MECHANISM OF TRANSLOCATION OF PAR-2 FROM THE PLASMA MEMBRANE TO THE NUCLEUS: IMPLICATIONS FOR ANGIOGENESIS AND NEOVASCULARIZATION

by

Satra Nim

Department of Pharmacology and Therapeutics

McGill University

Montreal, Quebec, Canada

A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2009

ABSTRACT

The existence of GPCRs at the cell nucleus is not universal and these nuclear receptors were reported to evoke functions related to gene transcription while plasma membrane receptors induce mostly acute non-genomic effects. Protease-activated receptor 2 (PAR-2) is a member of the GPCR family. Although many responses induced by PAR-2 can result from its activation at the cell surface, nuclear localization of PAR-2 has been detected in cell tissues. However, the origin of nuclear GPCRs and the mechanisms leading to such localization are not known. Therefore, we used PAR-2 as a model to evaluate whether peptide-activated GPCRs translocate to the cell nucleus. We investigated the possible contributions of PAR-2 nuclear translocation to gene induction and retinal angiogenesis. In addition, we aimed to study the mechanism of nuclear PAR-2 regulation of gene expression.

Cell surface PAR-2 translocated to the nucleus upon stimulation. PAR-2 nuclear translocation pathway is independent of clathrin and caveolin but requires intact microtubules. The C-terminal domain and both NLS sequences of PAR-2 are important for its nuclear translocation. Importinβ1 and SNX11 are shown to interact with PAR-2 and are essential for nuclear translocation of the receptor. VEGF expression induced by PAR-2 activation is dependent on nuclear translocation of the receptor. Silencing PAR-2 or SNX11 decreased mice retinal neovascularization while re-expression of PAR-2 in PAR-2 knockout mice was shown to restore normal neovascularization of the retina. Activation of PAR-2 resulted in the interaction of the receptor with Sp1 transcription factor in the nucleus. Sp1 is essential for the regulation of PRIM1 and VEGF gene expression induced by PAR-2 and for PAR-2 regulation of cell proliferation and migration.

In conclusion, PAR-2 at the cell nucleus contributes in serving distinct functions from PAR-2 at the cell surface, specifically induction of major proangiogenic genes both in *in vitro* and in *in vivo* model of angiogenesis. Sp1 transcription factor plays an important role in the regulation of gene expression induced by nuclear PAR-2 activation.

RÉSUMÉ

La présence de récepteurs couplés aux protéines G (RCPG) au noyau cellulaire n'est pas universelle et il a été suggéré que ces récepteurs nucléaires possèdent des fonctions de transcription de gènes, tandis que les récepteurs au niveau de la membrane plasmique semblent plutôt induire des effets aigus indépendants de la transcription de gènes. Le récepteur activé par protéase 2 (PAR-2) fait partie de la famille des RCPG. PAR-2 au noyau a été observée dans divers tissus cellulaires. L'origine des RCPG au noyau et les mécanismes menant à cette localisation ne sont pas connus. En utilisant PAR-2 comme modèle, le but de ce travail était d'évaluer si l'activation des RCPG par des peptides mène à leur translocation au noyau. Nous avons étudié les rôles possibles de la translocation nucléaire de PAR-2 dans l'induction de l'expression génique et l'angiogénèse rétinienne. De plus, nous voulions aussi étudier le mécanisme de régulation de l'expression génique du PAR-2 nucléaire.

PAR-2 était transloqué de la membrane plasmique au noyau suite à sa stimulation. Cette translocation est indépendante de la clathrine et des cavéolines, mais nécessite des microtubules intactes. Le domaine C-terminal et les deux séquences NLS de PAR-2 sont importants pour sa translocation nucléaire. L'importineβ1 et le SNX11 interagissent avec PAR-2 et sont essentiels pour la translocation nucléaire du récepteur. L'expression de VEGF induite par l'activation de PAR-2 est dépendante de la translocation nucléaire du récepteur. L'expression de PAR-2 et de SNX11 sont essentielles pour une néovascularisation normale de la rétine de souris. L'activation de PAR-2 résultait en une interaction du récepteur avec le facteur de transcription Sp1 au niveau du noyau cellulaire. Sp1 est essentiel pour la régulation de l'expression génique de PRIM1 et VEGF induite par la stimulation de PAR-2 et pour la régulation de la prolifération et migration cellulaire.

En conclusion, PAR-2 au niveau de la membrane plasmique diffère de PAR-2 au noyau dans la régulation des gènes pro-angiogéniques, plus spécifiquement dans l'induction des gènes pro-angiogéniques tant au niveau *in vitro* que *in vivo*. Sp1 joue un rôle important dans la régulation de l'expression des gènes induite par l'activation de PAR-2 nucléaire.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis supervisor, Dr. Sylvain Chemtob for welcoming me into his laboratory and for his support throughout my studies.

I wish to express my gratitude to the foundations that provided me with financial support over the course of my studies:

Ste-Justine Hospital Research Center Studentship Award (2009)
Canadian Institutes for Health Research (2005-2008)

I would like to thank every fellow members of the lab over the years for their guidance and their support. I have learned a lot of things from them and have grown as a scientist with their numerous advices.

I would like to thank deeply my parents, Sarong Nim and Sipho Khim, for their encouragement and their affection. They are my motivation for setting high goals in my life. They have always given me every opportunity to have a wonderful life and to grow happily. A special thank to my sister, Panha Nim, for her friendship and her kindness. She has always been there throught my joys and difficult times. I want to thank Kanha Rath for her generosity and her help whenever I needed her.

I would also like to extend my appreciation to my aunt and uncle, my cousins and nephews for their friendship and support. Last, but never least, my friends who are always there cheering me on.

TABLE OF CONTENTS

ABSTRACT…….. II
RÉSUMÉ……. III
ACKNOWLEDGMENTS……...IV
LIST OF TABLES ……...VIII
LIST OF FIGURES ……IX
LIST OF ABBREVIATIONS ……X
PREFACE – CONTRIBUTION OF AUTHORS ……XIII
CHAPTER 1 – INTRODUCTION ……..1
Study Rationale ……2
G-PROTEIN COUPLED RECEPTORS ……5
Structure and Functions of G-protein Coupled Receptors ……...5
G Proteins …….. 5
GPCRs Signalling …… 6
GTPases ……..7
GPCRs Endocytosis ……...8
***Endocytosis and Signal Induction* ……9**
***Microtubules and Protein Transport* ……9**
GPCRs Desensitization ……...10
GPCRs at the Cell Nucleus ……11
GPCR Nuclear Signalling Pathway ……...13
PROTEASE-ACTIVATED RECEPTORS ……...13
Structure of PAR-2 ……..15
PAR-2 Signalling ……16
PAR-2 Endocytosis and Desensitization ……17
Inflammtory Roles of PAR-2 ……..18
Roles of PAR-2 in Coagulation ……..20
PAR-2 Regulation of the Vascular Tone ……..21
PAR-2 and Angiogenesis ……21
SORTING NEXINS ……22

SNXs as Trafficking Mediators ……23

SNXs as Signalling Platform ……..24

ANGIOGENESIS ……24

Vascular Endothelial Growth Factor ……25

Angiopoietin and Tie Receptor ……..26

Retina and the Retinal Vasculature ……..28

THE GENERAL TRANSCRIPTIONAL MACHINERY ……...30

SP1 TRANSCRIPTION FACTOR ……31

Roles of Sp1 ……..32

Sp1 Interacting Proteins ……33

NF-1 TRANSCRIPTION FACTOR ……..34

OBJECTIVE ……....……35

HYPOTHESIS ……....35

CHAPTER 2 - FUNCTIONS OF GPCRs GOVERNED BY THEIR TRAFFICKING FROM THE PLASMA MEMBRANE TO THE CELL NUCLEUS ……36

Preamble ……..37

Abstract ……39

Introduction ……40

Materials and Methods ……...41

Results and Discussion ……49

Acknowledgements ……..55

References ……56

Figures and Legends ……..58

CHAPTER 3 – ACTIVATION OF PAR-2 ENHANCES PROANGIOGENIC PROCESSES VIA Sp1 ……74

Preamble ……..75

Abstract ……77

Introduction ……78

Materials and Methods ……...81

Results ……..89

Discussion …….95

Acknowledgements ……..97

References ……98

Figures and Legends …..……103

CHAPTER 4 – ORIGINAL CONTRIBUTIONS TO KNOWLEDGE, GENERAL DISCUSSION, CONCLUSION AND FUTURE PERSPECTIVES ……...120

Original Contributions to Knowledge …….121

General Discussion ……122

Conclusion ……..127

Future Perspectives ..…….127

CHAPTER 5 – BIBLIOGRAPHY ……...129

LIST OF TABLES

Chapter 3

Table 1. Proteins interacting with nuclear PAR-2 in BOSC23 cells by MS/MS. ……...108

Chapter 3

Table 2. Putative transcription factor binding sites in DNA fragments obtained by ChIP……..109

LIST OF FIGURES

Chapter 1

Figure 1. Activation of Protease-Activated Receptors …….14
Figure 2. Domain Analysis of Mammalian SNXs ……..……...23
Figure 3. Organization of adult human neural retina ……29
Figure 4. Structure of Sp1 and Sp3 transcription factors ……..31
Figure 5. Family of Sp transcription factors …….32

Chapter 2

Figure 1. Translocation of PAR-2 from the plasma membrane to the nucleus …….58
Figure 2. Domains and mechanism of translocation of PAR-2 to nucleus ……...60
Figure 3. Functionality of PAR-2 nuclear translocation ……...62
Figure 4. Functionality of PAR-2 nuclear translocation *in vivo* ……...64
Supplementary figure 1. ……66
Supplementary figure 2. ……68
Supplementary figure 3. ……70
Supplementary figure 4. ……72

Chapter 3

Figure 1. Functionality of nuclear PAR-2 ……..103
Figure 2. Preparation of nuclear PAR-2 interacting proteins for MS/MS ……..106
Figure 3. PAR-2-induced transcriptional activity is Sp1 dependent …… 110
Figure 4. Nuclear PAR-2 interacts with Sp1 protein ……..112
Figure 5. Proangiogenic functions of nuclear PAR-2 are dependent on Sp1 …….115
Supplementary figure 1 ……...118

LIST OF ABBREVIATIONS

Ang - angiopoietin
ARNO – ADP ribosylation factor nucleotide-binding site opener
ATF – activating transcription factor
βAR – beta adrenergic receptor
cAMP – 3',5'-cyclic adenosine monophosphate
CAT - chloramphenicol acetyl transferase
cGMP – 3',5'-cyclic guanosine monophosphate
CGRP – calcitonin-gene-related peptide
ChIP – chromatin immunoprecipitation
COX – cyclooxygenase
CTF – CAAT box transcription factor
CREB – camp response element-binding
CXCR – CXC chemokine receptor
CysLT1 – cysteinyl leukotriene receptor 1
DNA – deoxyribonucleic acid
EC – endothelial cell
EGF – epidermal growth factor
EGFR – epidermal growth factor receptor
EMSA – electrophoretic mobility shift assay
eNOS – endothelial nitric oxide synthase
ER – endoplasmic reticulum
ER – estrogen receptor
ERK – extracellular signal-regulated kinase
FACS – fluorescence-activated cell sorting
FGF - fibroblast growth factor
FGFR – fibroblast growth factor receptor
GABA – gamma-Aminobutyric acid
GAP – GTPase activating protein
GDP – guanosine diphosphate

GFP – green fluorescent protein

GH – growth hormone

GMP – guanosine monophosphate

GPCR – G protein-coupled receptor

GRB - growth factor receptors-bound protein

GRK – G-protein coupled receptor kinase

GTP – guanosine triphosphate

HA - hemagglutinin

HEK – human embryonic kidney

HUVEC – human umbilical vein endothelial cell

ICAM – intracellular adhesion molecule

IGF – insulin-like growth factor

IKK – I kappa B kinase

IL- interleukin

iNOS – inducible nitric oxide synthase

JNK – c-Jun N-terminal kinase

KLF – Krüppel-like factor

LPA – lysophosphatidic acid

LPS - lipopolysaccharid

MAP – mitogen activated protein

MAPK – mitogen activated protein kinase

MDM2 – murine double minute

NF1 – neurofibromatosis type 1

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NLS – nuclear localization signal

PAF – platelet activating factor

PAO – polyamine oxidase

PAR – protease-activated receptor

PDGF – platelet-derived growth factor

PDZ - PsD95/Dig/ZO1

PGE – prostaglandin E

PI3K – phosphoinositide 3-kinase

PKA – protein kinase A

PKB – protein kinase B

PKC – protein kinace C

PLC – phospholipase C

PRIM1 – DNA primase 1

PTX – pertussis toxin

PX – phox homology

RA – Ras association

RGS - regulator of G-protein signalling

RNA – ribonucleic acid

RNA Pol – ribonucleic acid polymerase

RT-PCR – reverse transcriptase polymerase chain reaction

SAPK – stress-activated protein kinase

SNX – sorting nexin

Sp – specificity protein

Tie - Tyrosine kinase with Immunoglobulin and epidermal growth factor homology domains

TF – tissue factor

TGF – transforming growth factor

TNF – tumor necrosis factor

VEGF – vascular endothelial growth factor

VEGFR – vascular endothelial growth factor receptor

VLDL – very low density lipoprotein

PREFACE – CONTRIBUTION OF AUTHORS

This thesis is written in manuscript form as permitted by the McGill University Faculty of Graduate Studies and Research. It is composed of two separate manuscripts, as listed below, with the contribution(s) of each author.

Nim S, Joyal JS, Zhu T, Zaniolo K, Bossolasco M, Hamel D, Shao Z, Chemtob S.
Functions of GPCRs Governed by their Trafficking from the Plasma Membrane to the Cell Nucleus. (Ready for submission)

In this study, the candidate produced all PAR-2 mutants except for PAR-2 E3, which was produced by Michela Bossolasco. Confocal imaging was performed by the candidate and Jean-Sébastien Joyal. Western Blot of cell fractions was carried out by the candidate. The candidate did FACS experiments and calcium mobility assays. Co-immunoprecipitation of PAR-2, importinβ1 and SNX11 were performed by the candidate. RT-PCR related to the expression of Tie2 and VEGF *in vitro* and *in vivo* induced by different PAR-2 mutants were done by the candidate. All mice manipulations were conducted by Jean-Sébastien Joyal and Zhuo Shao with the candidate performing data analysis. The candidate and Jean-Sébatien Joyal prepared the manuscript with the editorial assistance of Sylvain Chemtob.

Nim S, Zhu T, Zaniolo K, Bossolasco M, Joyal JS Chemtob S.
Activation of PAR-2 Enhances Proangiogenic Processes via Sp1 (Ready for Submission)

In this study, all experiments were carried out by the candidate with the exception of Mass Spectrometry and nuclear calcium mobility, which was performed by Tang Zhu. ChIP assay was conducted by the candidate with the assistance of Michela Bossolasco. EMSA experiments were done by the candidate with the assistance of Karine Zaniolo. Cell Migration and proliferation were done by the candidate. The candidate prepared the manuscript with the editorial assistance of Tang Zhu and Sylvain Chemtob.

CHAPTER 1 - INTRODUCTION

Study Rationale

In order to adapt to their extracellular environment, cells receive extracellular stimuli by receptors for hormones, growth factors, ligands and extracellular matrix. These molecules have been classically thought to bind mostly to cell surface receptors and trigger intracellular signalling. However, a number of independent studies have unveiled an "unusual" mode of cell signalling by peptide ligands via intracrine actions (Re and Parab 1984; Re 1999). Indeed, in addition to an activation of cell surface receptors, which elicits specific functions following internalization, the stimulation of the receptors can take place directly within the cell. The clearest evidence applies to G protein-coupled receptors (GPCRs) with lipids as ligands since these can be generated from intracellular membranes (Bhattacharya, Peri et al. 1998; Gobeil, Dumont et al. 2002; Marrache, Gobeil et al. 2002). Nonetheless, there is now supportive evidence for intracrine systems, classically known to be localized at the cell surface; such is the case for the renin-angiotensin system (Re and Parab 1984; Booz, Conrad et al. 1992; Eggena, Zhu et al. 1993; Eggena, Zhu et al. 1996). This type of intracellular signalling was also shown for insulin, growth hormone, fibroblast growth factor, prolactin, angiogenin as well as parathyroid hormone (Re and Parab 1984; Re 1999; Gobeil, Dumont et al. 2002; Gobeil, Bernier et al. 2003; Mertani, Raccurt et al. 2003; Reilly, Mizukoshi et al. 2004; Marrache, Gobeil et al. 2005).

GPCRs are 7 transmembrane receptors traditionally expressed at the plasma membrane. However, there are now increasing evidence showing the existence of perinuclear and/or nuclear localization of GPCRs. Nuclear localization of GPCRs has been suggested to be essential for gene regulation, enhancement or inhibition of effects elicited by cell surface receptor activation, maintenance of cell homeostasis and in the regulation of cell proliferation (Re 1999; Shibuta, Mori et al. 2002; Spano, Andre et al. 2004; Gobeil, Fortier et al. 2006; Zhu, Gobeil et al. 2006). Moreover, for some of the receptors, internalization was found to be necessary to elicit biological functions as demonstrated for parathyroid hormone and somatostatin receptors (Henderson, Amizuka et al. 1995; Sarret, Nouel et al. 1999). Presence of nuclear transmembrane receptors, specifically for prostanoids and platelet-activating factor, was previously reported by our group to evoke functions related to gene transcription while plasma membrane receptors

induced mostly acute non-genomic effects (Bhattacharya, Peri et al. 1998; Gobeil, Dumont et al. 2002; Marrache, Gobeil et al. 2002).

Further support for functionality of intracellular transmembrane receptors is revealed by adjacent localization of required signalling machinery coupled to the receptors. Along with the contiguity of the endoplasmic reticulum with the cell surface as well as outer nuclear membrane, one finds at the nuclear membrane heterotrimeric G proteins, small GTPases, phospholipases, protein kinases A and C, MAP kinases, adenylate cyclases, inositol phosphate receptors, and ion channels (Bhattacharya, Peri et al. 1998; Bkaily, Sleiman et al. 2003; Gobeil, Bernier et al. 2003; Marrache, Gobeil et al. 2005). Accordingly, direct stimulation of nuclear GPCRs with their specific ligands, leads to sequential signal transduction including calcium entry, activation of protein kinases (phosphorylation) and NF-κB (DNA binding), and gene transcription (Bhattacharya, Peri et al. 1998; Bhattacharya, Peri et al. 1999; Gobeil, Dumont et al. 2002; Marrache, Gobeil et al. 2002). Moreover, extracellular binding of peptide ligands to their cognate transmembrane receptors have also been shown to lead to cellular incorporation of the ligand along with its receptor, resulting in internalization of the ligand-receptor complex (Weitzmann and Savage 1992; Hofland, van Koetsveld et al. 1996). On the other hand, there is only convincing evidence for GPCRs with lipids as ligands to exhibit functionality at the nucleus (Bhattacharya, Peri et al. 1998; Bhattacharya, Peri et al. 1999; Gobeil, Dumont et al. 2002; Marrache, Gobeil et al. 2002). The physiological significance of the presence of nuclear or nuclear translocated GPCRs is not well understood. However, in certain diseases, accumulation of GPCRs at the nucleus has been documented. Indeed, an increased proportion of nuclear opioid κ receptors were observed in an experimental animal model of primary hereditary hypertrophic cardiomyopathy while in cancer cells, an increased proportion of CXCR4 has been observed (Ventura, Maioli et al. 1998; Shibuta, Mori et al. 2002; Spano, Andre et al. 2004).

The presence of GPCRs at the nucleus led to the assumption of an interaction of the receptors with different transcription factors possibly on the gene promoters necessary for activation of gene transcription. Indeed, GABA-B receptors can interact with transcription factors of the ATF family (Nehring, Horikawa et al. 2000; White, McIllhinney et al. 2000). Nuclear GPCRs have been reported to regulate the initiation of

transcription from RNA Pol I and RNA Pol II (Cavanagh and Colley 1989). However, the mechanisms of regulation of gene expression by these nuclear GPCRs are not well determined.

In this project, we planned to test for the first time if protease-activated receptor 2 (PAR-2), a member of the GPCR family, can translocate to the nucleus following its activation and play a role in the induction of proangiogenic genes as well as in the process of neovascularization. We aimed to identify the pathway for nuclear translocation and proteins interacting with PAR-2 that are essential in mediating nuclear translocation. Using an *in vivo* mice model of retinal neovascularization and aortic ring explants, we looked to find if nuclear translocation of PAR-2 could mediate physiological functions such as the release of proangiogenic factors and promoting vascularization. We also wanted to elucidate the mechanism of regulation of gene transcription by nuclear PAR-2 by analyzing its ability to interact with and/or to activate transcription factors and to initiate gene transcription.

G-PROTEIN COUPLED RECEPTORS

G-protein coupled receptors are part of the large superfamily of signalling transduction receptors. They are implicated in the regulation of diverse physiological processes including vision, olfaction, inflammation, immunity, cognition, pain perception, cardiac functions and neurotransmission (Lefkowitz 1993; Neer 1995; Surya, Stadel et al. 1998). Because of their numerous implications in these physiological processes, this type of receptors is a primary aim for drug development.

Structure and Functions of G-protein Coupled Receptors

GPCRs are composed of only one polypeptide and possess seven hydrophobic transmembrane domains. These transmembrane domains are characterized by alpha helices and are oriented perpendicularly to the plasma membrane. The N-terminal portion of the receptor is located at the extracellular matrix and contains glycosylation sites. The C terminus is located in the cytosol and is the subject of phosphorylation as well as other post-translational modifications. These receptors are responsible for the physiological actions of the majority of hormones, neurotransmitters and neuromodulators. Almost 80 % of signals induced by hormones are a consequence of GPCRs activation and more than 5 % of human genome encode these receptors. GPCRs enable the induction of signal transduction coming from the extracellular environment and activate a family of heterotrimeric proteins binding GTP, G proteins.

G Proteins

G proteins are known to regulate a number of different cytoplasmic effectors. These proteins are composed of three subunits: α, β and γ. In its inactive form α subunit is bound to GDP. Following the activation of a receptor, G protein is simulated and α subunit releases its GDP and exchanges it for a GTP. The exchange of GDP for GTP results in a dissociation of the α subunit from $\beta\gamma$ subunits. At the intracellular level, G proteins target many different enzymes or ionic channels. The hydrolysis of GTP by α subunit enables its re-association with $\beta\gamma$ subunits. The activation of G_{α} and $G_{\beta\gamma}$ proteins can results in a positive or negative regulation of different effectors like phospholipase, adenylyl cyclase and ionic channels. The family of G proteins comprises of many

members including G_s, G_i, G_q, G_t and G_{olf} proteins. G_s protein is known as an "activating" protein that can stimulate adenylyl cyclase and induce the formation of cAMP. cAMP is acting as a second messenger to activate or inhibit numerous enzymes or signalling cascades. G_i protein is often referred to as an "inactivating" protein that will inhibit adenylyl cyclase and probably possesses the same $\beta\gamma$ subunits as G_s protein but a different α subunit. G_q protein can activate phospholipase C_β and induce the formation of inositol triphosphates. Activation of G_t protein enables the activation of a phosphodiesterase responsible for the hydrolysis of cGMP to GMP. G_{olf} is expressed in sensorial olfaction neurons and is known to regulate olfaction signal transduction (Lefkowitz and Caron 1988; Levitzki 1988; Kaziro, Itoh et al. 1991; Majerus 1992). Activation of GPCRs can also activate proteins from the family of small G proteins (GTPase), Ras and Rho. These GTPases can influence the signalling response.

GPCRs Signalling

A great number of GPCRs are able to activate various signalling pathways that are necessary to regulate cell growth and differentiation. These signalling pathways include the activation of GTPases in the Ras family and the stimulation of the MAP kinase cascades. The mechanisms responsible for the activation of the MAP kinase cascades depend on the type of GPCRs and the cell type. Kinases activated by mitogens are serine/threonine kinases that are well conserved during evolution and are implicated in numerous signalling transductions coming from the extracellular environment. They are implicated in cell growth, cell division, differentiation and apoptosis. Mammary cells possess three major classes of MAP kinases, the class of ERK (extracellular signal-regulated kinase), the one of JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase), and the class of p38/HOG1 MAP kinases. Activation of ERK is important for the control of G0-G1 cell cycle transition, for the transition of cells into mitosis or meiosis and for cell survival by antiapoptotic regulation of cell. JNK/SAPK and p38/HOG1 are implicated in the regulation of cell growth arrest, apoptosis and activation of immune and reticulo-endothelial cells in response to environmental and hormonal stress (Pearson, Robinson et al. 2001). MAP kinases are regulated by a cascade comprising a series of kinases. Each pathway is composed of three kinases that phosphorylate and activate

themselves successively. Once activated, MAP kinases are able to phosphorylate components of the plasma membrane, the cytoplasm, the nucleus and the cytoskeleton. GPCRs can regulate MAP kinases by different mechanisms including the activation of signal transduction induced by small proteins like protein kinase A and C and by elevation of intracellular calcium. Tyrosine kinase receptors can also activate the MAP kinase cascades following their transactivation by GPCRs. GPCRs can also activate MAP kinases by interacting directly with members of the beta-arrestin family and the different components of the MAP kinase cascade (Lefkowitz and Whalen 2004; Shenoy and Lefkowitz 2005).

GTPases

The family of small G proteins includes more than 100 members classified into five different categories: the Ras, Rho, Arf, Rab and Ran GTPase family. These GTPases are found in monomeric state and their molecular masses are in the range of 20 to 30 kDa. In general, the family of Ras GTPases are known to regulate cell signals implicating in gene transcription. The family of Rho GTPases functions as a regulator of actin cytoskeleton and can also influence gene transcription. The Rab and Arf GTPases family regulates vesicules formation, fusion and movement between different cell compartments. Finally, Ran GTPases family are described to be regulators of nucleocytoplasmic transport of proteins and of microtubules organization. In their active form, GTPases are bound to GTP while in their inactive form they are bound to GDP (Bos 1998; Takai, Sasaki et al. 2001). Arf GTPase plays an essential role in the internalization of GPCRs. Arf6 is implicated in the regulation of the endocytosis of receptors and the recycling of endosomal vesicules. Two guanine nucleotide exchange factors are regulators of Arf6 activity, ARNO (ADP ribosylation factor nucleotide-binding site opener) and EFA6. Arf1 is another member of the Arf GTPase family that has been demonstrated to be important for GPCRs endocytosis (Mitchell, McCulloch et al. 1998; Claing, Chen et al. 2001). The Rab GTPases family comprises 60 members with very conserved structure and their roles are the regulation of endocyosis, exocytosis and endosomal fusions (Zerial and McBride 2001; Rosenfeld, Knoll et al. 2002; Seachrist and Ferguson 2003). Certain Rab GTPases like Rab1, Rab4, Rab5, Rab7 and Rab11 have important roles in the transport of

molecules between reticulum endoplasmic and the Golgi apparatus, in the endocytosis and the transport of GPCRs between endosomes and lysosomes (Seachrist, Anborgh et al. 2000; Gaborik and Hunyady 2004).

GPCRs Endocytosis

Endocytosis is a process that enables receptors to be internalized to intracellular compartments. GPCRs can be constitutively endocytosed or internalized following their activation by the binding of their ligands. Receptors can undergo many different endocytic pathways. The major internalization pathway is dependent on clathrin and receptors are internalized in vesicles containing clathrin and AP-2 complex. AP-2 complex is responsible for the polymerization of clathrin to the plasma membrane and enable the internalization of molecules following the detachment of vesicles by a process dependent on dynamin, a GTPase responsible for the scission of vesicles from the plasma membrane. Another important endocytic pathway is the caveolae-dependent internalization pathway. Caveolae are smooth invaginations of the plasma membrane and are enriched in cholesterol and sphingolipids (Palade 1953). Caveolins are proteins associated to cholesterol with a low density and are clustered on the cytosolic surface of caveolae. Three isoforms of caveolin have been discovered: caveolin 1, 2 and 3. Caveolin-1 and 2 are expressed in a variety of cellular types and tissues. Caveolin-3 is specifically expressed in muscular cells, astrocytes and chondrocytes (Song, Scherer et al. 1996; Tang, Scherer et al. 1996; Scherer and Lisanti 1997; Okamoto, Schlegel et al. 1998; Nishiyama, Trapp et al. 1999; Schwab, Galbiati et al. 1999). Cells that don't express or express very few caveolin-1 have a low concentration of caveolae at the cell surface. It has also been shown that the re-introduction of caveolin-1 in those cells enables the formation of caveolae (Fra, Williamson et al. 1995; Koleske, Baltimore et al. 1995; Mirre, Monlauzeur et al. 1996; Smart, Ying et al. 1996; Engelman, Wykoff et al. 1997; Orlandi and Fishman 1998; Le, Guay et al. 2002). In a similar fashion, endothelial cells from caveolin-1 knock out mice have no caveolae (Drab, Verkade et al. 2001; Razani, Engelman et al. 2001). Several novel pathways have been characterized to be independent of both clathrin and caveolae (Radhakrishna and Donaldson 1997; Lamaze, Dujeancourt et al. 2001; Sabharanjak, Sharma et al. 2002; Glebov, Bright et al. 2006; Orth, Krueger et

al. 2006). Moreover, as our knowledge of the different internalization pathways grows, it becomes clear that the endocytic vesicles internalized via distinct internalization pathways may eventually converge at the same endosomal structures and come under the regulation of the same recycling machinery.

Endocytosis and Signal Induction

Following the internalization of a receptor and its ligand, cells can generate a signalling cascade independent of G proteins. Indeed, internalization of a receptor can be another way to prolong signalling induced by the activation of the receptor at the plasma membrane (Griendling, Berk et al. 1987; Hunyady, Merelli et al. 1991). The complex of receptor-ligand in endosomes can also induce a new signalling cascade distinct of the one induced by activation of the receptor at the plasma membrane. In fact, studies have demonstrated that internalization of GPCRs enables a signalling transduction important for activation of MAPK cascades and regulation of transcription of certain genes (Luttrell, Daaka et al. 1997; Souaze, Rostene et al. 1997). A group of researchers have identified around 40 factors interacting with activated Rab5 at the endosome and internalization of receptors into the endosomes initiates a signalling event mediated by Rab5 effector (Zerial and McBride 2001; Miaczynska, Christoforidis et al. 2004). Inhibition of endocytosis dependent on clathrin in rat 1a fibroblasts results in the inhibition of ERK kinases (Luttrell, Daaka et al. 1997). There is increase evidence for connections between signalling molecules and endosomes.

Microtubules and Protein Transport

Molecular motors are important for the transport of vesicules and organelles within the cell. Recent progresses have revealed mechanistic links between membrane trafficking events like budding and fusion of vesicules and the transport of these vesicules along molecular motors. Microtubules form a dynamic and polarized cytoskeleton. Kinesins and dyneins are the two major superfamilies of microtubule motor proteins identified so far. In mammalian cells, kinesin superfamily contains 45 members that share a common motor domain but diverge in their cargo-binding tail domains (Miki, Okada et al. 2005). Dynein possess one major form of cytoplasmic domain for the transport of

many different cargos in the cell (Pfister, Shah et al. 2006). Most of dynein-mediated functions require an additional accessory complex known as dynactin, which is implicated in both cargo binding and motor processivity (Schroer 2004). Cytoplasmic dynein is the major motor for the minus end-directed transport of vesicles and organelles along microtubules, driving movement toward the cell center, such as traffic from the endoplasmic reticulum (ER) to the Golgi. The transport of vesicules from ER to Golgi along microtubules is dependent on the dynein-dynactin complex. In the cell, the dynamics of GFP-labeled proteins, such as Rab5, have demonstrated the long-range motility of endosomes along microtubules (Nielsen, Severin et al. 1999). Salman et al have shown that the presence of nuclear localization signals invokes active transport of proteins along microtubules in a cell-free Xenopus egg extract (Salman, Abu-Arish et al. 2005).

GPCRs Desensitization

Receptor internalization and relocalization are implicated in the regulation and desensitization of GPCRs. Rapid desensitization is characterized by the uncoupling of receptors to G proteins without affecting the number of receptors present in cells (Clark 1986). Desensitization of certain GPCRs is performed by a process called sequestration and is a result of a redistribution of receptors from the plasma membrane to intracellular compartments (Staehelin and Simons 1982; Toews and Perkins 1984; von Zastrow and Kobilka 1992). Slow desensitization of receptors is marked by a reduction of the total number of receptors and is induced over a long period of time of few hours or days following receptor activation. Activation of receptor will induce its phosphorylation by PKC, PKA and/or GPCR kinases (GRK). The binding of an adaptor protein, arrestin, to the phosphorylated receptor will induce the uncoupling of G protein from the receptor and the sequestration of the receptor in clathrin-coated vesicles. Following their activation, receptors are internalized in early endosomes and from there, they can be recycled back to the cell surface, transported to endosomal recycling compartment for eventually returning to the plasma membrane or fused to late endosomes. In early endosomes, receptors are dissociated from their ligands because of the low pH in the endosomal lumen (Presley, Mayor et al. 1997). It has been demonstrated that

ubiquitination of β_2AR and of CXCR4 is important for the transport of those receptors toward lysosomes and for their degradation (Marchese and Benovic 2001; Shenoy, McDonald et al. 2001). For certain receptors like δ-opioid receptors, ubiquitination is not implicated in their internalization toward lysosomes (Tanowitz and Von Zastrow 2002).

GPCRs at the Cell Nucleus

There are some studies showing the presence of growth factors, cytokines and their receptors at the cell nucleus. Indeed, fibroblast growth factor (FGF), epidermal growth factor (EGF), growth hormone (GH) and other ligands and their receptors are found at the nucleus or translocated to the nucleus. Because both ligands and their receptors have been found at the nucleus, researchers suggested that they are translocated together to the nucleus (Lobie, Wood et al. 1994; Maher 1996; Stachowiak, Maher et al. 1996; Lin, Makino et al. 2001; Wang, Lien et al. 2004). Other studies have also suggested that heptahelical receptors can localize at the plasma membrane and cell nucleus. Indeed, nuclear localization of endogenous chemokine receptor $CXCR_4$ has been detected in purified nuclei from cultured HeLa cells and in various cancer tissues (Shibuta, Mori et al. 2002; Spano, Andre et al. 2004). Our group has also demonstrated the presence of nuclear localization of functional GPCRs that are specific for prostaglandins (PGE_2), platelet-activating factor (PAF), lysophosphatidic acid (Citores, Wesche et al.) and apelin (Bhattacharya, Peri et al. 1998; Bhattacharya, Peri et al. 1999; Gobeil, Dumont et al. 2002; Marrache, Gobeil et al. 2002; Gobeil, Bernier et al. 2003). The mechanisms allowing the translocation of receptors at the plasma membrane and their ligands to the nucleus are not well established. It has been demonstrated that nuclear translocation of FGFR1 requires endosomal sorting and is dependent on dynamin and ARF6 protein (Bryant, Wylie et al. 2005). Studies have suggested that for some receptors, nuclear translocation is mediated by mechanisms dependent on lipids and/or caveolins (Citores, Wesche et al. 1999; Citores, Khnykin et al. 2001). The best-known mechanism of regulation of nuclear transport of proteins is the one dependent on nuclear localization signal (NLS), proteins in the family of importins and Ran GTPase (Pemberton and Paschal 2005). In some cases, NLS sequences may contain many basic amino acids as demonstrated for histones and ribosomal proteins. Proteins bound to RNA contain NLS

rich in arginine and glycine (Jakel and Gorlich 1998; Senger, Simos et al. 1998; Leslie, Zhang et al. 2004). NLS can also be enriched in glycine and deficient in basic amino acids (Pollard, Michael et al. 1996).

Nuclear transport of proteins occurs through the nuclear pore complex and is mediated by a superfamily of transport receptors known as karyopherins. Karyopherins can mediate nuclear import (importins) or nuclear export (exportins) of most proteins, RNA and ribosomal subunits by direct interaction or with the help of adapter proteins. The best-characterized member of the karyopherin family is karyopherin-α (importin-α) (Goldfarb, Corbett et al. 2004). Most karyopherin-β proteins (importin-β) bind directly to cargoes and do not need an adaptor (Fried and Kutay 2003; Weis 2003). Karyopherins that mediate the import of their cargoes to the nucleus recognize the nuclear localization signal (NLS). The complex karyopherin/cargo enters the nucleus via interactions with nucleoporins. Importin-α binds to its target via the NLS sequence and it forms a heterodimer with importin-β. The formation of this complex enables the translocation of the target molecule to the nucleus. In the nucleus, importins can associate with RanGTP and this association leads to the release of the cargo. Certain proteins or molecules can be translocated to the nucleus without having an NLS sequence or having an unidentified NLS sequence. In the nucleus, the cargo molecule can bind to response elements on the DNA and recruit factors leading to the assembling of the transcription initiation complex. A small number of GPCRs have been shown to contain functional NLS motif. Receptors for adenosine, angiotensin, bradikinin and endothelin have been reported to bear functional NLS (Lee, Lanca et al. 2004). Moreover, proteins containing non-classical NLS motifs were also shown to be imported to the nucleus (Christophe, Christophe-Hobertus et al. 2000). Alternatively, it is also possible that receptors may translocate to the perinuclear and (or) nuclear regions in association with carrier proteins containing NLS sequence. In fact, angiotensin II type 2 receptor requires an association with transcription factor promyelocytic zinc finger protein, which contains NLS for gene activation in mouse fibroblast-derived R3T3 cells (Senbonmatsu, Saito et al. 2003; Kelly, Cox et al. 2004).

GPCR Nuclear Signalling Pathway

Now studies of the intracellular trafficking of GPCRs revealed that some of these receptors are translocated to nuclear membrane for a prolonged period of time, compose distinctive signalling units and transduce nuclear transcriptional signals that are different from the same receptor located at the plasma membrane (Bkaily, Sleiman et al. 2003; Gobeil, Bernier et al. 2003; Gobeil, Fortier et al. 2006). It has been shown for example that activation of ERK1/2 from plasma membrane receptors induces signalling pathways that inhibit apoptosis while activation of nuclear ERK1/2 is coupled to cellular proliferation (Ajenjo, Canon et al. 2004; Gaumont-Leclerc, Mukhopadhyay et al. 2004; Jafri, El-Shewy et al. 2006). Nuclear GPCRs have been demonstrated to play an important role in the regulation of a number of physiological processes including inflammatory responses, cell proliferation and tumorigenesis (Faucheux, Horton et al. 2002; Shibuta, Mori et al. 2002; Gobeil, Bernier et al. 2003; Spano, Andre et al. 2004; Gobeil, Fortier et al. 2006; Waters, Saatian et al. 2006). Some studies have suggested that nuclear GPCRs can signal through activation of nuclear adenylyl cyclase and phospholipases. These receptors at the nucleus can also activate ERK and p38 MAPK as well as PKB and have direct effects on gene expression. In cultured brain microvascular endothelial cells, activation of nuclear receptors by their cognate ligands induces intranuclear Ca^{2+} mobility, protein kinase activity, and subsequent transcriptional regulation of target genes such as cfos, eNOS, COX-2, and iNOS (Bhattacharya, Peri et al. 1998; Gobeil, Dumont et al. 2002; Marrache, Gobeil et al. 2002; Gobeil, Bernier et al. 2003).

PROTEASE-ACTIVATED RECEPTORS

Protease-activated receptors (Gamble, Drew et al.) are part of the G protein-coupled receptor family. They are activated following a cleavage of the N-terminal part of the receptor by serine proteases and the binding of a tethered ligand on the extracellular domains of the receptor (Vu, Hung et al. 1991). These receptors can also be activated by a synthetic ligand containing the same sequence as the natural tethered ligand (Figure 1).

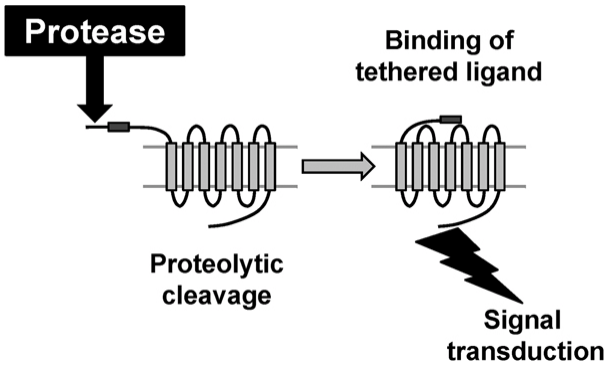


Figure 1: Activation of Protease-Activated Receptors (van der Poll 2008).

Four types of PARs have been discovered so far: PAR-1, PAR-2, PAR-3 and PAR-4. PARs are distributed in many tissues in the human body and are concentrated mainly in the intestine, kidney, colon and stomach. Studies have shown that PARs can induce mitogenic effects, more specifically neovascularization (Milia, Salis et al. 2002). PAR-1, -3 and 4 can be activated by thrombin, while PAR-2 is not activated by thrombin but by trypsin or mast cell tryptase. Trypsin is often related to the digestive enzyme secreted by the pancreas during digestion. On the other hand, trypsinogen is expressed by endothelial cells, epithelial cells, tumors and neurons and extra-pancreatic trypsin can also activate PAR-2 (Koivunen, Ristimaki et al. 1991; Wiegand, Corbach et al. 1993; Koshikawa, Yasumitsu et al. 1994; Koshikawa, Nagashima et al. 1997; Alm, Gagnemo-Persson et al. 2000)**.** An important number of enzymes from the family of endogenous or exogenous trypsin can cleave and activate PAR-2. A variety of human cancer cells secrete enzymes that have trypsin-like specificity and can activate PAR-2 (Koshikawa, Nagashima et al. 1997)**.** Endothelial trypsinogen 2, maptryptase, serine proteinase-1 and trypsinogen 4 generated by keratynocytes at the skin level are found among the members of the trypsin family that can activate PAR-2 (Koshikawa, Nagashima et al. 1997; Alm, Gagnemo-Persson et al. 2000; Takeuchi, Harris et al. 2000; Oberst, Anders et al. 2001; Steinhoff, Buddenkotte et al. 2005). Trypsin and tryptase induce a cleavage of the position Arginine[36] and Serine[37] of PAR-2. This cleavage will expose a tethered ligand on the N-terminal domain of the receptor and this ligand will bind to a conserved sequence on the second extracellular loop of PAR-2. PAR-2 can also be activated by an agonist peptide containing the same sequence as the tethered ligand: SLIGKV for human and

SLIGRL for mouse PAR-2. In contrast to trypsin, typtase is not able to completely activate PAR-2 in its glycosylated form (Compton, Renaux et al. 2001). The effect of glycosylation on the N-terminal domains of PAR-2 and PAR-4 has not been studied yet. PAR-3 serves has co-factor for PAR-4. PAR-4 has also been shown to be implicated in the activation of platelets (Hollenberg 2003).

Structure of PAR-2

PAR-1 and PAR-2 are encoded by genes localized on the chromosome 5q13 in human and these genes are separated by only 90 kb. PAR-3 gene is also located on the chromosome 5q13. The gene encoding for PAR-4 is located on the chromosome 19p12 but share the same common structure with other genes of PARs (Kahn, Hammes et al. 1998; Xu, Andersen et al. 1998; Al-Ani, Saifeddine et al. 1999). Human PAR-2 is a protein of 397 residus of amino acids and possesses 83 % of similarity in the sequence with mouse PAR-2 and 35 % of similarity in the sequence with human PAR-1. The extracellular N-terminal portion of the receptor is the least conserved region between human and mouse PAR-2 with only 65 % of similarity. Another region containing a high similarity between human and mouse PAR-2 is the region located at the N-terminal side of the first transmembrane domain. This part of the receptor is important for ligand binding (Bahou, Kutok et al. 1994). PAR-1 possesses a negatively charged sequence (WEDEEKNES) at the N-terminus and this sequence is distinct from the sequence responsible for the cleavage by thrombin (Vu, Hung et al. 1991). In contrast to PAR-1 and PAR-3, PAR-2 doesn't contain this negatively charged sequence. The presence of this sequence can explain the selectivity of thrombin toward PAR-1 and PAR-3 but not PAR-2. The N-terminal portion of PAR-2 is 29 amino acids shorter than the one of PAR-1 and doesn't contain thrombin binding domain. In fact, the N-terminal portion of PAR-2 contains a cleavage site (SKGR/SLIG) that can be cleaved by trypsin, mast cell tryptase and coagulation factors Xa and VIIa. This cleavage will expose an N-terminal sequence NH_2-SLIGRL (mouse) and NH_2-SLIGKV (human) that can bind to the second extracellular loop of the receptor and activate it. The N-terminus also contains consensus sites for glycosylation. Pancreatic trypsin is the strongest agonist of PAR-2 and it is found

in other cell types like epithelial cells (Koshikawa, Hasegawa et al. 1998; Cocks, Fong et al. 1999).

PAR-2 Signalling

In contrast to PAR-1, there are very few studies done on PAR-2 and its signalling pathways. Activation of PAR-1, -2 and -4 can activate $G_{q/11}$ protein and induce an increase of intracellular calcium. It has been shown that the second intracellular loop of PAR-1 is responsible for the coupling of the receptor to G_q protein (Verrall, Ishii et al. 1997). PAR-1 can also couple to G_i to inhibit adenylyl cyclase. It can activate many signalling pathways to regulate ionic channels and activate phospholipase Cβ and cSrc. The coupling of PAR-1 with either G_q, G_i or both of them depends on the cell type and the abundance of the two proteins (Hung, Wong et al. 1992; Swift, Sheridan et al. 2000). Studies have also suggested that PAR-1 can couple with members of $G_{12/13}$ family (Offermanns, Laugwitz et al. 1994). Until now, studies have demonstrated interaction of PAR-2 with G_q/G_{11} and with G_0/G_i. Indeed, activation of PAR-2 leads to G_0/G_i dependent transduction mechanism as demonstrated by the PTX-sensitive calcium modulation in Xenopus oocytes stimulated with trypsin (Schultheiss, Neumcke et al. 1997) and in HEK-293 transfected with PAR-2 stimulated with PAR-2 AP (Yu and Hinkle 1997). Because PAR-2 has been found to interact with G_q/G_{11}, it has been suggested that this receptor can activate PLC, PKC and the MAPK signalling cascade. Indeed, in neuronal cells, intestinal epithelial cells and smooth muscle cells, stimulation of PAR-2 by its agonist peptide was found to activate PLC and PKC (Berger, Tunon-De-Lara et al. 2001; Okamoto, Nishibori et al. 2001; van der Merwe, Moreau et al. 2009). Activation of PLC results in the formation of inositol triphosphate and diacylglycerol and this will induce the mobilization of intracellular calcium and the activation of PKC. It has also been shown that activation of PAR-2 in keratinocytes enables the activation of JNK and p38 MAPK (Kanke, Macfarlane et al. 2001). Activation of PAR-2 can also activate MAPK signalling pathway by activating ERK1/2 and in consequence enabling the transcription of genes like VEGFR-2 and DNA primase 1 (DeFea, Zalevsky et al. 2000). A group has reported that PAR-2 stimulation activated all three subtypes of mitogen-activated protein kinases (ERK, JNK, and p38 MAPK) and NO production by PAR-2 was blocked by inhibition of

ERK, p38, and JNK pathways (Park, Jeon et al. 2009). In addition, it has been described that activation of PAR-2 results in the activation of RhoA in HUVEC cells. These studies tend to demonstrate that PAR-2 is implicated in different physiological processes like cell proliferation and differentiation. Activation of PAR-2 by trypsin have also been demonstrated to stimulate the binding of NF-κB transcription factor to DNA and to activate IKKα and IKKβ kinases (Kanke, Macfarlane et al. 2001).

PAR-2 Endocytosis and Desensitization

Termination of signalling responses induced by PAR-2 activation is essential for a proper regulation of physiological processes implicating PAR-2. Studies have demonstrated recently that cellular responses initiated by PAR-2 activation are rapidly desensitized and its intracellular route is similar to the one of PAR-1. Termination of PAR-1 signalling is in part regulated by the phosphorylation of the receptor by GRKs. The overexpression of GRK3 and GRK5 has been shown to increase PAR-1 phosphorylation and thus lead to the inhibition of the accumulation of inositol phosphate. Studies have suggested that the C-terminal part of PAR-1 is a target for GRK phosphorylation and this region is responsible for signal termination (Ishii, Chen et al. 1994; Nanevicz, Wang et al. 1996). Indeed, a mutant of PAR-1 where serine and threonine residus have been converted to alanines possesses a better signalling capacity. This mutant displays a reduction in the phosphorylation state and is not inhibited by overexpression of GRKs. However, additional mechanisms for PAR-1 signalling arrest have been demonstrated. For example, the binding of PAR-1 to beta-arrestin independently of receptor phosphorylation is sufficient for receptor desensitization. In addition, PAR-1 desensitization is inhibited in cells that don't express beta-arrestin. It has also been described that phosphorylation of other sites on the receptor play an important role in the regulation of PAR-1 signalling. The C-terminal domain of PAR-2 contains many potential phosphorylation sites. The use of PKC inhibitors has allowed demonstrating that PKC is implicated in PAR-2 desensitization (Bohm, Khitin et al. 1996). Receptor internalization contributes to signalling arrest by dissociating receptors from G proteins and other effectors. It has been shown that PAR-2 is internalized via clathrin-coated pits toward endosomes and is redistributed to lysosomes. It is clear that

PAR-2 resensitization includes the synthesis of new receptors because this process is inhibited by cell treatment with cyclohexamide and brefeldin. It has also been demonstrated that during endocytosis PAR-2 can interact with β-arrestin and β2-adaptin from the AP-2 complex. The implication of PAR-2 ubiquitination in receptor endocytosis has not been shown to date. Because the use of PAO has little effect on intracellular calcium mobilization induced by PAR-2 activation, it seems that endocytosis is not the major process for PAR-2 desensitization.

Inflammatory Roles of PAR-2

Tryptase secreted by mast cells can activate PAR-2 in neurons, endothelial and epithelial cells. Because mast cells are implicated in inflammatory responses, hyper sensibility reactions and wound healing, this suggests that PAR-2 may also be implicated in these processes (Steinhoff, Corvera et al. 1999; Biedermann, Kneilling et al. 2000; Wang, Wang et al. 2009). Furthermore, tryptase can increase the expression of IL-1β and the secretion of IL-8. It can also increase the formation of intracellular adhesion molecules (ICAM) and selectin in endothelial cells. Tryptase is responsible for the accumulation of neutrophils and eosinophils. It causes vascular leakage and is mitogenic for epithelial cells, fibroblasts and smooth muscle cells (Brown, Jones et al. 1995; Brown, Tyler et al. 1995; Vergnolle 1999; Shpacovitch, Brzoska et al. 2002). PAR-2 is highly expressed in vascularized organs and at the level of blood vessels; they are expressed in endothelium and smooth muscle cells (Nystedt, Larsson et al. 1995; Bohm, Kong et al. 1996; D'Andrea, Derian et al. 1998). Inflammatory agents and tissue damages lead to the increase of PAR-2 expression in blood vessels. Indeed, tumor necrosis factor alpha (TNF-α), Interleukin-1 (IL-1) and lipopolysaccharid (LPS) induce the overexpression of PAR-2 mRNA and HUVEC (human umbilical vascular endothelial cells) cell immunoreactivity in between 4 to 20 hours following their exposition (Nystedt, Ramakrishnan et al. 1996). Because gastrointestinal tract inflammation enables the secretion of proteases that can activate PAR-2, it has been suggested that PAR-2 can play a role in inflammatory processes in the gastrointestinal tract. Indeed, the administration of trypsin, tryptase and agonist peptide of PAR-2 in mice colon led to generalized inflammatory responses in wild-type mice but not in PAR-2 knockout mice. In a model of allergic dermatite, there’s

a decrease in ear puffiness and a decrease in inflammatory cells infiltration in PAR-2 knockout mice (Kawagoe, Takizawa et al. 2002). PAR-2 activators can also send signals to afferent primary neurons inerving the skin to induce a release of neuropeptides SP and CGRP leading to a neurogenic inflammation (Krishna, Chauhan et al. 2001). Activation of PAR-2 can also activate NF-κB pathway in keratinocytes and myocytes. Agonist of PAR-2 can induce an increase of ICAM-1 mRNA in keratinocytes and an increase of IL-8 expression in endometrial epithelial cells and stromal cells (Hirota, Osuga et al. 2005). Activation of PAR-2 also stimulates the secretion of IL-1β, IL-8 and IL-6 in neutrophils (Shpacovitch, Varga et al. 2004; Hirota, Osuga et al. 2005). In addition, stimulation of PAR-2 with its agonist peptide results in the activation of certain protein kinases activated during cellular stress (JNK et p38) (Bretschneider, Kaufmann et al. 1999; Kanke, Macfarlane et al. 2001). At the level of respiratory tracts, PAR-2 can have pro and anti-inflammatory roles. PAR-1, PAR-2 and PAR-4 are expressed by epithelial cells of the respiratory tracts and activation of these receptors leads to the release of cytokines including IL-1, IL-8 and prostaglandin E_2 which are mediators regulating inflammation (Asokananthan, Graham et al. 2002). Moreover, PAR-2 activation induces an increase of vascular permeability, blood vessels relaxation, systemic hypotension, granulocytes infiltration and leucocytes adhesion (Damiano, Cheung et al. 1996; Hwa, Ghibaudi et al. 1996; Saifeddine, al-Ani et al. 1996; Kawabata, Kuroda et al. 1998; Vergnolle, Macnaughton et al. 1998; Al-Ani, Saifeddine et al. 1999; Vergnolle 1999; Vergnolle, Hollenberg et al. 1999). PAR-2 also possesses a role in the regulation of pain during inflammatory processes. Indeed, the presence of PAR-2 in the sensitive nerves has been detected and its activation resulted in mobilization of intracellular calcium. The detection of PAR-2 in neurons has incited researchers to identify a role for PAR-2 in nociception. In neurons, the activation of PAR-2 also leads to mobilization of intracellular calcium.

It has been shown in recent studies that PAR-2 can also have anti-inflammatory roles. Indeed, activation of PAR-2 with its agonist peptide offers a protection against inflammation in the colon by decreasing the level of cytokines and T cell proteins and by inhibiting myeloperoxidase activity (Fiorucci, Mencarelli et al. 2001). Pre-treatment with PAR-2 agonist can prevent gastric lesions induced by sub-cutaneous administration of indomethacin or by oral administration of a solution containing ethanol 60% and HCl 15

mM. This protection is due to the fact that PAR-2 activation induces gastric mucus secretion by stimulating the releasing CGRP and tachykinins from sensorial neurons (Kawabata, Kinoshita et al. 2001). PAR-2 is implicated in protection against lesions following reperfusion. Infusion of PAR-2 agonist peptide allows the functional reestablishment of the myocardium and a decrease of lipidic peroxydation of during blood flow (Napoli, Cicala et al. 2000). It has been demonstrated that injection of PAR-2 agonist peptide in mice results in a diminution of the synthesis of pro-inflammatory cytokines (IL-1β, IL-12 and interferon-γ) (Fiorucci, Mencarelli et al. 2001). Depending on the physiological or pathophysiological environment, PAR-2 can be implicated in pro- or anti-inflammatory processes.

Roles of PAR-2 in Coagulation

Coagulation can be generated by two processes: the intrinsic and extrinsic pathways. Although they are initiated by distinct mechanisms, the two converge on a common pathway that leads to clot formation. The extrinsic pathway is initiated by tissues damage and requires tissues factor (TF) or thromboplastin expressed by endothelial cells and monocytes during inflammation. During coagulation, TF binds to factor VIIa (FVIIa) and the complex TF/FVIIa interacts with factor X to activate the latter and to form factor Xa. Factor Xa binds to factor Va to induce the conversion of prothrombin to thrombin. Thrombin enables the conversion of fibrinogen to fibrin and can also activate PARs to induce platelet aggregation (Camerer, Kolsto et al. 1996; Kjalke, Monroe et al. 1998; Allen, Monroe et al. 2000). The intrinsic pathway is activated following the contact of blood with collagen due to endothelium damage and this pathway has low significance under normal physiological conditions. The intrinsic pathway can also be activated by vessel wall contact with bacteria. Most significant clinically is the activation of the intrinsic pathway by contact of the vessel wall with lipoprotein particles, very low-density lipoproteins (VLDLs) and chylomicrons. This process clearly demonstrates the role of hyperlipidemia in the generation of atherosclerosis. The intrinsic pathway starts with the conversion of factor XII to factor XIIa, a process catalyzed by kallikreins and kininogens. The conversion of this factor will generate a cacasde of events enabling the activation of factor XI and factor V. At this

point, the intrinsic pathway converges with the extrinsic pathway and result in the conversion of prothrombin to thrombin, a process dependent on factor Xa (Davie and Ratnoff 1964; Scott, Silver et al. 1985; Hoffman 2003). Factor VIIa and Xa can also bind to and activate members of the PAR family.

PAR-2 Regulation of the Vascular Tone

The expression of PAR-2 in endothelial and smooth muscle cells suggests that this receptor has an important role in vascular tone regulation. The predominant effect of PAR-2 agonists on the vasculature is the induction of blood vessels relaxation. Activation of PAR-2 in isolated aorta from rat and coronary artery from human and pig causes an endothelium-dependent relaxation (al-Ani, Saifeddine et al. 1995; Hwa, Ghibaudi et al. 1996; Hamilton, Nguyen et al. 1998; Roy, Saifeddine et al. 1998). A study reported that PAR-2 AP-induced endothelium-dependent relaxation is enhanced in mesenteric arteries isolated from type 2 diabetic GK rats at the chronic stage (Matsumoto, Ishida et al. 2009). In endothelial cells, arterial relaxation requires calcium moblization and nictric oxide synthase activation. The formation and the release of nictric oxide result in the relaxation of the vascular smooth muscle. Endothelin B receptor is also a mediator in the cardiovascular effects induced by PAR-2 agonist (Magazine, King et al. 1996). Even though the major effect of PAR-2 activation is vascular relaxation, trypsin and PAR-2 agonist peptide induce a vasoconstriction in isolated mice renal arteries when depleted of endothelium or when nitric oxide synthase inhibitors are used (Moffatt and Cocks 1998). In rat pulmonary arteries, low concentrations of PAR-2 agonist peptide induce an endothelium-dependent relaxation while high concentrations of the agonist peptides result in vasoconstriction. This constriction is independent of endothelin, angiotensin, noradrenalin or arachidonic acid metabolites (Roy, Saifeddine et al. 1998).

PAR-2 and Angiogenesis

PAR-1 and PAR-2 have been demonstrated to play functional roles in the vascular system. PAR-2 has been suggested to play an important role in angiogenesis. First of all, this receptor is widely expressed in highly vascularized organs such as kidney, small intestine, and stomach (Nystedt, Emilsson et al. 1994). PAR-2 also mediates endothelial

cell mitogenesis in vitro, and promotes vasodilation and microvascular permeability *in vivo* (Mirza, Yatsula et al. 1996; Hamilton, Nguyen et al. 1998). The expression of PAR-2 is upregulated by cytokines, including tumor necrosis factor, interleukin-1, and lipopolysaccharide, that are implicated in inflammatory angiogenesis (Nystedt, Ramakrishnan et al. 1996). Stimulation of PAR-2 with trypsin results in colon cancer cell proliferation (Darmoul, Gratio et al. 2004). In the model of ischemia of the mouse posterior member, activation of PAR-2 enables the formation of angiogenesis (Milia, Salis et al. 2002). Activation of PAR-2 leads to a proangiogenic response dependent on TNFα and subsequent induction of tie2 via the MEK/ERK pathway (Zhu, Sennlaub et al. 2006). In addition to angiogenesis, PAR-2 is also involved in endothelial cell mitogenesis in vitro and microvascular permeability in vivo (Milia, Salis et al. 2002). It has been shown that PAR-2 signalling is downstream to tissue factor (TF)-VIIa protease complex and it is involved in promoting tumour and angiogenesis in mice and human ocular tissue (Belting, Dorrell et al. 2004; Schaffner and Ruf 2009). Activation of PAR-2 also stimulates angiogenesis in a mouse model of hindlimb ischemia (Milia, Salis et al. 2002). Studies have indicated that PAR-2 is critical for MDA-MB-231 and BT549 breast cancer cell migration and invasion towards NIH 3T3 fibroblast conditioned medium (Morris, Ding et al. 2006). Another group has reported an important link between PAR-2 and Met receptor tyrosine kinase signalling in promoting hepatocellular carcinoma cell invasion (Kaufmann, Oettel et al. 2009). It has also been shown that PAR-1 is implicated in migration and metastasis and PAR-2 has unexpectedly a role in thrombin-dependent tumor cell migration and in metastasis (Shi, Gangadharan et al. 2004).

SORTING NEXINS

Sorting nexins (SNXs) are a large family of proteins proposed to regulate intracellular trafficking. To date, 29 mammalian SNXs and 10 yeast SNXs have been identified and they characterized by the presence of a SNX phox homology (PX) domain (SNX-PX), a subgroup of the PX domain superfamily (Teasdale, Loci et al. 2001). The SNX-PX domains enable SNXs to bind to phosphoinositide motifs on phosphoinositide-enriched membranes. Some members of the SNXs have been predicted to encode the Bin/Amphiphysin/Rvs (BAR) domain enabling these proteins to sense membrane

curvature and to increase their propensity to homo- and heterodimerise (Peter, Kent et al. 2004). Many of the mammalian sorting nexins contain protein:protein interaction domains, including SH3 domains, RGS (regulators of G-protein signalling) domains, RA (Ras association) domains and PDZ (PsD95/Dig/ZO1) domains (Figure 2). The presence of these domains led to the suggestion that SNXs function as trafficking mediators and/or scaffolds for the proper arrangement of intracellular signalling complexes.

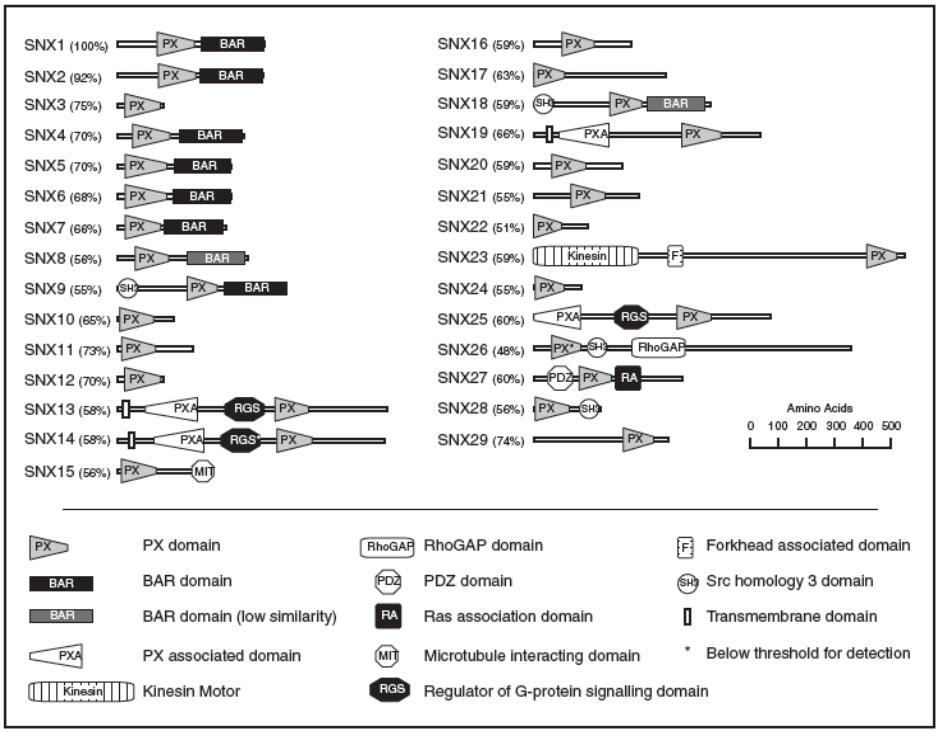


Figure 2: Domain Analysis of Mammalian SNXs (Carlton, Bujny et al. 2005).

SNXs as Trafficking Mediator

For the majority of the SNXs, their exact functions remain unknown. Mammalian SNXs have been suggested to function mostly in pro-degradative sorting, internalization, and endosomal recycling or sorting. Indeed, SNX1 was found in association with the sorting endosome and proposed to mediate the degradative sorting of the EGFR (Kurten, Cadena et al. 1996; Kurten, Eddington et al. 2001; Cozier, Carlton et al. 2002; Zhong, Lazar et al. 2002). Microinjection of SNX3 antisera prevented trafficking of transferring from the sorting endosomes to the recycling endosome, suggesting a role for SNX3 in the regulation of export along this pathway (Xu, Hortsman et al. 2001). Overexpression of

SNX15 caused endosomal retention of the CI-M6PR, internalization of tac-furin, and internalization of tac-TGN38 (Barr, Phillips et al. 2000; Phillips, Barr et al. 2001). Overexpression of SNX17 enhanced endocytosis of P-Selectin and retarded its delivery to lysosomes (Williams, Schluter et al. 2004). Other members of the SNX family have been shown to regulate retrieval of proteins from endosomes or Golgi (Pelham 2002). In most of these studies of SNXs, their overexpression resulted in endosomal retention of their interacting partners and in some cases prevented them from trafficking to lysosomes. These observations raise the possibility that SNXs are mostly implicated in the control of cargo removal from endosomes, rather than pro-degradative traffic through endosomes.

SNX as Signalling Platform

Given that SNXs contain multiple protein:protein interaction domains, these proteins are thought to function as scaffolds for the arrangement of intracellular signalling complexes. The RGS domain of SNX13 has been demonstrated to function as a $G_{\alpha s}$ GAP leading to the enhancement of GTP hydrolysis and a reduction of the production of cAMP (Zheng, Ma et al. 2001). SNX13 endosomal localization suggests that mechanisms exist to modulate signalling from endosomal membranes following activation and internalization of receptors. RGS proteins have been proposed as effectors, as well as negative regulators of GPCR signalling (Neubig and Siderovski 2002). The RGS domain of other SNXs remains to be established. BAR domains have been suggested to function as a small G-protein binding module. A group of researchers have demonstrated that the BAR domain of Arfaptin2 interacts with Arf, Arl and rac small GTPases (Peter, Kent et al. 2004). Thus, the potential for SNXs to play a role as signalling platform exists but whether they have their own signalling roles remains to be elucidated.

ANGIOGENESIS

Angiogenesis is a mechanism that has repercussions in a number of physiological and pathological situations. During embryonic development, a number of proangiogenic signalling pathways assure that all cells and tissues receive nutrients and oxygen contained in the blood. In adult tissue, angiogenesis is basically arrested and occurs only when needed, for example during the healing of wounds and bone fractures and the

female reproductive cycle. Angiogenesis is also activated in pathological situations. For example in the cardiovascular system, growth of new blood vessels occurs in ischemic regions of the heart. In artherosclerosis, angiogenesis can be deleterious and has been implicated in atherosclerotic plaque growth and disruption leading to myocardial infarction and ischemic stroke (O'Brien, Garvin et al. 1994; Inoue, Kanda et al. 1998; McCarthy, Loftus et al. 1999). Angiogenesis is also a major factor in tumor progression. In order to obtain oxygen and nutrients, tumors will activate angiogenesis and the formation of vessels. There are at least five initial steps in order to obtain a functional blood vessel: (1) the extracellular matrix must be degraded; (2) cell adhesion changes and disruption must occur; (3) cell permeability must be increased; (4) endothelial cells must proliferate, and (5) endothelial cells must migrate towards the site of blood vessel formation. These steps have been shown to be activated by a number of growth factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF) (Ferrara and Alitalo 1999).

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) was identified in 1989 as the first endothelial cell (EC)-specific growth factor, which had been described 6 years previously as vascular permeability factor (Senger, Galli et al. 1983). It is now assumed that the signalling cascade involving vascular endothelial growth factor (VEGF) is the critical event in the regulation of angiogenesis. VEGF stimulates growth of endothelial cells that originated from arteries, veins and lymphatic vessels and it also induces angiogenesis *in vivo* (Leung, Cachianes et al. 1989; Ferrara and Davis-Smyth 1997). The genes encoding human VEGF consists of eight exons separated by seven introns and the first 26 amino acids in VEGF constitute the signalling peptide (Houck, Ferrara et al. 1991; Tischer, Mitchell et al. 1991). Through alternative splicing, the VEGF protein produces four different isoforms-$VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, each of which differs in its heparin-binding properties (Tischer, Mitchell et al. 1991). The ability to bind heparin defines whether the secreted protein will be accumulated in extracellular matrix or will be released and become accessible for interaction with other cells. $VEGF_{121}$ does not bind heparin and is freely soluble, $VEGF_{165}$ adheres moderately, and $VEGF_{189}$ strongly binds

heparin and is almost completely accumulated in extracellular matrix (Houck, Ferrara et al. 1991). The most abundant isoform is the $VEGF_{165}$ isoform. Hypoxia is one of the key regulators inducing VEGF expression. Different pathological states have been shown to cause the increase the expression of VEGF mRNA under conditions of lowered oxygen content (Dor, Porat et al. 2001). Other factors have also been sown to increase the expression of VEGF like epidermal growth factor (EGF), transforming growth factors (TGFα and TGFβ), insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), etc (Ferrara and Davis-Smyth 1997; Neufeld, Cohen et al. 1999).

Other closely related factors of VEGF were detected, which formed a family consisting of growth factors VEGFA (VEGF), VEGFB, VEGFC, VEGFD, VEGFE, and placental growth factor PlGF and these factors exert their biological effect via receptors located on endothelial cell membranes. Indeed, these factors bind to three identified receptors: VEGFR1 (fms-like tyrosine kinase-1/Flt-1), VEGFR-2 (fetal liver kinase-1/Flk-1/kinase domain region) and VEGFR3 (Flt-4) (Shibuya, Yamaguchi et al. 1990; Alitalo and Carmeliet 2002).

Angiopoietin and Tie Receptor

The Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) receptors and their angiopoietin (Ang) ligands have been identified as the second vascular tissue-specific receptor Tyr kinase signalling system (Dumont, Yamaguchi et al. 1992; Partanen, Armstrong et al. 1992; Iwama, Hamaguchi et al. 1993; Maisonpierre, Goldfarb et al. 1993; Sato, Qin et al. 1993). Tie receptors are single transmembrane proteins having an extracellular ligand-binding domain and a split intracellular Tyr kinase domain. Tie1 and Tie2 have a similar overall structure and their intracellular domains are highly conserved with 76% identity (Schnurch and Risau 1993). The Tie receptors are co-expressed in both blood vascular and lymphatic endothelial cells, and in circulating haematopoietic cells (Hsu, Ema et al. 2000; Vart, Nikitenko et al. 2007). The angiopoietin is composed of 4 members: Angiopoietin 1 to angiopoietin 4 (Yancopoulos, Davis et al. 2000). Angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2) bind to Tie2 with similar affinities and bind to the second Ig-like loop (Fiedler, Krissl et al.

2003). Ang3 and Ang4 were subsequently cloned as human and mouse orthologues (Valenzuela, Griffiths et al. 1999). Ang1 and Ang4 are agonists while Ang2 and Ang3 are reported to inhibit Ang1-induced tyrosine phosphorylation of Tie2 (Davis, Aldrich et al. 1996; Maisonpierre, Suri et al. 1997). Ang proteins are comprised of an N-terminal Ang-specific superclustering domain, which contains two Cys molecules followed by a coiled-coil domain, a linker peptide and a carboxy-terminal fibrinogen-homology domain (Davis, Aldrich et al. 1996; Maisonpierre, Suri et al. 1997). The coiled-coil domain is necessary for dimerization or oligomerization whereas the fibrinogen-homology domain mediates receptor binding. Following the binding to its receptor, Ang1 induces Tie2 phosphorylation and leads to the recruitment of adaptor proteins, such as growth factor receptors-bound protein 2 (GRB2) and the p85 subunit of phosphoinositide 3-kinase (PI3K), which results in the activation of AKT. The activation of AKT leads to the activation of survival promoting pathways, such as endothelial nitric oxide synthase (eNOS) and survivin, and to the suppression of mediators of the apoptotic pathway, such as caspase 9 and BAD (Kim, Kim et al. 2000; DeBusk, Hallahan et al. 2004).

Ang-Tie signalling functions as a key regulator of adult vascular homeostasis and is essential during embryonic vessel assembly and maturation. Tie2-deficient mice can proceed through early steps of cardiovascular and angiogenic development but die between E10.5 and 12.5 (Sato, Tozawa et al. 1995; Patan 1998). By contrast, in Tie1-deficient mice, embryonic angiogenesis is not perturbed but the vessels lose their integrity and die later during development between E13.5 and birth (Puri, Rossant et al. 1995; Sato, Tozawa et al. 1995). Ang1-deficient mice show the same early embryonic lethal phenotype of Tie2-deficient mice indicating that Ang1 activity is non-redundant (Suri, Jones et al. 1996). Ang1/Tie2 signalling has been suggested to affect endothelial cells activities leading to the formation of more mature and stable vessel walls. It has been reported that Ang1 mediated Tie2 activation fails to stimulate mitogenesis of ECs. Ang1 was shown to mediate anti-inflammatory, anti-permeability, and anti-apoptotic effects (Gamble, Drew et al. 2000; Kim, Kim et al. 2000; Kim, Moon et al. 2001; Pizurki, Zhou et al. 2003). One group has suggested a potential use for exogenous Ang1 in reducing rather than increasing vascular density (Weber, Cai et al. 2005). Ang2-deficient mice show unperturbed developmental angiogenesis and newborn pups are born apparently

normal (Gale, Thurston et al. 2002). However, Ang2-deficient mice have persistent vascular defect as demonstrated with perturbation of the retinal vasculature (which grows postnatally in mice) (Gale, Thurston et al. 2002; Hackett, Wiegand et al. 2002). Overexpression of Ang2 has been shown to induce massive tumour vessel regression in 24 hours (Cao, Sonveaux et al. 2007). Ang3 and Ang4 seem to have negative or positive effects on angiogenesis in different conditions (Xu, Liu et al. 2004; Olsen, Ley et al. 2006).

Retina and the Retinal Vasculature

The innermost layer of the eye consists of the retina and its vasculature. The retina is composed of three layers of nerve cells and two of synapses. Many different types of cells reside in the retina and play specific roles for maintaining its proper functions (Figure 3). The retinal ganglion cells (RGC) lie innermost in the retina (inner plexiform layer or IPL) and the photoreceptive cells lie outermost (outer nuclear layer or ONL and outer plexiform layer or OPL). The ganglion axons group to form the optic nerve, which transmits the visual signal to the brain. Müller cells are a type of retinal macroglia and reside in the inner nuclear layer (INL). Microglia reside throughout the inner layers until the INL/OPL interface. Astrocytes are found in vascularized retinas and their precursors migrate into the retina via the optic nerve (Ling and Stone 1988). Endothelial cells and pericytes are part of the retinal microvasculature. During the retinal development, blood vessels that supply the inner portion of the retina are widely reorganized including vessel regression, sprouting angiogenesis, vascular remodelling and vessel differentiation. The vascular network spreads in the nerve fibre layer across the inner surface of the retina. It contains both arteries and veins that both enter and exit through the optic nerve. Blood vessels can arise via two processes, vasculogenesis and angiogenesis. Vasculogenesis is the formation of new blood vessels from vascular precursor cells (angioblasts) while angiogenesis occurs with the proliferation of mature endothelial cells (ECs) from pre-existing blood vessels (Risau and Flamme 1995; Risau 1997). The deeper networks of the retinal vasculature are believed to form by sprouting angiogenesis while the primary inner vascular plexus in the retina has been suggested to be formed by vasculogenesis (Ashton

1970; Chang-Ling, Vannas et al. 1990; Gariano, Iruela-Arispe et al. 1994; Sandercoe, Madigan et al. 1999; Hughes, Yang et al. 2000).

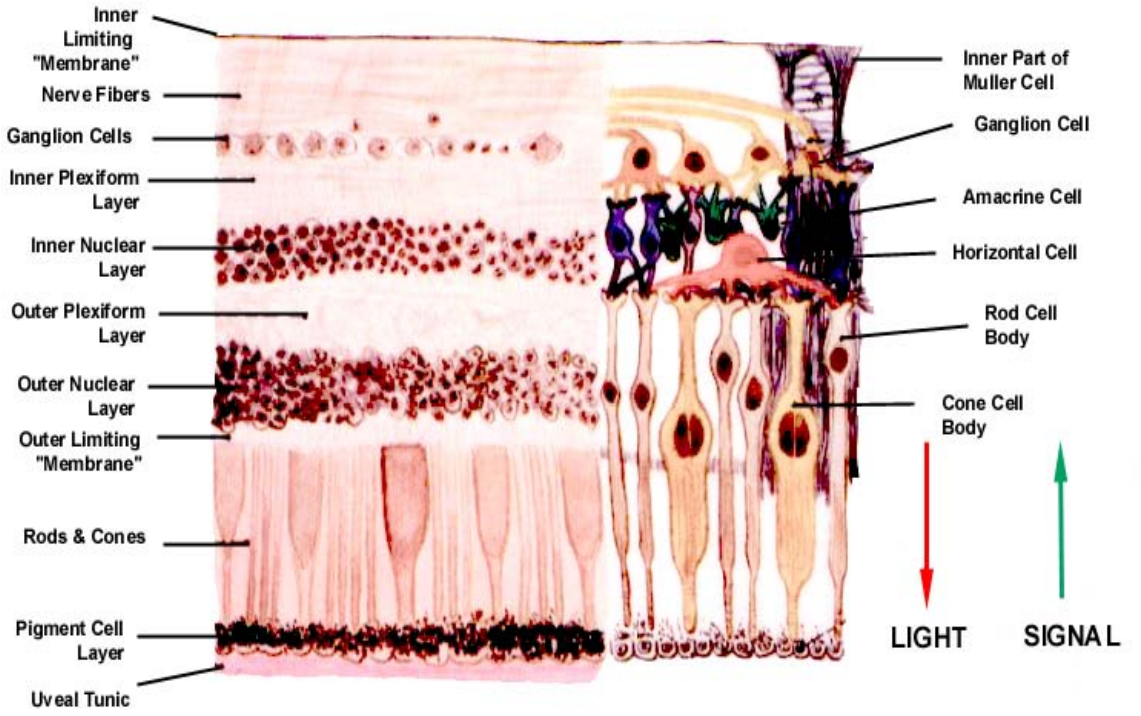


FIGURE 3: Organization of adult human neural retina.
(http://education.vetmed.vt.edu, accessed 19/01/10)

To date, vascular endothelial growth factor (VEGF) is the principal regulator of neovascularization and it mediates EC survival, migration, differentiation, and proliferation (Connolly, Heuvelman et al. 1989; Leung, Cachianes et al. 1989; Plouet and Gospodarowicz 1989; Gerber, McMurtrey et al. 1998). VEGF-A and both of its receptors VEGFR-1 (fms-like tyrosine kinase-1/Flt-1) and VEGFR-2 (fetal liver kinase-1/Flk-1/kinase domain region) are present in the retina (Kim, Ryan et al. 1999). Transforming growth factor beta (TGFβ) superfamily signalling has also been implicated in the control of vessel growth (Vargesson and Laufer 2001). Angiopoietins and the basic fibroblast growth factor are also important angiogenic factors (Lutsenko, Kiselev et al. 2003). Recent evidence suggests that RGCs are pivotal instigators of retinal angiogenesis. Indeed, mice (brn3bZ-dta/+;six3-cre mice) which do not have RGCs fail to develop a

retinal vasculature (Sapieha, Sirinyan et al. 2008). Proangiogenic effects of PAR-2 have been shown to be dependent on the receptor of angiopoietins, Tie2 (Zhu, Gobeil et al. 2006).

THE GENERAL TRANSCRIPTIONAL MACHINERY

Transcription is induced by many enzymes including RNA polymerase I which synthesises cytoplasmic RNAs (ribosomic RNAs), RNA polymerase II which is important for the synthesis of messenger RNAs (mRNAs) and certain snRNAs, and RNA polymerase III which synthesises small RNAs (tRNA, rRNA 5 S, snRNA, 7SL-RNA). Studies have shown that transcription initiation by RNA polymerase II (RNA pol II) requires at least the basal transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Thomas and Chiang 2006). These general transcription factors are important for promoter recognition and unwinding, and together with RNA pol II and promoter DNA comprise the preinitiation complex. The TATA element has been identified as a common basal promoter element and is recognized by the TATA-binding protein (TBP) subunit of TFIID (Smale and Kadonaga 2003). However, many promoters do not have a recognizable TATA element. Other basal elements have been identified and include the downstream promoter element (DPE), motif ten element (MTE), downstream core element (DCE), TFIIB recognition elements (BREs), and the initiator element (Inr) (Heinrich, Castell et al.) (Juven-Gershon, Hsu et al. 2008). Specific chromatin modifications may also play a role in basal factor recruitment. The histone acetyltransferase complex is recruited upstream the activating sequences. In addition to chromatin-modifying complex, a multitude of cofactors or coactivators are needed for activation of transcription. Three classes of general cofactors, including TBP-associated factors (TAFs), Mediator, and upstream stimulatory activity (Bretschneider, Kaufmann et al.)-derived positive cofactors (PC1/PARP-1, PC2, PC3/DNA topoisomerase I, and PC4) and negative cofactor (NC1/HMGB1), are important for promoter activity in a gene-specific or cell-specific manner. Cofactors, such as TAF1, BTAF1, and negative cofactor 2 (NC2) are capable of repressing basal transcription when activators are absent and can stimulate transcription in the presence of activators (Thomas and Chiang 2006).

SP1 TRANSCRIPTION FACTOR

The Sp family of transcription factors are characterized by three conserved Cys2His2 zinc fingers, which form the DNA-binding domain (Figure 4) (Suske 1999). They also contain a *trans*-activation domain consisting of two sub-domains (A and B), each of which can stimulate transcription. Domain C is a highly charged amino acids domain and domain D is a carboxyl-terminal domain required for synergistic activation along with sub-domains A and B (Pascal and Tjian 1991).

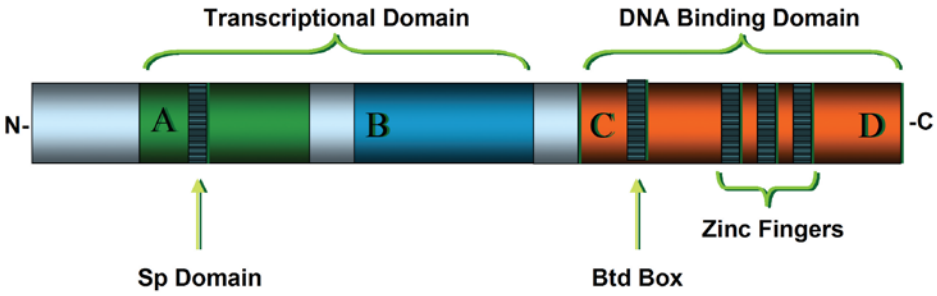


FIGURE 4: Structure of Sp1 and Sp3 transcription factors (Philipsen and Suske 1999).

At the N-terminus of the Sp proteins there's a highly conserved Sp box (SPLALLAATCSR/KI) containing an endoproteolytic cleavage site for Sp degradation. The general transcription factor Sp1 binds and acts through GC boxes to regulate gene expression. GC boxes are important *cis*-acting DNA regulatory elements required for the transcriptional regulation of many housekeeping, viral, tissue-specific and inducible genes (Philipsen and Suske 1999) (Suske 1999; Bouwman and Philipsen 2002). In contrast to other members of the family, Sp1 and Sp3 are ubiquitously expressed in mammalian cells and both share more than 90 % sequence homology in the DNA-binding domain although they have different functions (Figure 5). Sp1 and Sp3 genes encode 105- and 115 kDa proteins, respectively.

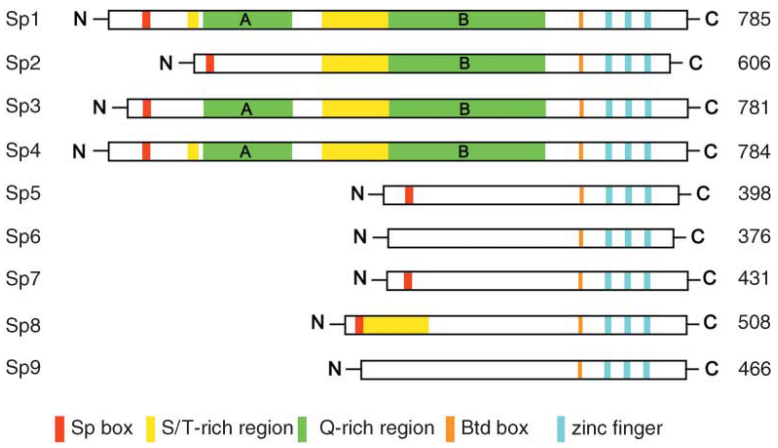


FIGURE 5: Family of Sp transcription factors (Zhao, Venkatasubbarao et al. 2003).

Sp1 is a transcriptional activator while Sp3 can activate or repress gene transcription (Hagen, Muller et al. 1994; Sun, Chen et al. 2002). Sp3 has similar affinity to Sp1 for the binding of GC and GT boxes and in the presence of Sp1 it can activate transcription synergistically. Sp1 and Sp3 are also competing for the binding on DNA (Hagen, Muller et al. 1994; Lania, Majello et al. 1997). Sp1 is subject to post-traductional modifications like phosphorylation, glycosylation, sumoylation and poly(ADP-risyl)ation (Dynan, Saffer et al. 1985; Jackson and Tjian 1988). Transcription factors of the Sp family are activated in majority by the MAPK cascade or PI3K. Once activated, ERK is translocated to the nucleus and phosphorylates Sp1 protein (Seger and Krebs 1995; Robinson and Cobb 1997; Kolch 2000; Pouyssegur, Volmat et al. 2002). Phosphorylation of Sp1 by MAPK can activate or repress gene transcription even though Sp1 is mostly known as an activator.

Roles of Sp1

Sp1 has been shown to regulate the expression of thousands of genes implicated in the control of diverse array of cellular responses, such as angiogenesis, cell growth, differentiation, apoptosis, and immune response (Jones, Kadonaga et al. 1986; Opitz and Rustgi 2000; Kaczynski, Cook et al. 2003; Mazure, Brahimi-Horn et al. 2003; Santiago, Ishii et al. 2007). In human umbilical vein endothelial cells, hypoxia enhances the amount of Sp1 protein while Sp3 levels remain unaltered (Xu, Ji et al. 2000). Expression

and gene-knockout studies revealed that Sp1-like/KLF proteins are involved in growth-regulatory or developmental processes of a large number of tissues. The knockout of *Sp1* gene leads to gross global morphological defects very early in development (Marin, Karis et al. 1997; Black, Black et al. 2001). Because, Sp1 regulates cell growth in a variety of cell types, it is not surprising that Sp1 also participates in mechanisms leading to carcinogenesis. Sp1 expression and activity have been demonstrated to be increased in epithelial carcinomas compared with benign tumors, such as papillomas, suggesting that Sp1 may be involved in tumor progression (Kumar and Butler 1999).

Sp1 Interacting Proteins

Sp1 and Sp3 regulate gene expression by cooperating with other proteins and protein-binding sites have been identified. They can directly or indirectly interact with transcription-associated proteins, sequence-specific DNA-binding proteins, transcriptional regulators and chromatin remodelling factors such as p300 and histone deacetylases (HDACs) (Chou, Chen et al. 2003; Chen and Jackson 2004). Sp1 can activate transcription synergistically because of its ability to form higher-order complexes through its D domain. Sp1 is organized has a multimer and presents multiple docking sites for interacting proteins (Mastrangelo, Courey et al. 1991). Estrogen can induce the expression of estrogen-responsive genes by forming complexes between the estrogen receptor (ER) and Sp1. ER and Sp1 recruit coactivators that interact with the transcription initiation complex to start transcription (Kim, Thu et al. 2003). The binding of proteins to Sp1 can also block its transactivation activity. For example, c-Myc can bind to the C-terminal domain of Sp1 and represses the transactivation activity of Sp1 (Gartel, Ye et al. 2001). Egr-1 can induce the displacement of Sp1/Sp3 binding to promoters and inhibits Sp1 transcriptional activity (Silverman, Khachigian et al. 1997). Sp1 can also act as a repressor by recruiting HDAC1, a corepressor (Sun, Chen et al. 2002). Sp1 and Sp3 can recruit and form complexes with many other factors, which can activate or repress the gene expression.

NF-1 TRANSCRIPTION FACTOR

NF-1 family is constituted of 4 members in vertebrates: NF-1-A, NF-1-B, NF-1-C and NF-1-X. These proteins are expressed by different genes and each gene can produce up to 9 different proteins due to post-translational modifications. RNA messengers for NF-1-A, -B, -C, and -X are 10.5, 9.7, 7.7 and 6.0 kilobases in size respectively (Nebl, Mermod et al. 1994; Chaudhry, Lyons et al. 1997). The number of NF-1 binding sites on the human genome is estimated to 75000 sites (Gronostajski, Adhya et al. 1985). At the beginning, NF-1 was discovered as a protein that binds to DNA on specific sites during adenoviral replication (Nagata, Guggenheimer et al. 1983). Later on, studies have shown that this transcription factor binds to typical sequence TTGGC(N5)GCCAA on double stranded DNA that is similar to CAAT box in sequence (Gronostajski, Adhya et al. 1985). For this reason, the sequence bound by NF-1 are called CAAT box Transcription Factor (CTF) (Jones, Kadonaga et al. 1987).

OBJECTIVE

Given that nuclear GPCRs have distinct functions from those at the plasma membrane, these studies were done to elucidate the mechanisms of translocation of PAR-2 from the plasma membrane to the nucleus. We looked to find the domains of PAR-2 and the proteins interacting with the receptor responsible for the translocation of PAR-2 to the nucleus. The impacts of nuclear translocation of PAR-2 on gene expression, angiogenesis, and on neovascularization were determined using HEK293 cells stably expressing PAR-2 GFP or PAR-2 mutants, RGC-5 cells, aortic ring explants, and the retinal vascular bed. The role of nuclear PAR-2 in the activation of transcription factors and in the regulation of transcriptional activity was also studied.

HYPOTHESIS

We hypothesized that nuclear localization of PAR-2 results from an activation and translocation of the receptor from the plasma membrane to the nuclear membrane. Translocation of PAR-2 to nucleus would require interactions with proteins to intracellular domains of the receptor. Nuclear relocalization of PAR-2 would induce gene transcription distinct from those induced by cell surface receptor. Translocation of PAR-2 to nucleus would results in the activation of transcription factors and gene expression. Specific gene transcription induced by nuclear translocated PAR-2 could result in physiologic contribution to retinal angiogenesis.

CHAPTER 2 – FUNCTIONS OF GPCRs GOVERNED BY THEIR TRAFFICKING FROM THE PLASMA MEMBRANE TO THE CELL NUCLEUS

Preamble

Some studies, including those from our lab, have demonstrated that nuclear transmembrane receptors from the family of G protein-coupled receptors are implicated in gene transcription while those at the plasma membrane seem to induce acute effects independent of gene transcription. This is the case for prostanoid receptors and platelet-activating factor receptors.

We proposed the existence of a new internalization pathway for PAR-2 resulting in the translocation of PAR-2 from the plasma membrane to the nucleus. In its inactivated form, PAR-2 is located at the plasma membrane. Following the activation of PAR-2 at the plasma membrane, a certain amount of this receptor is thought to be translocated to the nucleus to activate distinct signalling pathways from the traditional MAP kinase signalling pathway. The other portion of the activated PAR-2 at the plasma membrane follows an endocytic route resulting in its degradation. The nuclear translocation of the receptor would lead to new functionality of the receptor in regard of its ability to regulate gene expression. Since PAR-2 has been suggested to play a role in angiogenesis, we hypothesized that nuclear translocated PAR-2 plays an important role in vascularization using a mice model of retinal neovascularization.

Functions of GPCRs governed by their trafficking from the plasma membrane to the cell nucleus

Nim S[1,2], Joyal JS[1,2], Zhu T[1], Zaniolo K[1], Bossolasco M[1], Hamel D[1], Shao Z[1,2], Chemtob S[1,2].

[1]Departments of Pediatrics, Ophtalmology and Pharmacology, Research Centre, Hôpital Ste-Justine, Montreal, Quebec, Canada

[2]Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada

Corresponding author:

Dr. Sylvain Chemtob, M.D., Ph.D., F.R.C.P.C.

Department of pharmacology, Faculty of medicine, University of Montreal

Department of Pharmacology and Therapeutics, Faculty of Medicine, McGill University

Sainte-Justine Hospital Research center

3175, chemin de la Côte Sainte-Catherine

Montreal (Quebec), Canada H3T 1C5

Tel.: (514) 345-4692/Fax: (514) 345-4801

E-mail: sylvain.chemtob@umontreal.ca

ABSTRACT

G-protein coupled receptors (GPCRs) have been detected at the cell nucleus, wherein they evoke *in situ* gene induction. Yet, the sub-cellular origin of nuclear GPCRs and the mechanisms governing such localization are not known. Protease activated receptor 2 (PAR-2), which exerts important physiologic effects, was detected at the cell nucleus in tissues. We therefore investigated whether peptide-activated GPCRs translocate to the nucleus, using PAR-2 as a paradigm. The nuclear trafficking of PAR-2 requires the recruitment of Importin-β1 and previously unreported Sorting nexin 11 (SNX11). Translocation of PAR-2 to the nucleus was dependent on microtubule integrity and involved dynein, a microtubule carrier that drives the cargo towards the nucleus. Mutations of nuclear localization signals and truncation of the C-terminus of PAR-2 nearly abolished nuclear translocation, the expected gene induction and retinal angiogenesis. We hereby provide the first evidence for the mechanism of the translocation of a GPCR from the plasma membrane to the nucleus and its physiologic contribution to retinal angiogenesis.

INTRODUCTION

G-protein coupled receptors (GPCRs) represent more than half of the current pharmacologic targets. The sub-cellular distributions of GPCRs dictate in part their function. Although most GPCRs are known to be active at the plasma membrane, many have also been reported at the cell nucleus. Lipid-activated GPCRs were the first to be detected at the nucleus where they are activated by locally generated and membrane-permeable ligands(*1, 2*). More recently, several peptide-activated GPCRs have been described at the cell nucleus, but their ligands are mostly extracellular and membrane impermeable(*3*). How and why GPCRs reach the nucleus remains to be uncovered. Protease activated receptor 2 (PAR-2) exerts important pro-inflammatory and angiogenic effects(*4, 5*) and is stimulated by cleavage of N-terminus by serine proteases. PAR-2 is irreversibly self-activated by its own tethered peptide and commits to its final sub-cellular destination and function. Interestingly, we detected PAR-2 at the cell nucleus upon stimulation. Thus, we used PAR-2 as a model to investigate the translocation of peptide-activated GPCRs to the cell nucleus. Here we present three novel concepts providing a rationale for the origin and function of peptide-activated GPCRs at the nucleus. First, we demonstrate the translocation of PAR-2 from the plasma membrane to the cell nucleus upon stimulation. Second, we suggest a mechanism for the nuclear trafficking of PAR-2 involving SNX11 together with importinβ1. We provide evidence that endosomes containing PAR-2 are shuttled on microtubules with the carrier motor-protein dynein. Third, we uncover a PAR-2 translocation-specific gene induction pattern and its physiologic contribution to retinal angiogenesis.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibodies anti-PAR2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat polyclonal antibodies anti-karyopherin β1 (importin β1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies anti-SNX11 were a gift from Dr. Gregor Andelfinger (Ste-Justine Hospital, Montreal, Qc). Rabbit polyclonal antibodies anti-GFP were purchased from Invitrogen (Burlington, ON). Mouse monoclonal antibodies anti-FLAG M2 conjugated to FITC were purchased from SIGMA (Saint Louis, MO). Mouse monoclonal antibodies anti β-actin [C-15] were purchased from Abcam (Cambridge, MA). Antibodies anti-mouse IgG conjugated to Horseradish peroxidase were obtained from Pierce (Rockford. IL). Mouse monoclonal cytochrome C, and CD51 (Integrin αV) antibodies were from BD Biosciences, San Diego, CA. Mouse monoclonal Lamin A/C antibodies were obtained from Chemcon, Orlando, FL. HA mouse monoclonal-FITC conjugated antibodies (12CA5), colchicine and monodansyl cadaverin are from Sigma-Aldrich, St-Louis, MO. Rabbit monoclonal Lamin B receptor (LBR) antibodies were purchased from Epitomics, Burlingame, CA. Thy1.1 antibodies were purchased from Chemicon.

Animals

We used WT and PAR-2 knock-out mice from Jackson mice Lab (Maine, USA) in compliance with experimental procedures approved by the Animal Care Committee of the Centre Hospitalier Universitaire Ste-Justine (Montreal, Qc, Canada) and in accordance with the guidelines established by the Canadian Council on Animal Care. Following the injection of vehicle, SLIGRL or lentiviruses expressing GFP, PAR-2 GFP, PAR-2 NLSm, or containing vector for shRNAs. We extracted the eyes of mice after death in compliance with experimental procedures approved by the Animal Care Committee of the CHU Ste-Justine and in accordance with the guidelines established by the Canadian Council on Animal Care.

Cells

HEK 293 cells were human embryonic kidney cells (American Type Culture Collection; Manassas, VA) and were cultured in DMEM medium containing 10 % FBS and 1% of penicillin (100 units/mL) and streptomycin (100 μg/mL). Cell passages were

done when cells reached 80 % confluence. The RGC-5 cell line were a kind gift from N. Agarwal. We terminally differentiated these cells by exposing them to 1 mM staurosporine for 12 h, which effectively maintains RGC-5 cells in a post-mitotic, neuronal state57. We then stimulated RGC-5 cells (with or without treatment with shRNAs) with 20 μM SLIGRL for 12 h. We removed the SLIGRL-containing medium and replaced it with fresh DMEM (Invitrogen). We used stimulated RGC-5 cells to condition fresh medium for 16 h, at which point we collected the medium and filtered it through a 2-mm filter. We subsequently distributed this conditioned media to wells in both aortic explant and tube formation assays. For shRNA knockdown in RGC-5 cells, we transfected the cells (at 50–60% confluence) with 50 nM of either shRNA to PAR-2, SNX1, SNX2 or SNX11 with TransIT-TKO (Mirus).

Nuclei Isolation

HEK293 and RGC-5 cells were cultured in 15 cm plates until 80 % confluence. Cells were then washed 3 times with 10 mL cold PBS, scraped and transferred to a 15 mL tube. Cells were centrifuged to 1400 rpm for 5 minutes at 4^{o}C. Cells were resuspended with lysis buffer containing 10 mM TrisHCl pH 7.4, 10 nM NaCl, 3 mM $MgCl_2$, 300 mM sucrose and protease inhibitors. Cells were homogenized with sterile glass Teflon potter (pestle B; clearance 20-50 μm; Bellco Glass). Homogenate was centrifuged at 700 g for 10 minutes at 4^{o}C to pellet nuclei. Nuclei were washed with lysis buffer containing 0.1 % NP-49 and centrifuged at 700 g for 10 minutes at 4^{o}C. Mitochondria were obtained by centrifuging supernatant at 10 000 g for 15 minutes at 4^{o}C and plasma membrane was isolated by centrifuging at 120 000 g for 60 minutes at 4^{o}C.

Western blot

Cells were lysed with lysis buffer containing 150 mM NaCl, 150 mM KCl, 5 mM $MgCl_2$, 50 mM Sodium Phosphate pH 7.0, 1% Triton X-100, and a cocktail of protease inhibitors (Boehringer Mannheim. Mannheim, Germany). 30 μg of each sample was mixed with 4x Reducing Sample Buffer (Boston BioProducts Inc, Worcester, MA). Samples are boiled, cooled and put on a SDS-PAGE gel 10%. Migration of the proteins on the gel was done at 25 mA in a buffer containing 25 mM Tris, 192 mM glycine et 3.5 mM SDS. Proteins were transferred on a PVDF membrane (PerkinElmer; Boston, MA) at 100 volts for two hours or 30 volts for 24 hours at 4^{o}C in a buffer containing 25 mM Tris,

192 mM glycine and 20% methanol. Non-specific proteins were blocked by incubating the membrane with milk 5 % (ICN; Aurora, OH) diluted in the buffer TBS-T (10 mM tris-HCl pH 8.0, 150 mM NaCl and 0.05% tween-20) for 30 minutes. Primary antibodies were diluted with milk 1.5 % in TBS-T and incubated for 3 hours at room temperature for 24 hours at 4^{o}C. Reaction with secondary antibody was done with a mouse or rabbit anti-IgG conjugated to Horseradish and diluted in milk 1.5% TBS-T for one hour at room temperature. Specific proteins were revealed with a chemiluminescent agent (PerkinElmer Life Sciences, Boston, MA) and exposed on a ray X film.

PAR-2 Mutants Cloning Stategies

The cytosolic-tail of adenovirus type 5 E3-11.6k gene (amino acid 56-112) was commercially cloned into a pcDNA3 plasmid (Top Gene technologies, Canada). This 189 bp fragment was then sub-cloned into the BamH1-Age1 site of hPAR-2-eGFP-N1 vector (kindly provided by N. Bunnett) {Déry, 1999, p00108}, immediately following the cytosolic-tail of PAR-2 and preceeding the eGFP. PAR-2 C-truncated was cloned by PCR using ECORI-BamHI sites from hPAR-2-pCDNA3 (kindly provided by L. Fan) into hPAR-2-eGFP-N1 vector by removing the C-terminal part of the receptor at position amino acid 363. PAR-2 beta-arrestin1 Mutant was cloned by Kunkel Method using primer 5'-AACGCTCTCCTTTGCCGAGCCGTCCGCGCCGTAAAGCAGAT-GCAAGTATCC-3' containing mutations changing serine and threonine to alanines at position amino acids 363-366. PAR-2 NLSm 1iL and PAR-2 NLSm 3iL were cloned by PCR by changing lysines into asparagines at positions amino acids 105,106 and 107 for the first intracellular loop and amino acids 280, 281 and 283 for the third intracellular loop. Primer sense for PAR-2 NLSm 1iL is 5'-TCCGAACTAATAATAATCACCCTGCTGTGATT-3' and primer sense for PAR-2 NLSm 3iL is 5'-GGATGAAAACTCAGAGAATAATAGGAATAGGGCCATCAAA-3'. Primer anti-sense for both PAR-2 NLSm is 5'-CGGTGGGCCCCGATAGGAGGTCTTAA-3'.

RT-PCR

Total RNA was isolated with Trizol solution (Invitrogen, Molecular Probes, Burlington, Ontario). For the reverse transcription reaction, 5 μg of total RNA was heated at 70 ^{o}C for 10 minutes. After cooling on ice RNA was put in a reaction solution with

13.3 U/μL de M-MLV (enzyme for reverse transcription was from Invitrogen, Molecular Probes, Burlington, Ontario), 10 mM DDT, 0.129 U/μL RNAguard TM RNase Inhibitor (Amersham, Piscataway, NJ), 0.67 mM dNTP, 16.7 μg/mL d'oligo dT (Invitrogen, Molecular Probes, Burlington, Ontario) and First Strand buffer 1x in a total volume of 30 μL and incubated at 42 ^{o}C for 60 minutes. After inactivation of enzyme by incubating at 94 ^{o}C for 5 minutes, DNA was conserved at -80 ^{o}C. Oligonucleotides were conceived with Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Oligonucleotides sense sequence for human PAR-2 is $^{5'}$TGCTAGCAGCCTCTCTCTCC$^{3'}$ and anti-sense sequence is $^{5'}$CCAGTGAGGACAGATGCAGA$^{3'}$. Oligonucleotides sense sequence for mouse PAR-2 is $^{5'}$TGGGAGGTATCACCCTTCTG$^{3'}$ and anti-sense sequence is $^{5'}$GGGGAACCAGATGACAGAGA$^{3'}$. Oligonucleotides sense sequence for mouse SNX11 is $^{5'}$CGTCAAGGTCTCCAGCATTTCC$^{3'}$ and anti-sense sequence is $^{5'}$CGCCCTGATCTTGGAAGAAAGC$^{3'}$. Oligonucleotides sense sequence for GFP is $^{5'}$TGAACCGCATCGAGCTGAAG$^{3'}$ and anti-sense sequence is $^{5'}$AGAGTCGCGGCCGCTTTACTTATAC$^{3'}$. Oligonucleotides sense sequence for human VEGF is $^{5'}$AGGAGGAGGGCAGAATCATCA$^{3'}$ and anti-sense sequence is $^{5'}$CAGGGATTTTCTTGTCTTG$^{3'}$. Oligonucleotides sense sequence for mouse VEGF is $^{5'}$CAATGATGAAGCCCTGGAGT$^{3'}$ and anti-sense sequence is $^{5'}$AATGCTTTCTCCGCTCTGAA$^{3'}$. Oligonucleotides sense sequence for Ang1 is $^{5'}$GTGAGAGTGCGACAGAGCAG$^{3'}$ and anti-sense sequence is $^{5'}$GTGGTTTTGAACCGCATTCT$^{3'}$. Oligonucleotides sense sequence for Importin α1 is $^{5'}$CGCAGAATAGAGGTCAATGTG$^{3'}$ and anti-sense sequence is $^{5'}$CGGAGAAGTAGCATCATCAGG$^{3'}$. Oligonucleotides sense sequence for Importin α3 is $^{5'}$ATGCTTCAAGTGATAACCAGGG$^{3'}$ and anti-sense sequence is $^{5'}$CAAGACAATGGACTAAAATGG$^{3'}$. Oligonucleotides sense sequence for Importin α5 is $^{5'}$TCGCCTGAAAAGTTACAAGAA$^{3'}$ and anti-sense sequence is $^{5'}$AGAAGTGATGACACCACCTGG$^{3'}$. Oligoncleotides sense sequence for Importin β1 is $^{5'}$AAGCCGCAGATTCTGTCAGT$^{3'}$ and anti-sense sequence is $^{5'}$CGGGTGTACGTTCTCCTGAT$^{3'}$. Oligonucleotides QuantumRNA^{TM} universal 18S

Standard (Ambion, Austin, TX) was used as reference. PCR cycle is 94 ^{o}C for 15 seconds, 72 ^{o}C for 30 seconds and is repeated 30 times.

shRNA and siRNAs transfection

shRNA sequences against PAR2, SNX1, 2, 11 and controls obtained from OpenBiosystems (Huntsville, AL) were shPAR2: $^{5'}$GCTGTGATTTACATGGCCAATcgagATTGGCCATGTAAATCACAGC$^{3'}$, shSNX1: $^{5'}$CCTTCCAAGTAGCTGGGATTActcgagTAATCCCAGCTACTTGGAAGGT$^{3'}$, shSNX2 : ^{5}'CCCTAGAGTCAAGTCCATCATctcgagATGATGGACTTGACTCTAGGG^{3}', shSNX11 (A): $^{5'}$GCATTGCTCTACTCTTAGGTTctcgagAACCTAAGAGTAGAGCAATGCTTTTTTG$^{3'}$ shSNX11(B): $^{5'}$CCATTCTTCGATATGCTATGTctcgagACATAGCATATCGAAGAATGGTT TTTTG$^{3'}$, shSNX11(C): ^{5}'GCAAATTCTAAGCTGCCATATctcgagATATGGCAGCTTA-GAATTTGCTTTTTTG^{3}', shScrambled: $^{5'}$GACCGTAACTATTCGGTGCGTTGGG-CAgaagcttgTGCCCAACGCACCGAATAGTTACGGTC3'.

The siRNA sequences targeting importins α1, α3, α5 and β1 obtained from Ambion (Ambion Silencer TM siRNA Construction Kit) were CCAAGCUACUCAAGCUGCCAGGAAA for α1, CAGUGAUCGAAAU-CCACCAAUUGAU for α3, CCGGAAUGCAGUAUGGGCUUUGUCU for α5 and CAGUCUGGCUGAAGCUGCUUAUGAA for β1.

Immunofluorescence

Cells were placed in a 24 wells plate containing slides and were transfected with appropriate plasmids. 24 hours following transfection, cells were starved (DMEM 1% FBS) for 24 hours. After stimulation of cells with trypsin 100 nM (or SLIGKV 100 μM), they were fixed with paraformaldehyde 4% for 20 minutes at room temperature. Cells were blocked with blocking solution containing PBS, 5 % fœtal calf serum, 5 % goat serum and 0,02 % sodium azide for 45 minutes. Permeabilization of cells was done with Triton X-100 10 % diluted in blocking solution. Nuclei labeling was done with PI or DAPI diluted in blocking buffer for 5 minutes at room temperature.

Electron microcospy

Specimens for electron microscopy were prepared as previously described(*2*) . For immunolabeling assays, cells were incubated with 1 μg/mL mouse monoclonal PAR-2 antibodies, subsequently with a goat anti-mouse gold (1nm)-conjugated IgG

(1:100)(Electron Microscopy Sciences). Negative controls were analyzed by omitting primary antibodies. Specimens were examined with a transmission electron microscope (Philips 410LS).

Co-immunoprecipitation experiments

Endogenous PAR-2, SNX11, importinβ1 and dynein were immunoprecipitated respectively with 2 ug of antibodies (Santa Cruz) from clear lysates of HEK293 PAR-2 GFP or HEK293 SNX11 GFP cells treated with SLIGKV. Immune complexes were immobilized by adding 50 uL of protein A (SIGMA), washed three times wish lysis buffer and 30 uL of sample buffer was added. Western blot analyses for PAR-2, SNX11 and importinβ1 were performed.

Flow cytometry

HEK293 cells stably transfected with PAR-2 GFP, PAR-2 FLAG and different forms of PAR-2 mutants were treated with 20 μM SLIGKV or 100 ηM Trypsin for 45 minutes prior to nuclear isolation. HEK293 transfected with Flag-PAR-2-HA were incubated with Anti-Flag-FITC (1:200) for 15 min in DMEM (37^{o}C) and were activated for 45 minutes prior to nuclear isolation. The nuclear preparations were processed by flow cytometry using FACSCalibur (BD Biosciences, San Jose, CA) and results were analyzed by CellQuest software (BD Biosciences).

Measurement of Ca^{2+} Signals induced by Trypsin in HEK293 cells

Cellular Ca^{2+} signals were measured by the fura-2-AM technique as essentially described (HECHT *et al.*, 1996) with minor modifications. Briefly, cells (500 000 cells/mL) placed on a slide in HBSS/Calcium buffer (Hepes (0.02 M), $CaCl_2$ (1.5 mM), D-glucose (10 nM), NaCl (118 mM), KCl (4.6 mM)), were loaded in the dark with fura-2-AM (7 μM) (60 min at 4 °C). The [Ca^{2+}] was determined in 1 ml of fura-2-loaded cells (500 000 cells/ml) at 37 °C under constant stirring using the ratio of excitation wavelengths 350/380 nm with emission set at 500 nm. Cell calcium signals were measured by means of a spectrofluorometer (model LS50, PerkinElmer Life Sciences, UK). Calibration of maximal (*R*max) fluorescence signals was determined by sequential addition of triton (0.1%), whereas that of minimal (*R*min) fluorescence signals was obtained with EGTA (20 mM). The [Ca^{2+}] was calculated according to Grynkiewicz *et al. (GRYNKIEWICZ et al., 1985)* by the equation: [Ca^{2+}] =*Kd* (224 nM) [(*R* - *R*min)/(*R*max -

R)] (S_{f2}/S_{b2}), where *Kd* is the dissociation constant (224 nM for fura-2) and *S*f2/*S*b2 is the ratio of fluorescent intensity at 380-nm wavelength.

Lentivirus production

We produced infectious lentivirus by transiently transfecting lentivector and packaging vectors into HEK293T cells (ATCC) as previously described (Dull T., J Virol, 1998). We concentrated viral supernatants by ultra-centrifugation (>500-fold) and determined titers by ELISA for viral p24 antigen using a commercial kit (ZeptoMetrix).

Histochemistry

Eyes were enucleated and fixed in 4% paraformaldehyde. The corneas and lenses were removed and eyes transferred to 30% sucrose/PBS overnight (4°C), washed with PBS, and stored at −80°C until the time of cryosectioning (10 μm). Sections were washed with 0.1% Triton X-100/PBS and blocked for 1 h with 5% goat serum/1% BSA before primary incubation overnight (room temperature). The histochemical markers used, with their respective dilutions, were TRITC-conjugated rat and human lectin EC markers from *G. simplicifolia* and *Ulex europaeus***,** respectively (Sigma–Aldrich), anti-PAR-2 and anti-Thy1.1. Primary antibodies were visualized using appropriately titrated Alexa-conjugated secondary antibodies. Negative control experiments were performed in parallel by incubating sections with 0.1% Triton X-100/PBS alone followed by secondary antibody. Sections were assessed using epifluorescence microscopy.

Intravitreal injections and developmental growth assessment

We injected mice pup eyes with 20 μM SLIGRL (final intravitreal concentration), LV.GFP, LV. PAR-2 GFP, LV. PAR-2 NLSm, or LV.shRNA to PAR-2 GFP or SNX11 at P2 and determined vascular areas and densities at P4 for shRNA and P6 for lentiviral experiments. We visualized vasculature by incubating tissues with tetramethylrhodamine isothiocyanate-labeled lectin (Griffonia simplicifolia; 1:100, Sigma) overnight at 24 °C (Calza L, PNAS, 2001) and examining them by fluorescence microscopy (Nikon Eclipse E800).

Microvascular sprouting from aortic explants

We cut aortae from adult Sprague-Dawley rats into 1-mm-thick rings and placed them in growth factor–reduced Matrigel (BD Biosciences) in 24-well tissue culture plates and cultured them for 5 d in media conditioned by RGC-5 cells or in control DMEM.

Following transfection with shScrambled or shPAR-2, aortae were stimulated with SLIGRL. We took photomicrographs of individual explants and quantified microvascular sprouting by measuring the area covered by outgrowth of the aortic ring with ImagePro Plus 4.5 (Media Cybernetics).

Retinal Wholemounts

Eyes were fixed in 4% paraformaldehyde. Retinas were dissected free, subjected to 100% methanol (–20°C) for 10 minutes, and incubated overnight (room temperature) in 1% Triton X-100/phosphate-buffered saline (PBS) with either the TRITC-conjugated lectin EC marker Ulex europaeus (1:100; Sigma-Aldrich, St. Louis, MO). Labeled retinas were incubated with a monoclonal Alexa–conjugated secondary antibody (1:10 000; Molecular Probes, Burlington, ON, Canada) for 1 hour (room temperature) before all retinas were washed in PBS and mounted. Negative control experiments were performed in parallel by incubating retinas in 1% Triton X-100/PBS alone, followed by secondary antibody if one was used.

RESULTS AND DISCUSSION

Classically, the internalization of plasma membrane GPCRs was considered to play a role in their recycling or degradation. Because cleaved PAR-2 is permanently active, lysosomal degradation has been suggested to shut down its activity(*6*). Commitment to a sub-cellular localization is established early in the peripheral sorting endosomes. However, the ultimate destination of a given receptor might vary (*7, 8*). PAR-2 contains 2 lysine-rich nuclear localizing regions and was observed at the cell nucleus by electron microscopy (Fig 1G). Hence, we investigated whether PAR-2 may also be sorted for the cell nucleus upon activation as an alternative sub-cellular destination and a unique signalling pathway. Live confocal imaging video of stimulated PAR-2-GFP-containing cells supported the trafficking of PAR-2 from the plasma membrane to the nucleus (Fig. 1A and fig. S1A). In order to ascertain that activated PAR-2 originated from the plasma membrane, we transfected a PAR-2 construct containing a Flag epitope on its extracellular N-terminus and pre-incubated live cells with anti-Flag fluorescent antibodies. The agonist peptide (SLIGKV), but not trypsin which cleaves the Flag epitope, caused a clear nuclear redistribution of PAR-2 (FITC fluorescence). Tagged plasma membrane PAR-2 co-localized with the inner nuclear membrane marker Lamin B receptor (LBR) (Fig. 1B), as confirmed by line scan analysis (Fig. 1C) and isolated nuclei (Fig. 1D). In agreement with these observations, flow cytometry showed a 5-fold increase in fluorescence on nuclei from SLIGKV-stimulated cells, but not trypsin (Fig. 1E). In addition, time-dependant immunoblot of stimulated PAR-2-GFP cells revealed enrichment of PAR-2 on the nuclear fraction with a corresponding decreased expression at the plasma membrane (Fig.1F). The purity of our nuclear isolation was confirmed by electron microscopy and with immunoblot cellular fraction markers (fig. S1C). To our knowledge, these data provide the first unequivocal evidence for the translocation of peptide-activated GPCR from the plasma membrane to the nucleus and offer an explanation for their nuclear distribution.

In order to determine which receptor domains contribute to the nuclear translocation of PAR-2, we proceeded by selective mutagenesis of nuclear localizing regions from the first and third intracellular loops, the β-arrestin interaction domain and truncation of the C-terminus (position 363) (fig. S2A). Mutant PAR-2-GFP expressing

cell lines were studied using confocal microscopy and FACS of isolated nuclei following stimulation. The truncated C-terminus mutant of PAR-2 (C-trunc.), as well as the mutant of each nuclear localizing sequences [NLSm (3iL) more then NLSm-1iL], remained mostly at the plasma membrane upon stimulation (Fig 2A, B). Hence, the nuclear targeting sequences of PAR-2 seem essential for its nuclear trafficking, as well as the C-terminus, which could serve as a scaffold for assembly of the endosomes(*9*).

The destination of the endocytic cargo is influenced by receptor targeting sequences, its association with cargo-proteins and cytoskeletal-motors. The sorting of endosomes is location-dependent, taking place in segregated sorting compartments, as well as time-dependent, involving precisely orchestrated cargo-protein interactions(*10*). Importins are pivotal nuclear transport proteins of the karyopherin family that recognize and bind NLS targeting sequences. Although their essential role in protein import across the nucleopore complex is recognized, their role in the nuclear translocation of integral membrane protein was more recently demonstrated(*11*). Initially, adaptor protein importin-α was reported to recognize the NLS and recruit importin-β1, the latter granting passage through the NPC(*12*) However, importin-β1 alone is now believed to be sufficient for nuclear import(*13*). Silencing importin-β1 prevented the nuclear trafficking of plasma membrane Flag-PAR-2 (Fig2E, fig. S2J, K).

Like importins, sorting nexins (SNXs) are increasingly implicated in trafficking of endosomes. SNXs comprise a characteristic Phox domain (PX) and have been identified across phyla, from yeast to mammals, with preserved endocystosis, endosomal sorting, and signalling functions(*14*). Sorting nexin 1 and 2 (SNX1 & SNX2), along side other components of the mammalian retromer, contribute to endosome-to-trans-golgi network retrieval of PAR-1, preventing lysosomal degradation of this prototypical thrombin-activated protease activated receptor(*15*). Expanding the repertoire of their sorting destinations, SNX6 together with Pim-1 was reported to translocate to the cell nucleus(*16*). We hypothesized a similar nuclear distribution and trafficking for SNX11, a SNX of previously unreported function. SNX11 does not contain a BAR (Bin, amphiphysin, Rvs) domain but holds multiple nuclear localization motifs comparable to SNX6. Proteins involved in collaborative cellular functions are often genetically clustered; interestingly, SNX11 shares the same chromosomal locus with major nuclear

transport protein importin-β1 described earlier (17q21.32). Together, this raised the possibility of a role for SNX11 in nuclear trafficking in association with importin-β1.

We investigated the contribution of SNXs in PAR-2 nuclear trafficking by silencing SNX1, 2 and 11, using small hairpin RNA (shRNA); all three were found to interact with PAR-2 by co-immunoprecipitation (Fig. 2H, fig. S2C, E). Live cells transfected with Flag-PAR-2 and exposed to anti-Flag-FITC antibodies were used. FACS analysis of isolated nuclei revealed a marked inhibition of Flag-PAR-2 nuclear translocation using three different shRNA against SNX11, but not with shRNA against SNX1 or 2 (Fig. 2F**)**. We produced two polyclonal antibodies against SNX11 that were not working by immunocytochemistry; they were used successfully for immunoblots. Stable cell lines transfected with SNX11-GFP revealed a strong nuclear and peri-nuclear expression pattern by confocal microscopy (Fig2C, F, fig. S2B, H, I). Flag-PAR-2 expressed in SNX11-GFP cells readily relocalized from the plama membrane to the nucleus upon stimulation, together with SNX11-GFP and importin-β1 (Fig 2C); all three proteins were co-localized on isolated nuclei following stimulation (Fig. 2D). SNX11-GFP also co-localized with the late endosomal marker M6P (fig. 2SH). We confirmed visually that translocation of tagged plasma membrane Flag-PAR-2 was abrogated in cells silenced for SNX11 (SNX11kd) (Fig.2E, fig 2SF). The interaction of PAR-2 with Importin-β1 and SNX11 was confirmed by co-immunoprecipitations (Fig. 2G, fig S2E). Thus, SNX11 in association with importin-β1 were required for the translocation of PAR-2 from the plasma membrane to the cell nucleus.

Sorting nexins and importins have been reported to travel on microtubules; carrier-protein kinesins move cargos towards the plasma membrane and contribute to endosomal sorting, while dyneins(*17*) transit towards the juxtanuclear microtubule organizing center (MOC) and the nucleus(*18*). Sorting nexin 4 and dyneins were recently shown to facilitate transport of transferrin receptors to the endocytic recycling compartment. Dyneins are believed to allow transport of proteins from early-to-late endosomes and sorting to the endocytic recycling compartment (ERC) (*19*). Despite evidence of dynein contributing to the nuclear import of regulatory proteins, such as p53 tumor suppressor protein and parathyroid hormone-related protein (PTHrP)(*20*), the endocytic compartments it orginates before reaching the nucleus are unknown. Large

GPCRs, which comprise seven transmembrane domains, were also reported to bind dynein for endosomal cellular trafficking. Rhodopsin, a prototypical GPCR, can bind the light chain of dynein on its cytoplasmic tail, allowing the recycling of membrane receptors, otherwise leading to retinitis pigmentosa if disrupted(*21*). However, no one has yet implicated microtubules with dynein in the nuclear trafficking of a GPCR.

The intracellular distribution of PAR-2-GFP, SNX11-GFP and Importinβ-1 overlaps with the cells microtubule network (tubulin-α) (Fig 2G, fig2SI) and each co-immunoprecipitated with dynein. (Fig 2H, fig. S2E) Although clathrins with β-arrestin-1 may partake in the lysosomal degradation of PAR-2 (*22, 23*), dominant negative Dynamin cell lines (DynIK44A), clathrin inhibitor (cadaverin) (*24*) and mutation of the β-arrestin interaction domain (Fig2A, B), did not prevent the nuclear translocation of PAR-2 (Fig. 2H, I), suggesting an internalization mechanism independent of clathrin and caveolin. On the other hand, PAR-2 trafficking was abrogated by microtubule inhibitors colchicine and vinblastine (Fig 2I, J) (*25*). In short, the integrity of the microtubule highway is necessary for shuttling the endosomal complex containing PAR-2 together with cargo-protein dynein to the cell nucleus.

We carried on by investigating the functional purpose for GPCR translocation to the nucleus. The complexity of GPCR signalling is rapidly expanding; sub-cellular distribution and trafficking modulate their signals. We examined the gene induction signature of PAR-2 nuclear translocation. Because PAR-2 plays an important role in developmental and pathological angiogenesis(*5, 26*), we studied the induction pattern of vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1) (amongst others).

Native PAR-2 and PAR-2-β-arrestin mutant, which translocate to the nucleus, induced the expression of both VEGF and Ang1 following stimulation with agonist peptide (SLIGKV) (Fig.3A), Although inhibition of clathrin with Monodansyl Cadaverin did not prevent PAR-2 translocation and VEGF induction, it blocked increased Ang1 expression (Fig. 3C), suggesting its induction by protein complexes of early endosomes (*27*). Conversely, interfering with the nuclear translocation of PAR-2 (using PAR-2 C-truncated and NLS mutants expressing cells, silencing SNX11, or with microtubule inhibitor colchicine) prevented the induction of VEGF while preserving the ability to induce Ang1 (Fig. 3A-C). These observations suggested that the pool of PAR-2 (protein

complex) that translocate to the nucleus was responsible for VEGF induction. To further establish this postulate, we isolated nuclei of cells stimulated for 15 and 45 min with SLIGKV; we then treated these nuclei with PAR-2 agonist peptide. We noted an increase in VEGF expression but not in Ang1 (Fig. 3D), a distinction from the inherent basal nuclear response to PAR-2 agonist, which does not induce VEGF.

By adding a nuclear retention signal to the C-terminus of PAR-2, we wanted to explore further its function at the nucleus. The E3 region of adenovirus type 5 was shown to target transmembrane receptors to the nuclear membrane (*28, 29*). PAR-2-E3 was mostly retained at the nucleus, to the exclusion of the plasma membrane (Fig 3E). We compared the calcium transients and gene induction pattern of PAR-2-NLSm and PAR-2-E3 expressing cells. SLIGKV triggered immediate calcium release from PAR-2-NLSm cells with ensuing Ang1 expression, but had no effect on cells expressing nuclear PAR-2-E3 (Fig. 3F). In order to stimulate nuclear PAR-2-E3 across the plasma membrane, we fused the internalization peptide sequence tat to SLIGKV. Relative to the time course of the action of PAR-2-NLSm, tat-SLIGKV triggered a delayed response and lower nuclear calcium transient in PAR-2-E3 cells; this delay could be due to the time taken for the transit of the agonist to the nucleus possibly releasing a smaller pool of calcium *in situ* (Fig. 3F). Tat-SLIGRL induced the expression of VEGF only from nuclear PAR-2-E3 cells (Fig 3G). Despite interfering with receptor trafficking, mutants of PAR-2 were functional and released calcium transients comparable to native PAR-2 upon stimulation (measured by Fura-2-AM) (fig. S3A). Thus, a unique gene expression fingerprint can be associated with the translocation of PAR-2 to the nucleus, which determined the relative expression of VEGF and Ang1. To our knowledge, this represents a novel-signalling pathway for GPCRs.

Normal retinal vascular development requires a delicate balance between vascular expansion and maturation. Differentiation, proliferation and migration of the vascular endothelium (vasculogenesis) is attributed largely to VEGF, while Ang1 fosters newly formed vessels to remodel, mature and recruit pericytes (angiogenesis)(*30*). Although represented as a sequential process, the two phases occur simultaneously in the developing retina, allowing newly formed vessels to mature, while the vascular front expands. Disrupting the equilibrium between maturation and expansion leads to arrest in

vessel growth or pathologic angiogenesis. Retinal vessel development in mice occurs within the first 2 weeks of life. Blood vessels proceed from the optic nerve to the periphery and cover the superficial retina within 9 post-natal days (P9), followed by the formation of the deeper vascular plexus(*31*). Recent evidence suggests that RGCs are pivotal instigators of retinal angiogenesis. Mice (brn3bZ-dta/+;six3-cre mice) which do not possess RGCs fail to develop a retinal vasculature. It is postulated that retinal neurons sense the hypoxic stress of the avascular regions of the developing retina and liberate growth factors, such as VEGF and Ang1 (*32*). Concomitant increases in VEGFR2 and tie2 receptor are observed in the developing retinal endothelium (VEGF and angiopoietin receptors respectively). PAR-2 levels are also increased during that period and might contribute to the up-regulation of Tie2(*5*). Although PAR-2 was reported in endothelial cells, we found it is mostly expressed in retinal ganglion cells during development (Fig 4B).

In order to investigate the angiogenic role of PAR-2 in retinal neurons, we stimulated cultured RGCs (terminally differentiated) with PAR-2 agonist (SLIGRL 20uM) and used these cells to condition fresh medium. PAR-2 expression on these cells was confirmed by immunoblot (fig. S4C). The conditioned culture medium (containing factors secreted by SLIGRL-stimulated RGCs) was collected,filtered and used to assay vascular sprouting from aortic ring explants of PAR-2 knockout (PAR-2ko) mice. Medium conditioned by untreated RGCs or RGCs silenced for PAR-2 (shRNA), induced low levels of sprouting (Fig. 4A). However, conditioned medium obtained from RGCs primed with PAR-2 for 6 h produced a two-fold increase in sprouting (Fig. 4A), indicative of release of proangiogenic factors from PAR-2-treated RGCs.

We then explored the physiologic contribution of GPCR nuclear trafficking *in vivo*. Wild type mice (WT) injected with the agonist peptide of PAR-2 (SLIGRL in mice) showed increased retinal vessel growth and density compared to PAR-2 knockout mice (PAR-2ko), confirming a role of PAR-2 in retinal angiogenesis (Fig 4C). We used lentivirus (LV) vectors containing shRNA or PAR-2 mutants to examine their effects on the developing retina. We injected LV in mice vitreous body at P2 and observed a protein expression within 48 hours (P4). LV preferentially transfect superficial retinal vessels and ganglion cells, where native PAR-2 is naturally expressed (Fig 4B, fig. S4A). Silencing

PAR-2 and SNX11 with LV shRNA slowed the retinal vascular development of wild type (WT) mice compared to scrambled shRNA controls. Although PAR-2ko mice did not present a marked vascular phenotype, suggesting compensatory mechanisms, we used them to test our PAR-2 constructs without the confounding effects of endogenous PAR-2. Re-introducing native PAR-2 in knockout animals markedly increased both the retinal vascularization and the vascular density. More importantly, the expression of PAR-2-NLSm significantly curtailed the progression of vessel growth and vessel density in PAR-2ko retinas (Fig. 4C). Corroborating their *ex vivo* gene induction patterns, plasma membrane bound PAR-2-NLSm increased the retinal expression of Ang1 at the expense of VEGF, while native PAR-2 up-regulated both (Fig. 4D). Hence, PAR-2-NLSm tilted the balance towards early vascular maturation disrupting retinal vessel growth. This implies a functional role for the nuclear translocation of a GPCR *in vivo*.

In this study, we offered an explanation for the origin of peptide-activated GPCR at the nucleus. In our opinion, they may translocate from the plasma membrane to the nucleus upon stimulation. We also explored the mechanism and the functional purpose for nuclear trafficking. Finally, we verified this concept *in vivo*. We hope our data would expand our understanding of GPCR signalling and lead to novel pharmacologic targets.

Acknowledgements

The authors thank Hendrika Fernandez, Lenka Rihakova and Alexandra Zabeida for skillful technical assistance, Gregor Andelfinger for generously supplying SNXs antibodies and stably expressing SNXs cell lines, Nikolaus Heveker for his help constructing PAR-2 mutants, and Christian Beauséjour for producing lentiviral vectors. This work is supported by grants from the Canadian Institutes of Health Research (CIHR). S. Nim and JS Joyal are recipients of funding awards from the CIHR. S. Chemtob is a recipient of a Canada Research Chair.

REFERENCES

1. A. M. Marrache *et al.*, in *J Immunol*. (2002), vol. 169, pp. 6474-81.
2. F. Gobeil *et al.*, in *Can J Physiol Pharmacol*. (2006), vol. 84, pp. 287-97.
3. B. Boivin, G. Vaniotis, B. G. Allen, T. E. Hébert, in *J Recept Signal Transduct Res*. (2008), vol. 28, pp. 15-28.
4. V. S. Ossovskaya, N. W. Bunnett, in *Physiol Rev*. (2004), vol. 84, pp. 579-621.
5. T. Zhu *et al.*, in *Arteriosclerosis, Thrombosis, and Vascular Biology*. (2006), vol. 26, pp. 744-50.
6. O. Déry, M. S. Thoma, H. Wong, E. F. Grady, N. W. Bunnett, in *J Biol Chem*. (1999), vol. 274, pp. 18524-35.
7. A. C. Hanyaloglu, M. von Zastrow, in *Annu Rev Pharmacol Toxicol*. (2008), vol. 48, pp. 537-68.
8. P. Arora, T. K. Ricks, J. Trejo, in *J Cell Sci*. (2007), vol. 120, pp. 921-8.
9. J. Bockaert, P. Marin, A. Dumuis, L. Fagni, in *FEBS Lett*. (2003), vol. 546, pp. 65-72.
10. F. R. Maxfield, T. E. McGraw, in *Nat Rev Mol Cell Biol*. (2004), vol. 5, pp. 121-32.
11. M. C. King, C. P. Lusk, G. Blobel, in *Nature*. (2006), vol. 442, pp. 1003-7.
12. C. P. Lusk, G. Blobel, M. C. King, in *Nat Rev Mol Cell Biol*. (2007), vol. 8, pp. 414-20.
13. A. Harel, D. J. Forbes, in *Mol Cell*. (2004), vol. 16, pp. 319-30.
14. P. J. Cullen, in *Nat Rev Mol Cell Biol*. (2008), vol. 9, pp. 574-82.
15. A. Gullapalli, B. L. Wolfe, C. T. Griffin, T. Magnuson, J. Trejo, in *Mol Biol Cell*. (2006), vol. 17, pp. 1228-38.
16. Y. Ishibashi *et al.*, in *FEBS Lett*. (2001), vol. 506, pp. 33-8.
17. B. M. Paschal, R. B. Vallee, in *Nature*. (1987), vol. 330, pp. 181-3.
18. J. P. Caviston, E. L. Holzbaur, *Trends Cell Biol* **16**, 530 (Oct, 2006).
19. F. Aniento, N. Emans, G. Griffiths, J. Gruenberg, in *J Cell Biol*. (1993), vol. 123, pp. 1373-87.
20. D. M. Roth, G. W. Moseley, D. Glover, C. W. Pouton, D. A. Jans, in *Traffic*. (2007), vol. 8, pp. 673-86.

21. A. W. Tai, J. Z. Chuang, C. Bode, U. Wolfrum, C. H. Sung, in *Cell*. (1999), vol. 97, pp. 877-87.
22. K. A. DeFea *et al.*, *J Cell Biol* **148**, 1267 (Mar 20, 2000).
23. O. Dery, M. S. Thoma, H. Wong, E. F. Grady, N. W. Bunnett, *J Biol Chem* **274**, 18524 (Jun 25, 1999).
24. E. Ray, A. K. Samanta, *FEBS Lett* **378**, 235 (Jan 15, 1996).
25. J. Thyberg, S. Moskalewski, *Experimental cell research* **159**, 1 (Jul, 1985).
26. A. Maree, D. Fitzgerald, in *Circ Res*. (2002), vol. 90, pp. 366-8.
27. K. A. DeFea *et al.*, in *J Cell Biol*. (2000), vol. 148, pp. 1267-81.
28. K. Ghosh, H. P. Ghosh, in *Biochem Cell Biol*. (1999), vol. 77, pp. 165-78.
29. A. Scaria, A. E. Tollefson, S. K. Saha, W. S. Wold, in *Virology*. (1992), vol. 191, pp. 743-53.
30. G. Thurston, in *Cell Tissue Res*. (2003), vol. 314, pp. 61-8.
31. M. Fruttiger, in *Angiogenesis*. (2007), vol. 10, pp. 77-88.
32. P. Sapieha *et al.*, in *Nat Med*. (2008), vol. 14, pp. 1067-76.
33. J. H. Hecht, J. A. Weiner, S. R. Post, J. Chun, *J Cell Biol* **135**, 1071 (Nov, 1996).
34. G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J Biol Chem* **260**, 3440 (Mar 25, 1985).

FIGURES AND LEGENDS

Figure 1: **Translocation of PAR-2 to nucleus. (A)** Live confocal video of HEK293 cells stably transfected with PAR-2 GFP and stimulated with SLIGKV 20 μM for 0-45 minutes. Confocal images at different time points were taken from the live confocal video. **(B, C)** HEK293 cells were transfected with Flag PAR-2, incubated with anti-Flag FITC antibodies and cells were stimulated with SLIGKV 20 μM for 0-45 minutes. Nuclear membrane was stained using LBR antibodies. Co-localization was analysed by performing line scan analysis. **(D)** HEK293 cells were transfected with Flag PAR-2, incubated with anti-Flag antibodies for 30 min. and stimulated with SLIGKV 20 μM for 45 minutes. Nuclei were isolated. Nuclear membrane was stained using LBR antibodies. **(E, F)** HEK293 cells were transfected with Flag PAR-2. Following treatment of cells with the vehicle or 10 μM colchicine, cells were incubated with anti-Flag antibodies and stimulated with 20 μM SLIGKV for 45 minutes. Nuclei were isolated and FITC fluorescence was quantified by FACS. 30 ηg of proteins from the nuclei fraction and plasma membrane fraction were loaded on 10% SDS-PAGE gel. PAR-2 was stained using anti-PAR-2 antibodies. **$p<0.01$; ***$p<0.001$; n=3 or 4 **(G)** *In situ* localization of PAR-2 in endothelial cells as determined by immunogold electron microscopy.

Figure 1

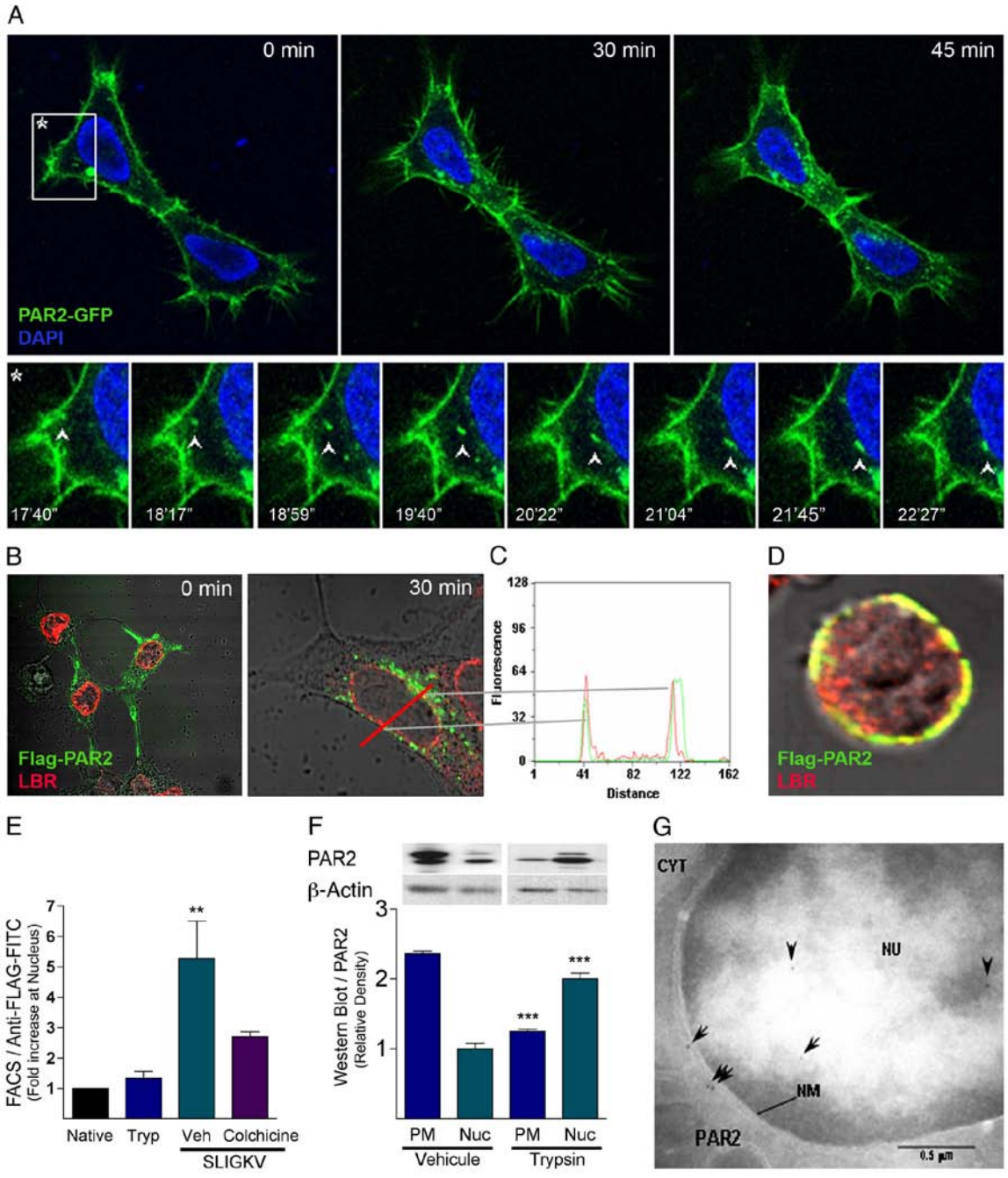

A
0 min
30 min
45 min
PAR2-GFP
DAPI
17'40"
18'17"
18'59"
19'40"
20'22"
21'04"
21'45"
22'27"
B
0 min
30 min
Flag-PAR2
LBR
C
Fluorescence
Distance
D
Flag-PAR2
LBR
E
FACS / Anti-FLAG-FITC
(Fold increase at Nucleus)
Native
Tryp
Veh
Colchicine
SLIGKV
F
PAR2
β-Actin
Western Blot / PAR2
(Relative Density)
PM
Nuc
PM
Nuc
Vehicule
Trypsin
G
CYT
NU
NM
PAR2
0.5 µm

Figure 2: **Domains and mechanism of translocation of PAR-2 to nucleus. (A, B)** HEK293 cells were stably transfected with PAR-2 GFP or PAR-2 mutants. Cells were stimulated with the vehicle or 20 μM SLIGKV for 45 min. Nuclei were isolated for FACS quantification of GFP fluorescence. **p<0.01; ***p<0.001; n=3 **(C)** HEK293 cells transfected with Flag PAR-2 and SNX11 GFP were stimulated with the vehicle or 20 μM SLIGKV for 45 min. PAR-2 and importinβ1 were stained using anti-Flag and anti-importinβ1 antibodies respectively **(D,E)** HEK293 SNX11 GFP cells were transfected with Flag PAR-2. Cells were transfected with shMOCK, shSNX1, shSNX2 or shSNX11 (A,B,C). Cells were stimulated with 20 μM SLIGKV for 45 min. FACS quantification was done on isolated nuclei from HEK293 SNX11 GFP cells transfected with shRNA. ***p<0.001; n=3. **(F)** HEK293 cells stably transfected with PAR-2 GFP or SNX11 GFP. Microtubules stained with anti-tubulin-α antibodies. **(G)** HEK293 SNX11 GFP cells were transfected with PAR-2 GFP, stimulated with 20 μM SLIGKV for 45 min. Proteins were immunoprecipitated with anti-PAR-2, -SNX11, -importinβ1 or -IgG antibodies. **(H, I)** HEK293 PAR-2 GFP cells were treated with 400 μM cadaverine, 10 μM colchicine or transfected with DynK44A. Cells were stimulated with the vehicle or SLIGKV 20 μM for 45 min. Nuclei labelled with DAPI. FACS quantification was done on isolated nuclei from HEK293 PAR-2 GFP cells treated with 400 μM cadaverine, 10 μM colchicine or transfected with DynK44A. *p<0.05; **p<0.01; n=3.

Figure 2

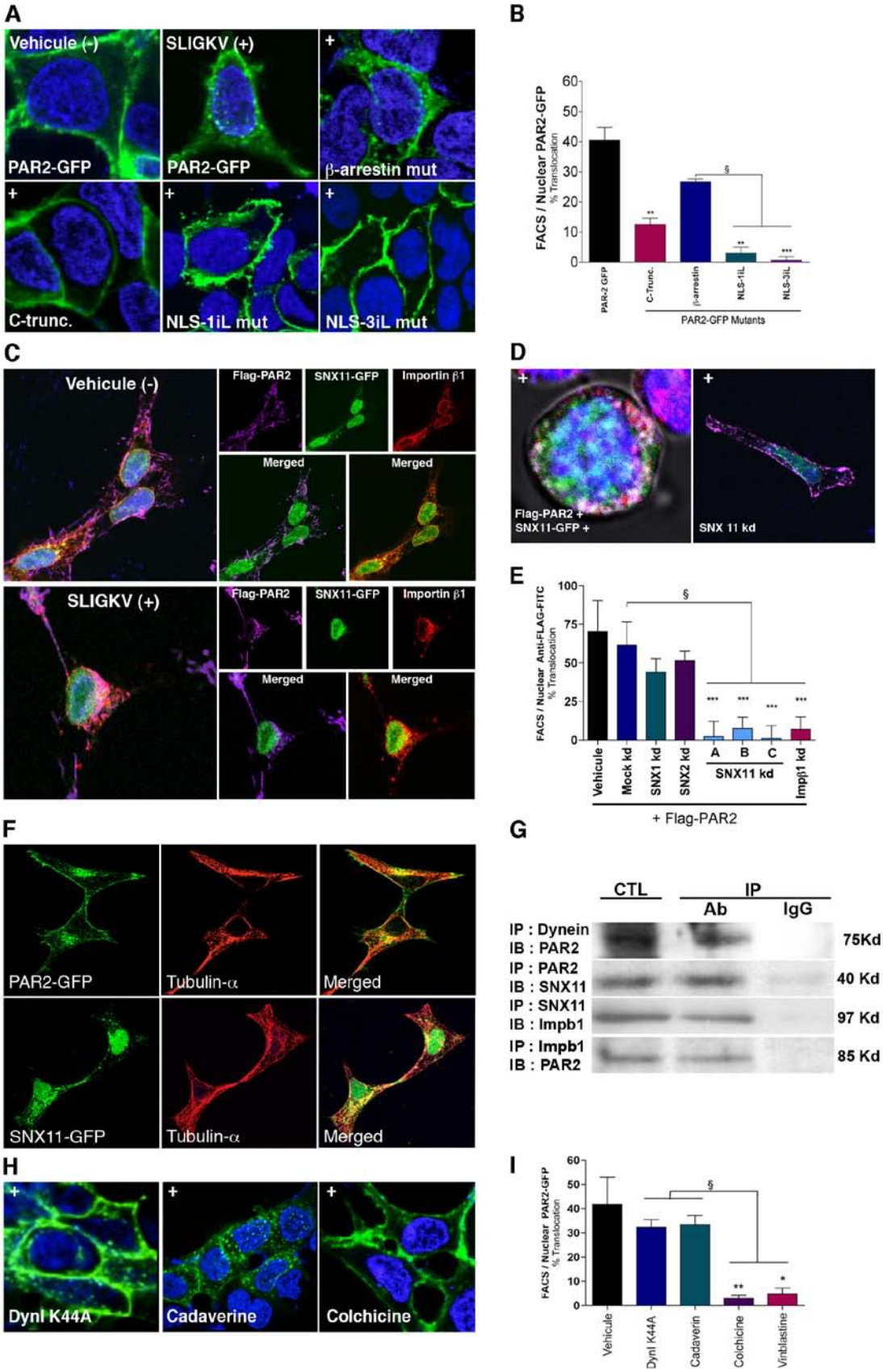

A
Vehicule (-)
SLIGKV (+)
+
PAR2-GFP
PAR2-GFP
β-arrestin mut
+
+
+
C-trunc.
NLS-1iL mut
NLS-3iL mut
B
FACS / Nuclear PAR2-GFP
% Translocation
60
50
40
30
20
10
0
§
**
**
***
PAR-2 GFP
C-Trunc.
β-arrestin
NLS-1iL
NLS-3iL
PAR2-GFP Mutants
C
Vehicule (-)
Flag-PAR2
SNX11-GFP
Importin β1
Merged
Merged
SLIGKV (+)
Flag-PAR2
SNX11-GFP
Importin β1
Merged
Merged
D
+
+
Flag-PAR2 +
SNX11-GFP +
SNX 11 kd
E
FACS / Nuclear Anti-FLAG-FITC
% Translocation
100
75
50
25
0
§
***
***
***
***
Vehicule
Mock kd
SNX1 kd
SNX2 kd
A
B
C
SNX11 kd
Impβ1 kd
+ Flag-PAR2
F
PAR2-GFP
Tubulin-α
Merged
SNX11-GFP
Tubulin-α
Merged
G
CTL
IP
Ab
IgG
IP : Dynein
IB : PAR2
75Kd
IP : PAR2
IB : SNX11
40 Kd
IP : SNX11
IB : Impb1
97 Kd
IP : Impb1
IB : PAR2
85 Kd
H
+
+
+
Dynl K44A
Cadaverine
Colchicine
I
FACS / Nuclear PAR2-GFP
% Translocation
60
50
40
30
20
10
0
§
**
*
Vehicule
Dynl K44A
Cadaverin
Colchicine
Vinblastine

Figure 3: Functionality of PAR-2 nuclear Translocation. (A) HEK293 PAR-2 GFP cells and HEK293 PAR-2 mutants cells were stimulated with 20 μM SLIGKV for 45 min. VEGF and Ang1 expression were analyzed by RT-PCR. *p<0.05; ***p<0.001; n=3 **(B)** RGC-5 cells were transfected with shRNA against SNX1, SNX2 or SNX11 and stimulated with 20 μM SLIGRL for 45 min. VEGF and Ang1 expression were analyzed by RT-PCR. *p<0.05; n=3 **(C)** RGC-5 cells were treated with 400 μM cadaverine or 10 μM colchicine and treated with 20 μM SLIGRL for 45 min. VEGF, Ang1 and PPARγ1 expression were analyzed by RT-PCR. *p<0.05; n=3 **(D)** RGC-5 cells were pre-stimulated with 20 μM SLIGRL and nuclei isolated prior to cell stimulation with SLIGRL for 15 and 45 min. VEGF and Ang1 expression were analyzed by RT-PCR. *p<0.05; n=3 **(E)** RGC-5 cells were transfected with PAR-2 E3 and microtubules stained using anti-β-III tubulin antibodies. Western blot analysis of cell fractions from HEK293 PAR-2 E3 GFP cells. **(F)** Calcium mobilization assay from HEK293 cells transfected with PAR-2 NLSm or PAR-2 E3. **(G)** HEK293 cells transfected with PAR-2 NLS mutant or PAR-2 E3 and stimulated with 20 μM tat, 20 μM SLIGKV or 20 μM tat-SLIGKV for 45 min. VEGF and Ang1 expression were analyzed by RT-PCR. **p<0.01; n=3.

Figure 3

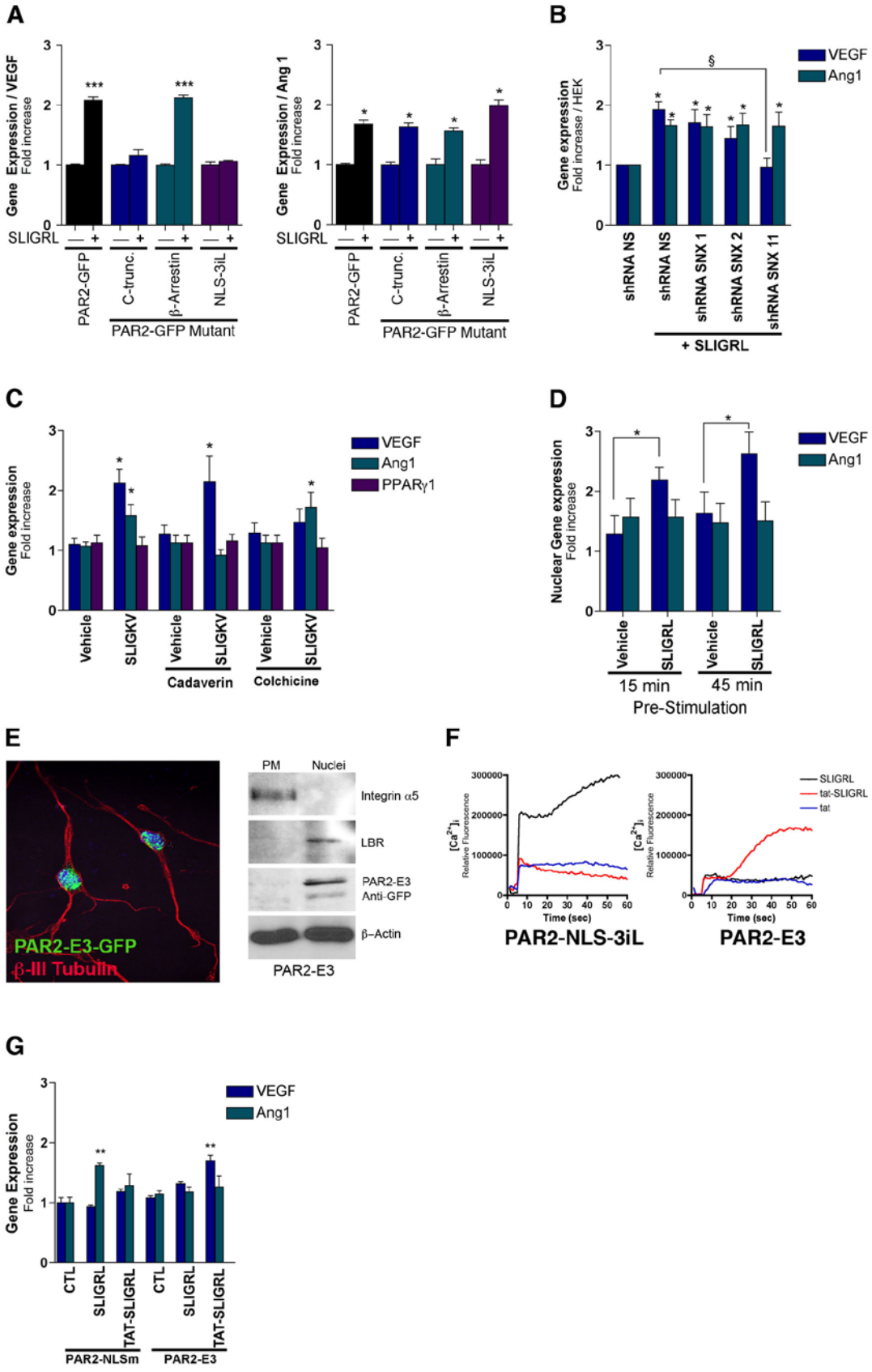
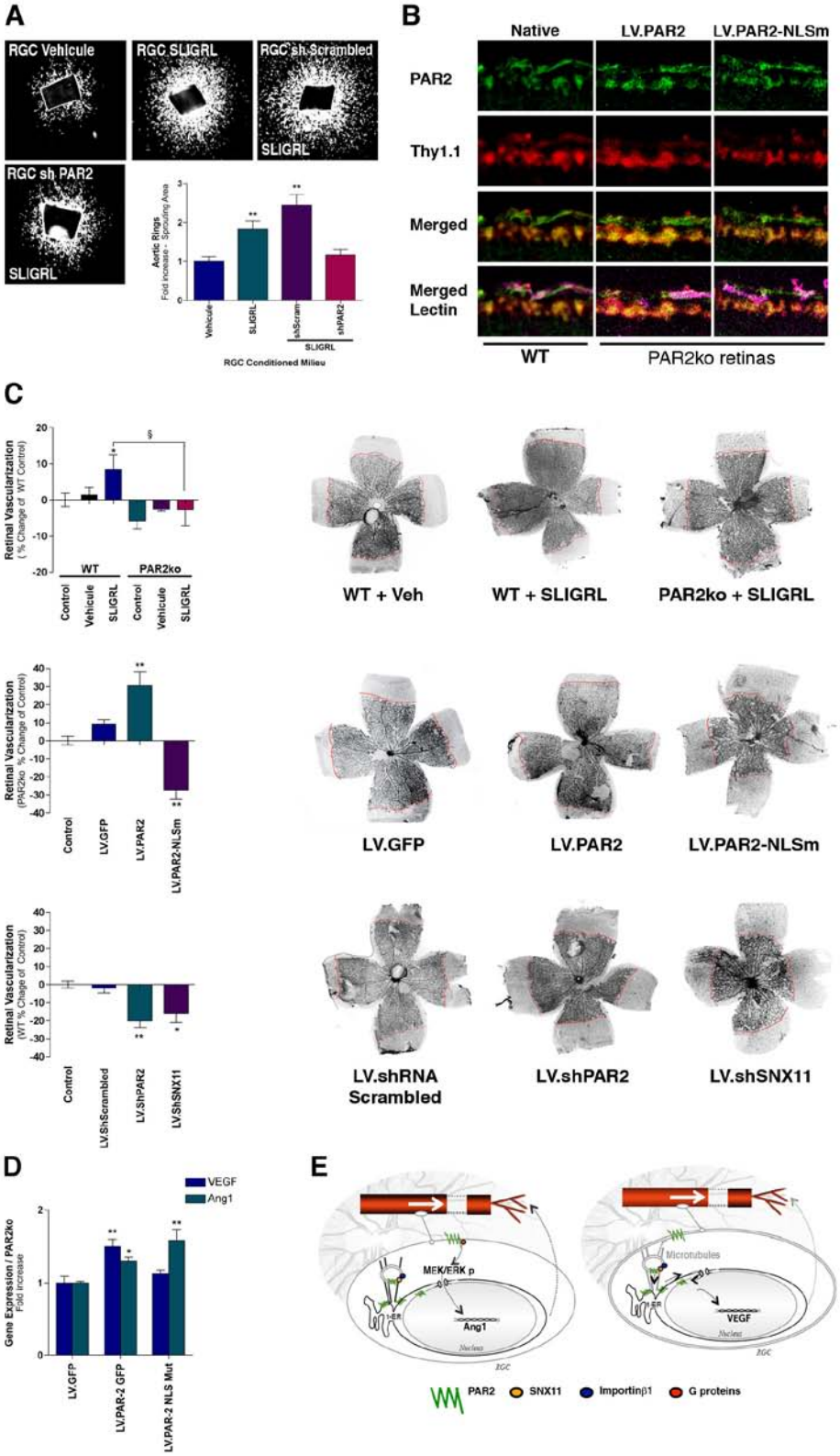

A
Gene Expression / VEGF
Fold increase
SLIGRL
PAR2-GFP
C-trunc.
β-Arrestin
NLS-3iL
PAR2-GFP Mutant
Gene Expression / Ang 1
Fold increase
B
Gene expression
Fold increase / HEK
VEGF
Ang1
shRNA NS
shRNA NS
shRNA SNX 1
shRNA SNX 2
shRNA SNX 11
+ SLIGRL
C
Gene expression
Fold increase
VEGF
Ang1
PPARγ1
Vehicle
SLIGKV
Cadaverin
Colchicine
D
Nuclear Gene expression
Fold increase
15 min
45 min
Pre-Stimulation
E
PAR2-E3-GFP
β-III Tubulin
PM
Nuclei
Integrin α5
LBR
PAR2-E3
Anti-GFP
β–Actin
PAR2-E3
F
[Ca2+]i
Relative Fluorescence
Time (sec)
SLIGRL
tat-SLIGRL
tat
PAR2-NLS-3iL
PAR2-E3
G
Gene Expression
Fold increase
CTL
SLIGRL
TAT-SLIGRL
PAR2-NLSm
PAR2-E3

Figure 4: Functionality of PAR-2 Nuclear Translocation *in vivo*. (A) Aortae from adult Sprague-Dawley rats were transfected with shPAR-2 or shScrambled and stimulated with 20 μM SLIGRL. Quantification of microvascular sprouting was done by measuring the outgrowth area. **p<0.01; n=4 **(B)** Histochemistry of PAR-2 (green) and Thy1.1 (red) with respect to lectin-labeled endothelial cells in WT and PAR-2 KO mice retinas transfected with LV.PAR-2 or LV.PAR-2NLSm. **(C)** Lectin-labeled retinal wholemounts at 6 days of WT or KO mice stimulated with vehicle or 20 μM SLIGRL or transfected with LV.GFP, LV.PAR-2, LV.PAR-2NLSm, LV.shRNAScrambled, LV.shPAR-2 or LV.shSNX11. Dotted lines delimit the vascular front. Area of vascularization was calculated. *p<0.05; **p<0.01; n=8 **(D)** PAR-2 KO mice were injected with LV.GFP, LV. PAR-2 GFP or LV. PAR-2 NLSm. VEGF and Ang1 expression were analyzed by RT-PCR. *p<0.05; **p<0.01; n=3 **(E)** Schematic representation of PAR-2 mechanisms of neovascularization regulation.

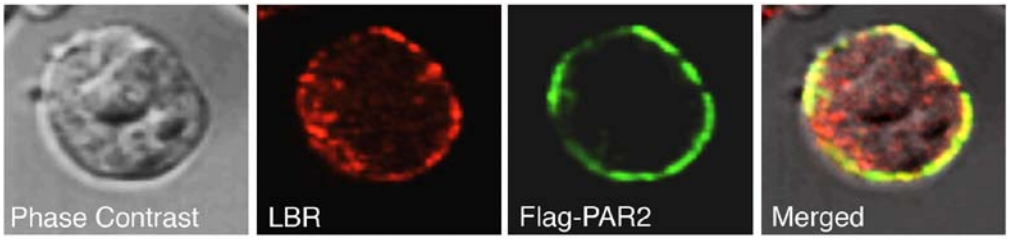
Figure 4


A
RGC Vehicule
RGC SLIGRL
RGC sh Scrambled
SLIGRL
RGC sh PAR2
SLIGRL
Aortic Rings
Fold increase - Sprouting Area
Vehicule
SLIGRL
shScram
shPAR2
SLIGRL
RGC Conditioned Milieu
B
Native
LV.PAR2
LV.PAR2-NLSm
PAR2
Thy1.1
Merged
Merged
Lectin
WT
PAR2ko retinas
C
Retinal Vascularization
(% Change of WT Control)
WT
PAR2ko
Control
Vehicule
SLIGRL
Control
Vehicule
SLIGRL
WT + Veh
WT + SLIGRL
PAR2ko + SLIGRL
Retinal Vascularization
(PAR2ko % Change of Control)
Control
LV.GFP
LV.PAR2
LV.PAR2-NLSm
LV.GFP
LV.PAR2
LV.PAR2-NLSm
Retinal Vascularization
(WT % Chage of Control)
Control
LV.ShScrambled
LV.ShPAR2
LV.ShSNX11
LV.shRNA
Scrambled
LV.shPAR2
LV.shSNX11
D
VEGF
Ang1
Gene Expression / PAR2ko
Fold Increase
LV.GFP
LV.PAR-2 GFP
LV.PAR-2 NLS Mut
E
MEK/ERK p
Ang1
Nucleus
EGC
Microtubules
VEGF
Nucleus
EGC
PAR2
SNX11
Importinβ1
G proteins

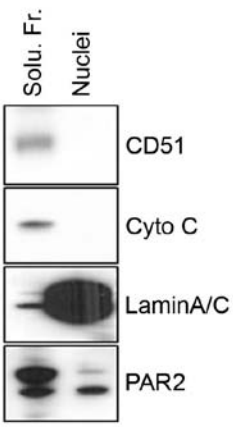
Supplementary Figure 1: (A) Video showing the translocation of PAR-2 from the plasma membrane to the nuclear membrane. **(B)** HEK293 were transfected with Flag PAR-2, incubated with Flag FITC antibody and cells were stimulated with 20 μM SLIGKV for 45 min. Nuclei were isolated. Nuclear membrane was stained using LBR antibody. **(C)** Western blot analysis of cell fractions (soluble fraction and nuclei) obtained from HEK293 PAR-2 E3 GFP cells. Equal amounts of denatured membrane proteins (25 μg) of each fraction were resolved by SDS-PAGE (10%) gel. Note the absence of plasma membrane microsomal, mitochondrial contamination in the nuclear preparation. **(D)** *In situ* localization of PAR-2 in endothelial cells and rat hepatocytes as determined by immunogold electron microscopy.

A - Video

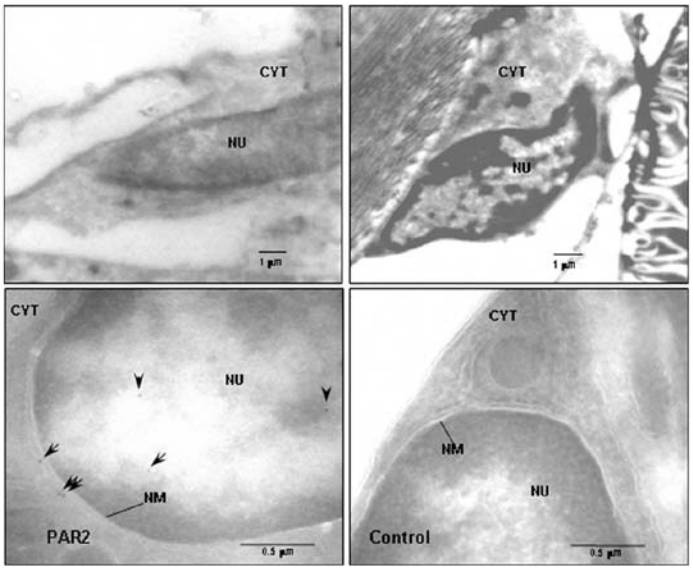
B


Phase Contrast
LBR
Flag-PAR2
Merged

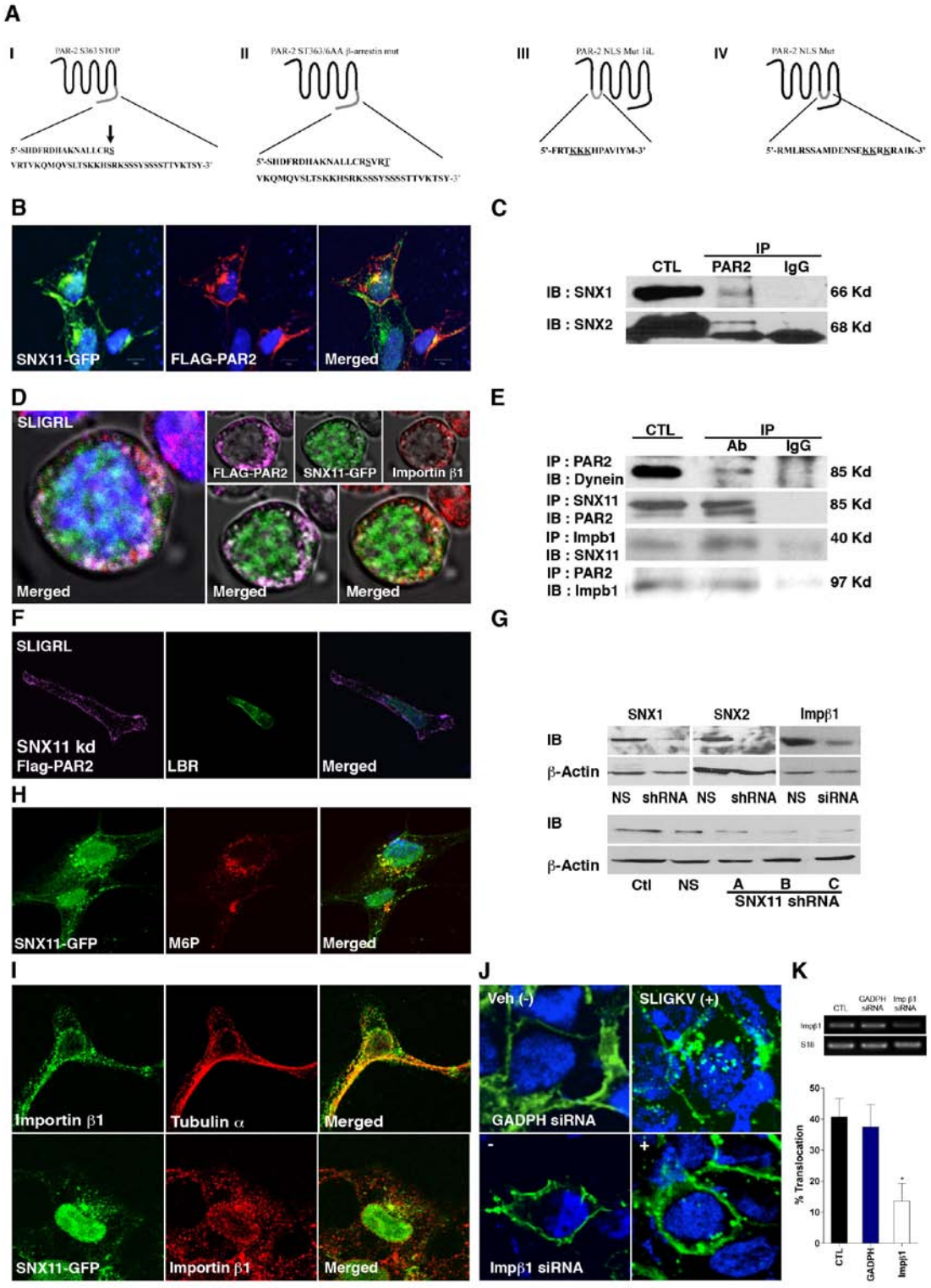
C


Solu. Fr.
Nuclei
CD51
Cyto C
LaminA/C
PAR2

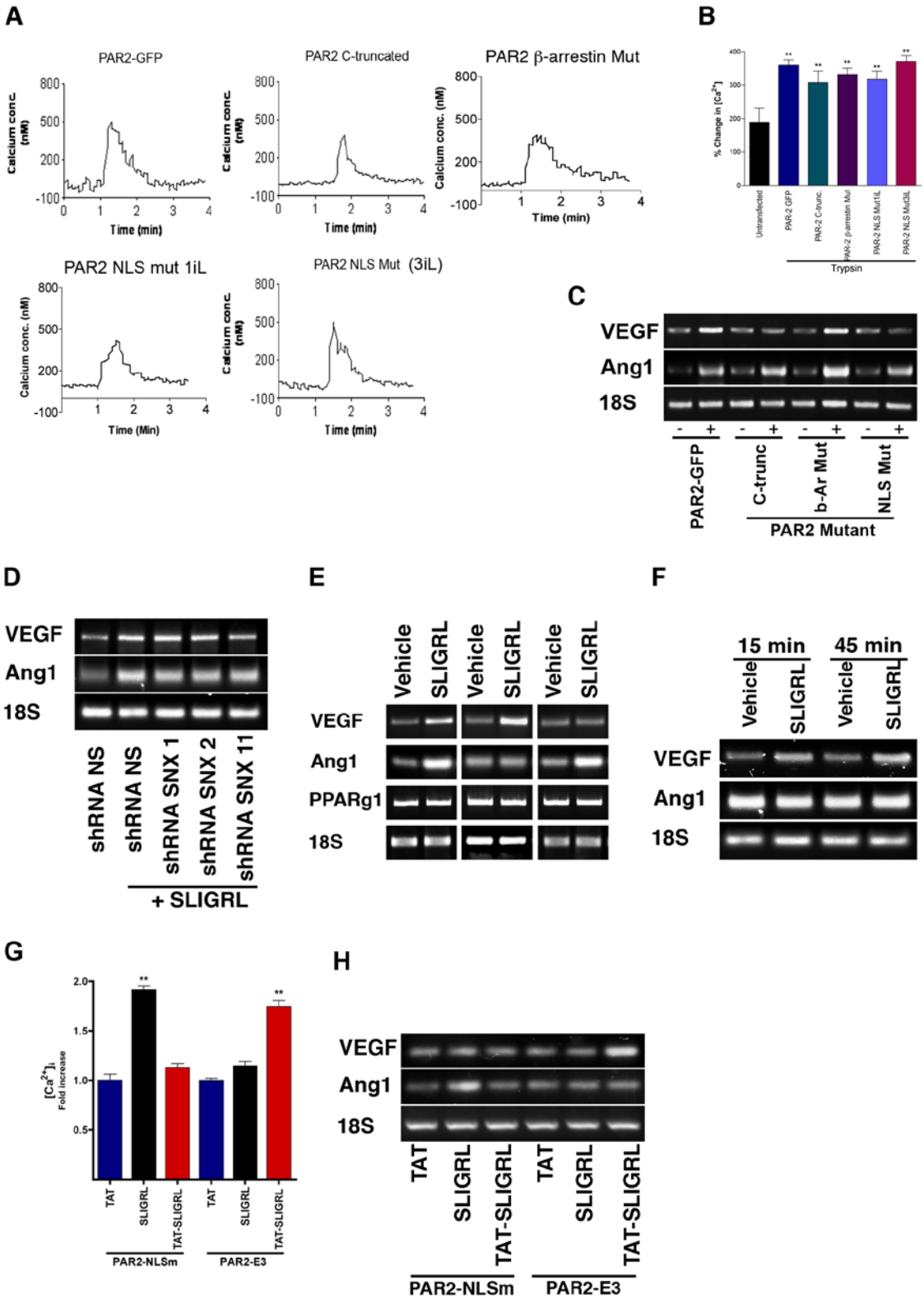
D


CYT
NU
CYT
NU
CYT
NU
NM
PAR2
CYT
NM
NU
Control

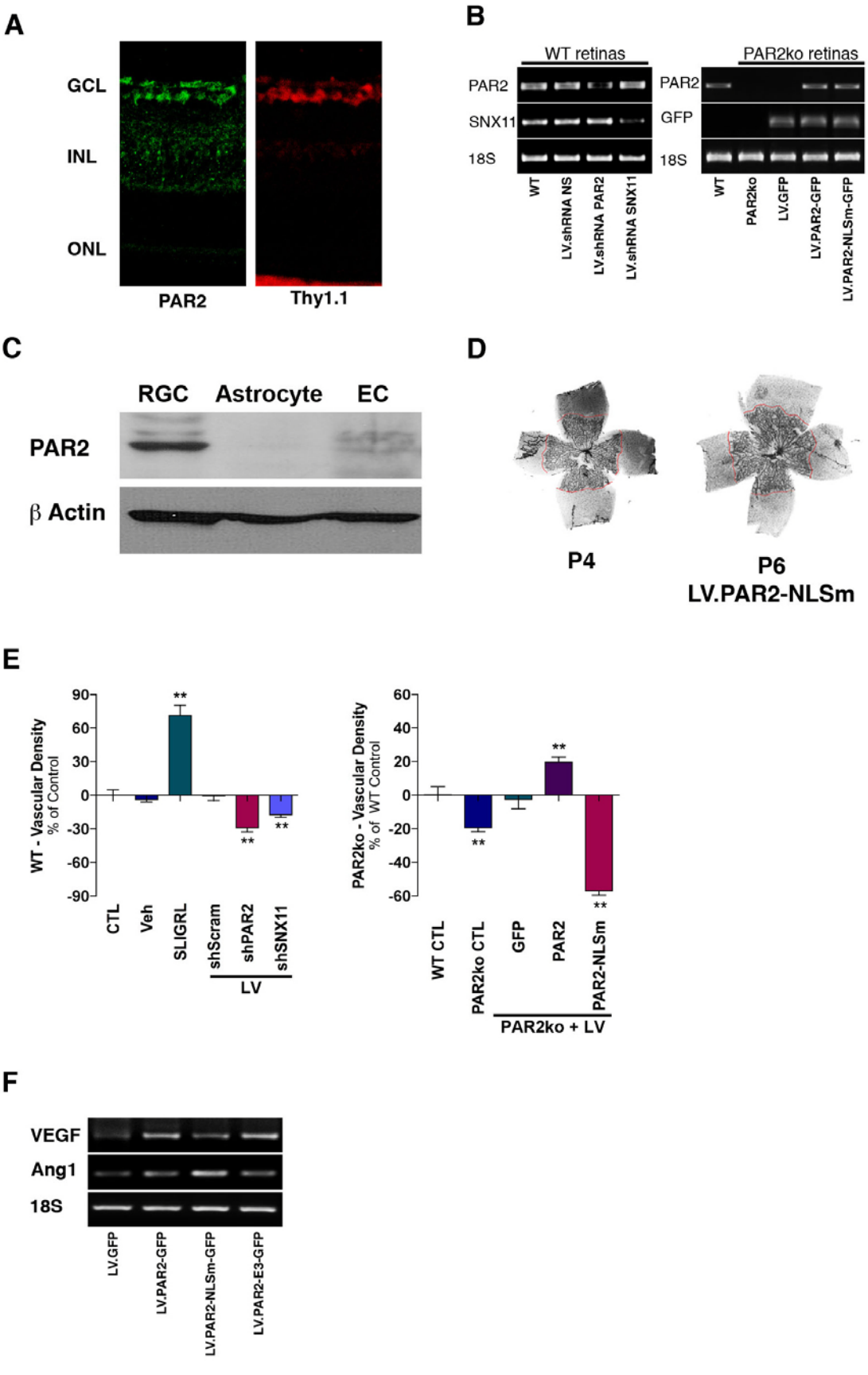
Supplementary Figure 2: (A) Mutational strategies for different PAR-2 mutants. **(B)** HEK293 SNX11 GFP cells were transfected with Flag PAR-2 **(C)** HEK293 SNX11 GFP cells were stimulated with 20 μM SLIGKV for 45 min. Cells were lysed and immunoprecipitated with anti-PAR-2 or anti-IgG antibodies. **(D)** HEK293 SNX11 GFP cells were transfected with Flag-PAR-2. Nuclei were isolated. PAR-2 and importinβ1 were stained with anti-Flag and anti-importinβ1 antibodies respectively. **(E)** HEK293 SNX11 GFP cells were stimulated with 20 μM SLIGKV for 45 min. Cells were lysed and immunoprecipitated with PAR-2, SNX11, importinβ1 or IgG antibodies. **(F)** HEK293 SNX11 GFP cells were transfected with Flag-PAR-2 and stimulated with 20 μM SLIGKV for 45 min. **(G)** Western blot analysis of SNX1, SNX2 and importinβ1 expression following transfection of HEK293 PAR-2 GFP cells with SNX1 shRNA, SNX2 shRNA, importinβ1 siRNA or different SNX11 shRNAs (A, B, C). **(H)** HEK293 SNX11 GFP cells stained using late endosomes marker anti-M6P antibodies. **(I)** HEK293 SNX11 GFP cells staining using anti-importinβ1 and anti-tubulin-α antibodies. **(J,K)** HEK293 PAR-2 GFP cells transfected with GAPDH siRNA or importinβ1 siRNA prior to cell stimulation with vehicle or 20 μM SLIGKV for 45 min. Expression of importinβ1 and S18 analyzed by RT-PCR. FACS quantification of isolated nuclei. *$p<0.05$; n=3.


A
I
PAR-2 S363 STOP
5'-SHDFRDHAKNALLCRS
VRTVKQMQVSLTSKKHSRKSSSYSSSSTTVKTSY-3'
II
PAR-2 ST363/6AA β-arrestin mut
5'-SHDFRDHAKNALLCRSVRT
VKQMQVSLTSKKHSRKSSSYSSSSTTVKTSY-3'
III
PAR-2 NLS Mut 1IL
5'-FRTKKKHPAVIYM-3'
IV
PAR-2 NLS Mut
5'-RMLRSSAMDENSEKKRKRAIK-3'
B
SNX11-GFP
FLAG-PAR2
Merged
C
IP
CTL
PAR2
IgG
IB : SNX1
66 Kd
IB : SNX2
68 Kd
D
SLIGRL
Merged
FLAG-PAR2
SNX11-GFP
Importin β1
Merged
Merged
E
CTL
IP
Ab
IgG
IP : PAR2
IB : Dynein
85 Kd
IP : SNX11
IB : PAR2
85 Kd
IP : Impb1
IB : SNX11
40 Kd
IP : PAR2
IB : Impb1
97 Kd
F
SLIGRL
SNX11 kd
Flag-PAR2
LBR
Merged
G
SNX1
SNX2
Impβ1
IB
β-Actin
NS shRNA NS shRNA NS siRNA
IB
β-Actin
Ctl NS A B C
SNX11 shRNA
H
SNX11-GFP
M6P
Merged
I
Importin β1
Tubulin α
Merged
SNX11-GFP
Importin β1
Merged
J
Veh (-)
SLIGKV (+)
GADPH siRNA
-
+
Impβ1 siRNA
K
CTL
GADPH siRNA
Impβ1 siRNA
Impβ1
Sln
% Translocation
50
40
30
20
10
0
CTL
GADPH
Impβ1

Supplementary Figure 3: (A,B) Intracellular calcium mobilization measurements following 20 ηM trypsin stimulation of HEK293 cells or HEK293 cells expressing PAR-2 GFP, PAR-2 C-truncated, PAR-2 β-arrestin muntant, PAR-2 NLS mut 1iL or PAR-2 NLS mut 3iL. **p<0.01; n=3 **(C)** HEK293 PAR-2 GFP cells and HEK293 PAR-2 mutants cells were stimulated with SLIGKV 20 μM for 45 min. VEGF and Ang1 expression were analyzed by RT-PCR. **(D)** RGC-5 cells were transfected with non-specific shRNA, SNX1 shRNA, SNX2 shRNA or SNX11 shRNA. Cells were stimulated with 20 μM SLIGRL for 45 min. VEGF and Ang1 expression were analyzed by RT-PCR **(E)** RGC-5 cells were treated with 400 μM cadaverine or 10 μM colchicine, and stimulated with 20 μM SLIGRL for 45 min. VEGF, Ang1 and PPARγ1 expression were analyzed by RT-PCR. **(F)** RGC-5 cells were pre-stimulated with 20 μM SLIGRL. Isolated nuclei were stimulated with 20 μM SLIGRL for 15 and 45 min. VEGF and Ang1 expression were analyzed by RT-PCR. **(G,H)** PAR-2 NLS mutant and HEK293 PAR-2 E3 cells stimulated with tat, SLIGKV or tat-SLIGKV. Intracellular calcium mobilization measurements. **p<0.01; n=3. VEGF and Ang1 expression were analyzed by RT-PCR.


A
PAR2-GFP
PAR2 C-truncated
PAR2 β-arrestin Mut
PAR2 NLS mut 1iL
PAR2 NLS Mut (3iL)
Calcium conc. (nM)
Time (min)
Time (Min)
B
% Change in [Ca2+]
Untransfected
PAR-2 GFP
PAR-2 C-trunc.
PAR-2 β-arrestin Mut
PAR-2 NLS Mut1iL
PAR-2 NLS Mut3iL
Trypsin
C
VEGF
Ang1
18S
PAR2-GFP
C-trunc
b-Ar Mut
NLS Mut
PAR2 Mutant
D
VEGF
Ang1
18S
shRNA NS
shRNA NS
shRNA SNX 1
shRNA SNX 2
shRNA SNX 11
+ SLIGRL
E
Vehicle
SLIGRL
VEGF
Ang1
PPARg1
18S
F
15 min
45 min
Vehicle
SLIGRL
VEGF
Ang1
18S
G
[Ca2+]i Fold increase
TAT
SLIGRL
TAT-SLIGRL
PAR2-NLSm
PAR2-E3
H
VEGF
Ang1
18S
TAT
SLIGRL
TAT-SLIGRL
PAR2-NLSm
PAR2-E3

Supplementary Figure 4: (A) Histochemistry of PAR-2 (green) and Thy1.1 (red) in mice retinas **(B)** WT mice were injected with LV.shRNA NS, LV.shPAR-2 and LV.shSNX11. KO mice were injected with LV.GFP, LV.PAR-2-GFP, and LV.PAR-2 NLSm-GFP. PAR-2, SNX11, GFP and 18S expression were analyzed by RT-PCR. **(C)** Western blot analyzing the expression of PAR-2 and beta-actin in RGC, astrocytes and EC cells. **(D,E)** Lectin-labeled retinal wholemounts at 4 and 6 days of WT or KO mice stimulated with vehicle or 20 μM SLIGRL or transfected with LV.GFP, LV.PAR-2, LV.PAR-2NLSm, LV.shRNAScrambled, LV.shPAR-2 or LV.shSNX11. Dotted lines delimit the vascular front. Density of vascularization was calculated. *$p<0.05$; **$p<0.01$; n=8 **(F)** KO mice transfected with LV.GFP, LV.PAR-2, LV.PAR-2 E3, and LV.PAR-2NLSm. VEGF, Ang1 and 18S expression were analyzed by RT-PCR.


A
GCL
INL
ONL
PAR2
Thy1.1
B
WT retinas
PAR2ko retinas
PAR2
SNX11
18S
PAR2
GFP
18S
WT
LV.shRNA NS
LV.shRNA PAR2
LV.shRNA SNX11
WT
PAR2ko
LV.GFP
LV.PAR2-GFP
LV.PAR2-NLSm-GFP
C
RGC
Astrocyte
EC
PAR2
β Actin
D
P4
P6
LV.PAR2-NLSm
E
WT - Vascular Density
% of Control
CTL
Veh
SLIGRL
shScram
shPAR2
shSNX11
LV
PAR2ko - Vascular Density
% of WT Control
WT CTL
PAR2ko CTL
GFP
PAR2
PAR2-NLSm
PAR2ko + LV
F
VEGF
Ang1
18S
LV.GFP
LV.PAR2-GFP
LV.PAR2-NLSm-GFP
LV.PAR2-E3-GFP

CHAPTER 3 – ACTIVATION OF PAR-2 ENHANCES PROANGIOGENIC PROCESSES VIA SP1

Preamble

Previous studies have suggested that some GPCRs can interact with transcription factors and regulate the initiation of transcription (Cavanagh and Colley 1989; Nehring, Horikawa et al. 2000; White, McIllhinney et al. 2000). Nuclear receptors including some members of the GPCR family and of the tyrosine kinase receptor family have been shown to mediate signalling through Sp1 transcription factors (Safe and Kim 2004).

We have demonstrated in our previous studies that activation of PAR-2, a member of the GPCR family, resulted in the translocation of the receptor from the plasma membrane to the nucleus. This translocation pathway is distinct from the degradation pathway of the receptor and nuclear translocation of PAR-2 plays an important role in the regulation of proangiogenic gene expression. Because PAR-2 is also found endogenously at the cell nucleus, which is thought to origin from a transclocation from the plasma membrane, we sought to find the role of nuclear PAR-2 in the regulation of gene expression possibly through the binding and/or the activation of transcription factors. We suggested that activation of gene expression by nuclear PAR-2 might be regulated by interaction of the receptor with transcription factors. These factors may play an important role in the regulation of cell proliferation and cell migration induced by PAR-2 activation.

Activation of PAR-2 Enhances Proangiogenic Processes via Sp1

Nim S[1,2], Zhu T[1], Zaniolo K[1], Bossolasco M[1], Joyal JS[1,2], Hamel D[1], Fan L[1], Chemtob S[1,2].

[1]Departments of Pediatrics, Ophtalmology and Pharmacology, Research Centre, Hôpital Ste-Justine, Montreal, Quebec, Canada

[2]Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada

Corresponding author:

Dr. Sylvain Chemtob, M.D., Ph.D., F.R.C.P.C.

Department of pharmacology, Faculty of medicine, University of Montreal

Department of Pharmacology and Therapeutics, Faculty of Medicine, McGill University

Sainte-Justine Hospital Research center

3175, chemin de la Côte Sainte-Catherine

Montreal (Quebec), Canada H3T 1C5

Tel.: (514) 345-4692/Fax: (514) 345-4801

E-mail: sylvain.chemtob@umontreal.ca

ABSTRACT

The protease-activated receptor 2 (PAR-2) belongs to the G protein-coupled receptor (GPCR) family and is activated by serine proteases, such as trypsin, or by synthetic peptides (SLIGKV or SLIGRL). PAR-2 is widely distributed in many cell types and tissues including those of the cardiovascular system. It partakes in many physiological and pathological processes, such as inflammation, tumor growth, vascular constriction and dilation. Although most of its effects are associated with the activation of the receptor at the cell surface, PAR-2 has also been demonstrated to be endogenously expressed at the nucleus in diverse tissues. Our previous studies have demonstrated the origin of nuclear PAR-2 by demonstrating the translocation of PAR-2 from the plasma membrane to the nucleus and the implication of this process in retinal angiogenesis. Therefore, the central aim of this present work was to elucidate the functionality of nuclear PAR-2, its mechanism of regulation of gene expression and its implication in the regulation of cell proliferation and migration. In this study, we confirmed endogenous PAR-2 nuclear localization by confocal microscopy. PAR-2 activation on isolated nuclei from Hela cells by SLIGKV induced intracellular calcium release and TNFα gene expression. We analyzed the molecular mechanism of nuclear PAR-2 in the regulation of gene induction by identifying nuclear PAR-2-interacting proteins by mass spectrometry (MS/MS) and transcription factors regulating its activity by chromatin immunoprecipitation (ChIP). Both techniques suggested the involvement of specificity protein 1 (Sp1) transcription factor in nuclear PAR-2 activity. This hypothesis was further confirmed by performing confocal microscopy, co-immunoprecipitation experiments, electrophoretic mobility shift assays (EMSA), cell migration assay and [^{3}H]thymidine incorporation assay. Sp1 gene silencing prohibited PAR-2-induced VEGF and PRIM1 gene expression, cellular migration and proliferation in porcine cerebral microvascular endothelial cells (pCMVEC). These results disclosed the molecular mechanism of nuclear PAR-2 activity particularly its implication in angiogenesis.

INTRODUCTION

The protease-activated receptors [1] are members of the G protein-coupled receptor (GPCR) family and are activated following the cleavage of the N-terminus and the binding of a tethered ligand on the extracellular domain of the receptor [2]. These receptors can also be activated by synthetic ligands having the same sequence as the natural tethered ligand. Four types of PARs have been discovered so far: PAR-1, PAR-2, PAR-3 and PAR-4. They are distributed in many human tissues especially in the intestine, kidney, colon and stomach. Studies have shown that PARs can induce mitogenic effects more specifically neovascularization [3]. PAR-1, 3 and 4 are activated by thrombin while PAR-2 is activated by trypsin, coagulation factors Xa and VIIa, and mast cell tryptase [4-6]. PAR-2 can also be activated by its agonist synthetic peptide, SLIGKV (for human) or SLIGRL (for mouse), which contains the same sequence as the natural PAR-2 tethered ligand. Studies have shown interaction between PAR-2 and G_q/G_{11} and probably G_0/G_i. Stimulation of PAR-2 by its agonist peptide induces activation of PLC and PKC as demonstrated in neuronal cells and smooth muscle cells [7, 8]. Activation of PLC results in the mobilization of intracellular calcium and activation of PKC. It has also been shown that activation of PAR-2 leads to the stimulation of JNK and p38 [9]. Activation of PAR-2 also activates the MAPK cascades through ERK1/2 and induces the transcription of VEGFR-2 and DNA primase 1 [10]. PAR-2 was reported to participate in many physiological and pathological processes, such as inflammation, tumor growth, vascular constriction and dilation. Indeed, stimulation of PAR-2 with trypsin induces cell proliferation in colon cancer cells [11]. Activation of PAR-2 stimulates angiogenesis in a mouse model of hindlimb ischemia [3]. Studies have indicated that PAR-2 is critical for MDA-MB-231 and BT549 breast cancer cell migration and invasion towards NIH 3T3 fibroblast conditioned medium [12]. It has also been shown that PAR-1 is implicated in migration and metastasis and PAR-2 has unexpectedly a role in thrombin-dependent tumor cell migration and in metastasis [13]. PAR-2 also promotes leukocyte rolling and infiltration into injured tissues as well as stimulates cell migration and neovascularization.[3, 14-17]. Numerous proinflammatory and proangiogenic agents are involved in activation of PAR-2 expression, for instance

tumor nectosis factor-α (TNFα), interleukin-1β, and lipopolysaccharide [18]. Our previous works have illustrated that TNFα is a pivotal intermediate in PAR-2-mediated up-regulation of proangiogenic tie2 receptor [14]. Proliferation of pCMVECs induced by PAR-2 activation is associated with up-regulation of downstream DNA primase 1 (PRIM1) [16].

Sp family of transcription factors are characterized by the presence of a conserved DNA-binding domain comprising three Krüppel-like zinc fingers [19]. Sp1 and Sp3 are ubiquitously expressed in mammalian cells and are both structurally similar but have strikingly different functions. These transcription factors are able to bind to GC-rich promoter elements [19, 20]. They are therefore implicated in the proper expression of a large variety of important cellular genes. Sp1 is capable of synergistic activation through direct protein-protein interaction while Sp3 is not [21, 22]. Specific activation and repression domains located within the N-terminal regions of Sp1 are responsible for these differences by facilitating interactions with various co-activators and co-repressors [23-25]. A minimum of three domains is required to comprise any of these site-specific transcription factors: namely, the previously mentioned DNA-binding domain, an NLS (nuclear localization signal) and a transcriptional regulatory domain [19, 20, 26, 27]. Similar to PAR-2, Sp1 has been linked to numerous angiogenic processes [28-32].

Our previous work has demonstrated that PAR-2 is endogenously expressed at the cell nucleus and its nuclear localization results from the translocation of the receptor from the plasma membrane to the nucleus. This nuclear import of PAR-2 enables vascular endothelial growth factor (VEGF) expression and the activation of proangiogenic processes (Nim S. 2009, ready for submission). The purpose of the present study is to elucidate the mechanism of regulation of gene expression induced by nuclear PAR-2. Using mass spectrometry (MS/MS) and chromatin immunoprecipitation (ChIP), we demonstrated in this study that Sp1 transcription factor is involved in nuclear PAR-2 biological activities. Indeed, analysis of DNA fragments found to co-immunoprecipitate with nuclear PAR-2 revealed putative binding sites for different transcription factors including Sp1. We showed that these DNA fragments contained promoter elements regulated by Sp1 activity. Different proteins were found to interact with nuclear PAR-2 as revealed by mass spectrometry including cofactors implicated in the regulation of Sp1

activity. Nuclear PAR-2 interacts with Sp1 following its activation and increases Sp1 binding activity to its consensus DNA sequence. Sp1 is implicated in the regulation of VEGF and PRIM1 gene expression induced by PAR-2 activation. Subsequently, Sp1 is important for PAR-2 regulation of cell proliferation and migration. These studies unveil a mechanism of regulation of gene expression induced by nuclear PAR-2 and show for the first time a mechanism of transcriptional activation by direct association of a transcription factor with a member of the GPCR family and the implication of this association in the activation of proangiogenic processes.

MATERIALS AND METHODS

Material

Trypsin (L-1-tosylamido-2-phenyl chloromethyl ketone-treated) was obtained from Worthington Biochemical Co. (Freehold, NJ). Mouse monoclonal antibodies anti-PAR2 (SAM-11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal PAR2 (H-99) and Sp1 (E-3) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal lamin B receptor (LBR) and mouse monoclonal anti-β-Actine were purchased from Abcam (Cambridge, MA). Mouse monoclonal Flag (M2), HA (HA-7), protein A-Sepharose 4B, and Lithium chloride were from Sigma-Adrich (Oakville, ON). Monoclonal anti-mouse IgG_{2B} antibodies were from R&D System (Minneapolis, MN). Antibodies anti-mouse IgG conjugated to Horseradish peroxidase were obtained from Pierce (Rockford. IL). PAR-2 agonist peptide (SLIGKV) was synthesized by Elim Biopharmaceuticals (Hayward, CA). Propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI), NTPs (ATP, CTP, GTP and UTP) and Trizol are purchased from Invitrogen-Molecular Probes, Burlington, Ontario. Complete EDTA-free protease inhibitor cocktail and FastStart Taq DNA polymerase were from Roche (Penzberg, Germany). SuperAse Inhibitor and QuantumRNA^{TM}universal 18S Standard primers were from Ambion (Austin, TX).

Cell Culture

HEK293 and BOSC23 are human embryonic kidney epithelial cell lines [American Type Culture Collection (ATCC); Manassas, VA]. Hela are human epithelium cells from adenocarcinoma (ATCC). Porcine cerebral microvascular endothelial cells (pCMVEC) were obtained as described previously [33]. All of these cells were cultured in complete medium of Dulbecco's Modified Eagle Medium (DMEM) (Gibico, Grand Isoland, NY, USA) containing 10% FBS, 100U/mL penicillin and 100μg/mL streptomycin and incubated in a 5% CO_2, 37^{o}C humidified tissue culture incubator. For synchronization, the cells were grown in starving medium (DMEM without FBS and supplemented with penicillin and streptomycin) for 4 h or overnight.

Immunofluorescence

Hela cells were seeded onto a 12-well culture dish with a coverslip and transfected with human PAR2-GFP/pEGFP-N1 [34] or Flag-PAR2-HA/pcDNA3 [35] [kindly provided by Dr. Bunnet NW (University of California, San Francisco)] using lipofectamine 2000 (Invitrogen, Burlington, Ontario). Starved cells were fixed with 4% (w/v) paraformaldehyde for 20 min. and permeabilized by adding 0.1% Triton X-100 for 45 min. Cells were incubated with 2μg/mL mouse monoclonal anti-PAR-2 (SAM-11) and rabbit polyclonal anti-LBR antibodies for 120 min. Secondary antibodies were incubated (Alexa Fluor 488 conjugated goat anti mouse IgG (1:200) and Alexa Fluor 594 conjugated goat anti rabbit IgG (1:200)) (Invitrogen, Burlington, Ontario) for 60 min. Nuclei were labelled with 50ng/mL DAPI for 5 min. The stained cells were analyzed with Zeiss *LSM 510 META* laser scanning confocal fluorescence microscopy (SEISS, Heidelberg, Germany).

Nuclei Isolation and gene expression

Hela, HEK293, or BOSC23 cells were cultured in 15cm plates until 90 % confluence. After starving for 4 hours, cells were then washed 3 times with 10mL cold PBS, scraped and transferred to a 15mL tube. Cells were pelletted by centrifugation at 1400 rpm for 5 minutes at 4^{o}C and resuspended with nuclear extracting buffer containing 10mM TrisHCl pH 7.4, 10mM NaCl, 3mM $MgCl_2$, 300mM sucrose and protease inhibitors cocktails. Cells were homogenized with glass Teflon potter (pestle B; clearance 20-50 μm; Bellco Glass) for about 45 min until over 95% nuclei were separated from cells by examining with trypan blue stain. The homogenate was then centrifuged at 700 x g for 10 minutes at 4^{o}C to pellet nuclei. The attached endoplasmic reticulum was cleared up by treating nuclear fractions with nuclear extracting buffer containing 0.1 % NP-40 for 5 min on ice. Isolated nuclei can be further used for calcium release assay, analysis of gene expression, and immunoprecipitation experiments. For gene expression analysis, the aliquot of nuclei was incubated in Nuclear Reaction Buffer [10mM TrisHCl pH 7.4, 140mM KCl, 0.5mM $MgCl_2$, 0.1μM CaCl2, 0.5mM NTPs (ATP, CTP, GTP, and UTP, from Invitrogen)] and 10U/μl RibLock RNase (Invitrogen) in a 37^{o}C incubator. Gene expression was induced by adding 20μM SLIGKV or 20nM trypsin for 60 min. Reaction

was terminated by adding 0.5ml trizol reagent (Invitrogen) to isolated total RNA according to the product instruction.

Calcium release assay in isolated Hela cell nuclei by Fluo-4NW kit.

Isolated Hela cell nuclei were re-suspended into Assay Buffer (from Fluo-4NW kit) and 2.5 x 10^6 nuclei/50μl were seeded into a 96-well culture dish. 50μl of 2 x Fluo-4NW dye (from Fluo-4NW kit) was loaded into each well and incubated at 37^{o}C for 30 min. Calcium release was then induced by injection with 10 μM ionomycin (Sigma), 20mM EGTA+20μM SLIGKV, or SLIGKV alone. The released calcium was bound to Fluo-4NW dye and recorded simultaneously at every second for a total of 80 sec by EnVison Microplate Screening Fluorescence Reader at 494 nm (excitation) and 516 nm (emission). Baselines were normalized to zero.

RT-PCR

Total RNA was isolated with Trizol solution (Invitrogen, Molecular Probes, Burlington, Ontario). For the reverse transcription reaction, 5 μg of total RNA was heated at 70 ^{o}C for 10 minutes. After cooling on ice RNA was put in a reaction solution with 13.3 U/μL de M-MLV (enzyme for reverse transcription is from Invitrogen, Molecular Probes, Burlington, Ontario), 10 mM DDT, 0.129 U/μL RNAguard TM RNase Inhibitor (Amersham, Piscataway, NJ), 0.67 mM dNTP, 16.7 μg/mL d'oligo dT (Invitrogen, Molecular Probes, Burlington, Ontario) and First Strand buffer 1x in a total volume of 30 μL and incubated at 42 ^{o}C for 60 minutes. After inactivation of enzyme by incubating at 94 ^{o}C for 5 minutes, DNA was conserved at -80 ^{o}C. Oligonucleotides for VEGF and Tie2 were conceived with Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Oligonucleotides sense sequence for human VEGF is $^{5'}$AGGAGGAGGGCAGAATCATCA$^{3'}$ and anti-sense sequence is $^{5'}$CAGGGATTTTCTTGTCTTG$^{3'}$. Oligonucleotides sense sequence for human Tie2 is $^{5'}$TACACCTGCCTCATGCTCAG$^{3'}$ and anti-sense sequence is $^{5'}$TTCACAAGCCTTCTCACACG$^{3'}$. The sense primer sequence for human TNFα is $^{5'}$AGACCCCTCCCAGATAGATG$^{3'}$ and anti-sense sequence is $^{5'}$GCCTGTAGCCCATGTTGTAG$^{3'}$. QuantumRNA^{TM} universal 18S Standard (Ambion, Austin, TX) was used as reference. PCR cycle is 94 ^{o}C for 15 seconds, 72 ^{o}C for 30 seconds and is repeated 30 times.

Immunoprecipitation and MS/MS analysis.

Ten 15-cm culture dishes of 90% confluent BOSC23 were employed for nuclear isolation as described above. Isolated nuclei were lysed with Lysis Buffer [(20mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, and 1 x Proteinase inhibitor cocktail (Roch, Mannheim, Germany)]. 300μg of the nuclear soluble fraction was reacted with 1μg mouse monoclonal anti-PAR2 (SAM-11) antibodies and 20μl Protein-A Sepharose 4B beads (Sigma) overnight at 4^{o}C. For Sp1 and PAR-2 co-immunoprecipitation experiments, we used mouse monoclonal anti-Sp1 and rabbit polyclonal anti-PAR-2 antibodies respectively. IgG_{2B} was used as negative control. After washing 6 times with Lysis Buffer, beads were re-suspended into 30μl SDS sample buffer. 5μl of the precipitant were used for Western blot analysis. For MS/MS analysis the beads from ten immunoprecipitation assays (PAR2 or IgG_{2B}) were put together and resuspended into 300μl SDS buffer. After boiling/ice treatment, the beads/SDS buffer mixture were loaded on a 12 x 12cm size 12% SDS-PAGE gel and separated by electrophoresis. The gel was stained by Coomassie blue. The gel from both PAR-2 and IgG_{2B} precipitant samples were then careful sliced into 12 pieces (avoiding IgG heavy and light chains) for MS/MS analysis (Technique Center of CHU Sainte-Justine, Montreal).

Chromatin Immunoprecipitation (ChIP)

BOSC23 cells were grown to confluence on 15 cm dishes and cells were stimulated with trypsin 20 nM for 45 minutes. For Cross-linking, we added formaldehyde to culture medium to a final concentration of 1%, mix and incubate for 10 minutes at 37^{o}C. Cells are washed with cold PBS, scraped, centrifuged at 3500 rpm for 5 min at 4^{o}C. Pellet is resuspended with warm SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Trs, pH 8.0) containing protease inhibitors and sonicated at 40% amplitude, 6 x 10 seconds. Centrifuge lysate for 10 minutes at 13,000 rpm at 4°C, keep supernatant. Supernatant was diluted 10x with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris, pH 8.0, 167mM NaCl) containing protease inhibitors. 100 ul of supernatant was kept as input. Sample is pre-cleared with 80 uL of proteinG/HS

slurry was added and incubated for 30 min at 4^{o}C. Sample is centrifuged at 3000 rpm 5 min and supernatant is collected. 10 ug of antibody was added to sample and incubated O/N at 4^{o}C. For a negative control, we performed an IgG (unspecific antibody) IP. 60 uL of proteinG/HS slurry is added to each sample and incubated 1h at 4^{o}C. Beads are pelleted at 2500 rpm 5 min and washed 1x with Low Salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris, pH 8.0, 150mM NaCl), 1x with High Salt wash buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris, pH 8.0, 500mM NaCl), 1x with LiCl (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris, pH 8.0) and 4x with TE buffer (10mM Tris, pH 8.0 and 1mM EDTA). The protein complex is eluted from the antibody by adding 250 µl elution buffer (1% SDS, 0.1M $NaHCO_3$) to the pelleted bead/antibody/protein complex. Reverse crosslink was done by adding 20 uL of 5M NaCl and incubated at 65^{o}C for 4h. Proteins were digested by adding 10 uL of 0.5M EDTA, 20 uL 1M Tris-HCl pH 6.5 and 2 uL of 10 mg/mL PK and incubated for 1h 45^{o}C. DNA was extracted with the addition of 275 uL of phenol and 275 uL of chloroform. DNA was precipitated with 100% EtOH and 5 uL tRNA. DNA fragments were cloned in TA vector (pCR2.1 from Invitrogen) for sequencing.

siRNAs transfection

Sp1 gene in HEK293 or pCMVEC cells was knocked down by siRNA transfection. The sequence for Sp1 siRNA sense oligonucleotide is $^{5'}$GCCACACAACTTTCACAGGCCTGTCTC$^{3'}$, antisense sequence is $^{5'}$GGTCATTTCTTTGCTTATGCCTGTCTC$^{3'}$. Cells were seeded 1 day before transfection with siRNAs. On day 0, transfection was carried out with Trans-IT (MIRUS) following the company protocol. After 2 days, second transfections were performed. After another two days, cells were harvested for further experiments. Transfection efficiencies for siRNAs were determined using RT-PCR and Western Blotting with anti-Sp1 antibodies.

Luciferase activity Assay

HEK293 PAR-2 GFP cells were maintained in 10% FBS DMEM. Cells were transiently transfected with empty PGL3 vectors or PGL3 vectors containing DNA fragment MP112, pCDNA3 LacZ and Sp1 or scrambled siRNA using the polycationic detergent Lipofectamine (Invitrogen-Gibco, On, Canada). Each Lipofectamine-

transfected plate received 1 μg of the PGL3 plasmids and 1 μg pCDNA3 LacZ. Dual-luciferase reporter assay system (Promega) was used to monitor luciferase activity in HEKS293 PAR-2 GFP cells as per the manufacturer's recommendations, using a Sirius single tube luminometer (Berthold). PCDNA3 LacZ vector was co-transfected in all transfections described to monitor transfection efficiency. Luciferase experiments were performed in triplicate or quadruplicate, repeated three independent times, and the data presented are representative experiments. All luciferase results are reported as relative light units (RLU): the average of the *Photinus pyralis* firefly activity observed divided by the average of the activity recorded from beta-galactosidase activity. Results are shown using luciferase activities from cells transfected with empty vectors as controls.

Electrophoresis mobility shift assay (EMSA)

Nuclei from PAR-2 GFP stably transfected HEK293 cells were stimulated by 20nM trypsin or 20μM SLIGKV for 60 min. Crude nuclear proteins were dialyzed against DNaseI buffer [50 mM KCl, 4 mM $MgCl_2$, 20 mM K_3PO_4 (pH 7.4), 1 mM β-mercaptoethanol, 20% glycerol]. Extracts were kept frozen in small aliquots at -80°C until use. EMSAs were carried out by incubating 3×10^4cpm of 5' end-labeled (^{32}P-γATP) double-stranded oligonucleotides bearing the high affinity binding sites for the transcription factors Sp1 (5'-GATCATATCTGCGGGGCGGGGCAGACACAG-3') with 5μg nuclear proteins in the presence of 25 ng poly(dI-dC). poly(dI-dC) (Pharmacia-LKD) in buffer D (5 mM HEPES; 10% glycerol; 0,05 mM EDTA; 0,125 mM PMSF). When indicated, unlabeled double-stranded oligonucleotides bearing various DNA target sequences for known transcription factors (Sp1, NFI) were added as unlabeled competitors (100- and 500-fold molar excesses) during the assay. Supershift experiments in EMSA were conducted by adding antibodies (400 ng) directed against Sp1 and PAR-2 to the above reaction mixtures. Incubation proceeded at room temperature for 5 min upon which time DNA-protein complexes were separated by gel electrophoresis through a 8% (for Sp1 and PAR-2) native polyacrylamide gel run against Tris-glycine buffer. Gels were dried and autoradiographed at -80°C to reveal the position of the shifted DNA-protein complexes.

Chloramphenicol acetyl-transferase Assay

PRIM1 promoter inserted in into pUMSVO-CAT vector was constructed in a previous study [16]. pCMVEC cells were transiently transfected with PRIM1-CAT vector and pCDNA3 LacZ, and Sp1 or scrambled siRNA using Lipofectamine (Invitrogen-Gibco, ON, Canada). Each Lipofectamine-transfected plate received 1 μg of the PRIM1-CAT plasmid and 1 μg pCDNA3 LacZ. Levels of CAT activity for all transfected cells were determined by liquid scintillation counting of CAT reaction products and normalized to the activity of beta-galactosidase. Each single value was expressed as 100 × (% CAT in 4 h)/100 μg protein/beta-gal.

Coverslip migration assay

Microscope cover glasses (12cir from Fisher Scientific) were sterilized by flaming and put on the bottom of each well of a 24-well culture dish. pCMVEC cells were seeded in each well at the density of 10^5 cells per well and incubated overnight in 0.5mL of DMEM complete medium. Cells were transfected with Sp1 or scrambled siRNA as indicated for 48h. Cells were then synchronized by incubating with DMEM starving medium for 4h. To discriminate between cell proliferation and migration, mitomycin C was supplied to the cells at 5μg/mL and incubated at 37^{o}C for 30 min. Then, coverslips were picked up carefully, rinsed with culture medium and placed in a 12-well culture dish, which provided sufficiently large space to allow cell migration outside of the coverslips. Cells were maintained in 2mL 2% FBS DMEM medium containing 20μM SLIGKV. pCMVECs were allowed to migrate outward from the coverslip. After 72 h incubation the original cell-covered coverslips were removed. Cells remaining in the culture dish were migrated cells. The cell rate migration was estimated by using the spectrophotometric yellow tetrazolium salt (MTT) assay and by reading samples in a spectrophotometer at an optical density of 545 nm with a reference wavelength of 690 nm as previously reported [14].

Cellular proliferation assay by [^{3}H]thymidine incorporation

pCMVEC cells were seeded at 4 x 10^4 cells per well in a 24-well plate with complete DMEM medium overnight. Cells were then transfected with scrambled or Sp1 siRNA. 48 hours later, cells were synchronized with serum-free DMEM medium for 4 hours. Cells were stimulated with 20μM SLIGKV for 6 hours and incubated with

[^{3}H]thymidine 1μCi/mL for 18 hours. Cells were then washed twiced with PBS to remove unincorporated [^{3}H]thymidine, and DNA was precipitated with lysis solution (0.1N NaOH, 0.1% Triton X-100) at 37°C for 20 min. Lysed cells were transferred into a scintillation flask and analyzed by a liquid scintillation counter. The original data are expressed as CPM [^{3}H]thymidine incorporation (mean ± SD of 3-4 different experiments).

RESULTS

PAR-2 functionality in Hela cell nuclei

In our previous studies, we observed localization of PAR-2 at the plasma membrane and also at the nuclear/perinuclear region in various cell types. Indeed, Hela and BOSC23 cells were observed to express a great amount of endogenous PAR-2 at the nucleus. We demonsrated that nuclear localization of PAR-2 results from a translocation of the receptor from the cell surface (Nim S.,2009, ready for submission). Herein, we confirmed the nuclear distribution of endogenously expressed PAR-2 in Hela cells by confocal immunofluorescence. Pre-starved Hela cells were stained with anti-PAR-2 monoclonal antibodies (Fig. 1A up panel). The staining corresponding to PAR-2 is distributed clearly not only at plasma membrane but also in the nuclear/perinuclear region. PAR-2 at the nucleus colocalized with lamin B receptor (LBR), which is a specific maker protein of the nuclear membrane. Furthermore, Hela cells were transfected with PAR-2 containing a GFP sequence at its C-terminus and fluorescence staining at the nucleus was also observed (Fig. 1A-2nd panel). In addition, Hela cells were transfected with another construct of PAR-2, which contained a Flag sequence in its N-terminus and a HA sequence at its C-terminus. By using anti-Flag and anti-HA antibodies, we confirmed that Flag-PAR2-HA is also expressed at the nucleus and colocalizes with LBR (Fig. 1A-3rd and -4th panels).

PAR-2 at the cell surface has been shown to be able to trigger calcium release through the activation of phospholipase C (PLC) and the formation of inositol 1,4,5-triphosphate (IP_3) [9]. Whether the nuclear PAR-2 is able to mobilize intracellular calcium following its activation has yet to be determined. Nuclear PAR-2 functionality was assessed in our study by analyzing its ability to induce mobilization of intracellular calcium and to regulate gene expression. We isolated Hela cells nuclei by using a teflon potter homogenizer. This technique was performed in our previous studies and its efficiency was proven by multiple techniques including electron microscopy, western blot, and confocal microscopy [33, 36]. Interestingly, PAR-2 specific agonist peptide, SLIGKV, induced calcium release from isolated Hela cells nuclei (Fig. 1B). The minimal response was obtained by treating the nuclei fraction with EGTA and the maximal response was induced by treating the nuclei fraction with ionomycin. The increase of the

SLIGKV-induced calcium mobility can reach up to 25% of the maximal response induced by ionomycin. This result indicated that nuclear PAR-2 is functional and is able to induce calcium release following its activation.

In addition, nuclear PAR-2 functionality was also analyzed by examining its ability to induce gene expression. Our previous studies have shown that PAR-2 activation leads to the expression of several proangiogenic and proinflammatory genes [14]. Here we showed that TNFα, an important inflammatory component, was induced by nuclear PAR-2 activation (Fig. 1C). TNFα expression was analyzed by time course RT-PCR after stimulation of isolated nuclei with 20μM SLIGKV. After 15 min stimulation, TNFα expression was significantly increased. TNFα expression level reached to a maximum at 30 min and then decreased slightly at 60 min. At same time, angiopoietin receptor tie2 expression was not significantly changed following stimulation of nuclear PAR-2 although we have demonstrated that tie2 expression is upregulated after PAR-2 stimulation in intact cells [14].

Identification of nuclear PAR-2 interacting proteins by MS/MS analysis

To investigate the mechanism of nuclear PAR-2 activity, we aimed to identify nuclear PAR-2 interacting proteins by performing mass spectrometry analysis on isolated nuclei from BOSC23 cells. We observed in our previous studies that Hela and BOSC23 cells express a high amount of endogenous PAR-2 at the nucleus. BOSC23 cells offer the advantage of having big nuclei, thus giving us a high quantity of proteins in the nuclear fraction. PAR-2 was immunoprecipitated by mouse monoclonal anti-PAR-2 antibodies (SAM11) from isolated BOSC23 nuclear fraction treated with SLIGKV. Meanwhile, mouse anti-IgG_{2B} antibodies were employed as negative control (Fig.2A). Samples from PAR-2 and IgG_{2B} immunoprecipitation were then separated on a SDS-PAGE gel and sliced into 12 pieces for MS/MS analysis (Fig. 2B). Totally, 2018 digested peptides were obtained. After analysis by MS/MS, these peptides were associated to 24 different proteins. By eliminating proteins found to be present in both samples from PAR2- and IgG_{2B}-immunoprecipitation, 17 proteins were exclusively found in PAR2-immunoprecipitant. These proteins were defined as candidates for nuclear PAR-2 interacting proteins and are listed in Table 1. Notably, two of the nuclear PAR2-

interacting proteins are cofactors required for Sp1 transcriptional ativation (CRSP6 and CRSP3) (table 1).

Nuclear PAR-2 binds to DNA containing promoter elements regulated by Sp1

To explore whether nuclear PAR-2 can regulated gene expression through the binding and/or the activation of transcription factors bound to promoter elements, we performed chromatin immunoprecipitation assay (ChIP). DNA fragments from BOSC23 cells stimulated with SLIGKV and crosslinked with paraformaldehyde were immunoprecipitated by monoclonal anti-PAR-2 antibodies (SAM11), and cloned into TA pCR2.1 vector for sequencing analysis. We selected 12 clones for sequencing in order to identify putative binding sites for transcription factors. One clone was named MP112 and used for further experiments. For most of the sequences from the DNA fragments obtained by ChIP, we found several putative binding sites for transcription factors including Sp1, Oct1, NF-E and GATA-1 binding sites. Different DNA fragments including MP112 were cloned into pGL3 vectors and the presence of promoter or enhancer elements was further analyzed by luciferase reporter gene assay using HEK293 cells stably expressing PAR-2 GFP (Fig.3A). HEK293 cells offer the advantage of efficiently expressing transfected components. pGL3-Enhancer vector contains an SV40 enhancer downstream of *luc+* and the poly(A) signal and aids in the verification of the presence of functional promoter elements. pGL3-Promoter vector contains an SV40 promoter upstream of the luciferase gene and is used to identify the presence of enhancer elements. pGL3-Basic vector lacks promoter and enhancer sequences. Interestingly, PAR-2 stimulation with SLIGKV or trypsin led to the highest increase in luciferase activity in HEK293 cells transfected with MP112/pGL3-Enhancer vector in comparison to cells transfected with MP112/pGL3-Basic and MP112/pGL3-Promoter vectors. This suggests that MP112 DNA fragment contains functional promoter elements. In addition, MP112 does not contain putative enhancer elements since the transcriptional activity of MP112/pGL3-Promoter is not stronger than that of empty pGL3-Promoter vector. Because PAR-2 is implicated in angiogenic processes and Sp1 is known to regulate the expression of angiogenic genes like VEGF, we sought to find if Sp1 could regulate the transcriptional activity induced by nuclear PAR-2 activation. In addition, based on our MS/MS analysis, two cofactors required for Sp1 transcriptional activity were found to

interact with nuclear PAR-2 (CRSP3 and CRSP6) (Table 1). MP112 DNA fragment, which was bound to nuclear PAR-2, also contains putative binding sites for Sp1 transcription factor. Overall, these experiments led to the suggestion that Sp1 could be implicated in nuclear PAR-2-induced transcriptional activity. Therefore, we silenced Sp1 gene expression using Sp1 siRNA in cells transfected with MP112/pGL3-Enhancer vector to see whether the absence of Sp1 could prevent PAR-2-induced transcriptional activity. The efficiency of Sp1 siRNA was tested by analysing Sp1 protein expression by Western blot. Sp1 protein expression was significantly decreased in Sp1 siRNA-transfected HEK293 cells compared to untransfected cells or scrambled siRNA-transfected cells (Supplemental fig.1A). Interestingly, Sp1 knock-down abolished PAR-2-induced transcriptional activity in MP112/pGL3-Enhancer transfected HEK293 cells in comparison to the untransfected or scrambled siRNA-transfected cells (Fig.3B). These results indicated that nuclear PAR-2-induced transcriptional activity in MP112/pGL3-Enhancer transfected cells is dependent on Sp1 transcriptional activity.

Nuclear PAR-2 interacts with Sp1

Because Sp1 is important for the regulation of transcriptional activity induced by PAR-2 activation, we suggested that PAR-2 could interact and/or activate Sp1. HEK293 cells stably expressing PAR-2 GFP was stimulated with SLIGKV in order to induce the translocation of the receptor to the nucleus and to increase the amount of the receptor at the nucleus for better visualization of the receptor. The interaction between Sp1 and nuclear PAR-2 was then verified by confocal microscopy using HEK293 PAR-2 GFP cells and by co-immunoprecipitation using native HEK293 cells. PAR-2 GFP is found to colocalize with Sp1 at the nucleus suggesting an interaction between the two proteins (Fig. 4A). The colocalization was also analyzed by performing line scan analysis. Additionally, PAR-2 and Sp1 interaction was confirmed as well by co-immunoprecipitation on isolate nuclei following PAR-2 stimulation (Fig. 4B). Neither PAR-2 nor Sp1 were found in immunoprecipitated samples with IgG_{2B} antibodies.

Activation of nuclear PAR-2 enhances binding activity of Sp1 to Sp1 consensus sequence

It is known that activation of Sp1 triggers the binding of Sp1 to different gene promoters including promoter of Sp1 gene and regulates gene transcription. We

performed EMSA experiments in order to show if activation of nuclear PAR-2 will induce the binding of Sp1 to its consensus sequence (Figure 4Ci). Nuclei from HEK293 PAR-2 GFP cells were isolated and stimulated with 20μM SLIGKV or 20nM trypsin. Crude nuclear proteins were incubated with 5'-end labeled, double-stranded oligonucleotides bearing the high affinity-binding site for either Sp1. DNA-protein complexes were resolved on native polyacrylamide gels and their position revealed by autoradiography. As shown on Figure 4Ci, Sp1 labeled probe yielded the appropriate DNA-protein complexes corresponding to the binding of Sp1 factor to their respective target sequence when incubated with the nuclear extracts from HEK293 PAR-2 GFP cells. The amount of this complex is increased by about 2 fold when nuclei were stimulated with SLIGKV or trypsin resulting in the activation of nuclear PAR-2. Formation of the Sp1 complex observed with the extracts from both stimulated or non-stimulated nuclei was found to be specific as it was entirely competed off by a 100-fold molar excess of the unlabeled Sp1 oligomer, but not at all by the unrelated NFI site (Figure 4Cii). The identity of the proteins that yielded the shifted complexes using the Sp1 labeled probe was further investigated by supershift analyses in EMSAs using antibodies directed against Sp1 and PAR-2 (Figure 4Ciii). The addition of anti-Sp1 antibodies was able to supershift the band corresponding to the complex of Sp1 transcription factor and Sp1 probes confirming the presence and binding of Sp1 transcription factor on the Sp1 consensus sequence. Surprisingly, we were not able to supershift the complex with PAR-2 antibodies even though we have shown that nuclear PAR-2 interacts with Sp1 following the activation of PAR-2.

Sp1 gene knock-down inhibits trypsin- or SLIGKV-induced VEGF and PRIM1gene expression, cell migration, and cell proliferation in pCMVECs

Previous results in this study indicated that nuclear PAR-2 interacts with Sp1 (Fig. 4A and B) and its activation promotes Sp1 transcriptional activity. Moreover, activated PAR-2 has been shown in our previous studies to translocate to the cell nucleus, to induce VEGF expression and to promote angiogenesis (Nim S., 2009, ready for submission). Therefore, we analysed the role of Sp1 in PAR-2 induced expression of proangiogenic genes and regulation of cell proliferation and migration in pCMVEC cells. pCMVEC cells are endothelial cells and are a good model for the analysis of cell migration and

proliferation. Activation of PAR-2 with 20ηM trypsin in pCMVEC cells increased the expression of VEGF and tie2 (Fig. 5A). Silencing Sp1 gene expression by transfecting cells with Sp1 siRNA abolished trypsin-induced VEGF expression but not tie2 expression. DNA primase 1 (PRIM1) is known as a nuclear DNA replication enzyme and was reported by us to be up-regulated by PAR-2 activation [16]. In order to determine if the up-regulation of PRIM1 gene expression following PAR-2 activation is dependent on Sp1, we used chloramphenicol acetyltransferase as a reporter gene to analyse the expression of PRIM1 in pCMVEC cells deprived of Sp1. Similarly to VEGF expression, activation of PAR-2 resulted in more than 2-fold increase of PRIM1-CAT and this increase is significantly reduced with the transfection of cells with Sp1 siRNA (Fig. 5B). To study the implication of Sp1 in the regulation of cell proliferation and migration induced by PAR-2 activation, we transfected pCMVEC cells with Sp1 siRNA and analysed their ability to migrate and to incorporate [^{3}H]thymidine. SLIGKV significantly increased pCMVEC migration rate, but this increase was completely diminished by Sp1 siRNA transfection as compared to the scrambled siRNA transfected group (Fig. 5C). The cellular proliferation was studied by analyzing [^{3}H]thymidine incorporation in pCMVEC cells (Fig.5D). Sp1 silencing significantly prevented SLIGKV-induced cellular proliferation in pCMVEC cells.

DISCUSSION

We have shown in our previous studies that activation of PAR-2 leads to the translocation of the receptor to the nucleus and this process is important for the induction of gene expression implicated in inflammatory and angiogenic processes (Nim S., 2009, ready for submission). Indeed, stimulation of PAR-2 increases the expression of tie-2, PRIM1 and VEGF, which are important factors in angiogenesis [16]. A number of transcription factors are known to be regulated by GPCRs and are responsible for transcription of gene implicated in numerous physiological processes. Sp1 transcription factor has been reported to regulate the expression of IL-1, COX-2 and VEGF; all of them are regulated by PAR-2 activity [37-39]. Since PAR-2 is endogenously expressed at the nucleus and this nuclear localization is suggested to origin from the translocation of the receptor from the cell surface, we analyzed the functionality of PAR-2 at the nucleus and its role in the regulation of gene expression. We proposed that activation of the receptor at the nucleus allows the receptor to bind and/or to activate transcription factors, and to regulate gene expression. Results by confocal microscopy confirmed that PAR-2 was endogenously expressed at the nucleus (Fig. 1A). In addition, the receptor at the nucleus demonstrated functionality since it was able to induce calcium release and to increase TNFα gene expression following its stimulation from isolated nuclei (Fig. 1B and C). The molecular mechanism of nuclear PAR-2 activity was investigated by MS/MS and ChIP. Sp1 co-factor proteins, CRSP3 and CRSP6, were found interacting with nuclear PAR-2 by mass spectrometry. Our ChIP experiments revealed that nuclear PAR-2 is found in a complex with cell chromatin after its activation. Analysis of sequences of the DNA fragments found to bind PAR-2 revealed numerous putative binding sites for transcription factors, notably Sp1, GATA1 and Oct1. We also sought to identify if these DNA fragments bound to activated nuclear PAR-2 contain promoter or enhancer elements. We proceeded by cloning these DNA fragments including MP112 into different PGL3 vectors and analysed the transcriptional activity (luciferase) induced by PAR-2 activation. Luciferase assays indicated that the DNA fragments bound to activated nuclear PAR-2 contain promoter elements as shown for MP112. Because sequencing analysis revealed that the different DNA fragments bound to nuclear PAR-2 contain putative Sp1 binding sites, we looked to find if Sp1 is implicated in the regulation of gene expression induced

by PAR-2 activation. Knock-down of Sp1 by transfection of cells with Sp1 siRNA inhibited the transcriptional activity of MP112-PGL3-Enhancer induced by PAR-2 activation compared to cells transfected with scrambled siRNA. These results clearly indicated that Sp1 is implicated in the regulation of gene expression induced by PAR-2 activation. It is therefore possible to imagine that PAR-2 is found in a complex with different proteins and transcription factors on cell chromatin possibly on promoter regions of different genes following the activation of the receptor at the nucleus or the translocation of the receptor to the nucleus. Indeed, we demonstrated that PAR-2 interacts with Sp1 at the nucleus following its activation. The stimulation of nuclear PAR-2 also increases Sp1 binding activity to Sp1 consensus sequence as revealed by EMSA experiments. Supershift assays in EMSA experiments did not support the presence of nuclear PAR-2 on Sp1 consensus sequence although the receptor was demonstrated to bind Sp1 possibly on gene promoters. It is therefore possible that PAR-2 interacts with Sp1 in a complex with other transcription factors on non-conventional Sp1 consensus sequences or simply on different promoter element sites. Sp1 has been shown to interact with numerous other transcription factors like Ets1, c-myc, c-Jun, Stat1, and Egr-1 and/or components of the basal transcriptional machinery [40-44]. Sp1 has also been linked to chromatin remodelling through interactions with chromatin-modifying factors such as p300 and histone deacetylases [45, 46]. Sp1 has been found to regulate multiple genes encoding angiogenic molecules, including VEGF, fibroblast growth factors (FGFs), epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGF-IR), and TNFα [28-32]. High level of Sp1 has been shown to correlate with high expression of VEGF protein and mRNA levels and has been linked to interactions of Sp1 with GC-rich motifs (-109 to -61) in the proximal region of the *VEGF* gene promoter [38]. Results from this study illustrated that PAR-2 activation by trypsin or SLIGKV triggered proangiogenic processes dependent on Sp1 in pCMVEC cells, such as: VEGF and PRIM1 gene expression, cellular migration and proliferation (Fig. 5).

In summary, we confirmed that PAR-2 is expressed endogenously at the nucleus and its functionality is demonstrated by its ability to induce TNFα gene expression and calcium release. Moreover, data illustrated that Sp1 proteins play an important role in nuclear PAR-2 regulation of proangiogenic processes as demonstrated using reporter

gene, RNA interference assays, cell migration and [^{3}H]thymidine incorporation assay. The activation of nuclear PAR-2 or its translocation to the nucleus is required for the increased activity of Sp1. The expression of VEGF and PRIM1 are regulated by Sp1 transcriptional activity. The role of Sp1 in nuclear PAR-2 regulation of angiogenesis can be extended and results could bring new concepts for the understanding of the regulation of gene expression by members of the GPCRs family. Future studies of the PAR-2/Sp1 pathway have a large potential for defining the machinery that not only regulates physiological processes but may also modulates human diseases.

Acknowledgements

This work is supported by grants from the Canadian Institutes of Health Research (CIHR). S. Nim is recipient of funding awards from the CIHR. S. Chemtob is a recipient of a Canada Research Chair.

REFERENCES

1. Gamble, J.R., et al., Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. Circ Res, 2000. **87**(7): p. 603-7.
2. Vu, T.K., et al., Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell, 1991. **64**(6): p. 1057-68.
3. Milia, A.F., et al., Protease-activated receptor-2 stimulates angiogenesis and accelerates hemodynamic recovery in a mouse model of hindlimb ischemia. Circ Res, 2002. **91**(4): p. 346-52.
4. Alm, A.K., et al., Extrapancreatic trypsin-2 cleaves proteinase-activated receptor-2. Biochem Biophys Res Commun, 2000. **275**(1): p. 77-83.
5. Koivunen, E., et al., Tumor-associated trypsin participates in cancer cell-mediated degradation of extracellular matrix. Cancer Res, 1991. **51**(8): p. 2107-12.
6. Wiegand, U., et al., Cloning of the cDNA encoding human brain trypsinogen and characterization of its product. Gene, 1993. **136**(1-2): p. 167-75.
7. Berger, P., et al., Selected contribution: tryptase-induced PAR-2-mediated Ca(2+) signaling in human airway smooth muscle cells. J Appl Physiol, 2001. **91**(2): p. 995-1003.
8. Okamoto, T., et al., The effects of stimulating protease-activated receptor-1 and -2 in A172 human glioblastoma. J Neural Transm, 2001. **108**(2): p. 125-40.
9. Kanke, T., et al., Proteinase-activated receptor-2-mediated activation of stress-activated protein kinases and inhibitory kappa B kinases in NCTC 2544 keratinocytes. J Biol Chem, 2001. **276**(34): p. 31657-66.
10. DeFea, K.A., et al., beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. J Cell Biol, 2000. **148**(6): p. 1267-81.
11. Darmoul, D., et al., Protease-activated receptor 2 in colon cancer: trypsin-induced MAPK phosphorylation and cell proliferation are mediated by epidermal growth factor receptor transactivation. J Biol Chem, 2004. **279**(20): p. 20927-34.

12. Morris, D.R., et al., Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells. Cancer Res, 2006. **66**(1): p. 307-14.
13. Shi, X., et al., Protease-activated receptors (PAR1 and PAR2) contribute to tumor cell motility and metastasis. Mol Cancer Res, 2004. **2**(7): p. 395-402.
14. Zhu, T., et al., Proangiogenic effects of protease-activated receptor 2 are tumor necrosis factor-alpha and consecutively Tie2 dependent. Arterioscler Thromb Vasc Biol, 2006. **26**(4): p. 744-50.
15. Hirota, Y., et al., Activation of protease-activated receptor 2 stimulates proliferation and interleukin (IL)-6 and IL-8 secretion of endometriotic stromal cells. Hum. Reprod., 2005. **20**(12): p. 3547-3553.
16. Fan, L., et al., Tissue factor enhances protease-activated receptor-2-mediated factor VIIa cell proliferative properties. J Thromb Haemost, 2005. **3**(5): p. 1056-63.
17. Frungieri, M.B., et al., Proliferative action of mast-cell tryptase is mediated by PAR2, COX2, prostaglandins, and PPARgamma : Possible relevance to human fibrotic disorders. Proc Natl Acad Sci U S A, 2002. **99**(23): p. 15072-7.
18. Nystedt, S., V. Ramakrishnan, and J. Sundelin, The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor. J Biol Chem, 1996. **271**(25): p. 14910-5.
19. Suske, G., The Sp-family of transcription factors. Gene, 1999. **238**(2): p. 291-300.
20. Philipsen, S. and G. Suske, A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res, 1999. **27**(15): p. 2991-3000.
21. Mastrangelo, I.A., et al., DNA looping and Sp1 multimer links: a mechanism for transcriptional synergism and enhancement. Proc Natl Acad Sci U S A, 1991. **88**(13): p. 5670-4.
22. Su, W., et al., DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1. Genes Dev, 1991. **5**(5): p. 820-6.
23. Kadonaga, J.T., et al., Distinct regions of Sp1 modulate DNA binding and transcriptional activation. Science, 1988. **242**(4885): p. 1566-70.

24. Pascal, E. and R. Tjian, Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev, 1991. **5**(9): p. 1646-56.
25. Courey, A.J., et al., Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell, 1989. **59**(5): p. 827-36.
26. Shou, Y., S. Baron, and M. Poncz, An Sp1-binding silencer element is a critical negative regulator of the megakaryocyte-specific alphaIIb gene. J Biol Chem, 1998. **273**(10): p. 5716-26.
27. Barth, N., et al., Identification of regulatory elements in the human adipose most abundant gene transcript-1 (apM-1) promoter: role of SP1/SP3 and TNF-alpha as regulatory pathways. Diabetologia, 2002. **45**(10): p. 1425-33.
28. Prasanna Kumar, S., et al., Butyrate-induced phosphatase regulates VEGF and angiogenesis via Sp1. Arch Biochem Biophys, 2008. **478**(1): p. 85-95.
29. Yuan, P., et al., Therapeutic inhibition of Sp1 expression in growing tumors by mithramycin a correlates directly with potent antiangiogenic effects on human pancreatic cancer. Cancer, 2007. **110**(12): p. 2682-90.
30. Santra, M., et al., Ectopic decorin expression up-regulates VEGF expression in mouse cerebral endothelial cells via activation of the transcription factors Sp1, HIF1alpha, and Stat3. J Neurochem, 2008. **105**(2): p. 324-37.
31. Novak, E.M., et al., Downregulation of TNF-alpha and VEGF expression by Sp1 decoy oligodeoxynucleotides in mouse melanoma tumor. Gene Ther, 2003. **10**(23): p. 1992-7.
32. Xie, K., D. Wei, and S. Huang, Transcriptional anti-angiogenesis therapy of human pancreatic cancer. Cytokine Growth Factor Rev, 2006. **17**(3): p. 147-56.
33. Gobeil, F., Jr., et al., Modulation of pro-inflammatory gene expression by nuclear lysophosphatidic acid receptor type-1. J Biol Chem, 2003. **278**(40): p. 38875-83.
34. Dery, O., et al., Trafficking of proteinase-activated receptor-2 and beta-arrestin-1 tagged with green fluorescent protein. beta-Arrestin-dependent endocytosis of a proteinase receptor. J Biol Chem, 1999. **274**(26): p. 18524-35.
35. Bohm, S.K., et al., Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. J Biol Chem, 1996. **271**(36): p. 22003-16.

36. Marrache, A.M., et al., Proinflammatory gene induction by platelet-activating factor mediated via its cognate nuclear receptor. J Immunol, 2002. **169**(11): p. 6474-81.
37. Xu, Q., Y.S. Ji, and J.F. Schmedtje, Jr., Sp1 increases expression of cyclooxygenase-2 in hypoxic vascular endothelium. Implications for the mechanisms of aortic aneurysm and heart failure. J Biol Chem, 2000. **275**(32): p. 24583-9.
38. Abdelrahim, M., et al., Role of Sp proteins in regulation of vascular endothelial growth factor expression and proliferation of pancreatic cancer cells. Cancer Res, 2004. **64**(18): p. 6740-9.
39. McDowell, T.L., J.A. Symons, and G.W. Duff, Human interleukin-1 alpha gene expression is regulated by Sp1 and a transcriptional repressor. Cytokine, 2005. **30**(4): p. 141-53.
40. Canaff, L., X. Zhou, and G.N. Hendy, The proinflammatory cytokine, interleukin-6, up-regulates calcium-sensing receptor gene transcription via Stat1/3 and Sp1/3. J Biol Chem, 2008. **283**(20): p. 13586-600.
41. Khachigian, L.M., et al., Egr-1-induced endothelial gene expression: a common theme in vascular injury. Science, 1996. **271**(5254): p. 1427-31.
42. McDonough, P.M., et al., Collaborative roles for c-Jun N-terminal kinase, c-Jun, serum response factor, and Sp1 in calcium-regulated myocardial gene expression. J Biol Chem, 1997. **272**(38): p. 24046-53.
43. Parisi, F., P. Wirapati, and F. Naef, Identifying synergistic regulation involving c-Myc and sp1 in human tissues. Nucleic Acids Res, 2007. **35**(4): p. 1098-107.
44. Rosmarin, A.G., et al., Sp1 cooperates with the ets transcription factor, GABP, to activate the CD18 (beta2 leukocyte integrin) promoter. J Biol Chem, 1998. **273**(21): p. 13097-103.
45. Suzuki, T., et al., Regulation of interaction of the acetyltransferase region of p300 and the DNA-binding domain of Sp1 on and through DNA binding. Genes Cells, 2000. **5**(1): p. 29-41.
46. Zhao, S., et al., Requirement of a specific Sp1 site for histone deacetylase-mediated repression of transforming growth factor beta Type II receptor

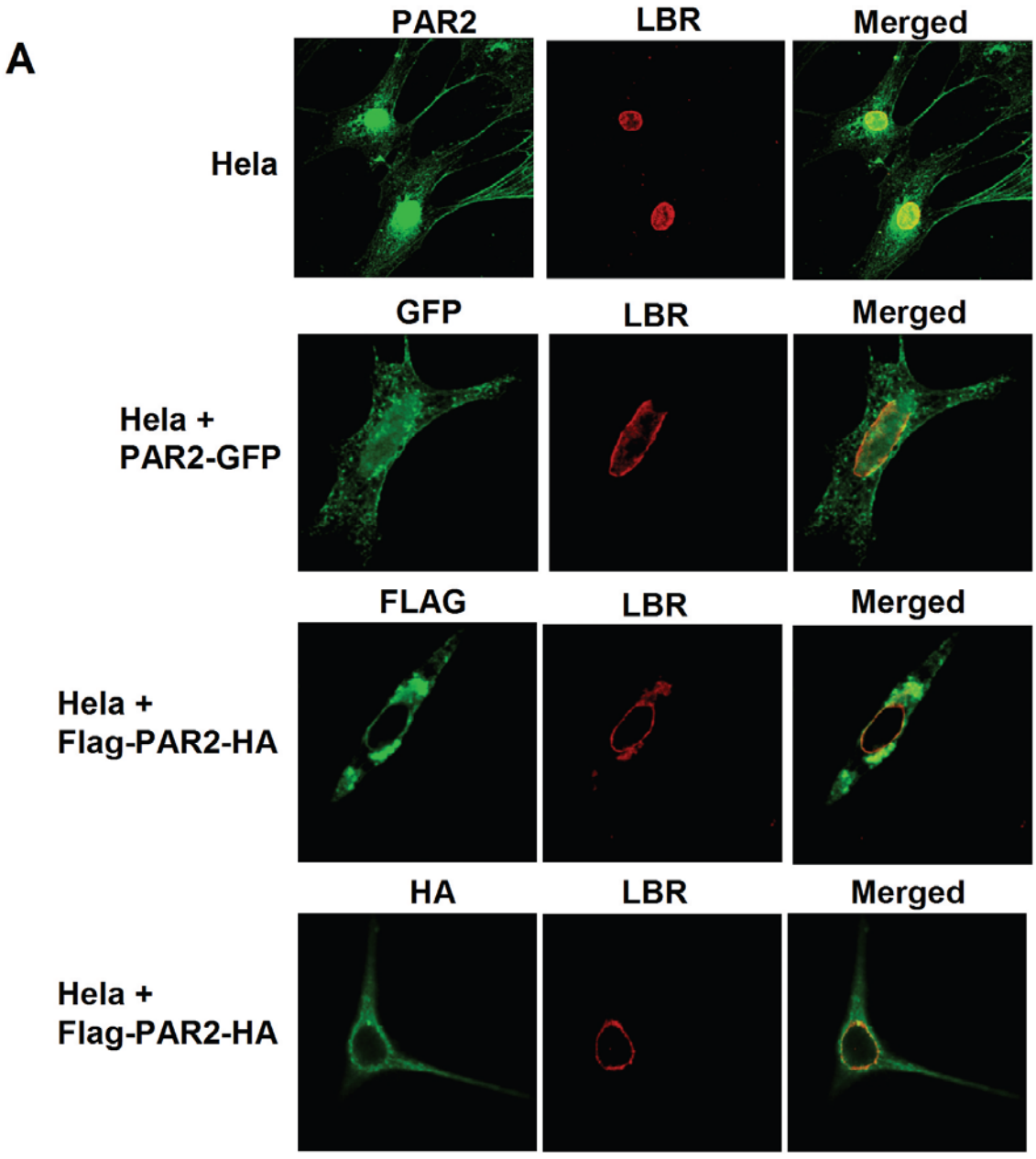
expression in human pancreatic cancer cells. Cancer Res, 2003. **63**(10): p. 2624-30.

47. Hunyady, L., et al., Agonist-induced endocytosis and signal generation in adrenal glomerulosa cells. A potential mechanism for receptor-operated calcium entry. J Biol Chem, 1991. **266**(5): p. 2783-8.

FIGURES AND LEGENDS

Figure 1. Functionality of nuclear PAR-2.

(A) Confocal immunofluorescence stainning of PAR-2 in Hela cells transfected with or without Flag-PAR2-HA/pcDNA3 (3^{rd} and 4^{th} panels) as indicated and PAR-2 was labeled with mouse monoclonal PAR-2 (SAM11), Flag, or HA antibodies. PAR-2 GFP was directly visualized in PAR-2 GFP/pEGFP-N1 transfected Hela cells (2^{nd} panel). Nuclei were stained with rabbit polyclonal anti-lamin B receptor (LBR) antibodies. The overlay colors corresponding to PAR-2 (green) and LBR (red) was revealed in merged pictures and indicate nuclear localization of PAR-2 (**B**) Calcium release assay in Hela nuclei. Isolated Hela cells nuclei were put in 96-well dish, loaded with Fluo-4NW dye mix and stimulated with 10μM ionomycin, 20mM EGTA+20μM SLIGKV or 20μM SKIGKV alone. Black arrow indicates the start of stimulation. The released calcium was recorded (for 80 sec total) by a fluorescence reader at 531nm (emission) and 480nm (excitation). Open circle, open square, and closed circle symbols corresponds to ionomycin, EGTA+SLIGKV, and SLIGKV stimulated groups, respectively. Each point was calculated upon 4-8 assays. (**C)** RT-PCR analysis. Isolated Hela nuclear fractions were stimulated by SLIGKV for indicated times (0, 15, 30, 60min). TNFα and tie2 gene expression were analyzed by RT-PCR. 18S was used as control gene reference. Left panel represent PCR blots, average data were put in right panel and were calculated with 3-5 independent assays. *$p<0.05$.


A
PAR2
LBR
Merged
Hela
GFP
LBR
Merged
Hela +
PAR2-GFP
FLAG
LBR
Merged
Hela +
Flag-PAR2-HA
HA
LBR
Merged
Hela +
Flag-PAR2-HA

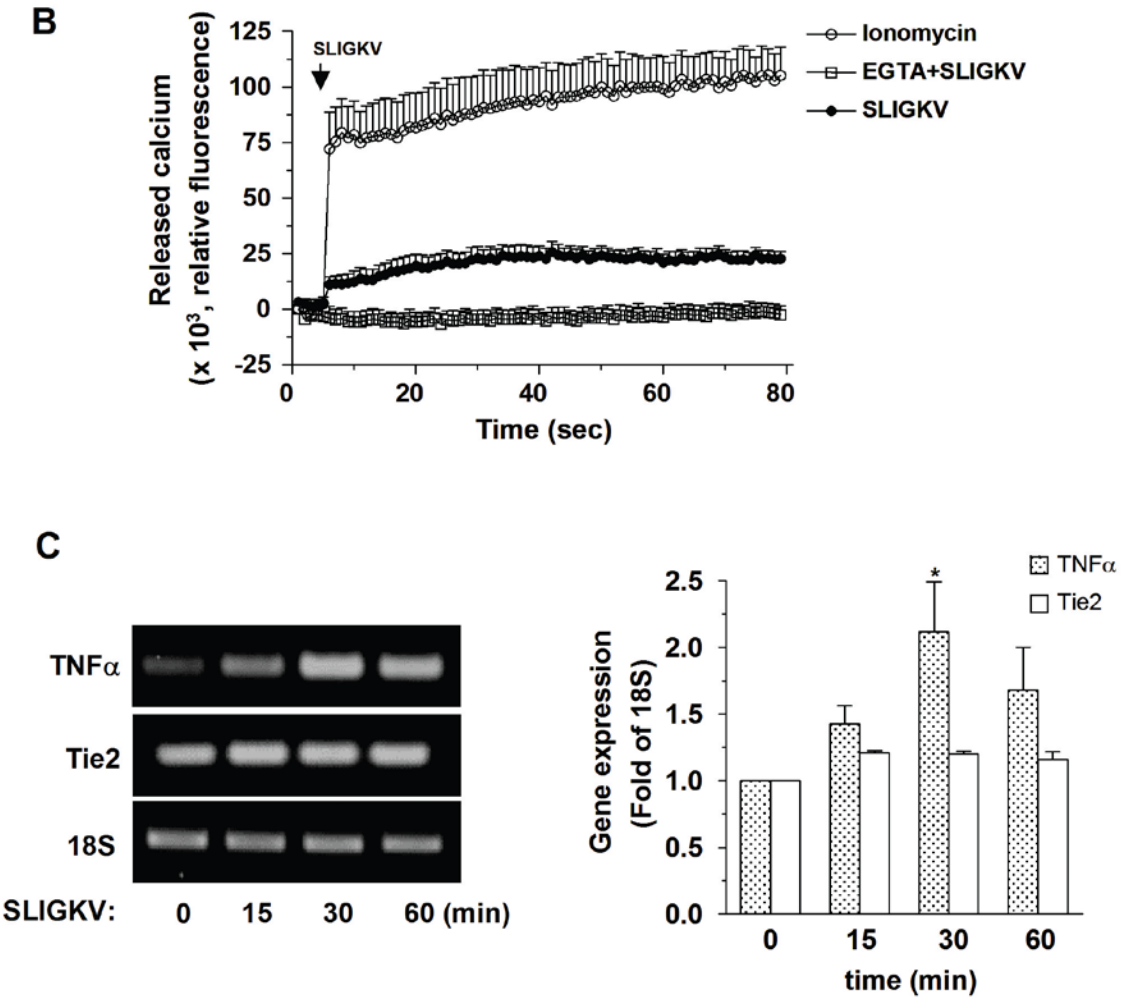

B
Released calcium
(x 10³, relative fluorescence)
SLIGKV
Ionomycin
EGTA+SLIGKV
SLIGKV
Time (sec)
C
TNFα
Tie2
18S
SLIGKV: 0 15 30 60 (min)
Gene expression
(Fold of 18S)
TNFα
Tie2
time (min)

Figure 2. Preparation of nuclear PAR-2 interacting proteins for MS/MS analysis. PAR-2 interacting proteins were precipitated by PAR-2 monoclonal antibodies and Protein-A Sepharose 4B from BOSC23 cells nuclear fraction. IgG_{2B} antibodies were used as well at same time as a negative control. (**A)** The specificity of the immunoprecipitant (IP) for PAR-2 was analyzed by Western blot with rabbit polyclonal anti-PAR-2 (H-99) antibodies. 20μg of whole cell lysate was loaded as positive control. 5μl of IgG_{2B} and PAR-2 antibodies were loaded for immunoprecipitation. PAR-2 specific bands are indicated by black arrow. Standard protein molecular weights are indicated on the right side. (**B)** PAR-2 immunoprecipitant was isolated on a 12% SDS-PAGE gel and stained with Coomassie blue. Number marked squares on the gel were sliced into pieces and sent for MS/MS analysis. Standard protein molecular weights are indicated on the left side.

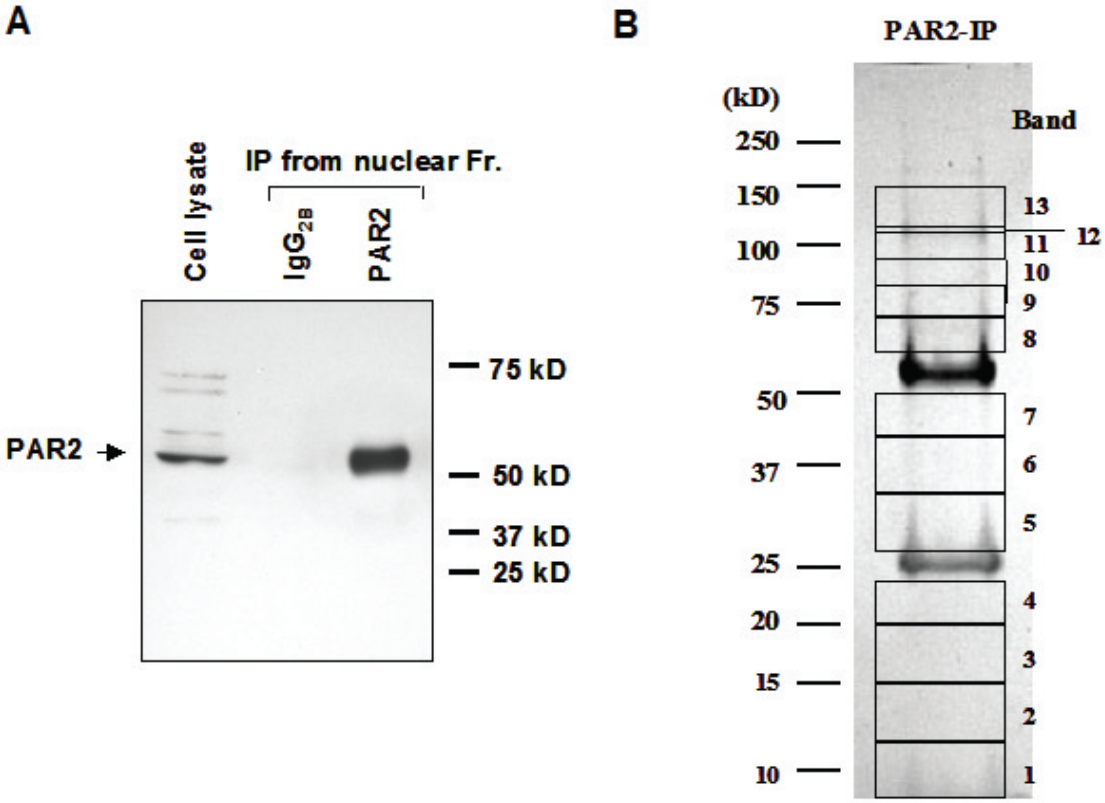

A
Cell lysate
IP from nuclear Fr.
IgG2B
PAR2
PAR2
75 kD
50 kD
37 kD
25 kD
B
PAR2-IP
(kD)
Band
250
150
100
75
50
37
25
20
15
10
13
11
12
10
9
8
7
6
5
4
3
2
1

Table 1: Proteins interacting with nuclear PAR-2 in BOSC23 cells by MS/MS.

Clathrin heavy chain 1
Heat shock 70kDa protein
Zinc finger and BTB domain containing 2
CRSP6 (Cofactor required for Sp1 transcriptional activation, subunit 6)
CRSP3 (Cofactor required for Sp1 transcriptional activation, subunit 3)
Thyroid hormone receptor-associated protein 4
Enthoprotin
TRF (TATA binding protein-related factor)-proximal protein
BMAL1e
MED25 protein (MED25 Mediator of RNA polymerase II transcription subunit 25 homolog)
Carboxypeptidase D (CPG)
USP6 N-terminal like protein
Mortalin-2
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3 variant
B lymphoid tyrosine kinase
Junction plakoglobin
Heterogeneous nuclear ribonucleoprotein H3 isoform a

Table 2: Putative transcription factor binding sites in DNA fragments obtained by ChIP.

CP1	fact	40 (-) CCAAT
GATA-1	fact	282 (-) nnnnTGATAnnnn
GR	fact	333 (-) CAGAG
NF-1	fact	199 (+) GCCA
NF-E	fact	302 (+) CTGTC
Oct1	fact	193 (+) TCTATGCCAA
Pit-1	fact	151 (-) TAAAT
Sp1	**fact**	**110 (+) TGCAC**
TBP	fact	194 (-) nnnnGTTTAGATTTCTTCGn

Figure 3. PAR-2-induced transcriptional activity is Sp1 dependent.

(A) BOSC23 cells were stimulated with 20μM SLIGKV for 60 minutes and were crosslinked using formaldehyde. DNA was fragmented by sonication. 250 ng of proteins were used for immunoprecipitation with 2 ng of PAR-2 antibody. DNA fragments (including MP112) were cloned in different PGL3 vectors and transfected in HEK293 PAR-2 GFP cells and analysed for the presence of promoter or enhancer elements using luciferase reporter gene assay. Results are shown for MP112 DNA fragment. The luciferase activities of empty vectors were used as controls and normalized to one-fold response. Expression of beta-galactosidase was used as transfection control. n=3, $^{**}p<0.01$ **(B)** HEK293 PAR-2 GFP cells were co-transfected with MP112/pGL3-Enhancer and Sp1 siRNA or scrambled siRNA. Cells transfection MP112/pGL3-Enhancer alone was used as controls (CTL). Luciferase activities were measured after 20μM SLIGKV or 20 nM trypsin stimulation. Activities of unstimulated cells (-) were normalized to one-fold response. Results of each group were calculated from three independent tests. $^{*}p<0.05$ and $^{**}p<0.01$, respectively.

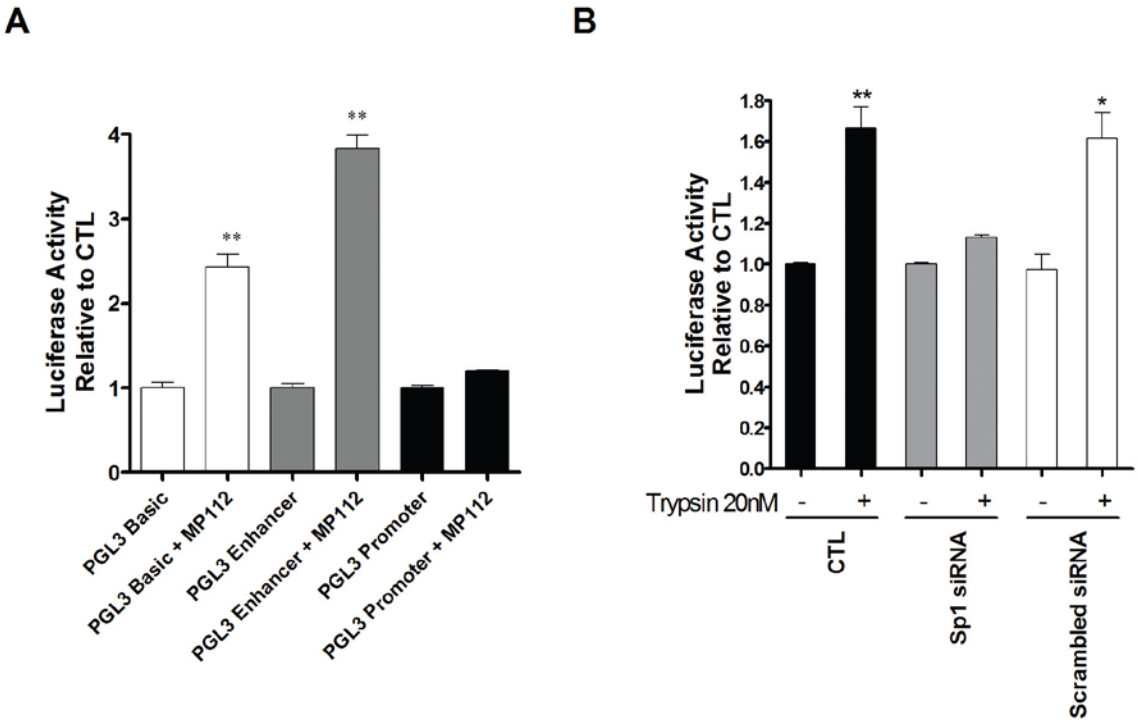
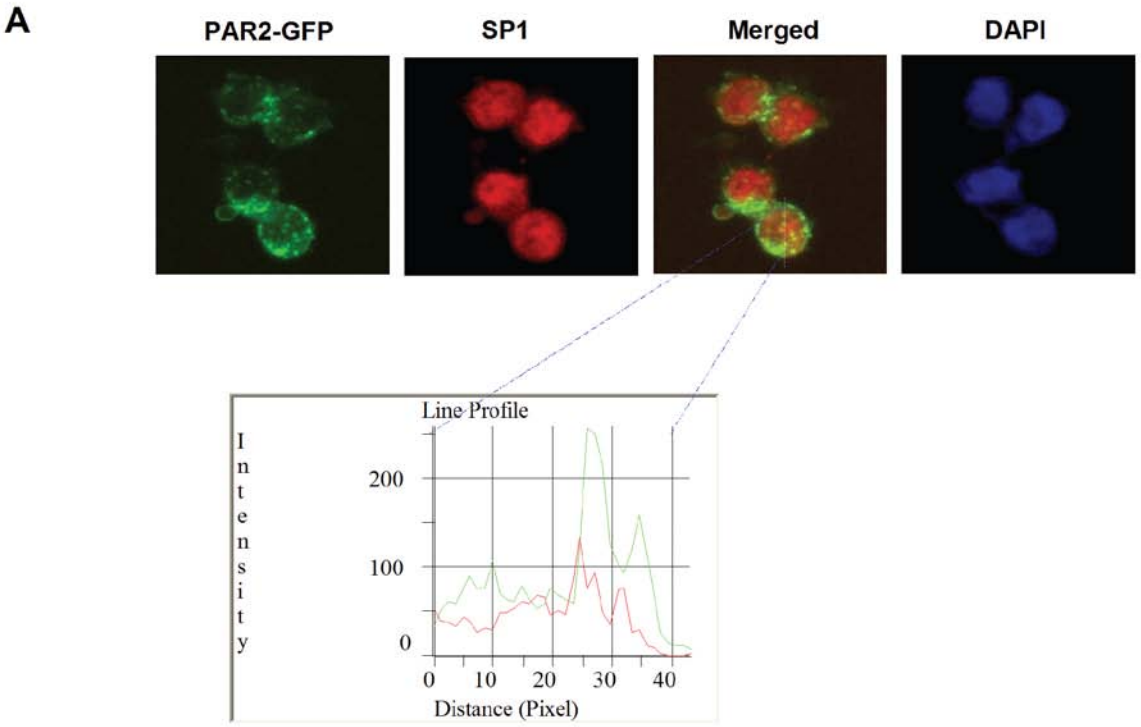
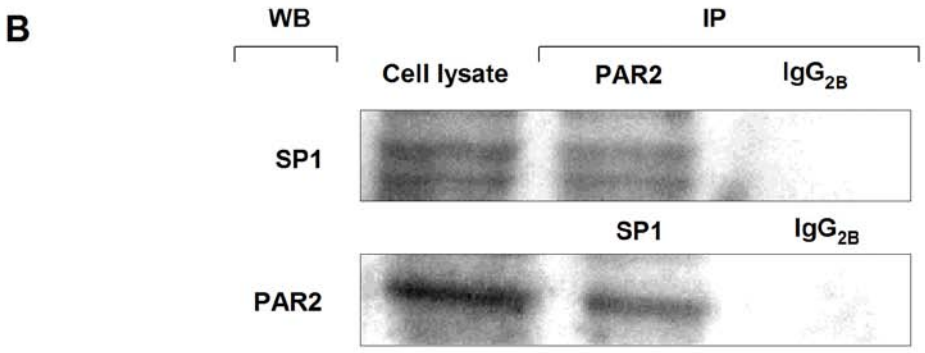

A
Luciferase Activity
Relative to CTL
0
1
2
3
4
**
**
PGL3 Basic
PGL3 Basic + MP112
PGL3 Enhancer
PGL3 Enhancer + MP112
PGL3 Promoter
PGL3 Promoter + MP112
B
Luciferase Activity
Relative to CTL
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
**
*
Trypsin 20nM
-
+
-
+
-
+
CTL
Sp1 siRNA
Scrambled siRNA

Figure 4. Nuclear PAR-2 interacts with Sp1 protein.

(A) PAR-2 GFP transfected HEK293 cells were stimulated with 20μM SLIGKV and were stained with monoclonal anti-Sp1 antibodies. PAR-2 GFP and Sp1 protein distribution was analyzed by confocal immunofluorescence microscopy. Co-localization between PAR-2 GFP and Sp1 is shown in merged image and was analyzed by performing line scan analysis (lower panel, green line represents PAR-2 GFP and red line corresponds to Sp1 proteins). The nucleus was stained with DAPI. **(B)** Isolated nuclei from HEK293 cells were stimulated with 20μM SLIGKV. PAR-2 (upper panel) and Sp1 (lower panel) immunoprecipitants (IP) were probed by Western blot (WB) with anti-Sp1 (upper panel) and anti-PAR-2 (lower panel) antibodies, respectively. Cell lysate was loaded on the left lane and used as a positive control. IgG_{2B} immunoprecipitant was used as a negative control. **(C)** i) Left panel, crude nuclear proteins (5 μg) from isolated nuclei of HEK293 PAR-2 GFP cells treated with 20nM trypsin or 20μM SLIGKV were incubated with a 5' end-labeled probe bearing the high affinity binding site for Sp1. Formation of DNA/protein complexes was then monitored by EMSA on an 8% (Sp1) native polyacrylamide gel and their position revealed through autoradiography. The position of both the Sp1/Sp3 DNA-protein complexes are shown, as well as that of the free probe (U). P: labeled probe alone. Right panel, quantification of binding activity of Sp1 in cells treated with Trypsin or SLIGKV for the activation of PAR-2. n=3, *$p<0.05$ ii) Sp1 competition experiment in EMSA. The Sp1 labeled probe used in panel A was incubated with nuclear proteins (5 μg) from both treated or non-treated cells in the presence of either no (-) or 100- and 500-fold molar excesses of unlabeled competitor oligonucleotides (either Sp1 or NFI). Formation of DNA/protein complexes was then monitored by EMSA on an 8% native gel. iii) Supershift experiment in EMSA. Crude nuclear proteins from either treated or non-treated cells were incubated with the Sp1 (5 μg proteins were used)) labeled probe in the presence of either no (-), or 2 μl of a polyclonal antibody directed against Sp1 (Sp1Ab) or PAR-2 (PAR-2Ab) and added either individually or in combination (PAR-2/Sp1Ab). Formation of the Sp1/Sp3 complexes, as well as their corresponding supershifted complexes (SSC) is indicated. P: labeled probe alone; U: unbound fraction of the labeled probe.


A
PAR2-GFP
SP1
Merged
DAPI
Line Profile
Intensity
200
100
0
0
10
20
30
40
Distance (Pixel)


B
WB
IP
Cell lysate
PAR2
IgG2B
SP1
SP1
IgG2B
PAR2

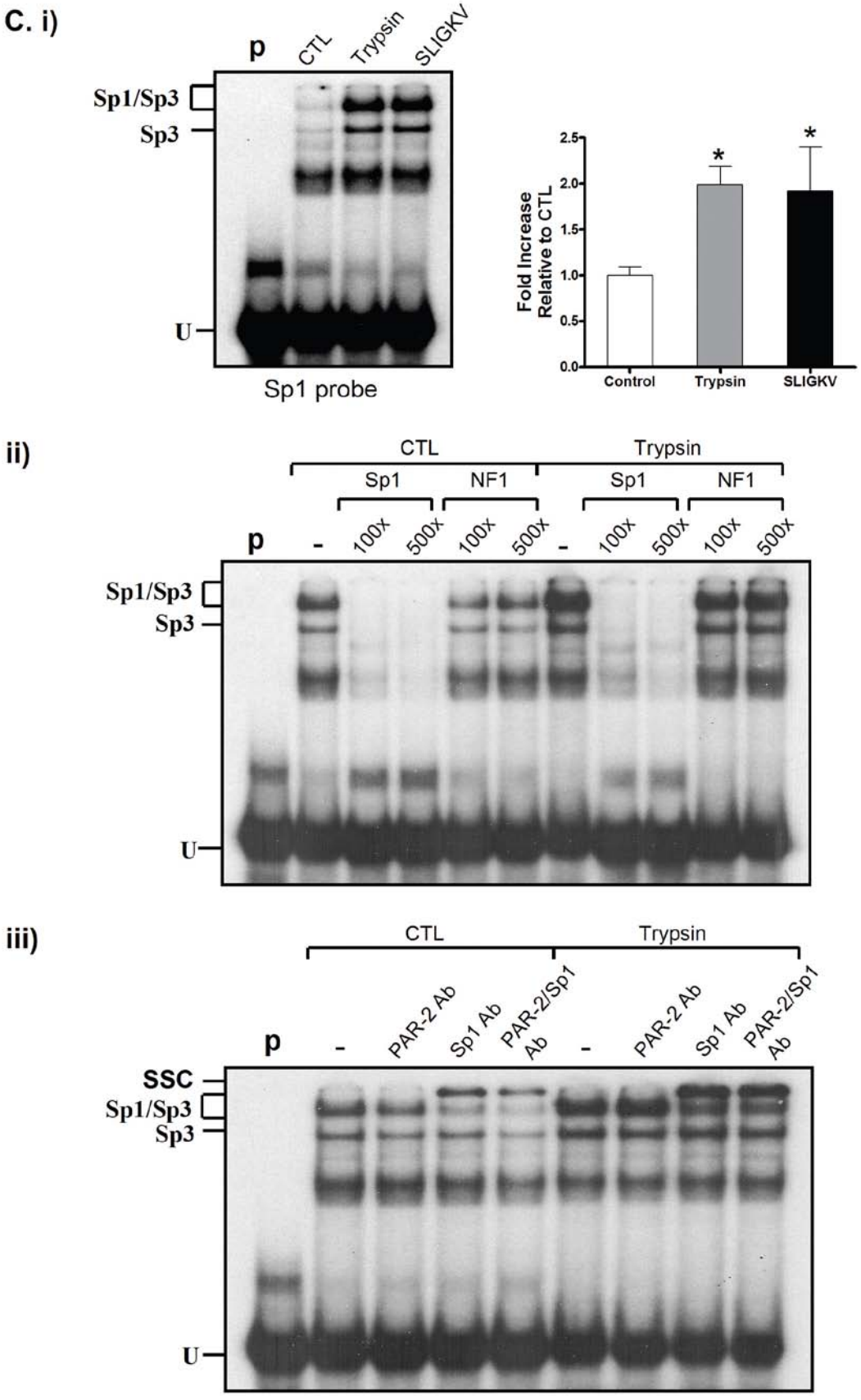
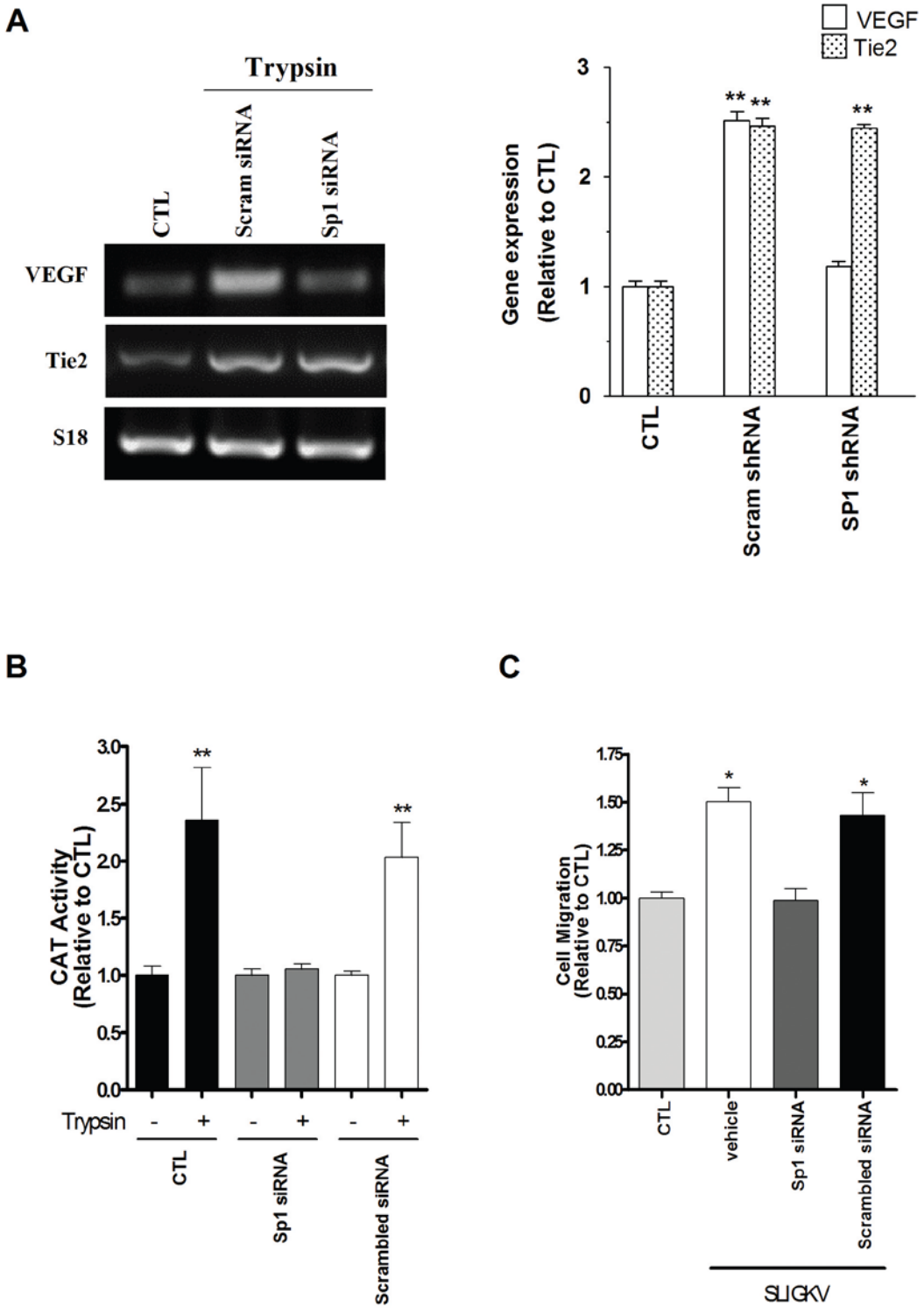
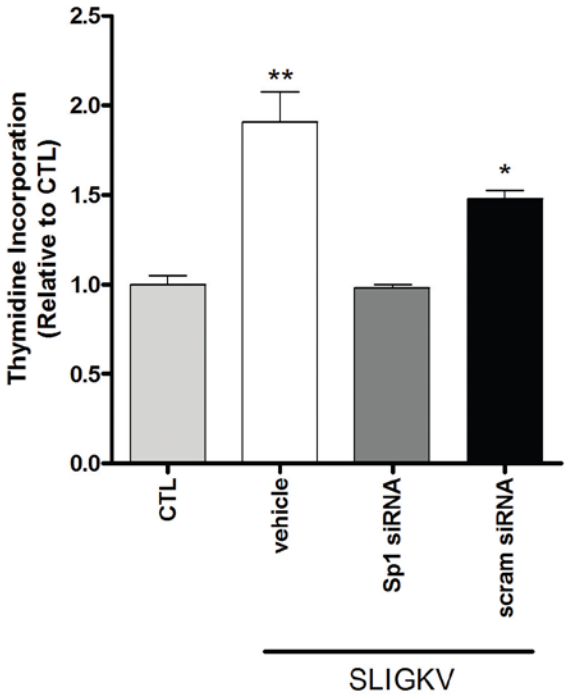

C. i)
p
CTL
Trypsin
SLIGKV
Sp1/Sp3
Sp3
U
Sp1 probe
Fold Increase Relative to CTL
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
Trypsin
SLIGKV
ii)
CTL
Trypsin
Sp1
NF1
Sp1
NF1
p
-
100x
500x
100x
500x
-
100x
500x
100x
500x
Sp1/Sp3
Sp3
U
iii)
CTL
Trypsin
p
-
PAR-2 Ab
Sp1 Ab
PAR-2/Sp1 Ab
-
PAR-2 Ab
Sp1 Ab
PAR-2/Sp1 Ab
SSC
Sp1/Sp3
Sp3
U

Figure 5. Proangiogenic functions of nuclear PAR-2 are dependent on Sp1.

(A) Sp1 knocking-down decreased PAR-2-induced VEGF gene expression in pCMVECs. Sp1 siRNA was transfected into pCMVEC cells two days before cell stimulation. Scrambled siRNA (Scram siRNA) was used as negative control. VEGF and tie2 gene expression were examined by RT-PCR. 18S was used as reference gene expression. Right graph shows quantification analysis from three independent assays. n=4, **p<0.01 (**B)** PRIM1 gene expression in pCMVECs was measured by chloramphenicol acetyl-transferase [47] reporting system. PRIM1 promoter inserted into pUMSVO-CAT vector and Sp1 siRNA or scrambled siRNA were transfected into cells 48 hours before stimulation. Cells transfected with PRIM1/pUMSVO vector alone were used as controls. After starvation, cells were stimulated with 20nM trypsin. CAT activity was then measured as described in *Methods*. Each value was calculated as CAT/100μg protein/β-galactose. n=3, **p<0.01. (**C)** Sp1 gene silencing diminished PAR-2-induced pCMVEC cell migration. Sp1 or scrambled siRNA were transfected into pCMVEC cells previously plated on coverslips in a 12-well culture dish. Cells were treated with 5 μg/mL mitomycin C for 30 min. for inhibition of cell proliferation. Cellular migration was induced by adding 20μM SLIGKV into the culture medium for 72 hours. After removing coverslips, the amount of migrated cells on the bottom of the culture dish was analyzed by MTT assay using a spectrophotometer (OD 545 nm with a reference wavelength of 690 nm). n=3 (**D)** Sp1 gene knock-down by Sp1 siRNA reduced SLIGKV-induced cellular proliferation in pCMVECs. Starved pCMVEC cells transfected with Sp1 or scrambled siRNA were stimulated with 20μM SLIGRL for 6 hours and incubated with 1μCi/mL [^{3}H]thymidine for 18 hours. Unstimulated cells were used as control. After removing the free [^{3}H]thymidine in the medium by washing with PBS, the incorporation of [^{3}H]thymidine was measured by liquid scintillation counter. CTL group was normalized to one-fold response and values were calculated as fold-increased to CTL. n=3,4 **p<0.01; *p<0.05.

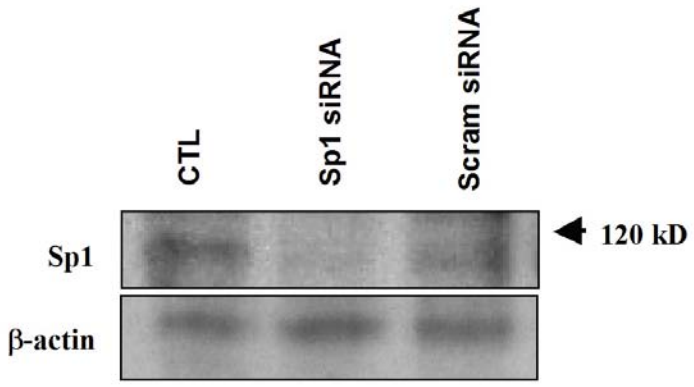

A
Trypsin
CTL
Scram siRNA
Sp1 siRNA
VEGF
Tie2
S18
VEGF
Tie2
Gene expression
(Relative to CTL)
CTL
Scram shRNA
SP1 shRNA
B
CAT Activity
(Relative to CTL)
Trypsin
CTL
Sp1 siRNA
Scrambled siRNA
C
Cell Migration
(Relative to CTL)
CTL
vehicle
Sp1 siRNA
Scrambled siRNA
SLIGKV

D


Thymidine Incorporation
(Relative to CTL)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
CTL
vehicle
Sp1 siRNA
scram siRNA
SLIGKV

Supplementary Figure 1: (A) Analysis of Sp1 protein expression in Hela cells transfected with Sp1 siRNA. Hela cells were transfected with Sp1 or scrambled siRNA for 48h. Sp1 and beta-actin protein expression were examined by Western blot with anti-Sp1 and anti-beta-actin antibodies.

A



CHAPTER 4 – ORIGINAL CONTRIBUTIONS TO KNOWLEDGE, GENERAL DISCUSSION, CONCLUSION AND FUTURE PERSPECTIVES

Original Contributions to Knowledge

In this thesis the following original contributions have been presented.

1) PAR-2 translocates to the nucleus following its activation by trypsin or its agonist peptide.
2) Neither clathrin nor caveolae internalization pathways are implicated in PAR-2 nuclear translocation.
3) Microtubules are required for the transport of PAR-2 to the nucleus.
4) C-terminus and NLS in the first and third intracellular loop of PAR-2 are important domains responsible for PAR-2 nuclear translocation.
5) Translocation of PAR-2 to the nucleus requires the formation of a complex comprising PAR-2, SNX11 and Importin β1.
6) Increased expression of VEGF induced by PAR-2 activation is dependent on PAR-2 nuclear translocation while increased expression of Ang1 is not.
7) Knock-down of PAR-2 and SNX11 in wild-type mice decreased retinal neovascularization.
8) Re-introduction of PAR-2 in PAR-2 knockout mice re-established normal retinal neovascularization.
9) Translocation of PAR-2 *in vivo* is required for the increase expression of VEGF and for normal retinal neovascularization.
10) PAR-2 binds to genomic DNA on promoter regions.
11) Sp1 binds to nuclear PAR-2 and is implicated in PAR-2 regulation of gene expression.
12) Sp1 is implicated in cell migration and cell proliferation induced by nuclear or nuclear translocated PAR-2 activation.

General Discussion

This study enabled us to identify the role of nuclear translocation of a member of the GPCR family, PAR-2, in inducing the transcription of genes implicated in angiogenic processes and in inducing angiogenesis *in vivo* using a mice model of retinal neovascularization. PAR-2 has been suggested to play an important role in angiogenesis and cell proliferation. First of all, PAR-2 is widely expressed in highly vascularized organs such as kidney, small intestine, and stomach (Nystedt, Emilsson et al. 1994). PAR-2 mediates endothelial cell mitogenesis *in vitro* and promotes microvascular permeability *in vivo* (Mirza, Yatsula et al. 1996; Hamilton, Nguyen et al. 1998). PAR-2 expression is also upregulated by cytokines, including tumor necrosis factor, interleukin-1 and lipopolysaccharide, that are implicated in inflammatory angiogenesis (Nystedt, Ramakrishnan et al. 1996). We used human embryonic kidney 293 (HEK293) cells stably expressing PAR-2 GFP and retinal ganglion (RGC-5) cells as *in vitro* models for the analysis of gene expression and a mice model of retinal neovascularization as an *in vivo* model to study the impact of PAR-2 nuclear translocation on vascularization. In our previous studies, we have determined that PAR-2 is located endogenously at the plasma membrane and also at the nucleus, as demonstrated by EM and confocal microscopy. The present study enabled us to find that a population of PAR-2 at the plasma membrane is translocated to the nucleus following the activation of the receptor. This process is rapid and occurs within minutes after the stimulation of the receptor. We looked to identify the pathway for PAR-2 nuclear translocation, the domains of the receptor implicated in this process and the proteins interacting with the receptor and mediating the translocation. Microtubules are essential not only for maintaining the cell shape but also for the trafficking of proteins and molecules to different compartments in the cell. Some studies revealed the importance of microtubules for internalization of certain GPCRs from the plasma membrane to endocytic vesicles and for their transport to other cellular compartments. Here we showed that microtubules are also critical for the nuclear translocation of PAR-2 from the plasma membrane. PAR-2 is known to be internalized via the clathrin-coated vesicles following the activation of the receptor and this route is used for its degradation and its signalling leading to the activation of the MAP kinase

signalling cascade. PAR-2 at the cell surface can also couple to $G_{\alpha q/11}$ protein leading to the activation of PLCβ and the mobilization of intracellular calcium. Our data indicated that the pathway for nuclear translocation of the receptor is distinct from its degradation pathway and is independent of both clathrin and caveolin.

The C-terminal domain and the third intracellular loop of many GPCRs are implicated in receptor internalization and trafficking. PAR-2 contains a site for beta-arrestin 1 binding in the C-terminus of the receptor and this interaction is essential for its internalization and desensitization. Previous studies have also shown that beta-arrestin 1 binds to PAR-2 following the activation of the receptor and this interaction enables the activation of the MAP kinase signalling cascade (Dery, Thoma et al. 1999; DeFea, Zalevsky et al. 2000). Beta-arrestin 1 has been reported to be able to translocate to the nucleus and to influence gene transcription by binding to transcription factors like CREB or p300. Beta-arrestin 1 can also interact with proteins interacting with transcription factors like IκB and MDM2 (Shenoy, McDonald et al. 2001; Gao, Sun et al. 2004; Witherow, Garrison et al. 2004; Kang, Shi et al. 2005). Nuclear translocation of PAR-2 does not depend on beta-arrestin 1 but requires an intact C-terminal domain of the receptor. A group has suggested that the carboxyl tail and the third intracellular loop of PAR-2 were mainly responsible for the interaction with Jun activation domain-binding protein 1 (Jab1), which was initially identified as a coactivator of c-Jun (Claret, Hibi et al. 1996; Luo, Wang et al. 2006). The C-terminal domain of PAR-2 may contain binding sites for other proteins implicated in the translocation of the receptor toward the nucleus. In addition, analysis of PAR-2 amino acid sequence revealed two potential NLS sequences. The first NLS is located in the first intracellular loop and the second is in the third intracellular loop. It is interesting to note that PAR-2 is not the first GPCR reported to have a functional NLS. Certain GPCRs such as the angiotensin type 1 receptor, B2 bradykinin receptor, CysLT1, apelin receptor, or endothelin receptor contain putative nuclear localization sequences (Lu, Yang et al. 1998; Lee, Lanca et al. 2004). Mutation of either one of PAR-2 NLS sites inhibited PAR-2 nuclear translocation. Mutation of NLS site in the third intracellular loop prevented interaction of PAR-2 with importin β1 and blocked the nuclear translocation of the receptor as demonstrated by confocal microscopy and FACS experiments. Importin β1 has been shown to interact directly with different

proteins and receptors and mediate their translocation to the nucleus with or without heterodimerization with members of the importin α family. Nevertheless, importin β1 has mostly been reported to form heterodimers with different members of importin α and to interact with nucleoporins in the nuclear pores. Importin α1, -3, and -5 are not implicated in the translocation of PAR-2 to the nucleus although they bind to similar NLS sequences as the third intracellular loop NLS of PAR-2 (Herold, Truant et al. 1998; Kovac, Emelyanov et al. 2000; Yeung, Chen et al. 2008). We have demonstrated that SNX11 is also implicated in the transport of PAR-2 to the nucleus. SNX11 interacts with PAR-2 and also with importin β1. Its seems that the formation of a complex comprising PAR-2, SNX11 and importin β1 is essentiel for the proper trafficking of PAR-2 to the nucleus following its activation. SNXs are proposed to regulate internalization, degradation, endosomal sorting or recycling of many proteins (Worby and Dixon 2002; Carlton, Bujny et al. 2005). SNX1 has been demonstrated to interact with PAR-1 and depletion of SNX1 impaired agonist-induced degradation of PAR-1 (Wang, Zhou et al. 2002). The function of SNX11 is not well understood. Members of the sorting nexin family are an interesting class of proteins and the improved understanding of their functions will help to elucidate the molecular mechanisms responsible for GPCR down-regulation and trafficking.

Because PAR-2 is able to translocate to the nucleus, we hypothesized that the translocation of the receptor has a predominant role in the induction of gene transcription. The role of nuclear translocation of PAR-2 has been investigated by identifying genes that are dependent or independent of the translocation of the receptor for their up-regulation. Since PAR-2 is implicated in angiogenesis, we analysed the expression of genes known to be essential for this process. Activation of PAR-2 induced the increased expression of Ang1 and VEGF but only the up-regulation of VEGF was dependent on the nuclear translocation of the receptor. This study revealed distinct roles of PAR-2 depending on its intracellular route in regard of its regulation of gene transcription. The expression of Ang1 induced by PAR-2 activation seems to be mediated by the classical activation of the MAP kinase signalling cascade while the expression of VEGF is mediated by the translocation of PAR-2 to the nucleus and the activation of nuclear signalling pathway.

The physiologic contribution of PAR-2 nuclear trafficking was analyzed by using an *in vivo* mice model of retinal neovascularization. The retina provides a good model system to study the molecular signals implicated in angiogenesis in embryonic development and in adult. In addition, the study of retinal angiogenesis has enormous clinical significance because retinal neovascularization resulting from diabetic retinopathy and retinopathy of prematurity are the most common causes of blindness in young patients (Klein, Meuer et al. 1995). We used wild-type and PAR-2 knockout mice in order to analyse the implication of PAR-2 nuclear translocation in angiogenesis *in vivo*. PAR-2 knockout mice showed a reduced level of neovascularization compared to wild-type mice. The re-expression of PAR-2 in knockout animals using lentiviral technology enabled to increase the level of retinal vascularization, confirming a role of PAR-2 in retinal angiogenesis. The re-expression of PAR-2 NLS mutant resulted in a decreased level of neovascularization compared to knockout mice and knockout mice re-expressing PAR-2. This result is explained by the fact that PAR-2 NLS mutant increased the retinal expression of Ang1 at the expense of VEGF. Ang1 has previously been demonstrated to have antiangiogenic properties. PAR-2 NLS mutant was also demonstrated in this study to be inabled to increase VEGF expression following its activation. These results suggested the importance of PAR-2 nuclear translocation in the normal neovascularization of the mice retina. Those results were further confirmed using wild-type mice in which PAR-2 and SNX11 were knocked-down. The reduced expression of PAR-2 and SNX11 decreased the level of neovascularization indicating the importance of PAR-2 expression and the nuclear translocation of PAR-2 in normal retinal vascular development. In addition, to investigate the angiogenic role of PAR2 in retinal neurons, vascular sprouting from aortic ring explants of PAR-2 KO mice was analysed. Conditioned medium obtained from RGCs primed with PAR2 produced a twofold increase in sprouting, indicative of release of proangiogenic factors from PAR2-treated RGCs. PAR-2 is therefore an important proangiogenic mediator and its activity is essential for the normal development of retinal neovascularization.

Although these results enabled us to elucidate the mechanism of translocation of PAR-2 from the plasma membrane to the nucleus and its implications in angiogenesis *in vitro* and *in vivo*, the mechanism of gene activation by the receptor at the nucleus was not

known. Therefore, we investigated the mechanism of gene activation induced by nuclear PAR-2 and we hypothesised that nuclear PAR-2 could participate in the interaction and/or activation of transcription factors leading to the activation of gene expression. PAR-2 at the nucleus was functional as demonstrated by its ability to mobilize intracellular calcium and to induce gene expression. Mass spectrometry enabled us to identify proteins interacting with activated nuclear PAR-2 and among those proteins we identified co-factors required for Sp1 transcriptional activation. PAR-2 and Sp1 have been shown to regulate the expression of genes implicated in angiogenesis and cell growth (Jones, Kadonaga et al. 1986; Opitz and Rustgi 2000; Kaczynski, Cook et al. 2003; Mazure, Brahimi-Horn et al. 2003; Santiago, Ishii et al. 2007). Because of their implications in angiogenesis and cell proliferation, we hypothesized that Sp1 could play a role in the regulation of gene expression induced by nuclear PAR-2. We showed that PAR-2 interacts with Sp1 at the nucleus. It is known that Sp1 binds to DNA on GC and GT boxes and regulate gene expression. Because there is an interaction between PAR-2 and Sp1 at the nucleus, we looked to find if PAR-2 could be found on DNA possibly bound to a complex of transcription factors on gene promoters. Chromatin immunoprecipitation assays revealed that PAR-2 binds to DNA fragments containing promoter elements at the nucleus following the activation of the receptor. The analysis of the sequences of these DNA fragments identified putative binding sites for many transcription factors including GATA-1, Oct-1 and Sp1. EMSA experiments demonstrated that activation of nuclear PAR-2 leads to an increase in Sp1 binding activity to Sp1 consensus sequence. Sp1 was shown to be essential for PAR-2 regulation of transcriptional activity and for the expression of VEGF. The VEGF promoters from different species share a lot of homology including consensus sites for Sp1/Sp3, AP-2, Egr-1, STAT-3, and HIF-1 (Levy, Levy et al. 1995; Shima, Kuroki et al. 1996; Buteau-Lozano, Ancelin et al. 2002). Sp1 has been shown to be a key modulator of VEGF expression and is a potent regulator of angiogenesis. Our results also indicated that Sp1 is implicated in PAR-2 induction of cell proliferation and migration. Because of its implication in angiogenesis, Sp1 appears to be a target protein for the action of many drugs affecting solid and haematological tumor development.

Conclusion

In the end, it seems that one receptor can trigger different effects depending on its subcellular localization and an impairment in its trafficking or its normal localization can have detrimental effects on the normal physiology of the cell. The findings described herein unveil the mechanism by which PAR-2 translocates to the nuclear membrane and regulate gene expression. This study will create a framework within which to examine how trafficking of GPCRs has an impact on the physiology of the cell. We hope this study may increase our understanding of GPCR trafficking and signalling, and lead to novel pharmacologic targets.

Future Perspectives

The understanding of the mechanisms of nuclear translocation of members of the GPCR family can open new avenues in the biology of heptahelical receptors regarding on how they control gene transcription. Disregulation of receptor trafficking can result in the loss or gain-of-function in GPCR signalling. Many questions remain concerning the topology of the receptor located at the nuclear membrane and how signalling is arrested following the nuclear translocation. In a set of preliminary experiments (data not shown) using PAR-2 containing a Flag sequence at the N-terminus and HA sequence at the C-terminus enabled us to hypothesize that the receptor exists in two conformations in the nuclear membrane. Indeed, fluorescence was detected when non-permeabilized isolated nuclei were incubated with anti-Flag antibodies or anti-HA antibodies. These experiments suggest that the first conformation would correspond to the receptor with the N-terminus facing the cytosol and the second one with the N-terminus facing the intraluminal space between the two nuclear lipid bilayers. PARs have been reported to form homo- or heterodimerization with members of the PARs family. It is possible that PAR-2 forms homo- and/or heterodimers with other members of the PARs family and that dimerization would allow the existence of two conformations of the receptor at the nuclear membrane. Further investigations are needed to better understand the association between the topology of the receptor and the activation of signal.

PAR-2 internalization and degradation in lysosomes are essential for signalling arrest. Questions remain on how nuclear PAR-2 signalling is arrested following the

translocation of the receptor. Similarly to the receptor at the cell surface, inactivation of PAR-2 at the nuclear membrane could be due to translocation of the receptor from the nuclear membrane to lysosomes for degradation. The presence of components of the endocytic machinery in or on the nucleus suggests this possibility. Nuclear beta-arrestin 1 could play a role in the signal arrest induced by the activation and translocation of PAR-2 to the nucleus. The presence of GRKs at the nucleus also suggests the possibility of inactivating nuclear PAR-2 through GRKs.

Further investigations are also needed for the understanding of their roles in physiological and pathological conditions. In certain diseases, accumulation of GPCRs at the nucleus has been demonstrated. Indeed, an increased proportion of nuclear opioid κ receptors were observed in an experimental animal model of primary hereditary hypertrophic cardiomyopathy (Ventura, Maioli et al. 1998). The expression of CXCR4 at the nucleus has been shown in cancer cells (Shibuta, Mori et al. 2002; Spano, Andre et al. 2004). This study unveils a crucial involvement of nuclear GPCRs in pro-survival and (or) proliferative pathways both *in vitro* and *in vivo*. Conclusions emerging from our study may have a major impact on therapeutic development: for example by promoting rapid normal revascularization of damaged tissues.

CHAPTER 5 – BIBLIOGRAPHY

Ajenjo, N., E. Canon, et al. (2004). "Subcellular localization determines the protective effects of activated ERK2 against distinct apoptogenic stimuli in myeloid leukemia cells." Journal of Biological Chemistry **279**(31): 32813-23.

al-Ani, B., M. Saifeddine, et al. (1995). "Detection of functional receptors for the proteinase-activated-receptor-2-activating polypeptide, SLIGRL-NH2, in rat vascular and gastric smooth muscle." Can J Physiol Pharmacol **73**(8): 1203-7.

Al-Ani, B., M. Saifeddine, et al. (1999). "Proteinase activated receptor 2: Role of extracellular loop 2 for ligand-mediated activation." Br J Pharmacol **128**(5): 1105-13.

Alitalo, K. and P. Carmeliet (2002). "Molecular mechanisms of lymphangiogenesis in health and disease." Cancer Cell **1**(3): 219-27.

Allen, G. A., D. M. Monroe, 3rd, et al. (2000). "The effect of factor X level on thrombin generation and the procoagulant effect of activated factor VII in a cell-based model of coagulation." Blood Coagul Fibrinolysis **11 Suppl 1**: S3-7.

Alm, A. K., R. Gagnemo-Persson, et al. (2000). "Extrapancreatic trypsin-2 cleaves proteinase-activated receptor-2." Biochem Biophys Res Commun **275**(1): 77-83.

Ashton, N. (1970). "Retinal angiogenesis in the human embryo." Br Med Bull **26**(2): 103-6.

Asokananthan, N., P. T. Graham, et al. (2002). "Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells." J Immunol **168**(7): 3577-85.

Bahou, W. F., J. L. Kutok, et al. (1994). "Identification of a novel thrombin receptor sequence required for activation-dependent responses." Blood **84**(12): 4195-202.

Barr, V. A., S. A. Phillips, et al. (2000). "Overexpression of a novel sorting nexin, SNX15, affects endosome morphology and protein trafficking." Traffic **1**(11): 904-16.

Belting, M., M. I. Dorrell, et al. (2004). "Regulation of angiogenesis by tissue factor cytoplasmic domain signaling." Nature Medicine **10**(5): 502-9.

Berger, P., J. M. Tunon-De-Lara, et al. (2001). "Selected contribution: tryptase-induced PAR-2-mediated Ca(2+) signaling in human airway smooth muscle cells." J Appl Physiol **91**(2): 995-1003.

Bhattacharya, M., K. Peri, et al. (1999). "Localization of functional prostaglandin E2 receptors EP3 and EP4 in the nuclear envelope." J Biol Chem **274**(22): 15719-24.

Bhattacharya, M., K. G. Peri, et al. (1998). "Nuclear localization of prostaglandin E2 receptors." Proc Natl Acad Sci U S A **95**(26): 15792-7.

Biedermann, T., M. Kneilling, et al. (2000). "Mast cells control neutrophil recruitment during T cell-mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2." J Exp Med **192**(10): 1441-52.

Bkaily, G., S. Sleiman, et al. (2003). "Angiotensin II AT1 receptor internalization, translocation and de novo synthesis modulate cytosolic and nuclear calcium in human vascular smooth muscle cells." Canadian Journal of Physiology & Pharmacology **81**(3): 274-87.

Black, A. R., J. D. Black, et al. (2001). "Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer." J Cell Physiol **188**(2): 143-60.

Bohm, S. K., L. M. Khitin, et al. (1996). "Mechanisms of desensitization and resensitization of proteinase-activated receptor-2." J Biol Chem **271**(36): 22003-16.

Bohm, S. K., W. Kong, et al. (1996). "Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2." Biochem J **314 (Pt 3)**: 1009-16.

Booz, G. W., K. M. Conrad, et al. (1992). "Angiotensin-II-binding sites on hepatocyte nuclei." Endocrinology **130**(6): 3641-9.

Bos, J. L. (1998). "All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral." Embo J **17**(23): 6776-82.

Bouwman, P. and S. Philipsen (2002). "Regulation of the activity of Sp1-related transcription factors." Molecular & Cellular Endocrinology **195**(1-2): 27-38.

Bretschneider, E., R. Kaufmann, et al. (1999). "Evidence for proteinase-activated receptor-2 (PAR-2)-mediated mitogenesis in coronary artery smooth muscle cells." Br J Pharmacol **126**(8): 1735-40.

Brown, J. K., C. A. Jones, et al. (1995). "Tryptase-induced mitogenesis in airway smooth muscle cells. Potency, mechanisms, and interactions with other mast cell mediators." Chest **107**(3 Suppl): 95S-96S.

Brown, J. K., C. L. Tyler, et al. (1995). "Tryptase, the dominant secretory granular protein in human mast cells, is a potent mitogen for cultured dog tracheal smooth muscle cells." Am J Respir Cell Mol Biol **13**(2): 227-36.

Bryant, D. M., F. G. Wylie, et al. (2005). "Regulation of endocytosis, nuclear translocation, and signaling of fibroblast growth factor receptor 1 by E-cadherin." Mol Biol Cell **16**(1): 14-23.

Buteau-Lozano, H., M. Ancelin, et al. (2002). "Transcriptional regulation of vascular endothelial growth factor by estradiol and tamoxifen in breast cancer cells: a complex interplay between estrogen receptors alpha and beta." Cancer Res **62**(17): 4977-84.

Camerer, E., A. B. Kolsto, et al. (1996). "Cell biology of tissue factor, the principal initiator of blood coagulation." Thromb Res **81**(1): 1-41.

Cao, Y., P. Sonveaux, et al. (2007). "Systemic overexpression of angiopoietin-2 promotes tumor microvessel regression and inhibits angiogenesis and tumor growth." Cancer Res **67**(8): 3835-44.

Carlton, J., M. Bujny, et al. (2005). "Sorting nexins--unifying trends and new perspectives." Traffic **6**(2): 75-82.

Cavanagh, H. D. and A. M. Colley (1989). "The molecular basis of neurotrophic keratitis." Acta Ophthalmol Suppl **192**: 115-34.

Chang-Ling, T., A. Vannas, et al. (1990). "Incision depth affects the recovery of corneal sensitivity and neural regeneration in the cat." Invest Ophthalmol Vis Sci **31**(8): 1533-41.

Chaudhry, A. Z., G. E. Lyons, et al. (1997). "Expression patterns of the four nuclear factor I genes during mouse embryogenesis indicate a potential role in development." Dev Dyn **208**(3): 313-25.

Chen, Q. Y. and N. Jackson (2004). "Human CD1D gene has TATA boxless dual promoters: an SP1-binding element determines the function of the proximal promoter." Journal of Immunology **172**(9): 5512-21.

Chou, S. F., H. L. Chen, et al. (2003). "Sp1 and Sp3 are involved in up-regulation of human deoxyribonuclease II transcription during differentiation of HL-60 cells." European Journal of Biochemistry **270**(8): 1855-62.

Christophe, D., C. Christophe-Hobertus, et al. (2000). "Nuclear targeting of proteins: how many different signals?" Cell Signal **12**(5): 337-41.

Citores, L., D. Khnykin, et al. (2001). "Modulation of intracellular transport of acidic fibroblast growth factor by mutations in the cytoplasmic receptor domain." J Cell Sci **114**(Pt 9): 1677-89.

Citores, L., J. Wesche, et al. (1999). "Uptake and intracellular transport of acidic fibroblast growth factor: evidence for free and cytoskeleton-anchored fibroblast growth factor receptors." Mol Biol Cell **10**(11): 3835-48.

Claing, A., W. Chen, et al. (2001). "beta-Arrestin-mediated ADP-ribosylation factor 6 activation and beta 2-adrenergic receptor endocytosis." J Biol Chem **276**(45): 42509-13.

Claret, F. X., M. Hibi, et al. (1996). "A new group of conserved coactivators that increase the specificity of AP-1 transcription factors." Nature **383**(6599): 453-7.

Clark, R. B. (1986). "Desensitization of hormonal stimuli coupled to regulation of cyclic AMP levels." Adv Cyclic Nucleotide Protein Phosphorylation Res **20**: 151-209.

Cocks, T. M., B. Fong, et al. (1999). "A protective role for protease-activated receptors in the airways." Nature **398**(6723): 156-60.

Compton, S. J., B. Renaux, et al. (2001). "Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase." Br J Pharmacol **134**(4): 705-18.

Connolly, D. T., D. M. Heuvelman, et al. (1989). "Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis." J Clin Invest **84**(5): 1470-8.

Cozier, G. E., J. Carlton, et al. (2002). "The phox homology (PX) domain-dependent, 3-phosphoinositide-mediated association of sorting nexin-1 with an early sorting

endosomal compartment is required for its ability to regulate epidermal growth factor receptor degradation." J Biol Chem **277**(50): 48730-6.

D'Andrea, M. R., C. K. Derian, et al. (1998). "Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues." J Histochem Cytochem **46**(2): 157-64.

Damiano, B. P., W. M. Cheung, et al. (1996). "Cardiovascular actions of thrombin receptor activation in vivo." J Pharmacol Exp Ther **279**(3): 1365-78.

Darmoul, D., V. Gratio, et al. (2004). "Protease-activated receptor 2 in colon cancer: trypsin-induced MAPK phosphorylation and cell proliferation are mediated by epidermal growth factor receptor transactivation." J Biol Chem **279**(20): 20927-34.

Davie, E. W. and O. D. Ratnoff (1964). "Waterfall Sequence for Intrinsic Blood Clotting." Science **145**: 1310-2.

Davis, S., T. H. Aldrich, et al. (1996). "Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning." Cell **87**(7): 1161-9.

DeBusk, L. M., D. E. Hallahan, et al. (2004). "Akt is a major angiogenic mediator downstream of the Ang1/Tie2 signaling pathway." Exp Cell Res **298**(1): 167-77.

DeFea, K. A., J. Zalevsky, et al. (2000). "beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2." J Cell Biol **148**(6): 1267-81.

Dery, O., M. S. Thoma, et al. (1999). "Trafficking of proteinase-activated receptor-2 and beta-arrestin-1 tagged with green fluorescent protein. beta-Arrestin-dependent endocytosis of a proteinase receptor." J Biol Chem **274**(26): 18524-35.

Dor, Y., R. Porat, et al. (2001). "Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis." Am J Physiol Cell Physiol **280**(6): C1367-74.

Drab, M., P. Verkade, et al. (2001). "Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice." Science **293**(5539): 2449-52.

Dumont, D. J., T. P. Yamaguchi, et al. (1992). "tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors." Oncogene **7**(8): 1471-80.

Dynan, W. S., J. D. Saffer, et al. (1985). "Transcription factor Sp1 recognizes promoter sequences from the monkey genome that are simian virus 40 promoter." Proc Natl Acad Sci U S A **82**(15): 4915-9.

Eggena, P., J. H. Zhu, et al. (1993). "Nuclear angiotensin receptors induce transcription of renin and angiotensinogen mRNA." Hypertension **22**(4): 496-501.

Eggena, P., J. H. Zhu, et al. (1996). "Hepatic angiotensin II nuclear receptors and transcription of growth-related factors." J Hypertens **14**(8): 961-8.

Engelman, J. A., C. C. Wykoff, et al. (1997). "Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth." J Biol Chem **272**(26): 16374-81.

Faucheux, C., M. A. Horton, et al. (2002). "Nuclear localization of type I parathyroid hormone/parathyroid hormone-related protein receptors in deer antler osteoclasts: evidence for parathyroid hormone-related protein and receptor activator of NF-kappaB-dependent effects on osteoclast formation in regenerating mammalian bone." J Bone Miner Res **17**(3): 455-64.

Ferrara, N. and K. Alitalo (1999). "Clinical applications of angiogenic growth factors and their inhibitors." Nat Med **5**(12): 1359-64.

Ferrara, N. and T. Davis-Smyth (1997). "The biology of vascular endothelial growth factor." Endocr Rev **18**(1): 4-25.

Fiedler, U., T. Krissl, et al. (2003). "Angiopoietin-1 and angiopoietin-2 share the same binding domains in the Tie-2 receptor involving the first Ig-like loop and the epidermal growth factor-like repeats." J Biol Chem **278**(3): 1721-7.

Fiorucci, S., A. Mencarelli, et al. (2001). "Proteinase-activated receptor 2 is an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis." Proc Natl Acad Sci U S A **98**(24): 13936-41.

Fra, A. M., E. Williamson, et al. (1995). "De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin." Proc Natl Acad Sci U S A **92**(19): 8655-9.

Fried, H. and U. Kutay (2003). "Nucleocytoplasmic transport: taking an inventory." Cellular & Molecular Life Sciences **60**(8): 1659-88.

Gaborik, Z. and L. Hunyady (2004). "Intracellular trafficking of hormone receptors." Trends Endocrinol Metab **15**(6): 286-93.

Gale, N. W., G. Thurston, et al. (2002). "Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1." Dev Cell **3**(3): 411-23.

Gamble, J. R., J. Drew, et al. (2000). "Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions." Circ Res **87**(7): 603-7.

Gao, H., Y. Sun, et al. (2004). "Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways." Mol Cell **14**(3): 303-17.

Gariano, R. F., M. L. Iruela-Arispe, et al. (1994). "Vascular development in primate retina: comparison of laminar plexus formation in monkey and human." Invest Ophthalmol Vis Sci **35**(9): 3442-55.

Gartel, A. L., X. Ye, et al. (2001). "Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3." Proc Natl Acad Sci U S A **98**(8): 4510-5.

Gaumont-Leclerc, M. F., U. K. Mukhopadhyay, et al. (2004). "PEA-15 is inhibited by adenovirus E1A and plays a role in ERK nuclear export and Ras-induced senescence." Journal of Biological Chemistry **279**(45): 46802-9.

Gerber, H. P., A. McMurtrey, et al. (1998). "Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation." J Biol Chem **273**(46): 30336-43.

Glebov, O. O., N. A. Bright, et al. (2006). "Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells." Nature Cell Biology **8**(1): 46-54.

Gobeil, F., A. Fortier, et al. (2006). "G-protein-coupled receptors signalling at the cell nucleus: an emerging paradigm." Canadian Journal of Physiology & Pharmacology **84**(3-4): 287-97.

Gobeil, F., Jr., S. G. Bernier, et al. (2003). "Modulation of pro-inflammatory gene expression by nuclear lysophosphatidic acid receptor type-1." J Biol Chem **278**(40): 38875-83.

Gobeil, F., Jr., I. Dumont, et al. (2002). "Regulation of eNOS expression in brain endothelial cells by perinuclear EP(3) receptors." Circ Res **90**(6): 682-9.

Goldfarb, D. S., A. H. Corbett, et al. (2004). "Importin alpha: a multipurpose nuclear-transport receptor." Trends in Cell Biology **14**(9): 505-14.

Griendling, K. K., B. C. Berk, et al. (1987). "Angiotensin II stimulation of vascular smooth muscle phosphoinositide metabolism. State of the art lecture." Hypertension **9**(6 Pt 2): III181-5.

Gronostajski, R. M., S. Adhya, et al. (1985). "Site-specific DNA binding of nuclear factor I: analyses of cellular binding sites." Mol Cell Biol **5**(5): 964-71.

Hackett, S. F., S. Wiegand, et al. (2002). "Angiopoietin-2 plays an important role in retinal angiogenesis." J Cell Physiol **192**(2): 182-7.

Hagen, G., S. Muller, et al. (1994). "Sp1-mediated transcriptional activation is repressed by Sp3." Embo J **13**(16): 3843-51.

Hamilton, J. R., P. B. Nguyen, et al. (1998). "Atypical protease-activated receptor mediates endothelium-dependent relaxation of human coronary arteries." Circ Res **82**(12): 1306-11.

Heinrich, P. C., J. V. Castell, et al. (1990). "Interleukin-6 and the acute phase response." Biochem J **265**(3): 621-36.

Henderson, J. E., N. Amizuka, et al. (1995). "Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death." Mol Cell Biol **15**(8): 4064-75.

Herold, A., R. Truant, et al. (1998). "Determination of the functional domain organization of the importin alpha nuclear import factor." J Cell Biol **143**(2): 309-18.

Hirota, Y., Y. Osuga, et al. (2005). "Evidence for the presence of protease-activated receptor 2 and its possible implication in remodeling of human endometrium." J Clin Endocrinol Metab **90**(3): 1662-9.

Hoffman, M. (2003). "Remodeling the blood coagulation cascade." J Thromb Thrombolysis **16**(1-2): 17-20.

Hofland, L. J., P. M. van Koetsveld, et al. (1996). "Internalisation of isotope-coupled somatostatin analogues." Digestion **57 Suppl 1**: 2-6.

Hollenberg, M. D. (2003). "Proteinase-mediated signaling: proteinase-activated receptors (PARs) and much more." Life Sci **74**(2-3): 237-46.

Houck, K. A., N. Ferrara, et al. (1991). "The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA." Mol Endocrinol **5**(12): 1806-14.

Hsu, H. C., H. Ema, et al. (2000). "Hematopoietic stem cells express Tie-2 receptor in the murine fetal liver." Blood **96**(12): 3757-62.

Hughes, S., H. Yang, et al. (2000). "Vascularization of the human fetal retina: roles of vasculogenesis and angiogenesis." Invest Ophthalmol Vis Sci **41**(5): 1217-28.

Hung, D. T., Y. H. Wong, et al. (1992). "The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase." J Biol Chem **267**(29): 20831-4.

Hunyady, L., F. Merelli, et al. (1991). "Agonist-induced endocytosis and signal generation in adrenal glomerulosa cells. A potential mechanism for receptor-operated calcium entry." J Biol Chem **266**(5): 2783-8.

Hwa, J. J., L. Ghibaudi, et al. (1996). "Evidence for the presence of a proteinase-activated receptor distinct from the thrombin receptor in vascular endothelial cells." Circ Res **78**(4): 581-8.

Inoue, M., T. Kanda, et al. (1998). "Impaired expression of atrial natriuretic peptide in diabetic rats with myocardial infarction." Res Commun Mol Pathol Pharmacol **100**(3): 327-38.

Ishii, K., J. Chen, et al. (1994). "Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase. Functional specificity among G-protein coupled receptor kinases." J Biol Chem **269**(2): 1125-30.

Iwama, A., I. Hamaguchi, et al. (1993). "Molecular cloning and characterization of mouse TIE and TEK receptor tyrosine kinase genes and their expression in hematopoietic stem cells." Biochem Biophys Res Commun **195**(1): 301-9.

Jackson, S. P. and R. Tjian (1988). "O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation." Cell **55**(1): 125-33.

Jafri, F., H. M. El-Shewy, et al. (2006). "Constitutive ERK1/2 activation by a chimeric neurokinin 1 receptor-beta-arrestin1 fusion protein. Probing the composition and

function of the G protein-coupled receptor "signalsome"." Journal of Biological Chemistry **281**(28): 19346-57.

Jakel, S. and D. Gorlich (1998). "Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells." Embo J **17**(15): 4491-502.

Jones, K. A., J. T. Kadonaga, et al. (1986). "Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1." Science **232**(4751): 755-9.

Jones, K. A., J. T. Kadonaga, et al. (1987). "A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication." Cell **48**(1): 79-89.

Juven-Gershon, T., J. Y. Hsu, et al. (2008). "The RNA polymerase II core promoter - the gateway to transcription." Curr Opin Cell Biol **20**(3): 253-9.

Kaczynski, J., T. Cook, et al. (2003). "Sp1- and Kruppel-like transcription factors." Genome Biol **4**(2): 206.

Kahn, M. L., S. R. Hammes, et al. (1998). "Gene and locus structure and chromosomal localization of the protease-activated receptor gene family." J Biol Chem **273**(36): 23290-6.

Kang, J., Y. Shi, et al. (2005). "A nuclear function of beta-arrestin1 in GPCR signaling: regulation of histone acetylation and gene transcription." Cell **123**(5): 833-47.

Kanke, T., S. R. Macfarlane, et al. (2001). "Proteinase-activated receptor-2-mediated activation of stress-activated protein kinases and inhibitory kappa B kinases in NCTC 2544 keratinocytes." J Biol Chem **276**(34): 31657-66.

Kaufmann, R., C. Oettel, et al. (2009). "Met receptor tyrosine kinase transactivation is involved in proteinase-activated receptor 2-mediated hepatocellular carcinoma cell invasion." Carcinogenesis.

Kawabata, A., M. Kinoshita, et al. (2001). "The protease-activated receptor-2 agonist induces gastric mucus secretion and mucosal cytoprotection." J Clin Invest **107**(11): 1443-50.

Kawabata, A., R. Kuroda, et al. (1998). "Increased vascular permeability by a specific agonist of protease-activated receptor-2 in rat hindpaw." Br J Pharmacol **125**(3): 419-22.

Kawagoe, J., T. Takizawa, et al. (2002). "Effect of protease-activated receptor-2 deficiency on allergic dermatitis in the mouse ear." Jpn J Pharmacol **88**(1): 77-84.

Kaziro, Y., H. Itoh, et al. (1991). "Structure and function of signal-transducing GTP-binding proteins." Annu Rev Biochem **60**: 349-400.

Kelly, D. J., A. J. Cox, et al. (2004). "Platelet-derived growth factor receptor transactivation mediates the trophic effects of angiotensin II in vivo." Hypertension **44**(2): 195-202.

Kim, I., H. G. Kim, et al. (2000). "Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion." Circ Res **86**(9): 952-9.

Kim, I., H. G. Kim, et al. (2000). "Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway." Circ Res **86**(1): 24-9.

Kim, I., S. O. Moon, et al. (2001). "Angiopoietin-1 reduces VEGF-stimulated leukocyte adhesion to endothelial cells by reducing ICAM-1, VCAM-1, and E-selectin expression." Circ Res **89**(6): 477-9.

Kim, I., A. M. Ryan, et al. (1999). "Constitutive expression of VEGF, VEGFR-1, and VEGFR-2 in normal eyes." Invest Ophthalmol Vis Sci **40**(9): 2115-21.

Kim, K., N. Thu, et al. (2003). "Domains of estrogen receptor alpha (ERalpha) required for ERalpha/Sp1-mediated activation of GC-rich promoters by estrogens and antiestrogens in breast cancer cells." Mol Endocrinol **17**(5): 804-17.

Kjalke, M., D. M. Monroe, et al. (1998). "The effects of activated factor VII in a cell-based model for tissue factor-initiated coagulation." Blood Coagul Fibrinolysis **9 Suppl 1**: S21-5.

Klein, R., S. M. Meuer, et al. (1995). "Retinal microaneurysm counts and 10-year progression of diabetic retinopathy." Arch Ophthalmol **113**(11): 1386-91.

Koivunen, E., A. Ristimaki, et al. (1991). "Tumor-associated trypsin participates in cancer cell-mediated degradation of extracellular matrix." Cancer Res **51**(8): 2107-12.

Kolch, W. (2000). "Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions." Biochem J **351 Pt 2**: 289-305.

Koleske, A. J., D. Baltimore, et al. (1995). "Reduction of caveolin and caveolae in oncogenically transformed cells." Proc Natl Acad Sci U S A **92**(5): 1381-5.

Koshikawa, N., S. Hasegawa, et al. (1998). "Expression of trypsin by epithelial cells of various tissues, leukocytes, and neurons in human and mouse." Am J Pathol **153**(3): 937-44.

Koshikawa, N., Y. Nagashima, et al. (1997). "Expression of trypsin in vascular endothelial cells." FEBS Lett **409**(3): 442-8.

Koshikawa, N., H. Yasumitsu, et al. (1994). "Identification of one- and two-chain forms of trypsinogen 1 produced by a human gastric adenocarcinoma cell line." Biochem J **303 (Pt 1)**: 187-90.

Kovac, C. R., A. Emelyanov, et al. (2000). "BSAP (Pax5)-importin alpha 1 (Rch1) interaction identifies a nuclear localization sequence." J Biol Chem **275**(22): 16752-7.

Krishna, M. T., A. Chauhan, et al. (2001). "Inhibition of mast cell tryptase by inhaled APC 366 attenuates allergen-induced late-phase airway obstruction in asthma." J Allergy Clin Immunol **107**(6): 1039-45.

Kumar, A. P. and A. P. Butler (1999). "Enhanced Sp1 DNA-binding activity in murine keratinocyte cell lines and epidermal tumors." Cancer Lett **137**(2): 159-65.

Kurten, R. C., D. L. Cadena, et al. (1996). "Enhanced degradation of EGF receptors by a sorting nexin, SNX1." Science **272**(5264): 1008-10.

Kurten, R. C., A. D. Eddington, et al. (2001). "Self-assembly and binding of a sorting nexin to sorting endosomes." J Cell Sci **114**(Pt 9): 1743-56.

Lamaze, C., A. Dujeancourt, et al. (2001). "Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway." Molecular Cell **7**(3): 661-71.

Lania, L., B. Majello, et al. (1997). "Transcriptional regulation by the Sp family proteins." Int J Biochem Cell Biol **29**(12): 1313-23.

Le, P. U., G. Guay, et al. (2002). "Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum." J Biol Chem **277**(5): 3371-9.

Lee, D. K., A. J. Lanca, et al. (2004). "Agonist-independent nuclear localization of the Apelin, angiotensin AT1, and bradykinin B2 receptors." J Biol Chem **279**(9): 7901-8.

Lefkowitz, R. J. (1993). "G protein-coupled receptor kinases." Cell **74**(3): 409-12.

Lefkowitz, R. J. and M. G. Caron (1988). "Adrenergic receptors. Models for the study of receptors coupled to guanine nucleotide regulatory proteins." J Biol Chem **263**(11): 4993-6.

Lefkowitz, R. J. and E. J. Whalen (2004). "beta-arrestins: traffic cops of cell signaling." Curr Opin Cell Biol **16**(2): 162-8.

Leslie, D. M., W. Zhang, et al. (2004). "Characterization of karyopherin cargoes reveals unique mechanisms of Kap121p-mediated nuclear import." Mol Cell Biol **24**(19): 8487-503.

Leung, D. W., G. Cachianes, et al. (1989). "Vascular endothelial growth factor is a secreted angiogenic mitogen." Science **246**(4935): 1306-9.

Levitzki, A. (1988). "From epinephrine to cyclic AMP." Science **241**(4867): 800-6.

Levy, A. P., N. S. Levy, et al. (1995). "Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia." J Biol Chem **270**(22): 13333-40.

Lin, S. Y., K. Makino, et al. (2001). "Nuclear localization of EGF receptor and its potential new role as a transcription factor." Nat Cell Biol **3**(9): 802-8.

Ling, T. L. and J. Stone (1988). "The development of astrocytes in the cat retina: evidence of migration from the optic nerve." Brain Res Dev Brain Res **44**(1): 73-85.

Lobie, P. E., T. J. Wood, et al. (1994). "Nuclear translocation and anchorage of the growth hormone receptor." J Biol Chem **269**(50): 31735-46.

Lu, D., H. Yang, et al. (1998). "Angiotensin II-induced nuclear targeting of the angiotensin type 1 (AT1) receptor in brain neurons." Endocrinology **139**(1): 365-75.

Luo, W., Y. Wang, et al. (2006). "Jab1, a novel protease-activated receptor-2 (PAR-2)-interacting protein, is involved in PAR-2-induced activation of activator protein-1." J Biol Chem **281**(12): 7927-36.

Lutsenko, S. V., S. M. Kiselev, et al. (2003). "Molecular mechanisms of tumor angiogenesis." Biochemistry (Mosc) **68**(3): 286-300.

Luttrell, L. M., Y. Daaka, et al. (1997). "G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases." J Biol Chem **272**(50): 31648-56.

Magazine, H. I., J. M. King, et al. (1996). "Protease activated receptors modulate aortic vascular tone." Int J Cardiol **53 Suppl**: S75-80.

Maher, P. A. (1996). "Nuclear Translocation of fibroblast growth factor (FGF) receptors in response to FGF-2." J Cell Biol **134**(2): 529-36.

Maisonpierre, P. C., M. Goldfarb, et al. (1993). "Distinct rat genes with related profiles of expression define a TIE receptor tyrosine kinase family." Oncogene **8**(6): 1631-7.

Maisonpierre, P. C., C. Suri, et al. (1997). "Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis." Science **277**(5322): 55-60.

Majerus, P. W. (1992). "Inositol phosphate biochemistry." Annu Rev Biochem **61**: 225-50.

Marchese, A. and J. L. Benovic (2001). "Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting." J Biol Chem **276**(49): 45509-12.

Marin, M., A. Karis, et al. (1997). "Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation." Cell **89**(4): 619-28.

Marrache, A. M., F. Gobeil, Jr., et al. (2002). "Proinflammatory gene induction by platelet-activating factor mediated via its cognate nuclear receptor." J Immunol **169**(11): 6474-81.

Marrache, A. M., F. Gobeil, et al. (2005). "Intracellular signaling of lipid mediators via cognate nuclear G protein-coupled receptors." Endothelium **12**(1-2): 63-72.

Mastrangelo, I. A., A. J. Courey, et al. (1991). "DNA looping and Sp1 multimer links: a mechanism for transcriptional synergism and enhancement." Proc Natl Acad Sci U S A **88**(13): 5670-4.

Matsumoto, T., K. Ishida, et al. (2009). "Mechanisms underlying enhanced vasorelaxant response to protease-activated receptor 2-activating peptide in type 2 diabetic Goto-Kakizaki rat mesenteric artery." Peptides **30**(9): 1729-34.

Mazure, N. M., M. C. Brahimi-Horn, et al. (2003). "Protein kinases and the hypoxia-inducible factor-1, two switches in angiogenesis." Curr Pharm Des **9**(7): 531-41.

McCarthy, M. J., I. M. Loftus, et al. (1999). "Angiogenesis and the atherosclerotic carotid plaque: an association between symptomatology and plaque morphology." J Vasc Surg **30**(2): 261-8.

Mertani, H. C., M. Raccurt, et al. (2003). "Nuclear translocation and retention of growth hormone." Endocrinology **144**(7): 3182-95.

Miaczynska, M., S. Christoforidis, et al. (2004). "APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment." Cell **116**(3): 445-56.

Miki, H., Y. Okada, et al. (2005). "Analysis of the kinesin superfamily: insights into structure and function." Trends in Cell Biology **15**(9): 467-76.

Milia, A. F., M. B. Salis, et al. (2002). "Protease-activated receptor-2 stimulates angiogenesis and accelerates hemodynamic recovery in a mouse model of hindlimb ischemia." Circ Res **91**(4): 346-52.

Milia, A. F., M. B. Salis, et al. (2002). "Protease-activated receptor-2 stimulates angiogenesis and accelerates hemodynamic recovery in a mouse model of hindlimb ischemia." Circulation Research **91**(4): 346-52.

Mirre, C., L. Monlauzeur, et al. (1996). "Detergent-resistant membrane microdomains from Caco-2 cells do not contain caveolin." Am J Physiol **271**(3 Pt 1): C887-94.

Mirza, H., V. Yatsula, et al. (1996). "The proteinase activated receptor-2 (PAR-2) mediates mitogenic responses in human vascular endothelial cells." J Clin Invest **97**(7): 1705-14.

Mitchell, R., D. McCulloch, et al. (1998). "Rhodopsin-family receptors associate with small G proteins to activate phospholipase D." Nature **392**(6674): 411-4.

Moffatt, J. D. and T. M. Cocks (1998). "Endothelium-dependent and -independent responses to protease-activated receptor-2 (PAR-2) activation in mouse isolated renal arteries." Br J Pharmacol **125**(4): 591-4.

Morris, D. R., Y. Ding, et al. (2006). "Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells." Cancer Res **66**(1): 307-14.

Nagata, K., R. A. Guggenheimer, et al. (1983). "Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA." Proc Natl Acad Sci U S A **80**(20): 6177-81.

Nanevicz, T., L. Wang, et al. (1996). "Thrombin receptor activating mutations. Alteration of an extracellular agonist recognition domain causes constitutive signaling." J Biol Chem **271**(2): 702-6.

Napoli, C., C. Cicala, et al. (2000). "Protease-activated receptor-2 modulates myocardial ischemia-reperfusion injury in the rat heart." Proc Natl Acad Sci U S A **97**(7): 3678-83.

Nebl, G., N. Mermod, et al. (1994). "Post-transcriptional down-regulation of expression of transcription factor NF1 by Ha-ras oncogene." J Biol Chem **269**(10): 7371-8.

Neer, E. J. (1995). "Heterotrimeric G proteins: organizers of transmembrane signals." Cell **80**(2): 249-57.

Nehring, R. B., H. P. Horikawa, et al. (2000). "The metabotropic GABAB receptor directly interacts with the activating transcription factor 4." J Biol Chem **275**(45): 35185-91.

Neubig, R. R. and D. P. Siderovski (2002). "Regulators of G-protein signalling as new central nervous system drug targets." Nat Rev Drug Discov **1**(3): 187-97.

Neufeld, G., T. Cohen, et al. (1999). "Vascular endothelial growth factor (VEGF) and its receptors." FASEB J **13**(1): 9-22.

Nielsen, E., F. Severin, et al. (1999). "Rab5 regulates motility of early endosomes on microtubules.[see comment]." Nature Cell Biology **1**(6): 376-82.

Nishiyama, K., B. D. Trapp, et al. (1999). "Caveolin-3 upregulation activates beta-secretase-mediated cleavage of the amyloid precursor protein in Alzheimer's disease." J Neurosci **19**(15): 6538-48.

Nystedt, S., K. Emilsson, et al. (1994). "Molecular cloning of a potential proteinase activated receptor." Proc Natl Acad Sci U S A **91**(20): 9208-12.

Nystedt, S., A. K. Larsson, et al. (1995). "The mouse proteinase-activated receptor-2 cDNA and gene. Molecular cloning and functional expression." J Biol Chem **270**(11): 5950-55.

Nystedt, S., V. Ramakrishnan, et al. (1996). "The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor." J Biol Chem **271**(25): 14910-5.

O'Brien, E. R., M. R. Garvin, et al. (1994). "Angiogenesis in human coronary atherosclerotic plaques." Am J Pathol **145**(4): 883-94.

Oberst, M., J. Anders, et al. (2001). "Matriptase and HAI-1 are expressed by normal and malignant epithelial cells in vitro and in vivo." Am J Pathol **158**(4): 1301-11.

Offermanns, S., K. L. Laugwitz, et al. (1994). "G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets." Proc Natl Acad Sci U S A **91**(2): 504-8.

Okamoto, T., M. Nishibori, et al. (2001). "The effects of stimulating protease-activated receptor-1 and -2 in A172 human glioblastoma." J Neural Transm **108**(2): 125-40.

Okamoto, T., A. Schlegel, et al. (1998). "Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane." J Biol Chem **273**(10): 5419-22.

Olsen, M. W., C. D. Ley, et al. (2006). "Angiopoietin-4 inhibits angiogenesis and reduces interstitial fluid pressure." Neoplasia **8**(5): 364-72.

Opitz, O. G. and A. K. Rustgi (2000). "Interaction between Sp1 and cell cycle regulatory proteins is important in transactivation of a differentiation-related gene." Cancer Res **60**(11): 2825-30.

Orlandi, P. A. and P. H. Fishman (1998). "Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains." J Cell Biol **141**(4): 905-15.

Orth, J. D., E. W. Krueger, et al. (2006). "A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization." Cancer Research **66**(7): 3603-10.

Palade, G. E. (1953). "An electron microscope study of the mitochondrial structure." J Histochem Cytochem **1**(4): 188-211.

Park, G. H., S. J. Jeon, et al. (2009). "Essential role of mitogen-activated protein kinase pathways in protease activated receptor 2-mediated nitric-oxide production from rat primary astrocytes." Nitric Oxide **21**(2): 110-9.

Partanen, J., E. Armstrong, et al. (1992). "A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains." Mol Cell Biol **12**(4): 1698-707.

Pascal, E. and R. Tjian (1991). "Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism." Genes & Development **5**(9): 1646-56.

Patan, S. (1998). "TIE1 and TIE2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth." Microvasc Res **56**(1): 1-21.

Pearson, G., F. Robinson, et al. (2001). "Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions." Endocr Rev **22**(2): 153-83.

Pelham, H. R. (2002). "Insights from yeast endosomes." Curr Opin Cell Biol **14**(4): 454-62.

Pemberton, L. F. and B. M. Paschal (2005). "Mechanisms of receptor-mediated nuclear import and nuclear export." Traffic **6**(3): 187-98.

Peter, B. J., H. M. Kent, et al. (2004). "BAR domains as sensors of membrane curvature: the amphiphysin BAR structure." Science **303**(5657): 495-9.

Pfister, K. K., P. R. Shah, et al. (2006). "Genetic analysis of the cytoplasmic dynein subunit families." PLoS Genetics **2**(1): e1.

Philipsen, S. and G. Suske (1999). "A tale of three fingers: the family of mammalian Sp/XKLF transcription factors." Nucleic Acids Res **27**(15): 2991-3000.

Phillips, S. A., V. A. Barr, et al. (2001). "Identification and characterization of SNX15, a novel sorting nexin involved in protein trafficking." J Biol Chem **276**(7): 5074-84.

Pizurki, L., Z. Zhou, et al. (2003). "Angiopoietin-1 inhibits endothelial permeability, neutrophil adherence and IL-8 production." Br J Pharmacol **139**(2): 329-36.

Plouet, J. and D. Gospodarowicz (1989). "Transforming growth factor beta-1 positively modulates the bioactivity of fibroblast growth factor on corneal endothelial cells." J Cell Physiol **141**(2): 392-9.

Pollard, V. W., W. M. Michael, et al. (1996). "A novel receptor-mediated nuclear protein import pathway." Cell **86**(6): 985-94.

Pouyssegur, J., V. Volmat, et al. (2002). "Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling." Biochem Pharmacol **64**(5-6): 755-63.

Presley, J. F., S. Mayor, et al. (1997). "Bafilomycin A1 treatment retards transferrin receptor recycling more than bulk membrane recycling." J Biol Chem **272**(21): 13929-36.

Puri, M. C., J. Rossant, et al. (1995). "The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells." EMBO J **14**(23): 5884-91.

Radhakrishna, H. and J. G. Donaldson (1997). "ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway." Journal of Cell Biology **139**(1): 49-61.

Razani, B., J. A. Engelman, et al. (2001). "Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities." J Biol Chem **276**(41): 38121-38.

Re, R. (1999). "The nature of intracrine peptide hormone action." Hypertension **34**(4 Pt 1): 534-8.

Re, R. and M. Parab (1984). "Effect of angiotensin II on RNA synthesis by isolated nuclei." Life Sci **34**(7): 647-51.

Reilly, J. F., E. Mizukoshi, et al. (2004). "Ligand dependent and independent internalization and nuclear translocation of fibroblast growth factor (FGF) receptor 1." DNA Cell Biol **23**(9): 538-48.

Risau, W. (1997). "Mechanisms of angiogenesis." Nature **386**(6626): 671-4.

Risau, W. and I. Flamme (1995). "Vasculogenesis." Annu Rev Cell Dev Biol **11**: 73-91.

Robinson, M. J. and M. H. Cobb (1997). "Mitogen-activated protein kinase pathways." Curr Opin Cell Biol **9**(2): 180-6.

Rosenfeld, J. L., B. J. Knoll, et al. (2002). "Regulation of G-protein-coupled receptor activity by rab GTPases." Receptors Channels **8**(2): 87-97.

Roy, S. S., M. Saifeddine, et al. (1998). "Dual endothelium-dependent vascular activities of proteinase-activated receptor-2-activating peptides: evidence for receptor heterogeneity." Br J Pharmacol **123**(7): 1434-40.

Sabharanjak, S., P. Sharma, et al. (2002). "GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway." Developmental Cell **2**(4): 411-23.

Safe, S. and K. Kim (2004). "Nuclear receptor-mediated transactivation through interaction with Sp proteins." Prog Nucleic Acid Res Mol Biol **77**: 1-36.

Saifeddine, M., B. al-Ani, et al. (1996). "Rat proteinase-activated receptor-2 (PAR-2): cDNA sequence and activity of receptor-derived peptides in gastric and vascular tissue." Br J Pharmacol **118**(3): 521-30.

Salman, H., A. Abu-Arish, et al. (2005). "Nuclear localization signal peptides induce molecular delivery along microtubules." Biophysical Journal **89**(3): 2134-45.

Sandercoe, T. M., M. C. Madigan, et al. (1999). "Astrocyte proliferation during development of the human retinal vasculature." Exp Eye Res **69**(5): 511-23.

Santiago, F. S., H. Ishii, et al. (2007). "Yin Yang-1 inhibits vascular smooth muscle cell growth and intimal thickening by repressing p21WAF1/Cip1 transcription and p21WAF1/Cip1-Cdk4-cyclin D1 assembly." Circ Res **101**(2): 146-55.

Sapieha, P., M. Sirinyan, et al. (2008). "The succinate receptor GPR91 in neurons has a major role in retinal angiogenesis." Nat Med **14**(10): 1067-76.

Sarret, P., D. Nouel, et al. (1999). "Receptor-mediated internalization is critical for the inhibition of the expression of growth hormone by somatostatin in the pituitary cell line AtT-20." J Biol Chem **274**(27): 19294-300.

Sato, T. N., Y. Qin, et al. (1993). "Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system." Proc Natl Acad Sci U S A **90**(20): 9355-8.

Sato, T. N., Y. Tozawa, et al. (1995). "Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation." Nature **376**(6535): 70-4.

Schaffner, F. and W. Ruf (2009). "Tissue Factor and PAR2 Signaling in the Tumor Microenvironment." Arterioscler Thromb Vasc Biol.

Scherer, P. E. and M. P. Lisanti (1997). "Association of phosphofructokinase-M with caveolin-3 in differentiated skeletal myotubes. Dynamic regulation by extracellular glucose and intracellular metabolites." J Biol Chem **272**(33): 20698-705.

Schnurch, H. and W. Risau (1993). "Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage." Development **119**(3): 957-68.

Schroer, T. A. (2004). "Dynactin." Annual Review of Cell & Developmental Biology **20**: 759-79.

Schultheiss, M., B. Neumcke, et al. (1997). "Endogenous trypsin receptors in Xenopus oocytes: linkage to internal calcium stores." Cellular & Molecular Life Sciences **53**(10): 842-9.

Schwab, W., F. Galbiati, et al. (1999). "Characterisation of caveolins from cartilage: expression of caveolin-1, -2 and -3 in chondrocytes and in alginate cell culture of the rat tibia." Histochem Cell Biol **112**(1): 41-9.

Scott, C. F., L. D. Silver, et al. (1985). "Cleavage of human high molecular weight kininogen by factor XIa in vitro. Effect on structure and function." J Biol Chem **260**(19): 10856-63.

Seachrist, J. L., P. H. Anborgh, et al. (2000). "beta 2-adrenergic receptor internalization, endosomal sorting, and plasma membrane recycling are regulated by rab GTPases." J Biol Chem **275**(35): 27221-8.

Seachrist, J. L. and S. S. Ferguson (2003). "Regulation of G protein-coupled receptor endocytosis and trafficking by Rab GTPases." Life Sci **74**(2-3): 225-35.

Seger, R. and E. G. Krebs (1995). "The MAPK signaling cascade." Faseb J **9**(9): 726-35.

Senbonmatsu, T., T. Saito, et al. (2003). "A novel angiotensin II type 2 receptor signaling pathway: possible role in cardiac hypertrophy." EMBO J **22**(24): 6471-82.

Senger, B., G. Simos, et al. (1998). "Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p." Embo J **17**(8): 2196-207.

Senger, D. R., S. J. Galli, et al. (1983). "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid." Science **219**(4587): 983-5.

Shenoy, S. K. and R. J. Lefkowitz (2005). "Seven-transmembrane receptor signaling through beta-arrestin." Sci STKE **2005**(308): cm10.

Shenoy, S. K., P. H. McDonald, et al. (2001). "Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin." Science **294**(5545): 1307-13.

Shi, X., B. Gangadharan, et al. (2004). "Protease-activated receptors (PAR1 and PAR2) contribute to tumor cell motility and metastasis." Mol Cancer Res **2**(7): 395-402.

Shibuta, K., M. Mori, et al. (2002). "Regional expression of CXCL12/CXCR4 in liver and hepatocellular carcinoma and cell-cycle variation during in vitro differentiation." Jpn J Cancer Res **93**(7): 789-97.

Shibuya, M., S. Yamaguchi, et al. (1990). "Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family." Oncogene **5**(4): 519-24.

Shima, D. T., M. Kuroki, et al. (1996). "The mouse gene for vascular endothelial growth factor. Genomic structure, definition of the transcriptional unit, and characterization of transcriptional and post-transcriptional regulatory sequences." J Biol Chem **271**(7): 3877-83.

Shpacovitch, V. M., T. Brzoska, et al. (2002). "Agonists of proteinase-activated receptor 2 induce cytokine release and activation of nuclear transcription factor kappaB in human dermal microvascular endothelial cells." J Invest Dermatol **118**(2): 380-5.

Shpacovitch, V. M., G. Varga, et al. (2004). "Agonists of proteinase-activated receptor-2 modulate human neutrophil cytokine secretion, expression of cell adhesion molecules, and migration within 3-D collagen lattices." J Leukoc Biol **76**(2): 388-98.

Silverman, E. S., L. M. Khachigian, et al. (1997). "Inducible PDGF A-chain transcription in smooth muscle cells is mediated by Egr-1 displacement of Sp1 and Sp3." Am J Physiol **273**(3 Pt 2): H1415-26.

Smale, S. T. and J. T. Kadonaga (2003). "The RNA polymerase II core promoter." Annu Rev Biochem **72**: 449-79.

Smart, E. J., Y. Ying, et al. (1996). "A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane." J Biol Chem **271**(46): 29427-35.

Song, K. S., P. E. Scherer, et al. (1996). "Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins." J Biol Chem **271**(25): 15160-5.

Souaze, F., W. Rostene, et al. (1997). "Neurotensin agonist induces differential regulation of neurotensin receptor mRNA. Identification of distinct transcriptional and post-transcriptional mechanisms." J Biol Chem **272**(15): 10087-94.

Spano, J. P., F. Andre, et al. (2004). "Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: pattern of expression and correlation with outcome." Ann Oncol **15**(4): 613-7.

Stachowiak, M. K., P. A. Maher, et al. (1996). "Nuclear localization of functional FGF receptor 1 in human astrocytes suggests a novel mechanism for growth factor action." Brain Res Mol Brain Res **38**(1): 161-5.

Staehelin, M. and P. Simons (1982). "Rapid and reversible disappearance of beta-adrenergic cell surface receptors." Embo J **1**(2): 187-90.

Steinhoff, M., J. Buddenkotte, et al. (2005). "Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response." Endocr Rev **26**(1): 1-43.

Steinhoff, M., C. U. Corvera, et al. (1999). "Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase." Exp Dermatol **8**(4): 282-94.

Sun, J. M., H. Y. Chen, et al. (2002). "The transcriptional repressor Sp3 is associated with CK2-phosphorylated histone deacetylase 2." J Biol Chem **277**(39): 35783-6.

Suri, C., P. F. Jones, et al. (1996). "Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis." Cell **87**(7): 1171-80.

Surya, A., J. M. Stadel, et al. (1998). "Evidence for multiple, biochemically distinguishable states in the G protein-coupled receptor, rhodopsin." Trends Pharmacol Sci **19**(7): 243-7.

Suske, G. (1999). "The Sp-family of transcription factors." Gene **238**(2): 291-300.

Swift, S., P. J. Sheridan, et al. (2000). "PAR1 thrombin receptor-G protein interactions. Separation of binding and coupling determinants in the galpha subunit." J Biol Chem **275**(4): 2627-35.

Takai, Y., T. Sasaki, et al. (2001). "Small GTP-binding proteins." Physiol Rev **81**(1): 153-208.

Takeuchi, T., J. L. Harris, et al. (2000). "Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates." J Biol Chem **275**(34): 26333-42.

Tang, Z., P. E. Scherer, et al. (1996). "Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle." J Biol Chem **271**(4): 2255-61.

Tanowitz, M. and M. Von Zastrow (2002). "Ubiquitination-independent trafficking of G protein-coupled receptors to lysosomes." J Biol Chem **277**(52): 50219-22.

Teasdale, R. D., D. Loci, et al. (2001). "A large family of endosome-localized proteins related to sorting nexin 1." Biochem J **358**(Pt 1): 7-16.

Thomas, M. C. and C. M. Chiang (2006). "The general transcription machinery and general cofactors." Crit Rev Biochem Mol Biol **41**(3): 105-78.

Tischer, E., R. Mitchell, et al. (1991). "The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing." J Biol Chem **266**(18): 11947-54.

Toews, M. L. and J. P. Perkins (1984). "Agonist-induced changes in beta-adrenergic receptors on intact cells." J Biol Chem **259**(4): 2227-35.

Valenzuela, D. M., J. A. Griffiths, et al. (1999). "Angiopoietins 3 and 4: diverging gene counterparts in mice and humans." Proc Natl Acad Sci U S A **96**(5): 1904-9.

van der Merwe, J. Q., F. Moreau, et al. (2009). "Protease-activated receptor-2 stimulates intestinal epithelial chloride transport through activation of PLC and selective PKC isoforms." Am J Physiol Gastrointest Liver Physiol **296**(6): G1258-66.

van der Poll, T. (2008). "Tissue factor as an initiator of coagulation and inflammation in the lung." Crit Care **12 Suppl 6**: S3.

Vargesson, N. and E. Laufer (2001). "Smad7 misexpression during embryonic angiogenesis causes vascular dilation and malformations independently of vascular smooth muscle cell function." Dev Biol **240**(2): 499-516.

Vart, R. J., L. L. Nikitenko, et al. (2007). "Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6 and G-protein-coupled receptor regulate angiopoietin-2 expression in lymphatic endothelial cells." Cancer Res **67**(9): 4042-51.

Ventura, C., M. Maioli, et al. (1998). "Nuclear opioid receptors activate opioid peptide gene transcription in isolated myocardial nuclei." J Biol Chem **273**(22): 13383-6.

Vergnolle, N. (1999). "Proteinase-activated receptor-2-activating peptides induce leukocyte rolling, adhesion, and extravasation in vivo." J Immunol **163**(9): 5064-9.

Vergnolle, N., M. D. Hollenberg, et al. (1999). "Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw." Br J Pharmacol **127**(5): 1083-90.

Vergnolle, N., W. K. Macnaughton, et al. (1998). "Proteinase-activated receptor 2 (PAR2)-activating peptides: identification of a receptor distinct from PAR2 that regulates intestinal transport." Proc Natl Acad Sci U S A **95**(13): 7766-71.

Verrall, S., M. Ishii, et al. (1997). "The thrombin receptor second cytoplasmic loop confers coupling to Gq-like G proteins in chimeric receptors. Additional evidence for a common transmembrane signaling and G protein coupling mechanism in G protein-coupled receptors." J Biol Chem **272**(11): 6898-902.

von Zastrow, M. and B. K. Kobilka (1992). "Ligand-regulated internalization and recycling of human beta 2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors." J Biol Chem **267**(5): 3530-8.

Vu, T. K., D. T. Hung, et al. (1991). "Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation." Cell **64**(6): 1057-68.

Wang, S. C., H. C. Lien, et al. (2004). "Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2." Cancer Cell **6**(3): 251-61.

Wang, Y., Y. Zhou, et al. (2002). "Down-regulation of protease-activated receptor-1 is regulated by sorting nexin 1." Mol Biol Cell **13**(6): 1965-76.

Wang, Z. Y., P. Wang, et al. (2009). "Role of mast cells and protease-activated receptor-2 in cyclooxygenase-2 expression in urothelial cells." Am J Physiol Regul Integr Comp Physiol.

Waters, C. M., B. Saatian, et al. (2006). "Integrin signalling regulates the nuclear localization and function of the lysophosphatidic acid receptor-1 (LPA1) in mammalian cells." Biochem J **398**(1): 55-62.

Weber, C. C., H. Cai, et al. (2005). "Effects of protein and gene transfer of the angiopoietin-1 fibrinogen-like receptor-binding domain on endothelial and vessel organization." J Biol Chem **280**(23): 22445-53.

Weis, K. (2003). "Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle." Cell **112**(4): 441-51.

Weitzmann, M. N. and N. Savage (1992). "Nuclear internalisation and DNA binding activities of interleukin-1, interleukin-1 receptor and interleukin-1/receptor complexes." Biochem Biophys Res Commun **187**(2): 1166-71.

White, J. H., R. A. McIllhinney, et al. (2000). "The GABAB receptor interacts directly with the related transcription factors CREB2 and ATFx." Proc Natl Acad Sci U S A **97**(25): 13967-72.

Wiegand, U., S. Corbach, et al. (1993). "Cloning of the cDNA encoding human brain trypsinogen and characterization of its product." Gene **136**(1-2): 167-75.

Williams, R., T. Schluter, et al. (2004). "Sorting nexin 17 accelerates internalization yet retards degradation of P-selectin." Mol Biol Cell **15**(7): 3095-105.

Witherow, D. S., T. R. Garrison, et al. (2004). "beta-Arrestin inhibits NF-kappaB activity by means of its interaction with the NF-kappaB inhibitor IkappaBalpha." Proc Natl Acad Sci U S A **101**(23): 8603-7.

Worby, C. A. and J. E. Dixon (2002). "Sorting out the cellular functions of sorting nexins." Nat Rev Mol Cell Biol **3**(12): 919-31.

Xu, Q., Y. S. Ji, et al. (2000). "Sp1 increases expression of cyclooxygenase-2 in hypoxic vascular endothelium. Implications for the mechanisms of aortic aneurysm and heart failure." J Biol Chem **275**(32): 24583-9.

Xu, W. F., H. Andersen, et al. (1998). "Cloning and characterization of human protease-activated receptor 4." Proc Natl Acad Sci U S A **95**(12): 6642-6.

Xu, Y., H. Hortsman, et al. (2001). "SNX3 regulates endosomal function through its PX-domain-mediated interaction with PtdIns(3)P." Nat Cell Biol **3**(7): 658-66.

Xu, Y., Y. J. Liu, et al. (2004). "Angiopoietin-3 inhibits pulmonary metastasis by inhibiting tumor angiogenesis." Cancer Res **64**(17): 6119-26.

Yancopoulos, G. D., S. Davis, et al. (2000). "Vascular-specific growth factors and blood vessel formation." Nature **407**(6801): 242-8.

Yeung, P. L., L. Y. Chen, et al. (2008). "Daxx contains two nuclear localization signals and interacts with importin alpha3." J Cell Biochem **103**(2): 456-70.

Yu, R. and P. M. Hinkle (1997). "Desensitization of thyrotropin-releasing hormone receptor-mediated responses involves multiple steps." Journal of Biological Chemistry **272**(45): 28301-7.

Zerial, M. and H. McBride (2001). "Rab proteins as membrane organizers." Nat Rev Mol Cell Biol **2**(2): 107-17.

Zhao, S., K. Venkatasubbarao, et al. (2003). "Requirement of a specific Sp1 site for histone deacetylase-mediated repression of transforming growth factor beta Type II receptor expression in human pancreatic cancer cells." Cancer Res **63**(10): 2624-30.

Zheng, B., Y. C. Ma, et al. (2001). "RGS-PX1, a GAP for GalphaS and sorting nexin in vesicular trafficking." Science **294**(5548): 1939-42.

Zhong, Q., C. S. Lazar, et al. (2002). "Endosomal localization and function of sorting nexin 1." Proc Natl Acad Sci U S A **99**(10): 6767-72.

Zhu, T., F. Gobeil, et al. (2006). "Intracrine signaling through lipid mediators and their cognate nuclear G-protein-coupled receptors: a paradigm based on PGE2, PAF, and LPA1 receptors." Can J Physiol Pharmacol **84**(3-4): 377-91.

Zhu, T., F. Sennlaub, et al. (2006). "Proangiogenic effects of protease-activated receptor 2 are tumor necrosis factor-alpha and consecutively Tie2 dependent." Arterioscler Thromb Vasc Biol **26**(4): 744-50.