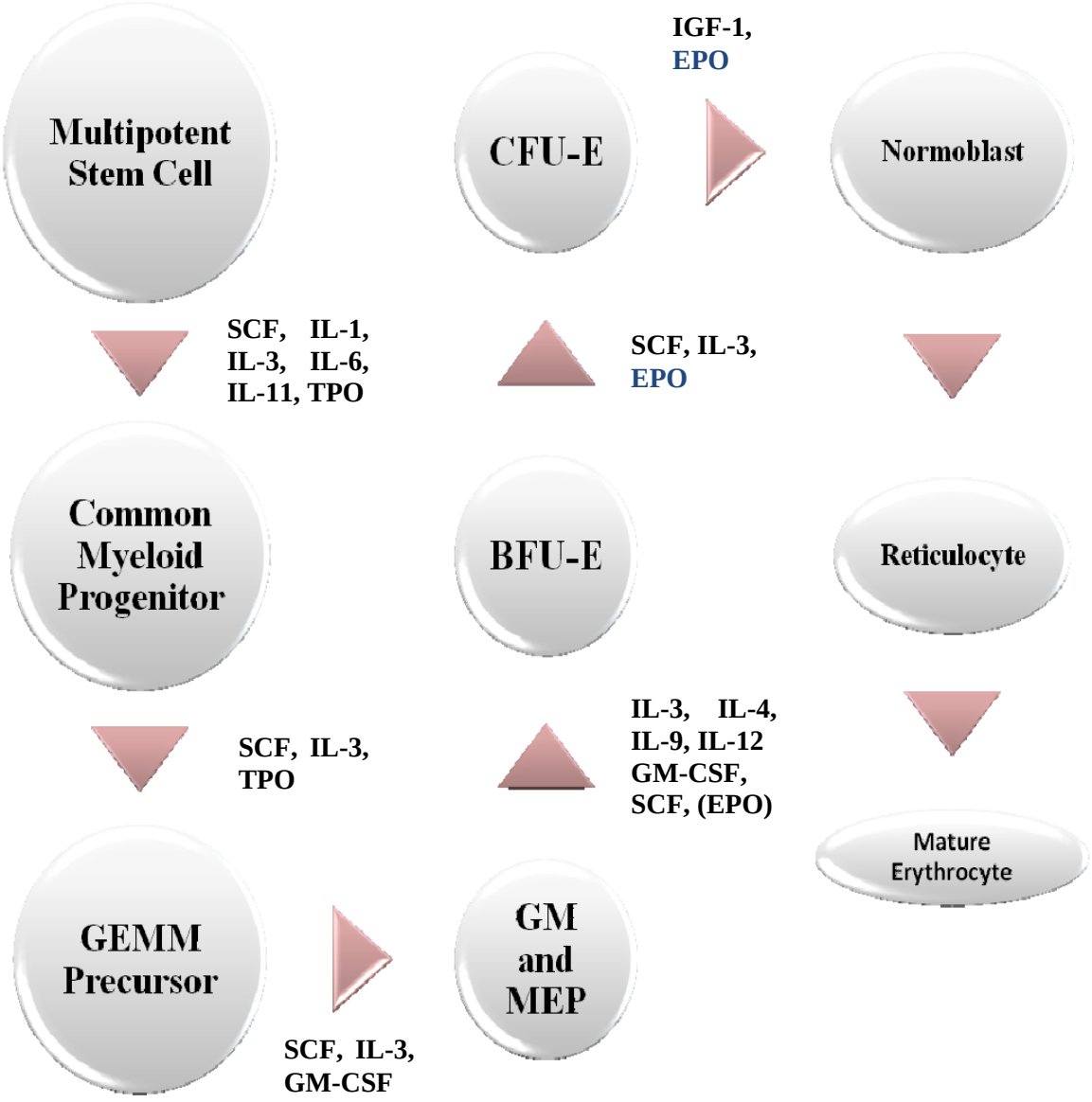
EPO is a highly glycosylated 30.4 kDa protein which contains 165 amino acids [1] and whose primary role is to sustain an adequate supply of erythrocytes throughout an individual's life. In order to achieve this, EPO must accommodate both proliferation and differentiation, two functions that are normally mutually exclusive. Erythropoiesis designates the maturation of erythrocytes from a multipotent stem cell under the action of various growth factors and interleukins, with EPO coming into play only at the later stages of development [5]. The first committed erythroid progenitor in this hierarchical process is the burst-forming unit erythroid (BFU-E). BFU-E's have few EPO receptors (EPO-R) and they require a large number of growth factors in order to proliferate. The most significant contribution is done by the stem cell factor (SCF), which synergizes with EPO in order to increase the size of BFU-E colonies and to secure their survival [8]. These colonies then give rise to colony-forming unit erythroid (CFU-E's), which are the most sensitive to and dependant

on EPO. Experiments involving the disruption of the EPO-R gene pointed out that the signaling through the EPO-R is not required for the proliferation of cells earlier than CFU-E, but that definitive erythropoiesis beyond the stage of CFU-E is inhibited by a mutation in EPO-R with the result that no mature erythrocytes are produced [9]. In other words, EPO acts both as a mitogen (in BFU-E) and a survival factor (in CFU-E) [10]. It maintains erythropoiesis by protecting CFU-E's from apoptosis, thus allowing them to mature through the pronormoblast and normoblast stages. The normoblasts loose their nucleus to become reticulocytes, which are converted to the circulating red blood cell (erythrocyte) responsible for oxygen transport [5]. **Fig. 2** illustrates a simplified schematic representation of erythropoiesis and points out the stages where EPO is known to act.

4. Signal transduction pathways of EPO

Initially considered a hormone, EPO is now recognized to be a multifunctional protein belonging to the pilotrophic cytokine superfamily [11]. Its receptor, EPO-R, belongs to the cytokine receptor superfamily and it consists of a single subunit, with one transmembrane domain and no intrinsic tyrosine kinase activity [12]. The binding of one EPO molecule induces the dimerization of the receptor, followed by an auto- or trans-phosphorylation of the Janus kinase 2 (Jak2) associated with the EPO-R's intracellular domain. The now activated Jak2 tyrosine kinases further

Fig. 2. A simplified model of erythropoiesis. The multipotent hematopoietic stem cell can either undergo self-renewal, or differentiate into either common lymphoid progenitors (not shown here) or common myeloid progenitors. The myeloid progenitor then gives rise to GEMM precursors (the common stem cell for Granulocytes, Erythrocytes, Monocytes and Megakaryocytes). These are the precursors for the more differentiated granulocytes and macrophages (GMs), and megakaryocytes and erythroid cells (MEPs). The earliest committed erythroid precursors, the burst-forming units erythroid (BFU-Es), have few EPO-Rs and require a large number of growth factors and interleukins to proliferate [8]. EPO synergizes with SCF to increase the size and numbers of BFU-Es and to prevent their apoptosis, but is not absolutely required at this stage. On the other hand, the direct descendant of BFU-E, the colony-forming unit erythroid (CFU-E), has an absolute requirement for EPO in order to avoid apoptosis and to mature through the normoblast stage. The normoblasts loose their nucleus to become reticulocytes, which are then converted to the mature erythrocytes (i.e. the circulating red blood cells). The various cytokines and growth factors involved in this process are shown at the specific stage where they are required. EPO denotes erythropoietin, GM-CSF granulocyte-macrophage colony-stimulating factor, IGF-1 insulin-like growth factor 1, IL interleukin, SCF stem cell factor and TPO thrombopoietin. (Adapted from [5] and [13]).


Multipotent Stem Cell
SCF, IL-1, IL-3, IL-6, IL-11, TPO
Common Myeloid Progenitor
SCF, IL-3, TPO
GEMM Precursor
SCF, IL-3, GM-CSF
GM and MEP
IL-3, IL-4, IL-9, IL-12 GM-CSF, SCF, (EPO)
BFU-E
SCF, IL-3, EPO
CFU-E
IGF-1, EPO
Normoblast
Reticulocyte
Mature Erythrocyte

phosporylate specific tyrosine residues on the receptor's cytoplasmic domain, allowing the binding of the monomeric cytosolic proteins STAT5 (signal transducer and activator of transcription 5) through their SH2-domains [14]. Once bound, STAT5 molecules are on their turn phosphorylated by Jak2 on tyrosine residues, which enables them to detach from the receptor and form homo- or heterodimers with other STAT molecules. The dimers are then able to translocate to the nucleus and induce the transcription of genes involved in survival and proliferation [12, 14], such as Bcl-X_L, for example [15].

The signaling pathways induced by EPO are quite complex. Aside from the Jak2/STAT5 pathway, EPO exerts its anti-apoptotic effects by activating the PI-3K/Akt and Ras/MAPK pathways as well [16-18]. Akt was found to have an extensive role in cytoprotection, in conditions such as ischemia or oxidative stress, since it triggers a series of events resulting in the stabilization of the mitochondrial membrane potential and the inhibition of cytochrome *c* release during anoxia or hypoxia [19-21]. Akt also phosphorylates and suppresses the activity of glycogen synthase kinase 3 (GSK-3), an enzyme involved in the regulation of apoptosis in human erythroblasts [22]. An activated EPO-R can induce phosphorylation of Shc, which forms a complex with the constitutively associated adaptor proteins Grb2-SOS. SOS can then activate Ras by exchanging a GDP on its surface to a GTP. Activated Ras can increase the kinase activity of Raf, which in turn activates the mitogen activated protein kinase (MAPK)

signalling cascade, which has a well-known role in mitogenesis [23, 24]. PI3-K activation by EPO binding to its receptor can trigger not only activation of Akt, but also the activation of certain isoforms of protein kinase C (PKC) [25]. PKC can activate mitogenic kinases (MEKs and ERKs) independently of Grb2 and Ras [26].

II. Endothelial contribution to erythropoiesis

Many studies have shown that endothelial cells can affect the process of erythropoiesis in different ways. Although it is known that the major supply of red blood cells in the first month of the embryo comes from the extra-embryonic blood islands of the yolk sac [27], Fennie C *et al.* showed that the coculture of CD34+ hematopoietic progenitor cells of the yolk sac with yolk sac-derived endothelial cells for 8 days increased up to 60-fold the total production of hematopoietic cells [28]. Bone marrow endothelial cells (BMEC) proved to have unique properties allowing them to sustain long-term proliferation of hematopoietic progenitors by secreting lineage-specific cytokines and permitting direct cell-to-cell interactions [29]. In our laboratory it was previously observed that bovine fetal liver stromal cells, sharing many characteristics with endothelial cells, produce low molecular weight (smaller than 3 kDa) erythroid stimulating factors that complement the action of EPO and are absolutely necessary for the *in vitro* CFU-E formation from cultures of bovine fetal liver cells [30]. It was

also noted that the simultaneous administration of interleukin 3 (IL-3) and EPO, two cytokines that act synergistically to increase hematopoietic cell proliferation *in vitro*, had the opposite effect (namely a reduction in red blood cell numbers) in mice with AZT-induced anemia [31]. Our hypothesis is that this antagonism is mediated by the endothelium, the major non-hematopoietic tissue with receptors for both IL-3 and EPO and whose mass is considerable (1.5 kg, 400m^2) *in vivo* [32]. Furthermore, it was found that BMECs produced low molecular weight erythroid stimulating factors whose levels are substantially increased following an incubation with EPO alone, but whose production is reduced to control levels after a simultaneous incubation with IL-3 and EPO [31]. One such factor was identified as being a fragment of thrombospondin-4 (TSP-4). Thrombospondins are multidomain extracellular glycoproteins that can bind calcium and are involved in cell-matrix and cell-cell interactions; they have a wide tissue distribution and perform diverse functions, which accounts for their impact on cardiovascular pathology [33, 34]. One interesting feature of this protein family is that small molecular fragments of thrombospondins can mimic the action of the intact protein at sufficiently high concentrations [35]. The C-terminal peptide of TSP-4 is acidic and comprises the last 21 amino acids arranged in the form of an amphipathic helix; also, this peptide (C21) was sufficient to stimulate thymidine incorporation into bovine erythroid cells as well as to stimulate the proliferation of human CD36+ erythroid precursors [36]. Interestingly

enough, this peptide also caused a small but significant increase in the proliferation of skin fibroblasts and in the same time significantly decreased cell numbers in cultures of human umbilical vein endothelial cells (HUVEC) [36]. Under the action of EPO, HUVEC cells produce thrombospodin-1 (TSP-1) and insulin-like growth factor binding protein 3 (IGFBP-3), two proteins found to have a direct effect on erythroid cell proliferation [37]. This is not at all surprising, since it is well known that EPO targets endothelial cells in tissues other than the hematopoietic ones, which allows it to exert both mitogenic and chemotactic effects [38]. The present study shows that EPO also stimulates the production of chaperonin 10 (cpn10) in HUVEC cells and describes the role of this protein in cell differentiation, while focusing mainly on its implication in the process of erythropoiesis.

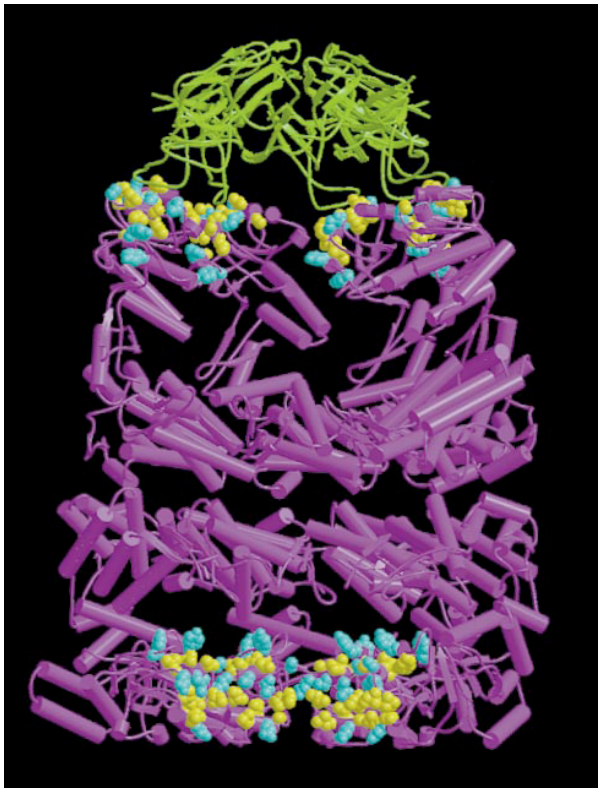
III. Description and functions of cpn10

1. Cpn10 and its role in protein folding

Molecular chaperones are a specialized family of proteins which are necessary *in vivo* for the correct folding and degradation of other proteins. They are ubiquitous and their function is necessary equally under normal conditions and under stress (such as high temperature, for example), which is why many of these chaperones are known as heat-shock proteins (Hsps) [39]. Chaperones are highly conserved throughout evolution, both

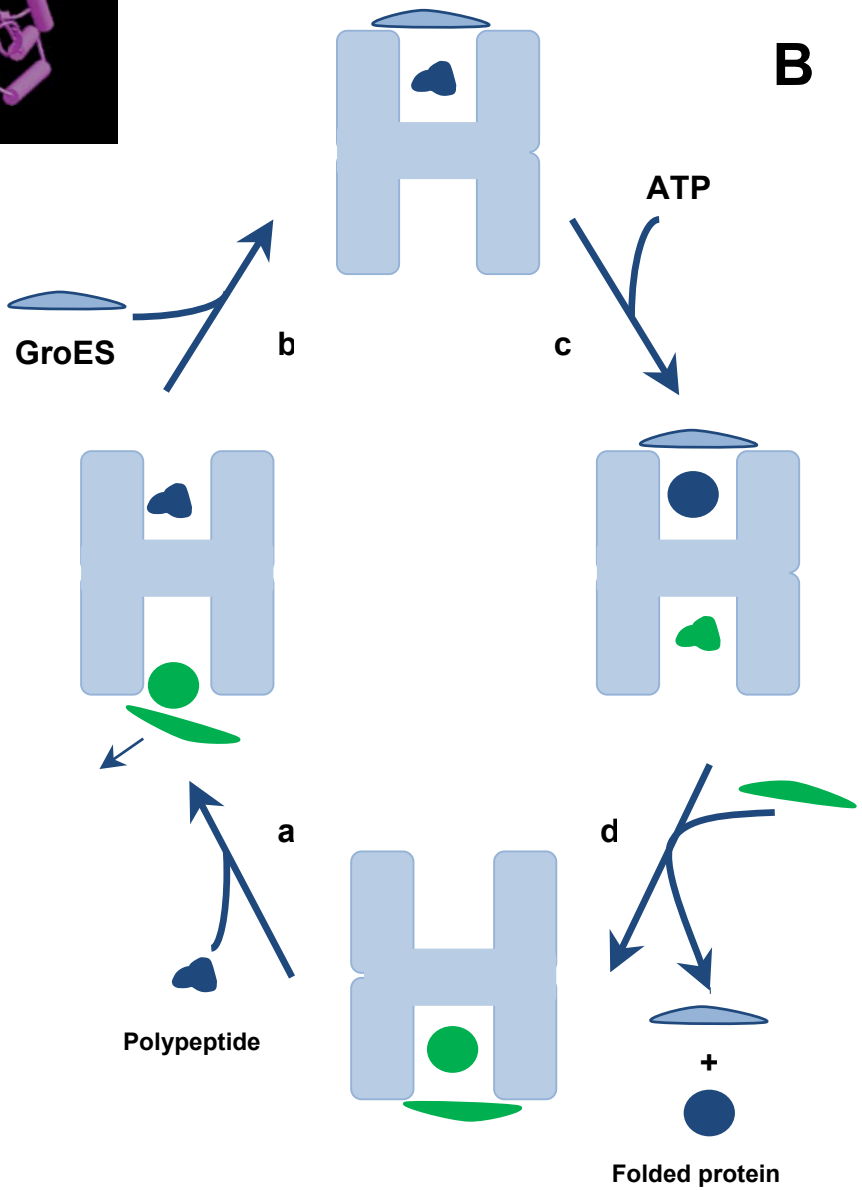
in sequence and in function. The best studied example of this set of proteins is the *E. coli* chaperonin GroEL and its co-chaperonin GroES, whose human homologues are Heat-shock protein 60 (Hsp60) or chaperonin 60 (cpn60) and Heat-shock protein 10 (Hsp10) or chaperonin 10 (cpn10), respectively [40]. GroEL is a tetradecamer, formed by two rings encompassing two central cavities, which combined form a cage-like structure lined with non-polar amino acids [41]. This inner hydrophobic surface is where the protein folding intermediates bind. The whole structure is capped by GroES, a dome-shaped ring formed by seven identical and symmetrical 10 kDa subunits. The entire folding process is dependent upon ATP hydrolysis and has been carefully reviewed by various authors [39, 41, 42]. The GroEL-GroES reaction cycle is decribed in **Fig. 3**. The two rings of GroEL are known as *trans* and *cis*; while a GroEL-GroES *cis* complex forms on one ring, another *cis* complex is disassembled on the opposite side. The misfolded protein substrate binds to the *trans* GroEL-ADP complex, which allows entry of an ATP on the *trans* side and triggers the disassembly of the GroEL-GroES *cis* complex on the other side, therefore allowing the GroES to detach and reassemble with the *trans* complex (the new *cis*). Inside the GroEL-GroES *cis* complex, hydrolysis of ATP drives the productive protein folding, followed by the disassembly of the complex, the substrate release and the regeneration of the *trans* complex, from where on the cycle can restart.

Fig. 3. (A) The crystal structure of the GroEL-GroES complex. GroEL is represented in purple and GroES in green. The space filling models denote substrate-binding sites, namely yellow for hydrophobic residues and blue for polar residues. Reproduced with permission from [39]. (B) The chaperonin-assisted protein folding. (a) The misfolded protein binds to the *trans* ring of the GroEL-GroES complex, and is most likely followed by the binding of ATP. (b) End-to-end exchange of GroES, which binds more rapidly and more efficiently to an ATP-occupied ring. This results in the encapsulation of the non-native protein in the new c*is* complex. (c) The longest step of the reaction (10 s), the hydrolysis of ATP drives the productive folding of the protein substrate and weakens the *cis* complex. (d) Entry of new polypeptide in the *trans* ring triggers the release of the *cis* ligands (properly folded protein and GroES), while allowing the protein folding cycle to restart. Figure adapted from [39] and [42].



A

B



Since conditions *in vivo* can often be unfavorable to adequate protein folding, the protected environment of the cage-like structure of the GroEL-GroES complex is essential for the chaperonins' function, especially under stress conditions when proteins have to be processed quickly in order for the cell to stay alive. Moreover, it was found that this "cage" is not merely a passive protective container, but that it actively and positively contributes to the protein folding process. Its net negative charge and its reduced size contribute to the crowding of the protein substrates inside the cage, which is key to the efficient and proper protein folding, as well as to the reduction in the crowding of misfolded proteins outside the cage [43].

2. Cpn10, a homologue of early pregnancy factor

Both cpn10 and cpn60 belong to the Type I family of chaperonins that reside in bacteria (such as GroEL and GroES) or in endosymbiotically aroused organelles such as the mitochondria (like is the case for cpn10 and cpn60) and chloroplasts (in the case of Rubisco) [42]. Given the conserved nature of intracellular chaperonins, the discovery that the early pregnancy factor (EPF) - an immunosuppressant and growth factor secreted in the serum of humans, mice and sheep within 6 to 24 hours of fertile mating [44-46] – is in fact identical in amino acid sequence (with one exception) to the rat mitochondrial cpn10 [47] was quite unexpected. EPF remains the earliest known indicator of pregnancy; it is secreted by tumor cells, normal proliferating cells, as well as by activated platelets [45, 48,

49]. The mechanism of secretion of EPF/cpn10 remains unknown, since this protein doesn't contain a secretory signal sequence and doesn't follow the classical ER-Golgi pathway [50]. Nevertheless, evidence for Hsp transport outside the cell is not unprecedented, since Hsp110, Hsp71 and Hsp73 were all shown to be released from cultures of rat embryo cells [51]. Several possible mechanisms of secretion other than the classical one are suggested by Multhoff G *et al*. [52]. This extracellular cpn10 is the main object of interest in the present investigation, since EPO seems to favor the secretion of this molecule from endothelial cells. It is still unknown at what level the regulation of the extracellular localization and activity of EPF/cpn10 occurs, but it was already suggested that this secreted cpn10 is able to act as an accessory molecule to proteins other than cpn60 and even to operate alone [47]. Furthermore, Fletcher BH *et al*. [53] performed a mouse genomic library screen and sequencing of cpn10 family members and found an intronless member, *Cpn10-rs1*, which encodes the full-length cpn10 protein, but with two modified amino acids. The product of the *Cpn10-rs1* gene was not expressed ubiquitously, like cpn10, but only selectively and according to previous evidence of EPF activity; moreover, they demonstrated that the purified recombinant gene product kept the same potency as the native protein in the EPF bioassay (the rosette inhibition test), which lead them to the conclusion that *Cpn10-rs1* is likely to encode EPF. The properties of EPF/cpn10 elucidated so far are very versatile and quite interesting. Also, they continue to be the object

of intense research due to cpn10's remarkable potential as both a therapeutic agent and a diagnostic marker.

3. Cpn10 as an immunosuppressant

Indeed, cpn10 was found to have some important therapeutic applications, and all of them seem to revolve around its ability to modulate the immune system. EPF/cpn10 was assigned an immunosuppressive role following its behavior in the rosette inhibition test [44], and it was found to be essential for the development and survival of the embryo both at pre- and post-implantation stages [54-56]. The action of EPF/cpn10 is mediated through the release of soluble mediators, EPF-S_1 and EPF-S_2, from murine as well as human lymphocytes [57]. In fact, EPF/cpn10 was shown to bind to a large number of lymphocyte subpopulations, such as CD4+ T cells (including naïve CD45RA+, memory CD45RO+ and regulatory CD4+CD25+ T cells), CD8+ T cells, CD14+ monocytes and CD56+ NK cells [58]. Moreover, cpn10 released by ovarian cancer cells and circulating through the patients' peripheral blood was shown to suppress the expression of the T cell CD3-zeta chain, thus inhibiting lymphocyte activation through the T cell receptor complex and allowing the tumor to evade immunosurveillance [59]. The demonstration of these diverse interactions between cpn10 and lymphocytes is consistent with various recorded activities of EPF/cpn10, namely the suppression of clinical signs of experimental autoimmune encephalomyelitis (EAE) and of

delayed-type hypersensitivity in mice, as well as the prolongation of skin graft survival time in rats [60-62]. In an attempt to investigate the immediate effects of EPF/cpn10 in rats with EAE, Harness J e*t al.* found an increase in spinal cord inflammatory cells mRNA levels of IL-4 and IL-10, and a decrease in the production of INF-γ, a cytokine spectrum consistent with a shift from a Th1 cell-mediated response towards a Th2 humoral response [63]. In contrast, no significant influence on the Th1/Th2 profile was found in mouse splenocyte-derived T cell populations by Johnson BJ *et al.*; however, this group discovered that recombinant cpn10 inhibited the lipopolysaccharide (LPS)-induced activation of NF-κB and reduced the secretion of TNF-α, RANTES and IL-6, while increasing the secretion of IL-10 in various human and murine *in vitro* systems as well as in two murine disease models [64]. These findings attest that cpn10 inhibits signaling through toll-like receptor 4 (TLR4), macrophage receptor that, when bound by LPS, increases the secretion of the aforementioned cytokines and chemokines. Since Hsp60 was previously shown to act as an agonist to both TLR4 and TLR2 [65, 66], the authors suggest that cpn10 blocks TLR signaling by binding to its co-chaperonin Hsp60 and preventing it from augmenting LPS-signaling through TLR [64]. Investigations centered upon the idea that EPF/cpn10 might have a membrane-bound form of cpn60 as its receptor are still in progress, even though the possibility of it having an alternative receptor has already been proposed [58].

With respect to the role of Hsps in the immune system, there is a phenomenon that DeMeester *et al.* called “the heat-shock paradox”, stating that a heat-shock stress prior to an inflammatory reaction can confer cytoprotection, whereas if the heat-shock stress occurs after inflammation it can precipitate cell death [67]. It was previously shown that death by necrosis causes a release of Hsps in the extracellular milieu, which can then activate the NF-κB pathway in antigen presenting cells (APCs) and thus trigger a response to cell death [68]. Therefore, location of the Hsp (inside or outside the cell) affects inflammation and the immune response differently. Intracellular Hsps have an anti-inflammatory effect, which they achieve by blocking the NF-κB pathway at various levels; on the other hand, extracellular Hsps modulate both the innate and adaptive immunity by priming APCs and interacting with T cells and NK cells directly [69]. Understanding this concept is important for the development of various anti-inflammatory treatments based on Hsps.

4. Cpn10 as a therapeutic agent

The most successful application of cpn10 as a therapeutic agent was a phase II randomized double-blind clinical trial in Australia in 23 patients with active rheumatoid arthritis [70]. The results of this study suggested that the recombinant cpn10 used (XToll) could be an attractive alternative treatment for this condition, due to its rapid decrease of inflammatory cytokine production, quality of life improvement, good

tolerability and absence of toxicity. More recently, an exploratory study used the same dosages (5, 7.5 and 10 mg of XToll) and same route of administration (intravenously, twice weekly for 12 weeks) as previously done in the rheumatoid arthritis trial, but this time in patients with chronic plaque psoriasis [71]. This short-term cpn10 treatment caused a decrease in disease parameters in all dose groups. Given the successful reduction of the inflammatory response and improvement of disease outcome in mice with EAE (the best animal model for multiple sclerosis) [60, 63], the same Australian group designed a study to explore the tolerability of recombinant cpn10 in a cohort of patients with relapse-remitting or secondary-progressive multiple sclerosis (MS) [72]. The findings suggested no significant difference in the frequency of adverse effects or in the clinical outcome measures between the different treatment arms. However, the study allowed concluding that the intravenous administration of 5 mg cpn10 twice weekly for 12 weeks was well tolerated and safe for the MS patients. Nonetheless, the promising results from the rheumatic arthritis study ensure cpn10 the status of a very attractive potential therapeutic agent in a variety of inflammatory diseases.

5. Role of cpn10 in growth regulation

As previously stated, cpn10 is localized mainly in the mitochondria, but has also been observed in zymogene granules and secretory granules in pancreatic cells, in growth hormone granules in anterior pituitary cells,

as well as in mature red blood cells which lack mitochondria [73]. As was the case for the immune system, the location of Hsps seems to affect cellular growth and protection differently. Whereas cpn10 is present mostly in the mitochondria of normal cells, cancer cells seem to have a higher cpn10 accumulation in their cytoplasm [74]. Indeed, cpn10 has been detected in a variety of tumors and pre-tumoral lesions, including malignant trophoblastic tumors [75, 76], germ tumor cells of the testis [77], ovarian cancer [59], large bowel, exocervical and prostate carcinomas [78-80]. In contrast, both cpn10 and cpn60 downregulation was linked to the development and progression of bronchial cancer in smokers with chronic obstructive pulmonary disease [81]. Therefore, enough evidence exists related to the involvement of cpn10 in cellular transformation and immortalization; however, its involvement in signal transduction pathways continues to be elusive.

Nevertheless, some insight into the chaperonin's mechanism of action has already been gained. It was shown that cpn10, in combination with cpn60 or alone, was able to maintain mitochondrial integrity and prevent apoptosis in neonatal cardiac myocytes undergoing ischemia-reperfusion injury [82]. Lim KA *et al.* suggest that this beneficial effect of cpn10 is due to an attenuation of the Ras GTP-ase pathway [83]. The authors found that cpn10 overexpression lead to the inactivation of Raf, ERK and p90Ribosomal kinase (p90RSK). Other interferences with the apoptotic pathway have been recorded for cpn10. Shan Y *et al.*

demonstrated that overexpression of cpn10 increased the anti-apoptotic Bcl-2 and Bcl-xl, reduced the pro-apoptotic Bax, stabilized the mitochondrial cross-membrane potential and inhibited Caspase 3 [84]. Also, cpn10 was shown to modulate insulin-like growth factor-1 (IGF-1) receptor signaling in cardiac muscle cells through post-translational modification [85]. Cpn10 overexpression increased the number of IGF-1 receptors, suppressed their polyubiquitination and amplified their signaling, thus stimulating Akt, MEK, ERK and p90RSK (which contrasts with the findings of Lim KA *et al.* mentioned above). Moreover, it has been proposed that, when released from proliferating cells, cpn10 can act in an autocrine and/or paracrine fashion to stimulate DNA synthesis [48]. Other interactions between cpn10 and both yeast and human proteins are summarized by Czarnecka AM *et al.* [74]. Although most of the current knowledge about cpn10 seems to point out that it is likely to increase cell proliferation and contribute to cancer establishment, some results are contradictory and impede the finding of a clear model for the tumorigenesis of cpn10 [86]. Cell transformation and subsequent immortalization are processes driven by very complex mechanisms. This may help explaining why cpn10 can serve as a tumor marker in some cancer types, while been downregulated in others, and why some groups were unable to confirm the tumor growth factor properties of this chaperonin [64].

6. Cpn10 and cancer vaccines

Given its implications in both carcinogenesis and the immune response, cpn10 (as well as its co-chaperonin cpn60) began to be considered a potential candidate for cancer therapy. This became apparent after the discovery that Hsps from one tumor can elicit an immune reaction specific for that tumor [87, 88]. Hsps are normally found inside the cell and even in the intercellular space, in which case they go undetected by the immune system; however, if they carry misfolded peptides, they become strong adjuvants in the cross-presentation of antigenic peptides and trigger off an immune response. These observations contributed to the concept of the “Hsp-based antitumor vaccine”, in which the vaccine can either designate a compound made of a Hsp and a tumor antigen, or a purified Hsp (or *hsp* gene) with antitumor potency, but lacking an antigen moiety [89]. In both cases, these vaccines are tumor- and patient- specific. The recent advances in purification of multiple Hsps from a single tumor [90] as well as the promising results from both preclinical and clinical trials have raised the hope that, within years, Hsp-based cancer vaccines could replace chemotherapy treatment [91-93]. The good characterization of cpn10 and its successful utilization against rheumatoid arthritis makes it an attractive antitumor agent [89].

IV. Aims of study

A negative synergy exists between anemia and renal insufficiency, due to the fact that anemia is not only a complication of this pathology, but can also result in its aggravation. Correcting the anemia by recombinant human EPO (rhEPO) administration is beneficial for patients suffering from kidney disease, since it significantly improves their physical performance, well being and quality of life [94]. The fact that cpn10 was shown to have immunosuppressive properties was of great importance for the present study, because chronic inflammation is very common in kidney failure patients, who become anemic and develop resistance to rhEPO therapy [95]. Inflammatory cytokines, such as IL-3, were shown to eliminate the EPO-dependent endothelial production of erythroid stimulating factors [31]. Therefore, the anti-inflammatory action of cpn10 would be beneficial for these patients. So far, only a weak and indirect correlation was found between cpn10 and red blood cell production *in vivo*: cpn10 production is increased in both pregnancy and hepatectomy, two conditions that also happen to be associated with increased erythropoiesis [48, 50, 96-98]. In this study we describe a new function for cpn10 as an EPO-dependent erythroid cell differentiation factor. We also show how this biological effect is mediated by GSK-3, an enzyme previously shown to regulate hematopoietic stem cell function *in vivo* [99].

V. Materials and methods

1. 2D-elecrophoresis of HUVEC lysates

HUVEC obtained from Cambrex/Lonza (Walkesville, MD) were expanded in complete EBM-2 medium (Lonza) supplemented with 10% fetal bovine serum. Cultures in T-75 flasks were starved in serum-free EBM-2 medium supplemented with 5µg/ml bovine transferrin for 8h and treated with EPO (2 units/ml) for 48h. At the end of the incubation lysates from control cell cultures and EPO-treated cells were lysed and separated by 2D-electrophoresis at the Proteome facility of the McGill University-Genome Quebec Innovation Centre. Electrophoretic spots that increased or decreased after EPO treatment were in-gel digested with trypsin and analyzed by mass spectrometry. Product ion spectra were submitted to Mascot (Matrix Science, London, UK) for identification.

2. Cpn10 determinations by immunoblot analysis

HUVEC were seeded at 1 x 10^6 cells per 25cm^2 flask in EBM-2 media supplemented with 10% fetal bovine serum, but without heparin. The day of treatment, the cells were starved for 8 hours in serum-free EBM-2 supplemented with 5µg/ml bovine transferrin. Different concentrations of EPO were added to the cultures. After 1, 2 or 3 days of incubation, the supernatants were collected and concentrated with 5K filters (Millipore-Amicon, Techno Fisher, Whitby, ON) up to approximately

100µl. The cells were washed with PBS and lysed in 200µl of a buffer consisting of 0.2% Triton X-100 in PBS and 1% protease inhibitor cocktail III (Calbiochem, San Diego, CA). The lysates were incubated for 30 minutes on ice and centrifuged for 30 minutes at 13,000 rpm at 4°C. The protein concentration of both lysates and supernatants was measured with a Qubit fluorometer (Invitrogen, Burlington, ON). 2µl of the concentrated samples and serial dilutions of the lysates and supernatants were loaded onto nitrocellulose membranes (Hybond C, GE Healthcare). The lysates blot was quenched for 10 minutes with Dual Endogenous Enzyme Block (Dako). Both membranes were incubated 1 hour in blocking solution (1% bovine serum albumin (BSA) in 0.05%-Tween-Tris-buffered saline (TBST, Sigma T-9063, Mississauga, ON). A rabbit polyclonal anti-cpn10 antibody (Stressgen, Cedarlane, Burlington, ON) was used as a primary antibody in a 1:1,000 dilution for 1 hour. The membranes were washed 3 times with TBST, incubated for 1 hour with a 1:5,000 dilution of anti-rabbit alkaline phosphatase conjugated IgG (Calbiochem), washed again and stained with BCIP/NBT (Sigma). Image analysis was performed using the UMAX PowerLook 2100XL scanner (Techville, Dallas, TX).

3. Cell proliferation assay

K562 cells were seeded in 24-well plates, 50,000 cells per well in 250µl of serum-free RPMI 1640 media supplemented with 100 µg/ml BSA (fatty-acid free, tissue culture grade, Sigma) and 5µg/ml bovine transferrin.

After an overnight starvation, the cells were incubated with cpn10 (Stressgen, Cedarlane, Burlington, ON), as well as with 1.25mM sodium butyrate (Sigma) as a positive control. After 4 days, cell proliferation was measured by a colorimetric assay with Alamar Blue, as previously described [100, 101]. TF-1 cell proliferation was measured both by Alamar blue or using a hemocytometer, required for accurate loading of cells in experiments to be described below.

4. Erythroid cell differentiation markers

TF-1 cells were maintained in culture as previously described [100, 101], but this time the cultures were supplemented with human IL-3 (R&D Systems) instead of GM-CSF. Cells were plated in 12-well plates at a density of 200,000 cells per well in 500µl Opti-MEM I (Invitrogen) and starved overnight. The cells were then incubated for 3 days with 0.1µg/ml cpn10 or 2U/ml EPO as a positive control. In another set of experiments, TF-1 cells were seeded at the same density, starved overnight in Opti-MEM as before, then incubated 3 days with 0.5nM, 5nM and 50nM GSK-3 Inhibitor IX (Calbiochem) in the presence or absence of 0.1µg/ml cpn10. The cells were washed with PBS, counted and resuspended in PBS. The samples were loaded onto nitrocellulose membranes so as to have 60,000 and 30,000 cells per dot. The cells were fixed on the membranes by incubating 20 minutes at 4°C with 4% paraformaldehyde in PBS. The membranes were then washed 2 times 5 minutes with TBST and 30

seconds with water. One membrane was stained with 0.2% benzidine in 0.5% acetic acid and 0.6% hydrogen peroxide, prepared according to Huang HM *et al.* [102]. Upon a blue color development, the reaction was stopped by rinsing with water and the membrane immediately scanned. A second membrane was quenched with Dual Endogenous Enzyme Block (Dako) for 10 min, washed as described above, incubated with Proteinase K (Dako) for 10 minutes, washed again then blocked overnight with 1% BSA in TBST at 4°C. The membrane was incubated for 1 h with a 1/3,000 dilution of monoclonal anti-Glycophorin A (Dako), washed 3 times with TBST and incubated for 1 h with goat anti-mouse IgG conjugated with alkaline phosphatase (Calbiochem) diluted 1:5,000. After washing the membranes were treated with BCIP/NBT and scanned.

5. Antibody microarray

K562 cells were seeded in 24-well plates at a density of 50,000 cells per well and starved overnight in 250µl serum-free RPMI 1640 media supplemented with 100 µg/ml BSA and 5µg/ml bovine transferrin. Cells were incubated for 10 minutes at 37°C with 0.1µg/ml cpn10. The incubation was stopped with 500µl ice cold PBS. The cells were washed and lysed with the lysis buffer recommended by Kinexus Bioinformatics Corporation, Vancouver, Canada. The lysates of cpn10-treated cells and control cell cultures were sonicated 4 times 10 seconds, with 10 seconds pauses in between, and then centrifuged for 30 minutes at 13,000 rpm at

4°C. The lysates were analyzed with an antibody microarray by Kinexus containing 273 phospho-site specific and 377 pan-specific antibodies. The 18 proteins with the most pronounced changes after cpn10 treatment were chosen for confirmation by an immunoblot analysis (Kinetworks), also performed by Kinexus. Out of these, only 6 proteins with noteworthy changes were chosen for a Kinetworks multiple sample screen immunoblot for statistical analysis.

6. Western Blot analysis of TF-1 cell lysates

200,000 TF-1 cells were seeded in 12-well plates in 500µl Opti-MEM I and starved overnight. The cells were then incubated with 0.1µg/ml cpn10 at 37°C. The reaction was stopped by adding 500µl per well ice cold PBS and by washing the cells with PBS. The pellets were then lysed in ice-cold lysis buffer (1% Triton X, 1mM DTT, 5mM EDTA, 2mM EGTA, 1% protease and phosphatase inhibitors cocktails III (Calbiochem)). The lysates were sonicated 4 times 10 seconds with 10 seconds pauses in between, then centrifuged for 30 minutes at 13,000 rpm at 4°C. Protein content of the lysates was determined with Qubit fluorometer (Invitrogen). The protein samples were then mixed with 4:1 v/v ratio with 4x sample loading buffer (Kinexus) + 5% β-mercaptoethanol. Samples were boiled at 95-100°C for 5 minutes then centrifuged. Equal amounts of protein for each sample were resolved by SDS-PAGE using pre-casted 15% gels (Bio-Rad). The proteins were then transferred to nitrocellulose membranes

by semi-dry blotting. The membranes were saturated overnight with 1% BSA in TBST at 4°C. The 1h primary antibody treatment (1:1000 dilution) was done with rabbit polyclonal anti-GSK-3 (Stressgen), which recognizes the phosphorylations at Tyr216/Tyr279 and rabbit monoclonal anti-cofilin-1 (Cell Signaling, New England Biolabs, Pickering, ON), which recognizes the phosphorylated serine at position 3. The membranes were washed, incubated with 1:5000 dilutions of the corresponding alkaline-phosphatase conjugated secondary antibodies. The membranes were stained and scanned as indicated above. Loading and transfer efficiencies were confirmed with reversible Ponceau Red staining for GSK-3 or actin for cofilin-1 [100].

7. Lentiviral transduction of skin fibroblasts

The cpn10 gene (Ultimate ORF collection, Invitrogen) was cloned into the pLenti6/V5-DEST plasmid using LR Clonase (Invitrogen) following the manufacturer's instructions. The expression plasmid was then co-transfected in 293FT cells with the ViraPower Mix (Invitrogen) according to the manufacturer's protocol. Three days later the supernatants containing the virions were collected, centrifuged 5 minutes at 1,000 rpm and filtered with 0.45µm filters (Millipore). Normal human skin fibroblasts (obtained from Lonza) were seeded at a density of 100,000 cells per 25cm^2 flask and starved with serum-free antibiotics-free DMEM. Infection with the cpn10-containing virions was done at a MOI of 7.8 in a total of 2 ml serum-

free antibiotics-free DMEM and the transformed fibroblasts selected with 6µg/ml blasticidin (Invivogen, Carlsbad, CA).

8. Collagen I determination

Normal skin fibroblasts and skin fibroblasts transduced with the lentiviral-associated cpn10 were seeded in a 96-well plate at different densities in 100µl DMEM containing 10% fetal bovine serum. The cells were then starved for either one or two days in serum-free DMEM supplemented with 5µg/ml bovine transferrin. The day of analysis, the cells' proliferation rate was measured according to the Alamar Blue technique previously described. Then the cells were fixed in 100µl per well of 4% paraformaldehyde for 30 minutes at 4°C, washed twice with PBS, dried and incubated overnight at 4°C. Collagen I in the monolayers was measured as previously indicated using an ELISA-type colorimetric method [103].

9. Statistical analysis

Analysis of variance using parametric and non-parametric methods was done with the GraphPad Instat software (San Diego, CA).

VI. Results

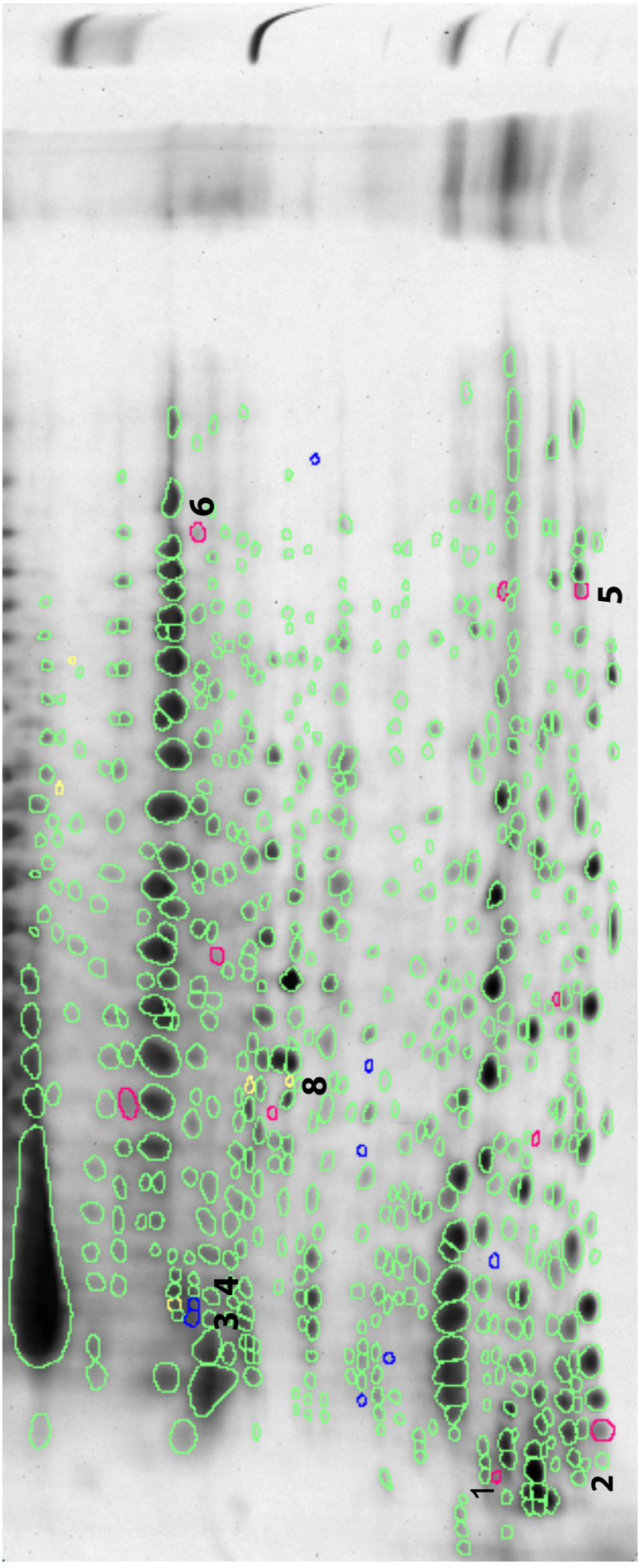
1. Identification of cpn10 as an EPO-dependent endothelial protein

HUVEC cultured in the presence of 2U/ml EPO and a control cell culture were collected after an incubation of 48 h, lysed and analyzed by 2D electrophoresis as previously described. Comparison of the two gels indicated that several spots had remarkable changes on staining intensity, pointing out that EPO increased or decrease the amounts of proteins present as compared with the control cell lysates. Eight spots showing the most impressive changes were chosen for in-gel digestion followed by mass spectrometry analysis, and they are illustrated in **Fig. 4**. Product ion spectra were analyzed with the Mascot program. Among the different identified proteins, two of them were heat shock proteins: Hsp27 and Hsp10. In a neonatal hypoxia-ischemia rat model, rhEPO was already shown to protect from apoptotic cell death, partially by activating Hsp27 [104]. We decided to study first Hsp10 or chaperonin 10 (cpn10) because, within the EPO-stimulated proteins, it had two characteristics considered essential as EPO-derived peptides in endothelial cells: it is secreted and immunosuppressive [47, 50]. The observation that cpn10 has anti-inflammatory activity caught our attention, because chronic inflammation is a common complication in patients with renal disease. In most cases, these patients also suffer from anemia and are resistant to rhEPO therapy [95]. Therefore, cpn10 could be an ideal agent to circumvent the

deleterious effects of proinflammatory cytokines known to antagonize the action of EPO at various levels [95] and to switch off the production of endothelial-derived erythroid stimulating factors [31].

Fig. 4. 2D electrophoresis of HUVEC lysates and identification of cpn10. (A) HUVEC cells were starved overnight in serum-free EBM-2 medium supplemented with 5μg/ml bovine transferrin, then incubated with 2U/ml EPO for 2 days. Cell lysates of EPO-treated and control cells were separated by 2D electrophoresis. The electrophoretic spots that increased (blue) or decreased (yellow) more than 5 times with EPO were in-gel digested with trypsin and analyzed by mass spectrometry. (B) Product ion spectra of spot 4 was identified by the Mascot program as being cpn10. The arrow indicates the Mowse score (87) obtained for this protein.

A



B MATRIX SCIENCE Mascot Search Results

Search title : LC03_08_06_T2; 192000; human_100; spot; 4_200606EPO_1; NA; NA; NA; NA; NA; 1; 15min_114; 1:7,F; micro_QTOF_1; wl_eqau4e85_a7
MS data file : /proline/data06/peaklist/REDISTILLER/20060822/LC03_08_06_T2.PRO_20_0.7/4_200606EPO_20_0.7_correctedv5.mgf
Database : NCBInr 20060803 (3841279 sequences; 1323634604 residues)
Taxonomy : Homo sapiens (human) (148835 sequences)
Timestamp : 22 Aug 2006 at 18:01:22 GMT
Significant hits: gi|4008131 chaperonin 10 [Homo sapiens]
gi|493852 Chain C, Hemoglobin Thionville Alpha Chain Mutant With Val 1 Replaced By Glu And An Acetylated Met

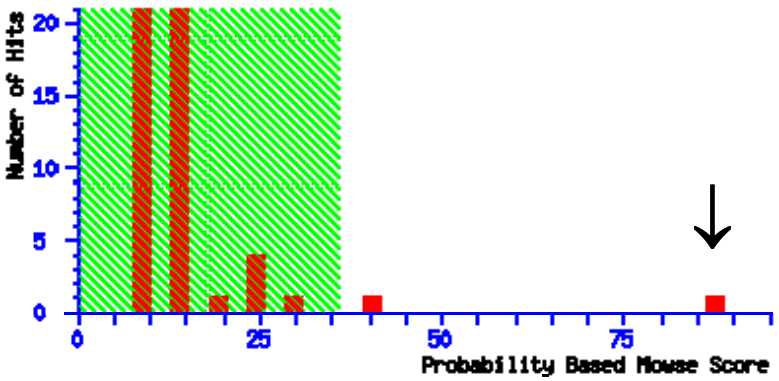
Probability Based Mowse Score

Score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 36 indicate identity or extensive homology (p<0.05).
1. gi|4008131 **Mass:** 10576 **Total score:** 87 **Peptides matched:** 3
chaperonin 10 [Homo sapiens]

☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Rank	Peptide
☑	16	454.22	906.42	906.50	-0.08	0	27	1	FLPLFDR
	18	507.25	1012.48	1012.56	-0.07	0	18	2	GGEIQPVSVK
☑	23	538.75	1075.49	1075.59	-0.10	0	42	1	VLLPEYGGTK

Proteins matching the same set of peptides:
gi|4028622 **Mass:** 10289 **Total score:** 87 **Peptides matched:** 3
chaperonin 10-related protein [Homo sapiens]
gi|23270723 **Mass:** 10925 **Total score:** 87 **Peptides matched:** 3
Heat shock 10kDa protein 1 (chaperonin 10) [Homo sapiens]



2. EPO stimulates the secretion of cpn10 in HUVEC cells

HUVEC were cultured with different concentrations of EPO, namely 0.05, 0.5, 2 and 4 U/ml. Cpn10 present in the cells or secreted into the medium was measured as previously indicated in Methods. **Fig. 5** shows that the most important increase of cpn10 concentrations after EPO treatment is detected in cell culture supernatants. The results, which are expressed as ratios of EPO-treated cells over control cell cultures, indicate that the most significant cpn10 secretion after EPO treatment is observed with the concentration of 2U/ml ($P<0.01$, Kruskall-Wallis ANOVA, Dunn's Multiple Comparison test). The amount of cpn10 released in the culture medium was approximately 2 times higher than in untreated controls in the presence of 0.05, 0.5 and 4 U/ml EPO and almost 3 times higher when 2U/ml EPO were added. The actual amounts of cpn10 present under these conditions were 9.2 ng and 7.1 ng cpn10 per µg of protein in the lysates and supernatants, respectively. In contrast, cpn10 levels in the cell lysates did not vary much with increasing EPO concentrations (the ratio remained constant at about 1). **Fig. 6** shows that the optimal production of EPO-mediated, secreted cpn10 occurs after 2 days, when it's over 3 times higher than in controls ($P<0.05$, one-way ANOVA, Student-Newman-Keuls Multiple Comparisons test). The actual intracellular cpn10 seems to be increased at day 1, but this stimulation was not statistically significant. After 3 days incubation, the production of cpn10 decreased.

Fig. 5. EPO increases the production of cpn10 in HUVEC. HUVEC were cultured with different concentrations of EPO and the amounts of cpn10 were measured in cell lysates and culture supernatants after 2 days incubation. The results are given as ratios of EPO-treated cells over control (untreated) cell cultures ± SE (n=5 for all concentrations except 2U/ml (n=8)). The secretion of cpn10 after treatment with 2U/ml EPO was significant. **P<0.01, Kruskall-Wallis ANOVA, Dunn's multiple comparisons test.

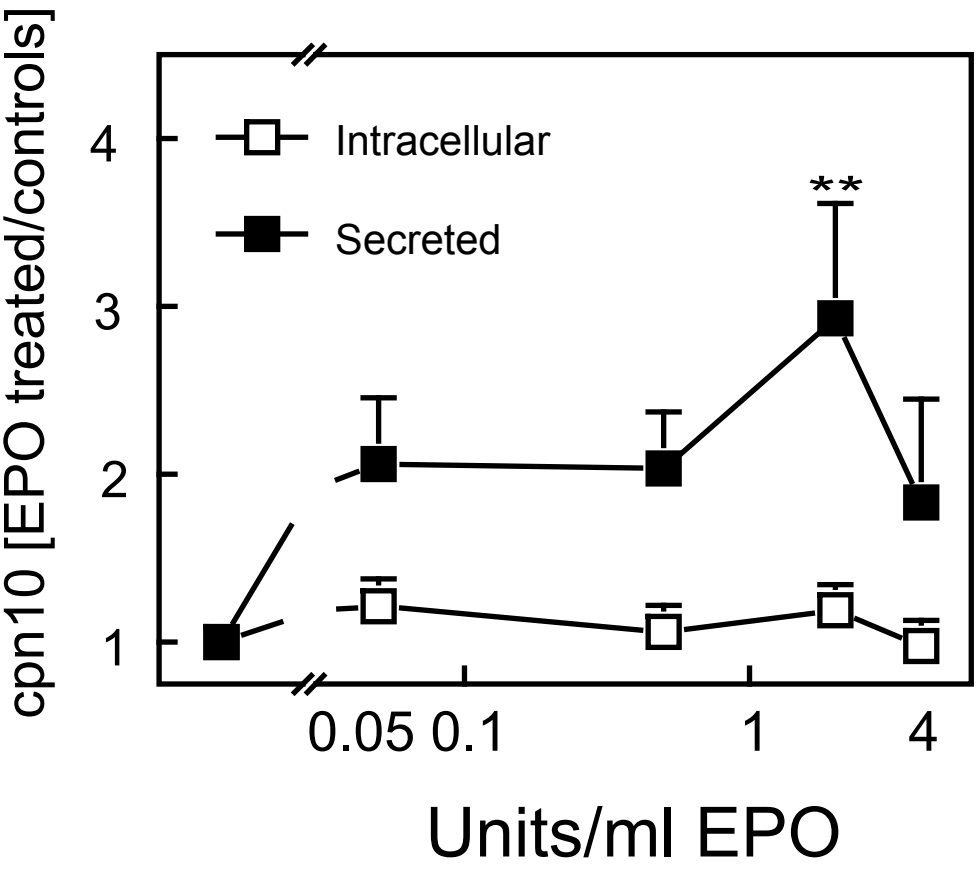
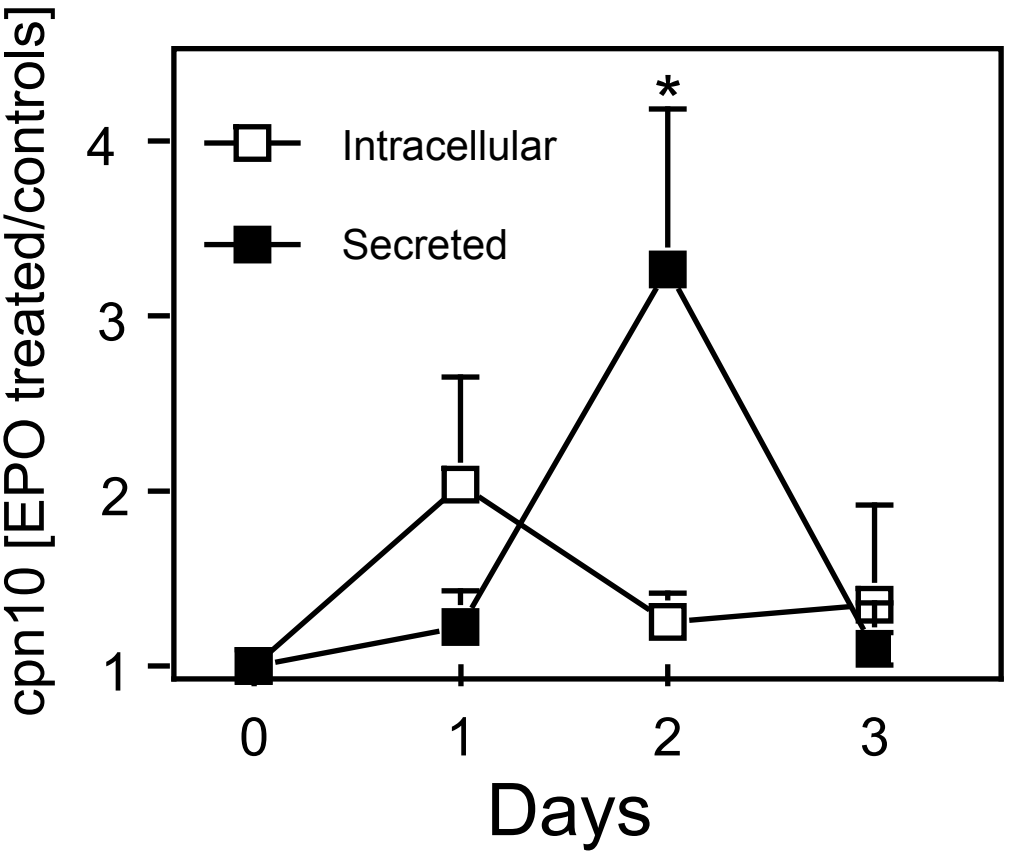

cpn10 [EPO treated/controls]
4
3
2
1
Intracellular
Secreted
**
0.05
0.1
1
4
Units/ml EPO

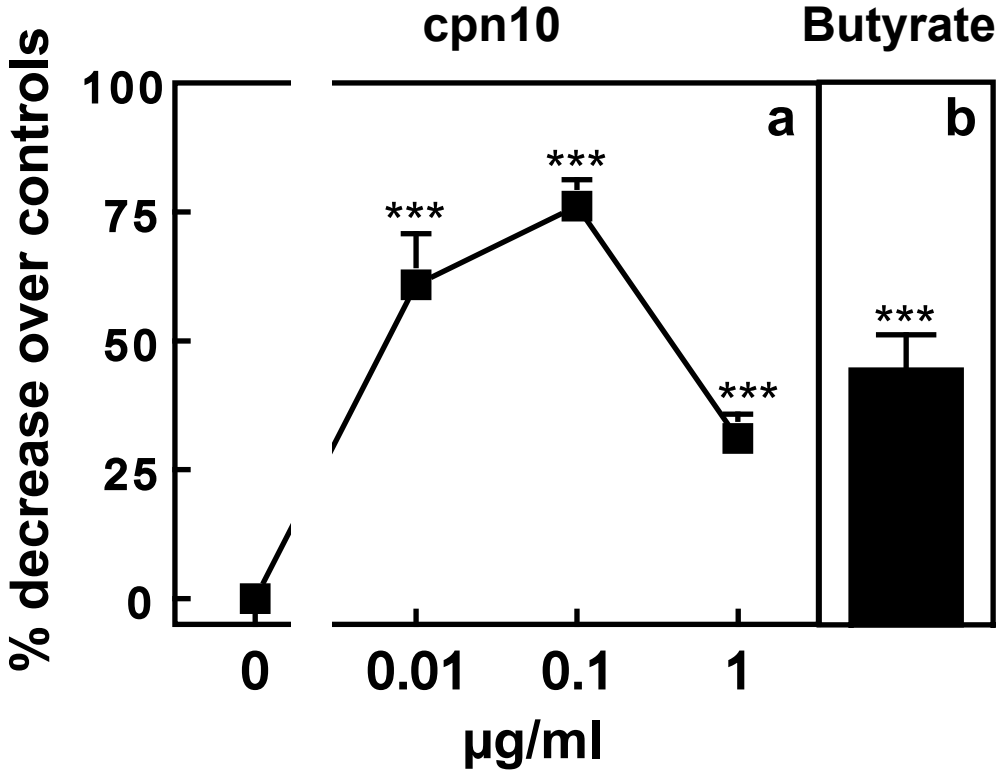
Fig. 6. Time course analysis of cpn10 production taking place in HUVEC. Cells were cultured with 2U/ml EPO and the cpn10 production in both lysates and supernatants was measured after 1, 2 or 3 days incubation. The results are given as ratios of EPO-treated cells over control (untreated) cell cultures ± SE (n=4 for all values except day 2 (n=8)). The increase observed after 2 days was significant. *P<0.05, one-way ANOVA, Student-Newman-Keuls Multiple Comparisons test.


cpn10 [EPO treated/controls]
4
3
2
1
Intracellular
Secreted
*
0
1
2
3
Days

3. Cpn10 inhibits K562 cell proliferation

If EPO stimulates the production of cpn10 by endothelial cells, does this secreted chaperonin have anything to do with erythroid cell proliferation or differentiation? We studied first the effects of cpn10 on K562 cells, a chronic myelogenous leukemia cell line. We used sodium butyrate as a positive control, since it is known to restrict growth and stimulate differentiation in this cell line. **Fig. 7** shows that cpn10 significantly decreased the cell numbers as compared to untreated cell cultures ($P<0.001$ for all cpn10 concentrations used, one-way ANOVA, Tukey-Kramer Multiple Comparisons test). The inhibitory action was more impressive (up to 76% decrease in cell numbers with 0.1 μg/ml cpn10) than that observed with sodium butyrate (only 53%). Since cpn10 is also a mitochondrial protein, internalization could result in interference with the redox indicator Alamar blue used to measure cell proliferation. Therefore, we confirmed the decrease of cell numbers at the concentration of 0.1μg/ml by measuring directly cell numbers with a hemocytometer. Cpn10 caused a decrease in cell numbers of 67±11% (n=3), which is in the range of the results with the redox indicator in **Fig. 7**.

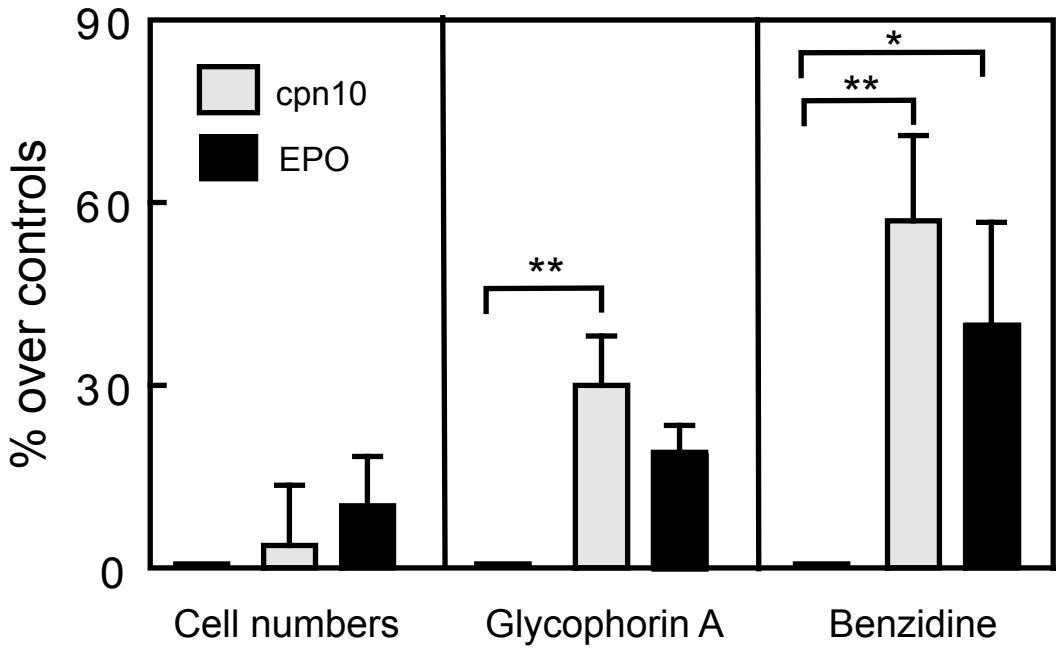
Fig. 7. Effect of cpn10 and sodium butyrate on K562 cell numbers. K562 cells were cultured in a serum-free medium as indicated in Methods for 4 days with different concentrations of cpn10 or 1.2mM butyrate. Cell numbers were assessed by the Alamar blue technique. The values are expressed as % decrease over control cell cultures ± SE (n=5 for all concentrations except 1μg/ml (n=4)). ***P<0.001, one-way ANOVA, Tukey-Kramer Multiple Comparisons test.


cpn10
Butyrate
a
b
% decrease over controls
100
75
50
25
0
***
***
***
***
0
0.01
0.1
1
µg/ml

4. Cpn10 stimulates TF-1 cell differentiation

We were unable to develop a good and consistent method to measure hemoglobin synthesis in K562 cells, even in the presence of sodium butyrate. Therefore, we turned our attention to the erythroleukemic cell line TF-1. This cell line is more difficult to maintain in culture but has a major advantage over K562 cells, in the sense that it has kept its capacity to respond to cytokines such as IL-3, GM-CSF and EPO [105]. We studied two different erythroid markers, namely glycophorin A and hemoglobin, which was measured by benzidine staining. **Fig. 8** depicts the similarity between the effect of cpn10 and EPO. The cpn10-mediated increase in glycophorin A was higher than that observed with EPO (30% vs. 19%) and was significantly higher than the untreated controls ($P<0.01$, one-way ANOVA, Student-Newman-Keuls Multiple Comparisons test). The effects were even more apparent in the case of hemoglobin, which was increased by 57% with cpn10 ($P<0.01$) and by 40% with EPO ($P<0.05$) as compared to controls. As opposed to its effect on EPO-independent K562 cells (**Fig. 7**), cpn10 caused a negligible increase in TF-1 cell proliferation, similar to that obtained with EPO (**Fig. 8**). *In vivo*, EPO is known to increase erythroblast proliferation and differentiation simultaneously [8, 10].

Fig. 8. Comparison of the effects of cpn10 and EPO on glycophorin A and hemoglobin levels in TF-1 cells. TF-1 cells were starved over night in a serum-free medium. They were then incubated with 0.1 μg/ml cpn10 or 2U/ml EPO for 3 days. Glycophorin A and benzidine-positive cells (hemoglobin) were estimated as explained in Methods. The values are expressed as mean percentage increases over control cell cultures ± SE. n=6 for benzidine determinations and cell numbers and n=5 for glycophorin determinations. *P<0.05 and **P<0.01, one-way ANOVA, Student-Newman-Keuls Multiple Comparisons test. The increases of cell numbers in the presence of cpn10 or EPO were not significantly different from control cell cultures.

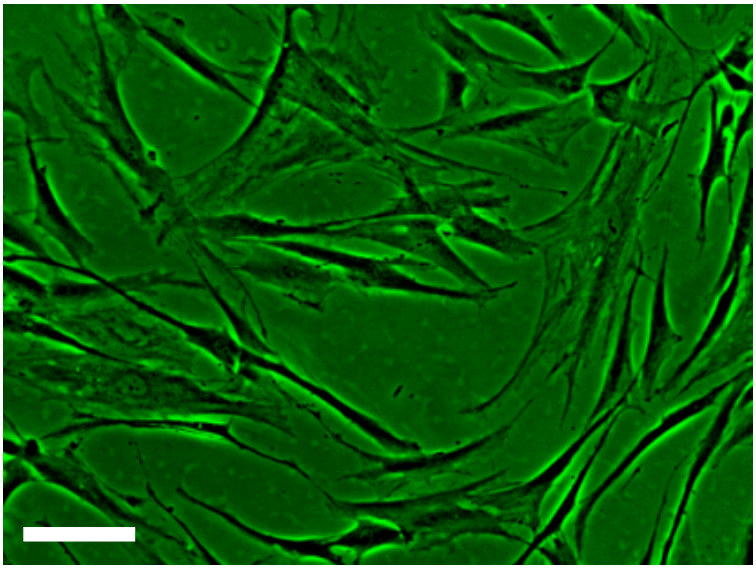

90
60
30
0
% over controls
cpn10
EPO
**
*
**
Cell numbers
Glycophorin A
Benzidine

5. Cpn10 overexpression promotes fibroblast differentiation

It is known that cpn10 stimulates collagen I synthesis in cultures of human osteoblasts, without affecting cell numbers [106]. Therefore, cpn10 probably acts as a differentiation factor not only in erythroid cells, but also in many other cell types. Another endothelial-derived EPO-dependent factor studied in our lab, namely C21 (the C-terminal peptide of thrombospondin-4), was shown to promote the growth of skin fibroblasts [36]. We were interested to know if, in a correlated way, cpn10 would also have an effect on fibroblast growth and eventually differentiation. Fibroblasts transduced with the Lentiviral system and overexpressing cpn10 had a very different morphology (as depicted in **Fig. 9**) and a much slower growth rate than the wild type fibroblasts. We estimated the collagen I production and divided the amounts obtained by the number of cells (determined with the Alamar Blue technique). The results shown in **Fig. 10** clearly indicate that cpn10 overexpression favors collagen I production at different cell densities, therefore promoting fibroblasts differentiation. We obtained significant results after one-day incubation with 1500 and 2000 cells, namely an increase in collagen of ratios 4.7 and 4.1 as opposed to 1.7 and 1.4, respectively, for untreated controls ($P<0.05$ for both, Student-Newman-Keuls Multiple Comparisons test). The increase was more drastic after 2 days incubation, when the ratios reached 6 and 5.9 vs. only 1.8 and 1.9 for controls with 1000 and 1500 cells, respectively ($P<0.05$ for both).

Fig. 9. Morphology of skin fibroblasts overexpressing cpn10. (A) Wildtype skin fibroblasts cultured in DMEM with 10% FBS and 2ng/ml FGF. (B) Lentivirus transduced skin fibroblasts overexpressing cpn10 cultured as indicated above and selected with 6μg/ml blasticidin. Cell cultures were visualized with a phase-contrast microscope. The white bar represents 0.1mm.

A



B

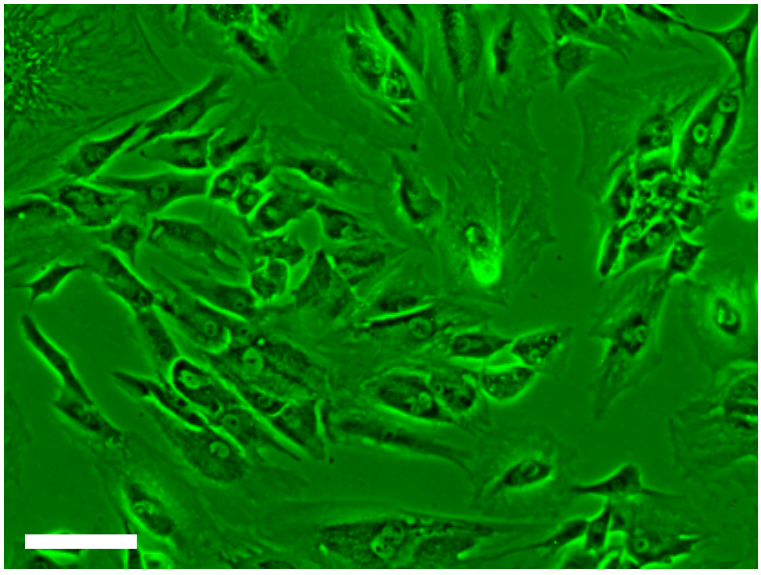
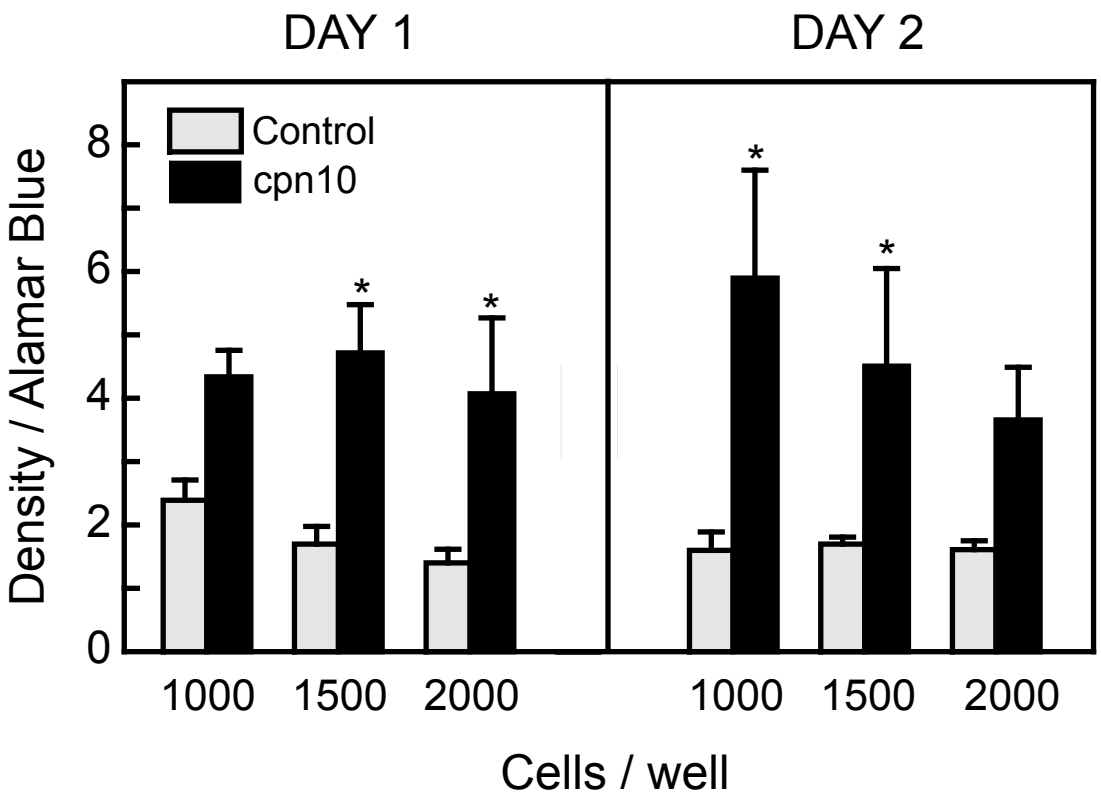


Fig. 10. Increased collagen I deposition in monolayers of skin fibroblasts overexpressing cpn10. Human skin fibroblasts were stably transduced with lentiviruses programmed for overproduction of cpn10 and the amount of collagen I in the monolayers were compared with the amounts present in normal skin fibroblasts after 1 or 2 day incubations. Cells were seeded on 96-well plates with increasing cell numbers. We observed significant increases in collagen deposition after one-day incubation in wells containing 1500 and 2000 cells and this difference was more pronounced after 2 days. The values are expressed as absorbance units of the color reaction observed in the ELISA plates and the absorbance of the Alamar blue determination in each well ± SE. Four wells were measured for each experiment. Total number of experiments: n=5 for day 1 and n=6 for day 2. *P<0.05, one-way ANOVA, Student-Newman-Keuls Multiple Comparisons test.


DAY 1
DAY 2
Control
cpn10
Density / Alamar Blue
0
2
4
6
8
*
*
*
*
1000
1500
2000
1000
1500
2000
Cells / well

6. Search for intracellular mediators of cpn10 with antibody microarrays

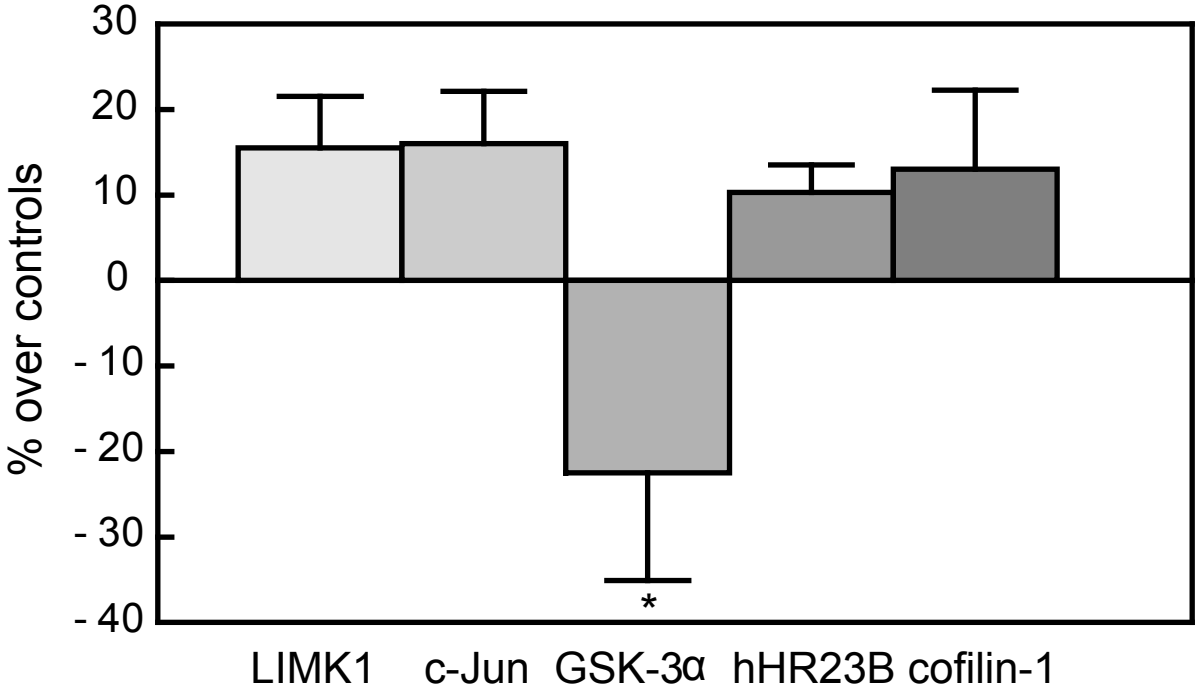
K562 cells were incubated for 10 minutes at 37°C with 0.1µg/ml cpn10 as indicated in Methods. The lysates of cpn10-treated and control cell cultures were analyzed with the antibody microarray by Kinexus containing 273 phospho-site specific and 377 pan-specific antibodies. Since this initial screen can produce false-positive results, 18 proteins with the most pronounced changes after cpn10 treatment were chosen for confirmation by an immunoblot analysis (Kinetworks), also performed by Kinexus. The results of the Kinetworks analysis are presented in **Table 1**. Only 6 proteins with noteworthy changes (namely with a 25% or higher difference as compared to the respective controls) were chosen for a Kinetworks multiple sample screen immunoblot for statistical analysis; these proteins are highlighted in the table. We kept cofilin-1 and included it in further investigations even if it only decreased by 17% in the cpn10-treated sample because of its involvement in cell cycle progression and its link with the LIM domain kinase 1 (LIMK1) [107, 108]. Of the 6 proteins and corresponding isoforms only 5 showed a detectable and consistent change in phosphorylation status and the results are depicted in **Fig. 11**. The only significant change observed in the presence of cpn10 was a decrease in GSK-3α phosphorylation at tyrosine 279. The major problem with the microarray analysis was not the method itself but the very variable responses of K562 cells. These cells proliferate very rapidly and their

responses vary tremendously, because the starvation period preceding cpn10 incubation does not always result in an efficient cell cycle arrest. Nevertheless, the most important message obtained from these experiments is that GSK-3α and GSK-3β play an important role on the action of cpn10. Both GSK-3 isoforms showed impressive changes in the initial screen and the second validation screen. Therefore, we decided to study the phosphorylation of these proteins in more detail with the more stable TF-1 cell line.

Table 1. Immunoblot analysis (Kinetworks) reports. Control and cpn10-treated K562 cell lysates were analyzed with an antibody microarray by Kinexus Bioinformatics Corporation, Vancouver, Canada. Eighteen proteins that showed important changes with cpn10 were used in a Western blot analysis (named Kinetworks and performed by the same company) for confirmation. In squared brackets is shown the phosphorylation site for which the antibody used was specific. If nothing is mentioned after the protein's name, it means a pan-specific antibody was used. The results of this analysis are shown in normalized counts per minute (C.P.M.), which represent the trace quantity (area under the band's intensity profile curve) of the band corrected to a scan time of 60 seconds and normalized to make up for differences in protein amount. The last column establishes the calculated percent increase or decrease in cpn10-treated samples as compared to controls.

Full name of protein	Abbreviation	Normalized C.P.M.		% cpn10
		Control	cpn10	
Aurora kinase C	Aurora C	0	0	0
B-cell lymphoma protein 2 alpha	Bcl2	0	0	0
Bcl2-antagonist of cell death [S91]	Bad	0	0	0
Cell division control protein 42 homolog	Cdc42	0	0	0
Cofilin 1	Cofilin 1	142	118	-17%
Epidermal growth factor receptor-tyrosine kinase [Y1148]	EGFR	0	0	0
Eukaryotic translation initiation factor 2 alpha [S51]	eIF2a	11125	13923	25%
Extracellular regulated protein-serine kinase 1 / 2 Extracellular regulated protein-serine kinase 2	Erk1/2 Erk2	4970 9064	4165 8662	-16% -4%
Fibroblast growth factor receptor-tyrosine kinase 2	FGFR2	0	0	0
FBJ murine osteosarcoma oncoprotein-related transcription factor [T232]	Fos-c	432	N/A	N/A
Glycogen synthase-serine kinase 3 alpha [Y279]	GSK-3a	3198	2062	-36%
Glycogen synthase-serine kinase 3 beta [Y216] Glycogen synthase-serine kinase 3 beta [Y216]	GSK-3b GSK-3b	3217 2170	1917 1421	-40% -35%
Histone H3.3 [T3]	Histone H3	2654	2373	-11%
Jun proto-oncogene-encoded AP1 transcription factor [S73]	Jun	3720	4769	28%
LIM domain kinase 1	LIMK1	128	162	27%
MAPK/ERK protein-serine kinase 1(MKK1) [T291]	MEK1	366	399	9%
MAPK/ERK protein-serine kinase 5 (MKK5)	MEK5	0	0	0
UV excision repair protein RAD23 homolog B	hHR23B	795	508	-36%

Fig. 11. Effect of cpn10 on the phosphorylation status of 5 regulatory proteins in K562 cells. K562 cells were incubated with 0.1μg/ml cpn10 and changes in the phosphorylation profile of 5 proteins (treated cells versus control cell cultures) were evaluated using a Kinetworks multiple sample screen immunoblot (Kinexus). The values are expressed as percent increase (or decrease) over control cell cultures ± SE. n=4. The only significant difference observed was the decrease of GSK-3α phosphorylation at tyrosine 279. *P<0.05, one-way ANOVA, Student-Newman-Keuls Multiple Comparisons test.


30
20
10
0
- 10
- 20
- 30
- 40
% over controls
*
LIMK1
c-Jun
GSK-3α
hHR23B
cofilin-1

7. Cpn10 increases GSK-3β phosphorylation, while decreasing cofilin-1 phosphorylation in TF-1 cells

We ultimately wanted to investigate the changes in the phosphorylation level of GSK-3α/β and cofilin-1, since these proteins are linked by the enzyme Cdc42 and might play a role in cell survival and apoptosis by two different mechanisms [109-111]. Furthermore, GSK-3α/β, Cdc42 and cofilin-1 were all present in the initial microarray screen with K562 cells. We incubated TF-1 cells with 0.1µg/ml cpn10 for 10, 20 and 30 minutes, lysed the cells as previously described and proceeded to a Western blot analysis of phosphorylation of GSK-3α at Y279, GSK-3β at Y216 and cofilin-1 at S3 (**Fig.'s 12** and **13**). No significant changes were detected in the case of GSK-3α; however, a small decrease in the phosphorylation of tyrosine 279 was observed after 10 and 20 minutes incubation with cpn10, which is consistent with the Kinexus findings in K562 cells (**Fig. 11**). In contrast, the phosphorylation of GSK-3β on tyrosine 216 increased gradually during the first 20 min incubation, but only the effect at 10 min was statistically significant ($P<0.05$, Wilcoxon test). In the case of cofilin-1, the phosphorylation levels of serine 3 decreased with time, reaching a significant decrease of 45% after 20 minutes incubation with cpn10 ($P<0.02$, Student's t-test).

Fig. 12. Western blots of TF-1 cell lysates. TF-1 cells were starved in a serum-free medium overnight and then incubated with 0.1 μg/ml cpn10 for 0 (control), 10, 20 and 30 min in 37°C, as represented by lanes 1, 2, 3 and 4, respectively. After incubation the cells were lysed as previously described in Methods, and the changes in phosphorylation status of GSK-3 and cofilin-1 were evaluated by Western blots. (A) Both phosphorylation of GSK-3α (Y279) and GSK-3β (Y216) are represented as single bands of approximately 51 and 47 kDa, respectively. (B) Loading and transfer efficiency for GSK-3 evaluated with a reversible Ponceau Red staining. (C) Phosphorylation of cofilin-1 (S3) observed as a 16 kDa band. (D) Loading and transfer efficiency for cofilin-1 confirmed with actin.

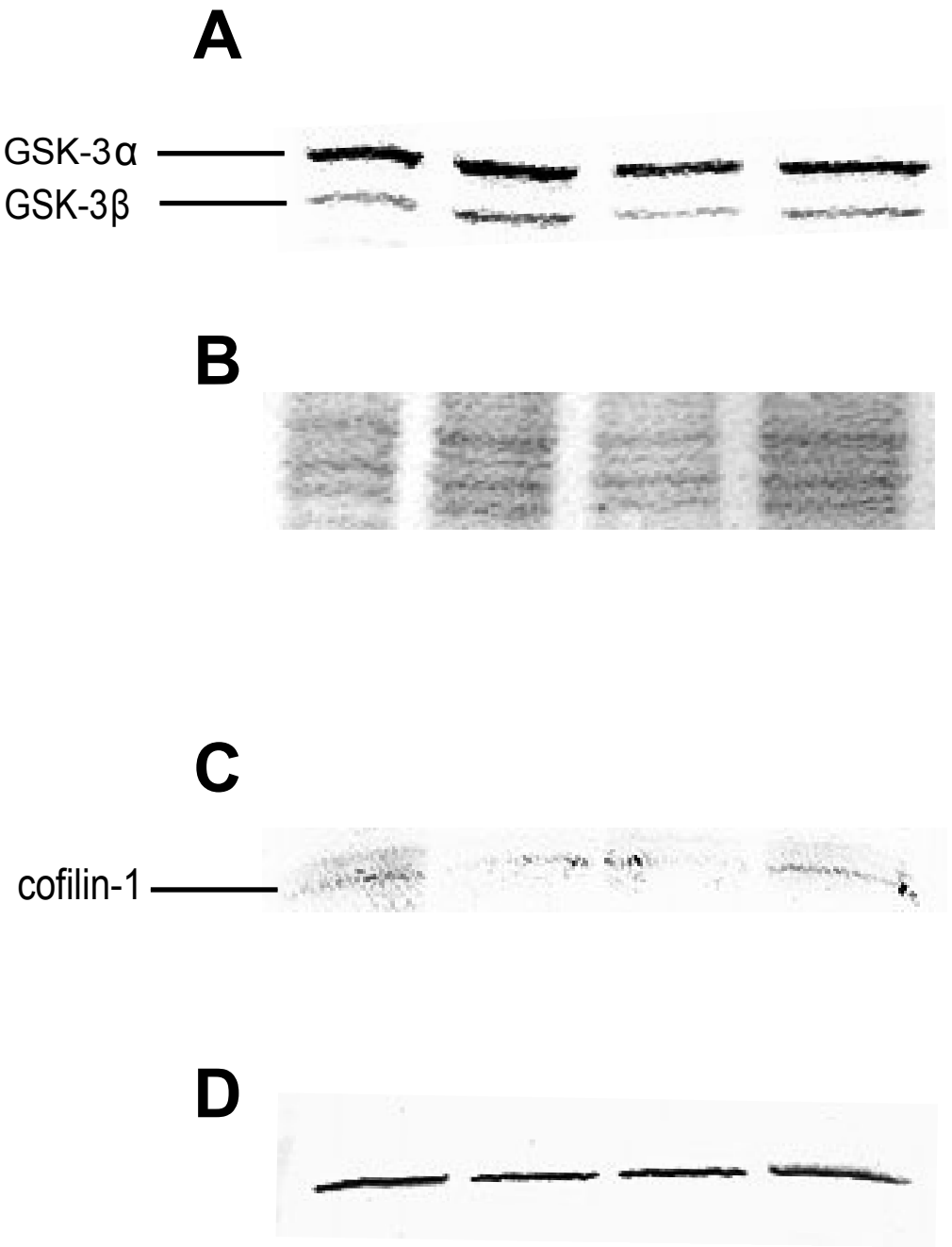
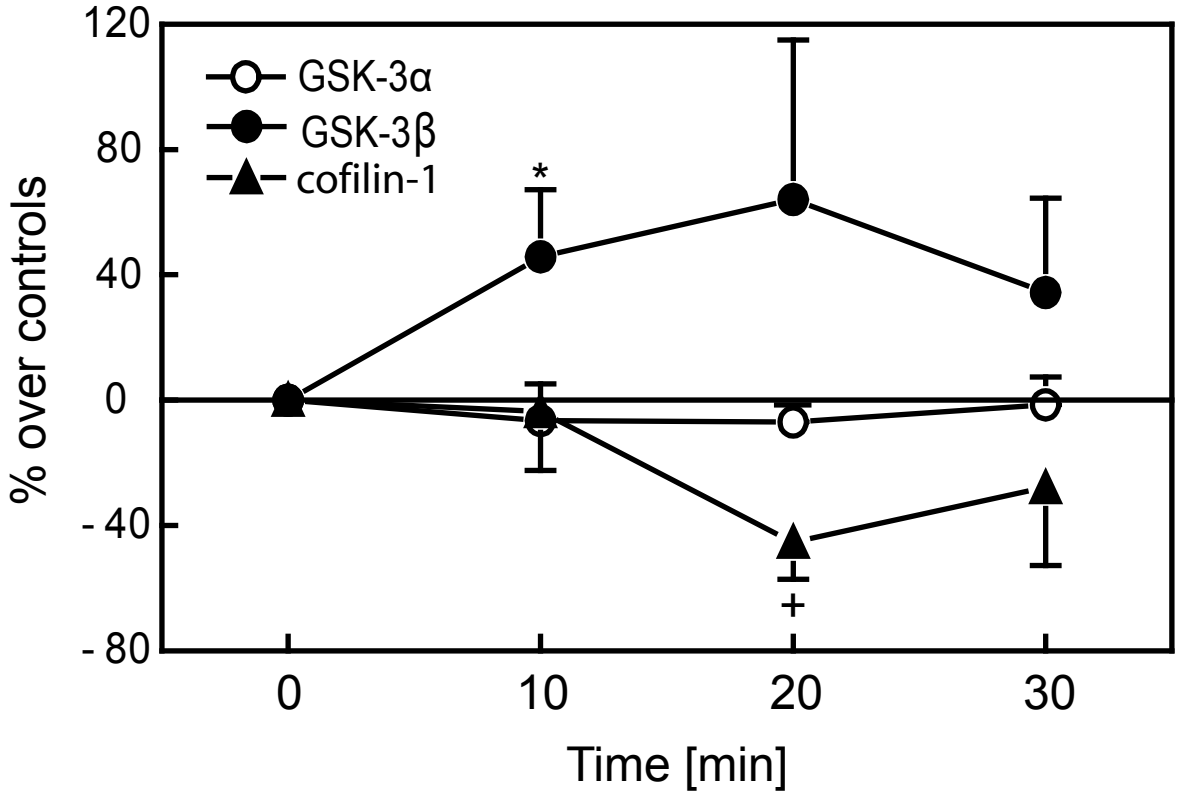

A
GSK-3α
GSK-3β
B
C
cofilin-1
D
1
2
3
4

Fig. 13. Effect of cpn10 on the phosphorylation levels of GSK-3 and cofilin1 in TF-1 cell lysates. TF-1 cells were starved in a serum-free medium overnight and then incubated for the different time periods indicated in the figure with 0.1 μg/ml cpn10. The cells were lysed and analyzed by Western blots as indicated in Methods. The obtained quantifications were normalized to correct for differences in protein loading and/or transfer by dividing the alkaline-phosphatase levels by the Ponceau Red or actin signal in the case of GSK-3 and cofilin-1, respectively. The results are expressed as increase (or decrease) over control cell cultures treated in identical way ± SE. n=6 for cofilin-1 and n=7 for both GSK-3s *P<0.05, Wilcoxon test. $^{+}$P<0.02, paired Student's t-test.


120
80
40
0
- 40
- 80
% over controls
GSK-3α
GSK-3β
cofilin-1
*
+
0
10
20
30
Time [min]

8. GSK-3 is involved in TF-1 cell differentiation, but also affects cell numbers

Given that the levels of phosphorylation of both GSK-3's (α and β) were affected by the addition of cpn10, we wanted to determine if this enzyme was involved in any way in the modulation of differentiation achieved by cpn10 in erythroid cells. We incubated TF-1 cells for 3 days with 0.5nM, 5nM and 50nM GSK-3 inhibitor, both in the presence and absence of 0.1µg/ml cpn10. This GSK-3 inhibitor is specific for both α and β isoforms. The differentiation of control TF-1 cells, as measured by the glycophorin A marker, was decreased by 51% when adding GSK-3 inhibitor at a concentration of 50 nM (**Fig. 14**). However, when cpn10 was added simultaneously with the inhibitor, essentially no change was observed in the differentiation status of the cells. At the highest concentration of GSK-3 inhibitor used (50 nM), the difference in glycophorin A levels between the control cells and the cpn10 treated ones was significant ($p=0.031$, paired t-test). When counting the cells, we noticed that there was an increase in proliferation with the highest inhibitor concentration used not only in the cpn10 treated cells, but also in controls (**Fig. 15**). Surprisingly, this increase in cell numbers was significant only in controls ($p<0.001$, one-way ANOVA, Student-Newman-Keuls Multiple Comparisons test). Moreover, the difference between the control and cpn10-treated cell numbers in the presence of 50 nM GSK-3 inhibitor was also significant ($p<0.05$, Student-Newman-Keuls test).

Fig. 14. Effect of GSK-3 inhibitor on the production of glycophorin A. TF-1 cells were starved and incubated as indicated in Methods. This time increasing concentrations of GSK-3 inhibitor IX were added in the presence (open circles) or absence (closed circles) of 0.1μg/ml cpn10. Mean ± SE, n=6. The difference between cpn10-treated cells and controls with 50nM inhibitor were statistically significant (+, P=0.031, paired t-test).

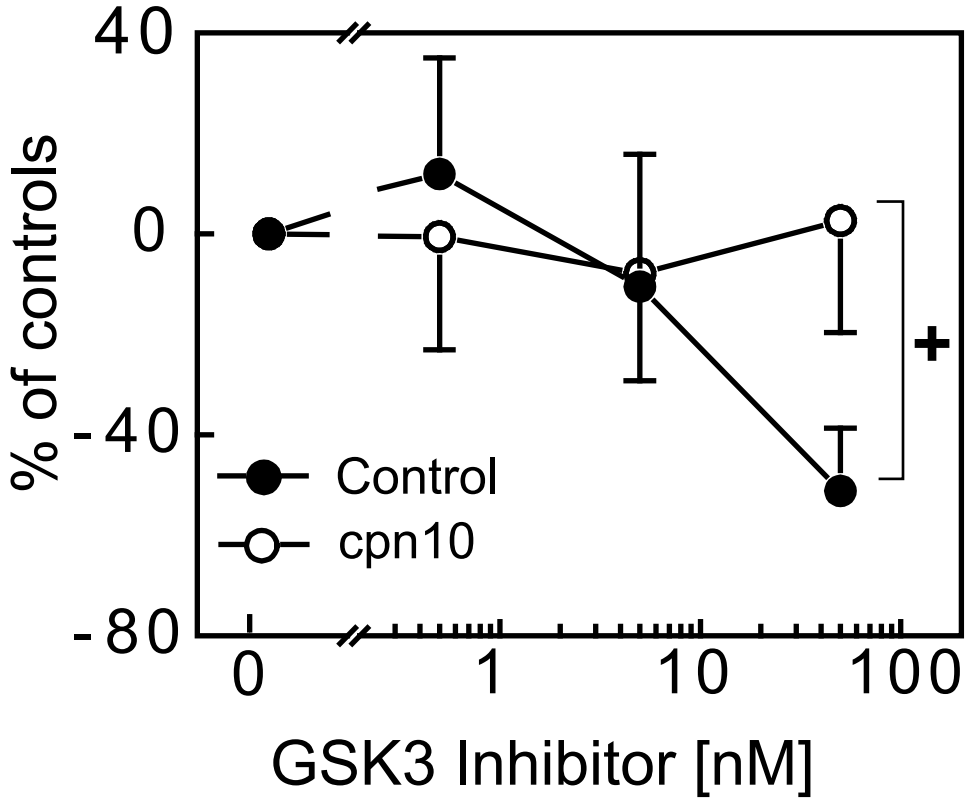
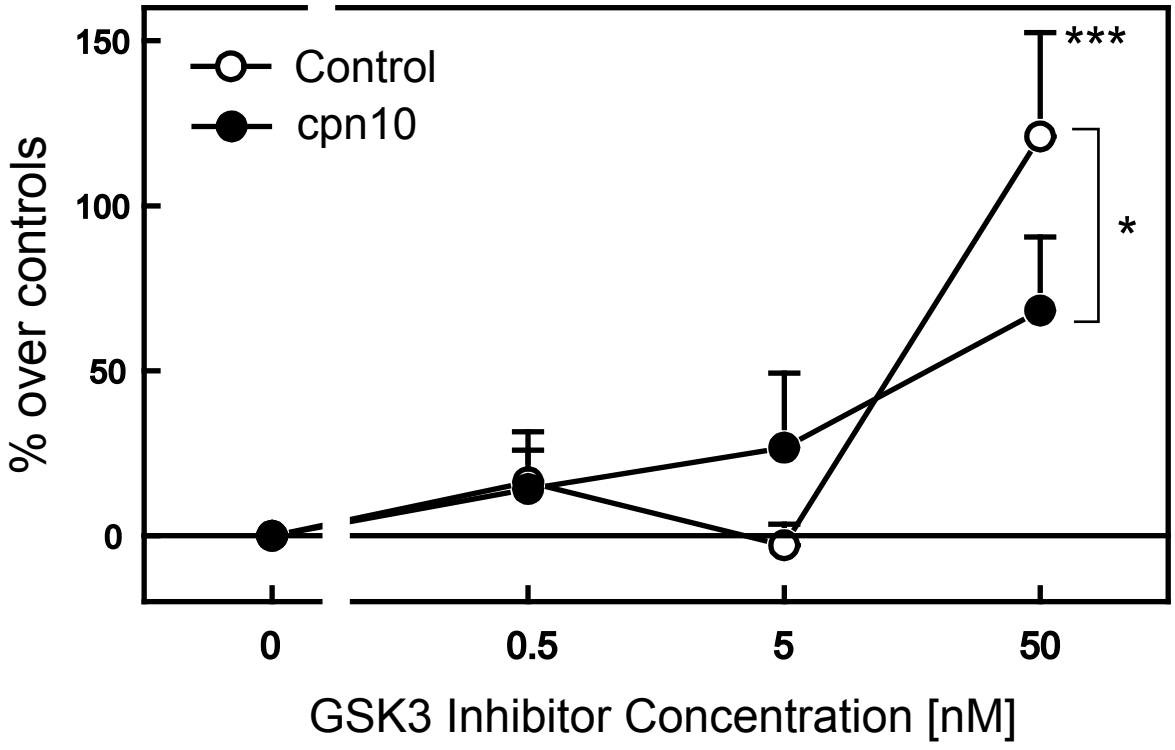

40
0
-40
-80
% of controls
Control
cpn10
+
0
1
10
100
GSK3 Inhibitor [nM]

Fig. 15. Effect of GSK-3 inhibitor on TF-1 cell proliferation. TF-1 cells were starved overnight and incubated with increasing concentrations of GSK-3 inhibitor IX in the presence (closed circles) or absence (open circles) of 0.1μg/ml cpn10. The results are expressed as percent increase over control cells cultured in the absence of inhibitor ± SE. n=8. The increase observed in control cell numbers in the presence of 50nM GSK-3 inhibitor was significant. The difference between cpn10-treated cells and controls with 50nM inhibitor was also statistically significant ***$p<0.001$, *$p<0.05$, one-way ANOVA, Student-Newman-Keuls Multiple Comparisons test.


150
100
50
0
% over controls
Control
cpn10
***
*
0
0.5
5
50
GSK3 Inhibitor Concentration [nM]

VII. Discussion

For many years, scientists' propensity for compartmentalization has confined EPO to the hematopoietic system alone. However, the discovery of the various pathways triggered by EPO-R activation has revealed the new and exciting role of EPO in mitogenesis, angiogenesis and tissue protection against apoptosis. The unraveling of EPO's numerous implications outside the process of hematopoiesis has raised a significant interest, in particular with regard to its cytoprotective role in the nervous and vascular systems. Although primarily secreted in the kidney, EPO can be found in a number of other organs, including the liver, brain, heart, reproductive organs and vascular system. Similarly, its receptor is also present in all these organs as well as in the gastrointestinal tract, pancreas and prostate [6]. EPO has a protective role in all those tissue types, mainly through the anti-apoptotic effect that it exerts by the activation of various pathways. First of all, activation of the EPO-R and the subsequent homodimerization of STAT5 were found to induce transcription of anti-apoptotic genes such as Bcl-X_L [15]. Second, EPO-R activation can also lead to the phosphorylation of PI3-K, a kinase that can trigger the phosphorylation and subsequent activation of the Akt enzyme, which has an extensive role in cytoprotection [16, 19-21]. In addition, Akt phosphorylation of GSK-3β helps protect cardiac myocytes from ischemia-reperfusion-induced necrosis, possibly by blocking the opening of the non-

specific mitochondrial permeability transition pores (MPTPs) [112]. Yang CW *et al.* [113] showed that EPO can also upregulate Hsp70 by the JAK/ STAT pathway and that this heat shock protein can have a protective effect on kidney cells following ischemia and reperfusion injury. Hsp70 overexpression was shown to bind to the apoptotic protease-activating factor 1 (Apaf-1) and inhibit it, therefore decreasing caspase-9 and 8 activity in response to hypoxia/ischemia injury in the brain [114]. Moreover, it seems that EPO preferentially employs the ERK1/2 pathway rather than the PI3-K/Akt or the STAT pathway in order to protect against retinal ganglion cells degeneration [115]. It was previously shown that, similarly to its action in the hematopoietic cells differentiation, EPO could also cause the production of neuronal progenitor cells from forebrain neural stem cells [116]. In addition to neurogenesis, rhEPO administration 24h after stroke was also shown to induce migration and proliferation of endothelial cells from other organs, formation of capillary-like tube structures on cerebral endothelial cells, as well as angiogenesis [117]. EPO‘s effect on neurogenesis and neovascularisation is believed to correlate with increased cerebral levels of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF), respectively [118].

Extensive research has been conducted so far with respect to EPO’s effects on both cardio- and neuro- protection, mainly because of its numerous potential therapeutic applications [119-123]. So far, recombinant EPO has been used to treat anemia associated with chronic

kidney disease, surgery, chemotherapy and zidovudine treatment in HIV-infected patients. Novel potential applications of EPO as a tissue-protective agent extend to a multitude of conditions, including ischemic heart failure, congestive heart disease, stroke, trauma, acute renal failure, wound healing, diabetic nephropathy, schizophrenia and addiction [123, 124].

From the many non-erythroid target cells of EPO, the endothelium is certainly most significant, given its large mass (1.5kg in humans [32]) and its immediate access to the bloodstream. For these same reasons, the EPO-dependent secretion of erythroid-regulatory factors from endothelial cells is likely to have an important contribution to erythropoiesis. As previously mentioned, some inflammatory cytokines (like IL-3) inhibit the endothelial-derived EPO-dependent secretion of stimulating factors (such as TSP-1, TSP-4 or IGFBP-3), which probably accounts for the inhibition of erythropoiesis *in vivo* when these cytokines and EPO are administrated simultaneously [31]. The 2D electrophoresis of HUVEC lysates cultured for 2 days in the presence or absence of 2U/ml EPO (**Fig. 4**) followed by mass spectrometry allowed the discovery of chaperonin 10 (cpn10) as a novel endothelial-derived EPO-dependant factor. This finding has been confirmed by an immunoblot analysis, which demonstrates that EPO significantly increases the secretion of cpn10 into the supernatants of HUVEC cultures after a 2 day incubation with 2U/ml EPO (**Fig. 5** and **6**). Cpn10 is an evolutionary conserved heat-shock

protein predominantly found in mitochondria, with roles in protein folding and cellular protection from stress [39, 40]. The general interest with respect to this chaperonin's functions has increased dramatically since it was discovered that cpn10 is a homologue of early pregnancy factor (EPF), an immunosuppressive agent secreted within 24 to 48h after fertilization in the serum and urine of all pregnant mammals tested [44, 47]. Cpn10 was later on shown to have various immunomodulatory properties, including the inhibition of LPS binding to TLR and the subsequent reduction in TNF-α and several other inflammatory mediators [64]. This anti-inflammatory property of cpn10 would be very beneficial in the context of erythropoiesis, which is inhibited by various cytokines, including TNF-α [125]. Kidney disease patients often become anemic and resistant to rhEPO therapy; in this case, they could very much profit from endothelial factors that promote erythropoiesis either directly (like C21) or indirectly, through a reduction in inflammatory cytokines (like cpn10).

The anti-inflammatory action of the cpn10 molecule has been carefully studied, because it could have important therapeutic applications. It has been the subject of clinical trials for the treatment of rheumatoid arthritis, psoriasis and multiple sclerosis [70-72]. It is also well established that cpn10 has growth factor properties [50]. However, no evidence to date links cpn10 to any erythropoietic activity. As mentioned in the introduction, the only potential correlation between cpn10 and red blood cell formation is that in two conditions associated with increased

erythropoiesis, namely pregnancy and hepatectomy, there is also an increased production of cpn10 [48, 50, 96-98]. **Fig. 7** shows that cpn10 significantly inhibits the proliferation of the erythroleukemic K562 cells, thus mimicking the effect of sodium butyrate, a known differentiation factor in this cell line. Moreover, cpn10 was shown to stimulate both hemoglobin and glycophorin A (two distinct erythroid differentiation markers) in the EPO-dependent and IL-3-responsive TF-1 erythroleukemic cell line (**Fig. 8**). This increase was even more pronounced in the cpn10-treated cells than in the EPO-treated, positive control cultures. In contrast with the effect obtained in K562 cells, TF-1 cell numbers remained almost unchanged after treatment with cpn10. These results might seem contradictory; however, they merely point out a certain resemblance between the actions of cpn10 and those of EPO. Cpn10 mimics the erythroid differentiation effects of EPO (**Fig. 8**), but without stimulating erythroid cell growth. The action of EPO is known to be anti-apoptotic [6, 8, 10, 15], whereas cpn10 inhibits K562 cell proliferation (**Fig. 7**). *In vivo*, EPO drives the erythrocyte maturation process, one of the few cases in development when proliferation and differentiation, two normally antithetical functions, are accommodated simultaneously [8, 10].

In the present investigation it was also observed that overexpressing cpn10 in skin fibroblasts triggers changes in morphology, a slower growth rate and a significantly higher collagen I deposition in the transformed cells (**Fig. 9** and **10**). These findings are in concordance with

previous studies that described cpn10's capacity to stimulate collagen I synthesis in osteoblasts [106]. It is therefore safe to assume that cpn10 might act as a more general differentiation factor, with its effects on differentiation not uniquely restricted to erythroid cells.

In spite of the intense research going on regarding the functions of secreted cpn10, not much is known about its receptor or its intracellular mediators with respect to cellular differentiation. Some evidence exists as to cpn10's potential to modulate the Ras/MAPK pathway, namely by altering the levels of Raf, MEK, Erk and p90RSK in myocytes overexpressing the chaperonin [83, 85]. Overexpression of cpn10 was also found to modulate the levels of Akt and the abundance of the anti-apoptotic factors Bcl-2 and Bcl-XL, as well as the pro-apoptotic Bax [84, 85]. Instead of testing these known cpn10 mediators, we decided to use antibody microarrays instead, since they allow the simultaneous analysis of multiple signal transduction pathways. Although no specific receptors were in the top hit list, the first microarray screen was useful in pointing out potential intracellular mediators of cpn10. Interestingly, among those were some of the already established cpn10-dependent signal transduction proteins, such as MEK1, MEK5, Erk1/2, Erk3 and Bcl-2. However, the subsequent validation of the results with immunoblots suggested that the changes induced by cpn10 in these proteins were insignificant (see **Table 1** for some of them). The discrepancy between the initial results and this validation is not unexpected, given the high possibility of false positives

when using antibody microarrays. Also, one should not expect to find the exact same effects as already documented for two main reasons. First, the previous experiments were done with myocytes, whereas this present analysis is conducted in erythroleukemic K562 cells, a completely different tissue. The second difference is that the myocytes used in those experiments were overexpressing cpn10, meaning that the chaperonin was produced inside the cell (endogenous), not added to the cell culture medium (exogenous) like was the case for K562 cells. It is not known if the endogenous cpn10 in overexpressing myocytes and skin fibroblasts is acting as a mitochondrial peptide or secreted into the medium, nor if the exogenous cpn10 is internalized.

The only consistently present element in the initial microarray data and the subsequent validations was the phosphorylation of GSK-3α/β. As seen in **Table 1**, the phosphorylation levels of tyrosine 279 in GSK-3α and tyrosine 216 in GSK-3β were decreased by over 35% in cpn10-treated cells as opposed to controls. We chose to test further six of the proteins that were over 25% different in cpn10 samples, including the two GSK-3 isoforms. In spite of the modest effect of cpn10 on cofilin-1 (17% decrease), we also included this protein in the last validation analysis due to its link with LIMK1 and GSK-3β, which will be discussed shortly. The results from the multiple immunoblots performed for these six proteins confirmed the significant decrease in phosphorylation of GSK-3α, as well as the increase (although not significant) in the phosphorylation of Jun and

LIMK1 (**Fig. 11**). GSK-3β and eIF2α do not appear on this figure because of the inconsistency of the results. This diversity in phosphorylation status as well as the change in the effect of cpn10 on cofilin-1 and hHR23B levels (**Table 1** vs. **Fig. 11**) are not due to the analysis method itself, but to the extreme variability of K562 cells. These cells proliferate very rapidly and their responses diverge tremendously, possibly because the starvation period preceding cpn10 incubation does not always result in an efficient cell cycle arrest. Therefore, we decided to study the phosphorylation of some of these proteins in more detail with the more stable TF-1 cell line.

We found two significant changes associated with cpn10 treatment in TF-1 cells, namely an increase in GSK-3β phosphorylation on tyrosine 216 and a decrease in cofilin-1 phosphorylation on serine 3 (**Fig. 13**). Although not significant, a decrease in tyrosine 279 phosphorylation of GSK-3α was noticed, which is consistent with the results previously observed in K562 cells. The role of GSK-3 in the cpn10-mediated differentiation of TF-1 cells was confirmed with the use of a GSK-3 inhibitor. As shown in **Fig. 14**, the production of the erythroid marker glycophorin A was reduced in TF-1 cells treated with increasing concentrations of the inhibitor. Furthermore, if the inhibitor and cpn10 were added simultaneously, the chaperonin prevented the action of the GSK-3 inhibitor on the production of glycophorin A. Although the effect of cpn10 on the phosphorylation status of GSK-3β in K562 cells differs from that in

TF-1 cells, the fact that the chaperonin can alter the effects of the GSK-3 inhibitor in the later cell line identifies this enzyme as an essential mediator in the action of cpn10 on cellular differentiation within the stable system conferred by the TF-1 cells. The results in **Fig. 15** also indicate that GSK-3 might be involved in TF-1 cell proliferation. The inhibitor contributed to a significant increase in cell numbers of control TF-1 cells at the highest concentration used (50nM). Surprisingly, the addition of cpn10 together with the inhibitor also contributed to an increase in cell proliferation, although not significant. The only possible explanation for this unexpected outcome is that the two isoforms, GSK-3α and GSK-3β, might act differently according to their concentration or availability. The GSK-3 inhibitor used in these experiments is directed against both kinases, therefore making it impossible to distinguish between an effect of GSK-3α or GSK-3β.

Taken together, all these results are very interesting, especially because of the indirect connection between cofilin-1 and GSK-3β conferred by Cdc42. Cdc42 was among the proteins whose phosphorylation increased in the first Kinexus antibody microarray, but unfortunately it gave no detectable result in the validation immunoblot. Cdc42 is a Rho GTP-ase involved in many cellular functions and recent findings suggest that it also has a role in the differentiation of hematopoietic progenitors. Yang *et al.* [126] showed that Cdc42 deficiency decreased erythroid burst-forming units (BFU-Es) and colony-forming units

(CFU-Es), thus blocking erythropoiesis at early stages. Interestingly, Cdc42 was also found to phosphorylate and subsequently inactivate GSK-3β through the intermediate of a Par6-atypical protein kinase C (Par6-PKCξ), with an effect on microtubule-associated proteins and cell polarity [109]. Hoeflich KP *et al.* [111] indicated that GSK-3β was required for the NF-κB-mediated survival of liver cells and that a homozygous deletion of GSK-3β in mice embryos resulted in severe liver damage and mid-gestation lethality. Nevertheless, GSK-3β was shown to have a dual role in cell survival. Inactivation of GSK-3 is correlated with an inhibition of mitochondrial-regulated apoptosis by sustained levels of MCL-1 and subsequent prevention of cytochrome c release [127], as well as by a limited mitochondrial permeability transition [128]. A particularity of GSK-3 is that it is inactivated when phosphorylated on serine 9, but constitutively phosphorylated on tyrosine residues 216 and 279, which promotes its kinase activity [129, 130]. The effect of Y216 phosphorylation of GSK-3α needs further investigation, but based on what is already known about the actions of GSK-3 we can speculate that the decrease in phosphorylation observed in **Fig. 11** might be related to the significant decrease in K562 cell numbers after incubation with cpn10.

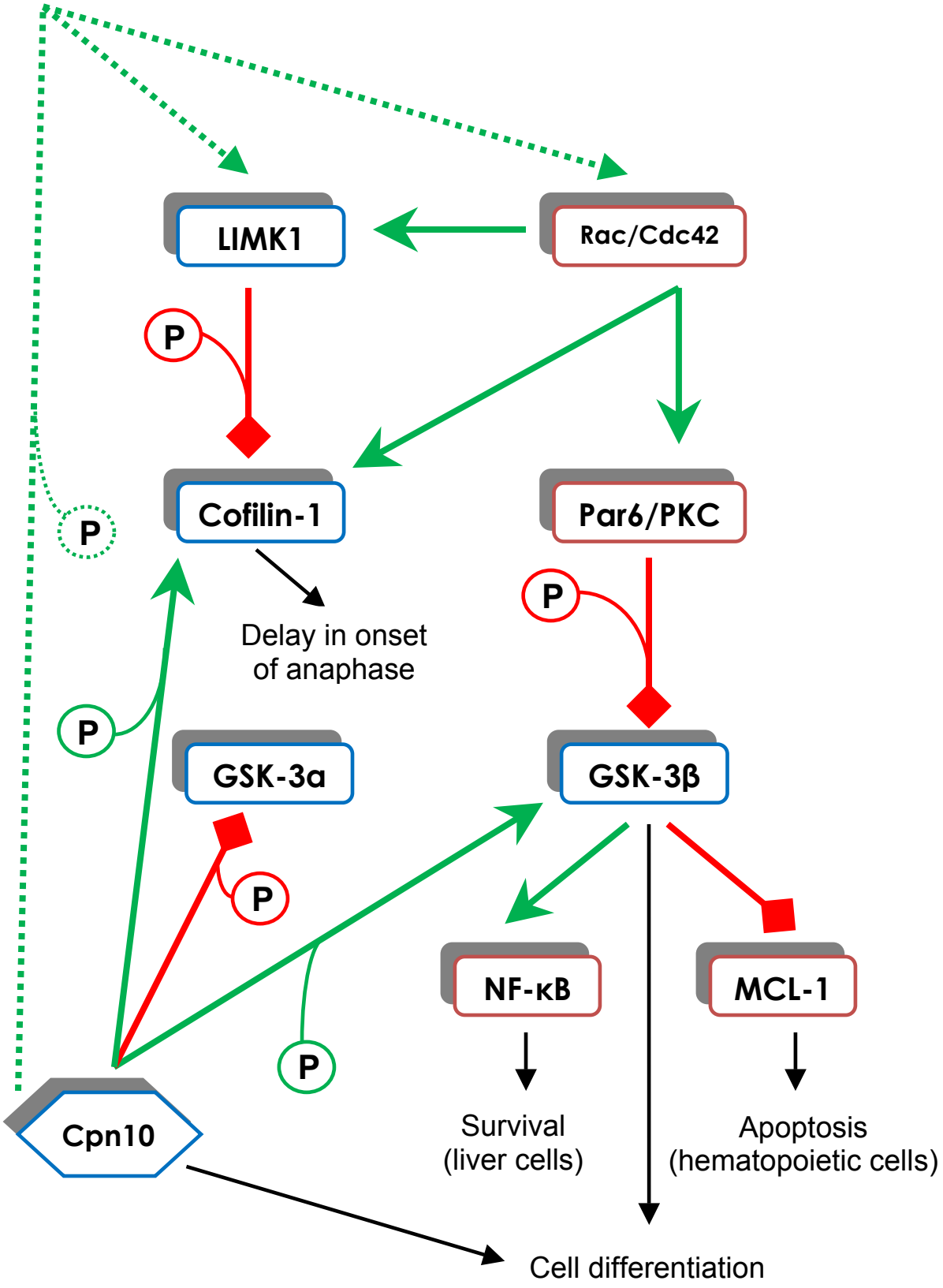
The other protein whose phosphorylation levels decreased after cpn10 incubation, namely cofilin-1, also turns out to be a Cdc42 effector. Cofilin-1 is an actin-depolymerizing protein which, in Cdc42 knock-out

cortices, was highly phosphorylated on serine 3 and subsequently became inactivated [110]. LIM domain kinase 1 (LIMK1) – mediated cofilin-1 inactivation following phosphorylation at serine 3 was also associated with the correct spindle orientation and actin networks stabilization during mitosis [108]. Moreover, LIM kinases LIMK1 and LIMK2 are regulated indirectly by Rho subfamily GTP-ases, including Cdc42 [131]. In **Fig. 13**, both cofilin-1 and GSK-3β are activated by a decrease in S3 and an increase in Y279 phosphorylation, respectively. All these proteins seem to be interrelated and to have direct effects on cell cycle progression and apoptosis. However, more work needs to be done before concluding that cpn10, through a pathway involving Cdc42, GSK-3, LIMK1 and cofilin-1, can affect the proliferation and differentiation in erythroid cells. The interconnection between all these proteins as well as the potential involvement of cpn10 in their regulation is depicted in **Fig. 16**.

In summary, cpn10 is a differentiation factor whose secretion from endothelial cells is stimulated by EPO. With respect to erythropoiesis, this is important in two ways. First, the EPO-dependent cpn10 can accelerate erythroid maturation. Second, given its immunosuppressive properties, cpn10 can facilitate the production of red blood cells in patients with kidney disorders, since they often experience chronic inflammation and anemia. Moreover, the effect of cpn10 on collagen I production in skin fribroblasts (and osteoblasts [106]) raises the possibility that its role as a differentiation factor could extend to other cell types and not be restricted

to erythroid cells only. The present study identifies GSK-3 as one of the key mediators in the action of cpn10 and points out that this kinase is not only important for the maintenance of stem cells [99], but might also be involved in the control of erythroid cells differentiation.

Fig. 16. The relationship between cpn10, cofilin-1 and GSK-3. Arrowheads (green) denote activation, whereas squared ends (red) denote inhibition. Dashed lines represent potential effects of cpn10 that remain to be confirmed. “P” stands for phosphorylation. Note that the phosphorylations by LIMK1 and PKC of cofilin-1 and GSK-3β, respectively, lead to the inactivation of their substrates. Further details and references are given in the text.


LIMK1
Rac/Cdc42
P
Cofilin-1
Par6/PKC
P
Delay in onset
of anaphase
P
GSK-3α
GSK-3β
P
NF-κB
MCL-1
P
Survival
(liver cells)
Apoptosis
(hematopoietic cells)
Cpn10
Cell differentiation

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Appendix

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LABORATORIES: L2.22, L2.19
PROJECT TITLE: Endothelial contribution to erythropoiesis

2. EMERGENCY: Person(s) designated to handle emergencies
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Name: Monica Dobocan Phone No: work: 35241 home: 450 761 6618

3. FUNDING SOURCE OR AGENCY (specify): Canadian Blood Services /CIHR
Grant No.: N/A Beginning date: 03/09 End date: 6/12

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Publications list

Luis Fernando Congote, Nyssa Temmel, Gulzhakhan Sadvakassova and **Monica C. Dobocan**. Comparison of the effects of serpin A1, a recombinant serpin A1-IGF chimera and serpin A1 C-terminal peptide on wound healing. *Peptides*. 2008 Jan;29(1):39-46.

Monica C. Dobocan, Gulzhakhan Sadvakassova and Luis Fernando Congote. Chaperonin 10 as an endothelial-derived differentiation factor: role of glycogen synthase kinase-3. *Journal of Cellular Physiology*. 2009. In press.