The Role of Protease-Activated Receptor-1 and -2 Signaling in Inflammation and Vascular Injury

A thesis

submitted by

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Abstract

Protease-activated receptors (PARs) play significant roles in various human diseases including thrombosis, sepsis, cancer, and inflammation. Attenuating the signaling of these G-protein coupled receptors holds promise for treatment of these ailments. This thesis examines the role of PAR1 and PAR2 signaling in vascular injury and inflammation.

PAR1 and PAR2 are diverse receptors that play intriguing roles in vascular injury. Both receptors are upregulated in injured human vascular smooth muscle cells and atherosclerotic plaques, however little is known about the functional activity of these receptors in vascular disease and injury. Previous studies have shown that PAR1 can transactivate PAR2 and that these receptors reside in close proximity. In the present studies, *in vitro* co-immunoprecipitation revealed that PAR1 and PAR2 associate as a heterodimer. To dissect the functional activity of PAR1/PAR2 heterodimers *in vivo*, I utilized a carotid arterial ligation injury model with C57BL/6 wild-type, $PAR1^{-/-}$, and $PAR2^{-/-}$ mice. I measured intimal and medial area 21 days after ligation injury in mice treated with vehicle or the PAR1 agonist, P1pal-13. Treatment of mice with P1pal-13 caused a massive increase in intimal hyperplasia in wild-type mice, which was significantly reduced in the PAR2 knockout strain. This suggests that both PAR1 and PAR2 mediate intimal hyperplasia in this model of carotid artery ligation.

PAR2, a key factor in inflammatory response, has advanced therapeutic possibilities for treating acute and chronic inflammatory diseases of the joints, lungs, gastrointestinal tract, and vascular systems. Despite considerable effort by the pharmaceutical industry, PAR2 has proven recalcitrant to targeting by small molecule

inhibitors. In this thesis, I developed a potent and specific cell-penetrating lipopeptide pepducin antagonist of PAR2, P2pal-18S, which specifically inhibits PAR2-dependent signaling in human neutrophils, colon adenocarcinoma cells, and in mouse models of inflammation. These data provide proof-of-concept that PAR2 pepducin, as exemplified by P2pal-18S, may be effective treatment for inflammation and other diseases related to PAR2 signaling. In this thesis, I employed newly developed PAR1 and PAR2 pepducins to demonstrate PAR1 and PAR2 signaling is important in inflammation and remodeling after vascular injury.

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Chapter 1: Introduction

1.1 General background

G-Protein Coupled Receptors (GPCRs):

GPCRs are seven helical transmembrane receptors that usually reside at the cell surface to allow the cell to respond to environmental changes by way of signal transduction. These receptors are the largest class of cell surface receptors and are involved in key physiological processes, making them attractive targets to the pharmaceutical industry. The mode of activation by GPCRs consists of the cognate ligand binding and causing a potential conformational change in the receptor that facilitates the exchange of GTP for GDP on a Gα subunit [1]. This exchange decreases the affinity of the Gα subunit for the Gβγ subunits, which thereby allows Gα to activate a multitude of downstream effector molecules.

The outcome of GPCR activation is diverse and often dependent on the family of the Gα subunit. GPCRs can couple to more than one class of Gα protein and therefore can activate distinct signaling pathways. The **$G\alpha_s$** pathway is classically known for adenylate cyclase activation that converts ATP to cAMP and subsequently activates protein-kinase A signaling [2]. Conversely, the **$G\alpha_i$** family inhibits adenylate cyclase thus decreasing the concentration and effects of cAMP [2]. Additionally, the $G\alpha_i$ subunit can activate Src, which in turn activates ERK signaling via the Rac pathway and increases the level of STAT3 transcription factor [2]. The **β** and **γ** subunits of the $G\alpha_i$ protein are thought to activate phosphoinositide 3-kinases (PI3K), mitogen-activated protein kinase pathways (MAPK), and transcriptional regulation [3]. The **$G\alpha_q$** family of alpha subunits

activates phospholipase C-β (PLC-β) that generates the second messengers inositol-3-phosphate (IP3) and diacylglycerol (DAG) [3]. These molecules promote protein kinase C (PKC) activation and release of Ca^{2+} from intracellular stores [4]. PKC is a known activator of NF-κB, which mediates the transcription of a host of genes involved in inflammation, maturation, and proliferation. The **$G\alpha_{12/13}$** family regulates cell proliferation, apoptosis, and the sodium-proton exchange. $G\alpha_{12/13}$ triggers Rho guanine nucleotide exchange factors (GEFs) to activate RhoA [2,3]. The Rho family consists of three primary members: Rho, Rac, and Cdc42. Rac activates assembly of contractile stress fibers, Rho activates lamellipodia and membrane ruffles, and Cdc42 causes filopodia to form. These events directly affect both cell shape and migration [4,5].

Some GPCRs have been shown to signal without using G proteins. For example, the ERK1/2 mitogen-activated protein kinase can be activated via β-arrestin-2 independent of G protein signaling [6]. The bradykinin B2 GPCR has been shown to directly interact with protein tyrosine phosphotases with no G protein involved [7].

The History of the Protease-activated Receptor Family

The thrombin receptor, later renamed protease-activated receptor-1 (PAR1), was first cloned by two separate laboratories in 1991 using direct expression cloning in Xenopus oocytes [8,9]. The objective of these studies was to find the critical receptor responsible for thrombin-induced platelet activation [8]. Since platelets have very little mRNA, human megakaryocyte-like cell lines (HEL and Dami cells) [8] or the Chinese hamster lung fibroblast (CCL39 cells) [9] were chosen for the task of identifying the thrombin-activated receptor. HEL, Dami, and CCL39 mRNA was fractionated by sucrose gradients and

assessed for thrombin induced calcium mobilization in Xenopus oocytes. A resulting 4 kB mRNA had an enriched 5-fold thrombin induced calcium response compared to unfractionated mRNA[8]. This mRNA encoded a 3.5 kB cDNA that was named *F2R* (PAR1). PAR1 was revealed to be a member of the seven transmembrane domain receptor family by hydropathy sequence plot analysis [8]. The long N-terminus extracellular domain of the receptor indicated a novel proteolytic mechanism of activation [8,9] (**see Figure 1.1, PAR1 sequence and domains)**. Both groups confirmed that expression of PAR1 cDNA caused calcium stimulation by the addition of thrombin, and moreover, it was observed that peptides mimicking the remaining N-terminus after the expected thrombin cleavage site (SFLLRN) could also stimulate calcium mobilization, unveiling an essential tool for studying the thrombin receptor [8,9].

PAR2 was discovered in 1994 in a mouse genomic library that was screened for GPCRs using a bovine substance K receptor sequence [10]. The receptor was found to be a single copy gene in mice that is expressed in the kidney, small intestine, stomach, and eye, but not in the brain, skeletal muscle, heart, liver, nor testis [10]. PAR2 has 30% sequence identity to PAR1 (28% to mouse PAR1), its closest relative [10]. Of these similarities in the receptors there were many conserved regions of 10 consecutive identical amino acids [10] (**See Figure 1.2, PAR1 & PAR2 sequence alignment**). An important difference between PAR1 and PAR2 is that the N-terminal PAR2 cleavage site lacks the acidic residues that allow for binding and cleavage of PAR1 by thrombin. Since proteolytic cleavage of PAR1 was a main mechanism of activation and since the two receptors had shared identity it was hypothesized that PAR2 would also be activated by a

Figure 1.1:

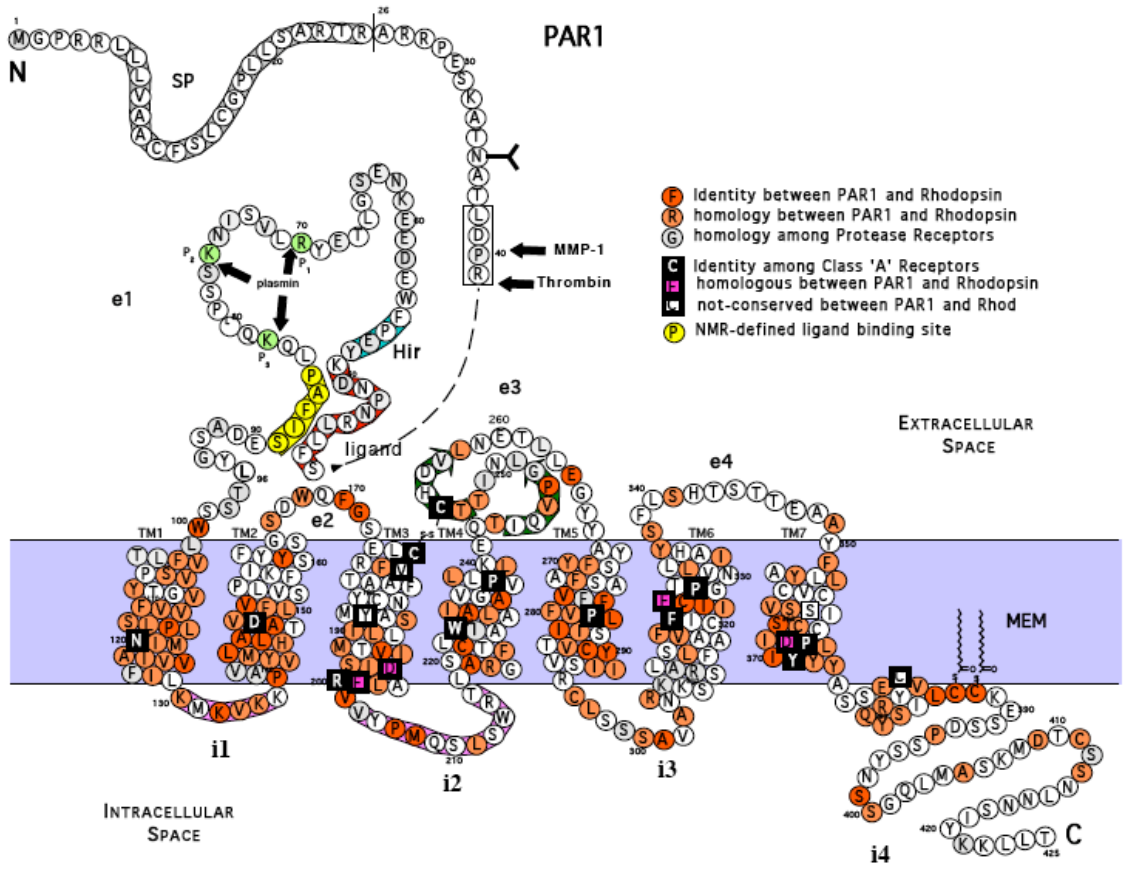

Figure 1.1. PAR1 sequence and domains. The amino acid sequence and spatial arrangement of the seven transmembrane helices of PAR1.

protease. Thrombin was not able to cleave the receptor, however trypsin could cleave and activate the receptor to generate calcium mobilization. Similarly to PAR1, it was found that the peptide corresponding to the cleaved N-terminus of the receptor (SLIGRL) also could stimulate PAR2 [10].

A second thrombin receptor, termed PAR3, was identified in 1997 [11]. PAR3 is robustly expressed in human heart, kidney, small intestine, lymph nodes, and bone marrow, and is absent from brain, lung, skeletal muscle, and spleen. The existence of a second thrombin receptor was hypothesized since no spontaneous bleeding was observed in PAR1 knockout mice (see Section 1.2) and because platelets from both wild-type and PAR1 knockout mice responded to thrombin but could not be activated by the PAR1 mouse peptide agonist SFFLRNPSE [12]. PAR3 was found using a PCR-based strategy with primers corresponding to conserved regions in the PAR1 and PAR2 sequences [11]. The resulting rat cDNA was used to isolate the human and mouse PAR3 clones [11]. The human PAR3 sequence shares 27% sequence similarity to PAR1 and 28% to PAR2 [11]. PAR3 contains a sequence similar to the PAR1 thrombin-binding domain, and PAR3 was confirmed as a thrombin substrate by cleavage in COS7 cells transfected with the receptor [11]. Interestingly, the peptide corresponding to the cleaved N-terminus of PAR3 does not cause activation as observed in PAR1 and PAR2 [11].

The most recent PAR to be discovered, PAR4, was independently cloned by two separate groups in 1998 [13,14]. The first group performed a homology BLAST search of the public database dbEST and the commercially available Incyte using the three previously identified PARs [13]. Indeed, PAR4 was recognized to share 34% identity to

Figure 1.2:

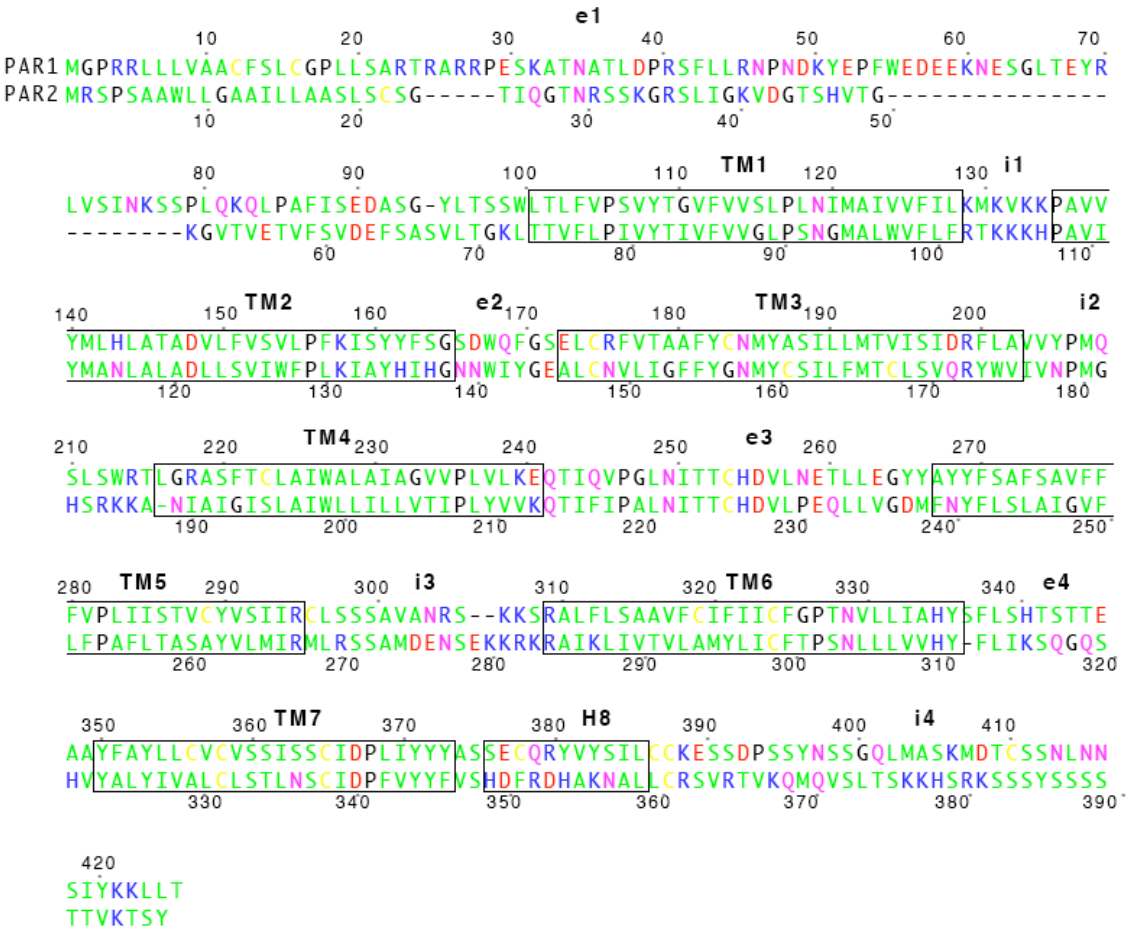

Figure 1.2. PAR1 and PAR2 sequence alignment. PAR1 and PAR2 amino acid alignment showing conserved and non-conserved regions between the two receptors. Colors indicate the type of amino acid: blue=basic amino acids, red=acidic amino acids, pink=amino acids with amide groups, black=glycine and proline (often found in turn and loop structures), yellow=cysteines, green=all others

PAR2 in the transmembrane regions [13]. The second group created a PAR3 knockout mouse and proposed the existence of another thrombin receptor since PAR3$^{-/-}$ platelets responded normally to thrombin [14]. This group did a BLAST search on the available mouse GenBank and found PAR4. Both groups confirmed that PAR4 responds to thrombin, albeit at much higher concentrations than PAR1, since a hirudin binding site, which facilitates thrombin binding, is lacking in PAR4. Additionally, it was observed that the peptide corresponding to the cleaved N-terminus of PAR4 (GYPGKF) could successfully activate the receptor in both mouse and human platelets, alluding to a dual thrombin receptor role in platelets of both species (See Section 1.4) [13,14]. PAR4 was robustly expressed in the lung, liver, and small intestine of human tissue samples, but not in the heart, brain, or bone marrow [13].

PAR1, PAR2, and PAR3 are tightly linked on human chromosome 5q13 [15,16]. PAR1 and PAR2 were mapped to be no more than 90 kb apart suggesting that PAR2 came from a recent gene duplication event [15]. The structural organization of PAR3 is nearly identical to that of PAR1 and PAR2 suggesting that this family of receptors evolved from a common ancestral gene [16]. PAR3 is located less than 25 kb upstream of the PAR1 gene [17]. PAR1, PAR2, and PAR3 all contain two exons, with the second exon containing most of the receptors' sequences [15,16]. Unlike the other PARs, PAR4 maps to human chromosome 19p12 [13]. It has been proposed that another gene cluster would be found at this location, but to date no other PARs have been discovered [13].

PAR activation and inhibition

PARs are proteolytically activated by serine proteases that cleave the NH_2-terminal extracellular domain thus creating a new N-terminus distinct to each receptor [8-11,13,14]. This cryptic ligand then binds to the extracellular domain of its receptor activating downstream signaling via G proteins [18]. Binding of the tethered ligand presumably occurs in the second extracellular domain which is highly conserved between the receptors; differences between GPCRs in this region have been shown to be important for receptor ligand specificity [19,20]. Of the four PARs identified, PAR1, PAR3, and PAR4 are activated by thrombin whereas PAR2 is not, but can be activated by trypsin, mast cell derived tryptase, coagulation proteases Factor VIIa and Factor Xa (FXa), and the recently discovered serine protease matriptase [10,21-24]. PAR1 can also be activated by other proteases such as activated protein C (APC) [25], FXa [26], and matrix metalloproteinase-1 (MMP-1) [27]. MMPs are zinc dependent endopeptidases that are traditionally known for cleavage and degradation of connective tissue matrices [28].

As previously described, PAR1, PAR2 and PAR4 can also be activated by synthetic peptide fragments based on their tethered ligand sequences [8,10,17]. The peptide TFLLRN specifically activates PAR1 while SLIGRL and SLIGKV, the respective murine and human PAR2 peptides activate PAR2 but not PAR1 as shown by calcium mobilization in Xenopus oocytes expressing these receptors [22] (see **Figure 1. 3, PAR1 & PAR2 agonists**). Interestingly, the peptide agonist SFLLRN (which corresponds to the PAR1 human sequence) activates both PAR1 and PAR2 [22]. PAR4 is specifically activated by the peptide sequences GYPGQV (human) and *A*YPGKF (mouse). These

peptide agonists are valuable tools for examining the effects of PAR1 and PAR2 without using proteases that can have non-specific biological activity.

Specific antagonists to PARs are also useful tools for characterizing receptor activity. Currently PAR1, PAR2, and PAR4 have functional inhibitors, however the efficacy and specificity of these inhibitors are not always satisfactory. PAR1 inhibitors include RWJ-56110, SCH 79797, P1pal-7, vorapaxar, E-5555, and JF5 which can inhibit GPCRs that contain a constrained 8th helix [29]. RWJ-56110 is a potent peptidomimetic antagonist that binds directly and specifically to PAR1 thereby preventing binding of the tethered ligand to the activation site [30]. This inhibitor does not block cleavage of PAR1 and does not interact with thrombin [30]. SCH 79797 is a small molecule antagonist that also inhibits binding of the tethered ligand [31]. P1pal-7 is a cell penetrating lipopeptide 'pepducin' that blocks PAR1 signaling [32,33]. Pepducins are amino acid sequences based on the intracellular domain of their cognate receptor with a cell penetrating, membrane tethering moiety attached at either the N-or C-termini (see Section 1.5) [33]. Pepducins have been successfully designed to turn on or to antagonize both PAR1 (P1pal-7) and PAR4 (P4pal-i1) and are specific for each receptor [33]. Two different PAR2 inhibitors have recently been developed. A PAR2 small molecule inhibitor, ENMD-1068, has been characterized in a model of joint inflammation [34]. This molecule requires high dosage (2.5 mM) to observe its effects *in vitro* and 145 mg/kg *in vivo*. The peptide antagonist K-14585 was shown to inhibit IL-8 production, NF-κB phosphorylation, and p38 signaling of SLIGRL. However, the compound also increased signaling of these pathways in the absence of SLIGRL, thus having a dual function of both antagonist and agonist of PAR2 [35,36]. There is also a specific small molecule antagonist for PAR4

called trans-cinnamoyl-YPGKF (YD-3) which binds to PAR4 to inhibit activation [37]. Refer to **Table 1.1**, **Summary of PAR agonists and antagonists**. This thesis will discuss the design and development of new PAR2 pepducin inhibitors, P2pal-18S and P2pal-14GQ, (see Chapter 3).

Figure 1.3: (adapted from Blackhart et al, 1996, *JBC*.)

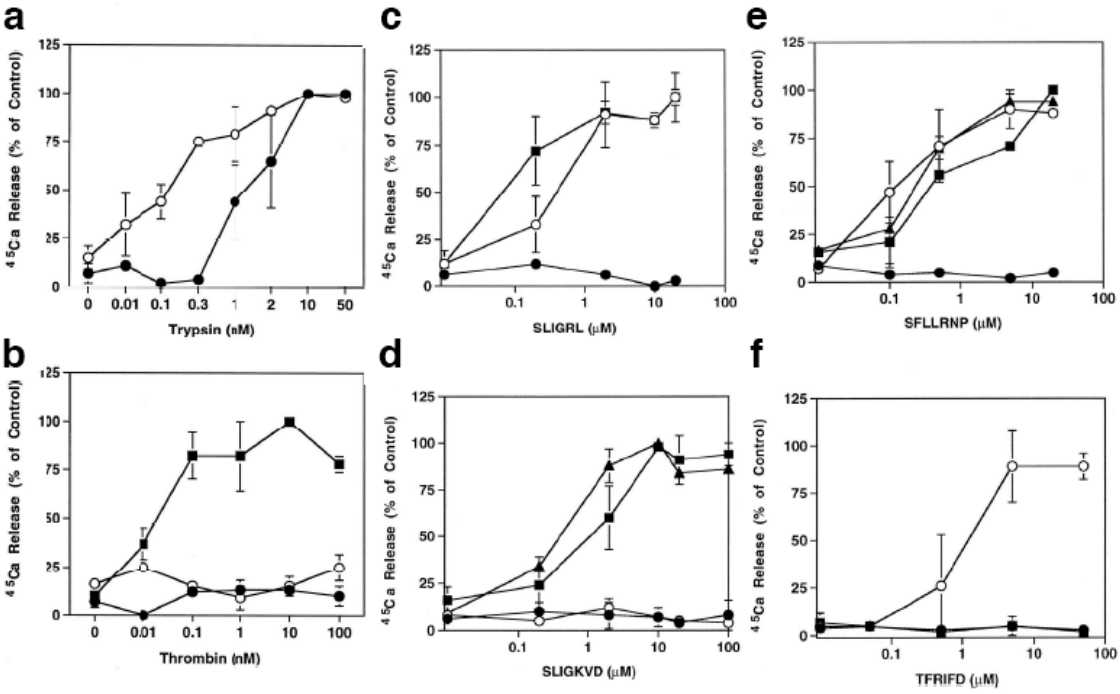

Figure 1.3. Protease and synthetic peptide calcium flux stimulation of PAR1 and PAR2 expressed in Xenopus oocytes. This research was originally published in the Journal of Biological Chemistry. Blackhart et al, *JBC*. 1996; 271: 16466-16471. © the American Society for Biochemistry and Molecular Biology. Oocytes were injected with H_20 (**a-f**: closed circles), PAR1 human cDNA (**a-b**: closed squares, **d-f**: open circle), human PAR2 (**a-c**: open circles, **d-f**: closed squares), or murine PAR2 (**c**: closed squares, **d-f**: closed triangles). Calcium flux was stimulated with indicated protease or peptide.

Table 1.1: Summary of PAR agonists and antagonists.

Receptor	Agonists	Antagonists
PAR1	Thrombin	P1pal-7
	MMP-1	P1pal-12S
	APC	RWJ-56110
	plasmin	SCH-79797
	Fxa, FVIIa	vorapaxar
	Trypsin	E-5555
	SFLLRN	JF5
	TFLLRN	F16618
	P1pal-13	BMS-200261
PAR2	Trypsin	P2pal-18S*
	Tryptase	P2pal-14GQ*
	Matriptase	ENMD-1068
	FXa, FVIIa	K-14585
	SFLLRN	
	SLIGRL /SLIGKV	
	P2pal-21F	
PAR3	Thrombin	none
PAR4	Thrombin	P4pal-10
	Trypsin	P4pal-i1
	Cathepsin G	cinnamoyl-YPGKF
	FXa,FVIIa	
	GYPGQV/AYPGKF	

*, Development and validation in Chapter 3

PAR signal transduction

PAR1 has been shown to activate $G\alpha_q$, $G\alpha_{12/13}$ and $G\alpha_i$ although the major signaling pathway is thought to be mediated by $G\alpha_q$ [38]. $G\alpha_q$ signaling is evident by generation of inositol triphosphate (IP3) and diacylglycerol, as well as calcium mobilization and protein kinase C signaling (PKC) [38]. Thrombin and PAR1 peptide induced IP3 generation in lung fibroblasts, that was partially sensitive to pertussis toxin (a known inhibitor of $G\alpha_i$ and $G\alpha_o$ subunits), suggesting signaling through the $G\alpha_i$ pathway and another $G\alpha$ protein [38]. $G\alpha_q$ antibodies inhibited calcium mobilization and GTPase activity in PAR1 activated cells [39]. $G\alpha_i$ coupling was evident by the fact that PAR1 agonist peptides decreased cAMP levels in cells and was pertussis toxin sensitive [38]. Furthermore, a dominant negative mutant of the $G\alpha_i$ subunit (Gly^{203} to Thr, G203T) selectively inhibited thrombin stimulation of archidonic acid release in CHO cells independently of adenyl cyclase inhibition [40].

$G\alpha_{12}$ and $G\alpha_{13}$ signaling has also been detected in response to thrombin stimulation of platelets, which is presumably mediated through PAR1 [41]. Thrombin stimulation of astrocytoma cells leads to Ras-dependent AP-1 mediated transcription activation and DNA replication [42]. A constitutively activate mutant of the $G\alpha_{12}$ subunit upregulated AP-1 dependent gene expression, and microinjection of an inhibitory $G\alpha_{12}$ antibody into these astrocytoma cells resulted in the concentration-dependent inhibition of thrombin-stimulated DNA synthesis [42]. This effect was not seen with an inhibitory antibody to the $G\alpha_o$ subunit, thereby confirming thrombin mediated activation of the $G\alpha_{12}$ subunit [42]. In an interesting study by Verrall et al., chimeras of PAR1 with the $G\alpha_s$ coupled β_2-adrenergic receptor (β_2AR) and with the $G\alpha_i$ coupled dopamine receptor

revealed that the second intracellular loop of PAR1 was sufficient to allow coupling of the β_2AR and the dopamine receptor to $G\alpha_q$ [43]. Not only does this confirm that PAR1 does indeed signal through this subunit, but also is evidence for a similar conformational change in GPCRs when activated despite having very different ligands [43].

PAR2 signaling has not been as thoroughly studied as PAR1, however multiple groups have shown calcium mobilization and the generation of IP3 by PAR2 stimulation and therefore the receptor is believed to couple to $G\alpha_q$. Calcium mobilization has been observed in response to trypsin stimulation in oocytes injected with PAR2 cDNA [10], in rat colonic myocytes stimulated with trypsin, SLIGRL, or mast cell tryptase [23], in human keratinocytes stimulated with trypsin, SLIGRL, or SFLLRN [44], as well as multiple other cell types. PAR2 has also been shown to couple to $G\alpha_i$ due to the observation that arachidonic acid release occurred in both enterocytes from the rat small intestine and PAR2 transfected cells after stimulation with trypsin or SLIGRL [45]. Treatment with SLIGRL stimulates c-fos mediated activation of tyrosine phosphorylation which was partially sensitive to pertussis toxin, showing that at least some of this mediated signal is through $G\alpha_i$ [46]. PAR2 stimulation in rat olfactory neuroepithelial cells caused $G\alpha_i$-mediated inhibition of cyclic AMP as well as stimulation of $G\alpha_q$ mediated phosphoinositide hydrolysis and activation of Rho [47]. PAR2 is also known to activate ERK1/2 and weakly stimulate MAP kinase p38 in rat aortic smooth muscle cells [48]. PAR2 activation of ERK and Akt was also observed in GI epithelial cells to cause the release of IL-8 that occurred by both the MEK/ERK and phosphoinositide-3-kinase (PI3K) pathways, independently of one another [49].

PAR3 is thought to be a 'dead' or decoy receptor despite the fact that it is widely expressed throughout human tissues. There is no known peptide agonist of PAR3 and COS7 cells transfected with PAR3 are not activated by thrombin [50]. However, when co-expressed with PAR4, PAR3 gave an enhanced sensitivity to thrombin stimulated phosphoinositide hydrolysis. This enhanced signal was not observed with a cleavage mutant of PAR4 suggesting that PAR3 may not directly activate G proteins but instead facilitate PAR4 cleavage by acting as a high-affinity thrombin binding site for PAR4 [50].

PAR4 couples to $G\alpha_q$ as shown by the ability of thrombin and PAR4 signal peptide GYPGKF to cause platelet aggregation. PAR4 signaling also generates calcium mobilization when PAR4 cDNA is injected into Xenopus oocytes [14] and mediates thrombin activation of phosphoinositide hydrolysis in transfected COS7 cells [50]. PAR4 generates the majority of intracellular calcium influx in human platelets with a prolonged signal compared to PAR1 calcium influx [51,14]. Interestingly, both PAR1 and PAR4 stimulation by SFLLRN and GYPGKF in platelets can inhibit cAMP production indicating signaling via $G\alpha_i$. However, this inhibition is completely dependent on PKC mediated ATP secretion ($G\alpha_q$ pathway) and the $G\alpha_i$ coupled-P2Y12 receptor, as shown by PKC inhibitor (RO-31-8820) and the P2Y12 selective inhibitor (AR-C66096) completely blocking the PAR1 and PAR4 stimulated cAMP inhibition [52]. Although PAR-signaling pathways have been extensively studied, much is still unknown about $G\alpha$ coupling preference of different receptor oligomers.

PAR desensitization and internalization

Since PAR activation is irreversible, receptor desensitization or endocytosis is required to regulate signaling shut-off. Most GPCR internalization is mediated by serine and threonine (Ser/Thr) phosphorylation at the C-terminal end and arrestin binding, though it has also been shown that covalent modifications of GPCRs with ubiquitin can signal internalization [53]. The most well characterized pathway of GPCR internalization is that of the β2-adrenergic receptor, which like PARs is a Class A GPCR. G protein-coupled receptor kinases (GRKs) phosphorylate the Ser/Thr residues in the C-tail of the receptor, which causes arrestins to bind [54]. Arrestins conformationally change upon binding, to expose their C-terminus, which then interacts with clathrin and the clathrin adaptor, AP-2 [55]. The complex is then internalized and arrestins dissociate.

C-terminal sequence motifs of a GPCR can indicate possible modes of receptor internalization. A bioinformatics search was performed to show GPCRs that had Ser/Thr clusters within their C-tail, since these clusters are thought to stabilize arrestin interactions [53]. Indeed PAR2 contains three Ser/Thr clusters in the C-terminal domain and agonist-induced desensitization of PAR2 involves the phosphorylation of the receptor and endocytosis via β-arrestin and clathrin-dependent mechanisms [56]. Later studies suggested that internalized β-arrestin/PAR2 can activate ERK 1/2 signaling, however only the signal observed at the cell surface (Ras dependent) was able to cause translocation of ERK to the nucleus and induce proliferation [57].

PAR1 has a tyrosine and dileucine-based motif ($Y^{420}KKL^{423}$) at the C-terminal end of the receptor [53]. This motif allows binding of AP-2 and internalization through clathrin coated pits (CCPs) [53,58]. This process occurs in the absence of ligand/activation

and independently from phosphorylation and arrestins [58]. Deletion or mutation of the $Y^{420}KKL^{423}$ motif inhibits PAR1 constitutive internalization [58]. Interestingly, mutation of a similar PAR1 C-terminal motif, $Y^{383}SIL^{386}$, inhibited receptor degradation but had little effect on endocytosis suggesting that the $Y^{420}KKL^{423}$ motif is responsible for constitutive internalization while $Y^{383}SIL^{386}$ regulates the lysosomal/degradation process. Additionally, bicaudal D1 (BicD1) has recently been identified to associate with the C-terminal tail of PAR1 [59]. Silencing of BicD1 expression impaired PAR1 internalization, suggesting that BicD1 is a novel adaptor protein involved in PAR1 endocytosis [59].

Internalization of PAR3 and PAR4 has not been as extensively studied. PAR3 has a considerably shorter C-terminal end than other PARs and contains five Ser/Thr residues, none of which cluster together [60]. No studies have been published on the receptor's regulatory mechanisms. PAR4 has been shown to have a more prolonged signal than PAR1 and fails to undergo agonist-triggered phosphorylation [61]. A mutation of all 13 Ser/Thr residues in the C-terminal tail of PAR4 to alanine did not significantly alter receptor signaling as compared to wild-type PAR4, suggesting that either PAR4 is not phosphorylated at these sites or that phosphorylation of these residues does not contribute to PAR4 signaling or regulation [61].

1.2 Development of PAR knockout mice

PAR1$^{-/-}$ mice

The PAR1 knockout mouse was developed in 1996 and to the surprise of many did not exhibit spontaneous bleeding. The knockout was made by homologous recombination that inserted a neomycin cassette to disrupt exon 2 [12]. PAR1 knockout mice were not born in Mendelian ratio and half of the embryos lost their heartbeat at E9.5. All PAR1$^{-/-}$ embryos were noticeably smaller at E9.0 and exhibited delayed placenta development [12]. Embryos that survived past E9.5 overcame all size differences by E11.5 and developed normally. Thrombin was able to activate platelets from PAR1$^{-/-}$ mice indicating that another thrombin receptor must be present. However, thrombin response was lost in fibroblasts isolated from the adult lung of the animal, as measured by both calcium flux and phosphoinositide hydrolysis [12].

The embryonic lethality of PAR1 deficiency in mice has been further investigated. Gross examination of embryos at E9.5 reveal blood in the exocoelomic and pericardial cavities of some embryos as well as a dilated pericardial sac indicative of cardiovascular failure [62]. Transgenic knock-in of mouse PAR1 with LacZ in the PAR1$^{-/-}$ background showed PAR1 expression in the endocardium, vascular endothelium of great and smaller vessels, and in a subset of mesenchymal appearing cells [62]. Due to the observation that PAR1 was highly expressed in endothelial cells, a second transgenic mouse was made to express PAR1 in endothelial cells driven by the endothelial specific promoter TIE2p/e in the PAR1$^{-/-}$ mice [62]. Expression of PAR1 in endothelial cells reduced embryonic lethality from 52% to 14%, demonstrating that PAR1 expression on endothelial cells of developing blood vessel is important in vascular development [62].

The relevant adult phenotypes for the PAR knockout mice will be discussed in **Section 1.4**.

PAR2$^{-/-}$ mice

The PAR2 knockout mouse was also made by homologous recombination that introduced a neomycin cassette to disrupt the second exon [63]. When breeding with heterozygous mice, PAR2$^{-/-}$ mice were not born in Mendelian ratio, and 20% were stillborn or died within 48 hours after birth. Deceased pups had no abnormalities apparent, so the cause of perinatal lethality is unknown [63]. Those mice that survived had no physiologic or histologic differences from wild-type or heterozygous littermates, and breeding of two knockout mice yielded normal litter sizes unlike PAR1 knockouts which had much smaller litters [12,63]. Arterial pressure, hypotension response, and hypertension response were all comparable to wild-type mice [22].

PAR1$^{-/-}$: PAR2$^{-/-}$ mice

Compensation between receptors was thought to be responsible for the lack of an obvious phenotype in adult PAR1 and PAR2 knockout mice. Dual knockout mice were generated by double targeting the PAR locus since the genes are too close together on chromosome 13 to allow for crossing the two mice strains [64]. The dual knockout is 95% embryonic lethal suggesting that compensation does occur in the single knockout mice [64]. Endothelial cells from surviving mice were completely insensitive to all PAR-activating proteases with one exception. High levels of thrombin generated a response in these cells which is most likely due to activation of PAR4 [64].

PAR3$^{-/-}$ mice

PAR3 knockout mice were generated by the substitution of a neomycin cassette for the mouse PAR3 gene (*Par3g*) in exon 2 [14]. These mice developed normally, had no spontaneous bleeding, and platelets responded to thrombin, though ATP secretion was slightly delayed [14]. Studies in these mice indicated that another thrombin receptor was present on mouse platelets that could mediate aggregation in response to thrombin. This receptor was later identified as PAR4.

PAR4$^{-/-}$ mice

PAR4 knockout mice were generated by the insertion of a LacZ coding sequence into the first exon of PAR4 resulting in the expression of β-galactosidase in its place [61]. PAR4 knockout mice were born in expected Mendelian ratios, survived to adulthood and were indistinguishable from wild-type littermates (in appearance and size) [61]. Platelets from PAR4$^{-/-}$ mice did not respond to AYPGKF or thrombin at any dose as measured by shape change, ATP secretion, aggregation or calcium flux [61]. Erythrocyte, leukocyte, and platelet counts were not different between PAR4$^{-/-}$ and wild-type mice, however tail bleeding times were markedly longer and mice lost 25 times more blood [61]. These results suggested that PAR4 activation of platelets by thrombin is necessary for normal hemostasis. PAR4$^{-/-}$ mice also had smaller thrombi in ferric chloride thrombosis injury indicating the importance of PAR4 in platelets during thrombosis[61].

PAR2$^{-/-}$: PAR4$^{-/-}$ mice

PAR2$^{-/-}$: PAR4$^{-/-}$ mice were made by intercrossing an existing PAR2$^{-/-}$ and a PAR4$^{-/-}$ mouse which was possible since these receptors are on different chromosomes [64]. These mice were made to explore possible redundancy among PARs in a model of endotoxemia. In this study, PAR2$^{-/-}$: PAR4$^{-/-}$ mice showed no difference in cytokine response compared to wild-type mice [64]. Though attempted, PAR1$^{-/-}$: PAR2$^{-/-}$: PAR4$^{-/-}$ mice had too high a level of embryonic lethality to allow for use of the strain.

1.3 Protease-Activated Receptor Dimerization

GPCR dimerization

A large number of GPCRs have been shown to interact as dimers and oligomers [65]. Though GPCR dimerization had been controversial a decade ago, it is a much more accepted concept today due to the advancement in detection assays. A direct and noninvasive biophysical technique such as fluorescence resonance energy transfer (FRET) is very useful to observe GPCR interactions at the membrane surface [66]. Unlike coimmunoprecipitations that require solubulization from protein membranes, live cells can be used and interactions of proteins tagged with acceptor and donor fluorophores (most commonly CFP and YFP) can be detected between molecules separated by less than 100 Å [66]. More recently an improved technique has emerged called bioluminescence resonance energy transfer (BRET) that uses a bioluminescent luciferase fused protein and a green fluorescent protein mutant to bypass the need of fluorescent excitation [67]. BRET uses the same concept as FRET, allowing interactions of less than 100 Å in distance to be detected, but does not have the same problems with background fluorescence, thus making it more specific [67]. The concept of dimerization has also been more readily accepted because structural data on the GPCR rhodopsin [68] and heterotrimeric G-protein [69] crystals suggests that a GPCR monomer surface area is too small to facilitate interaction with both the α and β/γ subunits, therefore it is likely that complexes or clusters of homo- and or hetero-oligomers signal to G proteins [70].

GPCR dimerization was first observed in 1979 with rhodopsin showing a faint homodimer band in an electrophoresis gel, and once cross-linked, the formation of dimers, trimers, tetramers, and higher oligomers was detected [71]. Interestingly, the

authors proposed that this observation was due to monomer collisions that were picked up by the cross-linking of the proteins [71]. Four years later, another group observed resonance energy transfer between two rhodopsin proteins thus showing an interaction or homodimer [72]. Multiple studies have confirmed this finding, including one that used atomic force microscopy to observe the higher oligomeric state of rhodopsin and opsin, showing that the interaction likely involves the IV and V helices of rhodopsin [73]. The muscarinic receptor in rat brain tissue has also been observed in a dimeric or oligomeric state through photoaffinity labeling detection showing 160,000 and 86,000-dalton bands that could be dissociated with alkali or hydroxylamine treatments to a single polypeptide chain of 40,000-daltons [74]. Moreover, β2-adrenergic receptors were observed in turkey erythrocytes to be present as a 109,000-dalton complex, suggesting a dimer from the 55,000-58,000-dalton protein [75]. Extensive chimera studies with the β2-adrenergic receptor and muscarinic receptor have shown that C-terminal portions of the receptor along with the VI and VII transmembrane regions are important for their dimerization [76] and that the third cytoplasmic loop is also necessary for detectable binding activity [77]. More recently, BRET studies have confirmed the (homo)dimerizations of both the muscarinic [78] and β2-adrenergic receptor [79].

Though a majority of receptors are thought to exist as homodimers, the number of reports of GPCR heterodimers has steadily increased in recent years. The most commonly reported heterodimers include the chemokine receptors, such as CCR5/CCR2b, CCR5/CXCR4, and CCR2/CXCR4, the dopamine receptors, and the opioid receptors [80]. Dimerization has been shown to occur at the level of synthesis in the endoplasmic reticulum or in the Golgi apparatus [81]. Preventing dimerization can

lead to effects in receptor trafficking and function, as with olfactory receptors that need β2-adrenergic receptor for efficient cell surface expression in transient systems [81,82]. Furthermore, heterodimerization is thought to mediate signal discrimination [70]. Formation of homo- versus heterodimers may lend signaling complexity to cells by modulating the affinity or selectivity of the signaling pathway [83].

PAR dimerization

There is considerable evidence that PARs can interact with each other and form homo- and hetero-dimers. Previous studies documented that PAR1 can transactivate an adjacent PAR1 [84], PAR1 can transactivate PAR2 in a possible PAR1-PAR2 heterodimer [85,86], and PAR1 can donate bound thrombin to cleave PAR4 in a stable PAR1-PAR4 complex [87,33]. FRET analysis has shown that PAR1 and PAR2 have the ability to form heterodimers in an endothelial cell line (EA.hy926 cells) using PAR1-YFP as the acceptor and PAR2-CFP as the fluorescent donor. PAR1 and PAR2 formed a substantial number of heterodimers that localize to the surface of the cell, and the FRET signal was increased when cells were stimulated by LPS [86] (see **Figure 1.4, PAR1 and PAR2 transactivation and FRET signal**). Thrombin stimulation of endothelial cells through PAR1 stimulated mostly $G\alpha_{12/13}$/Rho signaling while $G\alpha_i$/Rac signaling was mostly through PAR1/PAR2 demonstrating signaling differences between possible homo- and hetero-dimers. *In vivo* evidence for this dimer was shown by the loss of the protective effects of a PAR1 specific agonist pepducin (P1pal-13) on $PAR2^{-/-}$ mice receiving cecal ligation and puncture injury (CLP, a model of sepsis).

Figure 1.4: (from Kaneider et al, 2007, *Nat Immunol.*)

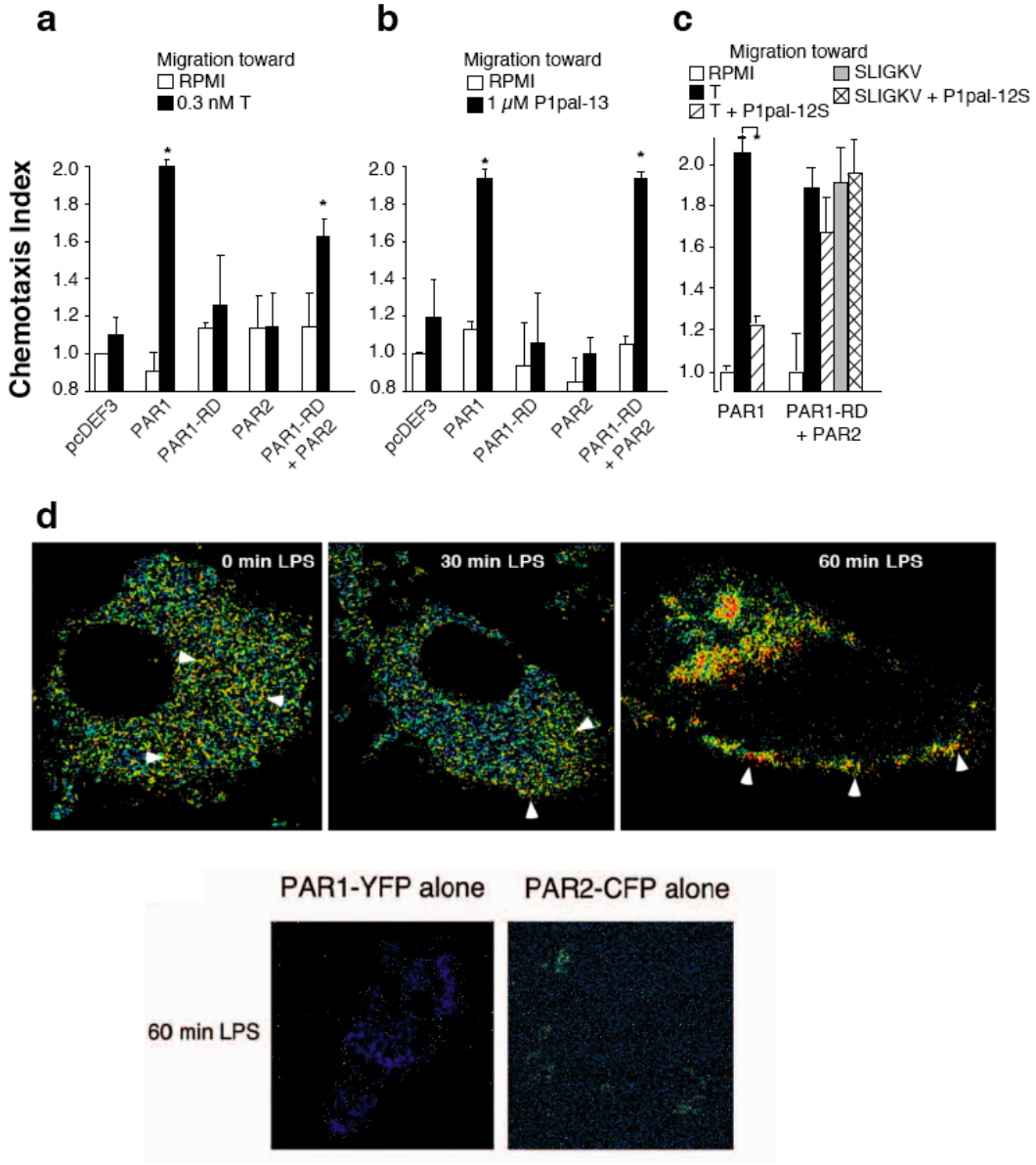

Figure 1.4. PAR1-PAR2 transactivation and heterodimers (reprinted with permission from Nature Publishing Group).
(**a-c)** Migration of HEK cells untransfected or transiently transfected with pcDEF3 vector, PAR1 (wild-type), PAR1-RD or PAR2 (wild-type) toward chemotactic gradients of thrombin (T; 0.3 nM), P1pal-13 (1 mM), SLIGKV (10 mM), P1pal-12S (3 mM) or RPMI, for 24 h in a transwell microchemotaxis apparatus. (**d)** LPS causes relocalization of PAR1-PAR2 complexes from internal stores to the periphery of endothelial cells. EAhy926 cells co-transfectd with PAR2Δ372CFP (cyanofluorescent) and PAR1Δ377-YFP (yellow fluorescents) and treated with 1μg/mL of LPS were assessed by confocal microscopy. Areas of blue represent low FRET

Other supporting data for the formation of heterodimers comes from O'Brien et al [85] who have shown PAR2 to be transactivated by thrombin-cleaved PAR1. In COS7 fibroblasts transfected with a cleavable but non-signaling PAR1-(L258P) mutant, only cotransfection of PAR2 could restore phosphoinositide hydrolysis in response to thrombin. This was suggested to be due to PAR1 donating its tethered N-terminal ligand to PAR2, which cannot be directly activated by thrombin [85]. Endogenous PAR2 transactivation was observed in human umbilical vascular endothelial cells (HUVECs) by the addition of a PAR1 specific inhibitor, BMS200261, which binds to PAR1 to prevent receptor activation by the tethered ligand but does not prevent cleavage [88]. This compound, which completely inhibited thrombin response in both platelets and a pre-B cell line that only expresses PAR1, could not inhibit thrombin-mediated calcium flux in PAR2 expressing HUVEC cells [85]. This was not due to the presence of PAR4 since HUVECs do not respond to GYPGKF. Cells were desensitized to PAR2 after thrombin mediated PAR1-PAR2 signaling, as observed by the lack of response to SLIGRL after the addition of thrombin [85]. PAR2 transactivation has also been demonstrated by using a signaling dead mutant of PAR1 ($D_{199}R_{200}\rightarrow RD$) [86]. This mutant did not give a chemotaxis response to thrombin when transfected into HEK cells, nor did PAR2 when transfected alone. However, when both receptors were co-transfected together a chemotaxis response was observed indicating that the PAR2 receptor has been transactivated (Figure 1.4a-c). The fact that PAR1 and PAR2 appear to be so tightly co-expressed may suggest that the two receptors interact with one another to mediate physiological events such as migration, mitogenesis, metastasis, and angiogenesis.

PAR1/1 and PAR3/3 homodimers and PAR1/PAR3 heterodimers have also been observed [89]. Using BRET^2 technology (BRET with a modified GFP-2), interactions were detected between PAR1-Rluc and PAR1-GFP^2, PAR1-Rluc and PAR3-GFP^2, and PAR3-Rluc and PAR3-GFP^2 [89]. Though the affinity for homodimerization over heterodimerization did not appear to be different, the coupling of the G protein did show selectivity with PAR1/1 coupling to $G\alpha_q$ and PAR1/3 coupling more to $G\alpha_{13}$ alpha subunits [89]. Not only does this support the theory of signaling discrimination, but also suggests that PAR3, while it does not itself interact with G-proteins, may have an important role in signal transduction.

Perhaps the most convincing evidence of PAR heterodimerization is the detection of endogenous PAR1/4 heterodimers in human platelets [87]. Based on the observation that PAR4 assists PAR1 in thrombin activation [90] it was proposed that these receptors reside in close proximity, perhaps in a complex. To determine whether a dimer was formed, PAR4 was immunoprecipitated from human platelets and indeed PAR1 was observed to be pulled down with PAR4 [87]. Furthermore, transfected COS7 cells showed a FRET signal with PAR1-CFP as the fluorescent donor and PAR4-YFP as the fluorescent acceptor [87]. PAR4 thrombin signaling can also be enhanced by the presence of PAR1 as shown by coexpression of a non-cleavable PAR1 mutant (F43A) with PAR4 in COS7 fibroblasts. A 2.9-fold increase of calcium signal is observed in the presence of PAR1, which was not effected by the PAR1 specific antagonist RWJ-56110 [87]. This effect is most likely due to PAR1 in heterodimers recruiting thrombin via a hirudin-like binding motif, and this thrombin then can cleave PAR4.

The formation of PAR heterodimers has been extensively examined and signal specification or discrimination between different heterodimers is an emerging theme. As previously mentioned, thrombin stimulation of endothelial cells through PAR1 strongly couples to $G\alpha_{12/13}$/Rho signaling while thrombin activation of PAR1/PAR2 heterodimers stimulates $G\alpha_i$/Rac signaling [86]. PAR1/PAR1 homodimers coupled to $G\alpha_q$ signaling while PAR1/PAR3 heterodimers stimulated $G\alpha_{13}$ signaling [89]. Dimer mediated activation of distinct signaling pathways would explain the need for heterogenous dimerization of PARs within a signaling cascade.

1.4 The Physiological role of PARs:

PARs are expressed in a wide variety of tissues and have roles in various normal physiological processes and diseases. They have been implicated in coagulation, cancer, vascular regulation, vascular disease and injury, as well as inflammation and pain. The following section will review the important physiological and pathological activity of PARs and discuss in detail the diseases that are relevant to this thesis project.

PARs in platelet aggregation

As previously described, PAR1 was first discovered during a search for a receptor responsible for platelet thrombin response [8]. Indeed, PAR1 and PAR4 are implicated in human platelet activation and aggregation, whereas in mouse platelets, PAR3 and PAR4 mediate aggregation since no PAR1 is present [91]. Guinea pigs, like humans, express PAR1 on their platelets (as well as PAR3 and PAR4) and therefore are commonly used in studies of PAR1 platelet aggregation and thrombosis [92].

Prothrombin is present in blood as an inactive zymogen precursor that is converted to active thrombin at sites of blood vessel injury or damage [93]. Conversion of prothrombin to an active protease can occur in response to exposure to tissue factor (TF), which is normally expressed in sub-endothelial tissues of the vessels [94]. When a blood vessel is injured, TF comes in contact with the blood and can convert inactive prothrombin to active thrombin [94]. Additionally, TF can activate the extrinsic pathway of coagulation leading to the formation of the TF/Factor VIIa complex, which cleaves and activates Factor Xa [94].

Thrombin triggers platelet aggregation by cleaving PAR1 to cause rapid activation of downstream G proteins [90]. This cascade stimulates $G\alpha_{12/13}$ which trigger the platelets to change shape and release platelet-dense granules [95] and $G\alpha_q$ to produce a rapid increase in intracellular calcium and activation of the fibrinogen receptor, GPIIb/IIIa [96]. Fibrin and GPIIb/IIIa facilitate thrombus formation by acting as a scaffold for platelet aggregates and subsequent thrombus at the site of vessel injury [97]. PAR4 is also activated by thrombin, but at higher concentrations, and slowly produces a robust intracellular calcium level that is sustained over time, thus strengthening platelet aggregate formation [90]. PAR1 and PAR4 in platelets can also activate the $G\alpha_i$-coupled P2Y12 ADP-receptor through $G\alpha_q$ mediated ADP secretion [52]. PAR3 has not been shown to activate platelets by itself in mice or rats, but rather is thought to serve as a cofactor that can sequester thrombin to a hirudin-like motif on the N-termini to then cleave and activate PAR4 as observed with PAR1 in human platelets [61,50,90].

PARs in cancer

PAR1 was first identified as a potential oncogene in a screen of cDNA libraries by Whitehead et al. in 1995 [98]. Overexpression of full length and C-terminally truncated PAR1 in NIH-3T3 cells conferred focus formation and loss of contact inhibition of proliferation [98]. PAR1 reemerged in 2001 in a search for novel oncogenes in B6SUtA mouse myeloid progenitor cells [99]. PAR1 overexpression induced focus formation to occur, as well as anchorage- and serum-independent growth. PAR1-mediated transformation occurred in the presence of the thrombin inhibitor hirudin, but did not occur when a PAR1 cleavage mutant was overexpressed in cells, thereby suggesting that

PAR1 cleavage was necessary but thrombin was not the protease involved in PAR1-mediated oncogenesis [99]. Treatment of DU-145 prostate cancer cells with thrombin showed an increase in secretion of the cytokines IL-8 and VEGF, known angiogenic factors, that was dependent on PAR1, indicating that thrombin may have signaling potential in PAR1-mediated oncogenesis, by stimulating angiogenesis [100].

Inhibition of PAR1-mediated transformation by the $G\alpha_i$ inhibitor, pertussis toxin, and the $G\alpha_{12/13}$ inhibitor, RGS domain of Lsc (a RhoGEF that contains a regulator of G protein signaling that functions as a GTPase and negative regulator of $G\alpha_{12/13}$), suggested that transformation is conferred, at least in part by, $G\alpha_i$ and $G\alpha_{12/13}$ signaling [99]. It was also observed that PAR1 transformation was partly dependent on RhoA activation since co-expression with a dominant negative RhoA significantly inhibited foci formation [99]. These preliminary studies, along with the observation of deregulation of heterotrimeric G-proteins and their receptors in transformation [101], were the foundation of the now widely studied role of PARs in tumorigenesis.

Further studies have shown that PAR1 is highly expressed in malignant breast tissue of human patients with much lower expression in normal breast tissue [102]. In established breast cancer cell lines, PAR1 expression correlates with the invasive potential of cells as assessed by chemotaxis and matrigel barrier assays, and PAR1 antisense inhibits the invasive response in breast cancer cells [102]. Kamath et al. found that PAR1, PAR2, and PAR4 were all highly expressed in the invasive breast cancer cell line, MDA-MB-231, whereas in the poorly invasive MCF-7 cell line PAR2 and PAR4 had low expression and expression of PAR1 was undetectable [103]. Pertussis toxin inhibited PAR1-thrombin mediated matrigel invasion, suggesting a role for $G\alpha_i$

signaling [103]. PAR2 and PAR4 activating peptides, but not PAR1, caused migration of the cancer cells regardless of their invasive potential suggesting a role for other PARs in cancer [103].

In recent years, it was discovered that matrix metalloprotease-1 (MMP-1) could cleave and activate PAR1 to mediate chemotaxis and invasion of breast cancer cells [27]. This was the first report of activation of PAR1 by a non-serine protease, and explained why the addition of hirudin did not effect PAR1 transformation potential [99]. MMP-1 is produced and secreted by the stromal fibroblasts therefore establishing a stromal-tumor relationship in which MMP-1 secreted by stromal cells cleaves and activates PAR1 on tumor cells to mediate chemotaxis, proliferation, and invasion [27]. MMP-1 has also been shown to robustly activate the Akt survival pathway, which can be inhibited by MMP-1 inhibitor, FN-439, and PAR1 inhibitor, P1pal-7 [104]. PAR1 tumor-stromal communication was also shown by the discovery that PAR1 on breast cancer cells induces expression of the angiogenic factor Cyr61 that subsequently induces MMP-1 expression on adjacent stromal fibroblasts [105]. Knockdown of Cyr61 suppressed MMP-1 induction and resulted in loss of migratory ability of cancer cells towards fibroblasts [105].

PAR1 over-expression in the non-invasive breast cancer cell line, MCF-7, caused tumorigenesis and angiogenesis in a mouse xenograft model [27]. Treatment of mice with a PAR1 specific pepducin antagonist, P1pal-7, reduced tumor volume thus confirming a role for PAR1 in tumorigenesis [27]. PAR1 and MMP-1 inhibitor treatments in an experimental metastasis model of highly invasive MDA-MB-231 cells also significantly

reduced the metastasis of these cells to mouse lungs indicating that the MMP1/PAR1 signaling is a potential target for metastatic cancer therapy [104].

PAR1 has been shown to play a role in ovarian cancer angiogenesis and tumor progression [106]. Both ascites from ovarian cancer patients and conditioned media of ovarian cancer cell lines induces PAR1-mediated migration of cancer cells. MMP-1 and PAR1 expression levels correlated with migratory ability of ovarian cancer cells that could be inhibited by PAR1 specific inhibitors, P1pal-7 and RWJ-566110, and MMP-1 inhibitor, FN-439 [106]. Endothelial permeability, which is important in tumor progression and invasion, was induced by the addition of MMP-1 and could be attenuated by PAR1 specific inhibitors [106]. When mice were treated with PAR1 antagonist P1pal-7 in a xenograft ovarian cancer model, a significant reduction in both ascites and tumor angiogenesis was observed, as well as an overall reduction in tumor progression as assessed by histological staging of invasiveness (**See Figure 1.5, Ovarian cancer and ascites treatment with P1pal-7**) [106]. Further confirming the role of PAR1 in angiogenesis, MMP-1induces angiogenic chemokine production in ovarian cancer cells through PAR1, including the CXCR1/2 chemokines IL-8 and Gro ($\alpha/\beta/\gamma$) and the CCR2 chemokine monocyte-chemoattractant protein-1 (MCP-1) [107]. MMP-1 inhibitor, FN-439, also reduced tumor angiogenesis and progression in an ovarian cancer xenograft model (Figure 1.5) [106]. Conditioned media from cancer cells treated with MMP-1 promoted endothelial tube formation, which could be inhibited by PAR1 antagonists P1pal-7 and RWJ-566110 [107].

MMP-1 was confirmed as a transforming protease by a study that observed the tumor derived soluble factors from melanoma and colon cancer cell lines could activate

endothelial cells into prothrombotic and proinflammatory states [108]. This activation was dependent on PAR1 activation as shown by inhibitors to PAR1, RWJ-58259 and

Figure 1.5:
(from Agarwal, A., Covic, L., **Sevigny, L.**, et al, 2008, Mol Cancer Ther.)

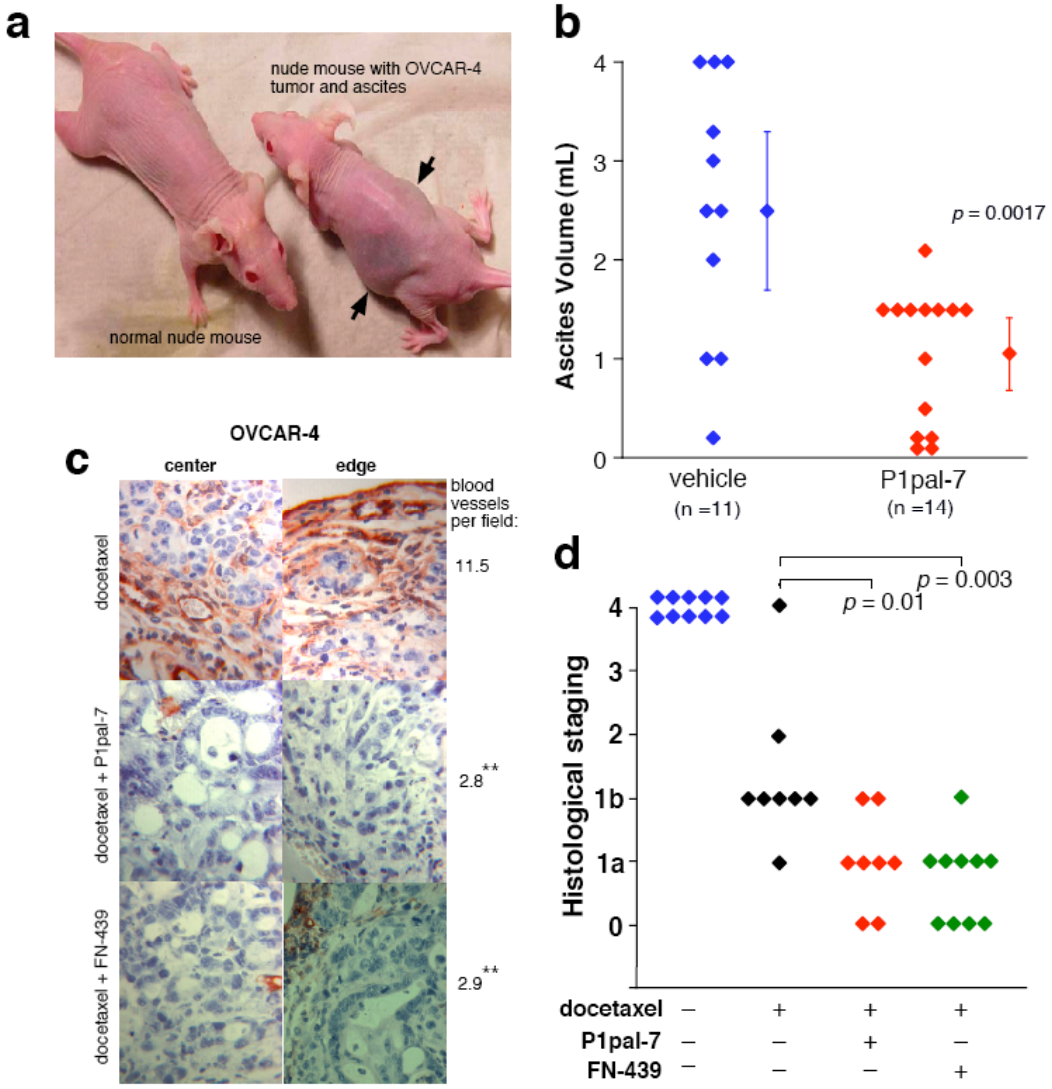

Figure 1.5. PAR1 antagonist, P1pal-7, and MMP-1 inhibitor, FN-439 reduce ovarian cancer tumor angiogenesis and progression (AACR Publications permits authors to use their articles or parts in doctoral thesis work).
(**a**) female NCR nu/nu mice were injected i.p. with RPMI (left) or 1.5 million OVCAR-4 cells (right) in RPMI. After 40 days, mice injected with cancer cells showed abdominal tumor masses and pronounced ascitic fluid (arrows). (**b**) mice were injected with 1.5 million OVCAR-4 cells and treated with either vehicle alone (20% DMSO) or P1pal-7 (10 mg/kg) for 40 days, ascites fluid was collected, and volume was determined. (**c**) P1pal-7 or FN-439 as dual therapy with docetaxel inhibited angiogenesis of OVCAR-4 cancer in mice. Weibel-Palade bodies within endothelial cells were stained using anti-von Willebrand factor and were scored from the tumor center or edge as shown. (**d**) P1pal-7 or FN-439 in combination with docetaxel inhibits invasion and metastasis of peritoneal ovarian cancer in mice.

SCH-79797 [108]. Activation of endothelial cells was attenuated when the melanoma supernatant was treated with MMP-1 inhibitor (FN-439) thus suggesting a new finding of tumor-endothelial cross-talk [108].

PAR2 is also expressed in multiple tumors and cancer cells including breast [103], pancreatic [109], colon [110], brain [111], and skin [112]. PAR2 can elicit a calcium flux signal and cause proliferation as well as cancer cell motility [110,112]. PAR2 expression in pancreatic cancer patient samples was only observed in malignant tissues, and was more robust in areas of severe fibrosis, suggesting that PAR2 may be involved in cancer cell invasion and induction of fibrosis [113]. Trypsin activation of PAR2 in the colon cancer cell line HT-29, caused MMP-dependent release of transforming growth factor (TGF-α) which in turn mediated activation of epidermal growth factor receptor (EGFR) and ERK1/2 to stimulate cell proliferation, suggesting a PAR2 transactivation mechanism of EGFR in these cells [114]. PAR2 has also been found to be essential for Factor VIIa and Xa induced signaling, migration, and invasion in breast cancer cells as determined by depleting PAR2 receptors in MDA-MB-231 cells; in this study, depletion of PAR1 had no effect on signaling [115].

PAR2 may have a proangiogenic effects as shown by the upregulation of VEGF message and protein in response to trypsin, FVIIa, and SLIGRL stimulation of MDA-MB-231 breast cancer cells, which was inhibited by a PAR2 cleavage blocking antibody and did not occur with PAR1, 3, or 4 agonist peptides or thrombin induction [116]. PAR2 induces an array of proangiogenic and immune modulating chemokines through TF/FVIIa activation, including CXCL1 which binds to CXCR2 on endothelial cells to promote angiogenesis [117]. Moreover, PAR2 significantly correlates

with ovarian cancer vascularization and rates of cell proliferation, making it a putative biomarker for poor patient prognosis [118]. PAR1 and PAR2 expression has also been detected in the alpha-smooth muscle cell actin (αSMA) positive stromal fibroblasts associated with carcinoma cells in human patients but not in those of normal or benign samples suggesting that PAR expression is not limited to the cancer cell but can also be found in the tumor-stromal microenvironment [119].

The roles of PAR3 and PAR4 have not been well established in cancer. Studies have shown that PAR3 is expressed in 12.6% of malignant samples (limited to kidney and liver cancer) and PAR4 in 54.9% with no correlation to tissue type [120]. PAR4 has been shown to elicit a breast cancer migratory response, but little more has been studied [103].

PARs in vascular endothelium and smooth muscle cells

All four PAR family members are expressed in arterial and/or venous endothelial cells and influence blood vessel tone and venomotor responses [121,122,16,123]. Endothelial PAR1 and PAR2 can react to their proteolytic environment to allow for contractility, proliferation, and inflammatory responses [4,124]. PAR1 endothelial activation stimulates intracellular calcium and secretion of von Willebrand Factor (vWF) and P-selectin from Weibel-Palade bodies, which subsequently can bind to collagen to promote platelet and leukocyte adhesion at the site of an injured vessel [125,126]. Moreover, PAR1 activation on endothelial cells can cause cell contraction, Rho-dependent cytoskeletal rearrangement, and cytokine release [127-129]. Activated PAR2 mediates endothelial cell proliferation and cytokine expression, and effects vasoreactivity [122,130-132]. PAR1 and

PAR2 appear to be important in blood vessel repair and injury response based on their ability to stimulate inflammatory responses and proliferative effects needed for repair. These functions raise the question of the effects of dysregulated receptor activity during these processes, as will be described in further detail below.

PAR1, PAR2, and PAR4 are all expressed on vascular smooth muscle cells (SMCs) and when activated, can elicit a calcium response and stimulate proliferation [133,92,134,135]. One study showed that PAR2, but not PAR1 or PAR4, could stimulate migration of vascular SMCs dose-dependently through trypsin, peptide agonist SLIGKV, or TF/FVIIa complex which was inhibited by a PAR2-blocking antibody (anti-sera to SLIGKV), but not by PAR1-blocking antibody [134]. PAR expression, especially PAR1 and PAR2 in highly vascularized tissues suggests a potentially critical role of these receptors in vascular biology and vascular repair.

PARs in thrombosis and myocardial ischemia/reperfusion injury

Proper regulation and temporal expression of PARs is important in the vascular system. Over-expression of PAR1 and PAR2 has been shown in the endothelium, vascular SMCs, and adventitial myofibroblasts (PAR2 only) of injured and diseased arteries [121,136,137,134,138]. This overexpression contributes to the physiological response of the artery to injury but can have detrimental effects leading to pathological conditions.

Hypertension is a common cardiovascular disease and it has been demonstrated that PAR1 and PAR2 stimulation can regulate blood pressure [63]. Both PAR1 and PAR2-deficient mice had no mean basal arterial pressure or heart rate differences compared to wild-type mice [63]. However, when given SFLLRN or TFLLRN wild-type

mice exhibited initial hypotension and decreased heart rate, with a secondary hypertensive phase. These effects were absent in PAR1$^{-/-}$ mice, confirming a PAR1 specific event, and enhanced in PAR2$^{-/-}$ mice suggesting cross-talk and compensatory response of PAR2 [63]. The PAR2 peptide agonist, SLIGRL, caused only initial hypotension, with no change in heart rate. These effects were not observed in PAR2$^{-/-}$ mice [63]. These initial vasoreactive responses initiated research into the role of PAR1 and PAR2 in vascular injury and disease.

As expected, PAR1 plays an important role in thrombosis. Though platelet aggregation and clots are necessary to avoid blood loss in normal hemostasis, a thrombus can cause occlusion of vessels thereby obstructing blood flow and ultimately lead to infarction of tissues deprived of oxygen. Dislodgment of thrombi and the resulting embolism can lead to stroke (cerebral infarction). The role of PAR1 in thrombosis was established in a model of arterial thrombosis in African green monkeys. A PAR1 blocking antibody abolished platelet-dependent cyclic flow reduction in 75% of the animals and reduced the frequency of this flow by 50% in the remaining animals [139]. This established PAR1 as a therapeutic target for thrombosis and has indeed led to the development of inhibitors that have been tested in models of arterial thrombosis including the Johnson & Johnson small molecule inhibitor RWJ-58259, which elicits anti-thrombotic effects by reducing thrombus weight in an A-V shunt model in guinea pigs [92]. The lack of full thrombotic inhibition was proposed to be due to the presence of the second thrombin receptor, PAR4, on the platelets. Indeed, the PAR4 antagonist, P4pal-10, also protected against thrombosis induced by $FeCl_3$ injury in mice carotid arteries, demonstrating that PAR4 plays a role in thrombi of mice platelets [140]. Dual

therapy to inhibit PAR1 and PAR4 in guinea pigs completely protected against thrombosis [87].

RWJ-58259 was further tested in cynomolgus monkeys and found to significantly extend occlusion time in a model of electrolytically injured carotid arteries. Examination of the resulting thrombi revealed that platelet deposition was significantly decreased [141]. Furthermore, RWJ-58259 completely inhibited thrombin-induced platelet aggregation in monkey platelets *ex vivo* [141]. Another PAR1 antagonist, SCH 602539, showed a similar effect of dose-dependent inhibition of cyclic flow reduction in a cynomolgus monkey model of arterial thrombosis which could be synergistically enhanced when used in combination with the P2Y12 ADP receptor inhibitor, cangrelor, suggesting that anti-thrombotic and anti-platelet therapy could be beneficial in diseased patients [142].

PAR1, PAR2, and PAR4 have been observed to have an effect in myocardial ischemia/reperfusion (I/R). Myocardial I/R is partly mediated by thrombin since functional inhibition reduces the infarct size after I/R, indicating a possible role for PAR1 [143]. Levels of the PAR1 agonist MMP-1 are elevated in the serum of myocardial infarction patients and are thought to increase as a function of myocardial damage [144]. PAR1 inhibition with SCH 79797 immediately before or during a rat model of myocardial I/R reduced myocardial necrosis and infarct size, and increased ventricular recovery [145]. The addition of a PAR1 selective agonist in this model abolished the observed protective effect but had no effect when given alone [145], suggesting that PAR1 signaling was partly responsible for myocardial damage and was maximally activated by proteases released during/after injury.

PAR2 activation by SLIGRL has been shown to have a protective effect against reperfusion injury in rat hearts by decreasing the ischemic risk zone (based on increase in coronary flow) and by decreasing creatine kinase release after treatment [146]. Interestingly, in experimental myocardial ischemia precondition in rat hearts, PAR2 activation decreased the neutrophil accumulation in the heart, improved ventricular function and coronary flow and again reduced creatine kinase release [147]. These results lead to the hypothesis that early PAR2 activation leads to endogenous protection. However, a recent study has suggested that PAR2 also has detrimental effects based on the observation that $PAR2^{-/-}$ mice had significantly smaller infarct size, less impairment of the heart, and reduced inflammatory cytokines IL-1β, TNF-α, KC, and IL-6 in heart lysates after injury [148]. The authors of this report suggested that PAR2 might have a dual role, with a protective effect on endothelial cells and a damaging effect on cardiomyocytes and infiltrating leukocytes [148]. In a hindlimb ischemia model, PAR2 activation by trypsin and SLIGRL increased the angiogenic response in ischemic tissue [149]. This accelerated hemodynamic recovery was based on the blood perfusion ratio and the enhanced capillarity in the adductor skeletal muscle of the animals, indicating that PAR2 activation may be beneficial in treating ischemic tissue [149]. Additionally, it is hypothesized that this effect was a result of PAR2 activation on the endothelial cells and not pro-inflammatory pathways [149].

Inhibition of PAR4 with the pepducin P4pal-10 has also been reported to mediate reduced infarct size in rat hearts and increased recovery of ventricular function, even when administered 15 min after the start of ischemia or 5 min after the start of reperfusion, suggesting that PAR4 also plays a role in vascular injury [150]. Overall,

PARs have an intriguing role in this injury process and therapeutic targeting of PAR1, PAR2, or PAR4 may be beneficial to patients.

PARs in cardiovascular disease

PAR activity in cardiovascular disease is not restricted to thrombosis and myocardial ischemia, but has also been reported in arterial injury models that lead to restenosis and neointima formation. Neointimal thickening involves an abnormal proliferation of smooth muscle cells on the intimal surface of a blood vessel. Two theories on the origins of these cells are held among most scientists, though they are not mutually exclusive. The first is that smooth muscle cells from the medial layer of the artery de-differentiate and migrate to the intima; there these cells continue to proliferate to cause neointimal hyperplasia [151]. The second theory is that injury stimulates homing of vascular progenitor or stem cells from the bone marrow to the site of injury and that these progenitors differentiate into a myofibroblast-like cell and proliferate to form the neointima [152,153].

Neointimal thickening usually occurs in areas of increased wall stress, endothelial damage, or chemical insult [154,151,155]. For example, in pulmonary arteries it has been shown that elevated pressure in the larger artery can lead to neointima formation [155]. Three commonly used animal models of vascular injury that induce neointimal formation are wire-injury, balloon angioplasty, and ligation injury. Wire injury involves the passage of a wire down the common carotid artery of an animal to denude the endothelial layer of the vessel [156]. Blood flow is restored to the common carotid artery after this procedure while the external carotid artery, which was used as an access point, is tied off.

Balloon angioplasty is performed by insertion of a balloon catheter via the external carotid into the common carotid. The balloon is then inflated and pulled back to damage the endothelial wall in a similar manner as human angioplasty which is used to reduce plaque size and occlusion [92]. Lastly, ligation injury, also known as a blood flow cessation injury, involves occlusion of the common carotid artery by suture ligation near the carotid bifurcation [157]. Each of these methods has their pitfalls and advantages, but all three are mechanical injuries that reproducably create an intimal response.

PAR1 has been shown to play a role in neointimal thickening mostly by using antagonists to the receptor. PAR1 blocking antibodies, in a balloon catheter model in rats, reduced neointima by 50% after 14 days and could inhibit rat smooth muscle cell proliferation *in vitro* [158]. In a mouse wire injury model, $PAR1^{-/-}$ mice had a trend of reduced intimal and medial area after 14 days of injury though no change in cellular proliferation was observed with bromodeoxyuridine (BrdU) being delivered subcutaneously with osmotic minipumps throughout the time of injury [156]. When PAR1 was inhibited in a model of rat balloon angioplasty by RWJ-58259, dose-dependent reduction on intima area was observed with no changes in media [92]. RWJ-58259 inhibited thrombin-induced calcium and proliferation from rat aortic smooth muscle cells [92]. The contractile response of arteries after injury has also been shown to be more responsive to thrombin, trypsin, PAR1 peptide agonist TFLLRN and PAR2 agonist SLIGRL after rabbit femoral artery balloon angioplasty [136]. These effects correlated with enhanced expression of both PAR1 and PAR2 in vascular lesions one week after injury. PAR1 and PAR2 expression decreased to basal levels 4 weeks after injury [136]. Recently, a new PAR1 antagonist, F16618, was used in a model of rat balloon

angioplasty to dose-dependently inhibit restenosis (intimal formation) which was hypothesized to be due to inhibition of human aortic SMC growth and thrombin mediated SMC migration [159]. It was noted that in the rat model, the PAR1 antagonist significantly reduced upregulation of *TNF-α* mRNA expression after injury and when given i.v. could reduce *MMP-7* mRNA upregulation, though no effect was seen on expression of *IL-6*, *TIMP1* (tissue inhibitor of metalloproteases), *TIMP2*, or *MCP-1*, which all increase with injury [159]. Therefore the authors suggest that PAR1 antagonism reduces restenosis by limiting early inflammatory events, MMP release, and SMC migration and proliferation [159]. (**See Figure 1.6, Neointima hyperplasia due to vascular injury**).

In rat balloon angioplasty, the damaged artery has upregulated expression of PAR2 especially in medial smooth muscle cells, proliferating adventitial myofibroblasts and SMC of the neointima [137]. This upregulation in PAR2 was observed by immunohistochemistry staining after 1 and 3 days of injury and also after 7 and 14 days, with the most intense staining at the luminal edge for the latter [137]. Furthermore, $PAR2^{-/-}$ mice had a reduction in neointima area after wire injury as well as reduced contractile response in the endothelial cells. It was also observed that lymphocyte adhesion was reduced in the $PAR2^{-/-}$ mice and therefore the reduction in neointima may be due to PAR2 modulation of inflammatory cell adhesion.

Blood vessel response to injury is not limited to the cells that are locally affected. Inflammatory cells are very important in the repair process that can become dysregulated to create intimal hyperplasia. Many vascular changes can occur due to inflammation such as vasodilation, edema, and cytokine increase [160]. Inflammatory cells have the ability to infiltrate the area of injury to cause these reactions. PAR1 and PAR2 have been

shown to play an important role in the inflammatory system, outlined below, and this function has to be taken into account when interpreting the studies previously described.

Figure 1.6:

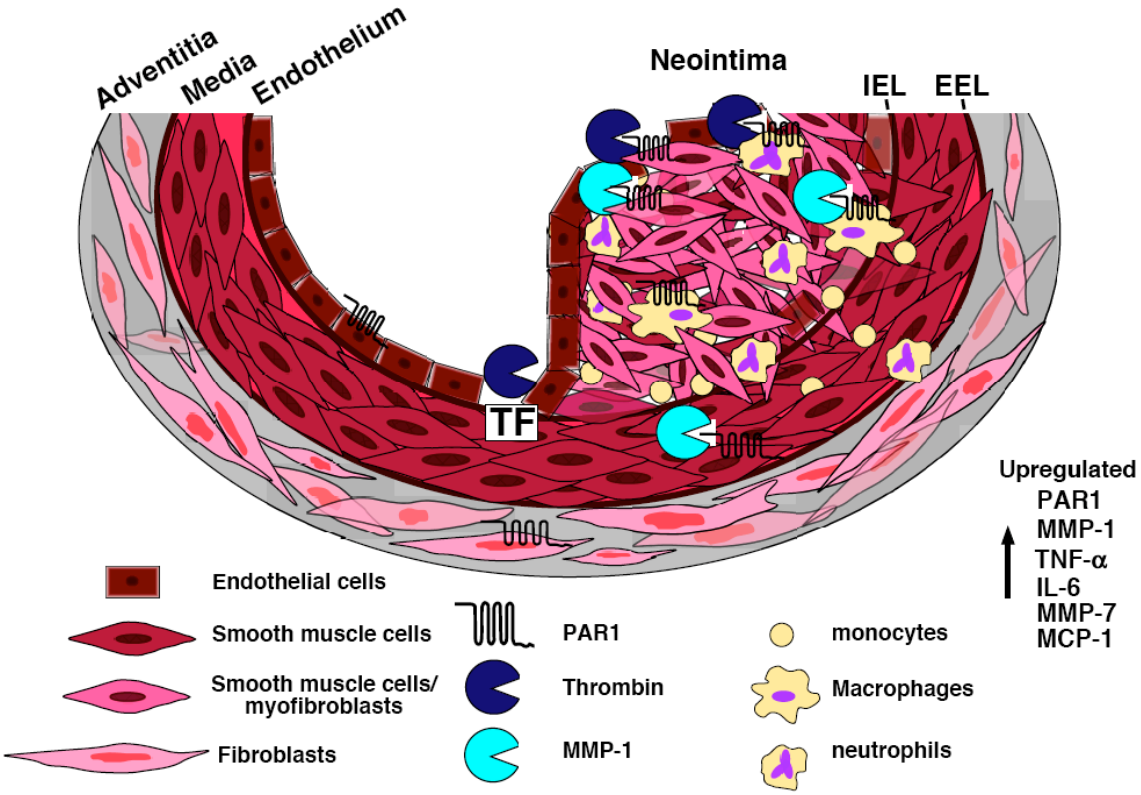

Figure 1.6. Proposed model of thrombin and MMP-1 activation of PAR1 contributing to neointima formation. PAR1 is expressed on endothelial cells, smooth muscle cells, fibroblasts, and inflammatory cells of the normal and diseased blood vessel. PAR1 activation by thrombin and possibly MMP-1 contributes to the migration and proliferation of smooth muscle cells to form intimal hyperplasia in the vessel after injury. Additionally, PAR1 stimulation can activate inflammatory mediators such as TNF-α and IL-6 to contribute to the diseased state.

The role of PAR1 and PAR2 in inflammation and pain

Inflammation is a normal physiological response to injury and pathogenic stimuli. It is necessary for the body to initiate the healing process and to remove unwanted irritants. Unfortunately, inflammation can also cause damage to the tissues that it serves to protect as in the cases of arthritis, atherosclerosis, some allergies (i.e. hayfever), and asthma.

The components that regulate inflammatory responses are all potential therapeutic targets for inflammatory related diseases.

PAR1, PAR2, and PAR4 have all been shown to play a role in inflammatory processes, with PAR2 possibly having a more prominent function. PAR1 is overexpressed in the colons of patients with IBS and patients with fibrotic lung disease [161,162]. PAR1 has a role in acute and chronic fibrotic lung injury as shown by the reduced infiltrating inflammatory cells, reduced bronchoalveolar lavage fluid, and a reduction in total lung collagen accumulation in PAR1 deficient mice [162]. Furthermore, PAR1 deficiency reduced the severity of adjuvant-stimulated arthritis in mice with less cartilage degradation, bone damage, synovial exudates, and a decrease in cytokine release including IL-1, IL-6, MMP-13, and serum IL-4. This suggests that PAR1 acts on inflammatory cells by increasing the production of cytokines [163]. Intraplantar injection of the PAR1 agonist peptide SFLLRN caused edema and vascular permeability, confirming PAR1 as an inflammatory mediator [164].

PAR1 also has been shown to have an interesting role in mouse models of sepsis [86]. Sepsis is inflammation of the whole body due to infection. It is a very deadly disease that is still prevalent in many intensive care units. Mice that have undergone cecal ligation and puncture (CLP) to induce septic shock and then given the PAR1 agonist P1pal-13 in the later phase of disease (4 hours) had increased survival rates, which was dependent on the ability of PAR1 transactivation of PAR2, showing a beneficial effect of PAR1 activation in inflammation [86]. However, PAR1 inhibition with a PAR1 antagonist P1pal-12S immediately following CLP increased survival, showing a benefit to inhibiting PAR1-mediated inflammation. This suggests that PAR1 can have a

dual role, first in early onset of inflammatory mediators which when reduced could be beneficial and a separate role in the later phase in which PAR1 activation is beneficial [86].

Activation of PAR4 has also shown proinflammatory effects. PAR4 agonism can induce leukocyte rolling and adhesion to vessel walls [165] as well as increase edema and vascular permeability in rat paws [166]. Edema and vascular permeability induced by carrageenan injection were both reduced when a PAR4 antagonist was administered [166]. This effect was dependent on neutrophil release of kallikreins, since mice that were immuno-depleted of neutrophils did not elicit a response to PAR4 agonist and inhibitors of plasma and tissue kallikreins reduced the PAR4 inflammatory response [166]. However, the PAR4 inflammatory response was not mediated by a neurogenic or mast cell-dependent mechanism [167].

PAR2 has been shown to regulate both the inflammatory and pain pathways through stimulation of NF-κB [4,130]. Inflammatory mediators such as TNF-α, IL-1α, and LPS are all known to strongly induce transcription of PAR2 [130]. Neutrophils express PAR2 on their surface, and activation of PAR2 on neutrophils causes shape change as observed by change in forward and side scatter in flow cytometry, and also induces intracellular calcium mobilization [168]. PAR2 activation also enhances these inflammatory signals with increased production of IL-1β, IL-6 and IL-8 by neutrophils coupled with increased migration through collagen lattices and increased L-selectin shedding (L-selectin is shed by proteolytic cleavage on neutrophils and is a marker for neutrophil activation) [169,170].

PAR2$^{-/-}$ mice have impaired leukocyte migration and rolling that delay the onset of inflammation, and also show deficiencies in allergic inflammatory response [171-173]. PAR2 agonist peptide SLIGRL induces inflammation when injected into mice and rat hindpaws, with redness, edema, and granulocyte infiltration observed [174,175]. Inflammation was reduced by mast cell depletion, and since PAR2 can be activated by tryptase from mast cells, a physiological pathway of activation is apparent [174,175].

Reducing PAR2-mediated inflammation is not always advantageous. As previously mentioned, PAR1 transactivation of PAR2 is beneficial for septic mice as shown by slightly decreased survival, and also by loss of protection from lung vascular permeability in PAR2$^{-/-}$ mice treated with PAR1 agonist P1pal-13 as compared to wild type mice [86]. Protective effects of an early dose (0 hours) of PAR1 antagonist were unchanged in PAR2$^{-/-}$ mice, as expected since the early adverse effects were thought to be largely PAR1 dependent [86]. Indeed, PAR2 is upregulated on neutrophils of septic patients, possibly increasing neutrophil activation and the clearing of bacteria [176]. In a model of lung inflammation and injury induced by *Pseudomonas aeruginosa*, a bacterium that can cause pneumonia, gastrointestinal and skin infection, and septic shock, PAR2$^{-/-}$ mice had more severe lung inflammation and injury as observed by higher bronchoalveolar lavage fluid and increased neutrophil numbers [177]. Interferon-gamma (IFN-γ), which is needed for proper clearing of the bacteria, was reduced in PAR2$^{-/-}$ mice and PAR2$^{-/-}$ neutrophils killed significantly less bacteria and had markedly less phagocytic efficiency [177]. The administration of lipopolysaccharides (LPS), the endotoxin that elicits the inflammatory response to Gram-negative bacteria including *Pseudomonas aeruginosa,* can increase the bronchorelaxant response of PAR2 agonist

SLIGRL in rat lungs [178]. This response was largely dependent on the increase in prostaglandin E2 after PAR2 activation, which is the most important prostaglandin in lung relaxation [178]. These studies suggest that although PAR2 inhibition may be beneficial in some cases, not all PAR2-mediated inflammation is negatively affecting the body, and PAR2 may be necessary to clear pathogens.

PAR2 has however been shown to have an important role in arthritis and therefore is a potential therapeutic target for the disease. Upregulation of PAR2 has been observed in chondrocytes of patients with osteoarthritis and synovial membranes of patients with rheumatoid arthritis (RA) [179,180]. IL-1β and TNF-α could both increase PAR2 expression while the regulatory cytokine TNF-β1 decreased PAR2 in cultured synovial fibroblasts [179]. In an adjuvant-induced chronic inflammation model of arthritis, $PAR2^{-/-}$ mice had significantly less joint swelling compared to wild-type mice [181]. Heterozygous animals with a single gene deletion ($PAR2^{+/-}$) showed an intermediate phenotype while PAR2 agonists injected intra-articular in wild-type animals induced prolonged swelling and hyperemia showing a role for PAR2 in mediating chronic inflammation [181]. Additionally, inhibition of PAR2 with the antagonist ENMD-1068 dose-dependently inhibited the release of IL-1β and TNF-α from the RA synovium and reduced joint swelling of mice intra-articular injected with carrageenan/kaolin [180,34]. Experimental osteoarthritis induced in mice was markedly decreased in $PAR2^{-/-}$ mice as assessed by reduced cartilage degradation and decreased subchondral bone formation. This decrease could be mimicked by a monoclonal antibody to PAR2 [182]. These studies indicate that PAR2 is potentially involved in the pathogenesis of arthritis by regulating

proinflammatory cytokines, suggesting PAR2 inhibition could be beneficial to arthritis patients.

Synovial tissue of RA patients has also been shown to have increased mast cell infiltration, which colocalizes with PAR2 staining [183] and chondrocytes from osteoarthritis patients have elevated gene expression of matriptase, a serine protease that can activate PAR2 [184]. Treatment of mice with the mast cell degranualting agent, 48/80, or human recombinant mast cell tryptase (β-tryptase) induces hyperemia as measured by laser Doppler imaging as well as visible joint swelling [183]. This vasodilation and tissue swelling in response to 48/80 and β-tryptase was not observed in $PAR2^{-/-}$ mice suggesting that mast cells contribute to arthritis through tryptase release and activation of PAR2 [183]. These studies confirm that PAR2 plays a substantial role in human arthritis that is most likely dependent on mast cells.

Skin disorders, such as psoriasis and inflammation of the skin, also link PAR2 with mast cell activation. Psoriasis patient samples exhibit an influx of tryptase-containing mast cells and highly increased PAR2 expression in both keratinocytes and endothelial cells of the inflamed human skin [185]. PAR1, PAR2, and PAR4 agonists (but not PAR3) induced scratching behavior in mice that could be suppressed by anti-histamine in the case of PAR1 and PAR4 agonists but not by PAR2, which also was the most potent inducer [186,187]. Indeed, PAR2 agonists fail to cause histamine release, suggesting that the effect is mediated by tryptase-dependent mechanisms [188].

Over the past decade, it has become apparent that PAR2 also plays a role in pain. This was first discovered by the observation that primary spinal afferent neurons expressed PAR2 and that trypsin, tryptase, and PAR2 peptide agonist SLIGRL could

induce release of the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) [189]. These neuropeptides act together to induce inflammatory pain. CGRP causes arteriolar vasodilation and increases blood flow to inflamed tissue to induce edema and SP acts on neurokinin receptor (NK_1R) on endothelial cells of postcapillary venules to cause gap formation and stimulate the adhesion of infiltrating neutrophils [189]. PAR2 agonists injected in rat hindpaws induced both thermal and mechanical hyperalgesia as measured by hindpaw withdrawal latency and nociceptive (pain receptors) threshold experiments [190]. PAR2-dependent withdrawal latency could be inhibited by a neurokinin-1 receptor antagonist. Furthermore, PAR2 agonists increased expression of Fos protein, a useful marker for nociceptive neurons [190]. PAR2 in rat dorsal root ganglia neurons enhances capsaicin, increases cAMP, and increases calcium flux. Protein kinase A (PKA) and PKC both colocalize with PAR2 which promotes translocation of these proteins to the plasma membrane in cultured cells [191]. PKC and PKA antagonists prevented sensitization of transient receptor potential vanilloid receptor-1 (TRPV-1) and suppress thermal hyperalgesia in rat hindpaws injected with PAR2 agonist suggesting that thermal hyperalgesia is induced by PAR2 sensitization of TRPV-1 [191].

Additional evidence of the contribution of PAR2 to pain was shown by mast cells promoting the expression of the P2X4R receptor in microglia cells [192]. This receptor releases brain-derived neutrophic factor (BDNF) which causes pain [192]. Mast cell-induced expression of P2X4R was abolished by antibodies blocking PAR2 and tryptase [192]. Most recently, pain associated with cancer has also been linked to PAR2. Supernatant from human head and neck squamous cell carcinomas injected into the

hindpaws of mice caused pain measured by hindpaw withdrawal latency, which was attenuated with serine protease inhibitors and mast cell depletion and was absent in $PAR2^{-/-}$ mice [193]. Together these studies suggest that PAR2 may be an effective target for the treatment of pain.

(See Table 1.2, Summary of the physiological roles of PARs).

Table 1.2: Summary of the physiological roles of PARs.

	Receptor			
	PAR1	**PAR2**	**PAR3**	**PAR4**
Physiolgocial Roles:	platelet aggregation thrombosis embryonic development vascular tone regulation pro-tumorigenic pro-metastatic pro-angiogenic inhibition reduces MIR* inhibition reduces restenosis pro-inflammatory: fibrotic lung injury sepsis (early) arthritis edema induction anti-inflammatory: sepsis (late phase)	vascular tone regulation cancer (pro-migratory) pro-angiogenic agonist reduces MIR* inhibition reduces restenosis induces pain pro-inflammatory: leukocyte migration lung inflammation arthritis lung fibrosis skin/psoriasis edema induction anti-inflammatory: lung bacterial/viral infection	cofactor for PAR4	platelet aggregation thrombosis inhibition reduces MIR* pro-inflammatory: leukocyte rolling/ leukocyte adhesion edema induction

*MIR=myocardial ischemia/reperfusion

1.5 Pepducin Technology, agonists and antagonists of GPCRs

Over the past decade, our lab has developed a novel technology to agonize and antagonize G-protein coupled receptors with cell-penetrating peptides, called pepducins. Pepducins are amino acid sequences designed based on the sequence of GPCR intracellular loops that have a cell penetrating, membrane tethering moiety, such as a lipid, at either the N- or C-termini. These lipidated peptides have the ability to cross the membrane and can act as either receptor agonists or antagonists [(33)]. Depending on the N-terminal lipid, the bioavailability, half-life, and efficacy of the pepducin may be manipulated. These lipid tags protect the peptide sequence from degradation and clearance [(140)]. Depending on the length and location of the sequence template, pepducins may be specific for a particular GPCR or also affect closely related or dimerizing receptors [(140)].

The proposed mechanism of action of pepducins is that they mimic and/or compete with contacts made by the GPCR with heterotrimeric G-proteins [(140)]. The lipid moiety on pepducins allows for the compound to "flip" across the plasma membrane and be anchored to the lipid bilayer, therefore increasing the maximal effective concentration by keeping the pepducin near the target receptor and G-protein interface. Cell penetration of pepducins has been confirmed using fluorescein to label a palmitoylated and a non-palmitoylated pepducin antagonist of PAR4 (P4pal-10). In both *in vivo* and *in vitro* assays, cells and platelets exhibited strong fluorescence after treatment with pronase to digest extracellular proteins or pepducin on the outside of the plasma membrane [(140)].

Pharmacokinetics and pharmacodynamics studies have demonstrated that pepducins are highly stable, effective, and specific. Intravenous administration in mice

results in plasma levels and platelet levels that were relatively high (5 μM) and maintained for 5 hours with a terminal elimination half-life of about 3.5 hours [140]. Pepducin biodistribution in mice after intravenous or subcutaneous injection of radioactively labeled P4pal-10C was measured and shown to be fairly evenly distributed between the kidney, liver, spleen, blood, heart, and lungs with much smaller amounts in the muscle and fat and none in the brain [194]. After one hour, radioactivity was observed to increase fivefold in the urine suggesting that the pepducin or byproducts are excreted in mice [194]. Pharmacodynamic assays demonstrated that the P4pal-10 pepducin delivered intravenously extended bleeding time in mice by 3-5 fold and this effect was maintained for 1-6 hours [140]. By 4.5 hours the protective effect was halved and by 24 hours bleeding was back to baseline levels. P4pal-10 treatment in baboons demonstrates that the pepducin could completely inhibit PAR4 platelet function without cross-inhibiting PAR1 [140].

Multiple pepducins targeting different GPCRs have been validated in animal models of platelet aggregation [32], thrombosis [140], inflammation [195] , sepsis [86], angiogenesis [107] and cancer [27,104]. The PAR4 pepducin P4pal-10, as mentioned above, effectively inhibited mouse systemic platelet activation in a similar manner as was observed in the $PAR4^{-/-}$ mice [32]. P4pal-10 has also been shown to protect mice from carotid artery occlusion in response to $FeCl_3$ injury, with 70% of treated animals not occluding by 30 minutes compared to only 10% in vehicle [140]. A PAR1 pepducin antagonist, P1pal-7, gave partial protection from arterial thrombosis in guinea pig carotid arteries [87]. PAR1 inhibition of thrombosis could be enhanced with dual therapy using the PAR4 specific antagonist P4pal-i1 and the PAR1 small molecule antagonist RWJ-

56110 [87]. P1pal-7 is also an effect treatment in cancer models, inhibiting breast cancer tumor growth with a reduction of blood vessel density in tumors [27], decreasing breast cancer cell metastasis [106,104], as well as reducing ascites, tumor progression and angiogenesis in ovarian cancer models [106]. Furthermore, dual therapy with taxotere and P1pal-7 synergistically inhibited xenograft growth of MDA-MB-231 by 95% compared to untreated animals [104].

Pepducins can be designed to target any GPCR, not just PARs. A pepducin targeted to CXCR1 and CXCR2 receptors (X1/2pal-i3) effectively inhibited systemic inflammation in mice with an 85% survival rate in CLP-induced sepsis [195]. Moreover this same pepducin inhibited MMP-1 dependent angiogenesis in endothelial tube formation and matrigel plugs [107]. The CXCR4 targeted pepducin, ATI-2341, induces dose-dependent recruitment of neutrophils from bone marrow and is effective in mobilizing hematopoietic stem/progenitor cells from the bone marrow in mice and cynomolgus monkeys [196].

A detailed understanding of pepducin technology has advanced our ability to design a PAR2 specific antagonist for this thesis project. The ideal PAR2 pepducin would safely and efficiently block pathological inflammation and potentially be an essential tool in further studying the physiological role of PAR2 in multiple disease models such as arthritis, asthma, pain, and cardiovascular diseases.

Chapter 2: PAR1 and PAR2 in vascular injury

Summary

PAR1 and PAR2 act together to regulate intimal growth and repair after vascular injury. Mouse vascular aorta smooth muscle cells (MOVAS) and primary smooth muscle cells respond to PAR1 and PAR2 agonists to induce proliferation and calcium mobilization, however the absence of PAR2 attenuates the observed PAR1 signal.

Co-immunoprecipitation studies reveal that PAR1 and PAR2 associate, suggesting that the receptors can form a heterodimer. Using a carotid arterial ligation injury, C57BL/6 wild-type, $PAR1^{-/-}$, and $PAR2^{-/-}$ mice were assessed for intimal and medial hyperplasia after 21 days of recovery. Mice were treated with vehicle or the PAR1 pepducin agonist, P1pal-13. P1pal-13 treatment resulted in a 13-fold increase in intimal hyperplasia in wild-type mice, which was significantly reduced in the PAR2 knockout strain, suggesting that deficiency of either receptor limits the resolution of cell growth in response to injury resulting in the observed hyperplasia. Additionally, the PAR1 agonist P1pal-13 stimulates de-differentiation of smooth muscle cells, allowing for increased migration and proliferation resulting in intimal hyperplasia.

Publication Status: (to be submitted)

Deficiency of PAR2 modulates PAR1 response in neointima hyperplasia

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2.1 Introduction

Cardiovascular disease is the leading cause of death in the United States. Vascular injury, remodeling and vascular inflammation are observed in a number of diseases ranging from atherosclerosis to restenosis. Smooth muscle cell hyperplasia, which results in neointimal thickening, is a contributing factor in a number of ischemic diseases and is often observed following interventions such as angioplasty. The detailed mechanisms underlying this abnormal proliferation of new layers of smooth muscle cells and myofibroblasts on the intimal surface of a blood vessel are poorly understood and preventing its occurrence has proven to be a challenge. Additionally, the recruitment of inflammatory cells is essential for vascular repair and the resolution of this inflammation is necessary to complete restoration of the blood vessel to its normal vascular function, illustrating the complexity of vascular injury.

As reviewed in the introduction of this thesis, accumulating evidence has shown that protease-activated receptors (PARs) are highly expressed in many of the cell types involved in the response to vascular injury, and that PAR1 and PAR2 have roles in inflammation and vascular remodeling. We sought to examine the signaling of PARs in smooth muscle cells and determine the effect of inflammatory mediators and injury on PAR signaling both *in vitro* and *in vivo*.

In these studies, we discovered that PAR1 functions cooperatively with PAR2 to mediate hyperplasia in an arterial injury model. PAR1 coimmunoprecipates with PAR2, providing evidence that these receptors associate in a heterodimer. We show that PAR agonists stimulate calcium mobilization in smooth muscle cells and induce proliferation. In a model of vascular injury and perivascular inflammation, PAR2 expression is

necessary for neointima formation in response to the PAR1 agonist pepducin, P1pal-13. P1pal-13 can induce de-differentiation of these cells indicating a mechanism for the observed increase in proliferation, as differentiated SMCs have much slower growth compared to those in a de-differentiated synthetic state.

2.2 MATERIALS & METHODS

Cell Culture

Mouse aorta vascular smooth muscle cells (MOVAS) were purchased from ATCC and maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.2 mg/mL G418 in 5% CO_2 at 37 °C. *Cercopithecus aethiops* kidney cells (COS7) were maintained in DMEM supplemented with 10% FBS, 1% P/S in 5% CO_2 at 37 °C. Primary SMCs from wild-type, PAR1$^{-/-}$, and PAR2$^{-/-}$ C57BL/6 mice were isolated from carotid arteries. Arteries were split and scraped to remove the intimal endothelial layer, and then plated on tissue culture plates pre-coated with Type I collagen (Sigma). SMCs were maintained in DMEM supplemented with 15% FBS, 1% P/S in 5% CO_2 at 37 °C.

Flow Cytometry

MOVAS or primary SMC cells were harvested by lifting with 3 mM EDTA/PBS. Cells were labeled with the PAR1 polyclonal Ab [103] (SFLLRNPNDKYEPFC), the PAR2 polyclonal Ab [86] (SLIGKVDGTSHVTGKGVC), or the PAR4 polyclonal Ab [103] (GYPGQVSANDSDTLELPC) and subsequently with FITC-labeled secondary Ab. PAR expression was quantified by mean fluorescence intensity relative to isotype control on the BD Canto II flow cytometer and results were analyzed using FlowJo Software (Tree Star).

Calcium Flux Assay

Intracellular Ca^{2+} flux was measured in MOVAS, labeled with fura-2AM, by the ratio of fluorescence excitation intensity at 340/380 nm on a LS 50B Luminescence Spectrometer (Perkin Elmer).

MTT Assay (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay

MOVAS were plated at 1000 cells/well in a 96-well plate and allowed to adhere overnight. Cells were incubated in starvation media (DMEM, P/S, 0.4% BSA) and subjected to specific treatment conditions, as specified in Figure 2.1c. After daily treatment for 4 days under low serum conditions MTT reagent (Sigma-Aldrich, Inc., St. Louis, MO) was added at a concentration of 0.5 mg/mL and allowed to incubate at 37 °C for 5 h. The resulting formazan crystals were dissolved in 100% DMSO and colorimetric readings were taken on the SPECTRAmax 340 microplate reader (Molecular Devices Corporation).

Immunofluorescence

Primary SMCs were plated at 1000-3000 cells/well on BD chamber Culture-Slides and allowed to adhere for 24-48 h. Media was aspirated and cells were washed with PBS. Cells were fixed with 4% formaldehyde in PBS for 20 min at 37 ºC. Cells were blocked with 1% BSA at 25 ºC for 30 min and 10% goat serum for 20 min, and then incubated with 1:50 or 1:100 dilutions of the indicated antibodies for 1 h at 25 ºC. Cells were then incubated with a 1:500 dilution of the appropriate secondary antibody (Alexa 488-rabbit

or Alexa 546-mouse, Invitrogen) in the dark for 1 h at 25 ºC. Cells were washed, ProLong antifade solution with DAPI (Invitrogen) was added, and coverslips were mounted on glass slides. Fluorescence was observed on a Nikon Eclipse 80i microscope and images were captured by a Spot 7.4 Slider camera (Diagnostic Instruments, Inc).

^{3}H Thymidine Incorporation

MOVAS or primary smooth muscle cells were lifted with 3 mM EDTA, plated at 2000 cells/well in 24-well plates, and allowed to attach overnight in complete media (DMEM, 10% FBS, P/S). Cells were starved overnight in DMEM, 0.4% BSA, P/S and treated as indicated in Figure 2.1d or Figure 2.2b once a day in starvation conditions. One day prior to harvesting, 1 μL of methyl ^{3}H-Thymidine (NEN) was added to each well. Cells were harvested 48-72 h after the first treatment. Media was aspirated, cells were washed with PBS, 2 mL of ice-cold 6% trichloroacetic acid (TCA) was used to fix cells and 1 mL of 0.2 N NaOH for 10 min was used to lift cells. The entire volume of each well was transferred to a scintillation vial, 7.5 mL of scintillation fluid was added, and vials were analyzed on a Tri-Carb 2900 TR Liquid Scintillation Analyzer (Packard).

Co-Immunoprecipitation

A pcDEF3-PAR1 construct was tagged at the N-terminus with a T7-epitope (MASMTGGQQMGT) as previously described [197]. A pcDEF3-PAR2 construct was tagged at the C-terminus with a myc-epitope tag (EQKLISEEDL) and a CXCR4 construct was tagged at the N-terminus with a HA epitope (MYPYDVPDYA). COS7 fibroblast cells were transiently transfected with pcDEF3 alone (vector), T7-PAR1 alone,

PAR2-myc alone, HA-CXCR4 alone, T7-PAR1 and PAR2-myc, T7-PAR1 and HA-CXCR4, or PAR2-myc and HA-CXCR4. Cell lysates were prepared 48 h after transfection in M-PER lysate buffer (Pierce) with Halt-Protease Inhibitor cocktail (Thermo Scientific) and protein was quantified using a Bradford assay. Cell lysates were pre-cleared with 25 μL of Protein A-Agarose (Calbiochem) for 1 h, then incubated overnight at 4 °C with 25 μL of either α-T7 agarose beads (Novagen) or α-cMyc Monoclonal Ab-agarose beads (Clontech) in a final volume of 500 μL. Agarose beads were collected by centrifugation and washed x3 with 1% of M-PER lysis buffer. Immunoprecipitated protein was eluted in 50 μL of 10 mM citric acid pH 2.2 in SDS loading buffer for 30 min at 37 °C. Samples were centrifuged to precipitate beads and 10 μL of neutralization buffer was added to the protein samples before gel electrophoresis.

Carotid Artery Ligation Injury

All animal experiments were performed in accordance with the US National Institutes for Health guidelines and approved by Tufts Medical Center Institutional Animal Care and Use Committee. Six-month old C57BL/6, PAR1$^{-/-}$, or PAR2$^{-/-}$ female mice were anesthetized with isoflurane and aseptically surgically prepared. A midline incision was made to expose the trachea and carotid artery. The left carotid artery was isolated and a 6-0 silk suture was placed around the common carotid and synched to completely restrict blood flow. The suture was squared and trimmed and the wound was closed with 6-0 nylon/monofilament non-absorbable sutures. Post-operative analgesia, buprenorphine, at 0.05 mg/kg was administered as needed. Pepducins (P1pal-13 at 2.5 mg/kg or P1pal-7 at

10 mg/kg) or vehicle (20% DMSO) were administered subcutaneously on a daily basis. After 21 days (or specified number of days in timecourse), mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (90-120 mg/kg, 10 mg/kg) and a midline incision was made to expose both carotid arteries. The chest was entered through anterior thoracotomy and a 25-gauge butterfly needle was inserted into the left ventricle of the heart. A pressurized bag at 125 mm Hg with 10% formalin was used to perfuse the animals. Left and right common carotid arteries were harvested and further fixed in 10% formalin. Samples were vertically embedded, slides were stained with H&E, elastin, or the proliferation marker Ki67 and cross-sections were analyzed.

Morphometry

Images of H&E carotid artery cross-sections were captured using a Nikon Eclipse 80i microscope and a Spot 7.4 Slider camera (Diagnostic Instruments, Inc) at a magnification of 10x and 40x. Images were analyzed for the intimal, medial, and adventitial layers by measuring area (um^2) of at least two images from each left carotid artery. Area was measured by weighing the portions of each arterial section of printed images and converted from mg to μm^2 by a standard curve of known sizes by capturing images of an objective micrometer. Area for each left carotid were averaged and reported for each group.

Bone Marrow Transfer and LacZ Staining

Bone marrow transplantation assays used LacZ or $PAR1^{-/-}$ mice as bone marrow donors and 8-10 week old wild-type C57BL/6 mice as recipients. Donors were euthanized by

CO_2 inhalation, followed by cervical dislocation and bone marrow was harvested from both femur and tibia. The bone marrow was then introduced by tail vein injection (30-gauge needle and 2 million cells in 0.2 mL of PBS) into syngenic recipient mice that were conditioned with ionizing radiation using a Shepherd Mark I gamma irradiator employing a ^137 Cs source. The dose of ionizing radiation was lethal (900cGy), which will cause mice to die from hematopoietic failure 10-12 d post-irradiation if not reconstituted with hematopoietic stem/progenitor cells. Post-transplantation recipients were given the antibiotic Sulfatrim (300 mg/kg) in their water for the first two weeks. Eight to 16 weeks post-transplantation, ligation was performed as described above. Arteries were extracted after saline perfusion and stained overnight at 37 °C in 1 mg/mL X-gal, 2 mmol/L $MgCl_2$, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$, 0.01% sodium deoxycholate, 0.02% NP40 in PBS. Arteries were then either fixed or frozen in O.C.T Compound Embedding Medium (Tissue-Tek) for histological analysis.

RT-PCR Analysis

Total RNA was extracted using the RNeasy mini kit (Qiagen) and 1 μg of RNA was reversed transcribed using Moloney murine leukemia virus reverse transcriptase and dNTPs (Invitrogen). Real-time PCR was conducted in 25 μL volumes with 12.5 μL of SYBR Green, 1.25 μL of each primer and 8 μL of RNA-free water. All reactions were performed in triplicate in a DNA Engine Opticon 2, Continous Fluorescence Detector (MJ Research). After 15 min of denaturation at 95 °C, 30 s at 94 °C, 1 h at 55 °C, 30 s at 72 °C, PCR was performed at 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 15 s for 40 cycles. The primers used are listed in **Table 2.1, RT-PCR primers**.

Statistical Analysis

All statistical analyses were performed using Graphpad Prism (version 5.0a) or Microsoft Exel (Version 11.6). Data are represented as mean ± SEM. For two group comparisons of parametric data, a two-tailed distribution unequal variance Student's *t* test was performed. For multiple-group comparisons, two-way ANOVA tests were performed followed by Bonferroni posttest analysis. Statistical significance was defined as

* $p < 0.05$ or ** $p < 0.005$

Table 2.1: Real-time PCR primers (for mouse SMCs).

Gene targeted	Official gene name	Direction	Primer Sequence
PAR1	*F2r*	forward	CTCCTCAAGGAGCAGACCAC
		reverse	CAAGAAAGAAGATGGCGGAG
PAR2	*F2rl1*	forward	CCACTGGTGGCGGATTGCCC
		reverse	GTTGCGTCCCGGTGCAAGGT
α-SMA	*Acta2*	forward	AGCCAGTCGCTGTCAGGAACCCT
		reverse	CACCAGCGAAGCCGGCCTTAC
myocardin	*Myocd*	forward	CAGCCAAGGGTGCGCACAGAACTC
		reverse	TGACTGCTGTCTGCTTGGGTGC
SM-22	*Tagln*	forward	GGCGGCCTTTAAACCCCTCACC
		reverse	GTTGAGGCAGAGAAGGCTTGGTCG
SM-MHC	*Myh10*	forward	TCTCAAGAACCGGCTCAGGCGGG
		reverse	GCGATGCCCCCTCAATGTGCAG
PDGF-B	*Pdgfb*	forward	CAGCGAGCCAAGACGCCTCAA
		reverse	ACACTCTTGCCGACGCCCCT
MMP-3	*Mmp3*	forward	GGGACTCTACCACTCAGCCAAGGC
		reverse	GGGGATGCTGTGGGAGTTCCATAGA
Type III collagen	*Col3a1*	forward	AGAGGGGCTCCTGGTGAGCG
		reverse	GGGCCAGGGGGACCAGGTT
GAPDH	*Gapdh*	forward	AGAACATCATCCCTGCATCC
		reverse	CACATTGGGGGTAGGAACAC

Results:

2.3 PAR1 and PAR2 signaling on vascular smooth muscle cells.

Mouse vascular aorta smooth muscle cells (MOVAS) were assessed for PAR cell surface expression by flow cytometry and calcium mobilization in response to PAR1, PAR2, and PAR4 agonists. The presence of PAR1, 2, and 4 on the cell surface was observed as well as a robust calcium signal from thrombin, the peptide agonist SFLLRN, and P1pal-13 with weaker signals from TFLLRN (PAR1) and SLIGRL (PAR2) agonist peptides (**Figure 2.1**). No calcium signal was observed from AYPGKF (PAR4). This indicates that MOVAS respond to PAR1, and to a lesser extent PAR2 agonists, to activate $G\alpha_q$ signaling. PAR1 agonists also increased cell proliferation of MOVAS as observed by MTT, a measure of metabolic activity and viability (Figure 2.1c), and by ^{3}H-thymidine incorporation into newly synthesized DNA. The PAR1 agonist thrombin increased cell growth, which was specifically inhibited by the PAR1 small molecule inhibitor, RWJ-56110 (Figure 2.1c). The PAR2 peptide agonist, SLIGRL, was able to modestly increase cell metabolism in MTT assays, however this PAR2 selective agonist did not increase mitosis as assessed by ^{3}H-thymidine incorporation (Figure 2.1c,d). The PAR4 peptide agonist, AYPGKF, gave no significant increase in proliferation compared to cells incubated with media alone. These results suggested that unlike PAR2, PAR1 agonists strongly stimulate calcium signaling and proliferation in murine SMCs.

To determine whether inflammatory mediators would enhance the proliferative response of MOVAS to PAR agonists, cells were incubated with low concentrations of TNF-α or IL-1β. TNF-α and IL-1β increased the proliferation of MOVAS beyond

Figure 2.1:

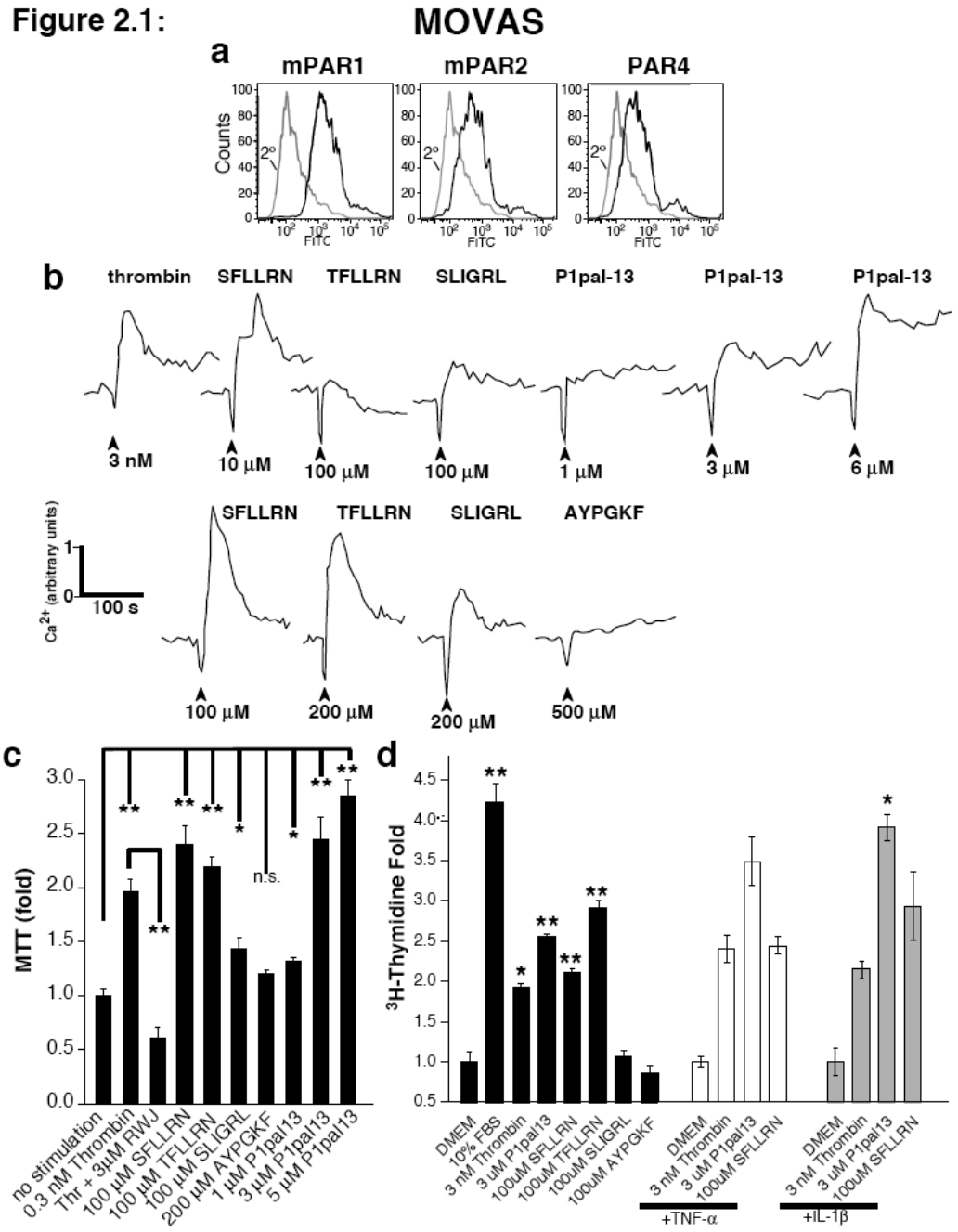

Figure 2.1. PAR1 and PAR2 agonists can stimulate calcium mobilization and proliferation of mouse smooth muscle cells. (**a**) Mouse vascular aorta smooth muscle cells (MOVAS) were analyzed for surface expression of mouse PAR1, PAR2, and PAR4 by flow cytometry with PAR-specific antibodies. (**b**) PAR agonist activity in calcium flux assays with endogenous expression in MOVAS. Intracellular Ca^{2+} flux was measured using cells labeled with fura-2AM by the ratio of fluorescence excitation intensity at 340/380 nm. (**c**) MOVAS were plated on a 96-well plate (1,000 cells/well) and treated daily as indicated for 4 days under low serum conditions. MTT was added after 4 d of growth and cell proliferation was quantified using colorimetric readings of the resulting formazan crystals. (**d**) MOVAS were plated on a 24-well plate (2,000 cells/well) and treated daily as described in **c**, methyl 3H-thymidine was added to wells 24 h prior to scintillation analysis. *, P <0.05 and **, P <0.005.

that of PAR1 agonist alone indicating that inflammatory costimulation could increase the response of PAR1 mitogenesis in SMCs (Figure 2.1d).

2.4 PAR2 is associated with PAR1, suggesting the formation of a heterodimer.

Transactivation and FRET data have shown that PAR1 and PAR2 reside in close proximity and can come within molecular contact to one another (≤100 Å) [85,86]. In this study, we performed co-immunoprecipitation using T7-epitope tagged PAR1 and PAR2-myc tagged constructs (**Figure 2.2**). We observed that PAR2-myc immunoprecipitated with T7-PAR1 when both receptors were present, demonstrating a direct physical interaction or complex (Figure 2.2a). Conversely, HA-CXCR4, a receptor that is not expected to bind PAR1 or PAR2, was used as a negative control and was not immunoprecipitated with T7-PAR1 (Figure 2.2b, right lane). T7-PAR1 immunoprecipitation with PAR2-myc was also observed using myc agarose beads (Figure 2.2c). These results suggest that PAR1 and PAR2 can form a heterodimer.

2.5 Timeline of arterial injury shows increase in inflammatory cells and proliferation.

To study the function of PAR1 and PAR2 in vascular remodeling and injury response, we performed ligation surgery of the left common carotid artery on wild-type C57BL/6, $PAR1^{-/-}$, and $PAR2^{-/-}$ mice of the same background. Ligation injury crushes the vessel and blocks all distal blood flow through the artery causing significant stress to the proximal occluded vessel. Inflammation of the vessel is also a large component of this

Figure 2.2:

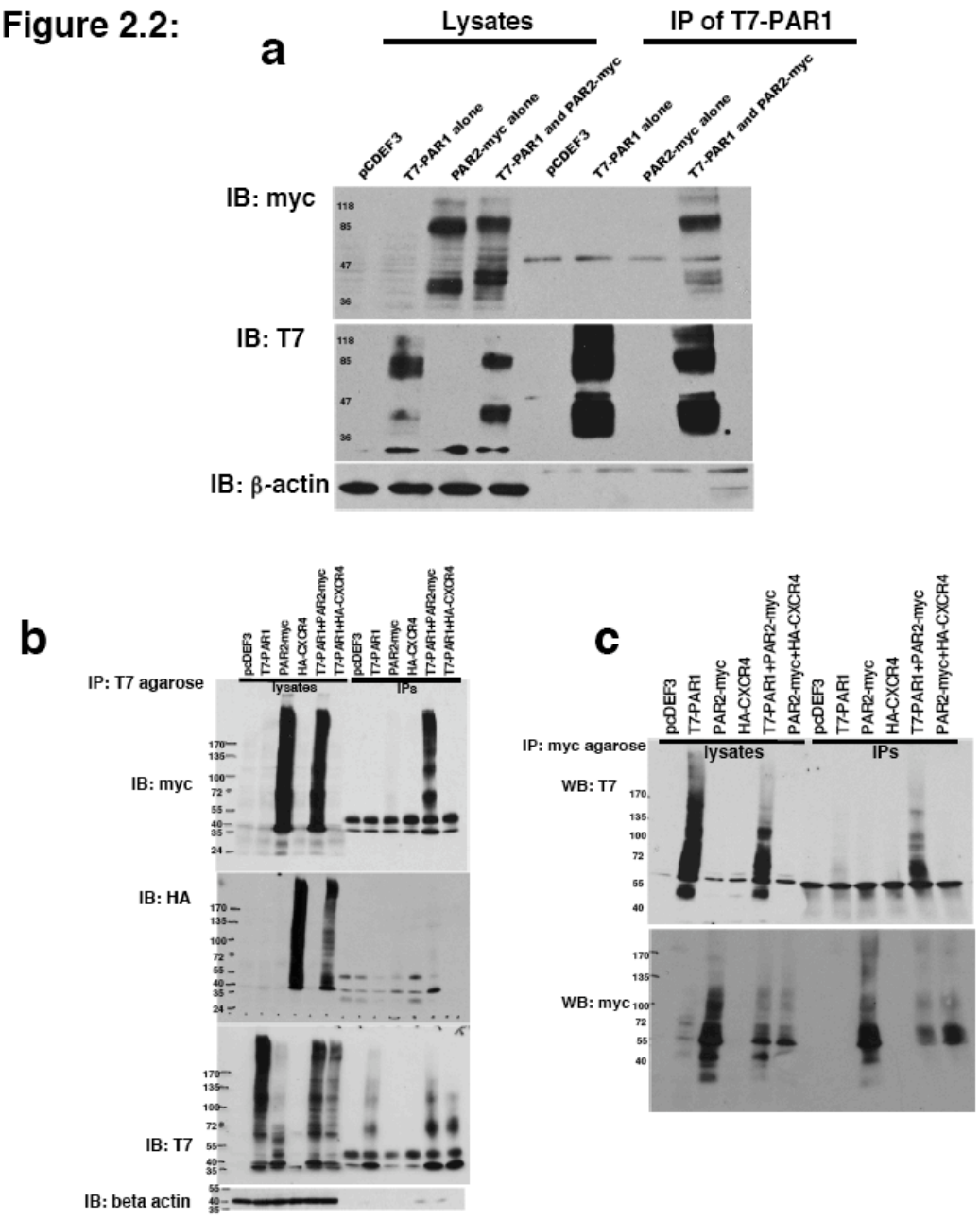

Figure 2.2. **Co-immunoprecipiation of PAR2-myc with T7-PAR1.**
(**a-c**) Tagged PAR1 and PAR2 constructs were generated to allow for immunoprecipitaion with (**a-b**) T7 agarose or (**c**) myc agarose beads revealing that PAR1 is associated with PAR2. (**b**) A negative control of the GPCR CXCR4 tagged with HA was not pulled down with T7-PAR1 (last lane).

injury model. To document the extent of stress and the response of the artery over the course of injury, we harvested carotid arteries at different time-points throughout the 21-day injury period.

According to our expert pathologist, Dr. Sheida Sharifi, in wild-type mice 2 hrs post-ligation, inflammatory cells were already noted in the lumen and adventitia of the artery (**Figure 2.3**). After 2 days of injury, the SMCs of the artery appearred more plump, indicative of edema, and inflammatory cells such as lymphocytes and neutrophils were present in increased numbers in the lumen and adventitia. Additionally, the enlarged nucleus of the smooth muscle cells suggested nuclear activity such as gene transcription. At 4 days, edema resolved and large numbers of lymphocytes were marginating the lumen of the injured artery. A considerable number of macrophages, lymphocytes, and neutrophils also were in the adventitia. Ki67 staining, a marker of cellular proliferation, revealed an increase in proliferation of cells in the adventitia (Figure 2.3, bottom panel). After 7 days, medial thickening was evident and more fibroblasts were apparent in the adventitia, though still mixed with numerous inflammatory cells. There were more macrophages than neutrophils compared to day 2, and lymphocytes had infiltrated into the media layer. At 7 days, Ki67 staining was strong throughout the artery and was most intense in the medial layer.

At 14 days of injury, inflammatory cells were not as prevalent though still present and the vessel appearred to be in a chronic state of injury (Figure 2.3). Proliferation was most noticeable in the medial and intimal layers based on the Ki67 staining (Figure 2.3, bottom panel). Finally, after 21 days of injury some vessels had a significant amount of intimal thickening. The adventitia still contained a mixture of fibroblasts and

macrophages but there were less neutrophils and lymphocytes, indicating that the stimulation of new inflammatory mediators was slowed or stopped and the vessel was in a chronic state of injury (**See Table 2.2**).

Figure 2.3:

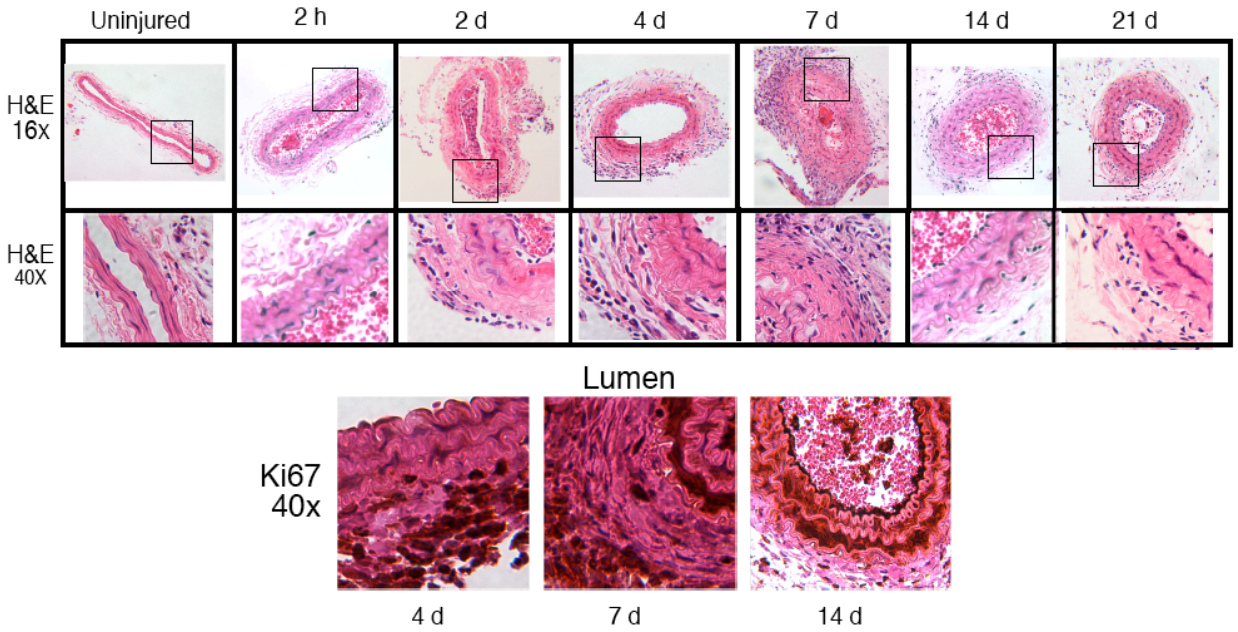

Figure 2.3. **Timeline of injury shows acute transmural edema followed by inflammatory and fibro-proliferative repair over time (done in collaboration with Karyn Austin)**. Ligated carotid arteries of wild-type C57BL/6 mice were observed at the indicated time point. In the beginning stages of vascular injury the arteries show increased edema and increased infiltration of neutrophils into the intimal area and neutrophils, lymphocytes, and macrophages into the adventitial area (2 h-2 d). By 7 d fewer neutrophils are observed, while lymphocytes and macrophages have increased dramatically, especially in the adventitia and can even occasionally be detected in the medial layer. By 14-21 d, though still present, fewer inflammatory cells are observed and more of a chronic state of injury has been reached. Ki67 staining (bottom panel, brown) indicates proliferation of medial cells at 4, 7, and 14 d.

Table 2.2: Inflammatory cells observed over 21 day ligation injury. (done in collaboration with Dr. Sheida Sharifi)
Quantification of the inflammatory cells that are observed after carotid artery ligation injury in H&E cross-sections. n=3 for each time-point.

Days after injury	Edema	Section of Artery	Neutrophils	Lymphocytes	Macrophages
2	++	Intima	+++	+	-
		Media	-	-	-
		Adventitia	+	+	++
4	+	Intima	-	++	-
		Media	-	-	-
		Adventitia	++	++	++
7	-	Intima	-	++	-
		Media	-	+	+
		Adventitia	+	+++	+++
14	-	Intima	-	+	-
		Media	-	+	+
		Adventitia	+	+	++
21	-	Intima	-	+	+
		Media	-	+	+
		Adventitia	-	+	+

2.6 PAR1 agonist causes medial and intimal hyperplasia in wild-type mice but not in PAR2 deficient mice.

To investigate the effect of PAR1 and PAR2 deficiency and activation on the chronic state of arterial injury, we performed ligation injury and allowed animals to recover for 21 days. Animals were treated daily with the PAR1 agonist, P1pal-13, or vehicle. After 21 days, mice were perfused and cross sections of the arteries were analyzed.

PAR1$^{-/-}$ and PAR2$^{-/-}$ vehicle treated mice showed no significant difference compared to wild-type counterparts in medial hyperplasia. However, P1pal-13 treated wild-type mice had a significant 1.62-fold increase in medial area compared to vehicle treated animals (**Figure 2.4**). As expected, P1pal-13 treated PAR1$^{-/-}$ mice did not exhibit

any difference in medial hyperplasia compared to PAR1$^{-/-}$ vehicle treated mice since the cognate receptor of the pepducin was absent. Medial hyperplasia showed a non-signficant increase of 1.3-fold when PAR2$^{-/-}$ mice were treated with P1pal-13, which was significantly less than the change observed in wild-type mice, suggesting that PAR1 and PAR2 cooperative signaling is necessary to mediate vascular remodeling. Representative elastin/Verhoeff's stained cross sections of P1pal-13 treated animals show the medial layer between elastic laminas (Figure 2.4, bottom panel).

Analysis of the intimal layer of injured arteries reveal that both PAR1$^{-/-}$ and PAR2$^{-/-}$ mice had a 6.6-fold increase in intimal hyperplasia compared to wild-type animals, suggesting a protective role of the receptors in this ligation injury model (**Figure 2.5**). When PAR1 signaling was stimulated with the PAR1 pepducin agonist P1pal-13, a significant 13-fold increase in intimal hyperplasia over vehicle treated animals was observed, suggesting that constant activation of PAR1 can play a harmful role in vascular remodeling. We did not observe any change in intimal hyperplasia when P1pal-13 was administered to PAR1$^{-/-}$ animals. PAR2$^{-/-}$ mice did not respond to P1pal-13 with increased intimal hyperplasia (Figure 2.5), which may indicate that the PAR1 response observed in wild-type mice requires signaling from both receptors, perhaps in a heterodimer, to cause this increase in hyperplasia.

To determine if PAR1 activation was most detrimental at a particular point during the 21 days of ligation injury, we treated animals with P1pal-13 for only the first 7 days of injury or only the last 14 days of injury (**Figure 2.6**). These timepoints correspond to an acute versus chronic stage of injury as observed in our timeline of injury. Interestingly, neither 7 day nor 14 day treatment resulted in increased intimal or medial

hyperplasia, suggesting that PAR1 activation is necessary throughout injury to increase the stenosis observed.

Figure 2.4:

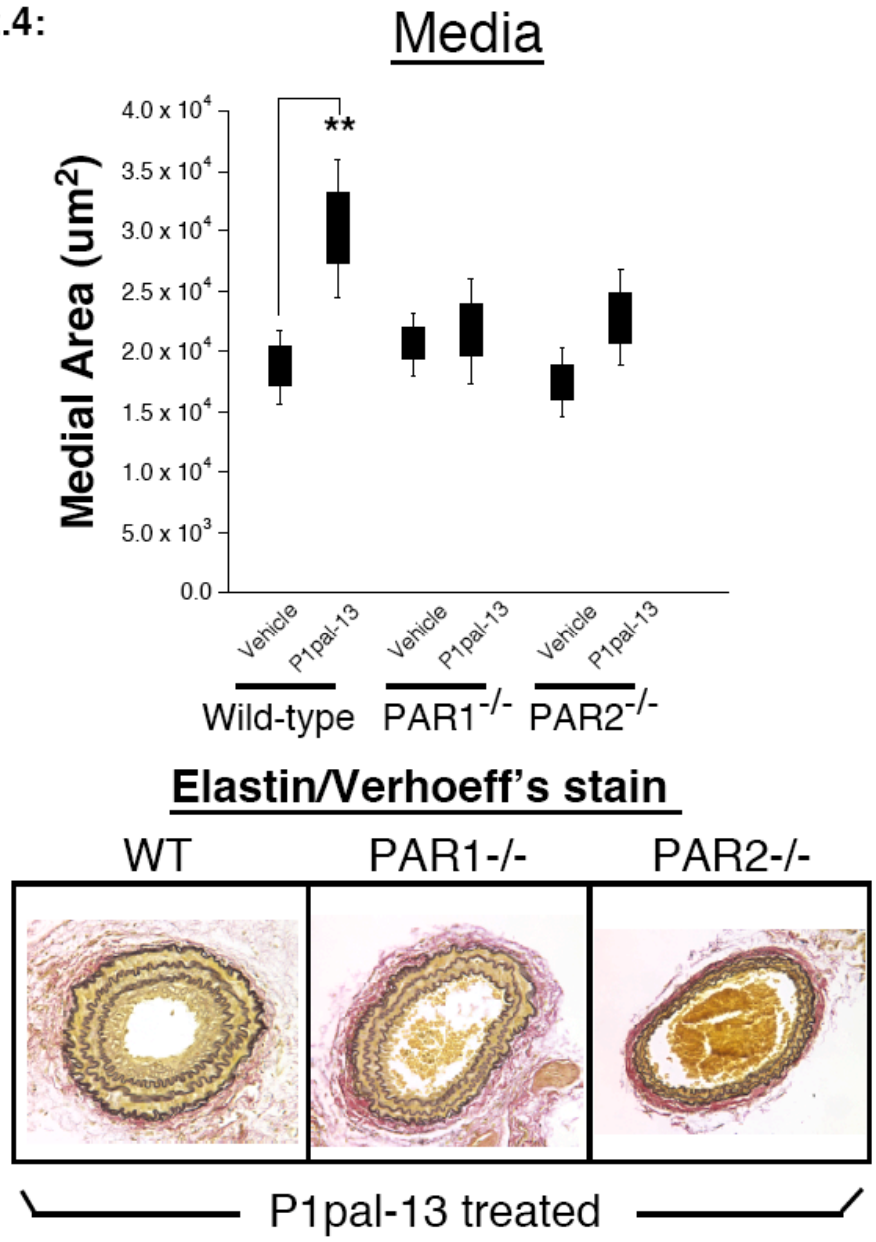

Figure 2.4. **PAR1 stimulation causes a significant increase in medial hyperplasia.** Media following carotid artery ligation revealed that P1pal-13 causes a significant increase in medial area, which is attenuated in PAR2$^{-/-}$ mice. Representative elastin/Verhoff's stained cross-sections of arteries (bottom). **, P <0.005.

Figure 2.5:

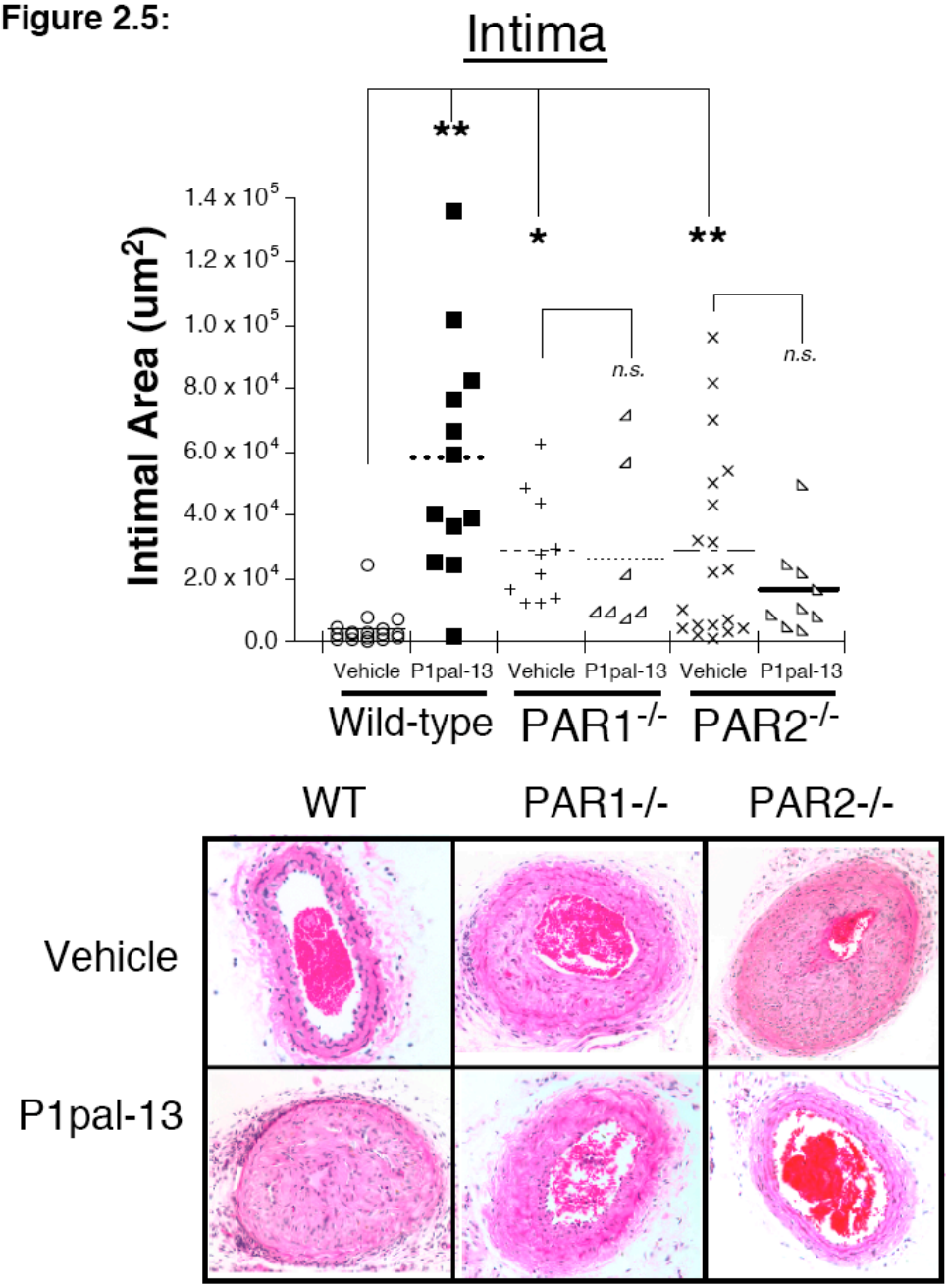

Figure 2.5. The PAR1 agonist, P1pal-13 caused a significant increase in intimal hyperplasia that is absent in PAR2 deficient mice. C57BL/6 wild-type, PAR1$^{-/-}$ and PAR2$^{-/-}$ mice underwent ligation surgery of the left common carotid artery and were allowed to recover for 21 days while being treated with P1pal-13 or vehicle. Resulting intimal hyperplasia was measured and plotted for each individual mouse. Representative H&E cross-sections show the differences observed between the genotypes and treatments (bottom).
*, P <0.05 and **, P <0.005.

Figure 2.6:

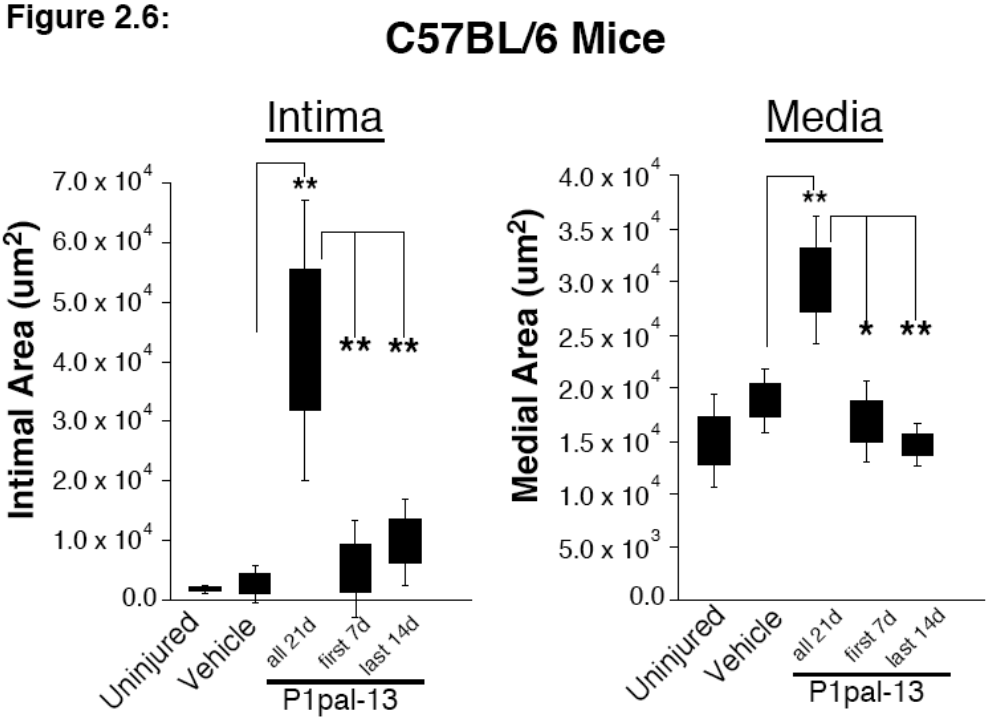

Figure 2.6. PAR1 stimulation is necessary for the full 21 days of injury for intimal hyperplasia. C57BL/6 wild-type mice were subjected to carotid artery ligation injury and treated with vehicle or P1pal-13 for all 21 days, the first 7 days, or the last 14 days after injury. A significant increase in intimal and medial area was observed only when P1pal-13 treatment was given for all 21 days. *, P <0.05 and **, P <0.005.

2.7 The effect of PAR1 antagonist, P1pal-7, on Intimal and Medial Hyperplasia.

Due to the observation that PAR1 activation causes an increase in intimal and medial area, we hypothesize that inhibition of PAR1 would cause a decrease in restenosis. The left common carotid of C57BL/6, PAR1$^{-/-}$ and PAR2$^{-/-}$ mice were ligated and mice were treated for 21 days with 10 mg/kg of the PAR1 antagonist pepducin, P1pal-7, or vehicle. Histological analyses of arteries from treated mice revealed that in both wild-type and PAR2$^{-/-}$ mice intimal area was non-significantly increased by 1.5 fold (**Figure 2.7**). As expected, there was no difference in PAR1$^{-/-}$ mice treated with vehicle or P1pal-7. Medial hyperplasia showed no change in wild-type or PAR1$^{-/-}$ mice, however a

significant 1.44 fold increase was observed in PAR2$^{-/-}$ mice, suggesting that in the absence of both PAR1 and PAR2 signaling there is a loss in regulation of the intimal growth. It must be stated that this is an inadequate mouse model in which to observe the therapeutic potential of drugs since the intimal and medial area produced by injury alone is minimal, and is therefore difficult to inhibit.

Figure 2.7:

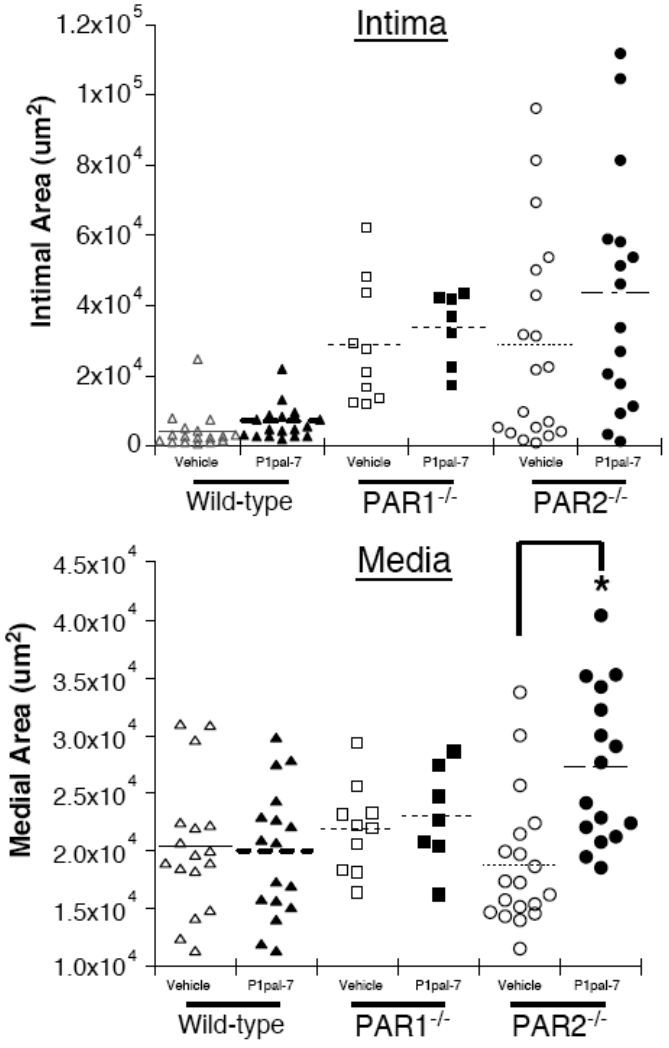

Figure 2.7. The PAR1 antagonist, P1pal-7 had no effect on intimal hyperplasia but increased the medial area of PAR2$^{-/-}$ ligation injured mice. C57BL/6 wild-type, PAR1$^{-/-}$ and PAR2$^{-/-}$ mice underwent ligation surgery of the left common carotid artery and were allowed to recover for 21 days while being treated with P1pal-7 or vehicle. Resulting intimal and medial hyperplasia was measured and plotted for each individual mouse.

2.8 Primary smooth muscle cell proliferation in response to P1pal-13.

We next examined the response to PAR1 agonist pepducin, P1pal-13, in primary SMCs isolated from wild-type, $PAR1^{-/-}$, and $PAR2^{-/-}$ mice. Immunofluroscence was performed on the primary cells with αSMA, SM-22, PAR1, and PAR2 which confirmed that the cultured cells were of SMC origin and that they lacked either PAR1 or PAR2 in the respective knockout mice (**Figure 2.8a**). Proliferation of the primary SMCs, as assessed by incorporation of ^{3}H-thymidine into newly synthesized DNA, exhibited a proliferative response to the PAR1 agonist P1pal-13 in wild-type cells. $PAR1^{-/-}$ and $PAR2^{-/-}$ cells did not respond to P1pal-13 treatment compared to wild-type cells, demonstrating that both receptors are necessary to elicit a proliferative signal from the PAR1 agonist pepducin P1pal-13 in these cells (Figure 2.8b).

Figure 2.8:

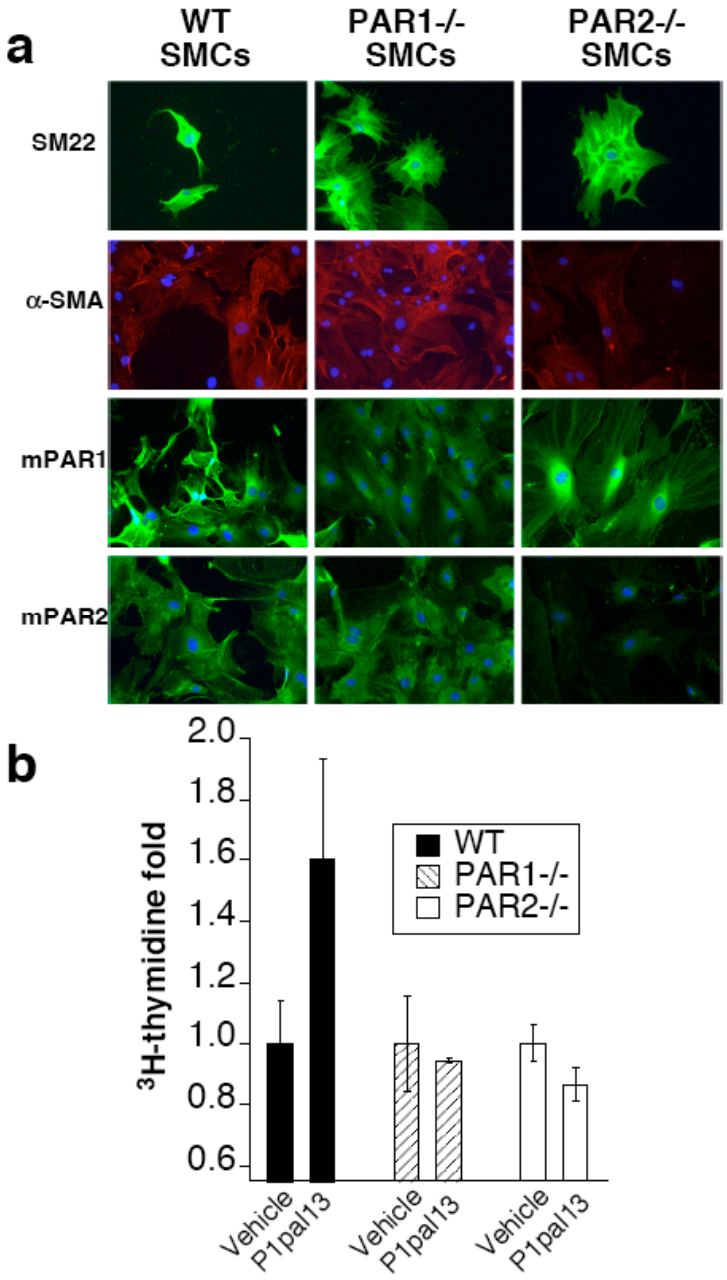

Figure 2.8. **Immunofluorescence and proliferation of primary SMCs.** (**a**) Primary smooth muscle cells were isolated from wild-type (WT), PAR1$^{-/-}$, and PAR2$^{-/-}$ mice. Cells were plated on culture slides and stained with DAPI (blue in all panels), SM-22 (top row, green/FITC), α-SMA (second row, red/TRITC), mouse PAR1 (third row, green/FITC), or mouse PAR2 (bottom row, green/FITC). (**b**) Primary SMCs were subjected to daily treatment of vehicle (0.2% DMSO) or P1pal-13 (3 μM) for 3 days. Methyl ^{3}H-Thymidine was added 24 hrs prior to lifting and cell proliferation quantified by a scintillation analyzer.

2.9 De-differentiation of smooth muscle cells.

If the intimal cells observed in the injured vessel originated from smooth muscle cells of the local artery's medial layer, then the ability to migrate to the intima and proliferate could result from the contractile differentiated SMCs converting to a more synthetic state possibly via de-differentiation [198,199].

Adult medial smooth muscle cells mediate contraction of blood vessel diameter to regulate pressure and blood flow and proliferate at an extremely low rate [199]. However, most SMCs are not terminally differentiated as shown by their ability to revert to a vascular repair/developmental state that exhibits an increase in proliferation and migration [199]. This phenomenon, termed phenotypic switching, is extremely important and is believed to play a role in vascular disease since signaling by environmental cues such as the damage caused by injury can trigger SMCs to revert to this proliferative state [199].

To distinguish between the contractile and synthetic states, differentiation markers have traditionally been used. Markers of SMC lineage include αSM-actin (αSMA), SM-22, and SM myosin heavy chain (MHC), as well as multiple other genes [199]. Some of these markers have been observed in other cell types during development (i.e. αSMA and SM-22) and virtually all markers for SMC differentiation can be found in myofibroblasts. It has been hypothesized that these myofibroblasts are representative of the alternative synthetic phenotype. However when examining gene expression, it is still expected that these markers of SMC differentiation, especially SM-MHC, will decrease if the cell is in a synthetic state. Additionally, markers that may have increased expression in the synthetic state could also be examined, such as PDGF-BB, Type III collagen, and

MMP-3. It has been observed that PDGF-BB is associated with downregulation of SMC differentiation markers and when SM-1 and SM-2 (isoforms of myosin heavy chain) are decreased, a correlative increase in PDGF and MMP-3 expression was observed in developing rabbit lesions [200]. Matrix metalloproteases are believed to be important in degradation and vascular remodeling since their presence would allow for cells to move/migrate through extracellular matrices [199]. De-differentiated SMCs exhibit a high rate of production of extracellular matrix components, such as Type III collagen, [199,201] a critical component for cardiovascular development in mice [202].

To determine if the SMCs in ligation injured arteries were in the contractile or synthetic state, real-time quantitative PCR was performed using primers to SMC differentiation markers (αSMA, SM-22, and SM-MHC) as well as the de-differentiation markers (PDGF, MMP-3, and Type III collagen). P1pal-13 and vehicle treated uninjured and injured arteries were assessed and normalized to GAPDH expression and the uninjured vehicle treated arteries were used for fold comparison.

RT-qPCR revealed that in injured arteries, vehicle or P1pal-13 treated, there was a significant decrease in expression of αSMA, SM-22, and SM-MHC (**Figure 2.9a**). Interestingly, arteries from uninjured animals treated with P1pal-13 also showed a decrease in these SMC markers, suggesting that PAR1 stimulation in the absence of injury can increase the de-differentiation of the smooth muscle cells. Since no differences in intimal or medial area were observed between the vehicle and P1pal-13 treated bilateral controls, this increase in the SMC differentiation state did not appear to affect healthy blood vessels. Ligation injury increased the expression of PDGF-B and Type III collagen, and P1pal-13 treatment significantly enhanced the expression of Type

III collagen and MMP-3 above the vehicle treated injured arteries (Figure 2.9b-d). These data suggest that P1pal-13 increased intimal hyperplasia in response to injury by stimulating de-differentiation of SMCs as indicated by reduced SMC lineage marker expression and increased Type III collagen and MMP-3 expression, markers of de-differentitation.

Figure 2.9:

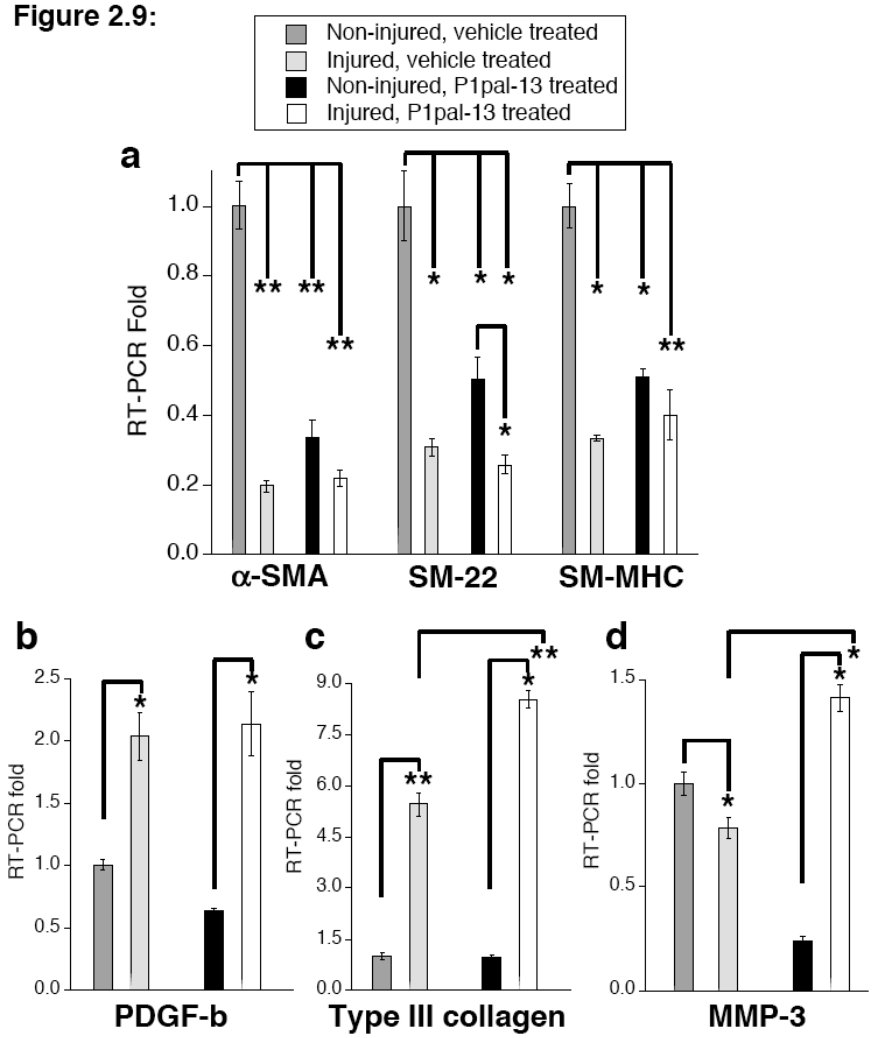

Figure 2.9. Markers of smooth muscle cells differentiation decrease and SMC de-differentiation markers increase when animals are treated with PAR1 agonist P1pal-13. RNA was extracted from uninjured and injured arteries of C57BL/6 wild-type mice treated with vehicle or P1pal-13. RNA was converted to cDNA and real-time PCR was performed with primers for (**a**) markers of SMC differentiation (**b-d**) or markers of SMC de-differentiaton. Gene expression was normalized to GAPDH expression and fold was determined by expression in uninjured vehicle treated arteries.

2.10 Identification and origin of neointimal cells

To confirm the presence of smooth muscle cells and infiltrating inflammatory cells in the neointimal lesions, immunohistochemistry (IHC) was performed on vertical cross-sections of injured carotids. Most of the cells comprising the intimal hyperplasia stained positive for smooth-muscle cell actin (αSMA) suggesting that these were SMCs and/or myofibroblasts (**Figure 2.10**). CD45 staining, which is specific for cells of hematopoietic origin, revealed positive cells mostly in the adventitia and lumen with a few positive cells in the intimal and medial layers. These cells are most likely inflammatory mediators responding to the injury (Figure 2.10a). F4-80, a marker of macrophages, was observed most prominently in the adventitia of the arterial cross sections. In conclusion, IHC has revealed that most of the cells of the intima are of SMC origin with some CD45 positive cells.

To distinguish if the neointima observed originated from the local arterial cells or from stem/progenitor cells of the bone marrow that are recruited to the site of injury, bone marrow transfer experiments were performed. Wild-type C57BL/6 mice were lethally irridated and bone marrow was transplanted to reconstitute the hematopoietic cells with an intravenous injection of two million cells from the bone marrow of donor LacZ mice, which express the LacZ gene under a Rosa-promoter in all cells. Mice were allowed to reconstitute for two or four months and peripheral blood was analyzed for the presence of β-galactosidase by flow cytometry (data not shown). Ligation surgery was performed and mice were treated with vehicle or P1pal-13 in an effort to induce robust intimal hyperplasia as previously observed. Mice were perfused 21 days post-ligation and arteries were incubated with a X-gal solution overnight to detect cells expressing

β-galctosidase. Arteries were then fixed or frozen for histological examination. In all experiments, intima formation was not observed and the majority of LacZ positive cells were marginating the lumen of the vessel and most likely inflammatory or blood cells (See Figure 2.10b). Concurrent experiments using $PAR1^{-/-}$ bone marrow in wild-type mice were also performed, but again intimal thickening was not observed and therefore no conclusion of the origin of these cells could be drawn from these experiments. Most likely, this was due to an unexpected suppressive effect of the lethal irradation.

As a second approach to test our hypothesis of the contribution of bone marrow cells to the neointima, ligations were performed and mice were treated for the first 7 days with a pepducin antagonist to CXCR4, termed PZ-218. In adult animals, hematopoietic stem cells are retained in the bone marrow niche and the chemotactic response and retainment of these cells are dependent on signaling of CXCR4 and its ligand SDF-1α [(203)]. Both agonists and antagonists of CXCR4 can cause mobilization of these stem/progenitor cells to the peripheral blood [(204)]. Injury, such as the case with ischemia, has been shown to recruit stem and progenitor cells via hypoxia-inducible factor-1 induction of SDF-1α to the damaged tissue [(205)]. We observed a massive increase in intimal hyperplasia in mice treated with CXCR4 antagonist, PZ-218 (Figure 2.10c). The neointima was similar in appearance to the hyperplasia observed in response to P1pal-13 treatment and in knockout mice. These data suggest that it is plausible that these intimal cells are derived from progenitors of the bone marrow niche, but does not exclude that intimal cells could be from the local artery.

Figure 2.10:

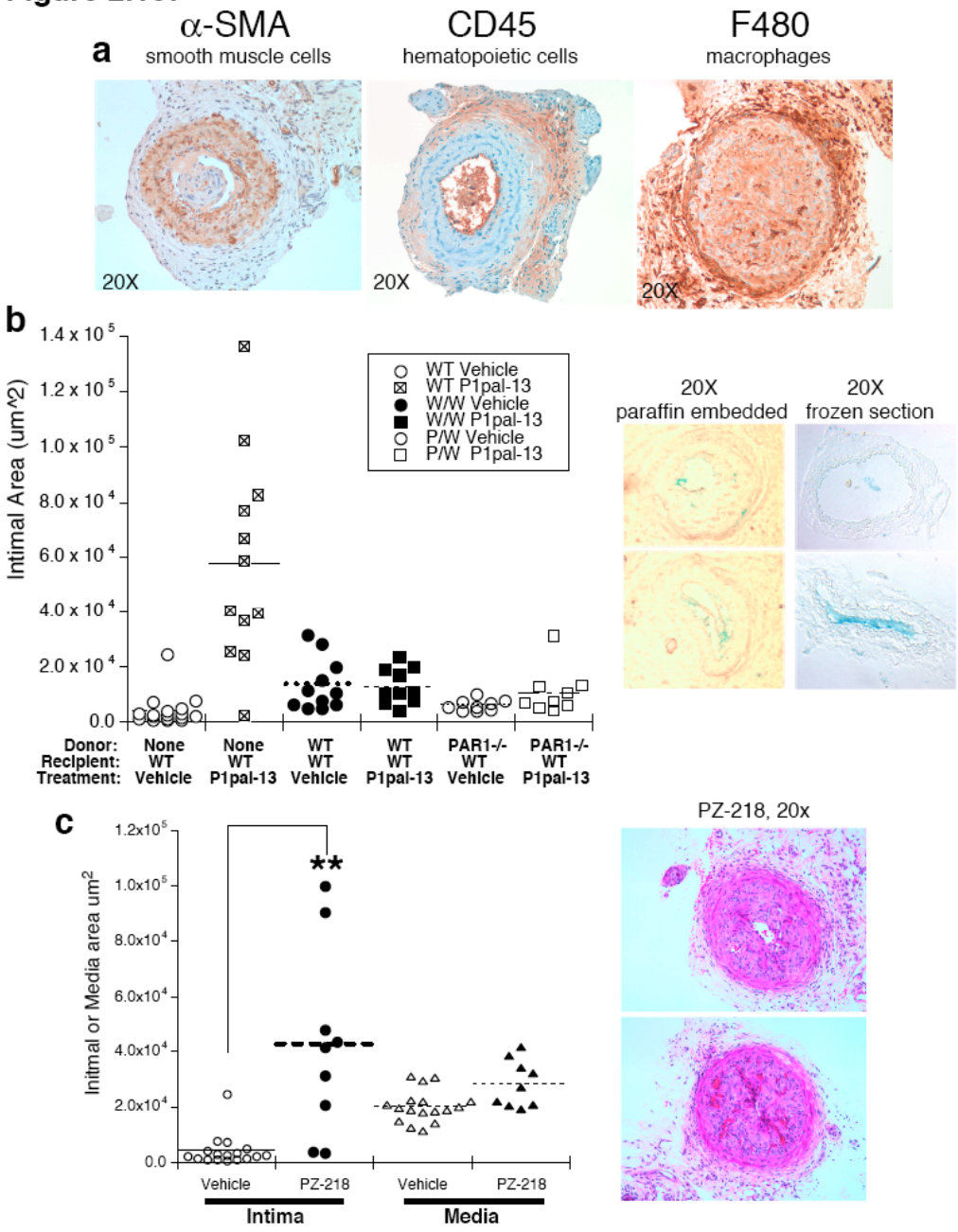

Figure 2.10. Investigating the origin of the cells of intimal hyperplasia.
(**a**) Immunohistochemistry was performed on cross-sections of injured arteries from experiments in Figure 2.5 for smooth muscle cells with anti-αSMC, hematopoietic cells using anti-CD45, or macrophages using anti-F480. (**b**) Bone marrow transplantation was performed using wild-type mice as recipients and either LacZ or PAR1$^{-/-}$ mice as donors. Mice then underwent ligation surgery of the carotid artery and were allowed to recover for 21 days while being treated with P1pal-13 or vehicle. Intimal hyperplasia was not observed in engrafted animals and LacZ staining appeared to be of hematopoietic origin (right). (**c**) C57BL/6 wild-type mice underwent ligation surgery and were allowed to recover for 21 days while being treated with CXCR4 antagonist, PZ-218 or vehicle. PZ-218 significantly increased intimal area of the injured arteries. Representative H&E cross-sections show the intimal hyperplasia (right). **, P <0.005.

2.11 Discussion.

In this study we demonstrate that PAR1 is closely associated with PAR2 and that the two receptors cooperatively signal in primary smooth muscle cells *in vitro* and in response to vascular injury *in vivo*. PAR1, PAR2, and PAR4 are expressed on the surface of SMCs and $G\alpha_q$ signaling, as observed by calcium mobilization, can be induced by PAR1 stimulation. Proliferation of SMCs is increased by the addition of PAR1 agonists, and in the absence of the PAR1 or PAR2 receptors proliferation rates in response to P1pal-13 are diminished, suggesting that signaling may be cooperative between these receptors. Consistent with previous reports of PAR1/PAR2 association and transactivation [85,86], we observed PAR1 and PAR2 association by coimmunoprecipitation indicating that PAR1 and PAR2 form a dimer.

It has been reported that PAR1 and PAR2 have both shared and separate downstream signaling which suggests that they may work both co-dependently and independently of one another [206]. Our study reveals that activation of PAR1 in vascular injury can be dependent on the presence of PAR2 since the intimal hyperplasia observed in wild-type mice treated with PAR1 agonist, P1pal-13, was not observed in $PAR1^{-/-}$ or $PAR2^{-/-}$ mice. Previous studies have shown that P1pal-13 is a specific agonist of PAR1 and does not activate PAR2, PAR4, CXCR1/2/4, S1P1/3, or CCR5 [86]. This suggests that PAR2 is not stimulated by P1pal-13 and that the lack of hyperplasia in $PAR2^{-/-}$ mice is due to the loss of the cooperative signal between PAR1 and PAR2.

Cooperative signaling between PAR1 and PAR2 *in vivo* has been demonstrated in a model of sepsis, where the protective effect of PAR1 activity was not observed without expression of PAR2 in endothelial permeability *in vitro* and survival and vascular

permeability of the lungs *in vivo* [86]. The protective effect of PAR2 in a model of inflammation has also been observed in resolving bacteria mediated inflammation in pulmonary pseudomonal infection [177]. However, models of acute inflammation [174], neurogenic pain [190], and arthritis [181] have all shown the damaging effects of PAR2 activation. These contradicting observations could be due to signaling differences between PAR1/PAR2 heterodimers and PAR2 homodimers (or monomers) [86]. The observation that PAR1 and PAR2 have been linked to overlapping G protein signaling as well as distinct subsets of Gα proteins suggests that signaling regulation may be a product of which receptor or dimer is being activated, giving the cell signaling discrimination [206].

We observe that $PAR1^{-/-}$ and $PAR2^{-/-}$ mice have increased intimal hyperplasia in response to ligation injury compared to wild-type mice. Possible explanations for this increase in intimal area are that the receptors are important for resolving inflammation in the later chronic stages of disease or possibly PAR1 and PAR2 are necessary for response to environmental signals that instruct the cells to differentiate out of the synthetic state of vessel repair and regeneration. PAR1 and PAR2 are thought to be important in vascular remodeling, and both receptors may need to be present to mediate decreased proliferation and differentiation to a contractile SMC state. More studies would need to be performed to test these hypotheses.

Previous studies have shown that PAR1 deficiency and PAR2 deficiency in mice have a non-significant protective effect in models of restenosis, more specifically angioplasty and wire injury [156,92,207]. It is important to call attention to the fact that these models are very different from ligation injury with angioplasty and wire injury

completely denuding the endothelium, with damage occurring mostly on the luminal side of the vessel. Ligation injury is a blood cessation and perivascular inflammatory injury, which brings together the components of massive inflammation in the blood vessel as well as obstruction of blood flow.

The ability of PAR1 activation to cause de-differentiation of smooth muscle cells is important in delineating the molecular events that take place in the smooth muscle cells of the artery. Ligation injury decreased the expression of differentiation markers α-SMA, SM22, and SM-MHC while the de-differentiation markers PDGF and Type III collagen were increased indicating a more synthetic SMC that can proliferate and migrate much more robustly [199]. In the already injured artery, P1pal-13 treatment further increased the expression of de-differentiated markers Type III collagen and MMP-3 explaining the exacerbated injury by SMC hyperplasia, even to the point of vessel occlusion. The role of PAR1 in de-differentiation may be necessary for vascular repair. However, here we show that this signaling can be detrimental to an injured vessel if not properly regulated.

In conclusion, PAR1 and PAR2 are essential in regulating intimal hyperplasia in our model of vascular ligation injury and perivascular inflammation. Increasing PAR1 signaling can cause smooth muscle cells to de-differentiate and proliferate to create a neointima. However, for PAR1 stimulation to cause intimal hyperplasia PAR2 must be present, suggesting transactivation and/or dimerization of PAR1 and PAR2.

Chapter 3: PAR2 pepducin antagonists

Summary

PAR2, a cell surface receptor for trypsin-like proteases, plays a key role in a number of acute and chronic inflammatory diseases of the joints, lungs, brain, gastrointestinal tract, and the vascular system. Despite considerable effort by the pharmaceutical industry, PAR2 has proven recalcitrant to targeting by small molecule inhibitors, which have been unable to effectively prevent the interaction of the protease-generated tethered ligand with the body of the receptor. Here, we report the development of a first-in-class cell-penetrating lipopeptide pepducin antagonist of PAR2. The design of the third intracellular (i3)-loop pepducins was based on a structural model of a PAR2 dimer and by mutating key pharmacophores in the receptor loops and analogous pepducins. Individual pharmacophores were identified that controlled constitutive, agonist and antagonist activities. This approach culminated in the identification of the P2pal-18S pepducin, which completely suppressed trypsin and mast cell tryptase signaling through PAR2 in neutrophils and colon cancer cells. The PAR2 pepducin was highly efficacious in blocking PAR2-dependent inflammatory responses in mouse models. These effects were lost in PAR2-deficient mice, thereby validating the specificity of the pepducin *in vivo*. These data provide proof-of-concept that PAR2 pepducin antagonists may afford effective treatments of potentially debilitating inflammatory diseases and provide a blueprint for developing highly potent and specific i3-loop based pepducins.

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Interdicting PAR2-driven inflammation with cell-penetrating pepducins

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3.1 Introduction.

Protease-activated receptor-2 (PAR2) mediates a number of (patho)physiological pathways involved in acute inflammation, arthritis, allergic reactions, sepsis, inflammatory pain, as well as cancer cell invasion and metastasis [174,171,190,34,112,195]. PAR2 is a member of the G protein-coupled protease-activated receptor subfamily, which includes the related PAR1, PAR3 and PAR4. Proteases such as trypsin [10], thrombin [8], and MMP-1 [27,208] cleave the N-terminal extracellular domain of the PARs, thereby unmasking a tethered ligand that binds to the outer surface of the receptor in an intramolecular mode, to activate intracellular signaling to G proteins [18]. The pleiotropic downstream pathways activated by PAR2 include calcium mobilization, phospholipase C-β-dependent accumulation of inositol phosphates and diacylglycerol, chemotaxis, MAP-kinase signaling, gene transcription, and Rho and Rac activation [4]. PAR2 itself is strongly upregulated by diverse inflammatory mediators such as TNF-α, IL-1α, bacterial endotoxin, and autoimmunity-inducing antigens, pointing to a role in the tissue response to inflammation [130,209].

As a cell surface sensor of proteases, PAR2 allows the cell to respond or over-respond to the rapidly changing microenvironment that occurs during inflammation. PAR2 is widely expressed in inflammatory cells, stroma, endothelium and intestinal epithelium [4]. PAR2-deficient mice exhibit reduced granulocytic infiltration and tissue damage, and suppression of inflammatory cytokines in models of intestinal inflammation, autoimmunity, and encephalomyelitis [210,209]. Reduced cardiac ischemia/reperfusion injury was also observed in PAR2 knockout mice, which correlated with a decline in inflammatory mediators [148]. Conversely, overstimulation of PAR2 leads to severe

edema, granulocyte infiltration, increased tissue permeability, tissue damage and hypotension [174,210]. Agonists of PAR2 including trypsin and the synthetic SLIGRL peptide also trigger the release of calcitonin and substance P from sensory neurons causing neutrophil infiltration, edema, hyperalgesia, and cancer pain [189,211,193]. PAR2 has been linked to arthritis as evidenced by significant decrease in joint inflammation in PAR2 knockout mice [181] and upregulated expression of the receptor in osteoarthritis and rheumatoid arthritis patient tissues [179,180,182].

A major pro-inflammatory serine protease, tryptase can also cleave and activate PAR2 [212]. Local or systemic release of high levels of mast cell-derived tryptase can have life-threatening consequences including acute asthma, systemic mastocytosis, and anaphylaxis [213]. A specific and effective pharmacological inhibitor of PAR2 has the potential to provide beneficial anti-inflammatory effects and reduce the detrimental activity of mast cells, neutrophils, and other PAR2-expressing leukocytes that contribute to tissue damage. To date, it has been challenging to identify an effective PAR2 antagonist that lacks agonist activity or is efficacious at sub-millimolar levels [35,34,33].

We have developed a novel way to inhibit G-protein coupled receptors on the inside surface of the lipid bilayer with cell-penetrating pepducins. Pepducins are derived from the sequence of the intracellular loops of their target receptor and comprise a lipid tether conjugated to the peptidic portion of an intracellular loop [33]. These lipidated peptides have the ability to rapidly flip across the membrane and inhibit receptor G protein signaling in a highly specific manner [33,86,140,214]. Pepducins targeting several GPCRs have been validated in models of inflammation, thrombosis, angiogenesis, and cancer [195,86,208,32,87,106,27,214].

In this chapter, we report the rational design and development of a potent and specific PAR2 antagonist pepducin based on the third intracellular loop, which effectively inhibits protease-PAR2 signaling induced by trypsin and tryptase. We demonstrate that the PAR2 pepducin provides salutary effects in reducing inflammation and edema. These data indicate that targeting PAR2 with pepducins may prove to be beneficial in inflammatory conditions and other diseases, which involve trypsin, tryptase and other protease agonists of PAR2.

3.2 MATERIALS & METHODS

Reagents

N-palmitoylated peptides and PAR1, PAR2, and PAR4 peptide agonists were synthesized with C-terminal amides by standard Fmoc solid-phase methods as previously described [32] by the Tufts University Core Facility. Pepducins were purified by C4- or C18-reverse-phase liquid chromatography. Trypsin, bovine pancreas and human mast cell tryptase (Calbiochem), recombinant IL-8 (Peprotech), thrombin (Haematologic Technologies Inc), myo-[^{3}H]-inositol (PerkinElmer), fura-2, AM and fluorescein goat anti-rabbit (Invitrogen), λ-Carrageenan, kaolin, and 48/80 (Sigma-Aldrich), mouse monoclonal (AA1) to Mast Cell Tryptase (abcam, ab2378), APC-366 (Tocris), Bovine Type II Collagen Solution (Chondrex), Freund's adjuvant (Sigma).

PAR2 Modeling

The model of the PAR2 monomer structure was constructed using the program Modeller using the solved structure of bovine rhodopsin (PDB code 1HZX) [215] as a template. The chemical geometry of the model was refined using Coot and Refmac5. To construct a model of the receptor dimer, we first constructed an initial model of the complex between PAR2 and the intact heterotrimeric G-protein based on the structure of opsin in complex with a C-terminal fragment of the Gα subunit (PDB code 3DQB) and the structure of the heterotrimeric G-protein transducin (PDB code 1GOT). We then constructed a two-fold symmetric model of the PAR2 receptor that permitted favorable contacts between the receptor monomers and positioned the G-protein such that regions of G-βγ known to interact with the receptor were in proximity to the second molecule of PAR2. Models

were refined using Coot and the molecular graphics figures were generated using PYMOL.

Cell culture

SW620 cells, a metastatic PAR2 expressing colorectal adenocarcinoma, were purchased from ATCC. The SW620 cells stably expressing PAR1 (SW620-PAR1) were generated in our lab as previously described [87]. *Cercopithecus aethiops* kidney cells (COS7) and human embryonic kidney cells (HEK 293T) were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin in 5% CO_2 at 37 °C. Human mast cells (HMC-1; Mayo Clinic, Rochester, MN) were maintained in IMDM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1.2 mM α−thioglycerol.

PAR cell surface expression

SW620 cells were harvested by lifting with 3 mM EDTA/PBS. Neutrophils were isolated as described [216]. Cells were labeled with the PAR1 polyclonal Ab [103] (SFLLRNPNDKYEPFC), the PAR2 polyclonal Ab [86] (SLIGKVDGTSHVTGKGVC), or the PAR4 polyclonal Ab [103] (GYPGQVSANDSDTLELPC) and subsequently with FITC-labeled secondary Ab. PAR expression was quantified by mean fluorescent intensity relative to isotype control on the BD Canto II flow cytometer and results were analyzed using FlowJo.

Transwell Migration

Chemotactic migration assays were conducted using an 8 μm pore size Transwell apparatus (Corning) as described previously [103]. Briefly, HEK 293T or SW620 cells were placed in the upper chamber and allowed to migrate toward agonists in the lower chamber of the apparatus. Migration was performed for 18 hours at 37 °C. Membranes were fixed, stained, and migrated cells were counted.

Paw Edema

All animal experiments were performed in accordance with the US National Institutes for Health guidelines and approved by Tufts Medical Center Institutional Animal Care and Use Committee. Eight-week-old C57BL/6 mice from Charles River Laboratories or age-matched PAR2 knockout mice in a C57BL/6 background were subcutaneously injected with vehicle (20% DMSO/80% water), P2pal-18S (10 mg/kg), or APC-366 (5 mg/kg). Mice were then challenged with a 30 μL intraplantar injection of the hindpaw with 2% λ-carrageenan and 4% kaolin, 100 μg SLIGRL, or λ-carrageenan/kaolin stimulated conditioned media from HMC-1 cells. Calipers were used to measure paw width and thickness to calculate area at indicated times and mice were sacrificed 48 hours post-injection.

Mast cell depletion

Eight-week-old C57BL/6 female mice were given 100 μL intraperitoneal injections of compound 48/80 dissolved in PBS (vehicle) according to the following schedule: 1st day,

0.6 mg/kg; 2nd day, 1.2 mg/kg; 3rd day, 1.2 mg/kg; 4th day, 2.4 mg/kg. Paw edema was performed 24 h after the last dose.

Neutrophil chemotaxis

Neutrophils were obtained from the peripheral blood of healthy volunteers as described [216], using informed consent procedures approved by the Institutional Review Board of the Tufts University School of Medicine. Mouse neutrophils were purified from blood collected from the vena cava. We measured migration using a 48-blindwell microchemotaxis chamber (Neuroprobe) equipped with 5 μm pore nitrocellulose filters for mouse and human neutrophils. Chemotactic ratio represents the distance of migration to a chemoattractant compared to the distance of random migration.

Cecal Ligation and Puncture

Eight to twelve-week-old C57BL/6 female mice were anesthetized with isoflurane and aseptically surgically prepared. A midline incision was made to expose the abdomen and the cecum was isolated. A 4-0 silk suture was placed around the cecum and synched, though not completely, as not to obstruct the bowel. An 18-gauge needle was then used to puncture the cecum once through both walls and forceps were used to compress the cecum until stool was visible. The abdomen wall was closed with 6-0 non-absorbable sutures, closing the muscle first with non-continuous stitches and subsequently the skin with a continuous stitch. Mice were treated immediately with 10 mg/kg of P2pal-18S or vehicle (20% DMSO) and monitored for survival. Surviving mice were treated daily.

Arterial Ligation

Arterial ligation was performed as described in **Section 2.2**. Briefly, six-month old C57BL/6 female mice were anesthetized and the left carotid artery was ligated with a 6-0 silk suture placed around the common carotid and synched to completely restrict blood flow. Pepducins (P2pal-18S at 10 mg/kg) or vehicle (20% DMSO) was administered subcutaneously on a daily basis. After 21 days, mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (90-120 mg/kg, 10 mg/kg) and perfused as previously described. Left and right common carotid arteries were harvested and further fixed in 10% formalin. Samples were vertically embedded and slides were stained with H&E. Morphometric analysis was performed as described in Section 2.2.

Collagen-Induced Arthritis

Eight-week-old DBA/1J female mice were subcutaneously pretreated with vehicle (20% DMSO), P2pal-14GF (10 mg/kg, a pepducin to PAR2 that showed no agonist or antagonist ability in a calcium flux assay), or P2pal-18S (10 mg/kg). Mice were challenged with an intraperitoneal injection of 100 μg of Type II collagen in Freund's Complete Adjuvant. Paw measurements were taken two-three times a week and mice were followed for 70 days post challenge. A booster immunization was given 7 d and 14 d after the first collagen injection.

Statistical analysis

All *in vitro* and *in vivo* assay results are presented as mean ± SD or ± SEM, as indicated. Comparisons were made with a two-tailed distribution unequal variance Student's *t* test. Statistical significance was defined as * $p < 0.05$ or ** $p < 0.005$.

RESULTS

3.3 Identification of critical pharmacophores in the intracellular i3 loop of PAR2

As has been shown with many GPCRs [217], there is considerable evidence that PARs can interact with each other and form homo- and hetero-dimers, and pepducins are postulated to mimic these dimeric interactions on the intracellular surface of the lipid bilayer [87]. To initiate the design of PAR2 pepducin antagonists, we constructed a molecular model of a PAR2 dimer. The model of the PAR2 monomer was based on the structure of rhodopsin [215] which shares 45% identity with PAR2. Residues 51-397 of PAR2 were substituted for residues 1–346 of bovine rhodopsin. Stereochemically reasonable positions were used for side chains that were later subjected to molecular dynamics and energy minimization. A series of two-fold symmetric PAR2 dimer models were then manually constructed with the aim of maximizing the surface area between the adjacent receptors. Among these, we selected the model that was most consistent with available data regarding interactions between the receptor pair and the G-protein (see methods, **Figure 3.1**).

To provide functional evidence that one PAR2 receptor can interact with an adjacent PAR2 receptor, we constructed a signaling dead PAR2-RQ mutant by transposing residues at Q172 and R173 located in the critical 'DRY' TM3 motif which is highly conserved in GPCRs (**Figure 3.2**) [218]. The PAR2-RQ mutant has an intact protease cleavage site and tethered ligand but cannot signal to G proteins. A noncleavable PAR2-R36A mutant [219] was also constructed which retains the ability to fully signal in response to SLIGRL ligand, but was not able to be proteolyzed and directly activated by trypsin (**Figure 3.3**). As expected, cells expressing PAR2-R36A or

PAR2-RQ alone were unable to migrate toward gradients of trypsin or to activate $G\alpha_q$-PLC-β signaling. However, when the two mutant receptors were co-transfected, chemotactic migration and signaling was restored (Figure 3.3a,c), consistent with a mechanism whereby PAR2-RQ can donate its tethered ligand to transactivate PAR2-R36A.

Figure 3.1:

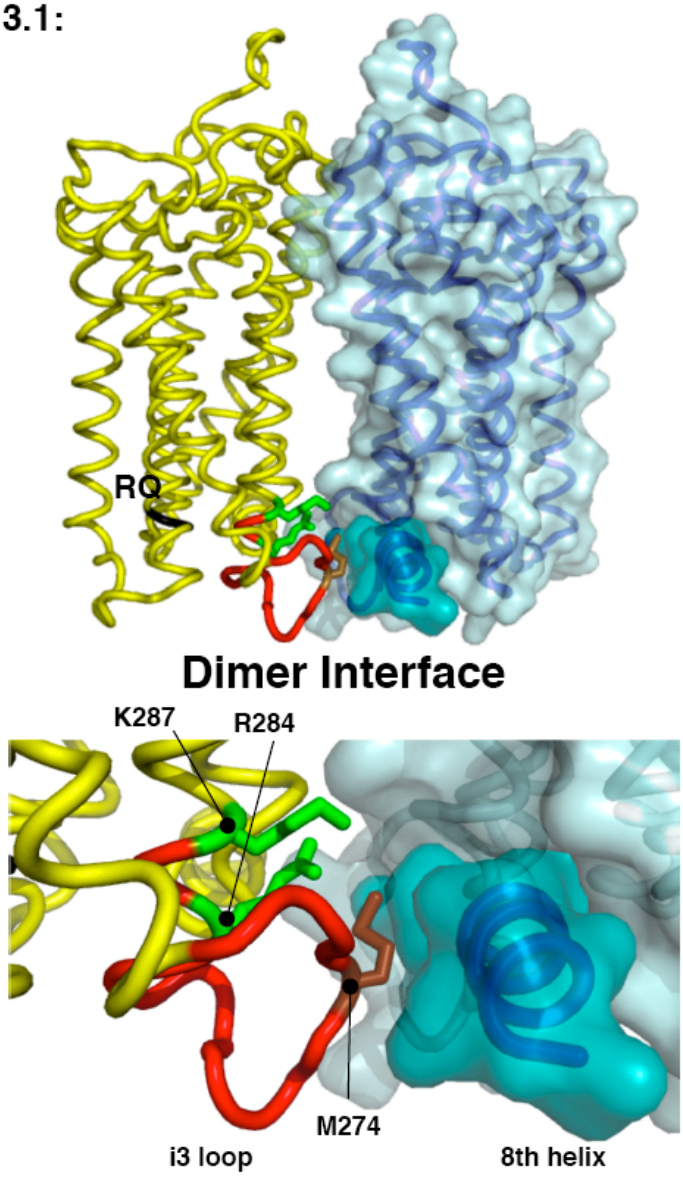


Figure 3.1. PAR2 model of the receptor dimer (done in collaboration with Dr. Andrew Bohm). The PAR2 model was constructed using x-ray structures of rhodopsin as described in the methods. This model predicts that the intracellular 3rd loop and the 8th helix reside at the dimer interface.

Figure 3.2:

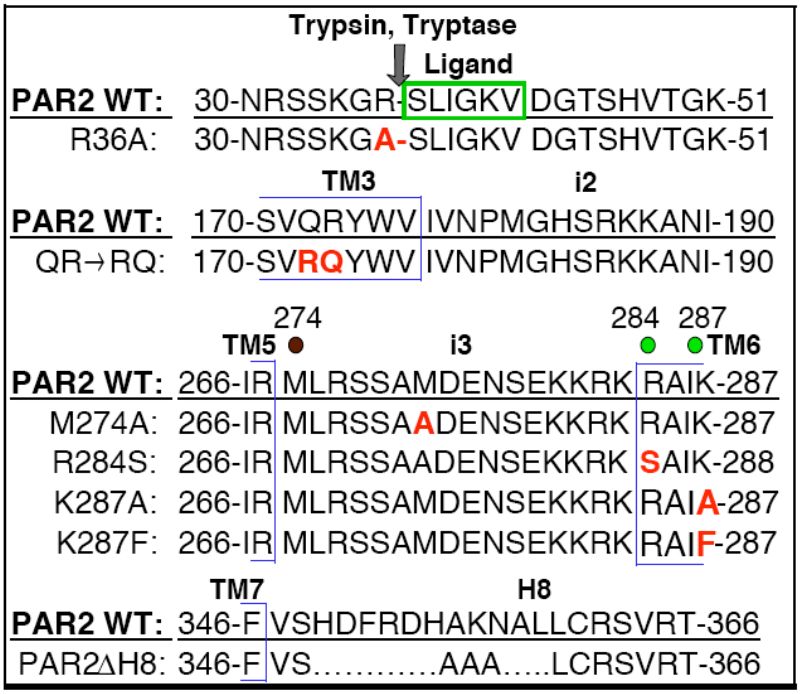


Figure 3.2. Sequence of PAR2 cleavage mutant, PAR2 activity mutant, and PAR2 key pharmacophores as predicted by the dimer model. Serine proteases trypsin and tryptase cleave the C-terminal side of extracellular residue R36 releasing the tethered ligand, SLIGKV—, which activates PAR2 either in an intramolecular mode or by transactivation of an adjacent PAR2. PAR2 key residues are predicted to reside in the intracellular 3rd loop and the 8th helix of the receptor.

Interestingly, we also found that wild-type PAR2 has constitutive activity in the absence of ligand (Figure 3.3e). Constitutive signaling has been observed in GPCRs and is often dependent on critical residues located in the C-terminal juxtamembrane region of the i3 loop [220,221]. The PAR2 homodimer model shown in Figure 3.1, predicts that the juxtamembrane i3 loop residues M274, R284, and K287 could potentially interact across the PAR2 dimer interface with the 8th helix region from an adjacent i4 domain. Mutation

Figure 3.3:

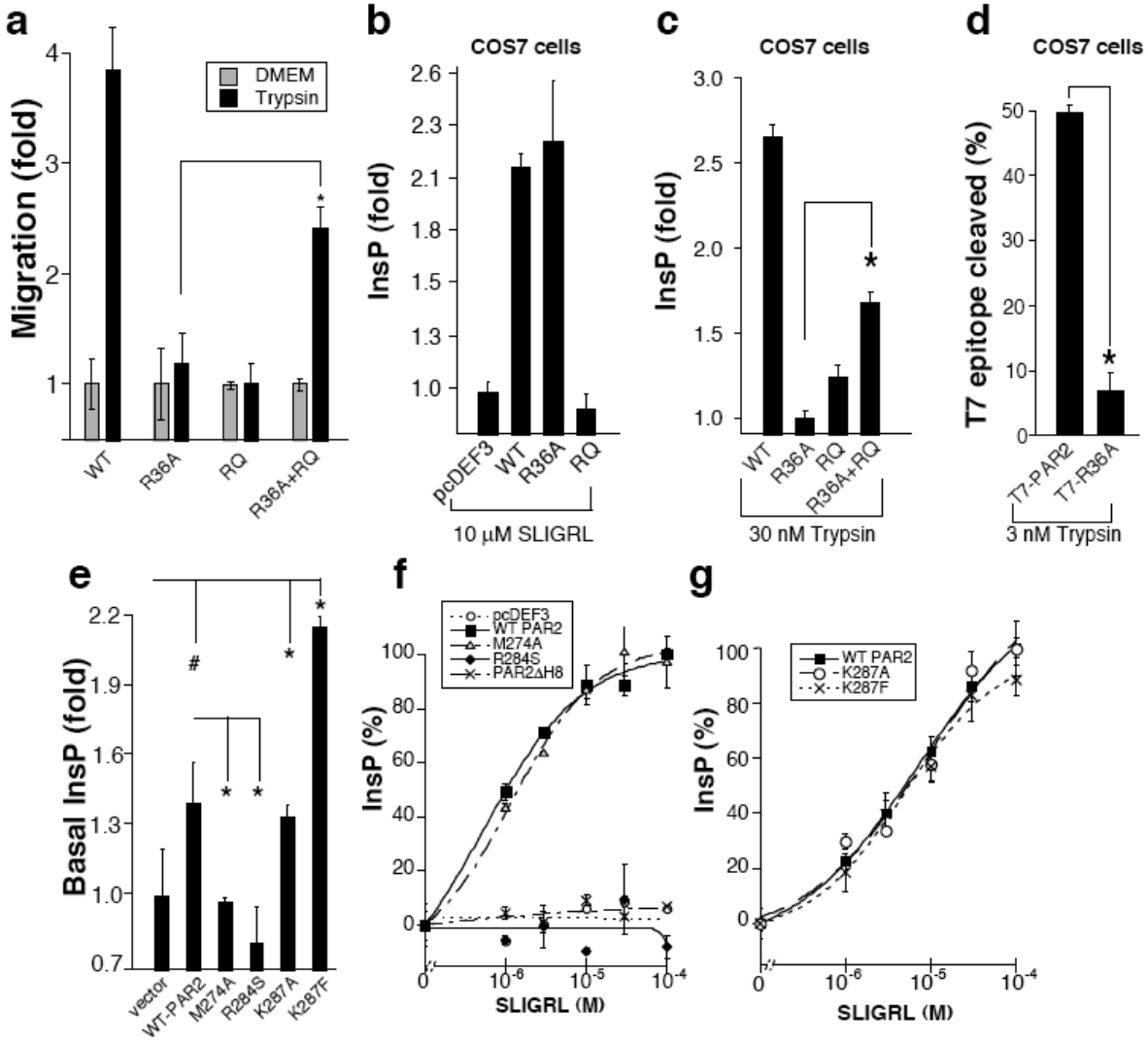


Figure 3.3. Constitutive and peptide ligand-dependent activity of PAR2 is regulated by juxtamembrane residues located in the third intracellular (i3) loop.
(**a**) Migration of HEK cells transiently transfected with PAR2 (wild-type, WT), PAR2-R36A, PAR2-RQ, or PAR2-R36A plus PAR2-RQ toward chemotactic gradients of 30 nM trypsin or DMEM media alone for 18 h in a transwell apparatus (8-μm pore), n=2, repeated three independent times.(**b,c,e,f,g**) Constitutive, SLIGRL-activated, or trypsin-activated signaling of PAR2 mutants to PLC-β. PLC-β activity was measured by [^{3}H]-InsP formation over 30 min. (**d**) T7 cleavage assay measured by flow cytometry of COS7 cells transiently transfected and subsequently incubated with trypsin (3 nM) confirms by loss of the T7 epitope that R36A has significantly less cleavage than wild-type PAR2. Data (n=2-8) represent the mean ± SD. *, P <0.05 and $^{\#}$, P=0.07.

of M274 to alanine ablated the constitutive signal, whereas mutation of K287 to alanine had no effect (Figure 3.3e). Strikingly, mutation of K287 to phenylalanine gave a ≥2-fold increase in the constitutive signal. Despite losing its constitutive activity, M274A was able to fully signal in response to agonist (Figure 3.3f). Likewise, K287A and K287F were also able to fully signal to agonist (Figure 3.3g). Conversely, the R284S mutant exhibited a loss of constitutive signal and was unable to be activated by even high concentrations of peptide ligand. Similarly, the PAR2ΔH8 mutant, which lacks the 8th helix in the i4 domain, was non-responsive to ligand (Figure 3.3f). Together, these data indicate that the juxtamembrane residues of the i3 loop of PAR2 play critical roles in both constitutive and ligand-dependent activity. We decided to further examine these residues for their functions as pharmacophores in the context of PAR2 i3 loop pepducins.

3.4 Receptor Mutagenesis Studies

In an attempt to learn which residues were important as well as which surfaces of the receptor interact in homodimerization, multiple mutations were made in different orientations of the PAR2 dimer model, termed FACE, BACK, and SIDE, as well as mutations in the C-terminal end (8th helix) and the intracellular loops that should change activity of the mutant by disrupting the interactions with the G protein (**Figure 3.4**). Initial observations of $G\alpha_q$ signaling of the mutants were made in COS7 fibroblasts by measuring the phosphoinositide hydrolysis. As depicted in **Table 3.1**, many of these mutants drastically decreased the maximal signal observed as well as the amount of agonist needed to stimulate the receptor (EC_{50} shift). However, no pattern of diminished activity was observed that revealed a possible orientation of the dimer and additionally

many mutations also had effects on the expression of the receptor to the cell surface, as assessed by flow cytometry, making it hard to draw any noteworthy conclusions from these experiments.

Figure 3.4:

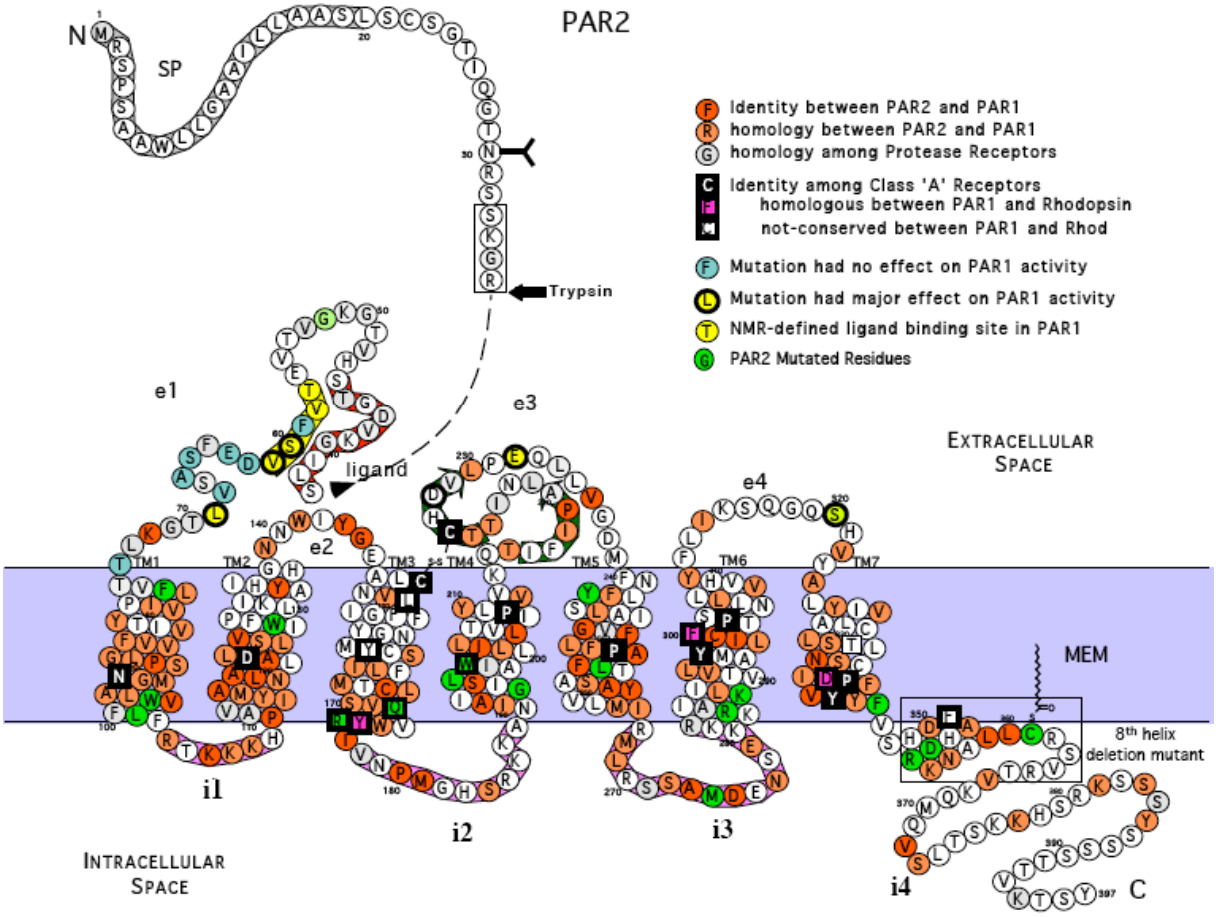


Figure 3.4. PAR2 domains mutational diagram. PAR2 mutants were made in each transmembrane by Quikchange site-directed mutagenesis (mutants in green). The results of each mutation on PAR2 activity can be observed in Table 3.1.

Table 3.1:
Effect of mutations on PAR2 activation of Gq-PLC-β signaling in COS7 fibroblasts.
COS7 fibroblasts were transiently transfected with PAR2 mutants and Gq activation was measured by the accumulation of inositol phosphate. Activation took place over 30 min following the addition of 10^{-9}-10^{-7} trypsin or 10^{-6} to 10^{-4} SLIGRL.

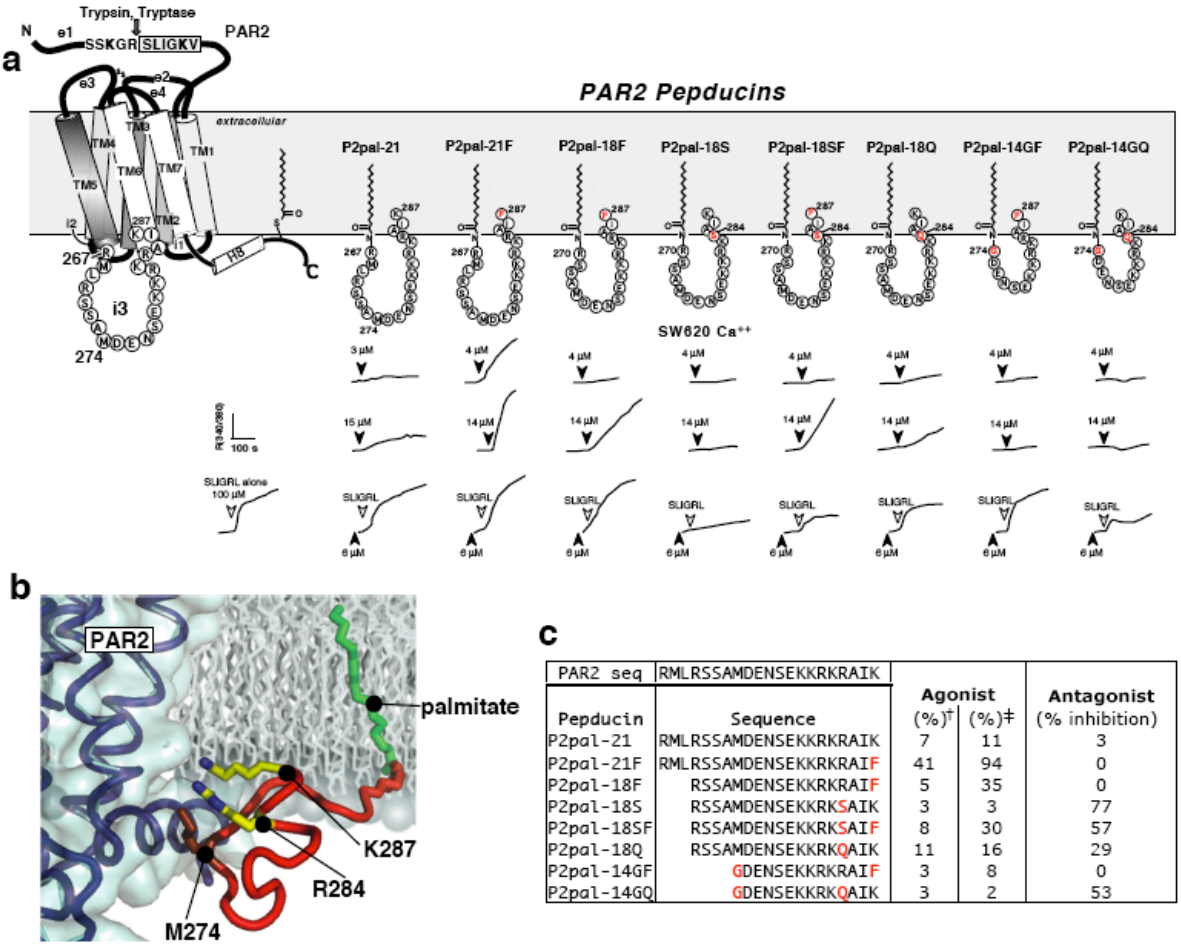
		SLIGRL		
mutation	Location of Mutation	EC_{50} shift	Maximal Signal	Receptor Expression
WT		1.0	1.0	1.0
Activity mutants:				
Q172R/R172Q	TM3-i2	3.4	0.1	0.7
Q172D	TM3-i2	2.6	0.0	0.5
Q172V	TM3-i2	1.1	0.3	0.6
M274A	i3	1.9	1.0	0.9
R284E	i3-TM6	1.8	0.2	0.7
R284S	i3-TM6	7.1	1.7	0.7
K287A	i3-TM6	1.0	1.0	1.4
K287F	i3-TM6	2.6	0.8	1.8
Face Mutants:				
F77A	TM1	2.6	1.4	0.5
F346A	TM7	1.7	2.0	0.5
Back Mutants:				
W98A/L101A	TM1	1.6	0.4	0.7
L101D	TM1	11.1	0.0	0.7
W127A	TM2	5.9	2.5	0.9
G193D	TM4	2.1	1.1	0.7
L196A/W199D	TM4	3.8	0.2	0.3
Side Mutants:				
Y242A	TM5	2.4	1.6	0.4
L257A	TM5	1.2	1.0	0.9
8^{th} Helix Mutants:				
C361S	8^{th} helix	1.2	0.5	0.4
R352A/D353A	8^{th} helix	1.2	0.8	0.9
PAR2ΔH8	8^{th} helix	14.3	0.1	0.2

3.5 Molecular engineering of PAR2 antagonist pepducins

We therefore continued with the knowledge of the juxtamembrane residues that were identified as important for the signaling of PAR2 in Section 3.3, by synthesizing a series of i3 loop-based pepducins with mutations of the critical M274, R284 or K287 pharmacophores (**Figure 3.5**). An initial screen of PAR2 pepducin agonist and antagonist activity was performed using the PAR2-expressing human colorectal adenocarcinoma cell line SW620. The wild-type full-length i3 loop pepducin, P2pal-21, gave a weak agonist signal but lacked antagonist activity against SLIGRL as assessed by calcium flux (Figure 3.5a,c). Consistent with the gain-of-constitutive activity observed in the PAR2-K287F mutant, the analogous P2pal-21F pepducin [33] gave full agonist activity but no antagonist activity in the SW620 cells (Figure 3.5a,c). Deletion of the first three residues to make P2pal-18F, gave a slight decrease in agonist activity, but still was devoid of antagonist activity.

The P2pal-18S pepducin, which replaces the critical R284 pharmacophore with serine, was a full antagonist of PAR2 and had no detectable agonist activity in the calcium flux assay (Figure 3.5a,c). Replacement of the C-terminal K287 of P2pal-18S to phenylalanine to make P2pal-18SF, restored agonist activity. The replacement of R284 with glutamine to make P2pal-18Q, had nearly no agonist activity but had partial antagonist activity. The N-terminally truncated P2pal-14GF had no agonist nor antagonist activity; however, substitution of R284 with glutamine to create P2pal-14GQ gained 53% antagonist activity (Figure 3.5a,c). From this initial calcium flux screen, we chose to further analyze the properties of the P2pal-18S antagonist pepducin for its ability to block other PAR2 functions.

Figure 3.5:



PAR2 seq	RMLRSSAMDENSEKKRKRAIK			
Pepducin	Sequence	Agonist (%)†	Agonist (%)‡	Antagonist (% inhibition)
P2pal-21	RMLRSSAMDENSEKKRKRAIK	7	11	3
P2pal-21F	RMLRSSAMDENSEKKRKRAIF	41	94	0
P2pal-18F	RSSAMDENSEKKRKRAIF	5	35	0
P2pal-18S	RSSAMDENSEKKRKSAIK	3	3	77
P2pal-18SF	RSSAMDENSEKKRKSAIF	8	30	57
P2pal-18Q	RSSAMDENSEKKRKQAIK	11	16	29
P2pal-14GF	GDENSEKKRKRAIF	3	8	0
P2pal-14GQ	GDENSEKKRKQAIK	3	2	53

Figure 3.5. Design and screening of agonist and antagonist PAR2 i3-loop pepducins. (**a**) Agonist and antagonist activity of third intracellular (i3) loop PAR2 pepducins using calcium flux assays with SW620 colon adenocarcinoma cells that endogenously express PAR2. Each column of three calcium flux traces corresponds to the i3 pepducin sequence shown above. The top row is the agonist activity of 3-4 µM pepducin and the middle row is the agonist activity of 14-15 µM pepducin. The bottom row depicts the calcium signal of 100 µM SLIGRL (open arrow) following 1 min pretreatment with 6 µM pepducin (closed arrow). Intracellular Ca^{2+} flux was measured using cells labeled with fura-2AM by the ratio of fluorescence excitation intensity at 340/380 nm. Final concentration of DMSO vehicle was 0.2%. (**b**) Model of the WT PAR2 i3 pepducin P2pal-21 bound to the intracellular surface of PAR2. The location of the i3 pepducin was derived by substituting the coordinates of the i3 loop on the intact receptor with the i3 pepducin using the PAR2 dimer model of Fig. 3.1. Key pharmacophores M274 (brown), R284 and K287 (yellow), and palmitate (green) are shown. (**c**) Agonist activity for each pepducin from **a** is reported as initial velocity of calcium flux at 4 µM pepducin (†), or at 14-15 µM pepducin (‡). Antagonist activity of 6 µM pepducin against 100 µM SLIGRL was measured by area under the curve of calcium flux from the bottom row of **a**. Experiments were repeated at least 2-3 times each and gave highly similar results.

3.6 P2pal-18S is a specific antagonist of PAR2 activity in neutrophils

Next, we tested the ability of P2pal-18S to antagonize PAR2-dependent activity of human neutrophils. Neutrophils were isolated from the peripheral blood of healthy volunteers and found to express high levels of surface PAR2 and PAR4 and lower apparent levels of PAR1 by flow cytometry (**Figure 3.6**). Neutrophils robustly migrated towards chemoattractant gradients of the PAR2 agonists trypsin and SLIGRL, which was completely blocked by P2pal-18S with IC_{50} values of 0.14-0.2 μM (Figure 3.6b). Likewise, 0.3 μM P2pal-18S completely blocked chemotactic migration of human neutrophils to 100 nM tryptase (Figure 3.6c). P2pal-18S also completely inhibited migration of mouse neutrophils toward 30 nM trypsin (Figure 3.6d). This was predicted, as human PAR2 shares 85% identity with mouse PAR2 and the mouse i3 loop retains all of the critical pharmacophores identified in the human PAR2 i3 loop.

Specificity of P2pal-18S for PAR2 was evident, as it had no antagonist activity to the closely related PAR1, PAR4, or CXCR1/2 IL-8 receptors in neutrophil chemotaxis assays (Figure 3.6e). P2pal-18S had no effect on PAR1-dependent platelet aggregation (**Figure 3.7**) and did not inhibit PAR1 or PAR4 by calcium flux assays in SW620 cells, nor inositol phosphate signaling in COS7 cells, despite providing effective inhibition to the PAR2 ligand SLIGRL (**Figure 3.8**). The P2pal-14GQ pepducin was also selective for PAR2 and not PAR1, but was not as efficacious in suppressing migration of SW620 cells to SLIGRL as compared to P2pal-18S (Figure 3.8a-d). Neither P2pal-18S nor P2pal-14GQ caused membrane disruption or apoptosis as assessed by propidium iodide uptake in SW620 cells with up to 30 μM concentrations of pepducin (**Figure 3.9**).

Figure 3.6:

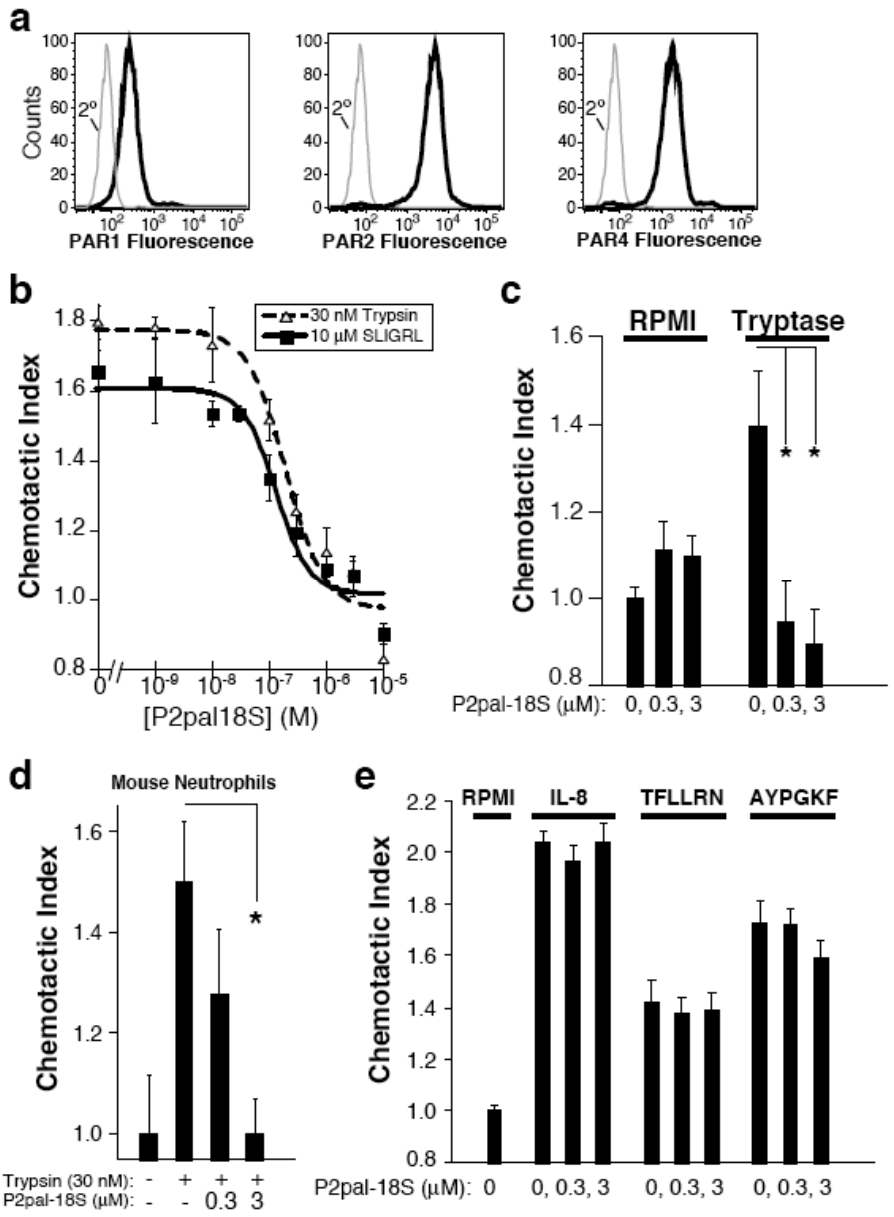


Figure 3.6. The P2pal-18S pepducin is a full antagonist of PAR2-dependent neutrophil chemotaxis. (**a**) Human neutrophils (n=4 normal volunteers) were analyzed for surface expression of human PAR1, PAR2, and PAR4 by flow cytometry with PAR-specific antibodies. (**b**) P2pal-18S inhibits human neutrophil chemotaxis to gradients of trypsin (30 nM) and SLIGRL (10 μM) with IC_{50} values of 0.14-0.2 μM. Chemotaxis index is the ratio of directed versus random migration over 30 min through a 5-μm pore filter. (**c**) P2pal-18S completely inhibits human neutrophil chemotaxis to tryptase (100 nM). (**d**) P2pal-18S completely inhibits mouse neutrophil (n=6) chemotaxis to 30 nM trypsin. (**e**) P2pal-18S does not affect human neutrophil chemotaxis to gradients of 100 nM IL-8 (CXCR1/CXCR2), 10 μM TFLLRN (PAR1), or 100 μM AYPGKF (PAR4). n=4-6, mean ± SEM. *, P <0.05.

Figure 3.7:

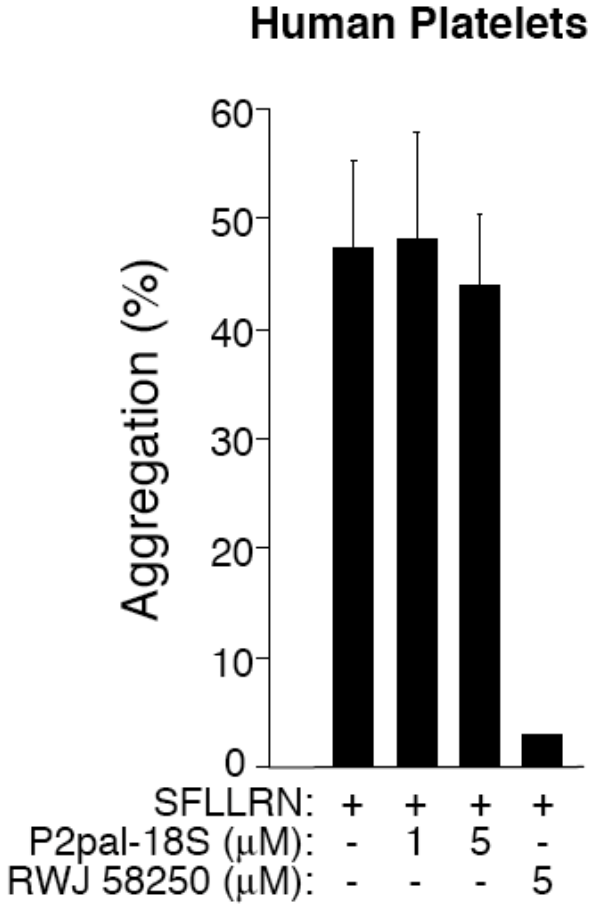


Figure 3.7. PAR1-dependent human platelet aggregation is not affected by P2pal-18S (done in collaboration with Dr. Ping Zhang). Gel-filtered platelets were incubated with 2.5 μM SFLLRN to induce PAR1-dependent platelet aggregation. Addition of 1 μM or 5 μM P2pal-18S did not affect the aggregation of the platelets. RWJ 58250, a small molecule PAR1 inhibitor, was used to show that specific blockade of PAR1 would in fact inhibit aggregation of the platelets. Error bars represent mean ± SD.

Figure 3.8:

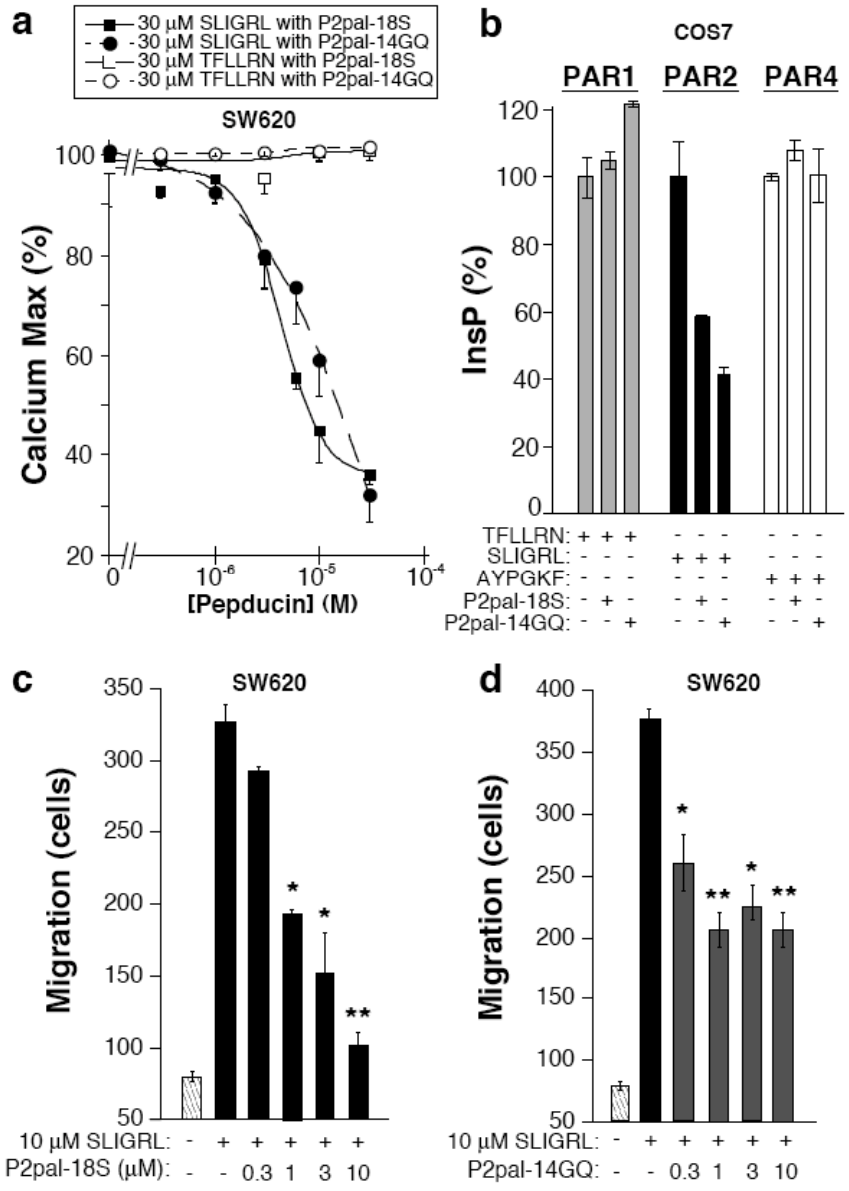


Figure 3.8. **Specificity of P2pal-18S and P2pal-14GQ pepducins for PAR2 but not the closely related PAR1 and PAR4 receptors.** (**a**) PAR2 antagonist pepducins P2pal-18S and P2pal-14GQ inhibit calcium flux induced by PAR2 (SLIGRL) but not PAR1 (TFLLRN) agonists. SW620 cells were stably transfected with PAR1 and pretreated for 1 min with various concentrations of P2pal-18S or P2pal-14GQ and then stimulated with 30 μM PAR agonist. (**b**) P2pal-18S and P2pal-14GQ do not inhibit InsP signaling from PAR1 or PAR4 transiently transfected into COS7 cells. Cells were stimulated with 1 μM TFLLRN, 1 μM SLIGRL or 100 μM AYPGKF and [^{3}H]-InsP formation measured over 30 min. (**c-d**) SW620 cells were treated with the indicated concentrations of (**c**) P2pal-18S or (**d**) P2pal-14GQ in the upper well of an 8-μm pore Boyden chamber and allowed to migrate for 18 h towards 10 μM of the PAR2 specific agonist SLIGRL in the lower chamber. The migration to RPMI / 0.1% FBS (—) represents random migration. Data (n=2-4) are mean ± SEM. *, P <0.05 and **, P <0.005.

Figure 3.9:

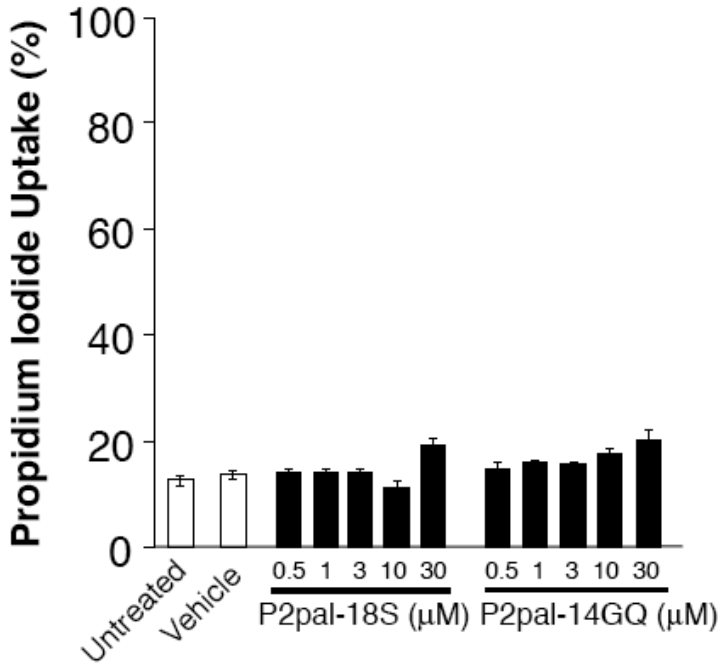


Figure 3.9. P2pal-18S and P2pal-14GQ do not induce apoptosis in SW620 cells. Cells were lifted and incubated for 30 min at 25 °C with 0.5-30 μM P2pal-18S or P2pal-14GQ. Propidium iodide (PI) was added directly to the tubes and after a 15 min incubation quantified by flow cytometry. PI uptake was not significantly different when pepducin was added to the cells as compared to untreated and 0.2% DMSO vehicle treated cells. Error bars represent ± SEM.

3.7 Efficacy of P2pal-18S in mouse models of inflammatory paw edema

To evaluate the *in vivo* efficacy and specificity of P2pal-18S, we first tested the ability of the pepducin to protect against inflammatory hindlimb paw edema using a well characterized mouse model in wild-type (WT) and PAR2-deficient mouse strains [63]. Acute inflammatory edema was induced by an intraplantar injection of λ-carrageenan and kaolin, irritants that cause a massive leukocytosis and hyperemic response, which leads to localized swelling [222]. We also directly assessed the PAR2-dependent activity of P2pal-18S by quantifying its inhibitory effects against the PAR2-specific agonist

SLIGRL when injected into the hind footpad of WT C57BL/6 mice. Acute inflammation induced by λ-carrageenan/kaolin resulted in a nearly 2-fold increase in paw edema with vehicle-treated WT mice, peaking 8 h after injection (**Figure 3.10**). The PAR2 agonist peptide, SLIGRL, also induced an increase in edema of WT mice, peaking 4 h after injection (Figure 3.10b). Systemic administration of P2pal-18S caused a significant 50% decrease in λ-carrageenan/kaolin-induced edema and an 85% decrease in SLIGRL-induced edema (Figure 3.10a,b). PAR2 deficiency conferred a 50% protective effect relative to WT mice following λ-carrageenan/kaolin injection, which was nearly identical to the protective effect observed in WT mice treated with P2pal-18S. Notably, treatment of $PAR2^{-/-}$ mice with P2pal-18S did not further reduce swelling confirming that the anti-inflammatory effects of the PAR2 pepducin required the presence of its cognate receptor.

Histological analysis of the inflamed footpads harvested 7 h post λ-carrageenan/kaolin injection revealed that P2pal-18S provided significant 60% protection ($P<0.005$) against the leukocytic infiltrates in the dermis of the footpads, which was identical to the protection observed in $PAR2^{-/-}$ relative to WT mice (Figure 3.10c). Together, these data demonstrate that the PAR2 pepducin P2pal-18S affords significant protection against acute leukocytic inflammation and edema, and these protective effects are dependent on the presence of PAR2.

Figure 3.10:

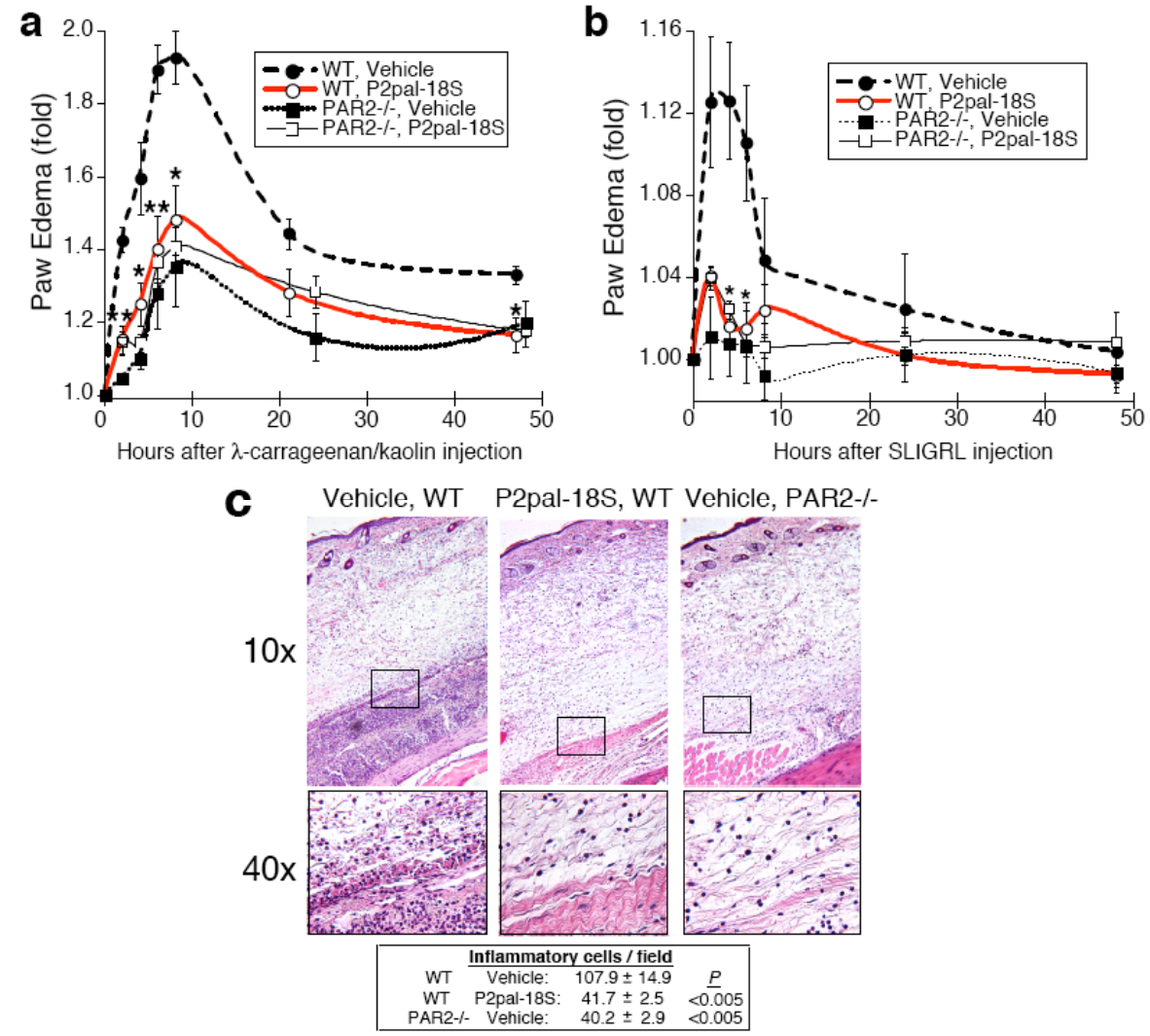


Figure 3.10. The PAR2 antagonist pepducin P2pal-18S significantly reduces mouse paw edema and inflammation in wild-type but not PAR2-deficient mice.
(**a**) λ-carrageenan/kaolin or (**b**) the PAR2 specific agonist SLIGRL was administered by intraplantar injection to the left hindpaw of C57BL/6 wild-type or PAR2$^{-/-}$ mice that were with subcutaneous (s.c.) injection of 10 mg/kg of P2pal-18S. Paw area was measured with calipers and calculated as fold-increase relative to baseline paw area.
(**c**) Histology of representative (n=5) H&E-stained footpads 7 h after λ-carrageenan/kaolin injection and quantification of the infiltrating inflammatory cells at 40X magnification. Data (n=4-6/group) mean ± SEM. *, P <0.05 and **, P <0.005.

3.8 P2pal-18S protects against mast cell tryptase-induced inflammation

Previous studies have established that mast cell tryptase cleaves and activates PAR2 signaling in human endothelium and keratinocytes and in mouse models of arthritis [212,183,34]. To determine whether mast cells and mast cell tryptase were contributing to the observed PAR2-dependent effects in the mouse models of paw inflammation, we stimulated mast cells with the degranulating agent 48/80, or λ-carrageenan/kaolin and collected conditioned media. As shown in Figure 3.11a, the stimulated mast cells secreted tryptase, which was then used as a chemoattractant source in neutrophil chemotaxis assays. The conditioned media from the stimulated mast cells gave comparable chemotactic migration as 100 nM tryptase (**Figure 3.11**). Treatment of human neutrophils with tryptase inhibitor, APC-366, or the PAR2 pepducin P2pal-18S, completely inhibited chemotactic migration toward the tryptase-containing mast cell media (Figure 3.11b). To provide *in vivo* evidence that mast cells and mast cell tryptase were activating PAR2 in the paw edema model, we depleted mice of mast cells by pretreatment with compound 48/80 [223]. We observed a decrease in λ-carrageenan/kaolin-induced edema in the 48/80-depleted animals as compared to non-treated controls (Figure 3.11c). Similarly, mice treated with tryptase inhibitor APC-366, gave a significant 40% protection in λ-carrageenan/kaolin-induced paw edema. An intraplantar injection of the tryptase-containing mast cell media resulted in a similar peak increase in paw edema as induced by the selective PAR2 agonist SLIGRL (**Figure 3.12**). Systemic treatment with P2pal-18S gave a 50% decrease in peak development of edema at 4 h and afforded complete protection at 8 h and thereafter. Together, these data

suggest that the P2pal-18S pepducin provides significant protection against inflammatory edema triggered by mast cell-derived tryptase.

Figure 3.11:

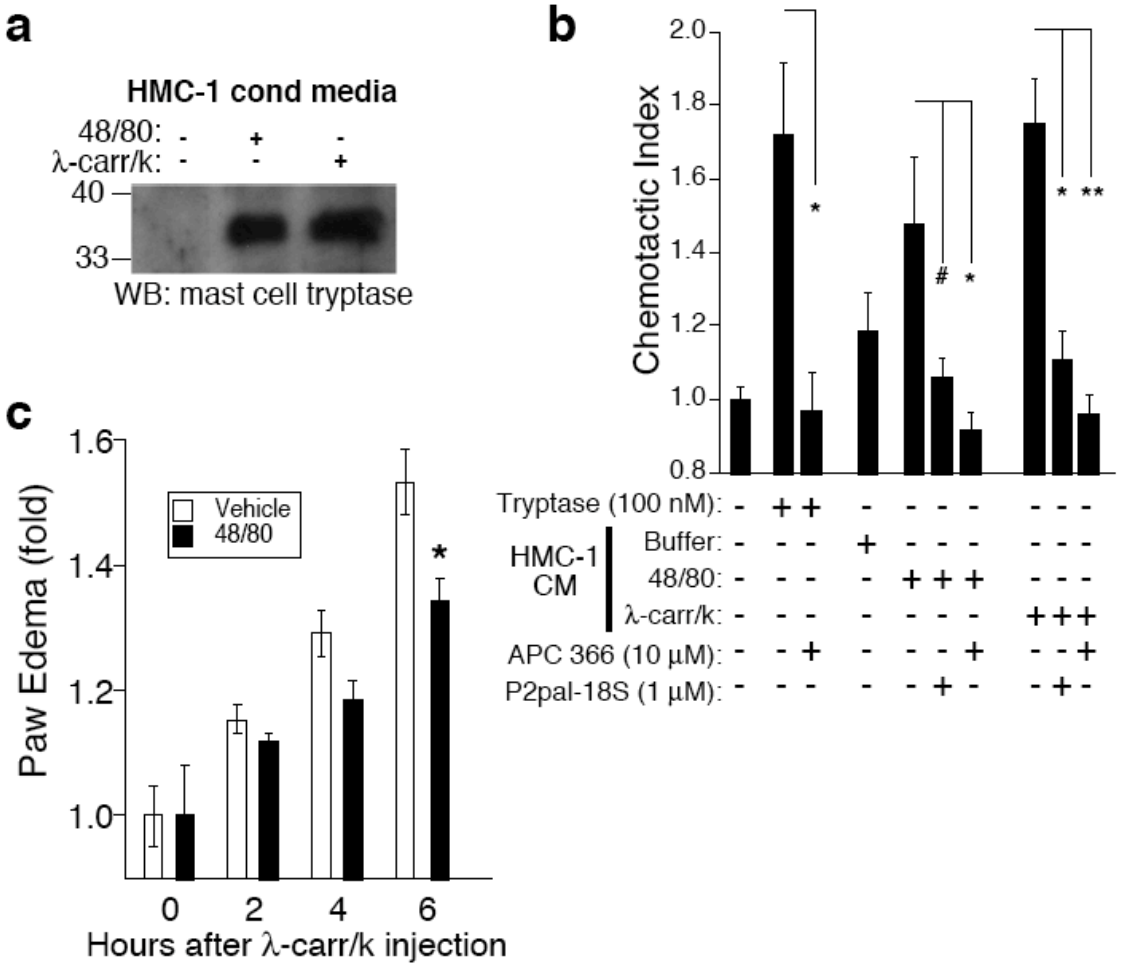


Figure 3.11. P2pal-18S significantly attenuates mast cell tryptase-dependent neutrophil migration and paw edema in mast cell depleted mice. (**a**) Mast cells were treated with 2 mg/mL of mast cell degranulating agent 48/80 or 2% λ-carrageenan/4% kaolin. Conditioned media (CM) was harvested at 24 h and a western blot of mast cell tryptase shows release of tryptase. (**b**) P2pal-18S inhibits human neutrophil chemotaxis (n=6) to mast cell media. Human neutrophils were incubated with 1 μM P2pal-18S, or 10 μM mast cell tryptase inhibitor APC-366 and allowed to migrate 30 min toward CM from mast cells. (**c**) Mast cell depletion in mice decreases paw edema induced by λ-carrageenan/kaolin. Mast cells were depleted from 8-week-old C57BL/6 female mice with 100 μL intraperitoneal injections of compound 48/80 twice daily for 4 days (Day 1 = 0.6 mg/kg, Day 2-3 = 1.2 mg/kg, Day 4 = 2.4 mg/kg). Paw edema was induced 24 h after the last dose of 48/80 with an intraplantar injection of λ-carrageenan/kaolin. A significant decrease in paw edema was observed in the mast cell-depleted mice 6 h post injection. Error bars represent mean ± SEM.

Figure 3.12:

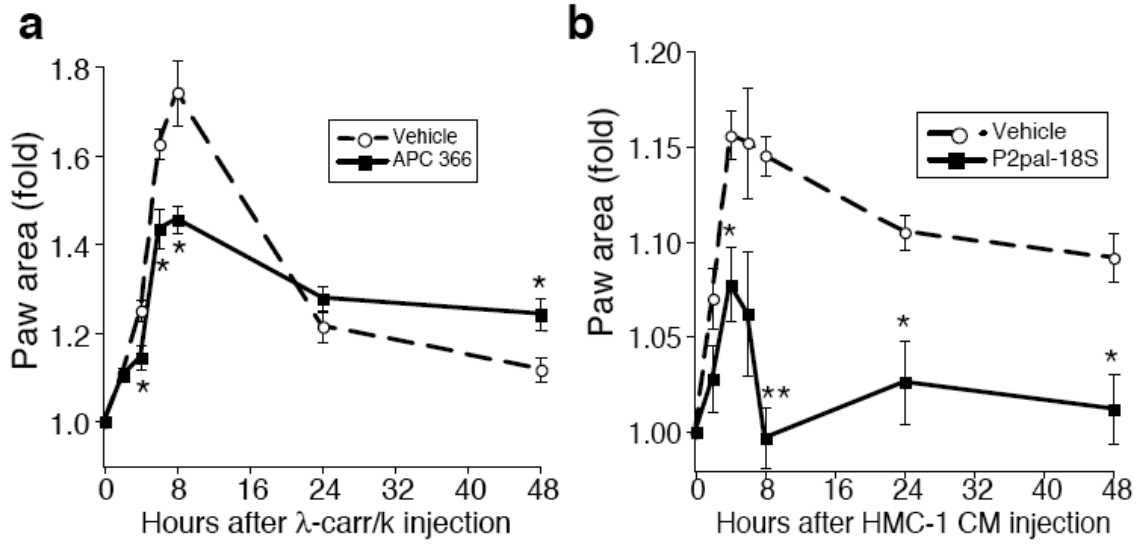


Figure 3.12. P2pal-18S significantly attenuates mast cell tryptase-dependent paw edema in mice (**a**) C57BL/6 mice (n=5) were pretreated with the tryptase inhibitor APC-366 (5 mg/kg, s.c.) or vehicle (20% DMSO) and then challenged with intraplantar injection of λ-carrageenan/kaolin. Paw area was measured over 2 days. (**b**) Mast cell conditioned media (30 μL of λ-carrageenan/kaolin stimulated media) was injected into the hindpaws of C57BL/6 mice (n=5). Mice were pretreated with 10 mg/kg P2pal-18S (s.c.) or vehicle. Data represent mean ± SEM. $^{\#}$, P =0.07, *, P <0.05 and **, P <0.005.

3.9 P2pal-18S in other pathophysiological models

P2pal-18S was tested in other pathophysiological models to observe if PAR2 inhibition would have an effect. In a model of sepsis that involves cecal ligation and puncture, the administration of P2pal-18S gave a small increase in survival after two days. This observation may suggest that PAR2 inhibition over time may be helpful in resolving the inflammatory response of the mice. To confirm this observation more mice and evaluations would need to be performed. These results do not contradict the previous finding that P1pal-13 treated PAR2$^{-/-}$ mice have a decrease in survival since that benefit is believed to be through PAR1/PAR2 signaling [86] (**Figure 3.13**).

In a model of arterial ligation injury, P2pal-18S increased intimal hyperplasia to an intermediate level between wild-type and PAR2$^{-/-}$ mice. No difference was observed in the medial area. Though this would not be a therapeutic indication for PAR2 inhibition, it was a confirmation that the pepducin was working to block some of PAR2 signaling in this model, though not completely, as compared to that of the PAR2-deficient animal (Figure 3.13b).

Lastly, a model of collagen-induced arthritis (CIA) was performed. DBA/1J mice which are sensitive to the induction of arthritis with collagen and adjuvant injections, were pretreated with either P2pal-18S, vehicle, or P2pal-14GF, a pepducin that showed no agonism or antagonism ability in calcium mobilization. Mice were then given an intraperitoneal injection of bovine Type II collagen and complete Freund's adjuvant. Pepducin treatments were continued daily throughout the experiment and booster immunizations of collagen/adjuvant were given on day 7 and day 14 to induce arthritis. Mice were monitored, and joints and paws were measured using calipers. Mice developed arthritis around day 30-40, but no significant differences between treatment groups were observed in the 80-day experiment (Figure 3.13c). After this observation, a study was published revealing that collagen induced arthritis was not significantly different in mice lacking mast cells compared to wild-type animals [(224)]. The authors believe that this observation is not contradictory to the observation that mast-cell deficient mice do not develop disease in other models of arthritis, or that mast cells are important in the disease severity in later phases. Instead they suggest that induction of arthritis with antigens such as Type II collagen is independent of mast cells [(224)]. Therefore, in hindsight, this arthritis model was a poor choice to examine PAR2-

dependent inflammation since as our results have shown mast cell tryptase is a very important component. Other models such as K/BxN, a transgenic mouse carrying a mutation in the MHC class II allele, which develops arthritis similar to human rheumatoid arthritis in chronic joint disease, cartilage and bone destruction, could be examined in the future [225].

Figure 3.13:

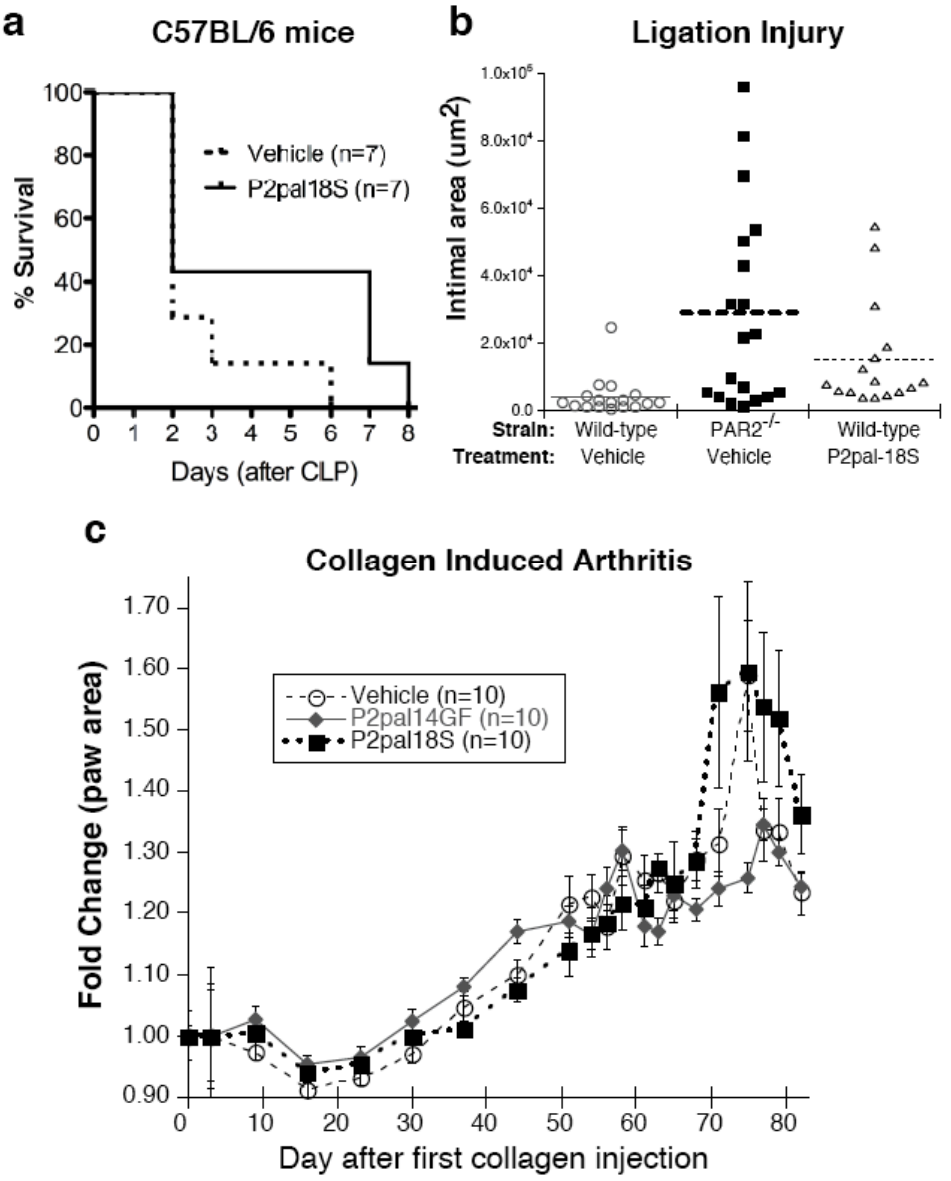


Figure 3.13. **Effect of PAR2 antagonist, P2pal-18S, in other pathophysiological mouse models.** (**a**) Survival of wild-type C57BL/6 mice (n=7) subjected to CLP, then given P2pal-18S (10 mg/kg) subcutaneously or vehicle immediately after; mice then received a daily dose of pepducin or vehicle. (**b**) C57BL/6 wild-type and PAR2$^{-/-}$ mice underwent ligation surgery of the left common carotid artery and were allowed to recover for 21 days while being treated with P2pal-18S or vehicle. Resulting intimal hyperplasia was measured and plotted for each individual mouse. (**c**) Eight-week old DBA/1J female mice were subcutaneously pretreated with vehicle, P2pal-14GF (10 mg/kg, a pepducin to PAR2 that showed no agonist or antagonist ability in a calcium flux assay), or P2pal-18S (10 mg/kg). Mice were challenged at day 7 with an intraperitoneal injection of 100 μg of Type II collagen in Freund's Complete Adjuvant. Paw measurements were taken two-three times a week and mice were followed for 70 days post challenge. A booster immunization was given 7 d and 14 d after the first collagen injection.

3.10 Discussion

In the present study, we describe the development of a first-in-class pepducin antagonist of PAR2. Pepducins are highly stable, lipidated peptides that modulate GPCR signaling in diverse disease models [33,32,195,27,86,106,87,208,140]. Pepducins comprise two components: a short peptide sequence derived from an i1-i4 intracellular loop of the target GPCR, and an acyl-chain fatty acid (e.g. palmitate) or other hydrophobic moiety conjugated to the peptide. Pepducins are targeted to the intracellular surface of their cognate GPCR and stabilize the receptor in either an active or inactive conformation, resulting in modulation of signal transduction [33,140]. Based on a structural model of a PAR2 receptor dimer and extensive mutagenesis of the receptor, we designed and tested a series of third intracellular loop pepducins to create full agonists and antagonists of PAR2. The most potent antagonist, P2pal-18S, fully ablated PAR2 signaling but did not inhibit the closely related PAR1 or PAR4 receptors, nor other GPCRs in neutrophils, platelets, or colon adenocarinoma cells.

The PAR2 pepducin antagonist had significant *in vivo* efficacy in suppressing leukocytic infiltration and edema induced by λ-carrageenan/kaolin or by a PAR2 selective agonist in mouse paw inflammation models. The anti-inflammatory effect of the P2pal-18S pepducin was lost in PAR2-deficient mice, providing further evidence that the pepducin was highly specific for PAR2. Moreover, the anti-inflammatory effect observed in the PAR2-deficient mice relative to wild type was nearly identical to that observed in wild-type mice treated with P2pal-18S. Together, these data indicate that P2pal-18S affords effective pharmacologic blockade of PAR2 in models of acute inflammation and that these effects require the presence of PAR2.

Many studies have implicated PAR2 as playing critical roles in a wide range of diseases including asthma [171], arthritis [182], hyperalgesia [190], neurogenic and cancer pain [189,193], and cancer invasion [112]. We provided several lines of evidence that the inflammatory response observed in the mouse footpad model was largely dependent on mast cells and mast cell-derived tryptase, an important agonist of PAR2-driven inflammation. We found that the PAR2 pepducin could completely suppress tryptase signaling through PAR2. However, the inhibitory effects conferred by the pepducin were greater than those accounted for by direct inhibition of tryptase alone with APC-366. This would suggest that the PAR2 pepducin was inhibiting signaling induced by other PAR2 agonists present in the inflammatory milieu. Indeed, PAR2 has been shown to be activated by other proteases including plasmin [226] and the TF-FXa-FVIIa complex [227].

Two other groups have disclosed PAR2 antagonists based on the tethered peptide ligand [34,36]. A PAR2 small molecule inhibitor, ENMD-1068, has been characterized in a model of joint inflammation [34]. ENMD-1068 requires millimolar concentrations to observe its effects *in vitro* and considerably higher doses *in vivo* to achieve similar protective effects afforded by P2pal-18S. The peptide antagonist K-14585 was shown to inhibit PAR2-dependent IL-8 production, NF-κB phosphorylation, and p38 signaling [35]. However, the K-14585 compound had partial agonist activity [35,36] as also observed with the wild-type PAR2 pepducin P2pal-21 [33]. In this regard, we discovered that wild-type PAR2 has constitutive activity indicating that certain extracellular or intracellular PAR2 ligands might trigger or stabilize the latent on-state. The realization that constitutive activity could be ablated or enhanced by mutation of critical i3 loop pharmacophores in the intact receptor led us to rationally design PAR2 pepducin antagonists that lost

residual agonist activity. The ability to design pepducin antagonists against difficult GPCR targets such as PAR2 is a valuable research and therapeutic approach which will aid in the delineation of complex mechanisms of GPCR signaling and pathophysiology and may lead to novel pharmacological agents for a potentially wide range of diseases.

Thesis Conclusion:

The purpose of this thesis was to explore the pathophysiological role of PAR1 and PAR2 in models of inflammation and cardiovascular disease, both important in current global health issues.

PAR1 and PAR2 have parallel and distinct signaling that may be regulated by the dimerization of the receptors. We have shown that PAR2 can homodimerize as well as heterodimerize with PAR1. These dimers are important in determining the possibility of PAR2 signaling discrimination; future studies determining the downstream Gα proteins that each dimer prefers would be of great interest. The PAR1/PAR2 heterodimer is necessary for the effect of PAR1 agonist, P1pal-13 in a blood cessation and perivascular inflammatory injury model, as observed by the lack of neointima if either receptor is absent. This suggests that PAR1/PAR2 heterodimers play a role in the pathophysiological response of vascular remodeling.

Activating PAR1/PAR2 heterodimers with PAR1 agonist P1pal-13 caused significant increases in both medial and intimal hyperplasia. The cells type responsible for this PAR1-dependent hyperplasia are most likely the smooth muscle cells from the medial layer or the endothelial cells that make up the intimal layer of the vessel. To determine if endothelial cells are involved in this process, one could utilize the PAR1 knock-in mouse with the Tie2p/e promoter [62]. As mentioned in the introduction, this mouse expresses PAR1 only in endothelial cells and resulting intimal hyperplasia would mean that activating PAR1 signaling on endothelial cells is sufficient to cause a blood vessel response. The lack of a response in these mice could mean that these processes

are not due to signaling on the endothelial cells or that more than one cell type is necessary.

Critical residues of PAR2, as predicted by a PAR2 dimer model, are important for receptor signaling, and allowed for the targeted molecular engineering of specific and effective PAR2 pepducin antagonists. Inhibition of PAR2 is sufficient to attenuate neutrophil chemotaxis *ex vivo* and in *in vivo* models of mouse acute inflammation. Mast cell tryptase plays an important role in PAR2-dependent inflammation and the PAR2 antagonist pepducin, P2pal-18S, also blocked neutrophil chemotaxis and mouse paw edema induced by mast cell tryptase. The next step for testing P2pal-18S would be to use the pepducin post-induction of acute inflammation. This model would be more physiologically relevant and would determine the beneficial effects of P2pal-18S on blocking inflammation after it has been initiated. The therapeutic applications for this pepducin remain to be explored, and could possibly include arthritis, asthma/dust allergens, pain, skin itch and psoriasis.

PAR2 expression has also been reported in macrophages and astrocytes of human multiple sclerosis patients and in a mouse model of MS: experimental autoimmune encephalomyelitis (EAE). In this model, the neuroinflammation and macrophage associated demyelination in the CNS was more severe in wild-type mice compared to $PAR2^{-/-}$ mice [209]. This study implies that a PAR2 antagonist could reduce demyelination and inflammation that lead to lesions in MS patients, and could subsequently reduce symptoms. More studies would be needed to determine whether PAR2 is involved in the onset of these chronic diseases and whether PAR2 activation is a factor in the ongoing

symptoms observed, which would help predict whether our antagonist could be therapeutically beneficial.

The mechanism of P2pal-18S is still being explored. Additional studies performed after the original submission of this thesis to the committee have revealed that P2pal-18S does not affect proteolytic cleavage of PAR2, MAPK signaling (ERK1/2 or p38), or endocytosis of the receptor. However, it has been observed that P2pal-18S blocks the transactivation of one PAR2 receptor on an adjacent PAR2 and one recent experiment suggests that P2pal-18S can change the conformation of the receptor. Further studies are underway to confirm this observation and to test whether the pepducin can also block transactivation of PAR2 by PAR1.

Of interest is the parallel that can be drawn between PAR2 antagonists and the inhibition of cyclooxgenase-2 (COX-2) with rofecoxib, more commonly known as Vioxx. COX-2 inhibition has been shown to be beneficial for reduction of pain and inflammation and was originally characterized in models such as edema, hyperalgesia, arthritis, and even the reduction of polyps in colorectal carcinoma tissues [(228)]. These models mirror those that PAR2 antagonists are characterized in. Additionally, selective COX-2 inhibitors were thought to reduce toxic side effects such as gastrointestinal ulceration, which is attributed to COX-1 inhibition [(228)]. However, after being approved for use in patients, Vioxx was withdrawn from the market due to an increase in cardiovascular risks, in particular myocardial infarction [(229)]. Though patients with severe rheumatoid arthritis can still receive Vioxx, the benefits and risks must be weighed on an individual basis [(230)].

PAR2 activation has been shown to upregulate the expression of COX-2 by ERK1/2 mediated activation of the β-catenin/Tcf-4 pathways [231]. It would be interesting to test if the reduction in inflammation in response to P2pal-18S is COX-2-dependent. Additionally, the increased intimal hyperplasia observed in both $PAR2^{-/-}$ mice and with P2pal-18S treatment may indicate that PAR2 antagonists have the potential to also have cardiovascular side effects, though more studies would need to be done before any conclusions can be drawn.

Lastly, the development of a pepducin by rational design and modeling has greatly advanced the specificity and effectiveness of PAR2 antagonists. It would be of great interest to repeat this methodology with other GPCRs to observe whether agonists and antagonists could be designed using this functional/structural approach.

Abbreviations:

APC	Activated Protein C
αSMA	alpha smooth muscle actin
BDNF	Brain-derived neutrophilic factor
BicD1	Bicaudal D homolog-1
BRET	bioluminescence resonance energy transfer
CCPs	clathrin coated pits
CGRP	calcitonin gene-related peptide
CLP	cecal ligation and puncture
COX-2	cyclooxgenase-2
DAG	diacylglycerol
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FRET	fluorescence resonance energy transfer
FVIIa	Factor VIIa
FXa	Factor Xa
γ-carr/k	γ-carrageenan/kaolin
GEFs	guanine exchange factors
GPCR	G-protein coupled receptors
GRKs	G-protein coupled receptor kinases
i.p.	intraperitoneal injection
i.v.	intravenous injection
I/R	ischemia/reperfusion
IFN-γ	interferon-gamma
IL-#	interleukin-_
InsP	inositol phosphate
IP3	inositol-triphosphate
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MMP	matrix metalloprotease
MOVAS	mouse vascular aorta smooth muscle cells
NK_1R	neurokinin receptor
P13K	phosphoinositide-3-kinase
PAR	Protease-Activated Receptor
PKA	protein kinase A
PKC	protein kinase C
PLC-β	phospholipase C-β
s.c.	subcutaneous injection
SDF-1α	stromal cell-derived factor-1 alpha
SMCs	smooth muscle cells
SM-MHC	smooth muscle-myosin heavy chain

SP	substance P
TGF-α	transforming growth factor-alpha
TNF-α	tumor necrosis factor-alpha
TRPV-1	transient receptor potential vanilloid receptor-1
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
WT	wild-type

Cell Line Appendix:

B6SUtA	mouse myeloid progenitor cells
CCL39	chinese hamster lung fibroblasts
CHO	chinese hamster ovary cells
COS7	African green monkey kidney fibroblasts
Dami	human megakaryocytic cell line
DU-145	human prostate carcinoma cells
EAhy926	fusion cell of HUVECs and A549, human lung carcinoma
HEK 293(T)	human embryonic kidney 293 cells (T=transformed with SV40, T-antigen)
HEL	human leukemia cell with some megakaryocyte features
HMC-1	human mast cell line, from the Mayo Clinic (Dr. J.H. Butterfield)
HT-29	human colon adenocarcinoma grade II cell line
HUVEC	human umbilical vascular endothelial cells
MCF-7	human breast adenocarcinoma cell line
MDA-MB-231	human breast adenocarcinoma cell line
MOVAS	mouse vascular aorta smooth muscle cell line
OVCAR-4	human ovarian adenocarcinoma cell line
SW620	human colorectal adenocarcinoma cell line

Bibliography

1. Ramachandran, S. and R. A. Cerione, (2006). How GPCRs hit the switch. *Nat Struct Mol Biol* **13** (9), 756-757.

2. Neves, S. R., P. T. Ram, and R. Iyengar, (2002). G protein pathways. *Science* **296** (5573), 1636-1639.

3. Coughlin, S. R., (2000). Thrombin signalling and protease-activated receptors. *Nature* **407** (6801), 258-264.

4. Ossovskaya, V. S. and N. W. Bunnett, (2004). Protease-activated receptors: contribution to physiology and disease. *Physiol Rev* **84** (2), 579-621.

5. Dudek, S. M. and J. G. Garcia, (2003). Rho family of guanine exchange factors (GEFs) in cellular activation: who's dancing? And with whom? *Circ Res* **93** (9), 794-795.

6. Wei, H., S. Ahn, S. K. Shenoy, S. S. Karnik, L. Hunyady, L. M. Luttrell, and R. J. Lefkowitz, (2003). Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci U S A* **100** (19), 10782-10787.

7. Duchene, J., J. P. Schanstra, C. Pecher, A. Pizard, C. Susini, J. P. Esteve, J. L. Bascands, and J. P. Girolami, (2002). A novel protein-protein interaction between a G protein-coupled receptor and the phosphatase SHP-2 is involved in bradykinin-induced inhibition of cell proliferation. *J Biol Chem* **277** (43), 40375-40383.

8. Vu, T. K., D. T. Hung, V. I. Wheaton, and S. R. Coughlin, (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64** (6), 1057-1068.

9. Rasmussen, U. B., V. Vouret-Craviari, S. Jallat, Y. Schlesinger, G. Pages, A. Pavirani, J. P. Lecocq, J. Pouyssegur, and E. Van Obberghen-Schilling, (1991). cDNA cloning and expression of a hamster alpha-thrombin receptor coupled to Ca2+ mobilization. *FEBS Lett* **288** (1-2), 123-128.

10. Nystedt, S., K. Emilsson, C. Wahlestedt, and J. Sundelin, (1994). Molecular cloning of a potential proteinase activated receptor. *Proc Natl Acad Sci U S A* **91** (20), 9208-9212.

11. Ishihara, H., A. J. Connolly, D. Zeng, M. L. Kahn, Y. W. Zheng, C. Timmons, T. Tram, and S. R. Coughlin, (1997). Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* **386** (6624), 502-506.

12. Connolly, A. J., H. Ishihara, M. L. Kahn, R. V. Farese, Jr., and S. R. Coughlin, (1996). Role of the thrombin receptor in development and evidence for a second receptor. *Nature* **381** (6582), 516-519.

13. Xu, W. F., H. Andersen, T. E. Whitmore, S. R. Presnell, D. P. Yee, A. Ching, T. Gilbert, E. W. Davie, and D. C. Foster, (1998). Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci U S A* **95** (12), 6642-6646.

14. Kahn, M. L., Y. W. Zheng, W. Huang, V. Bigornia, D. Zeng, S. Moff, R. V. Farese, Jr., C. Tam, and S. R. Coughlin, (1998). A dual thrombin receptor system for platelet activation. *Nature* **394** (6694), 690-694.

15. Schmidt, V. A., W. C. Nierman, T. V. Feldblyum, D. R. Maglott, and W. F. Bahou, (1997). The human thrombin receptor and proteinase activated receptor-2 genes are tightly linked on chromosome 5q13. *Br J Haematol* **97** (3), 523-529.

16. Schmidt, V. A., W. C. Nierman, D. R. Maglott, L. D. Cupit, K. A. Moskowitz, J. A. Wainer, and W. F. Bahou, (1998). The human proteinase-activated receptor-3 (PAR-3) gene. Identification within a Par gene cluster and characterization in vascular endothelial cells and platelets. *J Biol Chem* **273** (24), 15061-15068.

17. Kahn, M. L., S. R. Hammes, C. Botka, and S. R. Coughlin, (1998). Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. *J Biol Chem* **273** (36), 23290-23296.

18. O'Brien, P. J., M. Molino, M. Kahn, and L. F. Brass, (2001). Protease activated receptors: theme and variations. *Oncogene* **20** (13), 1570-1581.

19. Nystedt, S., K. Emilsson, A. K. Larsson, B. Strombeck, and J. Sundelin, (1995). Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur J Biochem* **232** (1), 84-89.

20. Lerner, D. J., M. Chen, T. Tram, and S. R. Coughlin, (1996). Agonist recognition by proteinase-activated receptor 2 and thrombin receptor. Importance of extracellular loop interactions for receptor function. *J Biol Chem* **271** (24), 13943-13947.

21. Ruf, W., A. Dorfleutner, and M. Riewald, (2003). Specificity of coagulation factor signaling. *J Thromb Haemost* **1** (7), 1495-1503.

22. Blackhart, B. D., K. Emilsson, D. Nguyen, W. Teng, A. J. Martelli, S. Nystedt, J. Sundelin, and R. M. Scarborough, (1996). Ligand cross-reactivity within the protease-activated receptor family. *J Biol Chem* **271** (28), 16466-16471.

23. Corvera, C. U., O. Dery, K. McConalogue, S. K. Bohm, L. M. Khitin, G. H. Caughey, D. G. Payan, and N. W. Bunnett, (1997). Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. *J Clin Invest* **100** (6), 1383-1393.

24. Seitz, I., S. Hess, H. Schulz, R. Eckl, G. Busch, H. P. Montens, R. Brandl, S. Seidl, A. Schomig, and I. Ott, (2007). Membrane-type serine protease-1/matriptase induces interleukin-6 and -8 in endothelial cells by activation of protease-activated receptor-2: potential implications in atherosclerosis. *Arterioscler Thromb Vasc Biol* **27** (4), 769-775.

25. Riewald, M., R. J. Petrovan, A. Donner, B. M. Mueller, and W. Ruf, (2002). Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science* **296** (5574), 1880-1882.

26. Camerer, E., H. Kataoka, M. Kahn, K. Lease, and S. R. Coughlin, (2002). Genetic evidence that protease-activated receptors mediate factor Xa signaling in endothelial cells. *J Biol Chem* **277** (18), 16081-16087.

27. Boire, A., L. Covic, A. Agarwal, S. Jacques, S. Sherifi, and A. Kuliopulos, (2005). PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell* **120** (3), 303-313.

28. Sternlicht, M. D. and Z. Werb, (2001). How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* **17**, 463-516.

29. Dowal, L., D. S. Sim, J. R. Dilks, P. Blair, S. Beaudry, B. M. Denker, G. Koukos, A. Kuliopulos, and R. Flaumenhaft, (2011). Identification of an antithrombotic allosteric modulator that acts through helix 8 of PAR1. *Proc Natl Acad Sci U S A*.

30. Andrade-Gordon, P., B. E. Maryanoff, C. K. Derian, H. C. Zhang, M. F. Addo, A. L. Darrow, A. J. Eckardt, W. J. Hoekstra, D. F. McComsey, D. Oksenberg, E. E. Reynolds, R. J. Santulli, R. M. Scarborough, C. E. Smith, and K. B. White, (1999). Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. *Proc Natl Acad Sci U S A* **96** (22), 12257-12262.

31. Ahn, H. S., C. Foster, G. Boykow, A. Stamford, M. Manna, and M. Graziano, (2000). Inhibition of cellular action of thrombin by N3-cyclopropyl-7-[[4-(1-methylethyl)phenyl]methyl]-7H-pyrrolo[3, 2-f]quinazoline-1,3-diamine (SCH 79797), a nonpeptide thrombin receptor antagonist. *Biochem Pharmacol* **60** (10), 1425-1434.

32. Covic, L., M. Misra, J. Badar, C. Singh, and A. Kuliopulos, (2002). Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nat Med* **8** (10), 1161-1165.

33. Covic, L., A. L. Gresser, J. Talavera, S. Swift, and A. Kuliopulos, (2002). Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. *Proc Natl Acad Sci U S A* **99** (2), 643-648.

34. Kelso, E. B., J. C. Lockhart, T. Hembrough, L. Dunning, R. Plevin, M. D. Hollenberg, C. P. Sommerhoff, J. S. McLean, and W. R. Ferrell, (2006). Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation. *J Pharmacol Exp Ther* **316** (3), 1017-1024.

35. Goh, F. G., P. Y. Ng, M. Nilsson, T. Kanke, and R. Plevin, (2009). Dual effect of the novel peptide antagonist K-14585 on proteinase-activated receptor-2-mediated signalling. *Br J Pharmacol* **158** (7), 1695-1704.

36. Kanke, T., M. Kabeya, S. Kubo, S. Kondo, K. Yasuoka, J. Tagashira, H. Ishiwata, M. Saka, T. Furuyama, T. Nishiyama, T. Doi, Y. Hattori, A. Kawabata, M. R. Cunningham, and R. Plevin, (2009). Novel antagonists for proteinase-activated receptor 2: inhibition of cellular and vascular responses in vitro and in vivo. *Br J Pharmacol* **158** (1), 361-371.

37. Wu, C. C. and C. M. Teng, (2006). Comparison of the effects of PAR1 antagonists, PAR4 antagonists, and their combinations on thrombin-induced human platelet activation. *Eur J Pharmacol* **546** (1-3), 142-147.

38. Hung, D. T., T. H. Vu, N. A. Nelken, and S. R. Coughlin, (1992). Thrombin-induced events in non-platelet cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor. *J Cell Biol* **116** (3), 827-832.

39. Baffy, G., L. Yang, S. Raj, D. R. Manning, and J. R. Williamson, (1994). G protein coupling to the thrombin receptor in Chinese hamster lung fibroblasts. *J Biol Chem* **269** (11), 8483-8487.

40. Winitz, S., S. K. Gupta, N. X. Qian, L. E. Heasley, R. A. Nemenoff, and G. L. Johnson, (1994). Expression of a mutant Gi2 alpha subunit inhibits ATP and thrombin stimulation of cytoplasmic phospholipase A2-mediated arachidonic acid release independent of Ca2+ and mitogen-activated protein kinase regulation. *J Biol Chem* **269** (3), 1889-1895.

41. Offermanns, S., K. L. Laugwitz, K. Spicher, and G. Schultz, (1994). G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. *Proc Natl Acad Sci U S A* **91** (2), 504-508.

42. Aragay, A. M., L. R. Collins, G. R. Post, A. J. Watson, J. R. Feramisco, J. H. Brown, and M. I. Simon, (1995). G12 requirement for thrombin-stimulated gene

expression and DNA synthesis in 1321N1 astrocytoma cells. *J Biol Chem* **270** (34), 20073-20077.

43. Verrall, S., M. Ishii, M. Chen, L. Wang, T. Tram, and S. R. Coughlin, (1997). The thrombin receptor second cytoplasmic loop confers coupling to Gq-like G proteins in chimeric receptors. Additional evidence for a common transmembrane signaling and G protein coupling mechanism in G protein-coupled receptors. *J Biol Chem* **272** (11), 6898-6902.

44. Santulli, R. J., C. K. Derian, A. L. Darrow, K. A. Tomko, A. J. Eckardt, M. Seiberg, R. M. Scarborough, and P. Andrade-Gordon, (1995). Evidence for the presence of a protease-activated receptor distinct from the thrombin receptor in human keratinocytes. *Proc Natl Acad Sci U S A* **92** (20), 9151-9155.

45. Kong, W., K. McConalogue, L. M. Khitin, M. D. Hollenberg, D. G. Payan, S. K. Bohm, and N. W. Bunnett, (1997). Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. *Proc Natl Acad Sci U S A* **94** (16), 8884-8889.

46. Yu, Z., S. Ahmad, J. L. Schwartz, D. Banville, and S. H. Shen, (1997). Protein-tyrosine phosphatase SHP2 is positively linked to proteinase-activated receptor 2-mediated mitogenic pathway. *J Biol Chem* **272** (11), 7519-7524.

47. Olianas, M. C., S. Dedoni, and P. Onali, (2007). Proteinase-activated receptors 1 and 2 in rat olfactory system: layer-specific regulation of multiple signaling pathways in the main olfactory bulb and induction of neurite retraction in olfactory sensory neurons. *Neuroscience* **146** (3), 1289-1301.

48. Belham, C. M., R. J. Tate, P. H. Scott, A. D. Pemberton, H. R. Miller, R. M. Wadsworth, G. W. Gould, and R. Plevin, (1996). Trypsin stimulates proteinase-activated receptor-2-dependent and -independent activation of mitogen-activated protein kinases. *Biochem J* **320 (Pt 3)**, 939-946.

49. Tanaka, Y., F. Sekiguchi, H. Hong, and A. Kawabata, (2008). PAR2 triggers IL-8 release via MEK/ERK and PI3-kinase/Akt pathways in GI epithelial cells. *Biochem Biophys Res Commun* **377** (2), 622-626.

50. Nakanishi-Matsui, M., Y. W. Zheng, D. J. Sulciner, E. J. Weiss, M. J. Ludeman, and S. R. Coughlin, (2000). PAR3 is a cofactor for PAR4 activation by thrombin. *Nature* **404** (6778), 609-613.

51. Leger, A. J., L. Covic, and A. Kuliopulos, (2006). Protease-activated receptors in cardiovascular diseases. *Circulation* **114** (10), 1070-1077.

52. Kim, S., C. Foster, A. Lecchi, T. M. Quinton, D. M. Prosser, J. Jin, M. Cattaneo, and S. P. Kunapuli, (2002). Protease-activated receptors 1 and 4 do not stimulate

G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling. *Blood* **99** (10), 3629-3636.

53. Marchese, A., M. M. Paing, B. R. Temple, and J. Trejo, (2008). G protein-coupled receptor sorting to endosomes and lysosomes. *Annu Rev Pharmacol Toxicol* **48**, 601-629.

54. Lefkowitz, R. J. and S. K. Shenoy, (2005). Transduction of receptor signals by beta-arrestins. *Science* **308** (5721), 512-517.

55. Goodman, O. B., Jr., J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen, and J. L. Benovic, (1996). Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* **383** (6599), 447-450.

56. Bohm, S. K., L. M. Khitin, E. F. Grady, G. Aponte, D. G. Payan, and N. W. Bunnett, (1996). Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *J Biol Chem* **271** (36), 22003-22016.

57. DeFea, K. A., J. Zalevsky, M. S. Thoma, O. Dery, R. D. Mullins, and N. W. Bunnett, (2000). beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* **148** (6), 1267-1281.

58. Paing, M. M., C. A. Johnston, D. P. Siderovski, and J. Trejo, (2006). Clathrin adaptor AP2 regulates thrombin receptor constitutive internalization and endothelial cell resensitization. *Mol Cell Biol* **26** (8), 3231-3242.

59. Swift, S., J. Xu, V. Trivedi, K. M. Austin, S. L. Tressel, L. Zhang, L. Covic, and A. Kuliopulos, (2010). A novel protease-activated receptor-1 interactor, Bicaudal D1, regulates G protein signaling and internalization. *J Biol Chem* **285** (15), 11402-11410.

60. Trejo, J., (2003). Protease-activated receptors: new concepts in regulation of G protein-coupled receptor signaling and trafficking. *J Pharmacol Exp Ther* **307** (2), 437-442.

61. Sambrano, G. R., E. J. Weiss, Y. W. Zheng, W. Huang, and S. R. Coughlin, (2001). Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature* **413** (6851), 74-78.

62. Griffin, C. T., Y. Srinivasan, Y. W. Zheng, W. Huang, and S. R. Coughlin, (2001). A role for thrombin receptor signaling in endothelial cells during embryonic development. *Science* **293** (5535), 1666-1670.

63. Damiano, B. P., W. M. Cheung, R. J. Santulli, W. P. Fung-Leung, K. Ngo, R. D. Ye, A. L. Darrow, C. K. Derian, L. de Garavilla, and P. Andrade-Gordon, (1999). Cardiovascular responses mediated by protease-activated receptor-2 (PAR-2) and thrombin receptor (PAR-1) are distinguished in mice deficient in PAR-2 or PAR-1. *J Pharmacol Exp Ther* **288** (2), 671-678.

64. Camerer, E., I. Cornelissen, H. Kataoka, D. N. Duong, Y. W. Zheng, and S. R. Coughlin, (2006). Roles of protease-activated receptors in a mouse model of endotoxemia. *Blood* **107** (10), 3912-3921.

65. Milligan, G., (2007). G protein-coupled receptor dimerisation: molecular basis and relevance to function. *Biochim Biophys Acta* **1768** (4), 825-835.

66. Overton, M. C. and K. J. Blumer, (2002). Use of fluorescence resonance energy transfer to analyze oligomerization of G-protein-coupled receptors expressed in yeast. *Methods* **27** (4), 324-332.

67. Xu, Y., D. W. Piston, and C. H. Johnson, (1999). A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. *Proc Natl Acad Sci U S A* **96** (1), 151-156.

68. Palczewski, K., T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. Le Trong, D. C. Teller, T. Okada, R. E. Stenkamp, M. Yamamoto, and M. Miyano, (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289** (5480), 739-745.

69. Lambright, D. G., J. Sondek, A. Bohm, N. P. Skiba, H. E. Hamm, and P. B. Sigler, (1996). The 2.0 A crystal structure of a heterotrimeric G protein. *Nature* **379** (6563), 311-319.

70. Maggio, R., G. Innamorati, and M. Parenti, (2007). G protein-coupled receptor oligomerization provides the framework for signal discrimination. *J Neurochem* **103** (5), 1741-1752.

71. Brett, M. and J. B. Findlay, (1979). Investigation of the organization of rhodopsin in the sheep photoreceptor membrane by using cross-linking reagents. *Biochem J* **177** (1), 215-223.

72. Borochov-Neori, H., P. A. Fortes, and M. Montal, (1983). Rhodopsin in reconstituted phospholipid vesicles. 2. Rhodopsin-rhodopsin interactions detected by resonance energy transfer. *Biochemistry* **22** (1), 206-213.

73. Liang, Y., D. Fotiadis, S. Filipek, D. A. Saperstein, K. Palczewski, and A. Engel, (2003). Organization of the G protein-coupled receptors rhodopsin and opsin in native membranes. *J Biol Chem* **278** (24), 21655-21662.

74. Avissar, S., G. Amitai, and M. Sokolovsky, (1983). Oligomeric structure of muscarinic receptors is shown by photoaffinity labeling: subunit assembly may explain high- and low-affinity agonist states. *Proc Natl Acad Sci U S A* **80** (1), 156-159.

75. Venter, J. C. and C. M. Fraser, (1983). beta-Adrenergic receptor isolation and characterization with immobilized drugs and monoclonal antibodies. *Fed Proc* **42** (2), 273-278.

76. Maggio, R., Z. Vogel, and J. Wess, (1993). Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors. *Proc Natl Acad Sci U S A* **90** (7), 3103-3107.

77. Maggio, R., P. Barbier, F. Fornai, and G. U. Corsini, (1996). Functional role of the third cytoplasmic loop in muscarinic receptor dimerization. *J Biol Chem* **271** (49), 31055-31060.

78. Itoh, M., K. Nagatomo, Y. Kubo, and O. Saitoh, (2006). Alternative splicing of RGS8 gene changes the binding property to the M1 muscarinic receptor to confer receptor type-specific Gq regulation. *J Neurochem* **99** (6), 1505-1516.

79. Angers, S., A. Salahpour, E. Joly, S. Hilairet, D. Chelsky, M. Dennis, and M. Bouvier, (2000). Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc Natl Acad Sci U S A* **97** (7), 3684-3689.

80. Birdsall, N. J., Class A GPCR heterodimers: evidence from binding studies. *Trends Pharmacol Sci* **31** (11), 499-508.

81. Bulenger, S., S. Marullo, and M. Bouvier, (2005). Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends Pharmacol Sci* **26** (3), 131-137.

82. Hague, C., M. A. Uberti, Z. Chen, C. F. Bush, S. V. Jones, K. J. Ressler, R. A. Hall, and K. P. Minneman, (2004). Olfactory receptor surface expression is driven by association with the beta2-adrenergic receptor. *Proc Natl Acad Sci U S A* **101** (37), 13672-13676.

83. Milligan, G. and J. C. McGrath, (2009). GPCR theme editorial. *Br J Pharmacol* **158** (1), 1-4.

84. Chen, J., M. Ishii, L. Wang, K. Ishii, and S. R. Coughlin, (1994). Thrombin receptor activation. Confirmation of the intramolecular tethered liganding hypothesis and discovery of an alternative intermolecular liganding mode. *J Biol Chem* **269** (23), 16041-16045.

85. O'Brien, P. J., N. Prevost, M. Molino, M. K. Hollinger, M. J. Woolkalis, D. S. Woulfe, and L. F. Brass, (2000). Thrombin responses in human endothelial cells. Contributions from receptors other than PAR1 include the transactivation of PAR2 by thrombin-cleaved PAR1. *J Biol Chem* **275** (18), 13502-13509.

86. Kaneider, N. C., A. J. Leger, A. Agarwal, N. Nguyen, G. Perides, C. Derian, L. Covic, and A. Kuliopulos, (2007). 'Role reversal' for the receptor PAR1 in sepsis-induced vascular damage. *Nat Immunol* **8** (12), 1303-1312.

87. Leger, A. J., S. L. Jacques, J. Badar, N. C. Kaneider, C. K. Derian, P. Andrade-Gordon, L. Covic, and A. Kuliopulos, (2006). Blocking the protease-activated receptor 1-4 heterodimer in platelet-mediated thrombosis. *Circulation* **113** (9), 1244-1254.

88. Seiler, S. M., (1996). Thrombin receptor antagonists. *Semin Thromb Hemost* **22** (3), 223-232.

89. McLaughlin, J. N., M. M. Patterson, and A. B. Malik, (2007). Protease-activated receptor-3 (PAR3) regulates PAR1 signaling by receptor dimerization. *Proc Natl Acad Sci U S A* **104** (13), 5662-5667.

90. Covic, L., A. L. Gresser, and A. Kuliopulos, (2000). Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry* **39** (18), 5458-5467.

91. Kahn, M. L., M. Nakanishi-Matsui, M. J. Shapiro, H. Ishihara, and S. R. Coughlin, (1999). Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest* **103** (6), 879-887.

92. Andrade-Gordon, P., C. K. Derian, B. E. Maryanoff, H. C. Zhang, M. F. Addo, W. Cheung, B. P. Damiano, M. R. D'Andrea, A. L. Darrow, L. de Garavilla, A. J. Eckardt, E. C. Giardino, B. J. Haertlein, and D. F. McComsey, (2001). Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. *J Pharmacol Exp Ther* **298** (1), 34-42.

93. Grand, R. J., A. S. Turnell, and P. W. Grabham, (1996). Cellular consequences of thrombin-receptor activation. *Biochem J* **313 (Pt 2)**, 353-368.

94. Mackman, N., (2006). Role of tissue factor in hemostasis and thrombosis. *Blood Cells Mol Dis* **36** (2), 104-107.

95. Offermanns, S., C. F. Toombs, Y. H. Hu, and M. I. Simon, (1997). Defective platelet activation in G alpha(q)-deficient mice. *Nature* **389** (6647), 183-186.

96. Andersen, H., D. L. Greenberg, K. Fujikawa, W. Xu, D. W. Chung, and E. W. Davie, (1999). Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proc Natl Acad Sci U S A* **96** (20), 11189-11193.

97. Shattil, S. J. and P. J. Newman, (2004). Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood* **104** (6), 1606-1615.

98. Whitehead, I., H. Kirk, and R. Kay, (1995). Expression cloning of oncogenes by retroviral transfer of cDNA libraries. *Mol Cell Biol* **15** (2), 704-710.

99. Martin, C. B., G. M. Mahon, M. B. Klinger, R. J. Kay, M. Symons, C. J. Der, and I. P. Whitehead, (2001). The thrombin receptor, PAR-1, causes transformation by activation of Rho-mediated signaling pathways. *Oncogene* **20** (16), 1953-1963.

100. Liu, J., P. Schuff-Werner, and M. Steiner, (2006). Thrombin/thrombin receptor (PAR-1)-mediated induction of IL-8 and VEGF expression in prostate cancer cells. *Biochem Biophys Res Commun* **343** (1), 183-189.

101. Gutkind, J. S., (1998). Cell growth control by G protein-coupled receptors: from signal transduction to signal integration. *Oncogene* **17** (11 Reviews), 1331-1342.

102. Even-Ram, S., B. Uziely, P. Cohen, S. Grisaru-Granovsky, M. Maoz, Y. Ginzburg, R. Reich, I. Vlodavsky, and R. Bar-Shavit, (1998). Thrombin receptor overexpression in malignant and physiological invasion processes. *Nat Med* **4** (8), 909-914.

103. Kamath, L., A. Meydani, F. Foss, and A. Kuliopulos, (2001). Signaling from protease-activated receptor-1 inhibits migration and invasion of breast cancer cells. *Cancer Res* **61** (15), 5933-5940.

104. Yang, E., A. Boire, A. Agarwal, N. Nguyen, K. O'Callaghan, P. Tu, A. Kuliopulos, and L. Covic, (2009). Blockade of PAR1 signaling with cell-penetrating pepducins inhibits Akt survival pathways in breast cancer cells and suppresses tumor survival and metastasis. *Cancer Res* **69** (15), 6223-6231.

105. Nguyen, N., A. Kuliopulos, R. A. Graham, and L. Covic, (2006). Tumor-derived Cyr61(CCN1) promotes stromal matrix metalloproteinase-1 production and protease-activated receptor 1-dependent migration of breast cancer cells. *Cancer Res* **66** (5), 2658-2665.

106. Agarwal, A., L. Covic, L. M. Sevigny, N. C. Kaneider, K. Lazarides, G. Azabdaftari, S. Sharifi, and A. Kuliopulos, (2008). Targeting a metalloprotease-PAR1 signaling system with cell-penetrating pepducins inhibits angiogenesis, ascites, and progression of ovarian cancer. *Mol Cancer Ther* **7** (9), 2746-2757.

107. Agarwal, A., S. L. Tressel, R. Kaimal, M. Balla, F. H. Lam, L. Covic, and A. Kuliopulos, (2010). Identification of a metalloprotease-chemokine signaling system in the ovarian cancer microenvironment: implications for antiangiogenic therapy. *Cancer Res* **70** (14), 5880-5890.

108. Goerge, T., A. Barg, E. M. Schnaeker, B. Poppelmann, V. Shpacovitch, A. Rattenholl, C. Maaser, T. A. Luger, M. Steinhoff, and S. W. Schneider, (2006). Tumor-derived matrix metalloproteinase-1 targets endothelial proteinase-activated receptor 1 promoting endothelial cell activation. *Cancer Res* **66** (15), 7766-7774.

109. Kaufmann, R., H. Schafberg, and G. Nowak, (1998). Proteinase-activated receptor-2-mediated signaling and inhibition of DNA synthesis in human pancreatic cancer cells. *Int J Pancreatol* **24** (2), 97-102.

110. Darmoul, D., J. C. Marie, H. Devaud, V. Gratio, and M. Laburthe, (2001). Initiation of human colon cancer cell proliferation by trypsin acting at protease-activated receptor-2. *Br J Cancer* **85** (5), 772-779.

111. Kaufmann, R., S. Patt, M. Zieger, R. Kraft, and G. Nowak, (1999). Presence of the proteinase-activated receptor-2 (PAR-2) in human brain tumor cells--trypsin- and SLIGRL-induced calcium response in primary cultured meningiomas. *Cancer Lett* **139** (1), 109-113.

112. Shi, X., B. Gangadharan, L. F. Brass, W. Ruf, and B. M. Mueller, (2004). Protease-activated receptors (PAR1 and PAR2) contribute to tumor cell motility and metastasis. *Mol Cancer Res* **2** (7), 395-402.

113. Ikeda, O., H. Egami, T. Ishiko, S. Ishikawa, H. Kamohara, H. Hidaka, S. Mita, and M. Ogawa, (2003). Expression of proteinase-activated receptor-2 in human pancreatic cancer: a possible relation to cancer invasion and induction of fibrosis. *Int J Oncol* **22** (2), 295-300.

114. Darmoul, D., V. Gratio, H. Devaud, and M. Laburthe, (2004). Protease-activated receptor 2 in colon cancer: trypsin-induced MAPK phosphorylation and cell proliferation are mediated by epidermal growth factor receptor transactivation. *J Biol Chem* **279** (20), 20927-20934.

115. Morris, D. R., Y. Ding, T. K. Ricks, A. Gullapalli, B. L. Wolfe, and J. Trejo, (2006). Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells. *Cancer Res* **66** (1), 307-314.

116. Liu, Y. and B. M. Mueller, (2006). Protease-activated receptor-2 regulates vascular endothelial growth factor expression in MDA-MB-231 cells via MAPK pathways. *Biochem Biophys Res Commun* **344** (4), 1263-1270.

117. Schaffner, F. and W. Ruf, (2009). Tissue factor and PAR2 signaling in the tumor microenvironment. *Arterioscler Thromb Vasc Biol* **29** (12), 1999-2004.

118. Jahan, I., J. Fujimoto, S. M. Alam, E. Sato, H. Sakaguchi, and T. Tamaya, (2007). Role of protease activated receptor-2 in tumor advancement of ovarian cancers. *Ann Oncol* **18** (9), 1506-1512.

119. D'Andrea, M. R., C. K. Derian, R. J. Santulli, and P. Andrade-Gordon, (2001). Differential expression of protease-activated receptors-1 and -2 in stromal fibroblasts of normal, benign, and malignant human tissues. *Am J Pathol* **158** (6), 2031-2041.

120. Elste, A. P. and I. Petersen, (2010). Expression of proteinase-activated receptor 1-4 (PAR 1-4) in human cancer. *J Mol Histol* **41** (2-3), 89-99.

121. Nelken, N. A., S. J. Soifer, J. O'Keefe, T. K. Vu, I. F. Charo, and S. R. Coughlin, (1992). Thrombin receptor expression in normal and atherosclerotic human arteries. *J Clin Invest* **90** (4), 1614-1621.

122. Mirza, H., V. Yatsula, and W. F. Bahou, (1996). The proteinase activated receptor-2 (PAR-2) mediates mitogenic responses in human vascular endothelial cells. *J Clin Invest* **97** (7), 1705-1714.

123. Kataoka, H., J. R. Hamilton, D. D. McKemy, E. Camerer, Y. W. Zheng, A. Cheng, C. Griffin, and S. R. Coughlin, (2003). Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells. *Blood* **102** (9), 3224-3231.

124. Coelho, A. M., V. Ossovskaya, and N. W. Bunnett, (2003). Proteinase-activated receptor-2: physiological and pathophysiological roles. *Curr Med Chem Cardiovasc Hematol Agents* **1** (1), 61-72.

125. Garcia, J. G., C. Patterson, C. Bahler, J. Aschner, C. M. Hart, and D. English, (1993). Thrombin receptor activating peptides induce Ca2+ mobilization, barrier dysfunction, prostaglandin synthesis, and platelet-derived growth factor mRNA expression in cultured endothelium. *J Cell Physiol* **156** (3), 541-549.

126. Hattori, R., K. K. Hamilton, R. D. Fugate, R. P. McEver, and P. J. Sims, (1989). Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. *J Biol Chem* **264** (14), 7768-7771.

127. Garcia, J. G., F. M. Pavalko, and C. E. Patterson, (1995). Vascular endothelial cell activation and permeability responses to thrombin. *Blood Coagul Fibrinolysis* **6** (7), 609-626.

128. Vouret-Craviari, V., C. Bourcier, E. Boulter, and E. van Obberghen-Schilling, (2002). Distinct signals via Rho GTPases and Src drive shape changes by thrombin and sphingosine-1-phosphate in endothelial cells. *J Cell Sci* **115** (Pt 12), 2475-2484.

129. Minami, T., A. Sugiyama, S. Q. Wu, R. Abid, T. Kodama, and W. C. Aird, (2004). Thrombin and phenotypic modulation of the endothelium. *Arterioscler Thromb Vasc Biol* **24** (1), 41-53.

130. Nystedt, S., V. Ramakrishnan, and J. Sundelin, (1996). The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor. *J Biol Chem* **271** (25), 14910-14915.

131. Luboshits, G., S. Shina, O. Kaplan, S. Engelberg, D. Nass, B. Lifshitz-Mercer, S. Chaitchik, I. Keydar, and A. Ben-Baruch, (1999). Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. *Cancer Res* **59** (18), 4681-4687.

132. Youngs, S. J., S. A. Ali, D. D. Taub, and R. C. Rees, (1997). Chemokines induce migrational responses in human breast carcinoma cell lines. *Int J Cancer* **71** (2), 257-266.

133. McNamara, C. A., I. J. Sarembock, L. W. Gimple, J. W. Fenton, 2nd, S. R. Coughlin, and G. K. Owens, (1993). Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. *J Clin Invest* **91** (1), 94-98.

134. Marutsuka, K., K. Hatakeyama, Y. Sato, A. Yamashita, A. Sumiyoshi, and Y. Asada, (2002). Protease-activated receptor 2 (PAR2) mediates vascular smooth muscle cell migration induced by tissue factor/factor VIIa complex. *Thromb Res* **107** (5), 271-276.

135. Bretschneider, E., R. Kaufmann, M. Braun, G. Nowak, E. Glusa, and K. Schror, (2001). Evidence for functionally active protease-activated receptor-4 (PAR-4) in human vascular smooth muscle cells. *Br J Pharmacol* **132** (7), 1441-1446.

136. Fukunaga, R., K. Hirano, M. Hirano, N. Niiro, J. Nishimura, Y. Maehara, and H. Kanaide, (2006). Upregulation of proteinase-activated receptors and hypercontractile responses precede development of arterial lesions after balloon injury. *Am J Physiol Heart Circ Physiol* **291** (5), H2388-2395.

137. Damiano, B. P., M. R. D'Andrea, L. de Garavilla, W. M. Cheung, and P. Andrade-Gordon, (1999). Increased expression of protease activated receptor-2 (PAR-2) in balloon-injured rat carotid artery. *Thromb Haemost* **81** (5), 808-814.

138. Napoli, C., F. de Nigris, J. L. Wallace, M. D. Hollenberg, G. Tajana, G. De Rosa, V. Sica, and G. Cirino, (2004). Evidence that protease activated receptor 2 expression is enhanced in human coronary atherosclerotic lesions. *J Clin Pathol* **57** (5), 513-516.

139. Cook, J. J., G. R. Sitko, B. Bednar, C. Condra, M. J. Mellott, D. M. Feng, R. F. Nutt, J. A. Shafer, R. J. Gould, and T. M. Connolly, (1995). An antibody against the exosite of the cloned thrombin receptor inhibits experimental arterial thrombosis in the African green monkey. *Circulation* **91** (12), 2961-2971.

140. Covic, L., Tchernychev B., Jacques, S., Kuliopulos, A. , in *Handbook of cell-penetrating peptides*, edited by Ulo Langel (Taylor & Francis, Boca Raton, 2007), pp. 245-257.

141. Derian, C. K., B. P. Damiano, M. F. Addo, A. L. Darrow, M. R. D'Andrea, M. Nedelman, H. C. Zhang, B. E. Maryanoff, and P. Andrade-Gordon, (2003). Blockade of the thrombin receptor protease-activated receptor-1 with a small-molecule antagonist prevents thrombus formation and vascular occlusion in nonhuman primates. *J Pharmacol Exp Ther* **304** (2), 855-861.

142. Chintala, M., J. Strony, B. Yang, S. Kurowski, and Q. Li, (2010). SCH 602539, a protease-activated receptor-1 antagonist, inhibits thrombosis alone and in combination with cangrelor in a Folts model of arterial thrombosis in cynomolgus monkeys. *Arterioscler Thromb Vasc Biol* **30** (11), 2143-2149.

143. Chong, A. J., T. H. Pohlman, C. R. Hampton, A. Shimamoto, N. Mackman, and E. D. Verrier, (2003). Tissue factor and thrombin mediate myocardial ischemia-reperfusion injury. *Ann Thorac Surg* **75** (2), S649-655.

144. Eckart, R. E., C. F. Uyehara, E. A. Shry, J. L. Furgerson, and R. A. Krasuski, (2004). Matrix metalloproteinases in patients with myocardial infarction and percutaneous revascularization. *J Interv Cardiol* **17** (1), 27-31.

145. Strande, J. L., A. Hsu, J. Su, X. Fu, G. J. Gross, and J. E. Baker, (2007). SCH 79797, a selective PAR1 antagonist, limits myocardial ischemia/reperfusion injury in rat hearts. *Basic Res Cardiol* **102** (4), 350-358.

146. Napoli, C., C. Cicala, J. L. Wallace, F. de Nigris, V. Santagada, G. Caliendo, F. Franconi, L. J. Ignarro, and G. Cirino, (2000). Protease-activated receptor-2 modulates myocardial ischemia-reperfusion injury in the rat heart. *Proc Natl Acad Sci U S A* **97** (7), 3678-3683.

147. Napoli, C., F. De Nigris, C. Cicala, J. L. Wallace, G. Caliendo, M. Condorelli, V. Santagada, and G. Cirino, (2002). Protease-activated receptor-2 activation improves efficiency of experimental ischemic preconditioning. *Am J Physiol Heart Circ Physiol* **282** (6), H2004-2010.

148. Antoniak, S., M. Rojas, D. Spring, T. Bullard, E. Verrier, B. Blaxall, N. Mackman, and R. Pawlinski, (2010). Protease-Activated Receptor 2 Deficiency Reduces Cardiac Ischemia/Reperfusion Injury. *Arterioscler Thromb Vasc Biol*. 30:2136-2142

149. Milia, A. F., M. B. Salis, T. Stacca, A. Pinna, P. Madeddu, M. Trevisani, P. Geppetti, and C. Emanueli, (2002). Protease-activated receptor-2 stimulates angiogenesis and accelerates hemodynamic recovery in a mouse model of hindlimb ischemia. *Circ Res* **91** (4), 346-352.

150. Strande, J. L., A. Hsu, J. Su, X. Fu, G. J. Gross, and J. E. Baker, (2008). Inhibiting protease-activated receptor 4 limits myocardial ischemia/reperfusion injury in rat hearts by unmasking adenosine signaling. *J Pharmacol Exp Ther* **324** (3), 1045-1054.

151. MacLeod, D. C., B. H. Strauss, M. de Jong, J. Escaned, V. A. Umans, R. J. van Suylen, A. Verkerk, P. J. de Feyter, and P. W. Serruys, (1994). Proliferation and extracellular matrix synthesis of smooth muscle cells cultured from human coronary atherosclerotic and restenotic lesions. *J Am Coll Cardiol* **23** (1), 59-65.

152. Asahara, T. and A. Kawamoto, (2004). Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol* **287** (3), C572-579.

153. Sahara, M., M. Sata, T. Morita, K. Nakamura, Y. Hirata, and R. Nagai, (2007). Diverse contribution of bone marrow-derived cells to vascular remodeling associated with pulmonary arterial hypertension and arterial neointimal formation. *Circulation* **115** (4), 509-517.

154. Humbert, M., N. W. Morrell, S. L. Archer, K. R. Stenmark, M. R. MacLean, I. M. Lang, B. W. Christman, E. K. Weir, O. Eickelberg, N. F. Voelkel, and M. Rabinovitch, (2004). Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* **43** (12 Suppl S), 13S-24S.

155. Mandegar, M., Y. C. Fung, W. Huang, C. V. Remillard, L. J. Rubin, and J. X. Yuan, (2004). Cellular and molecular mechanisms of pulmonary vascular remodeling: role in the development of pulmonary hypertension. *Microvasc Res* **68** (2), 75-103.

156. Cheung, W. M., M. R. D'Andrea, P. Andrade-Gordon, and B. P. Damiano, (1999). Altered vascular injury responses in mice deficient in protease-activated receptor-1. *Arterioscler Thromb Vasc Biol* **19** (12), 3014-3024.

157. Kumar, A. and V. Lindner, (1997). Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler Thromb Vasc Biol* **17** (10), 2238-2244.

158. Takada, M., H. Tanaka, T. Yamada, O. Ito, M. Kogushi, M. Yanagimachi, T. Kawamura, T. Musha, F. Yoshida, M. Ito, H. Kobayashi, S. Yoshitake, and I. Saito, (1998). Antibody to thrombin receptor inhibits neointimal smooth muscle cell accumulation without causing inhibition of platelet aggregation or altering hemostatic parameters after angioplasty in rat. *Circ Res* **82** (9), 980-987.

159. Chieng-Yane, P., A. Bocquet, R. Letienne, T. Bourbon, S. Sablayrolles, M. Perez, S. Hatem, A. M. Lompre, B. Le Grand, and M. David-Dufilho, (2010). Protease activated Receptor-1 antagonist, F 16618 reduces arterial restenosis by down-regulation of TNF{alpha} and MMP7 expression, and migration and proliferation of vascular smooth muscle cells. *J Pharmacol Exp Ther*.

160. Vergnolle, N., (2009). Protease-activated receptors as drug targets in inflammation and pain. *Pharmacol Ther* **123** (3), 292-309.

161. Vergnolle, N., L. Cellars, A. Mencarelli, G. Rizzo, S. Swaminathan, P. Beck, M. Steinhoff, P. Andrade-Gordon, N. W. Bunnett, M. D. Hollenberg, J. L. Wallace, G. Cirino, and S. Fiorucci, (2004). A role for proteinase-activated receptor-1 in inflammatory bowel diseases. *J Clin Invest* **114** (10), 1444-1456.

162. Howell, D. C., R. H. Johns, J. A. Lasky, B. Shan, C. J. Scotton, G. J. Laurent, and R. C. Chambers, (2005). Absence of proteinase-activated receptor-1 signaling affords protection from bleomycin-induced lung inflammation and fibrosis. *Am J Pathol* **166** (5), 1353-1365.

163. Yang, Y. H., P. Hall, C. B. Little, A. J. Fosang, G. Milenkovski, L. Santos, J. Xue, P. Tipping, and E. F. Morand, (2005). Reduction of arthritis severity in protease-activated receptor-deficient mice. *Arthritis Rheum* **52** (4), 1325-1332.

164. Cirino, G., C. Cicala, M. R. Bucci, L. Sorrentino, J. M. Maraganore, and S. R. Stone, (1996). Thrombin functions as an inflammatory mediator through activation of its receptor. *J Exp Med* **183** (3), 821-827.

165. Vergnolle, N., C. K. Derian, M. R. D'Andrea, M. Steinhoff, and P. Andrade-Gordon, (2002). Characterization of thrombin-induced leukocyte rolling and adherence: a potential proinflammatory role for proteinase-activated receptor-4. *J Immunol* **169** (3), 1467-1473.

166. Houle, S., M. D. Papez, M. Ferazzini, M. D. Hollenberg, and N. Vergnolle, (2005). Neutrophils and the kallikrein-kinin system in proteinase-activated receptor 4-mediated inflammation in rodents. *Br J Pharmacol* **146** (5), 670-678.

167. Hollenberg, M. D., M. Saifeddine, S. Sandhu, S. Houle, and N. Vergnolle, (2004). Proteinase-activated receptor-4: evaluation of tethered ligand-derived peptides as probes for receptor function and as inflammatory agonists in vivo. *Br J Pharmacol* **143** (4), 443-454.

168. Howells, G. L., M. G. Macey, C. Chinni, L. Hou, M. T. Fox, P. Harriott, and S. R. Stone, (1997). Proteinase-activated receptor-2: expression by human neutrophils. *J Cell Sci* **110 (Pt 7)**, 881-887.

169. Shpacovitch, V. M., G. Varga, A. Strey, M. Gunzer, F. Mooren, J. Buddenkotte, N. Vergnolle, C. P. Sommerhoff, S. Grabbe, V. Gerke, B. Homey, M. Hollenberg, T. A. Luger, and M. Steinhoff, (2004). Agonists of proteinase-activated receptor-2 modulate human neutrophil cytokine secretion, expression of cell adhesion molecules, and migration within 3-D collagen lattices. *J Leukoc Biol* **76** (2), 388-398.

170. Carvalho, R. F., G. Nilsson, and I. T. Harvima, (2010). Increased mast cell expression of PAR-2 in skin inflammatory diseases and release of IL-8 upon PAR-2 activation. *Exp Dermatol* **19** (2), 117-122.

171. Schmidlin, F., S. Amadesi, K. Dabbagh, D. E. Lewis, P. Knott, N. W. Bunnett, P. R. Gater, P. Geppetti, C. Bertrand, and M. E. Stevens, (2002). Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. *J Immunol* **169** (9), 5315-5321.

172. Lindner, J. R., M. L. Kahn, S. R. Coughlin, G. R. Sambrano, E. Schauble, D. Bernstein, D. Foy, A. Hafezi-Moghadam, and K. Ley, (2000). Delayed onset of inflammation in protease-activated receptor-2-deficient mice. *J Immunol* **165** (11), 6504-6510.

173. Major, C. D., R. J. Santulli, C. K. Derian, and P. Andrade-Gordon, (2003). Extracellular mediators in atherosclerosis and thrombosis: lessons from thrombin receptor knockout mice. *Arterioscler Thromb Vasc Biol* **23** (6), 931-939.

174. Vergnolle, N., M. D. Hollenberg, K. A. Sharkey, and J. L. Wallace, (1999). Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw. *Br J Pharmacol* **127** (5), 1083-1090.

175. Kawabata, A., R. Kuroda, T. Minami, K. Kataoka, and M. Taneda, (1998). Increased vascular permeability by a specific agonist of protease-activated receptor-2 in rat hindpaw. *Br J Pharmacol* **125** (3), 419-422.

176. Shpacovitch, V. M., S. Seeliger, M. Huber-Lang, S. Balkow, M. Feld, M. D. Hollenberg, V. J. Sarma, P. A. Ward, A. Strey, V. Gerke, C. P. Sommerhoff, N. Vergnolle, and M. Steinhoff, (2007). Agonists of proteinase-activated receptor-2 affect transendothelial migration and apoptosis of human neutrophils. *Exp Dermatol* **16** (10), 799-806.

177. Moraes, T. J., R. Martin, J. D. Plumb, E. Vachon, C. M. Cameron, A. Danesh, D. J. Kelvin, W. Ruf, and G. P. Downey, (2008). Role of PAR2 in murine pulmonary pseudomonal infection. *Am J Physiol Lung Cell Mol Physiol* **294** (2), L368-377.

178. Morello, S., V. Vellecco, F. Roviezzo, P. Maffia, S. Cuzzocrea, G. Cirino, and C. Cicala, (2005). A protective role for proteinase activated receptor 2 in airways of lipopolysaccharide-treated rats. *Biochem Pharmacol* **71** (1-2), 223-230.

179. Xiang, Y., K. Masuko-Hongo, T. Sekine, H. Nakamura, K. Yudoh, K. Nishioka, and T. Kato, (2006). Expression of proteinase-activated receptors (PAR)-2 in articular chondrocytes is modulated by IL-1beta, TNF-alpha and TGF-beta. *Osteoarthritis Cartilage* **14** (11), 1163-1173.

180. Kelso, E. B., W. R. Ferrell, J. C. Lockhart, I. Elias-Jones, T. Hembrough, L. Dunning, J. A. Gracie, and I. B. McInnes, (2007). Expression and proinflammatory role of proteinase-activated receptor 2 in rheumatoid synovium: ex vivo studies using a novel proteinase-activated receptor 2 antagonist. *Arthritis Rheum* **56** (3), 765-771.

181. Ferrell, W. R., J. C. Lockhart, E. B. Kelso, L. Dunning, R. Plevin, S. E. Meek, A. J. Smith, G. D. Hunter, J. S. McLean, F. McGarry, R. Ramage, L. Jiang, T. Kanke, and J. Kawagoe, (2003). Essential role for proteinase-activated receptor-2 in arthritis. *J Clin Invest* **111** (1), 35-41.

182. Ferrell, W. R., E. B. Kelso, J. C. Lockhart, R. Plevin, and I. B. McInnes, (2010). Protease-activated receptor 2: a novel pathogenic pathway in a murine model of osteoarthritis. *Ann Rheum Dis*.

183. Palmer, H. S., E. B. Kelso, J. C. Lockhart, C. P. Sommerhoff, R. Plevin, F. G. Goh, and W. R. Ferrell, (2007). Protease-activated receptor 2 mediates the proinflammatory effects of synovial mast cells. *Arthritis Rheum* **56** (11), 3532-3540.

184. Milner, J. M., A. Patel, R. K. Davidson, T. E. Swingler, A. Desilets, D. A. Young, E. B. Kelso, S. T. Donell, T. E. Cawston, I. M. Clark, W. R. Ferrell, R. Plevin, J. C. Lockhart, R. Leduc, and A. D. Rowan, (2010). Matriptase is a novel initiator of cartilage matrix degradation in osteoarthritis. *Arthritis Rheum* **62** (7), 1955-1966.

185. Steinhoff, M., C. U. Corvera, M. S. Thoma, W. Kong, B. E. McAlpine, G. H. Caughey, J. C. Ansel, and N. W. Bunnett, (1999). Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. *Exp Dermatol* **8** (4), 282-294.

186. Tsujii, K., T. Andoh, J. B. Lee, and Y. Kuraishi, (2008). Activation of proteinase-activated receptors induces itch-associated response through histamine-dependent and -independent pathways in mice. *J Pharmacol Sci* **108** (3), 385-388.

187. Shimada, S. G., K. A. Shimada, and J. G. Collins, (2006). Scratching behavior in mice induced by the proteinase-activated receptor-2 agonist, SLIGRL-NH2. *Eur J Pharmacol* **530** (3), 281-283.

188. He, S., A. Aslam, M. D. Gaca, Y. He, M. G. Buckley, M. D. Hollenberg, and A. F. Walls, (2004). Inhibitors of tryptase as mast cell-stabilizing agents in the human airways: effects of tryptase and other agonists of proteinase-activated receptor 2 on histamine release. *J Pharmacol Exp Ther* **309** (1), 119-126.

189. Steinhoff, M., N. Vergnolle, S. H. Young, M. Tognetto, S. Amadesi, H. S. Ennes, M. Trevisani, M. D. Hollenberg, J. L. Wallace, G. H. Caughey, S. E. Mitchell, L. M. Williams, P. Geppetti, E. A. Mayer, and N. W. Bunnett, (2000). Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat Med* **6** (2), 151-158.

190. Vergnolle, N., N. W. Bunnett, K. A. Sharkey, V. Brussee, S. J. Compton, E. F. Grady, G. Cirino, N. Gerard, A. I. Basbaum, P. Andrade-Gordon, M. D. Hollenberg, and J. L. Wallace, (2001). Proteinase-activated receptor-2 and hyperalgesia: A novel pain pathway. *Nat Med* **7** (7), 821-826.

191. Amadesi, S., G. S. Cottrell, L. Divino, K. Chapman, E. F. Grady, F. Bautista, R. Karanjia, C. Barajas-Lopez, S. Vanner, N. Vergnolle, and N. W. Bunnett, (2006). Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cepsilon- and A-dependent mechanisms in rats and mice. *J Physiol* **575** (Pt 2), 555-571.

192. Yuan, H., X. Zhu, S. Zhou, Q. Chen, X. Ma, X. He, M. Tian, and X. Shi, (2010). Role of mast cell activation in inducing microglial cells to release neurotrophin. *J Neurosci Res* **88** (6), 1348-1354.

193. Lam, D. K. and B. L. Schmidt, (2010). Serine proteases and protease-activated receptor 2-dependent allodynia: a novel cancer pain pathway. *Pain* **149** (2), 263-272.

194. Tressel, S. L., G. Koukos, B. Tchernychev, S. L. Jacques, L. Covic, and A. Kuliopulos, (2010). Pharmacology, Biodistribution, and Efficacy of GPCR-Based Pepducins in Disease Models. *Methods Mol Biol* **683**, 259-275.

195. Kaneider, N. C., A. Agarwal, A. J. Leger, and A. Kuliopulos, (2005). Reversing systemic inflammatory response syndrome with chemokine receptor pepducins. *Nat Med* **11** (6), 661-665.

196. Tchernychev, B., Y. Ren, P. Sachdev, J. M. Janz, L. Haggis, A. O'Shea, E. McBride, R. Looby, Q. Deng, T. McMurry, M. A. Kazmi, T. P. Sakmar, S. Hunt, 3rd, and K. E. Carlson, (2010). Discovery of a CXCR4 agonist pepducin that

mobilizes bone marrow hematopoietic cells. *Proc Natl Acad Sci U S A* **107** (51), 22255-22259.

197. Kuliopulos, A., L. Covic, S. K. Seeley, P. J. Sheridan, J. Helin, and C. E. Costello, (1999). Plasmin desensitization of the PAR1 thrombin receptor: kinetics, sites of truncation, and implications for thrombolytic therapy. *Biochemistry* **38** (14), 4572-4585.

198. Ross, R. and J. A. Glomset, (1976). The pathogenesis of atherosclerosis (first of two parts). *N Engl J Med* **295** (7), 369-377.

199. Owens, G. K., M. S. Kumar, and B. R. Wamhoff, (2004). Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* **84** (3), 767-801.

200. Aikawa, M., E. Rabkin, S. J. Voglic, H. Shing, R. Nagai, F. J. Schoen, and P. Libby, (1998). Lipid lowering promotes accumulation of mature smooth muscle cells expressing smooth muscle myosin heavy chain isoforms in rabbit atheroma. *Circ Res* **83** (10), 1015-1026.

201. Rzucidlo, E. M., K. A. Martin, and R. J. Powell, (2007). Regulation of vascular smooth muscle cell differentiation. *J Vasc Surg* **45 Suppl A**, A25-32.

202. Liu, X., H. Wu, M. Byrne, S. Krane, and R. Jaenisch, (1997). Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. *Proc Natl Acad Sci U S A* **94** (5), 1852-1856.

203. Burger, J. A. and T. J. Kipps, (2006). CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood* **107** (5), 1761-1767.

204. Faber, A., C. Roderburg, F. Wein, R. Saffrich, A. Seckinger, K. Horsch, A. Diehlmann, D. Wong, G. Bridger, V. Eckstein, A. D. Ho, and W. Wagner, (2007). The many facets of SDF-1alpha, CXCR4 agonists and antagonists on hematopoietic progenitor cells. *J Biomed Biotechnol* **2007** (3), 26065.

205. Ceradini, D. J., A. R. Kulkarni, M. J. Callaghan, O. M. Tepper, N. Bastidas, M. E. Kleinman, J. M. Capla, R. D. Galiano, J. P. Levine, and G. C. Gurtner, (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med* **10** (8), 858-864.

206. McCoy, K. L., S. F. Traynelis, and J. R. Hepler, (2010). PAR1 and PAR2 couple to overlapping and distinct sets of G proteins and linked signaling pathways to differentially regulate cell physiology. *Mol Pharmacol* **77** (6), 1005-1015.

207. Tennant, G. M., R. M. Wadsworth, and S. Kennedy, (2008). PAR-2 mediates increased inflammatory cell adhesion and neointima formation following vascular injury in the mouse. *Atherosclerosis* **198** (1), 57-64.

208. Trivedi, V., A. Boire, B. Tchernychev, N. C. Kaneider, A. J. Leger, K. O'Callaghan, L. Covic, and A. Kuliopulos, (2009). Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic ligand site. *Cell* **137** (2), 332-343.

209. Noorbakhsh, F., S. Tsutsui, N. Vergnolle, L. A. Boven, N. Shariat, M. Vodjgani, K. G. Warren, P. Andrade-Gordon, M. D. Hollenberg, and C. Power, (2006). Proteinase-activated receptor 2 modulates neuroinflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. *J Exp Med* **203** (2), 425-435.

210. Cenac, N., A. M. Coelho, C. Nguyen, S. Compton, P. Andrade-Gordon, W. K. MacNaughton, J. L. Wallace, M. D. Hollenberg, N. W. Bunnett, R. Garcia-Villar, L. Bueno, and N. Vergnolle, (2002). Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. *Am J Pathol* **161** (5), 1903-1915.

211. Dai, Y., S. Wang, M. Tominaga, S. Yamamoto, T. Fukuoka, T. Higashi, K. Kobayashi, K. Obata, H. Yamanaka, and K. Noguchi, (2007). Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. *J Clin Invest* **117** (7), 1979-1987.

212. Molino, M., E. S. Barnathan, R. Numerof, J. Clark, M. Dreyer, A. Cumashi, J. A. Hoxie, N. Schechter, M. Woolkalis, and L. F. Brass, (1997). Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J Biol Chem* **272** (7), 4043-4049.

213. Caughey, G. H., (2007). Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev* **217**, 141-154.

214. Wielders, S. J., A. Bennaghmouch, C. P. Reutelingsperger, E. M. Bevers, and T. Lindhout, (2007). Anticoagulant and antithrombotic properties of intracellular protease-activated receptor antagonists. *J. Thromb. Haemost.* **5**, 571-576.

215. Teller, D. C., T. Okada, C. A. Behnke, K. Palczewski, and R. E. Stenkamp, (2001). Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). *Biochemistry* **40** (26), 7761-7772.

216. Wiedermann, C. J., M. Niedermuhlbichler, H. Braunsteiner, and C. J. Widermann, (1992). Priming of polymorphonuclear neutrophils by atrial natriuretic peptide in vitro. *J Clin Invest* **89** (5), 1580-1586.

217. Breitwieser, G. E., (2004). G protein-coupled receptor oligomerization: implications for G protein activation and cell signaling. *Circ Res* **94** (1), 17-27.

218. Rovati, G. E., V. Capra, and R. R. Neubig, (2007). The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* **71** (4), 959-964.

219. Larsen, K. S., H. Ostergaard, O. H. Olsen, J. R. Bjelke, W. Ruf, and L. C. Petersen, (2010). Engineering of substrate selectivity for tissue factor.factor VIIa complex signaling through protease-activated receptor 2. *J Biol Chem* **285** (26), 19959-19966.

220. Kjelsberg, M. A., S. Cotecchia, J. Ostrowski, M. G. Caron, and R. J. Lefkowitz, (1992). Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation. *J Biol Chem* **267** (3), 1430-1433.

221. Beinborn, M., Y. Ren, M. Blaker, C. Chen, and A. S. Kopin, (2004). Ligand function at constitutively active receptor mutants is affected by two distinct yet interacting mechanisms. *Mol Pharmacol* **65** (3), 753-760.

222. Day, S. M., J. C. Lockhart, W. R. Ferrell, and J. S. McLean, (2004). Divergent roles of nitrergic and prostanoid pathways in chronic joint inflammation. *Ann Rheum Dis* **63** (12), 1564-1570.

223. Carvalho, M., C. Benjamim, F. Santos, S. Ferreira, and F. Cunha, (2005). Effect of mast cells depletion on the failure of neutrophil migration during sepsis. *Eur J Pharmacol* **525** (1-3), 161-169.

224. Pitman, N., D. L. Asquith, G. Murphy, F. Y. Liew, and I. B. McInnes, (2010). Collagen-induced arthritis is not impaired in mast cell-deficient mice. *Ann Rheum Dis*. [Epublish ahead of print]

225. Corr, M. and B. Crain, (2002). The role of FcgammaR signaling in the K/B x N serum transfer model of arthritis. *J Immunol* **169** (11), 6604-6609.

226. Loew, D., C. Perrault, M. Morales, S. Moog, C. Ravanat, S. Schuhler, R. Arcone, C. Pietropaolo, J. P. Cazenave, A. van Dorsselaer, and F. Lanza, (2000). Proteolysis of the exodomain of recombinant protease-activated receptors: prediction of receptor activation or inactivation by MALDI mass spectrometry. *Biochemistry* **39** (35), 10812-10822.

227. Camerer, E., W. Huang, and S. R. Coughlin, (2000). Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc Natl Acad Sci U S A* **97** (10), 5255-5260.

228. Prasit, P., Z. Wang, C. Brideau, C. C. Chan, S. Charleson, W. Cromlish, D. Ethier, J. F. Evans, A. W. Ford-Hutchinson, J. Y. Gauthier, R. Gordon, J. Guay, M. Gresser, S. Kargman, B. Kennedy, Y. Leblanc, S. Leger, J. Mancini, G. P. O'Neill, M. Ouellet, M. D. Percival, H. Perrier, D. Riendeau, I. Rodger, R. Zamboni, and et al., (1999). The discovery of rofecoxib, [MK 966, Vioxx, 4-(4'-methylsulfonylphenyl)-3-phenyl-2(5H)-furanone], an orally active cyclooxygenase-2-inhibitor. *Bioorg Med Chem Lett* **9** (13), 1773-1778.

229. Trelle, S., S. Reichenbach, S. Wandel, P. Hildebrand, B. Tschannen, P. M. Villiger, M. Egger, and P. Juni, (2011). Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *BMJ* **342**, c7086.

230. Bijlsma, J. W., (2010). Patient benefit-risk in arthritis--a rheumatologist's perspective. *Rheumatology (Oxford)* **49 Suppl 2**, ii11-17.

231. Wang, H., S. Wen, N. W. Bunnett, R. Leduc, M. D. Hollenberg, and W. K. MacNaughton, (2008). Proteinase-activated receptor-2 induces cyclooxygenase-2 expression through beta-catenin and cyclic AMP-response element-binding protein. *J Biol Chem* **283** (2), 809-815.