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Intracellular Trafficking of Protease – Activated Receptor 2 (PAR2) by Members of Sorting Nexins Family

par

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Intracellular Trafficking of Protease – Activated Receptor 2 (PAR2) by Members of Sorting Nexins Family

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RÉSUMÉ

Le dogme voulant que les récepteurs couplés aux protéines G (GPCRs) activent des voies de signalisation seulement lorsqu'ils sont localisés à la membrane plasmatique, a récemment été remis en question. Des données récentes indiquent que certains GPCRs peuvent également induire une réponse intracellulaire à partir des compartiments intracellulaires dont le noyau.

Les récepteurs activés par la protéase (PAR) sont des membres de la famille GPCR. Les PARs sont activés par le clivage de la partie N–terminale du récepteur ce qui permet au ligand attaché sur le récepteur de se lier à sa poche réceptrice. Quatre PARs ont été décrits : PAR1, PAR2, PAR3 et PAR4. PAR2 peut susciter des effets mitogéniques et participer aux processus comme l'angiogenèse et l'inflammation. Alors que beaucoup d'effets intracellulaires de PAR2 peuvent être expliqués lorsqu'il est localisé à la membrane plasmatique, une fonction intracrine de PAR2 a aussi été proposée. Pourtant les mécanismes par lesquels PAR2 peut provoquer l'expression de gènes ciblés sont toujours inconnus.

Le but de notre étude était de vérifier l'existence d'une population nucléaire de PAR2. Nous avons également émis l'hypothèse que les voies activées par l'activation de PAR2 dépendent de sa localization cellulaire. En utilisant des techniques de microscopie confocale et de "Western Blot" nous avons démontré la présence d'une population nucléaire de PAR2. À la suite de la stimulation de PAR2, nous avons observé une augmentation de la translocation du récepteur de la membrane plasmatique au noyau. En

utilisant la technique de "RT – PCR", nous avons observé des rôles différents de PAR2 à la surface de la cellule et du noyau dans l'initiation de l'expression des gènes.

Afin d'identifier les mécanismes responsables de la translocation nucléaire de PAR2, nous avons évalué l'implication des membres de la famille de "*Sorting Nexins* (SNX)" dans la translocation nucléaire de PAR2.

"*Sorting Nexins*" est un groupe de protéines avec des fonctions de transport bien établies. SNX1 et SNX2 ont été identifiés comme responsables du transfert de PAR1 vers les lysosomes. SNX11 n'a pas encore été étudié et nous avons émis l'hypothèse qu'il pourrait être un autre membre de la famille des SNX impliqué dans la signalisation de PAR2.

Pour ce faire, nous avons développé des "*knockdowns*" stables pour SNX1, SNX2 et SNX11 dans les cellules HEK293. En utilisant les essais d'immunofluorescence, "Western Blot" et de cytométrie en flux, nous avons déterminé que tous les trois membres du groupe SNX sont des partenaires d'interaction de PAR2. Toutefois, seul SNX11 se co-localise avec son partenaire au noyau et est responsable de sa translocation nucléaire. Les expériences de "RT - PCR" sur les lignées de cellule de SNXs "*knockdowns*" ont démontré que la fonction de PAR2 nucléaire dépend surtout de SNX11; néanmoins SNX1 et SNX2 peuvent aussi l'influencer, suggérant qu'ils font aussi partie du réseau signalétique de PAR2.

En conclusion, PAR2 est déplacé de la membrane plasmatique à la membrane nucléaire après sa stimulation avec un agoniste. La translocation nucléaire de PAR2 par un mécanisme impliquant SNX11, initie des effets intracellulaires différents de sa signalisation membranaire.

Mots clés : récepteurs couplés à la protéine G, “Sorting Nexins”, récepteurs activés par la protéase, translocation nucléaire, membrane nucléaire, signal nucléaire.

SUMMARY

During the recent years the existing statements that G – protein coupled receptors (GPCRs) are relaying signals only from the plasma membrane have been challenged. It has become clear that some GPCRs can also signal from intracellular compartments and the nucleus. The role and the function of these nuclear GPCRs are subject of intensive investigations.

Protease - activated receptors (PAR) are members of the GPCR family. PARs are activated by the cleavage of the N – terminus of the receptor followed by binding of the tethered ligand on to the receptor. Four PARs have been described: PAR1, PAR2, PAR3 and PAR4. PAR2 can induce mitogenic effects and participate in processes such as angiogenesis and inflammation. While many of the intracellular effects of PAR2 can be explained with its plasma membrane signalling pathway, intracrine effects of PAR2 have also been proposed. However the mechanisms by which PAR2 can induce its target gene expressions are still unknown.

The purpose of our study was to investigate whether a distinct nuclear population of PAR2 exists. We hypothesized that the roles of PAR2 at different cellular compartments are different since signalling pathways depend on subcellular context. Using confocal microscopy and Western blot techniques we were able to demonstrate the presence of a nuclear population of PAR2. Upon stimulation of the cell membrane PAR2, we observed significant translocation of the receptor from the plasma membrane to the nucleus. Using RT – PCR technique we detected diverse roles of cell surface and nuclear PAR2 on triggered gene expression.

In the current study we have attempted to reveal the mechanisms responsible for PAR2 nuclear translocation. We tested the hypothesis that members of the Sorting Nexin (SNX) family are involved in PAR2 nuclear translocation.

Sorting Nexins are a new, large group of proteins with well established cargo functions. SNX1 and SNX2 have been demonstrated to be responsible for lysosomal sorting of PAR1. SNX11 has not been studied yet, and we hypothesized that it may be another SNX involved in PAR2 signalling.

We developed stable knockdowns for SNX1, SNX2 and SNX11 in HEK293 cells. Using immunofluorescence, Western Blot analysis and FACS assays, we determined that all three members of SNX group are interaction partners of PAR2. However only SNX11 co-localized with its partner in the nucleus and is responsible for its nuclear translocation. RT – PCR experiments on SNXs knockdowns cell lines demonstrated that PAR2 nucleus function is mostly dependent on SNX11; nevertheless SNX1 and SNX2 knockdowns can also attenuate it, suggesting that they are part of PAR2 signalling network.

In conclusion, PAR2 is being translocated from the plasma membrane to the nuclear membrane after its stimulation with SLIGKV. PAR2 nucleus translocation triggers intracellular effects different from its cell membrane signalling. SNX11 is the major factor responsible for PAR2 nuclear sorting.

Keywords: G – protein coupled receptors, Sorting Nexins, Protease - activated receptors, nuclear translocation, nuclear membrane, nuclear signalling

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LIST OF ABBREVIATIONS

AP: Activating peptide

CI – M6PR: cation-independent mannose-6-phosphate receptor

CGRPR- Calcitonin Gene – related peptide receptor

GPCR: G – protein coupled receptor

ERK: Extracellular-signal regulated kinases

FACS: Fluorescence Activated Cell Sorting

ER: Endoplasmic reticulum

EGFR: Epidermal Growth Factor Receptor

IB: immuno blot

MAPK: Mitogen-activated protein kinases

PtdIns: Phosphatidylinositol phosphates

PX: Phox domain

PAR: Protease – activated receptor

RT: Room temperature

SP: Substance P

SNX: Sorting Nexins

TGN: Trans-Golgi network

TfnR: Transferrin receptor

TL: Tethered ligand

Vps: Vesicle protein sorting

DEDICATION

With all my profound gratitude to my family

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I. INTRODUCTION

During the last decades the pharmacological approach to creating new and more effective drugs has changed. Previous processes of discovering new chemical substances and testing for therapeutic effect have been removed from the rational drug design. Pharmacological studies have mostly concentrated on discovering new potential targets (Shaaban *et al.,* 2001). Cell surface receptors represent one of the most promising opportunities.

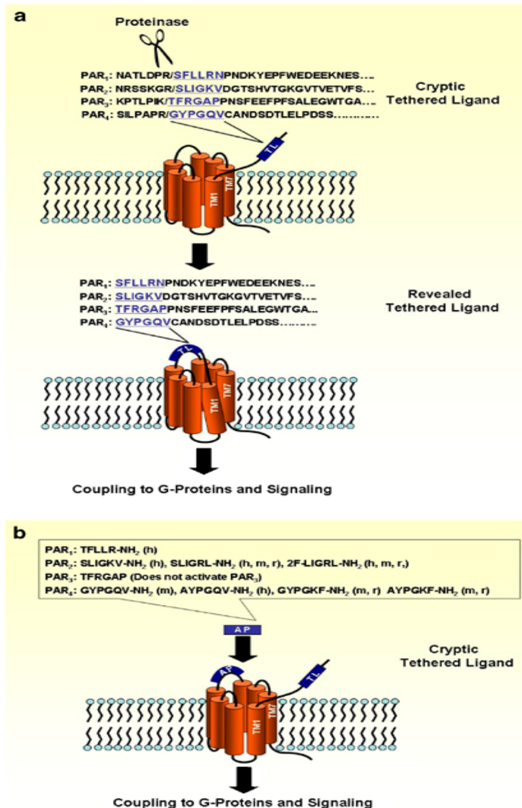
Molecular biology underwent remarkable progress in the methods for studying intracellular activity of the receptors. Numerous intensive investigations in this aspect have changed the old model of one way signalling pathway to a signalling network. Activation of one receptor with agonist can lead to different responses depending on its interactions with other receptors. Recently more and more studies revealed the importance of intracellular trafficking in receptor signalling (Claudinon *et al*., 2007)

G-protein coupled receptors (GPCRs) are the largest family of membrane-bound receptors. They play important roles in the intracellular signalling and are essential in all physiological systems. The last present therapeutic targets for curing pathological diseases such as cancer, cardiac dysfunction, diabetes, central nervous system disorders, obesity, inflammation, and pain (Premont *et al.,* 2007).

1. Protease - Activated Receptors

Protease – Activated Receptors (PARs) are a family of GPCRs. They have been intensively studied during the last 20 years. The family of PARs presently includes four members PAR1, PAR2, PAR3, and PAR4. The main agonist for PAR1, PAR3 and PAR4 in the human body is the coagulant protease thrombin. However, other proteases can cleave these receptors as well and may contribute to their function in vivo (Coughlin *et al.,* 2000). PAR2 is different from the other members. It is activated by multiple trypsin-like serine proteases including trypsin, tryptase, and coagulation proteases upstream of thrombin, factors VIIa and Xa, but not by thrombin (Coughlin and Camerer, 2003).

The hallmark of PAR receptors is their unique mechanism of irreversible activation: proteases cleave at specific site within the extracellular N-terminus to expose a tethered ligand (TL) domain which itself binds to conserved regions in extracellular loop II to initiate signalling. Trypsin cleaves PAR2 at $R^{34} \downarrow S^{35}$ LIGKV to reveal the TL SLIGKV in humans (Nystedt *et al.,* 1994; 1995). Itself SLIGKV can be used as an activation peptide (Figure 1). The PAR-activating peptides (PAR-APs) have been used with great value in investigations to determine specific receptor function in systems where more than one of the PAR family members is expressed. In addition to the cleavage/activation of PARs, proteinases can also negatively regulate functioning through the PARs by 'disarming' the receptor by cleavage at a non-receptor activating site to remove the TL (Tethered Ligand) (Ramachandran *et al.,* 2008) (Figure 1). These truncated receptors nonetheless remain responsive to PAR-APs but are unable to signal in a physiological environment (Ramachandran *et al.,* 2008).



Mechanisms of PAR activation.

(a) Activation of PAR signalling by proteinase-mediated cleavage of receptor N-terminus to reveal tethered ligand (TL).
(b) Activation of PAR signalling by exogenous application of synthetic PAR agonist peptide (AP) without the need for proteolytic revealing of the TL. PAR, proteinase-activated receptor

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Figure 1: Mechanisms of PAR activation

1.1 PAR signalling

Most of G protein – coupled receptors possess similar mechanism of activation and PARs as GPCRs do not differ from them. Upon ligand activation of PARs, conformational changes in the receptor induce interaction with heterotrimeric G proteins. PAR2 couples to Gαq/11, resulting in activation of phospholipase Cβ, production of inositol 1,4,5-trisphosphate and diacylglycerol, mobilization of Ca^{2+} and activation of

protein kinase C. These signalling events are rapidly attenuated and desensitized after repeated stimulation, indicative of receptor desensitization and down-regulation (Cottrell *et al*., 2003).

β-Arrestins 1 and 2 are cytosolic proteins that mediate desensitization and endocytosis of many GPCRs. PAR2 activation induces translocation of β-arrestins to the plasma membrane, where they interact with PAR2 to mediate both desensitization and endocytosis (Dery *et al.,* 1999; DeFea *et al.,* 2000a). The GTPase dynamin mediates detachment of PAR2-containing clathrin-coated pits, and Rab5a mediates distal steps in endocytic trafficking of PAR2 from clathrin-coated pits to early endosomes (Roosterman *et al.,* 2003).

Internalized GPCRs may be degraded or can be recycled. We can find receptors like PARs, which are activated by irreversible proteolysis and for that reason are single-use receptors which are targeted to the lysosomes for degradation (Bohm *et al.,* 1996). Receptors for neuropeptides such as the SP (substance P) receptor are activated by reversible peptide binding, which can be reused, internalized and recycled to the cell surface (Grady *et al.,* 1995).

The down-regulation of receptors is of fundamental importance in terminating signalling. However, little is known about the molecular mechanisms of post-endocytic sorting that target receptors for degradation. Ubiquitination mediates down-regulation of growth factor receptors (Strous *et al.,* 1996) and GPCRs (Hicke *et al.,* 1996; Shenoy *et al.,* 2001). Although ubiquitination of growth factor receptors is important for endocytosis and post-endocytic sorting through proteasome or the multivesicular

body/lysosome pathways, little is known about the mechanism and function of agonist-induced ubiquitination of GPCRs. Upon activation PAR2 is rapidly ubiquitinated within the C-terminus and ubiquitination is required for lysosomal trafficking but not endocytosis (Cottrell *et al.*, 2003).

Sustained signalling requires the mobilization of PAR2 from important stores in the Golgi apparatus or synthesis of intact receptors (Bohm *et al.,* 1996). Rab11a co-localizes in the Golgi apparatus with PAR2. Rab11a mediates both recovery of PAR2 at the cell surface and resensitization of PAR2 signalling (Roosterman *et al.,* 2003). Endocytosis and mitogenic signalling of PAR2 by MAPKs (mitogen-activated protein kinases) are organized into 'signalling modules' by tethering to scaffolding proteins and by direct interactions between the component kinases (Widmann *et al.,* 1999; Pouyssegur *et al.,* 2002). This organization ensures physical segregation of the pathways and allows the same kinase to be used in more than one MAPK module without affecting the function of other modules. Activation of PAR2 induces assembly of a MAPK signalling module by a mechanism that depends on β-arrestins (DeFea *et al.,* 2000A). PAR2 agonists activate ERKs (extracellular-signal regulated kinases) 1/2, and activation is clearly inhibited by dominant-negative β-arrestin319–418. ERK1/2 remain in the cytosol and do not translocate to the nucleus to stimulate proliferation. The cytosolic retention of ERK1/2 depends on the formation of a multiprotein signalling complex (apparent mass of 250–300 kDa) that contains PAR2, β-arrestin 1, Raf-1 and pERK1/2 (phosphorylated SRK1/2) (DeFea *et al.,* 2000A). Other GPCRs can similarly interact with β-arrestin/Src/MEK1 (MAPK/ERK kinase 1)/ERK1/2 modules that retain pERK1/2 in the cytosol (DeFea *et al.,* 2000A; Tohgo *et al.,* 2002).

Determining PAR function upon protease activation is a challenge especially in systems where more than one receptor is expressed. Peptides with analogue sequences of proteolytically revealed PAR TLs have been synthesized. These peptides have been used to stimulate the receptors without the necessity of proteolysis. The use of these peptides has contributed to the significant success to unravel cellular responses mediated by these receptors. Importantly, synthetic 'scrambled' TL peptide sequences that are not able to trigger activation of the PARs have been developed, which serve as appropriate 'control peptides' for studies done with cultured cells or tissues (Ramachandran *et al.,* 2008). Surprisingly, PAR3 stimulation, either with thrombin or with PAR-APs based on the thrombin-revealed PAR3 'tethered ligand sequence', was unable to mediate a cellular signal. Rather, peptides with sequences derived from the thrombin-revealed PAR3 N-terminus were able to activate either PAR1 or PAR2 (Hansen *et al.,* 2004). Studies have provided evidence for a possible cofactor- like role of PAR3 through its interaction with PAR1 and PAR4 (Nakanishi-Matsui *et al.*, 2000; McLaughlin *et al.*, 2007). Experiments conducted in tissues or cells, with PAR-selective activating peptides, along with the appropriate PAR-inactive peptide sequences, have served as key reagents to explore the impact of activating a specific PAR, without the need for protease-stimulated activation of the receptor. Such activation process might result in effects other than activating a specific PAR (Ramachandran *et al.,* 2008).

1.1.1 Physiological and Pathophysiological roles of PAR2

The discovery of PARs initiated intensive investigations into the function of these receptors. The general approach has been to map receptor distribution at tissue

and cellular level, and to examine the biological effects of PAR agonists in different systems. This approach has provided large information which suggests that proteases and their receptors play important role in tissue responses to injury, including inflammation, pain and healing (Coelho *et al*., 2003).

PAR1 was the first described member of the group and most of the initial studies were concentrated to reveal its function in the organism. However, gradually data has accumulated about the significant role of PAR2 in numerous important physiological processes. Hence, in the recent years PAR2 arose as a potential pharmacological target.

1.1.2 PAR2 is involved in the inflammation process

Most of the physiological agonists of PAR2 are released during inflammation which suggests that PAR2 actively participates in this process. PAR2 acts in inflammation by its up-regulation by tumour necrosis factor α, interleukin 1α and lipopolysaccharide (Nystedt *et al.,* 1996; Hamilton *et al.,* 2001).

Mutant deletion of PAR2 sequence results in the observed phenotype such as diminishing the inflammation processes in the respiratory tract and joints (Schmidlin *et al.,* 2002; Ferrell *et al*., 2003).

The proinflammatory effects of proteases can be mediated by activation of PAR2 on multiple cell types (Macfarlane *et al*., 2001). However, recent studies indicate an important role of PAR2-mediated inflammation in the nervous system. PAR2 is co-expressed with substance P (SP) and calcitonin gene-related peptide (CGRP) in rat dorsal root ganglia (Steinhoff *et al*., 2000). Activation of PAR2 on spinal primary afferent

neurons stimulates secretion of SP and CGRP from their projections in peripheral tissues and the spinal cord which cause neurogenic inflammation and thermal and mechanical hyperalgesia (Steinhoff *et al*., 2000). During inflammation, the proteases that activate PAR2 on sensory nerves remain to be identified. A possible protease is tryptase from mast cells. Mast cells containing tryptase are in close proximity to sensory nerve axons in uninflamed and inflamed tissues (Stead *et al.,* 1987). PAR2-deficient mice show diminished thermal hyperalgesia after mast cell degranulation (Vergnolle *et al.,* 2001). Inflammatory agents can potentiate responses to VR1 (vanilloid receptor-1), a member of the TRP (transient receptor potential) family of ion channels that is activated by protons, elevated temperature, certain lipids, and exogenous vanilloids such as capsaicin (Caterina *et al*., 1997; Julius *et al.,* 2001). TRPV1 (transient receptor potential vanilloid-like 1) mediates PAR2-induced thermal hyperalgesia in part by a protein kinase C dependent mechanism (Amadesi *et al.,* 2004).

1.1.3 PAR2 functions in angiogenesis

During the last years data have accumulated for the active participation of PAR2 in cell proliferation and migration which are essential processes for blood vessel formation. In vivo experiments show that PAR2 activation leads to angiogenesis stimulation in an ischemic mouse model (Milia *et al.,* 2002).

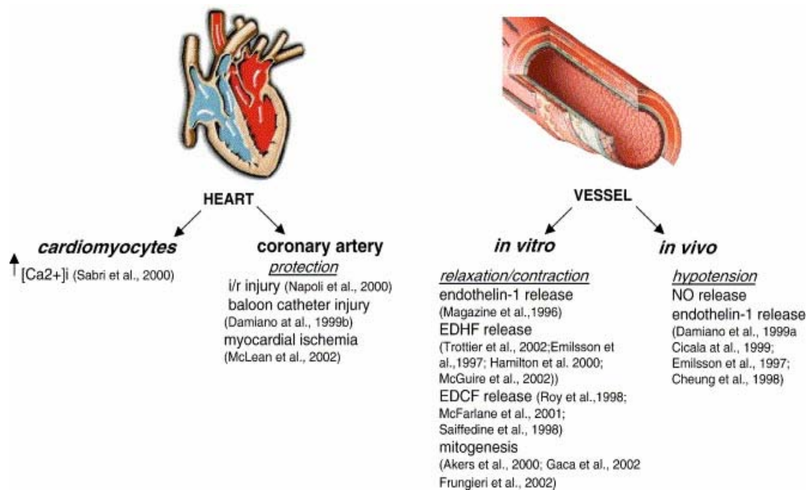
Al-Ani and colleagues have first described the functional role of PAR2 on endothelial cells (Al-Ani *et al.,* 1995). They have shown that trypsin and the PAR2 peptide agonist SLIGRL-NH2 induced an endothelium dependent vasorelaxation in rat aortic rings. The vasorelaxant effect was reduced by L-NAME, an inhibitor of nitric

oxide (NO) synthases. The last finding supports an involvement of the L-arginine/NO pathway (Al Ani *et al.*, 1995).

Successively, endothelium NO dependent responses following PAR2 activation have been demonstrated in several other blood vessels such as rabbit aorta (Roy *et al.,* 1998), porcine coronary arteries (Hwa *et al*., 1996; Hamilton *et al*., 1998) and porcine basilar arteries (Sobey and Cocks, 1998; Sobey *et al.*, 1999). Since inhibitors of the L-Arginine/NO pathway did not abrogate the vasodilatory response induced by PAR2, the possible involvement of other mediators has been investigated. On this basis endothelin has been proposed as a second messenger. Indeed, rapid release of nitric oxide induced by stimulation of aortic rings with PAR2 agonist, SLIGRL-NH_2, was reduced by pre-treatment with BQ-788, an ET_B endothelin receptor-specific antagonist (Magazine et al., 1996). Consistent with a role for endothelin-1 receptor activation in PAR2 AP-induced NO release, endothelin-1 levels were increased significantly after 5 min of treatment of aortic rings with PAR2 - AP. These results strongly support an involvement of ET_{1B} receptor in PAR2 response (Magazine et al., 1996). Similarly, it has been shown that NO production did not entirely account for vasorelaxant action of PAR2 - AP in resistance vessels (Hamilton and Cocks, 2000) such as rat femoral artery or vein (Emilsson *et al*., 1997; Roy et al., 1998) and isolated perfused normal rat kidney artery (Trottier *et al*., 2002). The multiple mechanisms underlying the PAR1 and PAR2-mediated vasodilatation confirm the involvement of NO, EDHF and prostanoids (Kawabata *et al*., 2004). This evidence indicates that redundant signalling pathways contribute to the vasodilatory response following PAR2 activation as it has been underlined by a recent study on afferent arterioles (Wang *et al*., 2005). On the other hand, this redundancy in

PAR2 signalling cascade could play a role in pathological settings such as inflammation or ischemia in which PAR2 is thought to be activated (Figure 2). In vitro studies simulating pathological conditions have suggested a possible protective role of PAR2. The protective role of PAR2 has also been proposed in myocardial ischemia/reperfusion (I/R) injury (McLean *et al.*, 2002). In isolated and perfused rat heart SLIGRL-NH_2 peptide has induced an endothelium-dependent coronary vasodilatation. Following I/R injury, PAR2 - AP-induced vasodilatation was selectively preserved as opposed to acetylcholine response. PAR2 response was not mediated by NO or prostanoids, but involved the release of an EDHF, possibly a lipoxygenase-derived eicosanoid, and the activation of vanilloid receptors on sensory C-fibers (McLean et al., 2002).

Figure 2: Cardiovascular effects of PAR2



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1.1.4 PAR2 role in tumorigenesis

We mentioned that PAR2 plays an important role in cell proliferation and migration. These processes are essential for tumor development, therefore it has been suggested that PAR2 may actively participate in tumorigenesis.

Darmoul *et al.(*2004) describe the role of PAR2 in colon cancer. They have found that activation of PAR2 by trypsin leads to transactivation of Epidermal Growth Factor Receptor (EGFR) through a pathway that includes matrix metalloproteinase – dependent cleavage and release of TGF – α. TGF – α in turn activates the EGFR and downstream MAPK signalling cascade, leading to cell proliferation (Darmoul *et al,* 2004). Another study with a STKM-1 gastric cancer cell line demonstrated that production of trypsin correlates with their malignant phenotype and invasive growth (Kato *et al.* 1998). In recent studies, PAR2 activation in IL-3- dependent murine lymphoma cell line BaF-3 resulted in cell proliferation (Mirza *et al.* 1997). Shimamoto *et al.* have shown that PAR2 agonist peptide SLIGKV and trypsin significantly increased cell proliferation in three pancreatic cancer cell lines SW1990, Capan-2, and Panc-1 (Shimamoto *et al.,* 2004). Ge *et al.* reported that secretion of trypsin-like protease and its autocrine activation of PAR2 in the breast cancer cell line MDA MB-231 influences cell migration (Ge *et al.,* 2004). Although PAR2 activation is predominantly considered as stimulating tumor cell growth, an inhibitory effect on cancer development has also been reported. Kaufmann *et al*. report that activation of PAR2 on CAPA2 pancreatic cell line decreases [^{3}H]-thymidine incorporation into the cell related to inhibition of tumor cell growth (Kaufmann *et al.,* 1998).

Similarly, PAR1, a receptor of the same family activated by thrombin, is a potential tumor cell proliferating and invading agent (Wojtukiewicz *et al.* 1993; Henrikson *et al.* 1999). Another study demonstrates that PAR1 signaling inhibits migration and invasion of breast cancer cells (Kamath *et al.* 2001). The findings of Yamashita *et al.* also support the notion that trypsin plays a tumor-suppressive role in human carcinomas, as they find reduced production of trypsinogen accompanied by reduced PAR2 expression in esophageal squamous cell carcinomas and 72 gastric adenocarcinomas (Yamashita *et al.,* 2003). Recently Rattenholl *et al.* show the role of PAR2 as an inhibitor of the development of keratinocyte-derived skin tumors (Rattenholl *et al.,* 2007). The controversial results point out the complex role of PAR2 in cell proliferation and migration and the necessity of more intensive investigations in this domain.

2. GPCR signalling into the nucleus

The existing theory of GPCR signalling states that receptors and their associated signalling molecules are independently trafficked to the plasma membrane where they become functional and responsive to agonist stimulation. These cell surface receptors are normally internalized during desensitization of the primary signalling pathway following prolonged or repetitive agonist stimulation. However, internalized receptors can activate signalling pathways that are quite distinct from those activated by the same receptors at the cell surface. Therefore, desensitization of primary G protein-dependent signalling pathways is followed by a second wave of arrestin-dependent signalling, which may be

both functionally and structurally G protein-independent (Shenoy *et al.,* 2005; Lefkowitz *et al.,* 2006; Smith *et al.,* 2006).

However, in recent years it has become clear that GPCRs can also signal from other intracellular locations as well. Furthermore, the notion that all GPCRs are initially trafficked to the plasma membrane has recently been challenged. For example, GABA B1 receptor subunits remain in the endoplasmic reticulum (ER) in the absence of GABA B2 subunits (Jones *et al.,* 1998; Margeta-Mitrovic *et al.,* 2000). The distribution of the GABA B1 receptor in the central nervous system is much broader than the GABA B2, suggesting that this receptor may have an intracellular function (Towers *et al.,* 2000; Ritter *et al.,* 2005). It has been demonstrated that a deorphanized GPCR30 receptor takes a unique place among other GPCR, as it is localized exclusively in the ER, where it functions as a receptor for estrogen (Revankar *et al.,* 2005). Also, on a parallel track, a number of studies show that heterotrimeric G proteins are localized to ER and Golgi compartments where they are involved in the regulation of anterograde protein trafficking and Golgi organization (Jamora *et al.,* 1999; Diaz Anel 2007). The receptors that control these latter events remain unknown at present. An increasing number of GPCRs are targeted to the nuclear membrane as well: recent articles describe lysophosphatidic acid receptors (LPA_1R) (Gobeil *et al.,* 2003), metabotropic glutamate receptors (mGluR5) (O'Malley *et al.,* 2003), apelin receptors (APJ) (Lee *et al.,* 2004), platelet-activating factor receptors (PAFR) (Marrache *et al.,* 2002), angiotensin II type 1 receptors (AT_1R) (Zhuo *et al*., 2002; Lee *et al.,* 2004), prostaglandin receptors (EP receptors) (Gobeil *et al*., 2002) endothelin receptors (ETR) (Boivin *et al.,* 2003) and β -adrenergic receptors (β -AR) (Boivin *et al.,* 2006) in the nuclear membrane.

3. Receptor trafficking

An essential requirement for the normal function of every life form is the ability of its cells to react to external stimuli. Therefore the adequate, fast and correct transmission of the signals, subsequent specific receptor activation and transmission via different signalling pathways are important for the survival of the whole organism. During the last years, lots of the studies that are being devoted to understanding receptor's signalling network indicate that proper trafficking of receptors to their appropriate destinations is crucial for the normal function of the cell.

Several distinct pathways for trafficking exist: one important pathway is the transport of already synthesized receptors from the endoplasmic reticulum to the Golgi network and from there to the cell surface. The other pathway is receptor internalization, which usually occurs subsequently to receptor activation, and sorting to the early endosome. From there, receptor destiny can be different. One possibility is that the receptor is recycled and sent again to the cell surface, another possibility is its degradation in the lysosomes. However, it is intriguing that more than one option is available, with opposite consequences for downstream signalling. Therefore, the major question arises: what drives the receptor fate? Different groups of trafficking proteins have been described, and the relatively new group called "Sorting Nexins" has attracted great interest.

3.1 Sorting Nexins, structure and homologues.

The sorting nexins are a large group of 33 members, which are mostly cytosolic or membrane - bound proteins. Their hallmark is the presence of a PX (Phox) domain – a sequence of approximately 120 amino acids (Xu *et al.,* 2001A). The name PX derives from the protein complex where it was originally identified—the *ph*agocyte NADPH *ox*idase (phox) (Ponting *et al.,* 1996; Xu *et al.,* 2001A). PX domain interacts actively with various phosphatidylinositol phosphates (PtdIns).

PtdIns are implicated in diverse cellular processes and they are an essential part of membrane structures in the cell. PtdIns (3,4,5) regulates many signalling processes related to cell growth and survival. PtdIns(4,5) serves as a localization signal for recruitment of specific proteins participating in endocytosis. The trans-Golgi network (TGN) is enriched in PtdIns(4), which functions to recruit effector proteins that regulate traffic to post-Golgi compartments. The sorting (early) endosome is particularly enriched in PtdIns(3) (Gillooly *et al.,* 2000); this PI serves as a homing/localizing signal for various endosomal proteins that contain PtdIns(3)-binding motifs. Therefore the PX domain figuratively is the driving engine of the SNXs. It allows them to interact with membrane structures and to serve their role in protein trafficking. In addition to the PX domain many SNXs have other domains crucial for protein-protein interactions that might participate in their subcellular localization or formation of complexes at particular lipid-enriched membranes. The first described member of the family was SNX1 (Kurten *et al.,* 1996). The possible function of SNX1 in intracellular trafficking was suggested based on the properties of its yeast homologue Vps5 (vacuolar protein sorting). Vps5 is a

PX containing protein involved in vacuolar protein sorting. In yeast a mutation in Vps5 results in the secretion of the hydrolase carboxypeptidase Y, which leads to inappropriate delivery of the hydrolase receptor Vps10 to the vacuole. Normally Vps10 binds carboxypeptidase Y in the TGN and transports it to pre-vacuolar endosomes where the hydrolase dissociates. The hydrolase is delivered to the vacuole, whereas Vps10 is recycled to the TGN for further rounds of hydrolase transportation. Vps5 carries out its biological functions by assembling into the 'retromer complex' – important for the retrieval of mannose – 6 phosphate receptor.

Grd19 is the yeast homologue of the group of SNXs which contains only a PX domain. It is a small hydrophilic protein that is predominantly localized in the cytosol. Grd19 has been shown to be a component of the retrieval machinery that functions by interacting directly with the cytosolic tails of certain resident TGN proteins during the sorting process at the pre-vacuolar compartment. Grd19 is important for the retrieval of Kex2 and A-ALP (alpha-actinin-associated LIM protein) (two Golgi-resident proteins), but not Vps10, and therefore does not have a role in sorting soluble enzymes to the vacuole (Lu *et al.,* 2002).

Mvp1 is the yeast homologue for SNX8. Mvp1 was isolated as a multicopy suppressor of Vps1 mutants that are deficient in trafficking of the carboxypeptidase Y receptor. Mvp1, in conjunction with Vps1, is involved in sorting proteins in the late Golgi for delivery to the vacuole. It might also function in the retrieval of proteins from the pre-vacuolar endosomes to the late Golgi (Ekena *et al.,* 1995).

3.1.1 Members of the SNX family

3.1.1.1 SNX1

As we mentioned above SNX1 was the first mammalian SNX to be characterized (Kurten *et al.,* 1996). SNX1 is both membrane associated and cytosolic, where it probably exists as a tetramer in large protein complexes (Kurten *et al.,* 2001). It was identified in a yeast two-hybrid screen as a partner for the core kinase domain of the EGFR. Yeast orthologue of SNX1 is Vps5p.

Vps5p is a component of the yeast retromer, a protein complex required for retrieval of the CPY receptor Vps10p from prevacuolar endosomes to the late-Golgi. Alongside Vps5p, the yeast retromer comprises Vps17p, Vps26p, Vps29p and the cargo selective subunit, Vps35p (Seaman *et al.,* 1998; Nothwehr *et al.,* 2000). Similarly to its yeast homologue SNX1 was found to be part of mammalian retromer.

Mammalian retromer is involved in mediating endosome-to-trans-Golgi-network retrograde transport of the cation-independent mannose-6-phosphate receptor. Together with SNX1 and SNX2, SNX5 and SNX6 have recently been proposed to be part of the retromer (Wassmer *et al.,* 2007).

Since SNX1 was discovered as an interaction partner of EGFR, it was proposed that it functions in retrieving this receptor. SNX1 was found to associate with the sorting endosome (Cozier *et al.,* 2002), from where it was suggested to increase the degradative sorting of the EGFR through an unknown mechanism. EGFR degradation is enhanced in cells overexpressing SNX1. Deletion mutants of SNX1 block EGFR degradation and fail

to inhibit receptor endocytosis (Kurten *et al.,* 1996; Zhong *et al.,* 2002). SNX1 interacts with hepatocyte growth factor (HGF)-regulated tyrosine-kinase substrate (HRS).

HRS is a FYVE-domain-containing protein that is localized to early endosomes and is a prominent target for phosphorylation by various receptors, including the EGFR (Raiborg *et al.,* 2002). HRS and SNX1 compete for the same binding site on the EGFR, and overexpression of HRS inhibits ligand-induced degradation of the EGFR, which indicates that HRS might modulate lysosomal trafficking of the receptor by sequestering SNX1 (Chin *et al.,* 2001). Recent studies demonstrated that both SNX1 and SNX2 are involved in regulating lysosomal sorting of internalized EGFR, but neither protein is essential for this process (Gullapalli *et al.,* 2006). SNX1 interacts with several other members of the receptor tyrosine kinase family, including the PDGFR and the insulin receptor (IR). In addition, SNX1 co-immunoprecipitates with the long form of the leptin receptor. Leptin receptor is a cytokine receptor that signals through the activation of Janus tyrosine kinases and transferrin receptors (TFR). These are receptors that internalize and recycle constitutively (Haft *et al.,* 1998; Dixon *et al.,* 2002). SNX1 interacts also with HRS (Chin *et al.,* 2001) and enterophilin-1 (Pons *et al.,* 2003).

Evidences for SNXs being involved in the trafficking and signalling of GPCRs present special interest. Heydorn *et al.* determined in *in vitro* experiments that SNX1 interacts with at least 10 different GPCRs (Heydorn *et al.,* 2004). The ability of SNX1 to traffick seven trans – membrane domain receptors was confirmed by Gullapalli *et al.* (2006). They demonstrated that SNX1 is important for the lysosomal degradation of PAR1. They also established that this function is independent of the retromer. SNX2 which usually dimerizes with SNX1, is not essential for lysosomal sorting of PAR1, but

rather can regulate PAR1 degradation by disrupting endosomal localization of endogenous SNX1 when ectopically expressed (Gullapalli *et. al.,* 2006). This study represents a great value for our research project, as this is the very first evidence for the participation of SNX member in trafficking of PARs.

3.1.1.2 SNX2

SNX2 and SNX1 share sixty-three per cent sequence identities and have the same localization in the cells (Dixon *et al.,* 2002). Similarly to SNX1, SNX2 can oligomerize with itself and form heteromeric complexes with SNX1, SNX4 and proteins of the retromer complex. Analogous to SNX1, both SNX2 and SNX4 interact to different degrees with several receptors, including the EGFR, PDGFR, IR and the long form of the leptin receptor (Dixon *et al.,* 2002). Gullapalli *et al.* determined that SNX2 plays a more important role in lysosomal sorting of EGFR (Gullapalli *et al.,* 2004). Also we mentioned above that SNX2 has a role in the trafficking of PAR1 (Gullapalli *et. al.,* 2006). Unlike SNX1, however, SNX2 does not interact with the TFR, which indicates different binding specificities. Despite the high sequence identity that is shared between their PX domains (70%), the SNX2 PX domain binds preferentially PtdIns(3), but not PtdIns(4,5) (Dixon *et al.,* 2002; Zhong *et al.,* 2002).

4. Trafficking of PAR2

As we mentioned before, the proper internalization and degradation of PAR2 has great importance for its role in the cellular signalling. Several studies have been devoted to understanding this process. Dery *et al.* (1999) explored the role of β – arrestins in

PAR2 endocytosis. β – Arrestins mediate uncoupling and endocytosis of certain neurotransmitter receptors, which are activated in a reversible manner. They examined for the first time the role of β – arrestin1 for the subcellular distribution of PAR2. Their results showed that β - arrestins mediate endocytosis of PAR2 and aid in uncoupling of PARs (Dery *et al.,* 1999). More recently Roosterman *et al.* evaluated the contribution of Rab5a and Rab11a to the trafficking and signalling of PAR2. They found that Rab5a is required for PAR2 endocytosis and resensitization, whereas Rab11a contributes to trafficking of PAR2 from the Golgi apparatus to the plasma membrane (Roosterman *et al.,* 2003). However there are still uncertainties for PAR2 intracellular trafficking, and how it impacts the receptor signalling.

5. Conclusion

Protease – Activated Receptors are a group of G – protein coupled receptors which have been intensively studied during the last 20 years. Recent investigations have demonstrated their essential role in numerous physiological processes. A member of this group, PAR2 has distinguished itself with its important functions in cardiovascular system, inflammation and tumorigenesis. Lately investigations have been devoted to understand PAR2 signalling pathways. New directions for GPCRs signalling from the cell nucleus have been established. Until now PAR2 has not been studied for such alternative signalling. Mechanisms of the nuclear trafficking of GPCRs remain unclear.

Sorting Nexins are large and diverse group of transporting proteins. The majority of SNX members are still not well studied. Some of them have a role in trafficking of various receptors. Until recently it was not clear if SNXs participate into the trafficking of

GPCRs. *In vitro* studies discovered that SNX1 interacts with at least 10 GPCRs. Lately it was discovered that SNX1 plays a key role into lysosomal degradation of PAR1 (the closest relative of PAR2). Logically it can be suggested that SNX1 and probably other members of the family may be involved in PAR2 intracellular trafficking.

II. HYPOTHESIS AND GOALS

1. Hypothesis:

Recent studies demonstrated that the G – protein coupled receptors can traffic from the plasma membrane to the nucleus.

We propose that two populations of PAR2 exist; the first is localized at the plasma membrane, the second at the nucleus. We hypothesize that the nuclear PAR2 population originates through translocation of PAR2 initially localized at the cell membrane. We hypothesize that the two distinct populations of PAR2 play different roles in signalling. We propose that members of Sorting Nexin family are involved in the intracellular trafficking and signalling pathways of PAR2. SNX1 and SNX2 are the most studied members of the group and they have been shown to participate in the intracellular trafficking of PAR1. Thus they could be suitable candidates for PAR2 trafficking too. SNX11 is a relatively not well studied member of SNX family. We hypothesize that it, like most members of its group, participates in trafficking of various proteins and receptors.

2. Goals:

1. To investigate the role of intracellular trafficking of PAR2 subsequent to its stimulation.

To evaluate the role of SNX1, SNX2 and SNX11 in nuclear trafficking of PAR2 and to establish the physical and functional components of this partnership.

III. MATERIALS AND METHODS

1. Materials

1.1 Chemicals and Reagents

- Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Wisent Inc. (St-Bruno, QC, Canada).

- Trypsin, TPCK treated was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA).

- SNX clones: Murine full-length SNX11 was cloned from embryonic murine heart cDNA.

- mycSNX1 and mycSNX2 clones were obtained through collaboration with Dr. J. Trejo (University of Chapel Hill, North Carolina, USA).

- PMSF (phenylmethanesulfonylfluoride), Aprotinin, Pepstatin A, Leupeptin, Na_3VO_4, NaF, DAPI, Igepal CA 630(NP-40) were purchased from Sigma-Aldrich (Oakville, ON, Canada).

- PEI (polyethylenimine) cat. № 23966 (Polyscineces, Warrington, PA, USA); T4 Polynucleotide Kinase, ATP, dNTP, *Pfu* polymerase, Calf Intestine Alkaline Phosphatase (CIAP), T4 ligase, Taq polymerase, and all restriction enzymes used in current work were purchased from Fermentas (Burlington, ON, Canada).

1.2 Antibodies

- Anti- actin cat. № sc-1616, anti- DsRed cat. № sc-32233, anti- GAPDH cat. № sc-33354, and anti- GFP cat. № sc-8334 were purchased from Santa-Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

- Anti- flag cat. № F 1804 and anti- GST cat. № G 1660 were purchased from Sigma-Aldrich (Oakville, ON, Canada).

- Anti- His cat. № sc-803, anti-c-Myc cat. № sc-40 and anti-PAR2 cat. № sc-13504 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

- Anti- CD49e cat. № 610634, alternate name – Integrin alpha 5, anti- SNX1 cat. № 611482 and anti-SNX2 cat. № 611308 were purchased from BD Biosciences (Mississauga, ON. Canada).

- Anti – SNX11 was designed specially for Dr. Andelfinger's laboratory from Open Biosystems (Huntsville, AL, USA). Epitope: CGWAQEERQSTSHLAKGDQ. The epitope was chosen such to be out of the Phox domain, to avoid cross-reactivity with other SNX family members. The chosen epitope allows that the antibodies identify rat, mouse and human SNX11.

- Anti- lamin B Receptor cat. № 1398-1 was purchased from Epitomics (Burlingame, CA, USA).

2. Methods

2.1 Phenol/Chloroform/Isoamyl alcohol (PCI)) protocol for DNA purification and precipitation

To obtain PCI emulsion, 25 parts of phenol were added to 24 parts of chloroform and one part of isoamylalcohol. Equal volume of PCI was added to the DNA aqueous phase (obtained by using Plasmid miniprep protocol), mixed and vortexed vigorously. The emulsion was spun at 10 000 g for 2 min, the upper aqueous phase was removed into a new centrifuge tube and mixed with equal volume of chloroform. The emulsion was vortexed vigorously and spun at 10 000 g for 1 min. The aqueous phase was removed into a new centrifuge tube and the chloroform purification step was repeated. Upper aqueous phase was removed into a new centrifuge tube and a volume of 3 M Sodium Acetate equal of 1/10 volume of the initial PCI volume and three volumes of 95 % v/v ethanol were added to the aqueous phase. The solution was mixed and incubated for 3 hours at -20 °C. The DNA solution was centrifuged at 10 000 g for 30 min and the supernatant was discarded and 0.5 ml of 70 % v/v ethanol was added to wash the pellet. The DNA pellet was incubated for 30 min and spun at 10 000 g. The supernatant was discarded and the precipitated DNA pellet was air dried for 5 min. The DNA was dissolved in an appropriate volume of water.

2.2 Electroporation protocol

A micro centrifuge tube and a 0.1 cm electroporation cuvette were placed on ice and DH10B cells (Invitrogen Corporation, Burlington, ON, Canada) were thawed on ice. Forty µl of the electrocompetent cell suspension were transferred in the cold micro centrifuge tube and mixed well with 1 to 2 µl of DNA corresponding to amount of 1 mcg of DNA (DNA should not be in a low strength buffer such as a Tris-EDTA buffer). All manipulations were performed on ice. The cell suspension was transferred to a cold electroporation cuvette placed in the chamber slide where one pulse was applied. Bio-Rad MicroPulser was set to “Ec1” when using 0.1 cm cuvette. Five hundred µl of warm LB (bacterial medium) (37 °C) was then gently mixed with the suspension in the cuvette. The cell suspension was transferred to a 17 x 100 mm polypropylene tube and was shaken at 37 °C for 1 hour at 225 rpm.

2.3 Preparation of glycerol stocks

Five hundred µl of bacterial culture (prepared as per protocol **2.2**) were added to 166.66 µl of 60% v/v sterile glycerol and mixed well. The glycerol stocks were kept at -80 ° C for long term storage.

2.4 Plasmid miniprep protocol

A GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Oakville, ON, Canada) was used.

The rest of bacterial culture (3.5 ml) from which 500 µl was used in 2.3 was pelleted at 12 000 g for 1 minute. The pellet was resuspended in 200 µl Resuspention buffer. Cells were vortexed thoroughly until a homogeneous suspension was obtained. Resuspended cells were lysed by adding 200 µl of the Lysis Solution. The contents were immediately mixed by gentle inversion (6–8 times) until the mixture became clear and viscous. The cell debris were precipitated by adding 350 µl of the Neutralization/Binding Solution. The tube was gently inverted 4–6 times. The cell debris were pelleted by centrifuging at 12 000 g for 10 minutes. GenElute Miniprep Binding Column was inserted into a microcentrifuge tube. Five hundred µl of the Column Preparation Solution were added to each miniprep column and centrifuged at 12 000 *g* for 30 seconds to 1 minute. The flow-through liquid was discarded. The cleared lysate was transferred to the column and centrifuged at 12 000 *g* for 30 seconds to 1 minute. The flow-through liquid was discarded. Seven hundred and fifty µl of the diluted Wash Solution were added to the column and centrifuged at 12 000 *g* for 30 seconds to 1 minute. The flow-through liquid was discarded, and the column was centrifuged again at 20 000 g for 1 to 2 minutes without adding any additional Wash Solution to remove excess ethanol. The column was transferred to a fresh collection tube and 100 µl of Elution Solution were added.

2.5 Yeast-two- hybrid assay (Y2H)

MatchMaker GAL4 Two-hybrid System 3 (Clontech, Palo Alto, CA, USA): a full-length SNX11 was cloned as bait into pGBKT7 downstream of the DNA-binding domain of GAL4. The vector was transformed into AH109 competent yeast cells. A lack of protein toxicity or transcriptional activation in AH109 cells was assayed in culture and protein expression confirmed by Western blot using an anti-c-Myc antibody. Transformed AH109 cells were mated with strain Y187 yeast pre-transformed with a Matchmaker 17 day mouse embryo library (Clontech, Palo Alto, CA, USA). Preys identified in this screen include cardiac actin, gamma actin, myosin binding protein C (slow type), Collagens 1A2, 5A1, 11A1, PLOD1 (dioxygenase involved in collagen synthesis), LASP1 (organizes actin filaments, involved in cytoskeleton and cell migration), and ITM2A (chondrogenic differentiation marker) (the results of 2.5 are Dr. Gregor Andelfinger`s unpublished data).

2.6 In vitro translation of prey clones

Ten selected prey clones were translated *in vitro* using the MagneGST system (Promega, San Luis Obispo, CA USA). Their protein interactions with GST controls and GST-SNX11 were confirmed by GST pull-down assay. (Dr. Gregor Andelfinger’s unpublished data).

2.7 RNA extraction from cells and tissues.

RNA from tissues and cells was isolated using Trizol (Invitrogen Corporation, Burlington, ON, Canada). The cells were lysed directly in the culture dish (10 cm) by adding 2 ml of Tryzol LS Reagent and mixed using a pipette. The homogenized samples were incubated for 5 min at RT in order to permit complete dissociation of nucleoprotein complex from the RNA. The sample was transferred into two tubes of 1 ml and 0.2 ml of chloroform was added to each of the tubes and shaken vigorously by hand for 15 seconds. The samples were incubated at RT for 15 minutes and centrifuged at 12 000 g for 15 minutes at 4 °C. The aqueous phase containing the RNA was transferred to a clean tube, precipitated with 0.5 ml isopropyl alcohol and incubated at RT for 10 minutes. The samples were centrifuged at 12 000 g for 15 minutes at 4 ° C. The supernatant was removed and the RNA pellet was washed with 1 ml 75 % v/v ethanol. Samples were vortexed, centrifuged at 7 500 g for 5 minutes at 4 ° C, and the RNA pellet was briefly air dried. The RNA pellet was dissolved in RNase free water and incubated for 10 min at 55-60°C.

2.8 First strand cDNA synthesis

First strand cDNA was synthesized following manual protocol of Super Script ™ II Reverse Transcriptase (Invitrogen Corporation, Burlington, ON, Canada).

2.9 Reverse-transcription polymerase chain reaction (RT-PCR)

For RT-PCR, primers were designed and synthesized as follows:

Primer name	Sequence 5'-3'
SNX11_F	GTGGCTGAGAAAGCAGCTACAGA
SNX11_R	TTTGCAGGAATAGG TGCAACTGGC
iNOS_F	GTGGAAACGGTAACAAAGGA
iNOS_R	TGCCGTTGTTGGTAGAGTAA
tie2_F	CCCCAACATCATCAACTTC
tie2_R	TCAGGTACTGCATGCCATTG
PPAR1_F	TGCTGTGGGGATGTCTCATA
PPAR1_R	TTGGGCTCCATAAAGTCACC

Quantum RNA™ universal 18S Standard primers (Ambion, Austin, TX, USA) were used as internal standard references. *Taq* DNA polymerase was used for the RT-PCR reactions. For the PCR cocktail 1µl cDNA, 1 µM of each primer, 1 µM dNTP, 2.5 u *Taq*, 2.5 mM $MgCl_2$, 1x Taq buffer was used. The PCR amplification was performed as initial denaturation at 94°C for 2 minutes; 35 repetitive cycles including 94°C for 45 seconds, primer annealing at 62°C for 45 seconds, extension at 72°C for 1 minute and 72 °C for 10 minutes as final extension step.

2.10 Cloning

In order to study the intracellular localization of SNX11, clones with different tags were constructed. Murine SNX11 was cloned in EGFP and DsRED vectors (Invitrogen, Burlington, ON, Canada). These constructs contain a chromatophore group and allow overexpression and visualization of the intracellular distribution of the protein of interest, under fluorescent or confocal microscopy.

Blunt-end cloning was performed using *Sma*I enzyme with restriction site 5'-C C C^G G G-3'. A 3 µg plasmid DsRed and EGFP digestion was performed through incubation for 5 hours at 37 °C in buffer Tango containing *SmaI* – 25 units in final volume of 100 µl. Consequently dephosphorylation of the digested plasmids (100 µl) was carried out using 1 unit CIAP in final volume of 250 µl buffer CIAP.

Forward and reverse primers were 5' phosphorylated: forward primer 5'GCC ACC ATG GGC TTG TGG TAT AGG ATG 3'; reverse primer 5' GGT CTA TAG AGT GAG TTC CAG GAC AGC 3'. To 1 µM of each primer, 1 x buffer A, 5 µM ATP, 1x T4 polynucleotide kinase were added. The reaction was performed in a 10 µl final volume at 37 °C for not more than 30 minutes.

10 µM TOPO-mSNX11 was used as a template; 10 mM phosphorylated primers were mixed with: 1µM dNTP; 1x buffer *Pfu* (Fermentas, Burlington, ON, Canada) with $MgSO_4$; P*fu* – 2.5 units in final volume of 100 µl. The PCR amplification was performed as initial denaturation at 95 °C for 5 minutes, 35 repetitive cycles of denaturation at 95 °C for 30 seconds, primer annealing at 60 °C for 30 seconds and extension at 72 °C for 2 minutes and 72 °C for 10 minutes as final extension step.

The plasmids and the PCR product of SNX11 were extracted by PCI extraction (2.1) and loaded on an agarose gel to verify the quality and the concentration. The ligation reaction was performed overnight at 16 °C in a molar ratio of insert / plasmid 5:1, 0.5 unit *T4 ligase*, 0.25 unit *SmaI*, 1x ligase buffer, 0.5 x PEG 4000 were present in final volume of 20 μl.

On the next day, DH10B (E. coli) cells were transformed by electroporation (2.2). The cells were spread on a petri dish with agar medium and an appropriate antibiotic for selection was used. Positive and negative controls were set up. As a positive control we used transformation of pure plasmid (DsRed or EGFP, directly taken from maxi prep and diluted 1/1000 or 1/10 000). Two negative controls were established: the first contained digested plasmid without insert and the second control contained only competent cells without ligation mixture. The two controls were used for transformation of DH10B cells. All the dishes were incubated overnight at 37 °C. On the next day, the plates were checked for the presence of single colonies and the absence of such colonies in the negative controls. From each dish several colonies were picked and were processed for analysis. With a single tip, colonies were picked up and the tip was dropped into a polypropylene sterile tube with LB medium and an appropriate antibiotic selection. The bacteria were incubated overnight at 37 °C with shaking at 225 rpm. Next day a glycerol stock (2.3) was prepared for each sample and miniprep (2.4) was performed (Sigma kit, Sigma - Aldrich, Oakville, ON, Canada).

In order to confirm the presence of the insert the isolated plasmid DNA was digested with the appropriate enzymes. In a case of a positive result after verifying them on an agrose gel, the DNA was sent out for sequencing to confirm the nucleotide

accuracy of the construct. In case of successful result we proceeded to maxi-prep (Qiagen, Mississauga, ON, Canada).

2.11 Cell cultures

HEK293 cells were grown in DMEM with sodium pyruvate 110 mg/L, penicillin G 100 U/ml, streptomycin sulphate 100 μg/ml and 10% FBS. Cells were serum - starved for 6 hours prior to stimulation with Trypsin. The HEK 293 EGFP-PAR2, EGFP-SNX11 and DsRed-SNX11 stable cell lines were grown in DMEM complete medium with 200 μg/ml G 418 for selection.

2.12 Establishing knockdown stable cell lines.

The following vectors were used for establishing knockdown stable lines:

- human GIPZ LENTIVIRAL shRNAmir SNX1 V2LHS_153303
- GIPZ LENTIVIRAL shRNAmir SNX2V2LHS_153307
- human GIPZ LENTIVIRAL shRNAmir SNX11 V2LHS_65515
- Non-silenced LENTIVIRAL shRNAmir negative control – RHS 4346

The lentiviral constructs were purchased from Open Biosystems (Huntsville, AL, USA). The vectors contain GFP tag which allows measurement of transfection and transduction efficiency.

On day 0, 10 x 10^4 cells per well in a 12 well plate were plated using full medium (with 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin) and incubated

overnight. On day 1, transfection was performed using "PEI Transfection Protocol". In a sterile tube 100 µl of warm DMEM free of serum, pyruvate and antibiotics, were added; 1 µg of DNA was transfected and mixed by pipetting. In another sterile tube, 100 µl of warm DMEM free of serum, pyruvate and antibiotics was mixed by pipetting with 8 µl of PEI. PEI solution was added to DNA solution, mixed by pipetting, and incubated 15 minutes at RT. After that, medium from cell culture was aspirated and replaced with fresh warm DMEM free of serum, pyruvate and antibiotics. Transfection solution was added to cell culture, mixed by gentle swirling and incubated at 37° C in 5% CO_2. After 6 hours the medium was replaced with fresh warm complete DMEM without pyruvate.

On day 2 the efficiency of the transfections was verified under fluorescence microscope (transfected cells fluorescence in green). A puromycin efficiency curve was established and the concentration of 2 µg/ml was determined as the most appropriate.

On day 3 the selection with puromycin 2 µg/ml was started. Every second day the rate of GFP-positive cells was examined. When cells reached more than 90% of transfection, the concentration of puromycin was reduced to 1 µg/ml. The stable level of transfection was maintained constantly with 1 µg/ml puromycin.

The same procedure was performed to generate the EGFP – SNX11 HEK293 stable line. In this case the antibiotic for selection was 400 µg/ml G 418. Once the stable line was established, 200 µg/ml G 418 was used.

2.13 Immunoblotting

For the purpose of Immunoblot analysis the following buffers were used:

- Laemmli (0.0625 M Tris-base, pH 6.8; 10 % Glycerol; 2 % SDS; 14.4 M 2 – β – mercaptoethanol, 0.01 % bromphenol blue) added 1/6 of the final volume of protein sample.
- Running buffer (123.82 mM Tris-base, 1M Glycine, 5 % SDS) diluted 1/5 in water to the final volume.
- Transfer buffer (25 mM Tris-base, 192 mM Glycine, 20 % (v/v) Methanol).
- TBS-T1 pH 7.5 (199.77 mM Tris-base, 1.5 mM NaCl, 1 % Tween 20) diluted 1/10 in water to the final volume.
- Separation gel: 0.375 mM Tris pH 8.8, 0.1 % SDS, Acrylamide / bis-acrylamide (Bio-Rad, Mississauga, ON, Canada), 10 %, 0.05 % ammonium persulfate (Bio-Rad), 5 % TEMED (Invitrogen Corporation, Burlington, ON, Canada).
- Stacking gel**:** 0.125 mM Tris pH 6.8, 0.1 % SDS, 4 % Acrylamide, 0.05 % ammonium persulfate, 5 % TEMED.

Extracted proteins were quantified by the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Mississauga, ON, Canada). Equal amounts of protein fractions (20 µg) were resuspended in sample buffer for SDS-PAGE electrophoresis and loaded on 10% Acrylamide gels. Protein standard was loaded on each gel. Electrophoresis was conducted for 1 hour at 150 V **(**Kaleidoscope^{TM}, Bio-Rad, Mississauga, ON, Canada). The proteins from the gels were transferred onto nitrocellulose membranes (Trans Blot Protein Assay, Bio-Rad, Mississauga, ON, Canada) at 100 V for 1 hour (two gels apparatus, Bio-Rad) or

30 min (four gels apparatus, Bio-Rad). Furthermore, membranes were incubated in 5% milk in TBS-T for 1 hour at RT. Each washing step was performed in TBS-T for 5 minutes and was repeated three times. The membranes were incubated overnight at 4°C with the appropriate primary antibodies. On the next day the membranes were washed three times in TBS-T buffer, the first wash for 15 minutes followed by two washes of 5 minutes each. The membranes were incubated at RT with the appropriate secondary antibodies for 1 hour. Membranes were washed again three times in TBS-T buffer, the first wash for 15 minutes followed by two washes of 5 minutes each. Membranes were treated with Chemiluminescence reagent Plus (Western lighting™, PerkinElmer, Woodbridge, ON, Canada) and exposed on Bioflex scientific imaging films (Clonex Corp, Markham, ON, Canada) at different time points in order to reach optimal clearance of the picture. The results were analyzed by comparing the molecular size of the obtained band with the reference standard.

All the membranes were reblotted for actin using anti-actin antibody which was used as a loading control. The intensity of the bands was analyzed on Quantity One™ software (Bio-Rad, Mississauga, ON, Canada). Densitometry protein data were expressed as the ratio to actin signal.

2.14 Subcellular fractionation and trypsin stimulation

The subcellular fractionations were performed using two different protocols with similar efficiency.

Protocol I

Cells (sixty to seventy per cent confluence) were starved in DMEM medium without FBS for 6 hours for cell synchronization (reach phase G0/quiescence). The synchronized cells were stimulated with 20 nM Trypsin or 20 µM SLIGKV for 30 minutes. Consequently subcellular fractionation was performed. The cells were harvested in a solution of PBS/1mM EDTA and centrifuged for 5 minutes at 500 g, washed in cold PBS and centrifuged again. The pellet was resuspended in 1 ml hypotonic buffer (10 mM Hepes pH 8.0, 1,5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) with proteinase inhibitors (1 mM PMSF, 1 µg/ml Leupeptin, 1 µg/ml Aprotinin, 1 µg/ml Pepstatin, 1mM Na_3VO_4, 1mM NaF). The pellet was incubated for 15 minutes to allow cells to swell up. One hundred µl Nonidet P-40 was then added to the cell suspension and mixed vigorously for 10 seconds. The lysate was centrifuged at 12 000 g for 3 minutes at 4°C. The supernatant (cytosolic and membrane fractions) was collected in a fresh pre-chilled tube. The nuclear pellet was resuspended in 175 µl ice-cold nuclear extraction buffer I (20 mM Hepes pH 8.0; 25 mM $MgCl_2$; 25% glycerol; 100 mM NaCl; 0.5 M EDTA; 5 mM DTT) and the cocktail of proteinase inhibitors was added. Subsequently, the nuclei suspension was vortexed vigorously for 30 seconds and shaken on a turn-and-over shaker at 4°C for 30 minutes followed by centrifugation at 12 000 g for 15 minutes. The supernatant was collected as a nuclear extraction fraction and transferred in a new pre-chilled centrifuge tube.

Protocol II

Cells from two 15 cm dish cells at approximately eighty per cent confluence were starved for 6 hours in DMEM medium without FBS. Following stimulation with 20 nM Trypsin or 20 µM SLIGKV for 30 minutes cells were harvested by scraping in cold PBS. The cell suspension was transferred into two 15 ml tubes, centrifuged for 5 minutes at 2000 g, resuspended and washed two times in cold PBS. The cell pellet was resuspended in 4 ml ice cold nuclear extraction buffer II (20 mM HEPES pH 7.5, 20 mM NaCl, 3 mM $MgCl_2$, and 300 mM Sucrose) and the cocktail of protease inhibitors was added to the buffer immediately before use. The cell suspension was homogenized with a Teflon pestle for 20-40 minutes. Five µl of the cell suspension were pipetted, mixed with 10 µl 0.4% Trypan Blue stain and 35 µl PBS. The sample was observed under the microscope for the presence of intact nuclei and complete lysis of the cells. After confirmation of successful cell lysis, the suspension was centrifuged at 700 g for 10 minutes to pellet the nuclear fraction. The supernatant, which is the non- nuclear fraction, was kept for further investigation. The nuclear fraction was resuspended in 2 ml of cold nuclear extraction buffer and 0.1 % Nonidet P-40 was added to the solution, which was kept on ice for 5 minutes, washed twice with 2 ml nuclear extraction buffer and centrifuged at 800 g for 10 minutes. At this stage, the isolated nuclei can be used for immunofluorescence, FACS studies or used for nuclear protein extraction.

For the nuclear protein extraction, the nuclear pellet was resuspended in 100-200 µl extraction buffer (40 mM Tris pH 7.4, 2 mM EDTA, 2mM EGTA, 300 mM NaCl, 1mM Na_3VO_4, 2% Triton). Samples were shaken on a turnover shaker at 4°C for 30

minutes and centrifuged at 12 000 g for 15 minutes. The supernatant contained the nuclear proteins.

2.15 Protocol FACS (Fluorescence Activated Cell Sorting)

HEK293 cells were grown in a 15 cm petri dish until they reached eighty per cent confluence. Subsequently the cells were transiently transfected with a FLAG – PAR2 – HA construct (generous gift from Dr. Nigel Bunnett, Roosterman *et al.,* 2003). Twenty-four hours post transfection, the cells were split into four 10 cm dishes. Forty-eight hours after transfection, the cells were starved for 6 h. Where applicable, 10 µM colchicine was added for 3 hours after 3 hours of starving. Cells were then treated with anti – Flag antibody (1: 200) for 15 minutes, the cell medium was changed and 20 µM SLIGKV was added for 30 minutes. Subsequently, intact nuclei were isolated and examined by FACS (BD LSR II Flow Cytometer)**.**

2.16 Immunofluorescence assay

In order to determine the levels of co-localization between PAR2 and members of the SNX family, different cell lines were used:

a) HEK293

b) HEK293 EGFP - hPAR2 stable cell line.

HEK293 cells were transfected with EGFP – hPAR2 construct. The efficiency of the transfection was maintained with 200 µg/ ml G 418.

c) HEK293 EGFP - mSNX11 stable cell line.

HEK293 cells were transfected with EGFP – mSNX11 construct. The efficiency of the transfection was maintained with 200 µg/ ml G 418.

On the first day, cells were incubated with trypsin, and 60 000 cells were plated per well (12 well plate) on polylysine or collagen treated cover-slips.

On day two, cells with fifty to sixty per cent confluence were starved for 6 hours. The cells were separated in two groups: the first group was stimulated for 30 minutes with SLIGKV, and the second was not stimulated. Subsequently the medium was aspirated and cells were fixed with 4% paraformaldehyde for 20 minutes. The coverslips were washed three times with PBS for 5 minutes each time and were treated with 0.5 ml blocking buffer (90% PBS, 5% goat serum and 5% FBS) for 30 minutes; 0.1% Triton was then added. The Triton functions to permeabilise the plasma membrane and to allow the antibodies to penetrate. After 15 minutes the Triton was removed and the cells were washed three times for 5 minutes with PBS. The coverslips were then treated with the appropriate primary antibodies (SNX1 1/100 in blocking buffer, SNX2 1/100 in blocking buffer, GFP 1 / 100 in blocking buffer) and incubated overnight at 4 °C.

On the next day coverslips were washed 3 times with PBS for 5 minutes, incubated for one hour at RT, after addition of secondary antibodies Alexa Fluor 594 (Invitrogen Corporation, Burlington, ON, Canada) and washed 3 times with PBS for 5 minutes. Nuclei staining was performed with DAPI 1 / 1000 in PBS for 1 minute and washing 3 times with PBS. Coverslips were mounted on a microscope glass using 15 µl mounting buffer ProLong (Invitrogen Corporation, Burlington, ON, Canada). The same procedure was followed for isolated intact nuclei. The images were obtained with LSM 510 Zeiss microscope at 63x magnification.

2.17 Co-immunoprecipitation

For the co-immunoprecipitation assays we used the protocol provided by the manufactoring company. Immunoprecipitation kit (Protein G) (Roche Applied Science, Laval, QC, Canada) was used. One petri dish with cells was scratched, washed and the cells were homogenized using a Dounce homogenizer. The cell suspension was centrifuged at 12 000 g for 10 minutes. The supernatant was carefully transferred to a fresh microcentrifuge tube. Five hundred µg protein G – agarose suspension was added to the sample and the mixture was incubated for 3 hours at 4 ºC on a rocking platform. Agarose beads were pelleted at 12 000 g for 20 seconds. The supernatant was transferred to a new tube, 2 µg of anti - PAR2 or anti – His antibodies (CTL Ig G – 2 µg) were added. Five hundred µg protein G – agarose suspension was added and the mixture was incubated for 3 hours at 4 º C on a rocking platform. The agarose – antibody – antigen complex was collected at 12 000 g for 20 seconds, resuspended in 1 ml washing buffer I and incubated for 20 minutes at 4 º C on a rocking platform. The last step was repeated and the complex was collected as described before. The complex was resuspended in 1 ml washing buffer II and the procedure was repeated as described above for washing buffer I. Finally the complex was washed with washing buffer III, pelleted and the washing was repeated. The pellet was resuspended in 75 µl gel loading buffer, the protein complex was denatured at 100 ºC for 3 minutes and protein G – agarose was removed at 12 000 g for 20 seconds. The supernatant was transferred in a new tube and used for SDS – electrophoresis and consecutive Western blot study.

2.18 Statistical analysis

The statistical analysis was performed using GraphPad Software (La Jolla, CA, USA). One-way analysis of variance (ANOVA) test was performed for the calculations of the P values. If the results from the ANOVA test were significant than multiple comparison tests were performed. In some cases we used Bonferroni test to determine the differences between all groups and in other cases we used Dunnet test to compare with the control group. The results of the ANOVA are presented in an ANOVA table, followed by the F statistic and associated P value. If the P value is less than 0.05, then the hypothesis is accepted that the means of at least two of the subgroups differ significantly.

IV. RESULTS

1. Expression analysis and subcellular localization of PAR2

The first aim was to visualize the subcellular localization of PAR2 using fluorescence microscopy and confocal microscopy techniques. PAR2 antibodies on HEK293 cells were used to determine PAR2 expression (Figure 3). The basic levels of PAR2 in native cells and non – stimulated condition were low. For more precise results and visualization of PAR2 intracellular localization, we used a stable overexpression system with GFP tag (Figure 4). The images of HEK293 – PAR2 stable line cells with confocal microscope demonstrated as well the translocation of PAR2 from the plasma membrane to the endosomes and perinucleus upon activation of PAR2 receptor. In a detailed study, where a marker for the nuclear membrane -Lamin B receptor was used, we discovered co-localization of PAR2 and Lamin B receptor, which suggested, that part of the cell membrane PAR2 is also translocated to the nucleus (Figure 4).

The initial images demonstrated that PAR2 is localized preferentially in the plasma membrane, however a small population of endogenous PAR2 was observed in perinucleus and in the nuclear membrane.

Figure 3: Intracellular distribution of native PAR2 in HEK293 cells

The cells were grown on glass coverslips until they reached sixty – seventy per cent confluence, starved for 6 hours and fixed with 4 % paraformaldehyde for 20 min. Consequently they were treated with primary anti – PAR2 antibody diluted 250 times in PBS buffer, and secondary antibodies in red, diluted 20 000 times in PBS buffer. The nuclei were visualized using DAPI staining. The images were taken on a Zeiss LSM 510 confocal microscope at 63x magnification.

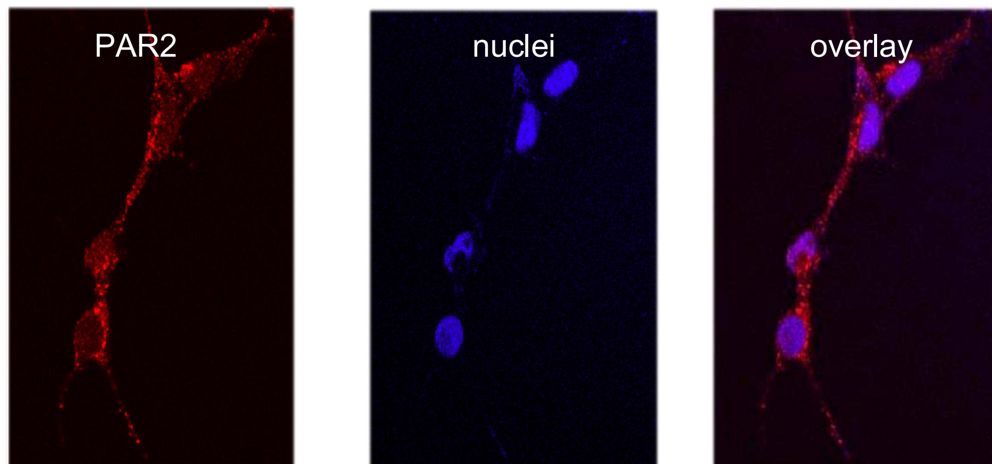


Figure 4: Internalization and nuclear translocation of PAR2 upon activation in HEK293 cells

Stable HEK293 cell line transfected with PAR2-GFP was used. The cells were grown on glass coverslips until they reached sixty-seventy per cent confluence, starved for 6 hours and used either as unstimulated cells or cells stimulated with 20 µM SLIGKV for 30 minutes. Cells were fixed with 4 % paraformaldehyde for 20 minutes. Anti – Lamin B Receptor antibody was used to visualize the nuclear membrane. Nuclei were visualized with DAPI staining. Images were taken with a Zeiss LSM 510 confocal microscope at 63x magnification.

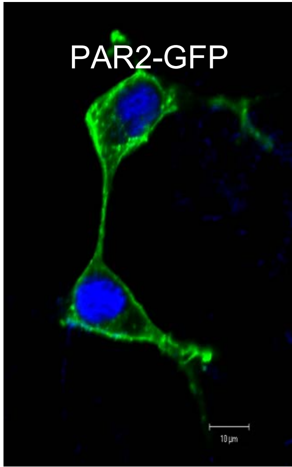
Colours:

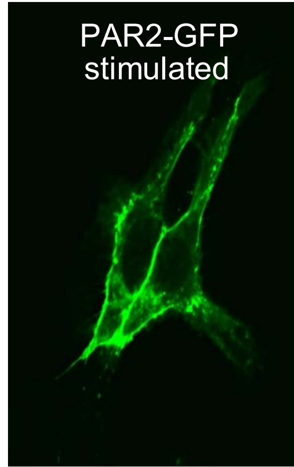
Red: Lamin B receptor

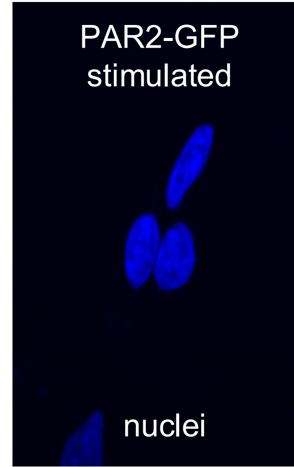
Blue: Nucleus

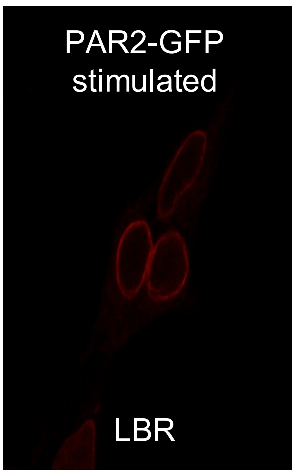
Green: PAR2-GFP

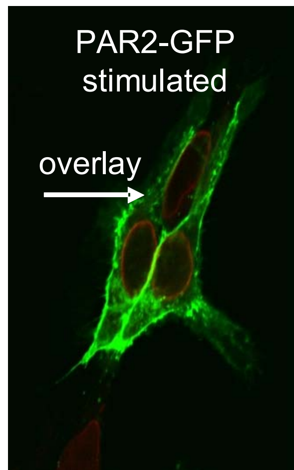
Orange: Overlay

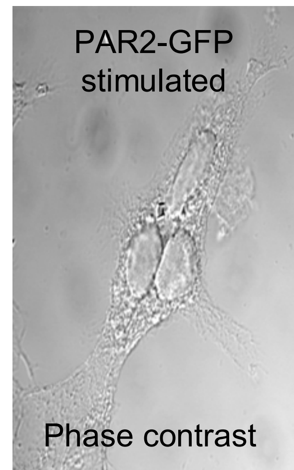

PAR2-GFP


PAR2-GFP
stimulated


PAR2-GFP
stimulated
nuclei


PAR2-GFP
stimulated
LBR


PAR2-GFP
stimulated
overlay


PAR2-GFP
stimulated
Phase contrast

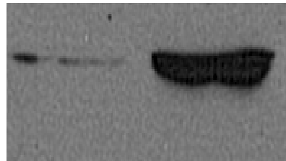
2. Agonist stimulation of PAR2 and Western blot analysis

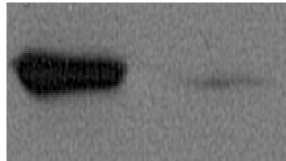
In order to confirm the results obtained from confocal microscope with Western blot we needed first to establish a technique to ensure that we could isolate pure nuclear fractions. It was necessary that our nuclear fraction did not contain any significant traces from other cellular fractions and especially from the plasma membrane fraction which is extremely rich in PAR2. Once we had developed a reliable method for nuclear isolation (Figure 5) we were able to demonstrate significant augmentation of PAR2 in the nucleus fraction (Figure 6). In comparison with the control experiments (non-stimulated cells) and an actin loading control, a stable average pattern of twofold increased nuclear PAR2 expression upon stimulation was observed (Figure 6).

.

Figure 5: Determining the purity of non-nuclear and nuclear cell fractions

HEK293 cells were stimulated with 20 nM trypsin for 30 minutes. Subsequently, they were lysed and centrifuged in order to separate the different cellular fractions. 20 µg protein from each fraction were loaded on 10 % SDS gel for PAGE separation. The antibodies were diluted as follow: 250 times for Lamin B receptor, 500 times for GAPDH and 4000 times for Integrin α 5. The reaction with secondary antibodies was carried out with antibodies from the appropriate IgG species conjugated to Horseradish peroxidase, diluted 20 000 times in 1,5 % milk in TBS – T during one hour at RT.


non - nuclear
nuclear
Lamin BR


non - nuclear
nuclear
GAPDH

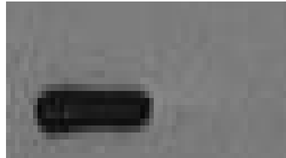
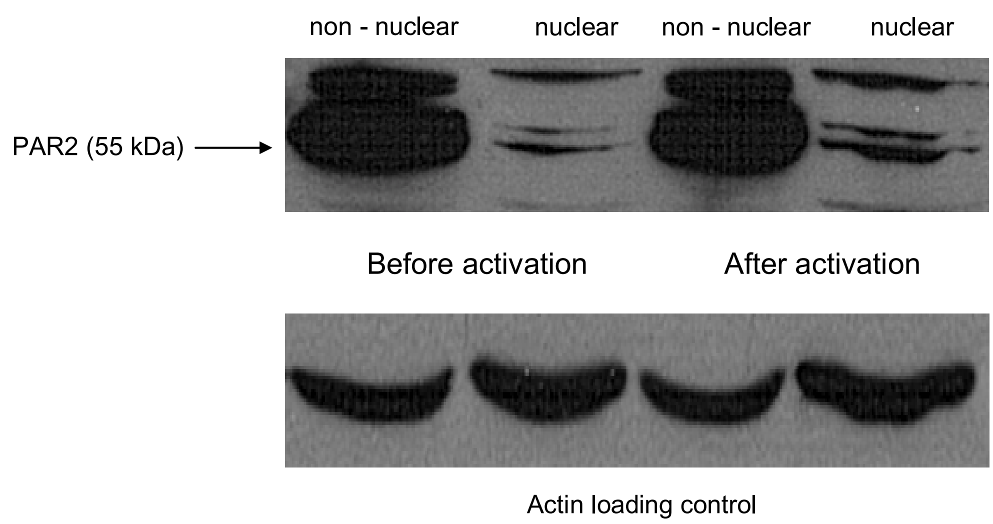

non - nuclear
nuclear
Integrin α5

Figure 6: Translocation of PAR2 from the plasma membrane to the nuclear membrane upon stimulation with 20 nM trypsin in HEK293 cells

HEK293 cells were stimulated with 20 mM trypsin for 30 minutes. Subsequently they were lysed and centrifuged in order to separate the different cellular fractions. Twenty µg proteins from each fraction were loaded on 10 % SDS – gel in order to separate the proteins by PAGE. Primary anti – PAR2 antibody was diluted 250 times in 1,5 % milk diluted in buffer TBS – T and incubated 24 hours at 4 º C. The reaction with secondary antibodies was carried out with antibodies from the appropriate species IgG conjugated to Horseradish peroxidase, diluted 20 000 times in 1,5 % milk in TBS – T during one hour at RT. PAR2 appeared on the blot at 55 kDa, as two separate lines. The band over 55 kDa is unspecific (as shown on the specifications from the manufacturer).


non - nuclear
nuclear
non - nuclear
nuclear
PAR2 (55 kDa)
Before activation
After activation
Actin loading control

3. Fluorescence-activated cell sorting analysis

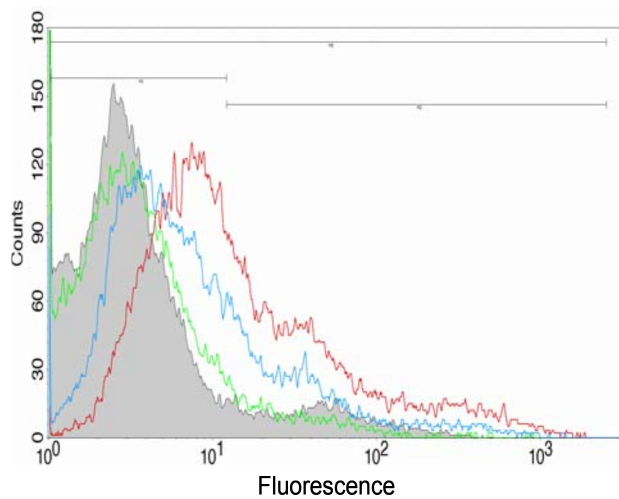
Western blot is not highly precise as a method for quantitative analyse. Therefore we used FACS (Fluorescence-activated cell sorting) technique. HEK293 cells were transiently transfected with a FLAG – PAR2 – HA construct. The cells were stimulated with 20 μM SLIGKV, which does not cleave the N – terminal upon stimulation and therefore leaves the antibody-labelled FLAG tag intact. Intact nuclei were isolated, and immunofluorescence assay against FLAG was performed. Subsequently the nuclei of stimulated and control cells were examined under FACS, and the results showed an approximately fivefold increase of PAR2 under stimulation with SLIGKV peptide. (Figure 7).

PAR2 nuclear translocation was significantly decreased when blocking the intracellular trafficking with colchicine (Figure 7, Table 1).

These observations confirm that PAR2 translocates from the plasma membrane to the nucleus upon SLIGKV stimulation.

Figure 7: Nuclear translocation of PAR2

HEK293 cells were transiently transfected with FLAG – PAR2 – HA construct. 48 hours post transfection cells were starved for 6 hours. Ten μM Colchicine was added for the last 3 hours of starvation. Subsequently, cells were treated with 200 times diluted anti – FLAG antibodies for 15 minutes and were stimulated with 20 μM SLIGLKV for 30 minutes. Nuclear fraction was isolated and intact nuclei were examined on FACS for the intensity of fluorescence signal. All results were normalized to the untransfected portion.



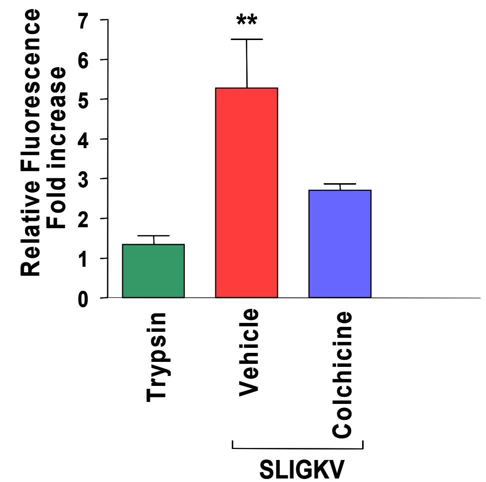


Table 1: Statistical analysis of nuclear translocation of PAR2

One-way analysis of variance				
P value	0.0027			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	7.761			
R squared	0.6245			
ANOVA Table	SS	df	MS	
Treatment (between columns)	52.02	3	17.34	
Residual (within columns)	31.28	14	2.234	
Total	83.29	17		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary
Trypsin vs Vehicle	-3.935	3.925	Yes	**
Trypsin vs Colchicine	-1.369	1.365	No	ns
Vehicle vs Colchicine	2.566	2.714	No	ns

4. iNOS and Tie2 genes activated by PAR2 expression

We aimed at identifying if there was any difference in the function of the two distinct PAR2 receptor populations in the plasma membrane and in the nucleus.

We investigated the expression pattern of the genes iNOS and Tie2 which are known to be upregulated by PAR2. For this purpose, HEK293 cells were stimulated with 20 μM SLIGKV for 30 min, and at the same time intracellular trafficking was unspecifically blocked with 10 μM colchicine. RNA extraction and RT-PCR on the genes of interest was then performed. PPARγ1 gene expression was used as a control. This is a nuclear receptor and it is not influenced by intracellular trafficking.

We observed that when the cells were stimulated with SLIGKV, Tie2 and iNOS nuclear expressions increased similarly. When we blocked intracellular trafficking with colchicine and stimulated with SLIGKV only Tie2 nuclear expression increased, whereas iNOS levels remained unaltered. These results revealed that iNOS is dependent on PAR2 intracellular trafficking, whereas Tie2 is independent (Figure 8). With the results described above we were able to confirm that nuclear sorting plays an important role in the signalling of PAR2.

Figure 8: Functional characterization of PPARγ1 iNOS, and Tie2 genes after PAR2 internalization upon stimulation

HEK293 cells were starved for 6 hours (in case of colchicine treatment – after the third hour of cell starvation, 10 µM colchicine was added for 3 hours). The cells were stimulated with 20 µM SLIGKV for 30 minutes. RT – PCRs were conducted. As a control PPARγ1 was used, which is a nuclear receptor whose expression is not dependent of intracellular trafficking. iNOS and Tie2 are genes whose expression is up regulated by PAR2. 18S housekeeping gene was used as a control for normalization. All results were normalized to 18S expression levels.

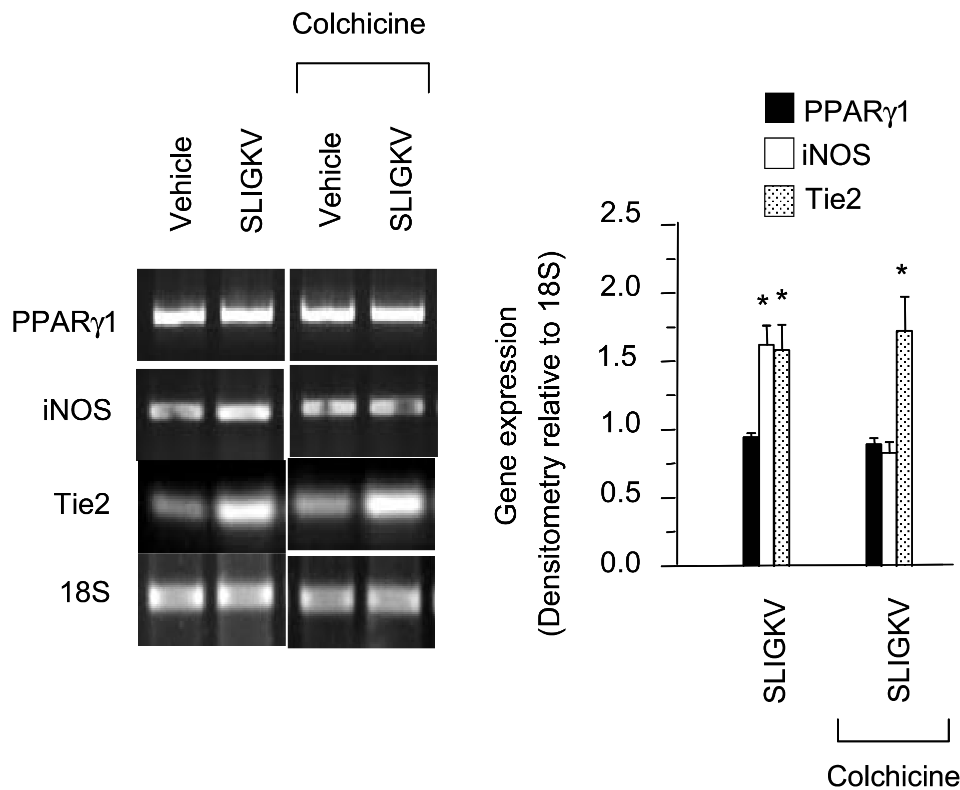


Table 2: Statistical analysis of functional characterization of PPARγ1 iNOS, and Tie2 genes after PAR2 internalization upon stimulation

iNOS

Table Analyzed	Data 1				
One-way analysis of variance					
P value	0.0018				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	9.327				
R squared	0.6998				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2.648	3	0.8827		
Residual (within columns)	1.136	12	0.09465		
Total	3.784	15			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant P < 0.05	Summary	95% CI of diff
Vehicle vs SLIGKV	-0.8375	3.850	Yes	**	-1.421 to -0.2539
Vehicle vs Colchicine	0.0000	0.0000	No	ns	-0.5836 to 0.5836
Vehicle vs Colchicine+AP	0.2300	1.057	No	ns	-0.3536 to 0.8136

Tie2

Table Analyzed	Data 1				
One-way analysis of variance					
P value	0.0098				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	7.642				
R squared	0.7413				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2.455	3	0.8184		
Residual (within columns)	0.8567	8	0.1071		
Total	3.312	11			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant P < 0.05	Summary	95% CI of diff
Vehicle vs SLIGKV	-0.9357	3.502	Yes	*	-1.705 to -0.1662
Vehicle vs Colchicine	0.0000	0.0000	No	ns	-0.7694 to 0.7694
Vehicle vs Colchicine+AP	-0.8713	3.261	Yes	*	-1.641 to -0.1019

PPARγ1

Table Analyzed	Data 1				
One-way analysis of variance					
P value	0.0485				
P value summary	*				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	4.119				
R squared	0.607				
ANOVA Table	SS	df	MS		
Treatment (between columns)	0.02272	3	0.007574		
Residual (within columns)	0.01471	8	0.001839		
Total	0.03743	11			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Vehicle vs SLIGKV	0.05533	1.58	No	ns	-0.04549 to 0.1562
Vehicle vs Colchicine	0	0	No	ns	-0.1008 to 0.1008
Vehicle vs Colchicine +AP	0.1043	2.98	Yes	*	0.003512 to 0.2052

5. SNX1 and SNX2 implication in PAR2 translocation to the nucleus

In order to test the hypothesis that SNX1 and SNX2 are implicated in PAR2 translocation to the nucleus, HEK293-EGFP-PAR2 stable line was used.

Cells were stimulated with SLIGKV, while as a control we used unstimulated cells (Figure 9). The same method was employed for the isolated nuclei (Figure 10). The results showed that SNX1 and SNX2 co-localize with PAR2 (Figure 9) as expected from the data shown in literature about PAR1 (Wang *et al.,* 2002). In this study the authors observed that PAR1 and endogenous SNX1 colocalize with EEA1-positive early endosomes. In our results the pattern of co-localization suggests probable spatial interaction in perinuclear zone and probably in endosomes (Figure 9). On the other hand no co-localization was detected at nuclear level (Figure 10).

Figure 9: Intracellular co-localization of PAR2 with SNX1 and SNX2

A. PAR2 - GFP stable transfected HEK293 cells were grown on glass coverslips, starved for six hours, fixed with 4 % paraformaldehyde for 20 minutes, treated with anti – SNX1 antibodies diluted 200 times and DAPI stained to visualize the nuclei. Images were obtained on a Zeiss LSM 510 confocal microscope at 63x magnification.

B. PAR2 - GFP stable transfected HEK293 cells were grown on glass coverslips, starved for six hours, fixed with 4 % paraformaldehyde for 20 minutes, treated with anti – SNX2 antibodies diluted 200 times and DAPIstained to visualize the nuclei. Images were obtained on a Zeiss LSM 510 confocal microscope at 63x magnification.

Colours:

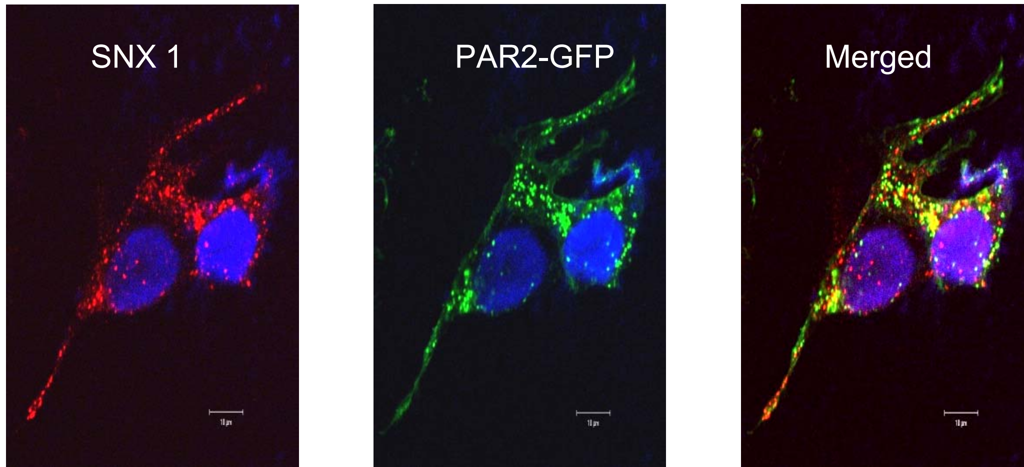
Red: SNX1 (A); SNX2 (B)

Blue: Nucleus

Green: PAR2-GFP

Yellow: Merged

A



B

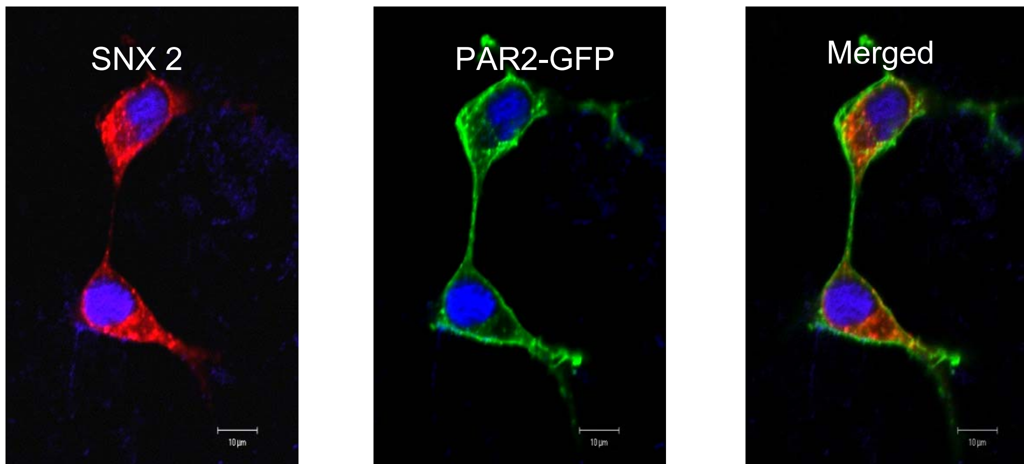


Figure 10: Lack of nuclear localization of SNX1 and SNX2

A. PAR2 – GFP stable transfected HEK293 cells were starved for 6 hours, and treated with 20 μM SLIGKV for 30 minutes. Nuclei were isolated, fixed with 4 % paraformaldehyde for 20 minutes, treated with anti –SNX1 antibodies diluted 100 times and DAPIstained. Images were obtained on a Zeiss LSM 510 confocal microscope at 63x magnification.

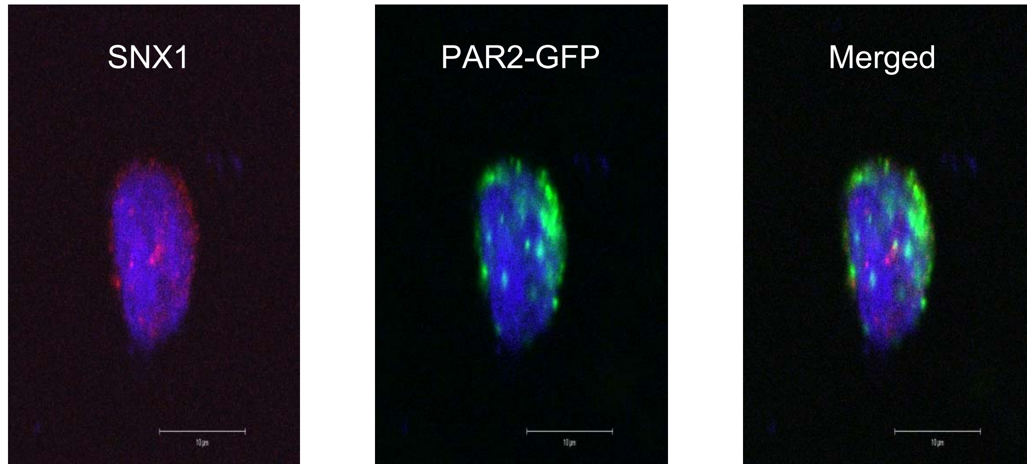
B. PAR2 – GFP stable transfected HEK293 cells were starved for 6 hours, and treated with 20 μM SLIGKV for 30 minutes. Nuclei were isolated, fixed with 4 % paraformaldehyde for 20 minutes, treated with anti –SNX2 antibodies diluted 200 times and DAPI stained. Images were obtained on a Zeiss LSM 510 confocal microscope at 63x magnification.

Colours:

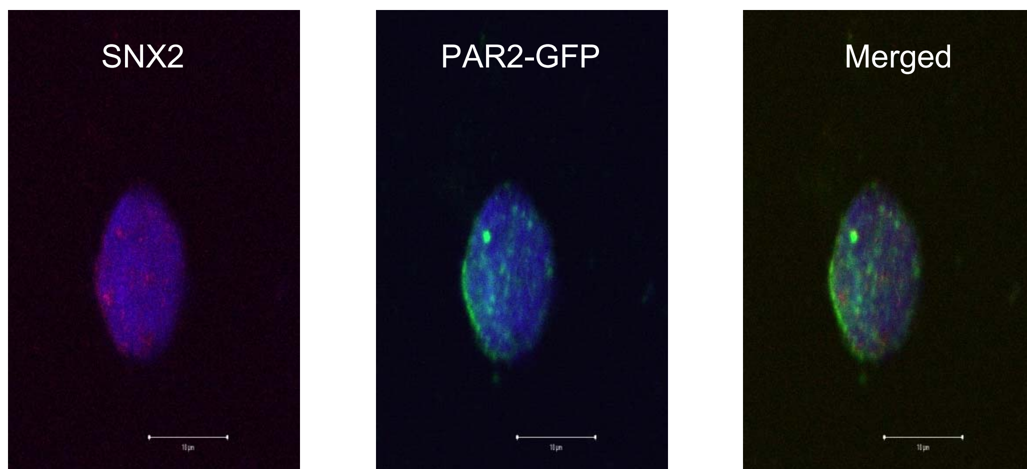
Green: PAR2-GFP

Blue: Nucleus

A



B



6. Subcellular localization of SNX11 and PAR2

In order to study the cellular function of SNX11, GFP and HIS tagged clones of murine SNX11 were created and stable overexpression in HEK293 cells was achieved. We used the available mSNX11 clone to carry out a cloning in EGFP expression vector. A transient transfection of HEK293-EGFP-SNX11 cell lines with FLAG-PAR2-HA construct was performed. Subsequently the experiment was conducted with unstimulated cells and cells stimulated with AP (20 μM SLIGV) for 30 minutes. The cells were fixed and observed under a confocal microscope (Figure 11). The same procedure was used for the extracted nuclei (Figure 12). Images revealed two interesting observations:

1. SNX11 was shown to be present in the nucleus.

2. We were able to demonstrate that under stimulation PAR2 and SNX11 co-localized in the nuclear membrane.

The co-localization of SNX11 with Lamin A/C after SLIGKV stimulation provides evidence for presence of SNX11 in the nuclear membrane (Figure 12).

Figure 11: Intracellular distribution of SNX11 and co – localization with PAR2 upon stimulation

A. SNX11 – GFP stable transfected HEK293 cells were transiently transfected with FLAG – PAR2 – HA construct and grown on glass coverslips until they reached sixty to seventy per cent confluence. Cells were then starved for six hours, fixed with 4 % paraformaldehyde for 20 minutes, treated with anti – FLAG antibodies diluted 200 times and nuclei were visualized with DAPI staining. Images were obtained on a Zeiss LSM 510 confocal microscope at 63x magnification.

B. SNX11 – GFP stable transfected HEK293 cells were transiently transfected with FLAG – PAR2 – HA construct and grown on glass coverslips. 24 hours post transfection cells were starved for six hours and treated with 20 µM SLIGKV for 30 minutes. Cells were fixed with 4 % paraformaldehyde for 20 minutes, treated with anti – FLAG antibodies diluted 200 times, nuclei were visualized with DAPI. Images were obtained on a confocal Zeiss LSM 510 microscope at 63x magnification.

Colours:

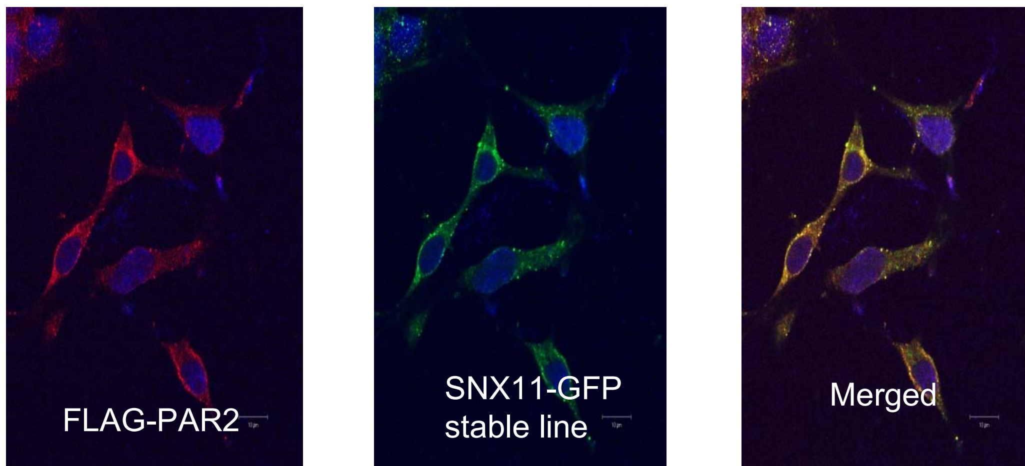
Red: FLAG-PAR2

Green: SNX11-GFP

Blue: Nucleus

Yellow: Merged

A



B

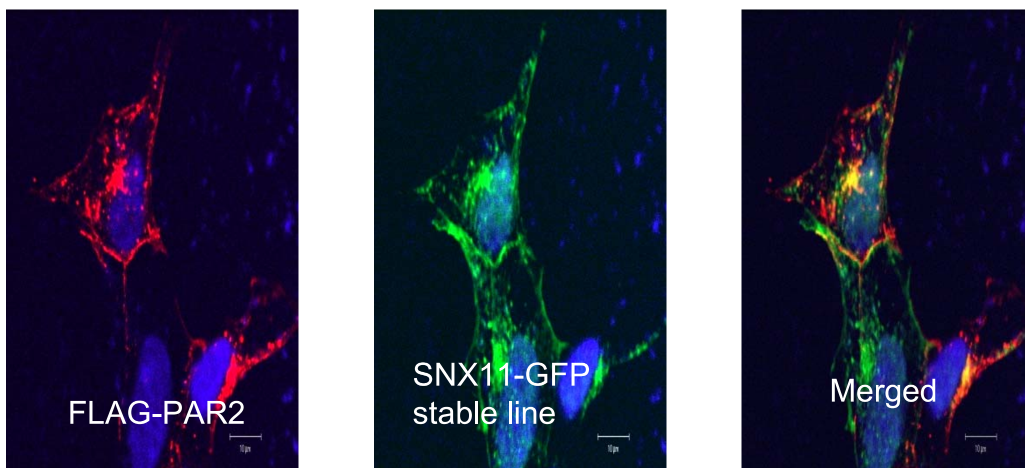


Figure 12: Nuclear expression and co-localization of SNX11/ PAR2 and SNX11/ Lamin A/C receptor

A. SNX11 – GFP stable transfected HEK293 cells were transiently transfected with FLAG – PAR2 – HA construct. 24 hours post transfection cells were starved for six hours and treated with 20 µM SLIGKV for 30 minutes. Nuclei were isolated, fixed with 4 % paraformaldehyde for 20 minutes, treated with anti – FLAG antibodies diluted 200 times and process for DAPI staining. Images were obtained on a Zeiss LSM 510 confocal microscope at 63x magnification

B. SNX11 – GFP stable transfected HEK293 cells were starved for six hours and treated with 20 µM SLIGKV for 30 minutes. Nuclei were isolated, fixed with 4 % paraformaldehyde for 20 minutes, treated with anti – Lamin A/C antibodies diluted 30 times and DAPIstained. Images were obtained on a Zeiss LSM 510 confocal microscope at 63x magnification.

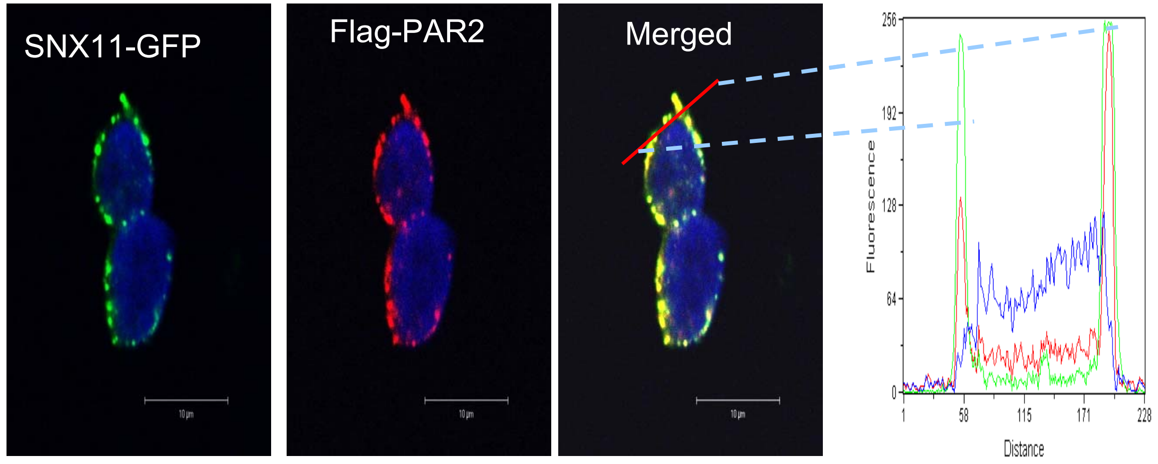
Colours:

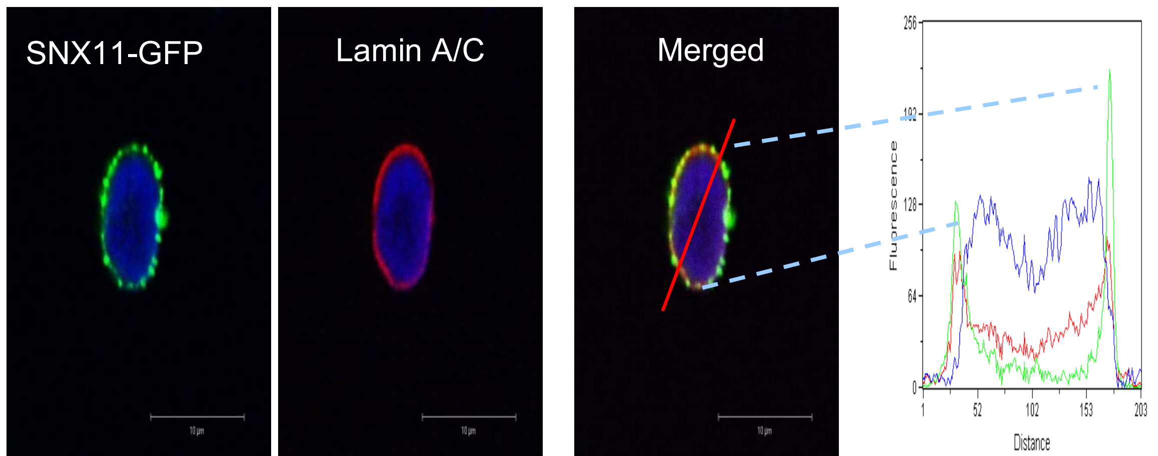
Red: FLAG-PAR2 (A) / Lamin A/C (B)

Green: SNX11-GFP

Blue: Nucleus

Yellow: Merged


A
SNX11-GFP
Flag-PAR2
Merged
Fluorescence
Distance


B
SNX11-GFP
Lamin A/C
Merged
Fluorescence
Distance

7. Co-immnunoprecipitation analysis

7.1. Co-immunoprecipitation of PAR2 and SNX1 and SNX2

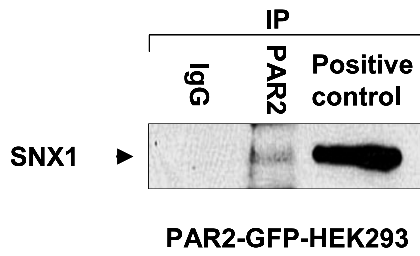
For the detection of an interaction between PAR2 and SNX1 and SNX2, PAR2-GFP HEK293 stable cell lines were used. Anti –PAR2 antibodies were used to precipitate PAR2 protein and its partners. After protein separation and transfer on a membrane we detected successfully PAR2-SNX1 and PAR2-SNX2 interaction using SNX1 and SNX2 antibodies (Figure 13). Pull down with IgG only was used as negative control for the antibodies; Western blot on a whole cell lysate of HEK293 was used as a positive control for the antibodies.

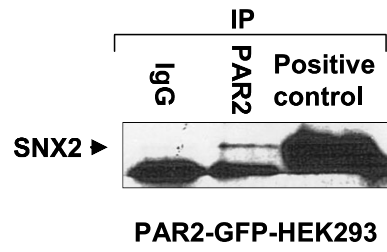
7.2. Co-immunoprecipitation of PAR2 and SNX11

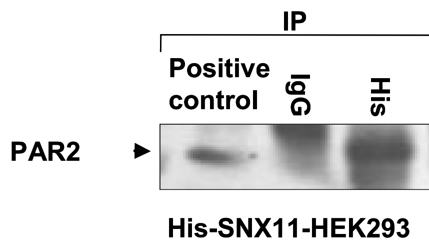
For the detection of an interaction between PAR2 and SNX11, His-mSNX11 and PAR2-GFP HEK293 stable cell lines were used. Anti - His antibodies were used to precipitate SNX11 protein and its partners. After protein separation and transfer on a membrane we detected successfully PAR2-SNX11 interaction using PAR2 antibody (Figure 13). Reverse co-immunoprecipitation was also performed. In PAR2-GFP HEK293 stable cell line, PAR2 antibody was used for pull-down and SNX11 was detected with specific antibody (Figure 13). The interaction between SNX11 and PAR2 was significantly stronger than those between PAR2 and SNX1 and SNX2. Pull down with IgG only was used as negative control for the antibodies; Western blot on a whole cell lysate of was used as a positive control for the antibodies.

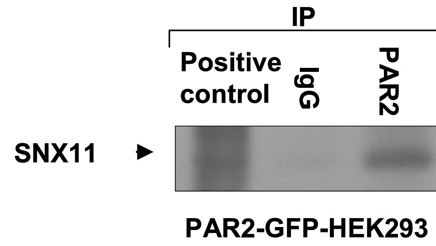
Figure 13: Co – immunoprecipitation of PAR2 with SNX1, SNX2 and SNX11

PAR2 – GFP and His – SNX11 stable HEK293 cell lines were used, following the general protocol for Protein G-Agarose provided by the manufacturing company (Roche Applied Science, Laval, QC, Canada). 2 µg of anti - PAR2, anti – His (CTL Ig G – 2 µg) and 500 µg protein G – agarose suspension were used. For the Western blot analysis anti – SNX1 and anti – SNX2 antibodies were diluted 200 times, anti – SNX11 antibodies were diluted 500 times in 5 % milk in TBS – T buffer. Pull down with IgG only was used as negative control for the antibodies; Western blot on a whole cell lysate was used as a positive control for the antibodies.


IP
IgG
PAR2
Positive control
SNX1
PAR2-GFP-HEK293


IP
IgG
PAR2
Positive control
SNX2
PAR2-GFP-HEK293


IP
Positive control
IgG
His
PAR2
His-SNX11-HEK293

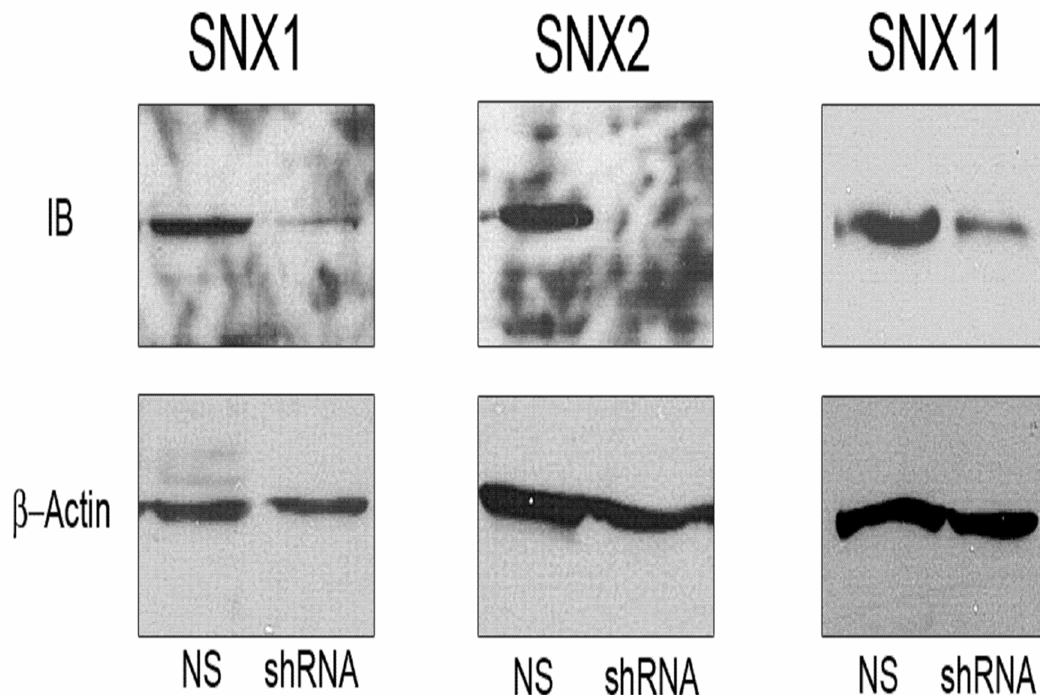

IP
Positive control
IgG
PAR2
SNX11
PAR2-GFP-HEK293

8. SNXs knockdown experiments

In order to conduct functional studies of SNX1, SNX2 and SNX11 we created knockdown cell lines. The knockdowns were performed with sh RNAs. The cells were transfected with shSNX1, shSNX2 and shSNX11 and control sh RNA NS (non-sense). The rate of knockdown efficiency was observed under a fluorescent microscope for the expression of GFP (green fluorescent protein) which was tagged to the above mentioned expression vectors. Knockdowns were confirmed using Western blot analysis. The shNS cell line was used as a control (Figure 14). We achieved very high efficiency of the knockdowns – almost 100 % for SNX2, 80 % for SNX1 and SNX11.

Figure 14: Establishing "knockdown" HEK293 cell lines of SNX1, SNX2 and SNX11 genes

HEK293 cells were transfected with the appropriate shRNA vector using the manufacturer's protocol. Efficiency of transfection was confirmed using epifluorescence microscopy. The knockdowns were confirmed with Western blot. Cells were lyzed, proteins were extracted, separated on a 10 % acrylamide gel, transferred on a nitrocellulose membrane and treated with the appropriate antibodies. Anti – SNX1 and anti – SNX2 antibodies were diluted 200 times, and anti – SNX11 was diluted 500 times in 5 % milk in TBS – T. Secondary antibodies were diluted 20 000 times.

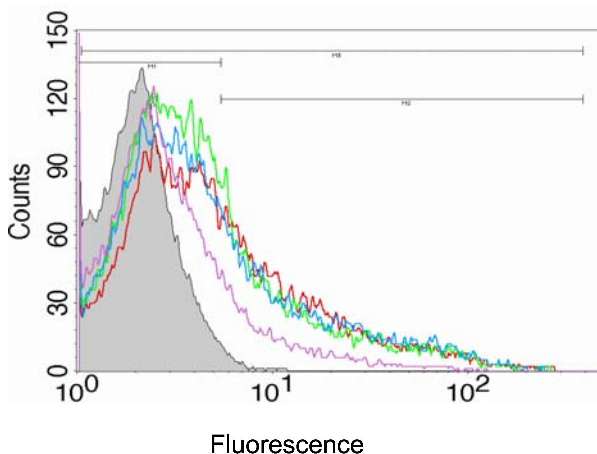


The knockdown cell line was used to perform experiments with AP stimulation followed by nuclear isolation. For each experiment four groups of cells were used: CTL-non-stimulated, CTL-stimulated, SNX-knockdown non-stimulated and SNX-knockdown stimulated. The goal of the experiment was to demonstrate that in control cells after stimulation the nuclear PAR2 increases and that such an augmentation cannot be detected in the knockdown cells.

We carried out several series of experiments using FACS on isolated nuclei from cells transiently transfected with the FLAG-PAR2-HA construct (Roosterman *et al.,* 2003). After cell transfection, the cells were stimulated with 20 μM SLIGKV for 30 minutes, treated with anti - FLAG antibodies, and the nuclei were isolated. FACS analysis of the nuclei revealed that the knockdown of either SNX1 or SNX2 does not abolish the increase of nuclear PAR2 after stimulation. Conversely, in SNX11 knockdowns we were able to detect a two-fold decrease in PAR2 nuclear localization after stimulation, compared to the stimulated control cells (Figure 15). We were able to confirm the effect of SNX11 knockdown on Western blot analysis (Figure 16). In control cells stimulated with trypsin (shNS stable line) nuclear PAR2 was increased, whereas in shSNX11 stable cell line stimulation resulted in a two-fold decrease of nuclear PAR2. Using confocal microscopy (Figure 17) we obtained similar results. In SNX11 stable knockdown HEK293 cells, PAR2 is exclusively membrane localized and no nuclear pattern was detected after SLIGKV stimulation.

Figure 15: Effect of SNXs knockdowns on PAR2 nuclear translocation

Knockdown HEK293 cell lines of SNX1, SNX2, SNX11 and control were used. Cells were transfected with FLAG – PAR2 – HA construct. 48 hours post transfection, cells were starved for 6 hours, treated with anti – FLAG antibodies diluted 200 times in PBS for 15 minutes and stimulated with 20 μM SLIGKV for 30 min. Nuclei were isolated and examined on FACS.



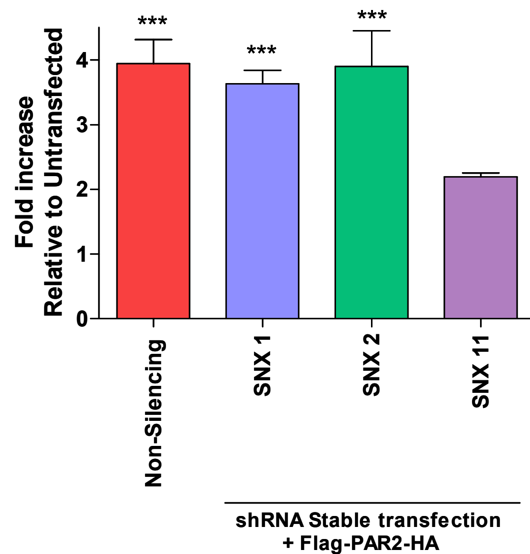


Table 3: Statistical analysis of effect of SNXs knockdowns on PAR2 nuclear translocation

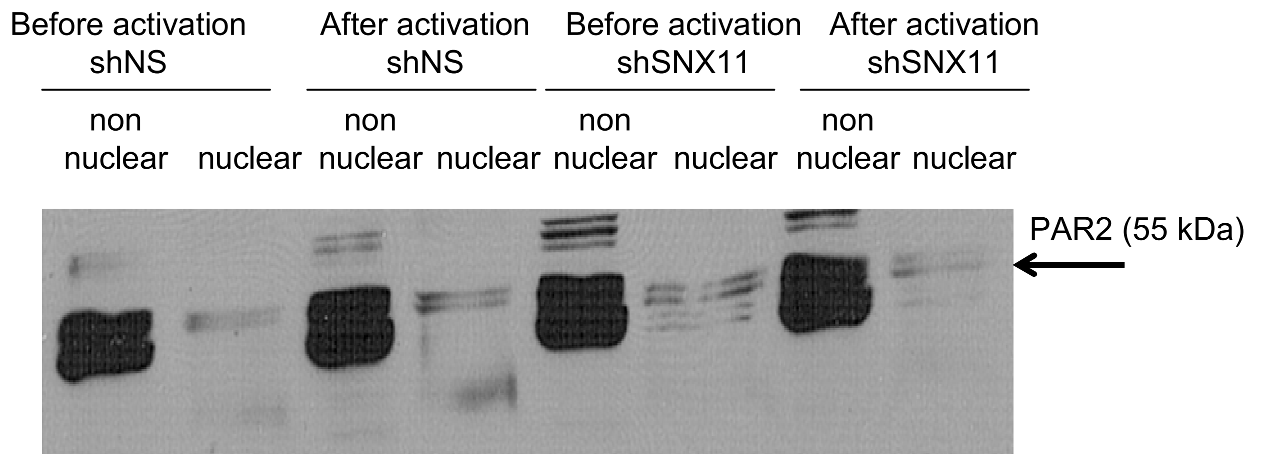
One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different (P < 0.05)	Yes
Number of groups	5
F	19.89
R squared	0.8689

ANOVA Table	SS	df	MS
Treatment (between columns)	24.91	4	6.227
Residual (within columns)	3.756	12	0.3130
Total	28.66	16	

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant P < 0.05	Summary
Untransfected vs Non-Silencing	-2.945	7.444	Yes	***
Untransfected vs SNX 1	-2.633	6.163	Yes	***
Untransfected vs SNX 2	-2.897	6.779	Yes	***
Untransfected vs SNX 11	-1.190	2.785	No	ns

Figure 16: SNX11 knockdown decreases PAR2 nuclear translocation

SNX11 knockdown HEK293 and control cells were grown to eighty per cent confluence and starved for 6 hours. Cells were stimulated with 20 nM Trypsin for 30 minutes and cellular fractions were isolated. Proteins were separated on 10 % acrylamide gel, and examined on Western blot. Anti - PAR2 antibodies were diluted 250 times in 5 % milk in TBS – T buffer.



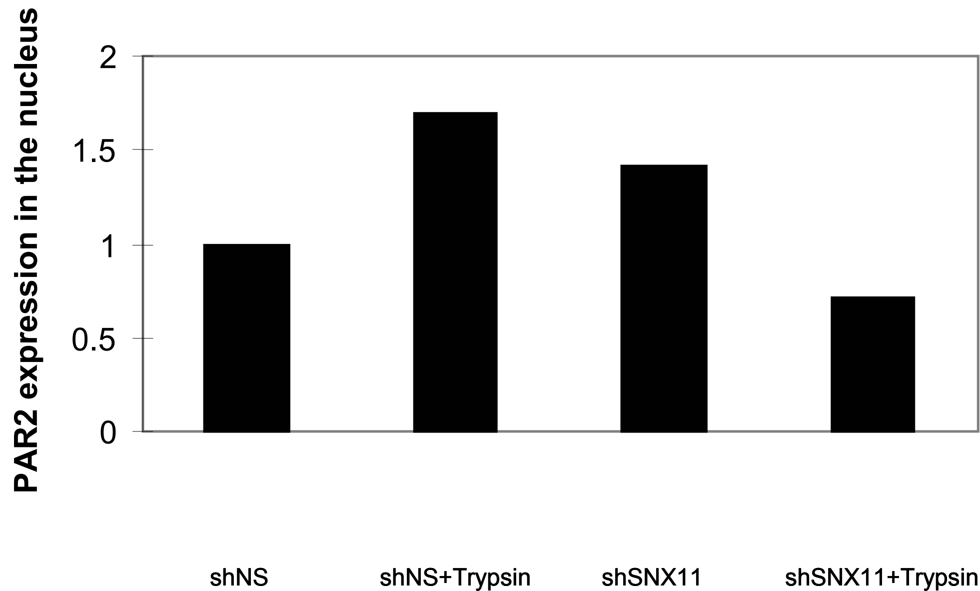


Figure 17: Immunofluorescence studies of PAR2 cellular localization in SNX11 knockdown HEK293 cells

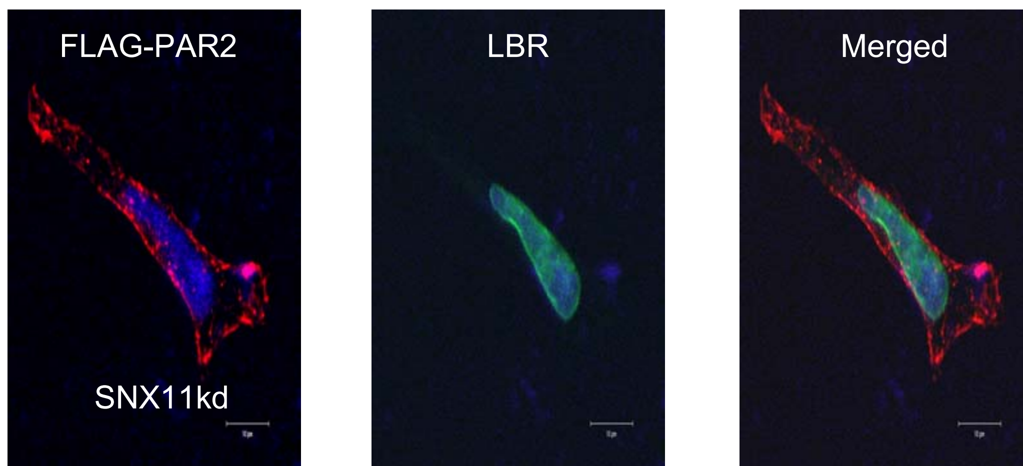
SNX11 knockdown HEK293 cell were transfected with FLAG – PAR2 –HA construct and cells were grown on glass coverslips. 48 hours after the transfection the cells were starved for 6 hours, stimulated with 20 μM SLIGKV for 30 minutes, fixed with 4 % paraformaldehyde for 20 minutes, treated with anti – FLAG and anti – LBR antibodies diluted 200 times in PBS and DAPI. Images were obtained on a LSM 510 Zeiss confocal microscope at 63x magnification.

Colours:

Red: FLAG-PAR2

Green: LBR

Blue: Nucleus



9. iNOS gene as a marker for PAR2 nuclear trafficking

Final objective was to investigate if there was any functional relationship of PAR2 and SNXs members to iNOS. iNOS up regulation is dependent on nuclear trafficking of PAR2. Therefore we used knockdown cell lines of SNX1, SNX2 and SNX11 to investigate how they influence iNOS expression. As a control we used Tie2 gene whose regulation is not dependent of PAR2 translocation. The cells were stimulated with 20 μM SLIGKV for 30 minutes, RNA was extracted and RT – PCR was performed. As expected, knockdown of SNX11 diminished the expression of iNOS to the level observed in the control experiments without SLIGKV. This result confirmed the functional role of SNX11 in PAR2 nuclear signalling. Interestingly, SNX1 and SNX2 were also able to attenuate iNOS expression though not to the same extent as SNX11. Expression levels of iNOS were attenuated by 15 % and 24% for SNX1 and SNX2 knockdowns respectively (Figure 18). Tie2 expression was not affected by knockdown of any of the three SNX family members.

Figure 18: Effect of SNXs knockdowns on the expression of PPARγ1, iNOS, and Tie2 genes after PAR2 stimulation

HEK293 stable knockdowns of SNX1, SNX2 and SNX11 cells were starved for 6 hours. The cells were stimulated with 20 μM SLIGKV for 30 minutes. RT – PCRs were conducted. As a control PPARγ1 was used, which is a nuclear receptor whose expression is not dependent of intracellular trafficking. iNOS and Tie2 are genes which expression is up regulated by PAR2. 18S housekeeping gene was used as a control for normalization. All results were normalized to 18S expression levels.

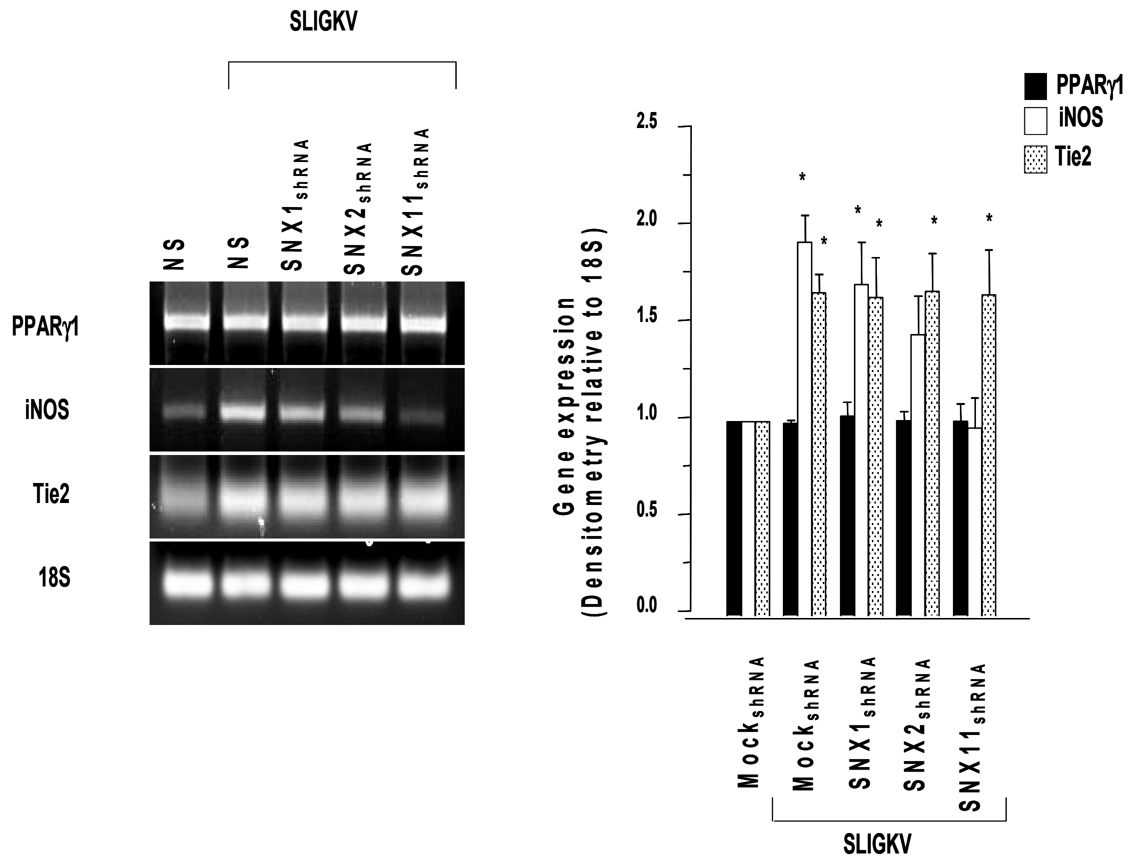


Table 4: Statistical analysis of effect of SNXs knockdowns on the expression of PPARγ1, iNOS, and Tie2 genes after PAR2 stimulation

iNOS

One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	5
F	1118
R squared	0.9978

ANOVA Table	SS	df	MS
Treatment (between columns)	1.848	4	0.4621
Residual (within columns)	0.004133	10	0.0004133
Total	1.853	14	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
MockshRNA vs MockshRNA+AP	-0.8767	52.81	Yes	***	-0.9326 to -0.8208
MockshRNA vs SNX1shRNA+AP	-0.7033	42.37	Yes	***	-0.7592 to -0.6474
MockshRNA vs SNX2shRNA+AP	-0.41	24.7	Yes	***	-0.4659 to -0.3541
MockshRNA vs SNX11shRNA+AP	-0.03	1.807	No	ns	-0.08591 to 0.02591
MockshRNA+AP vs SNX1shRNA+AP	0.1733	10.44	Yes	***	0.1174 to 0.2292
MockshRNA+AP vs SNX2shRNA+AP	0.4667	28.11	Yes	***	0.4108 to 0.5226
MockshRNA+AP vs SNX11shRNA+AP	0.8467	51	Yes	***	0.7908 to 0.9026

Tie2

One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	5
F	652.6
R squared	0.9962

ANOVA Table	SS	df	MS
Treatment (between columns)	1.096	4	0.2741
Residual (within columns)	0.0042	10	0.00042
Total	1.101	14	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
MockshRNA vs MockshRNA+AP	-0.68	40.64	Yes	***	-0.7364 to -0.6236
MockshRNA vs SNX1shRNA+AP	-0.6533	39.04	Yes	***	-0.7097 to -0.5970
MockshRNA vs SNX2shRNA+AP	-0.7133	42.63	Yes	***	-0.7697 to -0.6570
MockshRNA vs SNX11shRNA+AP	-0.6467	38.65	Yes	***	-0.7030 to -0.5903

MockshRNA+AP vs SNX1shRNA+AP	0.02667	1.594	No	ns	-0.02969 to 0.08303
MockshRNA+AP vs SNX2shRNA+AP	-0.03333	1.992	No	ns	-0.08969 to 0.02303
MockshRNA+AP vs SNX11shRNA+AP	0.03333	1.992	No	ns	-0.02303 to 0.08969

PPARγ1

One-way analysis of variance	
P value	0.0343
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	5
F	4
R squared	0.6154

ANOVA Table	SS	df	MS
Treatment (between columns)	0.000427	4	0.0001067
Residual (within columns)	0.000267	10	0.00002667
Total	0.000693	14	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
MockshRNA vs MockshRNA+AP	-0.01333	3.162	No	ns	-0.02753 to 0.0008682
MockshRNA vs SNX1shRNA+AP	-0.01333	3.162	No	ns	-0.02753 to 0.0008682
MockshRNA vs SNX2shRNA+AP	-0.01333	3.162	No	ns	-0.02753 to 0.0008682
MockshRNA vs SNX11shRNA+AP	-0.01333	3.162	No	ns	-0.02753 to 0.0008682
MockshRNA+AP vs SNX1shRNA+AP	0	0	No	ns	-0.01420 to 0.01420
MockshRNA+AP vs SNX2shRNA+AP	0	0	No	ns	-0.01420 to 0.01420
MockshRNA+AP vs SNX11shRNA+AP	0	0	No	ns	-0.01420 to 0.01420

V. DISCUSSION

G – protein coupled receptors represent the largest and the most diverse group of membrane receptors. Recent and detailed analysis of the human genome reveals over 800 unique GPCRs (Kobilka, 2007). These receptors are the widest source of targets for the pharmaceutical industry. Today, approximately one-quarter of the best-selling drugs on the market target GPCRs (Schultz et al., 2007).

In the recent years a group of GPCRs called Protease – activated receptors (PAR) emerged as an interesting new target for pharmacological studies. It was demonstrated that PAR members have significant role in diverse pathophysiological processes. Among PAR members, PAR2 lately distinguished itself as a promising pharmacological target (Bunnett *et al.,* 2003).

Several lines of evidence suggested its active contribution in important processes such as cell migration, proliferation and coagulation (Bunnett *et al.,* 2003). Bunnett *et al.* revealed that PAR2 has significant importance for the normal function of major physiological processes such as angiogenesis and the appropriate development of the cardiovascular system (Bunnett *et al.,* 2003). Current studies showed active participation of PAR2 in tumorigenesis, together with Epidermal Growth Factor Receptor (EGFR) and Tissue Growth Factor Receptor (TGFR). PAR2 is overexpressed in different type of cancers such as breast cancer, colon cancer and other type of malignant transformations (Darmoul *et al,* 2004; Ge *et al.,* 2004; Shimamoto *et al.,* 2004). At the same time some data suggest its protective role in certain types of tumors causing skin cancer (Laburthe *et al.,* 2004; Matej *et al.,* 2006; Steinhoff *et al.,* 2007). The dual role of PAR2 as tumorigenesis factor for some diseases and as a factor in cancer protection in other diseases is being intensively studied. Therefore to be able to better understand the

physiological role of PAR2 we need to understand its intracellular function and its cellular pathway.

PAR receptors take a unique place among the other GPCRs with their mechanism of activation. Trypsin is the agonist of PAR2. It cleaves the N-terminus of the receptor and opens a new tethered ligand, which itself activates the receptor and causes an irreversible receptor activation. Once stimulated, the activated receptor continuously triggers intracellular signals. Therefore, the right coordination of lysosomal sorting is highly important to end PAR2 signalling. Eventual defects in this mechanism can lead to pathophysiological changes such as a cancer.

For a long period of time it was an enigma how PAR2 undergoes degradation until a very recent study discovered the role of Rab5a and Rab11a in its intracellular trafficking (Roosterman *et al.,* 2003). They established that Rab5a is required for PAR2 endocytosis and resensitization, whereas Rab11a contributes to trafficking of PAR2 from the Golgi apparatus to the plasma membrane. However there are still lots of questions to be answered about how PAR2 signaling is being relayed in the cell. It is not clear if Rab5a is the only factor responsible for the endocytosis of PAR2 and if it contributes directly to PAR2 lysosomal degradation. It remains unclear how PAR2 is being transported to the nucleus as we discovered in our study.

In the recent years significant data have been accumulated for GPCRs which demonstrate that under activation these receptors are being translocated from the plasma membrane to the nucleus (Boivin *et al.,* 2008). Receptors such as EGFR were discovered to translocate from the plasma membrane to the nucleus where they function as a transcription factor (Lo *et al.,* 2006).

In our study we demonstrated that PAR2 is also involved in an alternative signalling pathway. We showed that two endogenous populations of PAR2 receptors are available in the cell, the first being on the plasma membrane and the second being on the nuclear membrane. The quantity of receptors located in the nucleus is relatively small in comparison with those on the plasma membrane. We have demonstrated that after stimulation with trypsin or its active peptide SLIGKV, PAR2 which is located in the plasma membrane is internalized and translocated to the perinuclear membrane (Figures 4, 6 and 7). The process of translocation is rapid and is completed within one hour after receptor stimulation (Figure 4).

The mechanisms of PAR2 translocation from the plasma membrane to the nuclear membrane are still not known. It is evident that two pathways for PAR2 internalization exist. One population of the receptors will be degraded after stimulation and the other will be translocated to the nuclear membrane where it will probably play a role in the induction of certain genes involved in cell proliferation, angiogenesis and inflammation processes. The presence of two different pools of PAR2 suggests that they play different roles. For example we have established that PAR2 nuclear translocation leads to increased levels of iNOS but not Tie2 (genes which are up-regulated by PAR2). Upon stimulation with SLIGKV, Tie2 levels are still increased when blocking unspecifically the intracellular trafficking with colchicine, in contsrast to the iNOS levels which are no longer increased (Figure 8), although the two genes are up-reagulated by PAR2. Thus the question arises which trafficking proteins determine this receptor fate?

In our study we explored the role of three proteins which belong to less well studied gene family of Sorting Nexins.

In recent years members of this family emerged as important partners in the intracellular trafficking of various receptors including GPCRs. It has been discovered that SNX1 is involved in lysosomal degradation of PAR1 (Gullapalli *et al.,* 2006) and in *in vitro* studies SNX1 interacts with PAR2 (Heydorn *et al.,* 2004).

In previous experiments in our lab it was discovered that SNX11 interacts with various proteins, most of them involved in cytoskeleton organisation and extracellular matrix (Table 5). Based on these data we presumed that SNX11 can be implicated in the trafficking of receptors.

In co – immunoprecipitation assay we were able to show that a physical interaction between PAR2 and SNXs exists (Figure 13). We detected a strong interaction between SNX11 and PAR2, and significantly weaker ones between SNX1/2 and PAR2. Confirming that PAR2 interacts with SNXs was an important result because we were able to show for the first time that SNX11 has the ability to interact with GPCRs and particularly with PAR2 membrane receptor.

SNX1 and SNX2 are well explored proteins and known to have the cargo ability to drive the receptors to the lysosomal degradation pathway.

At first, SNX1 was discovered to interact with EGFR and to play a role in its sorting to the lysosomes (Kurten *et al.,* 1996). Later on, it has been described that SNX2 may play an even more important role in the cellular transport of EGFR than SNX1 (Gullapalli *et al.,* 2004). SNX1 and SNX2 have been shown to have plasma membrane localization and vesicle localization (Gullapalli *et al.,* 2004). This cellular localization was reconfirmed in our studies (Figure 9). SNX1 and SNX2 have similar structure, possessing Phox domain and BAR domain, which allow them to dimerize (Bonifacino *et*

Table 5: SNX11 interaction partners

GENE	GROUP / DESCRIPTION	BIOLOGICAL FUNCTION	HUMAN PHENOTYPE
	CYTOSKELETON, CELL ADHESION, ECM		
Actc	Cardiac Alpha actin	Cardiac actin	Dilated and hypertrophic cardiomyopathy
Actg	Gamma actin	Cytoskeletal, Cell adhesion	Deafness
MYBPC1	Myosin binding protein C, slow type	Cell contractility	
Col1a2	Procollagen, type I, alpha 2	ECM	Ehlers-Danlos type VIIb, Osteogenesis imperfecta II, III, IV
Col5a2	Procollagen, type V	ECM	Ehlers-Danlos type I, II; autosomal recessive ED valvular type
Col11a1	Procollagen, type XI	ECM	Marshall syndrome, Stickler syndrome type II
Plod1	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	Collagen synthesis	Ehlers-Danlos syndrome type Via, Nevo syndrome
Itm2a	Integral membrane protein 2A	Myogenic, chondrogenic differentiation	
Lasp1	LIM and SH3 protein 1	Cytoskeleton, focal adhesions	
	LYSOSOME		
Ctsl	Cathepsin L	Proteolytic enzyme, lysosome	

Abbreviations: ECM= extracellular matrix

al., 2006). SNX1 and SNX2 have been shown to play a role in retrieving PAR1 (Gullapalli *et al.,* 2006); this study established that SNX2 is not required for PAR1 degradation, but can regulate PAR1 lysosomal sorting through its ability to disrupt endosomal localization of endogenous SNX1.

SNX11 shows lots of structural differences from SNX1 and SNX2. It belongs to a subfamily which is characterized only with a Phox domain, but not BAR domain. In addition the BAR domain in SNX1 and SNX2 functions as a curvature sensor, which allows proteins to interact with membrane structures. This could suggest that the intracellular function of SNX1 and SNX2 in trafficking receptors is different than that of SNX11.

SNX11 possesses a long C-terminal. We speculate that this structure is important to interact directly with receptors or other proteins. In our work we showed the predominant cytosolic and perinuclear distribution of SNX11. We investigated in which cellular compartment interaction between PAR2 and SNXs can be detected.

In immunofluorescence assays we were able to confirm the results from co – immunoprecipitation and we showed co-localization of SNX1, SNX2 and SNX11 with PAR2 (Figures 9, 11). The three SNXs showed different distribution pattern of co – localization with PAR2. SNX1 and SNX2 together with PAR2 were predominately localized in the vesicles, endosomes, and also perinuclear. This pattern of co-localization was more pronounced for SNX1 than for SNX2. These images demonstrate remarkable similarity with the previous photos for endosomal co-localization of PAR2 with Rab5a and Rab11a (Roosterman *et al.,* 2003). Based on these publications we speculate that in parallel to their interactions with PAR1, SNX1 and perhaps SNX2 are probably implicated in lysosomal sorting of PAR2.

The objective of our research was to determine the genes responsible for PAR2 nuclear translocation, and for that reason we did not concentrate further on the investigation of its endosomal pathway.

As compared to SNX1 and SNX2, SNX11 co-localized with PAR2 predominantly in perinuclear structures and may be in the nuclear membrane. In experiments with whole cell and using confocal microscopy it was very difficult to confirm the nuclear co-localization therefore we used a method for nuclei isolation, and performed immunofluorescence analysis. We were able to demonstrate that SNX11 also shows nuclear distribution. This result was confirmed in convincing manner with co-localization

of SNX11 with Lamin A/C, which is a marker for the nuclear envelope. SNX1 and SNX2 failed to reveal a nuclear pattern of distribution.

At this stage we were able to confirm with great confidence the ability of SNX members to interact with PAR2. We also distinguished that SNX1 and SNX2 co-localized with PAR2 predominately in endosomes, whereas only SNX11 localizes together with PAR2 in the nuclear membrane. The strength of these interactions was different: a much stronger interaction for SNX11 and weaker interactions for SNX1 and SNX2 were noted. Now the challenge was to establish if SNX members would show the capability to transport PAR2 in functional studies.

In order to evaluate the functional relationship of PAR2 and SNXs we performed loss of function studies. We created stable SNX1, SNX2 and SNX11 knockdown HEK293 cell lines. The knockdown cell lines are a powerful tool to evaluate the exact implication of each of the investigated SNX in the nuclear transport of PAR2. Since the levels of PAR2 expression are relatively low, we transfected the stable knockdown cells with a FLAG-PAR2-HA construct. After 30 min of stimulation with SLIGKV, intact nuclei were isolated and investigated with FACS and Western blot analysis. The nuclear translocation of PAR2 after stimulation in SNX11 knockdown cells was decreased twofold compared to control cells, confirming the importance of SNX11 in nuclear transportation of PAR2 (Figure 15). In comparison SNX1 and SNX2 knockdowns did not appear to demonstrate any significant implication in PAR2 nuclear translocation. We were thus able to demonstrate distinct regulation of PAR2 intracellular trafficking and to identify that SNX11 is a key factor for PAR2 nuclear localization. However does this make any difference in its signalling pathway?

We mentioned that iNOS expression is dependent on PAR2 expression. When blocking unspecifically the intracellular traffick with colchicine, iNOS expression was decreased. Most likely the translocation of PAR2 to the nucleus was abolished (Figure 7).

Using the knockdown cell lines we were able to investigate how each of the studied SNX members can influence iNOS expression after PAR2 stimulation. The results from the RT-PCR clearly indicated a drastic decrease of iNOS expression in SNX11 knockdown HEK293 cells (Figure 18). Surprisingly, the SNX1 and SNX2 knockdown also seemed to diminish iNOS expression, though not to the same extent as the SNX11 knockdowns. At first glance this finding might lead to the controversial conclusion that SNX1 and SNX2 do not interfere with nuclear trafficking of PAR2, but influence iNOS expression which depends on PAR2. We mentioned before that SNX11 and PAR2 are localized predominately in the cytosol and in the perinucleus. Therefore we suggest that SNX11 does not transport directly PAR2 from the plasma membrane to the nucleus. Instead we suggest that this is a much more complicated process. Probably the first stage is the internalization of the receptor where SNX1 and SNX2 play an important role. However they are not the only factors, as we mentioned before the function of Rab5a and Rab11a (Roosterman *et al.,* 2003). Thus the single knockdown of SNX1 or SNX2 is perhaps not sufficient to block the first stage of PAR2 intracellular translocation from plasma membrane to the endosomes and consequently its nuclear localization. On the other hand PAR2 nuclear signalling is probably a much more specific process which demands fine regulation. For that reason the role of SNX11 is possibly crucial.

During the recent years different studies demonstrated the presence of GPCRs at nuclear level, but PAR2 had not been detected so far. In the current study we discovered that PAR2 is localized on the plasma membrane and on the nuclear membrane. Stimulation of PAR2 leads to its translocation preferentially to the nuclear membrane. We established that the nuclear localization is part of the PAR2 signalling pathway. In order to determine the mechanisms involved in nuclear trafficking we examined members of the SNX family as promising candidates for this regulation. Our results show that SNX11 plays a crucial role in translocation of PAR2 to the nucleus and nuclear signalling. SNX1 and SNX2 are perhaps involved in the first stage of receptor internalization. With its role in inflammation and tumorigenesis PAR2 arises as a promising pharmacological target. Many aspects of PAR2 signalling remain unclear. Our work demonstrated that SNXs are important factors in PAR2 intracellular trafficking.

VI. FUTURE DIRECTION

In the present study we demonstrated a role of SNX11 to traffic receptors in the cell. We showed its specific function to translocate PAR2 to the nuclear membrane. Still many questions remain to be answered. One important issue is to understand in details how SNX11 functions in transporting receptors to the relevant cellular targets in the cell. For that reason we propose to use markers for early endosomes (EEA1), lysosomes (LAMP1), late endosomes (M6PR, cation-independent Mannose-6-phosphate receptor), Golgi and ER to search for possible localization of SNX11 in these cell compartments. These results will unravel at what cellular compartment SNX11 functions in receptor sorting. We can use the same approach for PAR2 and determine its intracellular pathway. Consequently we can use the knockdown cell lines of SNX1, SNX2 and SNX11 to establish at what point each of them abolishes PAR2 trafficking.

As we discussed in the introduction, PAR2 is involved in numerous physiological processes including angiogenesis, cell migration and cancer development. Once we established the presence of the two populations of PAR2, it would be interesting to answer the question of what is the physiological role of each of these populations. In our study we demonstrated that SNX11 can influence significantly PAR2 nuclear trafficking. The knockdown cell lines of SNX11 will be interesting model to evaluate its role in PAR2 - induced cell migration. Revealing the mechanisms of trafficking and the physiological role of nuclear PAR2 can give researchers a tool for developing highly specific antagonists for controlling such processes as cell migration, involved in the development of numerous tumors. In this regard it will be important to determine what the binding domain is for SNX11 and PAR2 interaction. We propose that the long C – tail

of SNX11 plays a role in this process. Therefore it is possible to create several mutants and study how they influence the nuclear trafficking of PAR2.

It is hard to believe that SNX11 functions only in the nuclear trafficking of PAR2. Some receptors like EGFR have a documented nuclear trafficking, but no transporting protein has been identified so far. Actually the first member of SNX family, SNX1 was discovered as an interaction partner of EGFR. However as in the case of PAR1, and probably PAR2, SNX1 is responsible for its lysosomal degradation. We suggest that this can be a general paradigm for the function of SNX proteins and it is very possible that SNX11 is involved in EGFR nuclear trafficking too.

It is important to study SNX11 not only at cell level, but to establish what role it plays in tissue and organ development. For this purpose we will need animal models. In this respect the frog *Xenopus leavis* is a very useful animal model. The eggs of *Xenopus leavis* will be injected with specific morpholino sequences of SNX11, which will create temporary knockout of SNX11 gene expression. This will allow us to study the effect of SNX11 in early embryo development. When a function of a protein is studied the creation of loss – of – function mouse model has high value. The SNX11 deficient mice will be studied for any pathophysiological phenotype.

Our work discovered the role of SNX11 as an important factor in PAR2 nuclear translocation. The future investigations will allow us to identify its contribution in various events in intracellular trafficking. As pharmacological aspect, SNX11 will arise as an interesting target to modulate receptor activity.

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