

**DELINEATING AND RATIONALLY PERTURBING SIGNALING MECHANISMS**

**INVOLVED IN METASTASIS**

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**Abstract**  
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**Delineating and rationally perturbing signaling mechanisms**  
**involved in metastasis**  
**(Under the direction of Drs. JoAnn Trejo and Pilar Blancafort)**

Metastasis is the dissemination of tumor cells from the primary locus of formation to other organs. During migration from the primary tumor, tumor cells need to traverse the vasculature, a process termed extravasation. Extravasation is a critical step in the metastatic cascade and nevertheless a poorly understood phenomenon. Endothelial cells form a barrier, which prevents cells and plasma constituents from moving into interstitial tissues. The disruption of the endothelial barrier leads to increased barrier permeability resulting in enhanced cancer cell extravasation.

Protease-activated receptor-1 (PAR1) is a G protein-coupled receptor uniquely activated by proteases. PAR1 increases endothelial permeability when activated by the protease thrombin. Strikingly, PAR1 signaling can also mediate decreases in endothelial permeability when activated by activated protein C (APC), an anti-coagulant protease. In the first two chapters of this dissertation I examined the mechanism responsible for protease-selective signaling by PAR1. I specifically examined the effect of APC and thrombin on the activation of RhoA and Rac1 that differentially regulate endothelial permeability. In chapter 2 of this dissertation I also investigated whether compartmentalization of PAR1 in caveolae was critical for APC selective signaling and I demonstrated that caveolae are required for APC-selective signaling to Rac1 activation and endothelial barrier protection. Furthermore, in chapter 3 of this dissertation I asked whether

APC protection from thrombin-induced increased permeability involved desensitization of PAR1. And I reported that APC induces PAR1 phosphorylation and desensitizes endothelial cells to thrombin.

The metastatic process also requires degradation of extracellular matrices by proteases present in the tumor microenvironment, especially serine proteases. Inhibition of these proteases has remarkable therapeutic effects against tumor progression.

Maspin is an atypical member of the family of serine proteases inhibitors. Maspin inhibits the serine protease urokinase activated plasminogen and suppresses tumor growth and metastasis. Interestingly, maspin is silenced by epigenetic mechanisms in cancer cells. In chapter 4 of my dissertation I used artificial transcription factors (ATFs) as a novel strategy to re-activate maspin in breast cancer cells. I showed that re-expression of maspin by ATFs leads to reduction of tumor growth and metastasis in an *in vivo* xenograft animal model.

### **Dedication**

To my parents Assunta Falco and Alfonso Russo who have always supported me through my life and career. I am especially thankful for the confidence they have always had in me and for their understanding of all my choices. To my husband Saverio Gentile and my daughter Vittoria Gentile, I am grateful for their love and patience as I pursue my dreams.

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## Preface

The work presented in Chapters 1 of this dissertation has been published as a review in the journal “Molecular Intervention”:

Proteases display biased agonism at protease-activated receptors: location matters!

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The work presented in Chapters 2 and 3 has been published in the journal “Proceedings of the National Academy of Sciences”.

Caveolae are required for protease-selective signaling by protease-activated receptor-1.

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## List of abbreviation

$\alpha$ , alpha

$\beta$ , beta

AP, adaptor protein

AR, adrenergic receptor

ADAM, proteins containing a disintegrin and a metalloprotease domain

APC, activated protein C

$\text{Ca}^{2+}$ , calcium

ATF, artificial transcription factor

ATP, adenosine triphosphate

BLI, bioluminescence

CAV1, caveolin-1

CCR, chemokine receptor

DBD, DNA binding domain

Dox, doxycycline

ECM, extracellular matrix

EPCR, endothelial protein C receptor

EGFR/Erb/HER, epidermal growth factor receptor

ERK, extracellular signal regulated kinase

Fig, figure

FGF, fibroblast growth factor

GPCR, G protein coupled receptor  
GRK, G-protein dependent kinase  
GST, glutathione-S-transferase  
GTP, guanosine triphosphate  
HDAC, histone deacetylase  
HT2AR, hydroxytryptamine 2A receptor  
IFN, interferon  
MAPK, mitogen-activated protein kinase  
MMP, matrix metallo-protease  
MOR,  $\mu$ -opiate receptor  
PAK, p21-activated kinase  
PAR, protease-activated receptor  
PBD, p21-activated kinase binding domain  
PI, phosphoinositides  
PLC, phospholipase C  
RBD, rhotekin binding domain  
SAHA, suberoylnide hydroxamic acid  
SDS, sodium dodecyl sulfate  
TF, tissue factor  
TGF- $\alpha$ , transforming growth factor  
Th, thrombin  
TM, thrombomodulin  
TPA, tissue plasminogen activator  
TSA, trichostatin A

uPA, urokinase-plasminogen activator

UTP, uridine triphosphate

VEGF, vascular endothelial growth factor

ZF, zinc finger.

## CHAPTER 1. INTRODUCTION

### 1.1 Protease-activated receptor 1 implications in tumor growth and metastasis

Metastasis involves the spread of cancer cells from a primary tumor site of formation to distant organs in the body. Metastasis is the leading cause of cancer patient deaths. Invasion is a critical step in the metastatic cascade and involves the degradation of extracellular matrix (ECM) proteins. ECM degradation is mediated by a network of pericellular proteolytic enzymes, and is tightly controlled by protease activators and protease inhibitors (Jedinak A. and Malnar T. 2005).

An important group of receptors that elicit cellular responses to extracellular proteases is the family of G-protein coupled protease-activated receptors (PARs). Protease-activated receptor 1 (PAR1) is the prototype of the PAR family and responds to the coagulant protease thrombin (Th). PAR1 is overexpressed in aggressive melanoma, colon cancer, prostate cancer and invasive breast cancer (Even-Ram S. 1998; Darmoul D. 2004). Overexpression of PAR1 results in NIH 3T3 fibroblasts transformation (Martin C.B. 2001) and induces hyperplasia of mammary gland epithelial cells (Nicolai S. and Blasi F. 2003).

In addition PARs can also be activated by tumor-generated proteases. Tumors are replete with proteases, including urokinase-plasminogen activator (uPA) and matrix metalloproteases (MMPs). Tumor cells up-regulate uPA expression, which is associated



with poor prognosis (Nicolai S. and Blasi F. 2003). UPA binds to its cell surface receptor uPAR (Bissell M.J. and Radisky D. 2001) and cleaves plasminogen to generate plasmin. Plasmin can proteolytically activate PAR1 (Kuliopulos A. 1999). Plasmin also degrades extracellular matrix proteins and cleaves and activates MMPs. The tumor microenvironment is also enriched with several types of MMPs, including MMP-1, which can cleave and activate PAR1 (Boire A. 2005).

The mitogenic activity of PAR1, induced upon thrombin activation, is associated with prolonged activation of extracellular signal-regulated kinase 1,2 (ERK1,2) (Kahan C. 1992; Trejo J. 1996). The sustained increase in ERK1,2 signaling stimulated by thrombin not only promotes cell cycle progression but also contributes to cellular transformation, migration and survival. Our recent work demonstrated that proteolytic activation of PAR1 by thrombin causes sustained ERK1,2 signaling and promotes breast carcinoma cell invasion via persistent transactivation of ErbB family members (Arora P. 2008). The ErbB family of receptor tyrosine kinases includes epidermal growth factor (EGF) receptor (EGFR)/ErbB1, ErbB2/HER2, ErbB3 and ErbB4. ErbB family members undergo ligand-induced dimerization, which provokes intrinsic kinase activation, transphosphorylation of cytoplasmic tyrosine residues and recruitment of signaling molecules. ErbB2/HER2 is overexpressed in ~20–30% of human invasive breast cancers and is correlated with increased metastatic potential and decreased patient survival (Cobleigh M.A. 1999). Transactivation of EGFR by GPCRs results from the activation of the proteins containing a disintegrin and a metalloprotease domain (ADAMs) and/or matrix metalloprotease (MMPs) family members, which release membrane-anchored ligands such as heparin-binding-EGF or transforming growth factor- $\alpha$  (TGF- $\alpha$ ) (Prenzel N. 1999; Gschwind A. 2003).

PAR1 is also expressed in the vascular endothelial cells (Coughlin S.R. 2005) (Fig. 1). Endothelial cells lining the intima of blood vessels form a functional barrier that controls exchange of proteins and cells between blood and the interstitial space. Disruption of the endothelial barrier contributes to pathological conditions such as vascular leakage, septic shock and cancer cell extravasation. Conversely, preservation of a tight intact endothelial barrier prevents processes that contribute to such pathological conditions.

In response to thrombin, PAR1 promotes pro-inflammatory responses and increases permeability of the endothelial barrier (Bogatcheva N.V. 2002; Feistritzer C. 2005). Under normal conditions, pro-inflammatory responses associated with thrombin are counteracted by activated protein C (APC). APC inactivates up-stream coagulant proteases diminishing thrombin generation and elicits anti-inflammatory responses that preserve the integrity of the endothelial barrier (Feistritzer C. 2005). Previous studies showed that the opposing effects of thrombin and APC on endothelial cell permeability are both mediated by the activation of PAR1 (Feistritzer C. 2005). Moreover the anti-coagulant and anti-inflammatory properties associated with APC have been shown to be effective in treatment of septic shock, a severe inflammatory disorder (Baillie J.K. 2007). Recombinant APC is the only drug approved by FDA to treat severe sepsis. However, the molecular mechanisms by which APC and thrombin elicit distinct cellular responses through the same receptor remain poorly understood. Therefore, understanding the role of PAR1 in APC-signaling may provide new insight into the molecular mechanism of inflammatory disorders and cancer cell extravasation. The observation that different ligands acting at the same receptor can elicit distinct signaling responses has been reported for many GPCRs and is a process termed “functional selectivity” or “biased agonism” (Urban J.D. 2007). The molecular basis of functional selectivity appears to involve ligand-induced stabilization of distinct active

receptor conformations. New studies now indicate that compartmentalization of GPCRs within membrane microdomains also facilitate stabilization of distinct active receptor conformations and promote receptor coupling to specific signaling effectors (Zheng H. 2008). The proteolytic irreversible activation of PARs, which results in the unmasking of a tethered ligand, is distinct from most normal ligand-activated GPCRs and questioned whether proteases displayed biased agonism at PARs. The studies described in chapter 2 and 3 of this dissertation reveals that compartmentalization of PAR1 in caveolae is critical for protease-selective activation and signaling by PAR1 (Russo A. 2009).

## **1.2 The family of PARs**

PARs are a unique class of GPCRs that signal in response to extracellular proteases. There are four PARs encoded by distinct genes in the mammalian genome. The discovery of PAR1 in 1991 resulted from a search for a receptor that conferred thrombin signaling and was originally dubbed the “thrombin receptor” (Vu T.K. 1991a). PAR1 is considered the family prototype and is the predominant mediator of thrombin signaling in most cell types. PAR3 and PAR4 were later discovered and also shown to elicit cellular responses to thrombin (Ishihara H. 1997) (Xu W.F. 1998). PAR2 was identified in a mouse genomic library screen using probes homologous to the transmembrane regions of the substance K receptor (Nystedt S. 1994). Unlike other PARs, PAR2 is activated by trypsin-like serine proteases but not by thrombin.

Thrombin, the main effector protease of the coagulation cascade, drives fibrin deposition and signals through PARs to promote platelet activation, which is critical for hemostasis and thrombosis (Coughlin S.R. 2005). Activation of PARs by thrombin also contributes to inflammatory and proliferative responses triggered by vascular injury and

thrombotic disease. PARs are expressed primarily in cells of the vasculature including platelets, immune cells, endothelial cells, fibroblasts and smooth muscle cells and exhibit species-specific differences in expression patterns. PAR1 and PAR4 are the functional thrombin receptors present on human platelets (Kahn M.L. 1999), whereas PAR3 and PAR4 mediate thrombin signaling in murine platelets (Kahn M.L. 1999) (Sambrano G.R. 2001). In human endothelial cells, PAR1 is predominantly expressed together with PAR3 (O'Brien P.J. 2000), whereas PAR4 is co-expressed with PAR1 in murine endothelial cells (Kataoka H. 2003). PAR2 is widely distributed and expressed in endothelial cells, fibroblasts, intestinal epithelial cells as well as in airway cells and mediates inflammatory and proliferative responses associated with tissue injury (Ramachandran R. 2008). PAR1 and PAR2 are also expressed in sensory neurons and glial cells and initiate neurogenic inflammation, edema and hyperalgesia, however, the proteases that activate PARs in these particular cell types *in vivo* have yet to be identified (Traynelis S.F. and Trejo J. 2007). Indeed, with the exception of coagulant proteases and vascular cells, the particular proteases that function as the physiological regulator of PAR activation in a given tissue or cellular setting are not well defined.

### **1.3 Activation and signaling by PARs**

The model for proteolytic activation of PARs posits that proteases cleave at a specific peptide bond within the N-terminus of the receptor, which results in the formation of a new N-terminus that acts like a tethered ligand by binding intramolecularly to the receptor to trigger transmembrane signaling (Fig. 2) (Vu T.K. 1991a; Vu T.K. 1991b). Consistent with this mode of activation, synthetic peptides that mimic the tethered ligand sequence of the newly exposed N-terminus can activate PARs independent of proteolytic cleavage, with the

exception of PAR3 (Ishihara H. 1997). Although typically proteolytic cleavage leads to activation of the same receptor, there is evidence of crosstalk between different PARs. In murine platelets and transfected cells, PAR3 binds to and localizes thrombin to facilitate activation of PAR4, a low affinity thrombin receptor (Nakanishi-Matsui M. 2000). PAR3 is efficiently cleaved by thrombin, but is less efficacious than other PARs at eliciting G protein-dependent cellular responses in vascular cells. However, recent work indicates that activation of PAR3 by thrombin induces rapid  $Ca^{2+}$ -dependent release of ATP from lung epithelial A549 cells, a cell line that does not express detectable PAR1 or PAR4 (Seminario-Vidal L. 2009). In addition, PAR3 has been shown to dimerize with PAR1 and consequently potentiates thrombin signaling in endothelial cells, suggesting that PAR3 functions as an allosteric modulator of PAR1 signaling in certain cell types (McLaughlin J.N. 2007). Another type of PAR crosstalk occurs in endothelial cells, where the tethered ligand domain of signaling defective cleaved PAR1 transactivates PAR2 (O'Brien P.J. 2000), although the physiological significance of this type of intermolecular transactivation remained elusive. New studies indicate that during the progression of sepsis, a systemic inflammatory condition with disseminated intravascular coagulation activated PAR1 switches from endothelial disruptive signaling to protective signaling via transactivation of PAR2, a receptor upregulated in endothelial cells during severe sepsis (Kaneider N.C. 2007). Thus, the formation of heterodimeric complexes between PARs appears to modulate certain signaling responses but is unlikely to be critical for monomeric PAR coupling to heterotrimeric G-protein signaling (Whorton M.R. 2007). However, distinct PAR dimeric complexes might have other functions such as facilitating distinct protease-selective signaling, but this remains to be determined.

Once activated, PARs undergo conformational changes that facilitate coupling to heterotrimeric G-proteins. Activated PAR1 couples to multiple heterotrimeric G-protein subtypes including  $G_q$ ,  $G_i$  and  $G_{12,13}$  and promotes diverse cellular responses (Fig. 2). Several early studies indicated that PAR1 couples to inhibition of cAMP accumulation through  $G_i$  and stimulates phospholipase C (PLC)-catalyzed hydrolysis of phosphoinositides (PI) and  $Ca^{2+}$  mobilization through  $G_q$  (Baffy G. 1994; Benka M.L. 1995), whereas activation of ERK1,2 occurs through both  $G_q$  and  $G_i$  signaling (Trejo J. 1996). Other studies have illustrated coupling of PAR1 to  $G_{12/13}$ , which leads to activation of Rho GEFs, induction of cytoskeletal changes and PLC activation (Lopez I. 2001; Gohla A. 1999).

Previous studies questioned whether PAR3 is capable of signaling autonomously (Nakanishi-Matsui M. 2000). However, new work indicates that PAR3 can elicit cellular responses to thrombin typical of G-protein signaling in particular cell types (Seminario-Vidal L. 2009). Although there is no direct evidence linking PAR2 to heterotrimeric G-protein activation, numerous studies demonstrate that activation of PAR2 with proteases and/or synthetic peptide agonists increase second messenger responses suggestive of  $G_q$ ,  $G_i$  and perhaps  $G_{12/13}$  signaling. Previous studies also indicate that activated PAR2 binds to and internalizes with  $\beta$ -arrestin, a multifunctional adaptor protein (DeFea K.A. 2000; Stalheim L. 2005). Once internalized, the PAR2- $\beta$ -arrestin complex functions as a scaffold to recruit ERK1,2 on endocytic vesicles and is thought to sustain ERK1,2 signaling in the cytoplasm independently of G-protein activation. The activation of distinct G-protein subtypes as well as non-G-protein effectors by PARs is crucial for eliciting cell type-specific responses. The extent to which PARs couples to distinct G-protein subtypes in a particular cell type

depends in part on the G-protein and effector repertoire expressed in the cells but other mechanisms are likely to exist.

#### **1.4 Multiple proteases activate PAR signaling**

In addition to thrombin, other proteases activate PAR1. Tissue factor (TF), a single transmembrane protein, initiates coagulation by generating thrombin through activation of coagulation factor VIIa and FXa and also promotes cellular signaling via activation of PARs. The upstream coagulation protease FXa can cleave and activate PAR1 as a monomer or in a complex with TF and FVIIa (Fig. 3) (Camerer E. 2000; Riewald M. 2001). Activated protein C (APC) bound to the endothelial protein C receptor (EPCR), a single spanning transmembrane protein, cleaves and inactivates FVa and VIIa diminishing thrombin generation and induces cellular responses through the activation of PAR1 (Riewald M. 2002). APC, an anti-coagulant protease, is generated on the endothelial cell surface via activation of protein C (PC) by the thrombin-thrombomodulin complex (Fig. 3) (Stearns-Kurosawa D.J. 1996). The plasma enriched zymogen plasminogen is cleaved by urokinase and tissue plasminogen activator (TPA) to generate the active enzyme plasmin, which degrades fibrin and also cleaves PAR1 at multiple sites, which either activates or incapacitates the receptor, depending on the cleavage site (Mannaioni G., (2008)) (Kuliopulos A. 1999). More recently, matrix metalloproteases-1 (MMP-1), also known as interstitial collagenase, was shown to activate PAR1 in invasive breast carcinoma but precisely how MMP-1 acts on PAR1 to generate a functional ligand and/or cellular signaling has not been clearly established (Boire A. 2005).

Several proteases can activate PAR2 including trypsin, FVIIa in complex with TF and FXa, neutrophil protease-2, mast cell tryptase, membrane-tethered serine protease-1

and others but not thrombin (Ramachandran R. 2008). The TF-FVIIa complex can activate PAR2 either directly as a binary complex or indirectly through generation of FXa. FXa may signal more efficiently in a ternary complex with TF-VIIa than it does as a monomer. Interestingly, Ahamed *et al.* showed that a TF-VIIa complex formed with a distinct “cryptic” form of TF in which a specific disulfide bond is reduced fails to support coagulation, i.e. thrombin generation, but retains its ability to signal via PAR2 (Ahamed J. 2006). However, it has not been determined whether activation of PAR2 by TF-VIIa and TF-VIIa-Xa differentially promote distinct cellular responses and hence, questioned the physiological relevance of these findings (Camerer E. and Trejo J. 2006). Similar to other PARs, PAR2 appears to be modified by N-linked glycosylation, a process that affects activation of PAR2 by tryptase but not trypsin nor synthetic peptide agonists (Compton S.J. 2002). Interestingly, mutants of PAR2 in which the tethered ligand sequence SLIGRL was mutated to SLAAA or SAIGRL displayed robust increases in Ca<sup>2+</sup> mobilization when activated proteolytically by trypsin (Al-Ani B. 2004). However, neither SLAAA nor SAIGRL synthetic peptides could elicit cellular responses comparable to SLIGRL, the native tethered ligand sequence, when added exogenously to cells. These findings suggest that cleavage of the receptor rather than unmasking of the entire ligand sequence is critical for proteolytic activation of PAR2, whereas other structural determinants are required for activation of PAR2 by synthetic peptide agonists.

Similar to other PARs, PAR4 is cleaved and activated by multiple serine proteases including thrombin, trypsin, plasmin and cathepsin G (Coughlin S. R. 2005). Interestingly, both the kinetics of PAR4 activation and shut-off of signaling responses are slow resulting in sustained signaling, in marked contrast to other PARs (Shapiro M.J. 2000; Holinstat M. 2006). Mechanistically how this occurs is not known. In addition, the signaling effectors and



cell-type specific responses triggered by activated PAR4 remain poorly characterized. Besides thrombin, it remains to be determined whether other proteases are capable of proteolytically activating PAR3, a receptor that displays autonomous signaling only in certain cell types. Clearly many different proteases cleave and activate PARs, however, whether these proteases stabilize distinct active PAR conformations to promote coupling to specific signaling effectors and cell-type specific responses has not been thoroughly investigated. However, new studies have revealed that activation of PAR1 by two different proteases promotes distinct cellular signaling, a phenomenon termed functional selectivity or biased agonism (Urban J.D. 2007).

### **1.5 PAR1 displays biased agonism**

The finding that different ligands are capable of promoting distinct signaling responses through the activation of the same receptor has now been reported for many GPCRs (Urban J.D. 2007). In many cases, differences in signaling have been observed in studies comparing synthetic ligands to natural ligands, questioning the physiological significance of such findings. Indeed, early studies suggested that distinct cellular responses could be evoked by PAR1 when activated proteolytically by its tethered ligand *versus* untethered “free” synthetic peptide agonists (Fig. 4) (Blackhart B.D. 2000). McLaughlin *et al.* more recently demonstrated using human endothelial cells that activation of PAR1 with thrombin favors  $G_{12/13}$  signaling and induction of endothelial barrier permeability over  $G_q$ -dependent  $Ca^{2+}$  mobilization (McLaughlin J.N. 2005). In contrast, synthetic PAR1 peptide agonists SFLLRN and TFLLRNPNDK caused an opposite rank order of activation favoring  $G_q$ -triggered  $Ca^{2+}$  increases rather than  $G_{12/13}$  signaling, but

whether different endogenous proteases promote distinct signaling through the activation of PARs remained an open question.

Several recent studies have now reported that two different proteases, thrombin and activated protein C, cleave and activate PAR1 but cause opposite effects on endothelial barrier permeability (Feistritzer C. 2005; Finigan J.H. 2005). In general, thrombin functions as a pro-inflammatory mediator and disrupts endothelial barrier permeability through the activation of PAR1 (Komarova Y.A. 2007). In contrast to thrombin, however, activation of PAR1 by APC elicits anti-inflammatory and promotes endothelial barrier stabilization (Feistritzer C. 2005; Finigan J.H. 2005). APC is generated on the endothelial cell surface and is poised to signal via direct activation of PAR1 (Riewald M. 2002; Feistritzer C. 2006). The cytoprotective and anti-inflammatory responses induced by APC also require the co-factor function of EPCR, a single transmembrane protein (Riewald M. 2002). Moreover, APC has been shown clinically to reduce mortality of patients with severe sepsis (Bernard G.R. 2001). The molecular mechanisms by which APC distinctly activates PAR1 signaling are not clearly understood.

Proteolytic activation of PAR1 requires unmasking of a tethered ligand sequence that binds intramolecularly to the body of the receptor stabilizing an active receptor conformation that triggers transmembrane signaling. Previous studies have shown that APC has the capacity to cleave PAR1, albeit with considerably less efficiency than thrombin (Ludeman M.J. 2005). Thus, the extent of PAR1 activation by APC should be different than thrombin, which efficiently cleaves the receptor. However, whether APC cleavage of PAR1 is the only critical determinant that facilitates PAR1 selective signaling and endothelial barrier protection is not known. If cleavage of PAR1 by APC is solely responsible for endothelial barrier protective signaling then quantitative not qualitative differences in

signaling would be observed following activation of PAR1 by thrombin *versus* APC. To test this possibility, in this dissertation we examined the effect of thrombin and APC on the activation of RhoA and Rac1, small GTPases that differentially regulate endothelial barrier permeability. We initially found that thrombin and APC signaling were lost in PAR1-deficient endothelial cells, indicating that PAR1 is the major effector for protease signaling in these cells (Russo A. 2009). Surprisingly, we also found that thrombin caused robust RhoA signaling but not Rac1 activation, whereas APC stimulated a marked increase in Rac1 activation but not RhoA signaling, consistent with the opposing functions of these proteases on endothelial barrier integrity (Russo A. 2009). Thus, the activation of PAR1 by APC likely results in a distinct active receptor conformation that selectively couples to effectors that mediate endothelial barrier protective signaling rather than disruption.

### **1.6 Membrane microdomains and protease-dependent biased agonism**

How can activation of the same receptor by two different proteases elicit distinct cellular responses? Herein we showed that the underlying mechanisms that regulate coupling of proteolytically activated PAR1 to distinct signaling effectors involve localization to caveolae, a specific type of plasma membrane microdomain. Caveolae are lipid rafts enriched in cholesterol and caveolins and function as microdomains that facilitate receptor-effector coupling and signal transduction. Indeed, we recently showed that caveolin-1 expression is essential for APC but not thrombin activation of PAR1 signaling and endothelial barrier protective effects (Russo A. 2009), suggesting that PAR1 localization to caveolae is critical for protease-selective signaling. Previous studies also showed that the APC co-factor EPCR, PAR1, G<sub>q</sub> and G<sub>i</sub> partition into lipid rafts and associate with caveolin-1 (Bae J.S. 2007; Li S. 1995). Caveolin-1 is a structural protein essential for caveolae

formation in endothelial cells (Razani B. 2001). The barrier protective signaling induced by APC is also blocked by pertussis toxin, suggesting a role for  $G_{i/o}$  in this process (Bae J.S. 2007). Moreover, the localization of APC bound to EPCR in lipid rafts facilitates efficient PAR1 cleavage and activation, suggesting that perhaps caveolae localization stabilizes a distinct active receptor conformation that elicits barrier protective signaling (Bae J.S. 2008). Taken together these data suggest that PAR1, EPCR,  $G_{i/o}$  proteins localize to caveolae and exist as a preassembled complex poised to signal following APC generation on the cell surface. In contrast, caveolin-1 is not essential for thrombin activation of PAR1 signaling (Russo A. 2009), indicating that caveolin-1 only modulates PAR1 signaling when selectively activated by APC and not by thrombin in endothelial cells. Interestingly, a function for lipid rafts but not caveolae in thrombin-induced cytoskeletal changes, a cellular process controlled predominantly by  $G_{12/13}$  signaling, in endothelial cells has previously been reported (Carlie-Klusacek M.E. and Rizzo V. 2007). Moreover, caveolae are also required for TF-VIIa but not for peptide agonist activation of PAR2 but whether TF-VIIa, agonist peptide or trypsin promote distinct signaling responses was not examined (Awasthi V. 2008).

The molecular determinants that specify the targeting of PAR1 and signaling components to caveolae are largely unknown but may involve post-translational modifications. Caveolin-1 is palmitoylated and together with cholesterol and sphingolipids form caveolar microdomains, which sequester other lipid-modified proteins within the plasma membrane. A large number of GPCRs appear to be modified by palmitoylation, which occurs through the covalent attachment of a C16 fatty-acid chain via a thioester linkage to cysteine residues localized within the cytoplasmic tail of the receptor. PAR1 and PAR2 have cytoplasmic cysteine residues that could serve as sites for palmitoylation but to

our knowledge this has not been reported. Previous studies have shown that palmitoylation of tissue factor (TF) facilitates its localization to caveolae and prevents protein kinase C dependent phosphorylation, a process that controls tissue factor pro-coagulant activity (Dorfleutner A. and Ruf W. 2003). Thus, the modulation of TF with palmitoylation may facilitate localization of TF-FVIIa and Xa with PAR2 in caveolar microdomains to promote cellular signaling. Whether EPCR is similarly palmitoylated is not known. Interestingly, several studies suggest that protein palmitoylation protects proteins from ubiquitination and subsequent degradation (Valdez-Taubas J. 2005; Abrami L. 2006). Modification of proteins with ubiquitin occurs through the covalent attachment of ubiquitin, a 76-amino acid protein, to lysine residues on the target protein. Both PAR1 and PAR2 are ubiquitinated (Wolfe B.L. 2007; Jacob C. 2005) but whether PAR ubiquitination facilitates receptor palmitoylation and/or targeting of proteins to caveolae has not been examined.

In addition to post-translational modifications, recent work suggests that localization of the  $\mu$ -opiate receptor (MOR) to lipid rafts is regulated by its interaction with the heterotrimeric  $G_{i2}$  protein (Zheng H. 2008). The localization of MOR to lipid rafts was disrupted by cholesterol depletion as well as by altering the expression of  $G_{i2}$ . In the absence of agonist, MOR directly associated with  $G_{i2}$ , which is mediated by a G protein-interaction motif, upon the deletion of this motif, the receptor redistributed out of the lipid microdomain. Interestingly, etorphine promoted MOR interaction with  $\beta$ -arrestin, which in turn facilitated receptor translocation out of the lipid raft, a process that required receptor dissociation from  $G_{i2}$ . By contrast, stimulation of MOR with morphine induced an activate receptor conformation that preferentially bound to  $G_{i2}$  and showed a low affinity for arrestin, consistent with minimal receptor phosphorylation and lack of internalization. Thus, membrane microdomain association, and interaction with heterotrimeric  $G_{i2}$  protein

regulates agonist-selective signaling by MOR. Whether PAR1 interaction with  $G_{i2}$  is important for caveolae association and protease-selective signaling will be important to determine.

The expression of  $\beta$ -arrestin-2 is also critical for maintaining and/or stabilizing distinct assemblies of the G-protein coupled serotonin 2A receptor (5-HT<sub>2A</sub>R) with signaling effectors (Schmid C.L. 2008). The ablation of  $\beta$ -arrestin-2 expression caused selective loss of responses to the endogenous ligand serotonin, but not to a distinct synthetic ligand 2,5-dimethoxy-4-iodoamphetamine (DOI) (Schmid C.L. 2008). Interestingly, caveolin-1 was previously shown to interact with 5-HT<sub>2A</sub>R and to modulate its capacity to couple to  $G_q$  signaling (Bhatnagar A. 2004). However, whether  $\beta$ -arrestin affects 5-HT<sub>2A</sub>R localization to lipid rafts was not evaluated.

### **1.7 Protease-selective mechanisms of PAR desensitization**

The ability of different ligands to promote distinct signaling responses through the activation of the same receptor suggests that unique active receptor conformations can be induced. These findings raise intriguing questions regarding the mechanisms that mediate desensitization of distinctly activated GPCRs. In the classic paradigm, GPCRs are initially desensitized by rapid phosphorylation of activated receptors by G-protein dependent kinases (GRKs) (Marchese A. 2008). Phosphorylation enhances receptor affinity for arrestin, and arrestin binding prevents receptor-G-protein interaction, thereby uncoupling the receptor from signaling. Arrestin also interacts with components of the endocytic machinery to facilitate GPCR internalization, and thereby removes activated receptor from signaling effectors at the plasma membrane. Within endosomes, receptor dissociates from their ligands, become dephosphorylated, and then returns to the cell surface in a state

capable of responding to ligand again. Several recent studies indicate that GPCRs activated by different ligands are desensitized through distinct mechanisms (Kelly E. 2008).

Desensitization of the  $\mu$ -opioid receptor (MOR) following activation by morphine occurs predominantly through protein kinase C-dependent phosphorylation, whereas DAMGO activated MOR phosphorylation is largely dependent on GRK. Interestingly, activation of the CC chemokine receptor 7 (CCR7) with the endogenous chemokine CCL19 induces robust phosphorylation and arrestin-dependent desensitization (Kohout T.A. 2004). In contrast, a different endogenous ligand CCL21 fails to promote phosphorylation or arrestin-dependent desensitization of activated CCR7, suggesting that different ligands induce distinct desensitization of CCR7.

The desensitization of PAR1 signaling is controlled by multiple regulatory mechanisms. The first involves agonist-induced PAR1 phosphorylation and the second involves interaction with  $\beta$ -arrestin. However, signaling by PAR1 appears to be more effectively regulated by  $\beta$ -arrestin-1 rather than  $\beta$ -arrestin-2 through a process that does not require receptor phosphorylation (Chen C.H. 2004; Paing M.M. 2002). In addition, activated PAR1 internalization and lysosomal degradation are also critical for termination of receptor signaling (Trejo J. 1998). Interestingly, activated PAR1 internalization occurs independently of arrestins (Paing M.M. 2002) (Fig. 5). Whether desensitization of PAR1 differs when activated proteolytically by thrombin *versus* synthetic peptide agonists has not been rigorously examined. Interestingly, however, we recently found that APC promotes PAR1 phosphorylation and desensitization but causes negligible receptor internalization and degradation (Russo A. 2009).

## **1.8 The tumor suppressor serine protease inhibitor maspin**

Maspin is a multifaceted protein affecting a variety of cellular processes. Maspin is a tumor suppressor that exerts its function by increasing cell adhesion, inducing apoptosis and decreasing motility and angiogenesis (Sheng S. 1996; Seftor R.E.B. 1998; Zhang M. 2000). The function of maspin in reducing tumor progression has been extensively studied *in vitro*, in animal models and also by assessing cancer patient survival (Shi H.Y. 2002; Sheng S. 1996) *Maspin* is expressed by normal epithelial breast cells but not in invasive breast cancer (Fig. 6). Maspin is silenced during metastatic progression by transcriptional regulation and aberrant promoter methylation (Futscher B.W. 2002; Futscher B.W., 2004). Maspin re-expression is a marker of improved prognosis. Indeed, evidence shows that restoring the expression of maspin in invasive carcinoma cells results in increased rate of spontaneous apoptosis, more prominent actin cytoskeleton, reduced invasive capacity, and altered proteasomal function. Animal studies have validated the *in vitro* observations. In this dissertation I examined whether reactivation of maspin using artificial transcription factors (ATFs) *in vivo* reduced tumor growth and metastasis.

## **1.9 Subcellular localization and function of maspin**

Maspin localizes into different subcellular compartments. Maspin is predominantly cytoplasmic, with some membrane association, partial secretion, and nuclear localization (Fig. 7). The differential localization of maspin dictates its functions. The differential localization of maspin is determined by specific binding partners such as interferon regulatory factor 6 (IRF6) (Seftor R.E.B. 1998; Liu J. 2004), histone deacetylase 1 (HDAC1), glutathione S-transferase (GST), heat shock proteins HSP70, and HSP90 (Zhang H. 2003). It has been proposed that the interaction of maspin with other proteins influences



maspin function in response to stress and to induce apoptosis. Induction of apoptosis is one of the most interesting effects of maspin and implicates its use as therapeutical tool in inhibiting tumor growth. Intracellular maspin is implicated in apoptotic responses. Ectopic expression of maspin leads to high levels of pro-apoptotic Bax expression (Zhang W. 2005; Liu J. 2004) decreased levels of Bcl-2 (Zhang W. 2005), and activation of Caspase-3 (Li Z. 2005) and/or caspase-9 (Liu J. 2004). Some evidence indicates that maspin can function as an inhibitor of angiogenesis. Secreted maspin can inhibit the migration of cultured endothelial cells toward fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which act as important chemoattractants during angiogenesis (Zhang M. 2000). Maspin successfully blocks neovascularization and reduces tumor-associated microvessels *in vivo*. In addition, expression of maspin has been shown to reduce metastatic dissemination of tumor cells (Zhang M. S. 2000; Watanabe M. 2005). Extracellular maspin blocks cell detachment, motility, and invasion. Extracellular maspin interacts with the components of extracellular matrix such as collagens type I and III. This interaction contributes to the tumor-suppressive property of maspin. Furthermore, maspin acts as unconventional serine-protease inhibitor by binding and inhibiting the serine protease urokinase plasminogen activator (uPA) without affecting uPA proteolytic function. UPA is the principal participant of ECM degradation. Therefore maspin inhibition of uPA is thought to be responsible for maspin ability to block tumor invasion and metastasis (Mc.Gowen R. 2000; Sheng S. 1998). Silencing of maspin is associated with higher risk of distant metastasis in breast carcinomas (Maass N. 2001).

### 1.10 Regulation of maspin expression

The most important regulatory mechanism for maspin involves p53. p53 is a well-known tumor suppressor that regulates cell-cycle and has also roles in apoptosis and angiogenesis. It has been demonstrated that adenoviral delivery of wild-type p53 to breast and prostate cancer cell lines induces maspin expression (Zhou Z. 2000). The regulation of maspin by p53 is believed to be one of the most important mechanisms elucidating p53 function in inhibiting cell invasion and metastasis. This regulation occurs primarily through the hormone response element located within the maspin promoter. Several studies use delivery of cDNA using viral vectors (Watanabe M. 2005) or liposomes (Li Z. 2005) to overexpress maspin. It has been reported that ectopic Maspin expression reduced tumor growth and metastasis *in vivo* (Shi H.Y. 2002). However, novel strategies are developed for the up-regulation of the endogenous *maspin* in tumor cells. Tamoxifen (TAM) can also up-regulate endogenous maspin (Khalkhali-Ellis Z. 2004). TAM is a drug commonly used for the treatment and prophylaxis of breast cancer which acts by competing with estrogen for estrogen receptor binding.

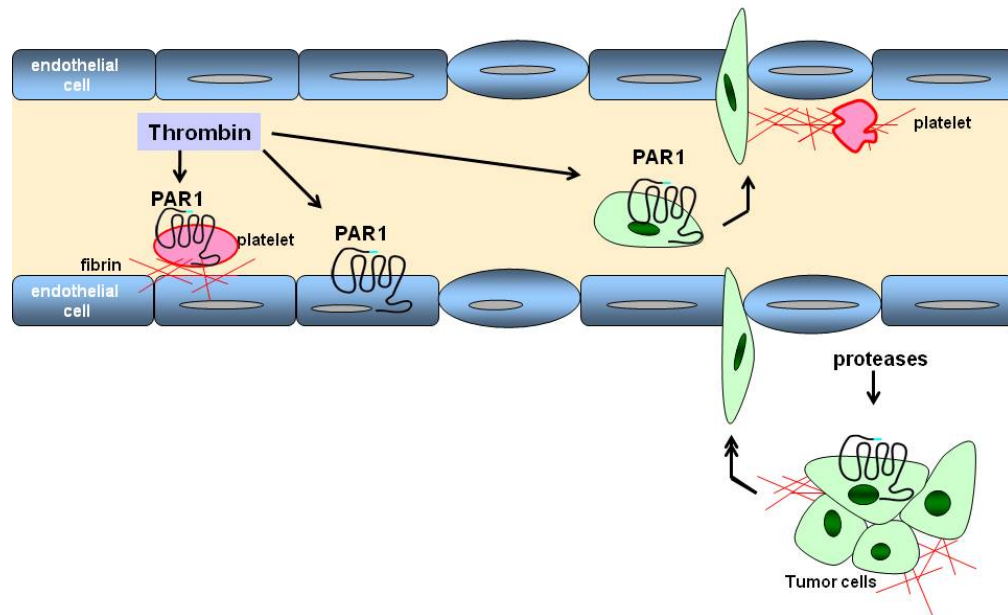
Epigenetic mechanisms are responsible for maspin silencing in a variety of tumor cells. Chromatin remodeling drugs have been used to re-activate silenced endogenous maspin (Kulp S.K. 2006). These agents could function by loosening up the chromatin and allowing the access of TFs and polymerase, facilitating transcription. Examples of these types of drugs are methyltransferase inhibitors and histone deacetylase inhibitors. The methyltransferase inhibitor 5-aza-2'-deoxycytidine has been recently approved for therapeutic treatment (Samlowski W.E. 2005). *Maspin* has also been re-activated by treatment with HDAC inhibitors, such as Trichostatin A (TSA), Dipeptide and suberoylanide hydroxamic acid (SAHA). The latest has been approved in clinical trials for the treatment of

solid and hematological tumors (Juttermann R. 1994). However, chromatin remodeling drugs are often not selective and result in high toxicity and acquired drug resistance in cancer patients (Eyupoglu I.Y. 2005). Herein I present a novel molecular strategy for the controlled re-activation of maspin. Specifically I designed a new inducible system that allows controlled ATF expression *in vivo*. This strategy will allow evaluating the therapeutic potential of ATFs in reducing tumor growth and metastasis.

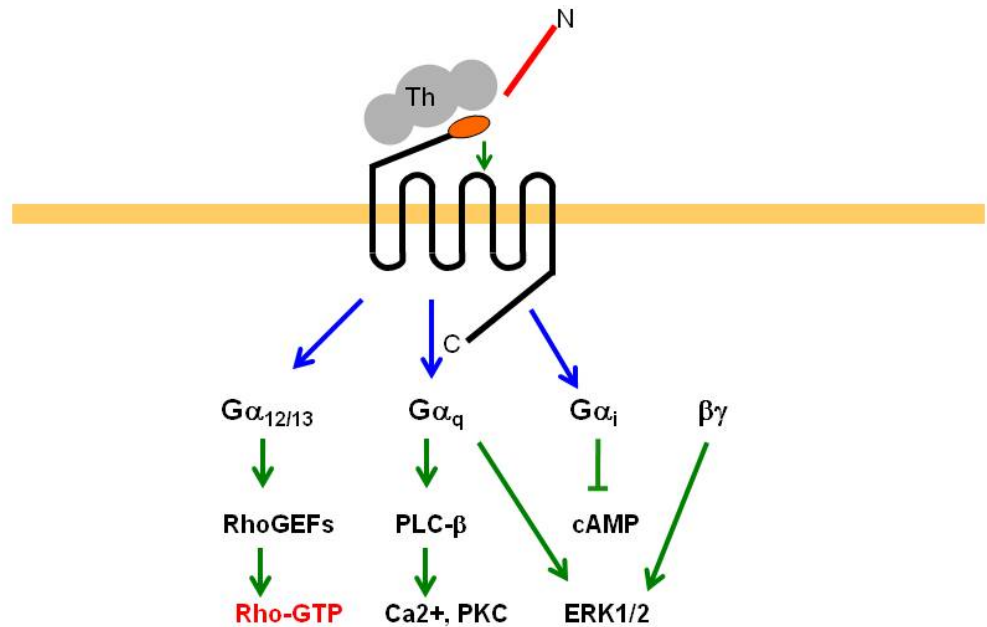
### **1.11 Artificial transcription factors (ATFs) design**

Artificial transcription factors (ATFs) are made of DNA-binding domains (DBDs) fused with transcriptional effector domains, such as activator or repressor domains (Fig. 8) (Blancafort P. 2004). The DBD is made of zinc fingers (ZFs) of the type of Cys2-His2. The ZF consist of ~30 amino acids that fold into two antiparallel  $\beta$ -strands and a recognition  $\alpha$ -helix, which makes specific contacts with the target DNA. Each ZF domain specifically interacts with 3 base pairs (bp) (or triplet of recognition) of DNA (Fig. 9 A) (Pavletich N.P. 1991). ZFs bind DNA in a quasi-independent manner (there are modular) (Fig. 9 B). This property allows investigators to engineer “polydactyl” ZF proteins by linkage of individual ZF domains. A six-ZF protein will recognize 18 bp with high selectivity and affinity. An 18-bp site is potentially unique in the human genome and thus, 6ZF proteins have the potential to recognize single genes. ZF domains that recognizing specifically most of the 64 DNA triplets have been isolated by phage display (Segal D.J. 1999). Thus, a collection of ZF alpha-helical “lexicons” recognizing all these triplets is now available. Engineered ATFs can be generated by simply grafting the recognition alpha helix (designed to bind the corresponding triplet) into a given 6ZF protein that serves as “backbone”. A very useful database for the construction of 6ZF domains (zinc finger tools) is available to predict the

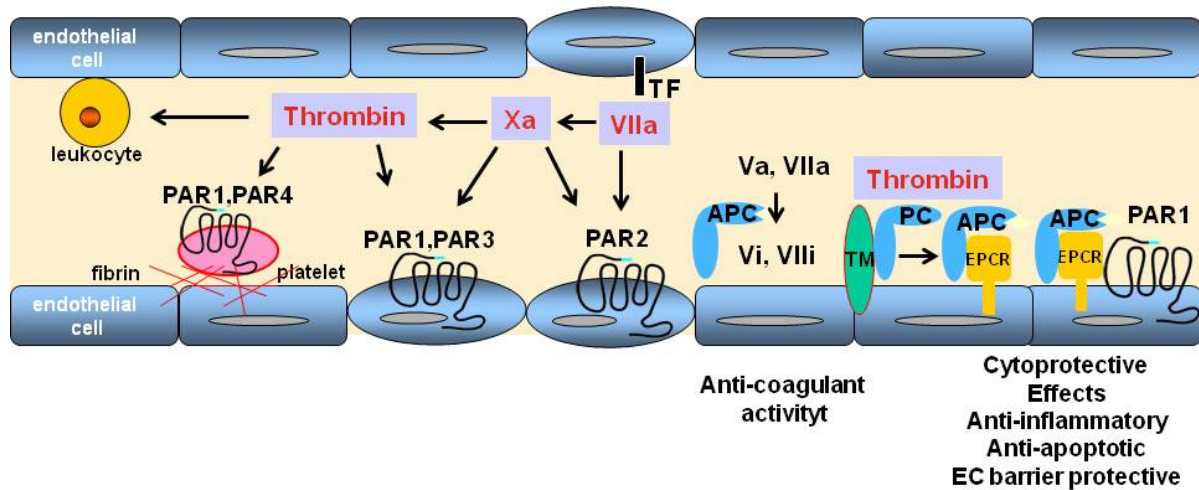
amino acid sequence for a zinc finger protein expected to bind to a desired target site (Rebar E.J. 1994). The ZFs are fused with activator or repressor domains and thus, can regulate specifically endogenous genes (Fig. 10 A and B) (Blancafort P. 2004) (Beerli R.R. 1998). 6ZF proteins targeting 18-bps are able to regulate their target genes with unique specificity (Segal D.J. 2003). The Blancafort lab has constructed ATFs able to up-regulate the tumor suppressor maspin that is silenced in metastatic breast cancer cells (Beltran A. 2007). In addition, these ATFs were able to synergize with current methyltransferase and HDAC inhibitors, increasing the targeting efficiency and specificity of these small molecules. Herein, I describe the development of an inducible vector system to express the ATF in an animal model of breast cancer.



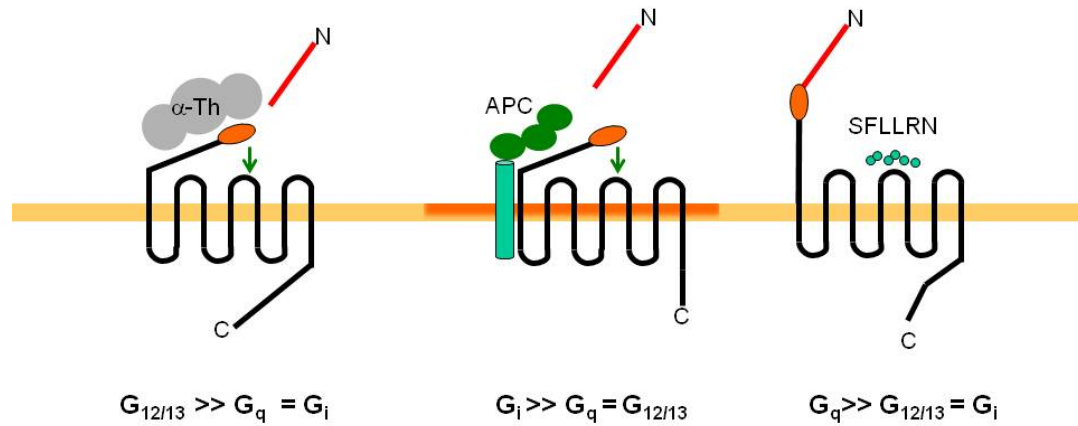
**Fig. 1. Protease-activated receptor-1 functions in the vasculature and in cancer progression.** Thrombin, the main effector protease of the coagulation cascade, activates PAR1, to elicit signaling in a variety of cell types. Thrombin activates PAR1 in human platelets and generates fibrin, which is important for thrombus formation and tumor cell survival and metastasis. PAR1 is also cleaved and activated by tumor-generated proteases, which contribute to tumor cell growth, invasion and metastasis. PAR1 is expressed in endothelial cells and responds to thrombin leading to increased endothelial permeability.



**Fig. 2. Protease-activated receptor-1 signals to different G proteins.** Thrombin binds to and cleaves the extracellular amino-terminal domain of PAR1, exposing a new N-terminal that acts like a tethered ligand to elicit transmembrane signaling. Upon thrombin activation, PAR1 couples to multiple heterotrimeric G-protein subtypes including G<sub>q</sub>, G<sub>i</sub> and G<sub>12,13</sub> and promotes diverse cellular responses. PAR1 couples to inhibition of cAMP accumulation through G<sub>i</sub> and stimulates phospholipase C (PLC)-catalyzed hydrolysis of phosphoinositides (PI) and Ca<sup>2+</sup> mobilization through G<sub>q</sub>. PAR1 also couple to G<sub>12/13</sub>, which leads to activation of Rho GEFs, induction of cytoskeletal changes and PLC activation.

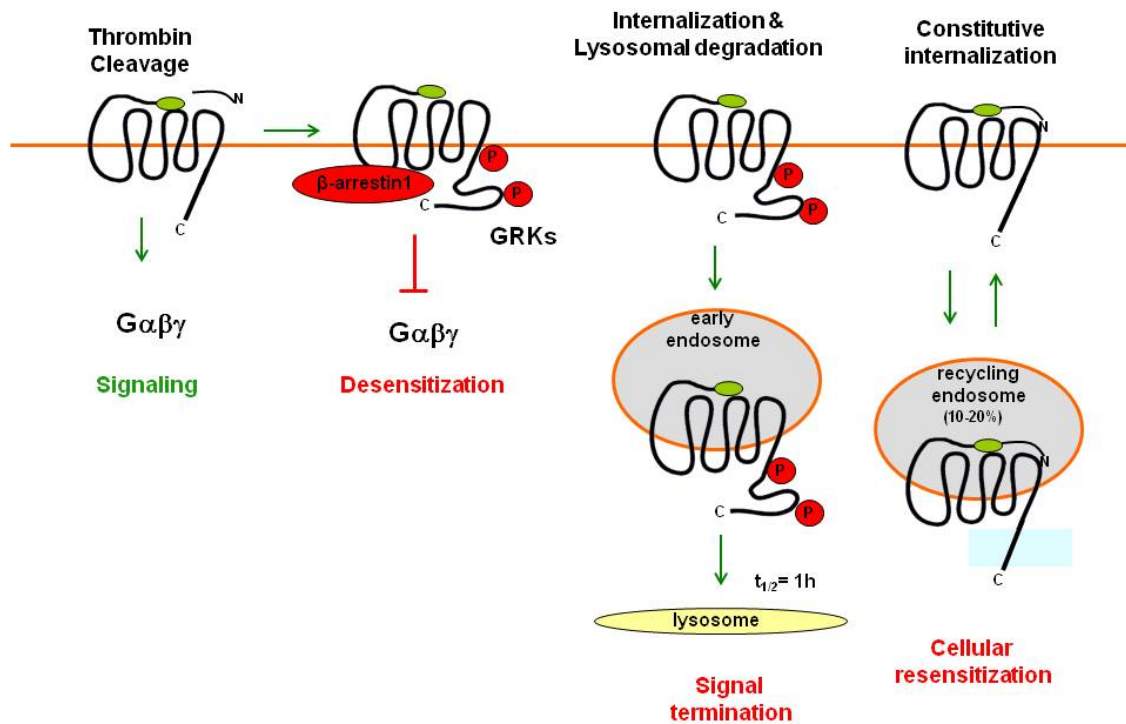


**Fig. 3. Activated protein C (APC) signals through PAR1 to promote endothelial cell barrier protection.** Tissue factor (TF), a single transmembrane protein, initiates coagulation by generating thrombin through activation of coagulation factor VIIa and FXa through the extrinsic pathway. Thrombin elicits responses through activation of PAR1. Thrombin is rapidly sequestered by thrombomodulin (TM) which facilitate cleavage of protein C (PC) to generate activated protein C (APC). APC bound to endothelial protein C receptor (EPCR), a single spanning transmembrane protein, cleaves and inactivates FVa and VIIa diminishing thrombin generation. APC also exerts endothelial barrier protection through activation of PAR1.



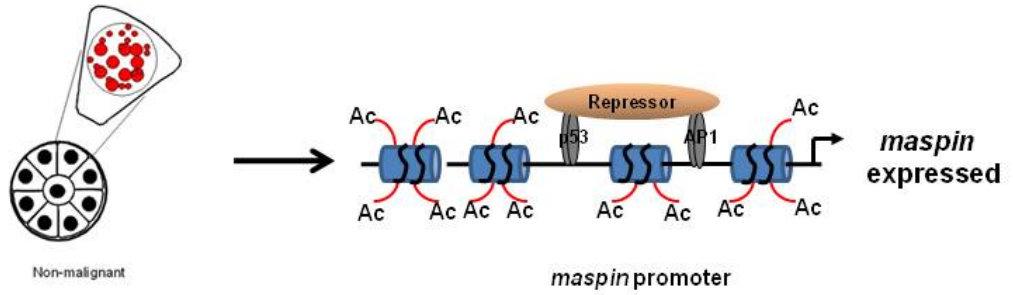
**Fig. 4. Proteases and peptide agonist display biased agonism at protease-activated receptor 1.** Thrombin-activated PAR1 favors  $G_{12/13}$  signaling and induction of endothelial barrier permeability over  $G_q$ -dependent  $Ca^{2+}$  mobilization. In contrast, synthetic PAR1 peptide agonists SFLLRN and TFLLRNPNDK caused an opposite rank order of activation favoring  $G_q$ -triggered  $Ca^{2+}$  increases rather than  $G_{12/13}$  signaling. Activated protein C (APC), cleaves and activates PAR1 and acts through  $G_i$ , causing opposite effects on endothelial barrier permeability. APC



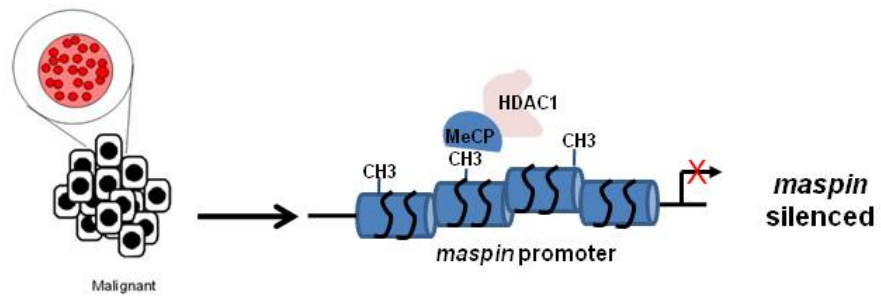


**Fig. 5. Mechanisms of PAR1 desensitization and down-regulation** The desensitization of PAR1 signaling is controlled by multiple regulatory mechanisms. The first involves agonist-induced PAR1 phosphorylation and the second involves interaction with  $\beta$ -arrestin. In addition, activated PAR1 internalization and lysosomal degradation are also critical for termination of receptor signaling. Interestingly, activated PAR1 internalization occurs independently of arrestins whereas arrestins are required for receptor desensitization.

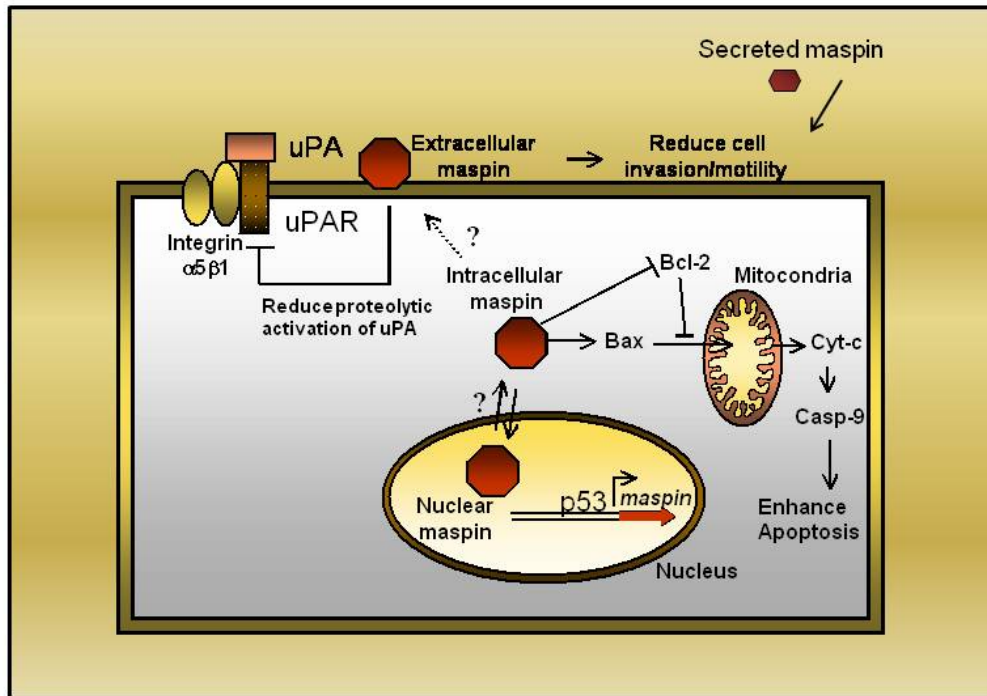
### Normal breast epithelial cells



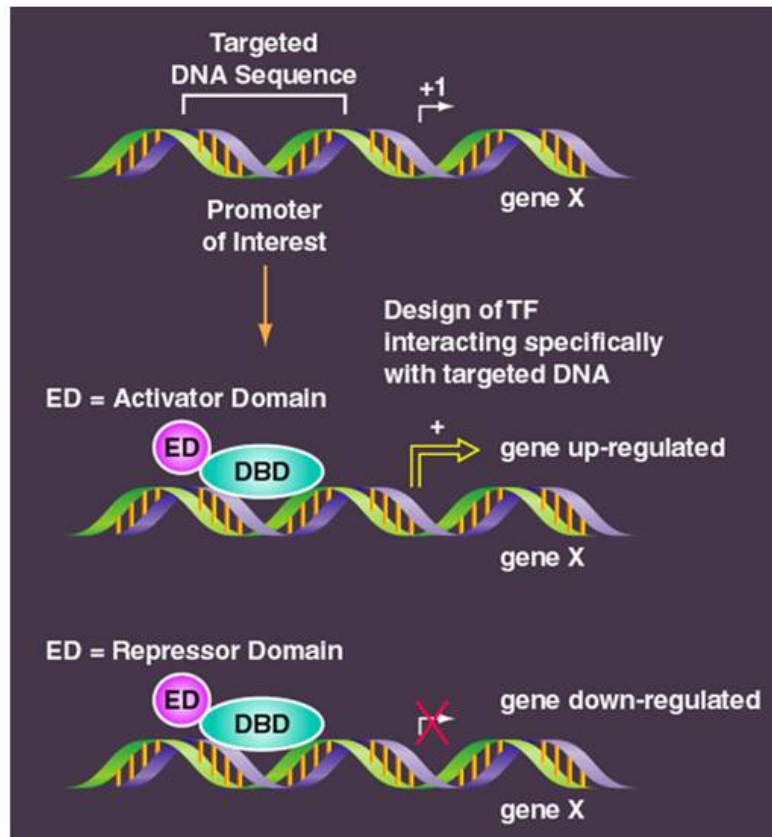
### MDA-MB-231 breast cancer cells



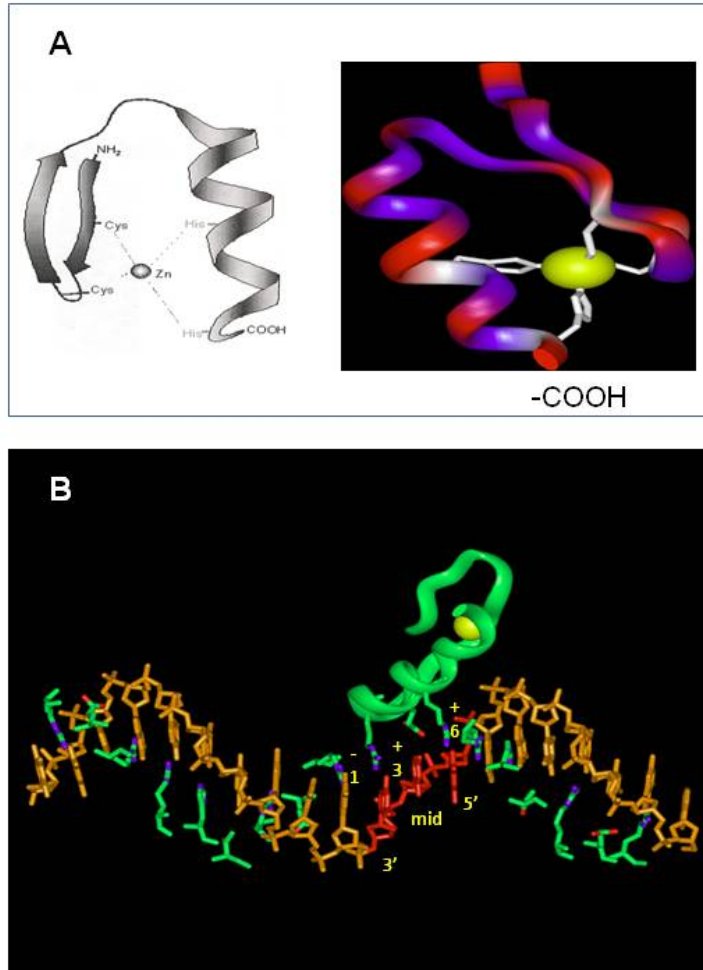
**Fig. 6. Maspin is silenced by epigenetic mechanisms in invasive breast cancer cells.** Maspin promoter is acetylated and not methylated in normal breast cells resulting in relaxed chromatin that allows maspin expression. Conversely, *Maspin* promoter is de-acetylated and methylated in invasive breast cancer cells (MDA-MB-231) resulting in condensed chromatin that does not allow maspin expression.



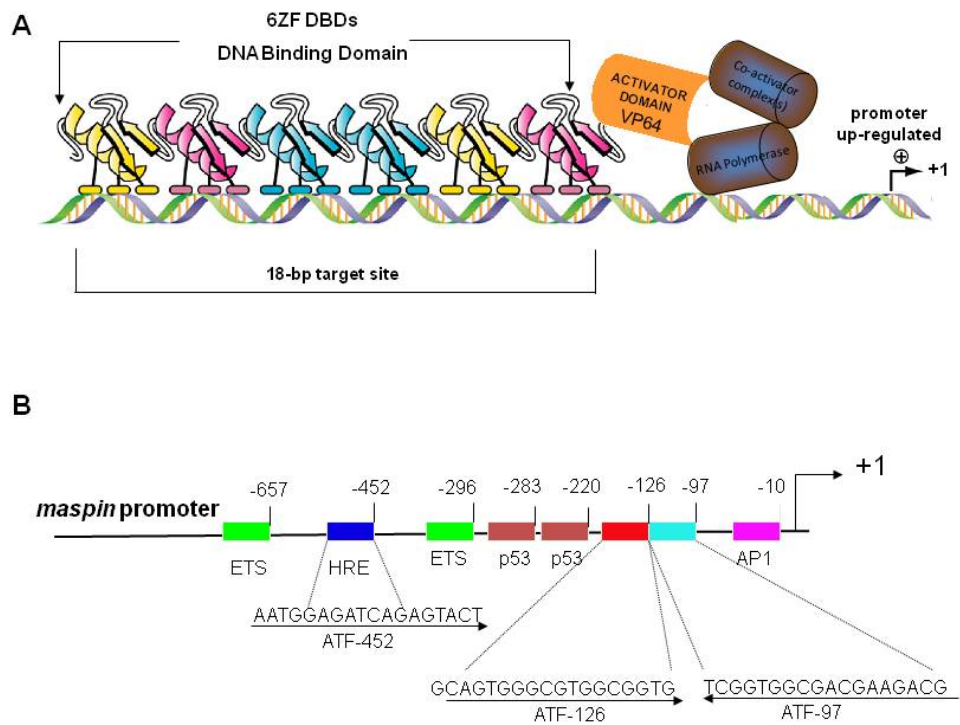
**Fig. 7. Maspin functions are dictated by protein localization.** Extracellular and secreted maspin reduces cell invasion and motility by inhibiting extracellular matrix degradation. Intracellular maspin is responsible for cancer cell selective apoptosis.



**Fig. 8. Artificial Transcription Factors (ATFs) function.** ATFs are made of DNA binding domain (DBD) responsible for target specificity and effector domain (ED). The effector domain can be an activator or a repressor which will dictate whether the target gene will be up-regulated or down-regulated.



**Fig. 9. The Cys<sub>2</sub>-His<sub>2</sub> Zinc Finger domain structure and its interaction with DNA. (A)** A zinc finger is a protein containing a Zn<sup>++</sup> cation chelated to two cysteine amino acids each on an antiparallel  $\beta$  sheet, and two histidine amino acids on a single  $\alpha$  helix. **(B)** Each ZF interacts with 3bp on the DNA.



**Fig. 10. Designing Artificial Transcription Factors (ATFs) to up-regulate tumor suppressor gens. (A)** ATFs are made of six zinc finger domains fused to the activator domain VP64. **(B)** Each 6ZF recognizes a 18bp sequence in the promoter. ATF-126 has been shown to be the most effective in regulating maspin promoter in breast cancer cell lines. Adapted from (Beltran A. 2006.)

## **CHAPTER 2. APC PROTECTIVE EFFECT ON ENDOTHELIAL BARRIER REQUIRES CAVEOLIN-1**

### **2.1: Abstract**

PAR1 is a G protein-coupled receptor uniquely activated by proteolysis. Thrombin, a coagulant protease, induces inflammatory responses and endothelial barrier permeability through the activation of PAR1. Activated protein C (APC), an anti-coagulant protease, also activates PAR1. However, unlike thrombin, APC elicits anti-inflammatory responses and protects against endothelial barrier dysfunction induced by thrombin. We found that thrombin and APC signaling were lost in PAR1-deficient endothelial cells, indicating that PAR1 is the major effector of protease signaling. To delineate the mechanism responsible for protease-selective signaling by PAR1, we examined the effect of APC and thrombin on the activation of RhoA and Rac1, small GTPases that differentially regulate endothelial barrier permeability. Thrombin caused robust RhoA signaling but not Rac1 activation, whereas APC stimulated a marked increase in Rac1 activation but not RhoA signaling, consistent with the opposing functions of these proteases on endothelial barrier integrity. Strikingly, APC signaling and endothelial barrier protection effects were abolished in cells lacking caveolin-1, whereas thrombin signaling remained intact. These findings suggest that compartmentalization of PAR1 in caveolae is critical for APC selective signaling to Rac1 activation and endothelial barrier protection.

## 2.2: Introduction

The coagulant protease thrombin is generated in response to vascular injury and in thrombotic disease and drives fibrin deposition and platelet activation, which are critical for hemostasis and thrombosis (Coughlin S.R. 2005). Thrombin promotes pro-inflammatory responses and disrupts endothelial barrier permeability (Komarova Y.A. 2007). PAR1 is the predominant mediator of thrombin responses in cells. Thrombin activates PAR1 by cleaving the N-terminal domain generating a new N-terminus that binds intramolecularly to the receptor to trigger transmembrane signaling (Vu T.K. 1991a). Synthetic peptides that represent the newly formed N-terminus of PAR1 can activate the receptor independent of thrombin and proteolytic cleavage. Interestingly, thrombin and peptide agonists differ in their capacity to promote endothelial barrier permeability and  $Ca^{2+}$  mobilization (McLaughlin J.N. 2005). These studies suggest that distinct cellular responses can be evoked by the same receptor when activated proteolytically by the tethered ligand versus untethered “free” synthetic peptide agonists. Similar phenomena have been reported for other GPCRs activated by different ligands and this is a poorly understood process termed functional selectivity (Urban J.D. 2007).

APC, an anti-coagulant protease, displays cytoprotective and anti-inflammatory activities and has been shown clinically to reduce mortality of patients with severe sepsis (Bernard G.R. 2001). APC is generated on the endothelial cell surface via activation of protein C (PC) by the thrombin-thrombomodulin complex (Stearns-Kurosawa D.J. 1996). APC bound to endothelial protein C receptor (EPCR) cleaves and inactivates factors Va and VIIa diminishing thrombin generation and induces cellular responses through the activation of PAR1 (Mosnier L.O. 2003; Riewald M. 2002). In contrast to thrombin, however,



APC elicits anti-inflammatory responses and promotes endothelial barrier stabilization (Feistritzer C. 2005; Finigan J.H. 2005). The mechanism by which APC exerts anti-inflammatory and cytoprotective signaling in endothelial cells is not fully understood.

Previous studies suggest that most endogenous PC is bound to EPCR on the endothelial cell surface and cleaved by the thrombin-thrombomodulin complex (Feistritzer C. 2006). The newly formed APC is then poised to signal via direct activation of PAR1. Thus, APC generation on the endothelial cell surface is linked mechanistically to PAR1 protective signaling. The cytoprotective and anti-inflammatory responses induced by APC also require the co-factor function of EPCR (Riewald M. 2002; Taylor F.B. 2000). Interestingly, thrombomodulin, EPCR and PAR1 partition into lipid rafts and co-fractionate with caveolin-1, a structural protein essential for caveolae formation in endothelial cells (Razani B. 2001), suggesting that these proteins reside at least partially in caveolar microdomains, a subtype of lipid rafts (Bae J.S. 2007a; Bae J.S. 2007b). However, whether caveolae are required for APC activation of PAR1 signaling and endothelial barrier protective effects is not known.

Our studies here reveal a critical role for caveolae in APC, but not thrombin, activation of PAR1 signaling and endothelial barrier protection. These findings are the first to demonstrate an essential role for caveolae in agonist selective signaling by PAR1.

## **2.3: Results**

### **2.3.1: APC signals through PAR1 to activate the signal-regulated kinases ERK1,2 and the small GTPase Rac1.**

To determine whether PAR1 is essential for protease-selective signaling, we examined thrombin and APC signaling in human endothelial cells stably expressing a PAR1-specific shRNA (Arora P. 2008). In control cells, thrombin and APC induced a similar biphasic increase in extracellular signal-regulated kinase 1, 2 (ERK1,2) activity (Fig. 11 A). ERK1,2 activation by APC required APC catalytic activity (Fig. 12).

Endothelial cells expressing PAR1 shRNA displayed minimal PAR1 expression and signaling, whereas PAR2 signaling remained intact (Fig. 13 A and B), indicating loss of functional PAR1.

However, cells lacking PAR1 displayed minimal ERK1,2 activation in response to various concentrations of thrombin or APC compared to control cells (Fig. 14 A and B). Thrombin stimulated p38 MAP kinase activation (Fig. 11 B) which was also lost in cells deficient in PAR1 expression (Fig. 13 C). In contrast to thrombin, APC failed to stimulate p38 MAP kinase signaling (Fig. 11 B). These findings suggest that PAR1, and not another receptor or factor, is critical for thrombin and APC signaling in endothelial cells.

We next examined endothelial barrier permeability. Thrombin-stimulated endothelial barrier permeability was blocked in cells preincubated with APC (Fig. 15 A). These findings are consistent with a role for APC in stabilization of endothelial cell-cell junctions and protection against endothelial barrier dysfunction induced by thrombin (Feistritz C. 2005). We also evaluated the effect of thrombin and APC on the activation of endogenous RhoA and Rac1. Activation of RhoA promotes endothelial barrier dysfunction, whereas Rac1 signaling has

been implicated in endothelial barrier stabilization (Komarova Y.A. 2007). Thrombin induced RhoA activation but not Rac1 signaling (Fig. 15 B and C). In contrast, APC stimulated Rac1 activation and minimal RhoA signaling (Fig. 15 B and C). Thus, thrombin and APC have the capacity to elicit distinct signaling pathways and differentially regulate endothelial barrier permeability. Moreover, APC-stimulated increase in Rac1 activation was lost in endothelial cells deficient in PAR1 expression (Fig. 15 D).

These studies demonstrate that endogenous PAR1 is essential for APC signaling and raises the question of how activation of the same receptor by two different proteases elicits distinct signaling responses in endothelial cells.

### **2.3.2: Caveolae are required for APC signaling through PAR1 and APC-mediated signaling and endothelial barrier protection.**

PAR1 and EPCR localize to lipid rafts and associate with caveolin-1 (Bae J.S. 2007a), but whether caveolae are essential for APC signaling and endothelial barrier protection has not been determined. To examine the role of caveolae in thrombin and APC signaling we generated endothelial cells stably expressing a caveolin-1 (CAV1) shRNA to ablate caveolin-1 expression (Fig. 16 A) (Schuck S. 2004). Importantly, the amount of cell surface PAR1 and EPCR was similar in control and caveolin-1 lacking cells (Fig. 16 B and C), suggesting that caveolae deficiency does not globally disrupt protein expression at the cell surface. Interestingly, thrombin activation of ERK1,2 was comparable in control and caveolin-1 deficient endothelial cells (Fig. 17 A), indicating that caveolae are not essential for thrombin signaling. Remarkably, however, activation of ERK1,2 by APC was lost in caveolin-1 deficient endothelial cells examined at early and late times (Fig. 17 B and C).

These findings suggest that caveolae are critical for activation of PAR1 signaling by APC but not thrombin.

We next investigated the function of caveolae in APC-induced Rac1 activation and endothelial barrier protection. APC stimulated a marked increase in Rac1 activation in control cells that was virtually abolished in endothelial cells lacking caveolin-1 (Fig. 18 A). These findings provide further evidence that caveolae are essential for APC activation of PAR1 signaling. Moreover, APC pretreatment failed to protect caveolin-1 deficient endothelial cells from thrombin-induced endothelial barrier permeability (Fig. 18 B), consistent with loss of APC signaling in caveolin-1 defective cells. Thus, the compartmentalization of PAR1 in caveolae is essential for APC activation of PAR1 protective signaling in endothelial cells.

## **2.4: Discussion**

In the present study, we define a novel function for caveolae in protease-selective signaling by PAR1. We show that endogenous PAR1 is required for thrombin and APC signaling in endothelial cells. We further demonstrate that caveolin-1 is essential for activation of PAR1 signaling by APC but not thrombin, indicating that caveolae are critical for protease-selective signaling by PAR1. Caveolae are also required for activation of PAR2 by tissue factor-factor VIIa but not the synthetic peptide agonist in transformed cells (Awasthi V. 2008), consistent with a role for caveolae in protease-selective signaling. Moreover, a function for lipid rafts but not caveolae, in thrombin-induced cytoskeletal changes in endothelial cells has previously been reported (Carlie-Klusacek M.E. and Rizzo V. 2007).

Thrombin activated PAR1 couples to  $G\alpha_q$ ,  $G\alpha_{12/13}$ , and RhoA signaling, which induces endothelial barrier dysfunction (Komarova Y.A. 2007). In contrast, we show that APC activated PAR1 stimulates Rac1 but not RhoA, signaling and promotes endothelial barrier protection. Thus, the activation of PAR1 by APC may result in a distinct active receptor conformation that selectively couples to different signaling pathways. We further show that caveolin-1 is essential for APC but not thrombin activation of PAR1 signaling and endothelial barrier protective effects, suggesting that PAR1 localization to caveolae is critical for protease-selective signaling. Previous studies have shown that the APC co-factor EPCR, PAR1,  $G\alpha_q$  and  $G\alpha_i$  partition into lipid rafts and interact with caveolin-1 (Bae J.S. 2007a) (Bae J.S. 2007b) (Li S. 1995). The barrier protective signaling induced by APC is also blocked by pertussis toxin, suggesting a role for  $G\alpha_{i/o}$  proteins in this process (Bae J.S. 2007a). Moreover, the binding of APC to EPCR facilitates efficient PAR1 cleavage in lipid rafts and endothelial barrier signaling, suggesting that caveolae localization may induce a distinct active receptor conformation that elicits barrier protective signaling (Bae J.S. 2008). Thus, PAR1, EPCR and  $G\alpha_{i/o}$  proteins localize to caveolae and may exist as a preassembled complex poised to signal following PC binding to EPCR and generation of APC. In contrast, caveolin-1 is not essential for thrombin activation of PAR1 signaling, indicating that caveolin-1 only modulates PAR1 signaling when selectively activated by APC and not by thrombin in endothelial cells.

Our studies provide new insight into the molecular mechanisms responsible for protease-selective signaling by PAR1. Evidence presented here suggests that compartmentalization of PAR1 in caveolae facilitates selective endothelial barrier protective signaling (Fig. 19). The molecular determinants that specify the targeting of PAR1 to

caveolae are not known but may involve unique posttranslational modifications. The novel regulation of PAR1 signaling by APC is also critical for endothelial barrier protection.

## **2.5: Materials and methods**

### **Reagents and Antibodies**

Human  $\alpha$ -thrombin was purchased from Enzyme Research Laboratories. Human APC and active site blocked APC-DEGR (dansyl-EGR chloromethyl ketone) were from Hematologic Technologies (Essex Junction, VT). The peptides agonists, TFLLRNPNDK and SLIGKV were synthesized at the UNC Peptide Facility, Chapel Hill, NC. Hirudin and actin antibody were obtained from Sigma (St. Louis, MO). Caveolin-1 antibody was from BD Biosciences (San Jose, CA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and -rabbit secondary antibodies were from Bio-Rad (Richmond, CA).

[*myo*-<sup>3</sup>H]inositol was purchased from American Radiolabelled Chemicals Inc. Monoclonal anti-phospho-p44/42 mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase (ERK 1,2) antibody and polyclonal anti-p44/42 MAPK (ERK1,2) antibody were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-phospho-p38 and anti-p38 were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-actin antibody was purchased from Sigma-Aldrich. Anti-caveolin1 and Anti-Rac1 antibodies were from Transduction Laboratory. Anti-RhoA was from Santa Cruz Biotech. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies were from Bio-Rad (Richmond, CA).

### **Cell lines**

EA.hy926 cells were obtained from Coragene Edgell (UNC-Chapel Hill). Cells were maintained in DMEM plus 10 % FBS and passaged once a week.

### **EA.hy926 cells expressing PAR1 shRNA or CAV1 shRNA**

The short hairpin RNAi (shRNA) 5'- AGAUUAGUCUCCAUCAUA-3' targeting PAR1 and non-specific siRNA 5'-CUACGUCCAGGAGCGCACC-3' were subcloned into *pSilencer* 5.1-U6 Retro (Ambion, Austin, TX) as described (Coughlin S.R. 2005). The caveolin-1 shRNA (5'-AAGATGTGATTGCAGAACCAGA-3') construct in pRVH1-puro was obtained from K. Simons and scrambled shRNA 5' -GTAAATGCCATACCTTATA-3' of PAR1 siRNA sequence was inserted into pSUPER.retro.puro vector (Oligoengine, Seattle, WA). Retroviruses were generated using PA317 packaging cells and used to infect EA.hy926 cells. Mass populations of cells stably transduced with PAR1 shRNA, caveolin-1 shRNA, non-specific, scrambled shRNAs and vector control constructs were selected with 0.6 µg/ml of puromycin.

### **RhoA and Rac1 Activity Assays**

GST-rhotekin RBD and PAK-PBD were purified and assays were conducted as described below. EA.hy926 endothelial cells were plated in 6-well dishes at  $5 \times 10^5$  cells per well, grown for two days, deprived of serum and then treated with or without agonists for various times at 37°C. To assess RhoA activation, cells were lysed in buffer containing 50 mM Tris-HCL, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 20 mM MgCl<sub>2</sub> with protease inhibitors. Endogenous RhoA activity was then measured in pull-down assays using a GST fusion of the Rho binding domain (RBD) of Rhotekin. To monitor Rac1 activity, cells were lysed in buffer containing 50 mM Tris-HCL, pH 7.6, 150 mM NaCl, 1% Triton X-100, 20 mM MgCl<sub>2</sub> with protease inhibitors and then incubated with GST-p21-activated kinase (PAK-1) binding domain (PBD) fusion protein. The GST PAK-PBD and Rhotekin-RBD fusion constructs were transformed into BL21 (DE3) *E. coli* and fusion

proteins were induced and prepared using standard techniques. GST- PAK-PBD (120 mg) or Rhotekin-RBD (90 mg) bound to glutathione-Sepharose beads were then incubated with cell lysates for 1 h at 4°C and washed. GTP-bound RhoA or Rac1 were eluted in 2X SDS-sample buffer containing 100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue, resolved by SDS-PAGE and transferred to membranes. The amount of endogenous activated RhoA and Rac1 were then detected by immunoblotting using a monoclonal anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or a monoclonal anti-Rac1 antibody from BD Biosciences, respectively. Immunoblots were developed with enhanced chemiluminescence (ECL) (Amersham Biosciences, Inc., Arlington, IL), imaged by autoradiography, and quantitated using a Bio-Rad Fluor-S-Multimager (Richmond, CA).

#### **ERK1,2 and p38 activation**

EA.hy926 cells were plated in 24-well dishes at a density of  $0.7 \times 10^5$  per well. Cells were serum-starved in 0.2% FBS over-night. The cells were pre-incubated in the absence of serum for 2 hrs and then treated with 10nM thrombin or 5 nM APC plus 0.5U/ml of Hirudin or left untreated. Then the cells were lysed in 2x SDS-gel loading buffer [100 mmol/L Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.02% bromophenol blue]. Cell lysates were resolved by SDS-PAGE, transferred to membranes, and immunoblotted with an anti-phospho-p44/42 MAPK (ERK1,2) or anti-phospho p38 antibodies (Cell Signaling). To detect total p44/42 MAPK (ERK1,2), membranes were stripped and reprobbed with an anti-p44/42 MAPK (ERK1,2) or anti p38 antibodies. Immunoblots were developed, imaged, and quantitated using a Bio-Rad Fluor-S Multimager.



### **Cell surface ELISA**

To follow cell surface PAR1 expression, EA.hy926 endothelial cells were plated at 70% confluence on 24-well culture dishes (Falcon), grown for two days and then incubated with or without agonist for various times at 37 °C. fixed with 4% paraformaldehyde for 5 min at 4 °C, washed, and incubated with anti-PAR1 antibody C54 33, washed incubated with secondary horseradish peroxidase-goat anti-rabbit GAR-HRP. The amount of bound secondary antibody was determined by incubation with 1-Step ABTS (2,2'-azinobis-3-ethylbenz-thiazoline-6-sulfonic acid) (Pierce) substrate for 10–20 min at 25 °C. An aliquot was removed, and the optical density determined at 405 nm using a Molecular Devices SpectraMax Plus microplate reader.

### **Immunoblotting**

EA.hy926 endothelial cells were plated at 70% density on 24-well culture dishes (Falcon). The cells were starved overnight in 0.2% FBS. The cells were pre-incubated in the absence of serum for 2 hrs and then treated with 10nM thrombin or 5 nM APC plus 0.5U/ml of Hirudin or left untreated. The cells were lysed in Laemmli lysis buffer. Lysates were resolved by SDS-PAGE, transferred, and immunoblotted with anti-caveolin1 antibody (BD). Immunoblots were developed with ECL-PLUS (Amersham Biosciences, Inc.), developed by the enhanced chemiluminescence ECL system (Amersham Biosciences) and visualized by exposure to film. Membranes were then stripped and reprobed with anti-actin antibody.

### **Phosphoinositide Hydrolysis**

Cells plated in 12-well dishes and labeled with 2 µCi/ml of [*myo*-<sup>3</sup>H]inositol in serum-free DMEM containing 1 mg/ml BSA overnight. Cells were washed and then incubated in the

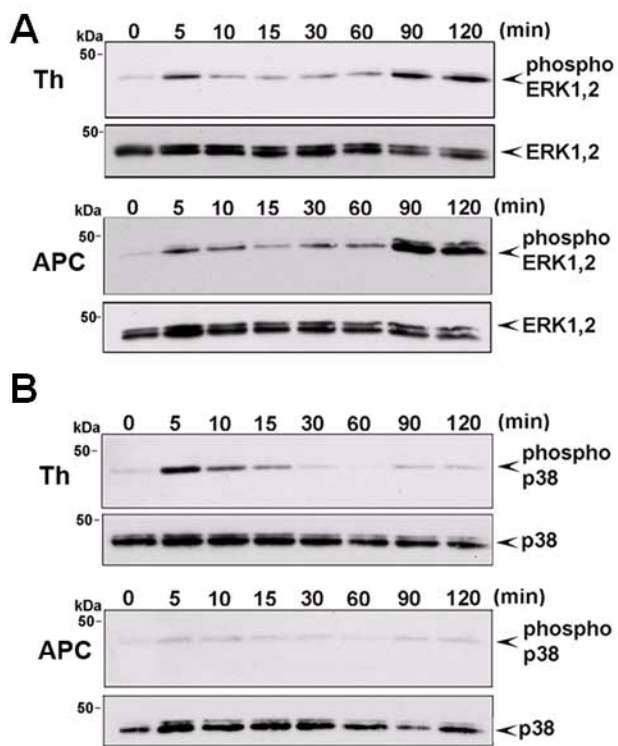
absence or presence of agonist diluted in DMEM, 1 mg/ml BSA containing 20 mM lithium chloride for various times at 37 °C. Cell incubation medium was removed, and total cellular [3H]inositol phosphates ([3H]IPs) were extracted, isolated, and quantitated as described previously (Paing M.M. 2002).

### **Permeability Assay**

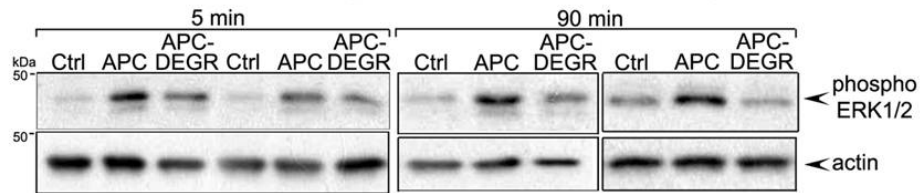
Endothelial barrier permeability was quantified by measuring the flux of Evans blue-bound bovine serum albumin (BSA) (Sigma, St. Louis, MO). Briefly, EA.hy926 cells ( $5 \times 10^4$  cells per well) were plated on 12-mm diameter transwell dishes coated with 2% gelatin (3- $\mu$ m pore size polycarbonate filter; Costar, Corning, NY) and grown for 4-6 days at 37°C. The upper and lower chambers contained 500- $\mu$ L and 1500- $\mu$ L growth media, respectively. The day before the experiment, the growth medium was replaced with starvation medium (DMEM containing 0.2% FBS). On the day of the experiment, cells were washed and then incubated with or without 10 nM APC for 3 h at 37°C, added to the upper chamber. Cells were washed, and then incubated with or without 10 nM thrombin or 10 nM APC for 10 to 20 min at 37°C added to the upper chamber. The medium in the upper chamber was then replaced with 0.67 mg/mL Evans blue-BSA diluted in growth medium containing 4% BSA (Sigma) and after 10-20 minutes the optical density (OD) at 650 nm was measured in a 1:3 diluted 50  $\mu$ L sample from the lower chamber using a Molecular Devices Plate Reader (Sunnyvale, CA).

### **Data Analysis**

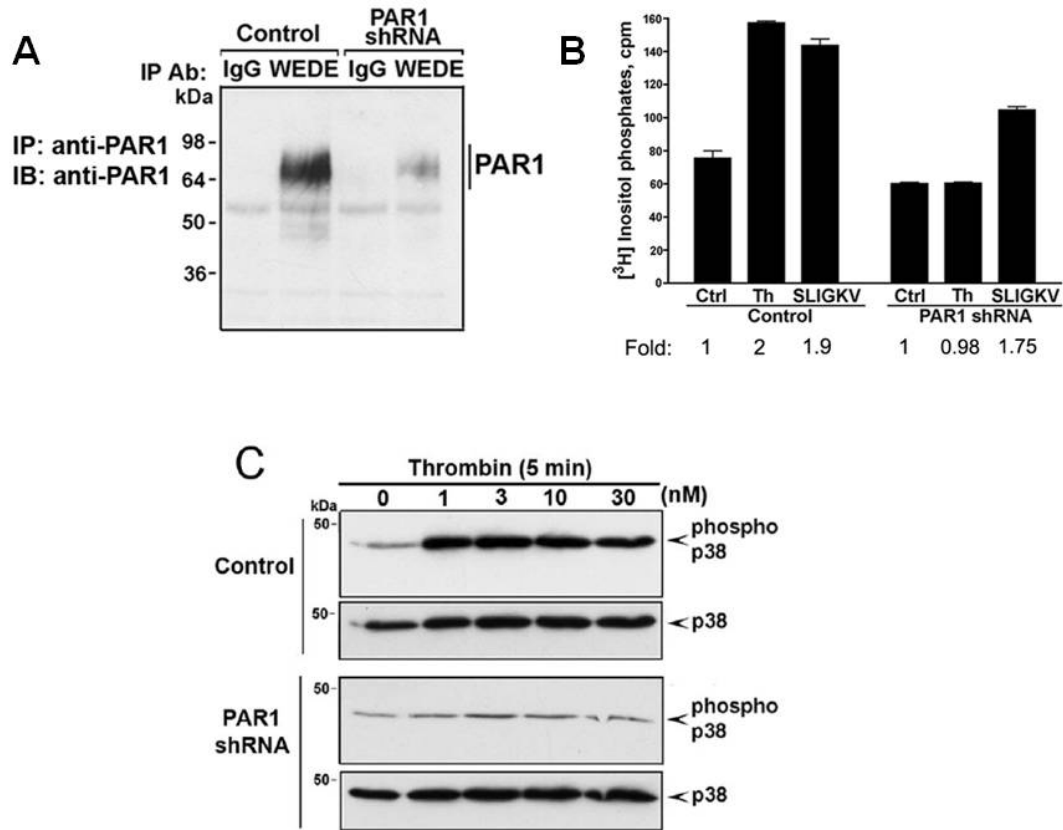
Data were analyzed using Prism 4.0 software (GraphPad), and statistical significance was determined using InStat 3.0 (GraphPad). Group comparisons were made using an unpaired t-test.



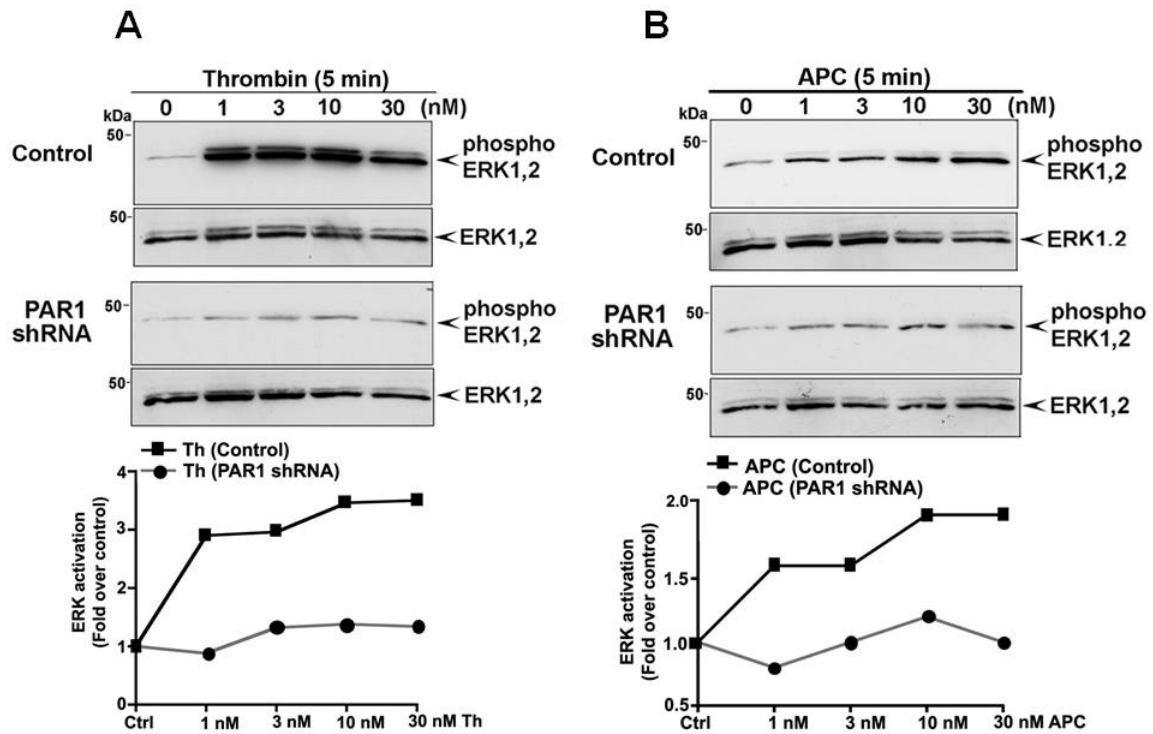
**Fig. 11. Activation of ERK1,2 and p38 signaling by thrombin and APC. (A) and (B)** Serum-starved endothelial cells were incubated in the absence or presence of 10 nM thrombin (Th) or 10 nM APC (with 0.5 U/ml hirudin) for various times at 37°C. ERK1,2 and p38 activation were then determined using specific anti-phospho mitogen-activated protein kinase (MAPK) antibodies. Membranes were stripped and reprobbed for total MAPK.



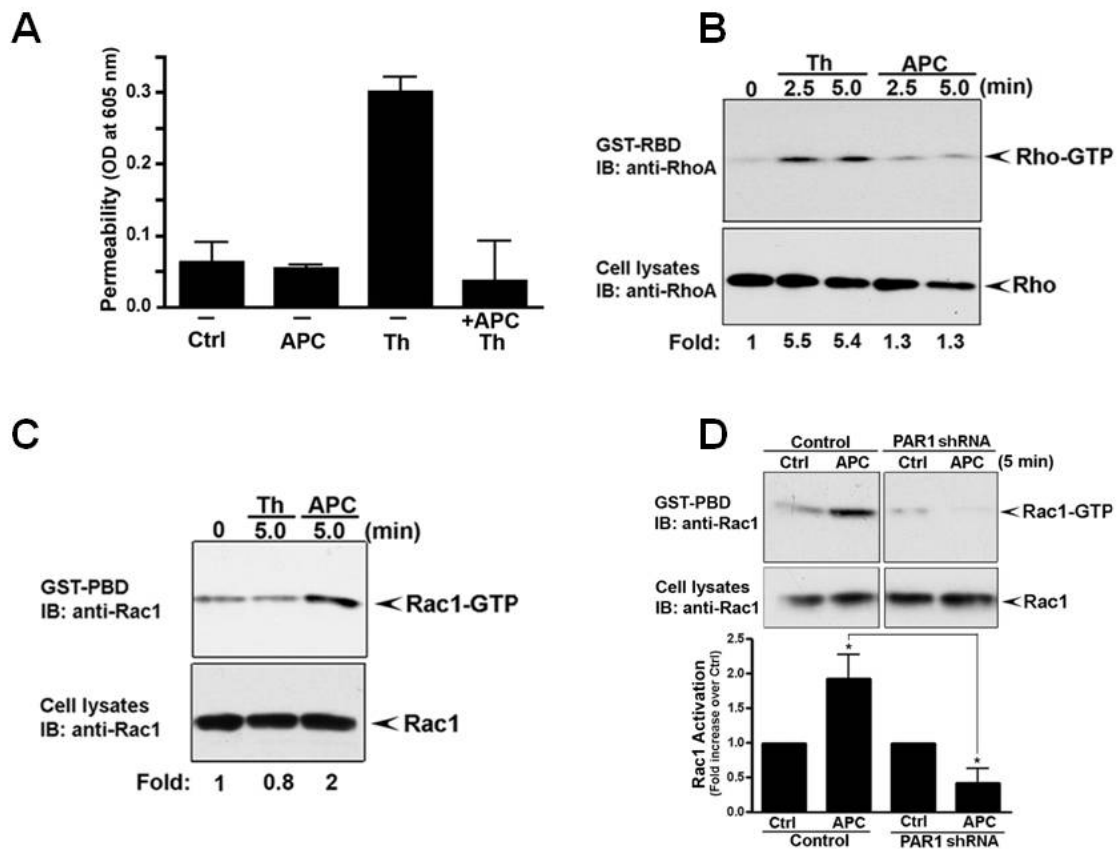
**Fig. 12. Catalytic activity of APC is required for ERK1,2 activation.** Serum-starved wild-type EA.hy926 endothelial cells were incubated in the absence or presence of 10 nM APC or active site blocked 10 nM APC-dansyl-EGR chloromethyl ketone (DEGR) for 5 min or 90 min at 37°C. Cells were lysed and ERK1,2 activity was determined by immunoblotting. Membranes were stripped and re-probed with anti-actin antibody as a control for loading. These findings were observed in several separate experiments.



**Fig. 13. Thrombin signaling is lost in EA.hy926 endothelial cells expressing PAR1 shRNA.** (A) Equivalent amounts of lysates prepared from control and PAR1 shRNA expressing cells were immunoprecipitated with monoclonal anti-PAR1 antibody or IgG control. Immunoprecipitates were immunoblotted with anti-PAR1 polyclonal antibody to detect PAR1 expression. (B) Control and PAR1-deficient endothelial cells labeled with *myo*-[<sup>3</sup>H]inositol were incubated in the presence or absence of 10 nM thrombin (Th) or 100  $\mu$ M SLIGKV (PAR2agonist peptide) for 60 min at 37°C in medium containing lithium chloride. The amounts of accumulated [<sup>3</sup>H]IPs were then measured. The data are shown as total [<sup>3</sup>H]inositol phosphates (cpm) accumulated and expressed as fold-increase over untreated control. (C) Serum-starved control and PAR1 deficient cells were incubated with or without various concentrations of thrombin (Th) for 5 min at 37°C. Cells were lysed and activation of p38 was determined using anti-phospho p38 antibody. Membranes were re probed with total p38 antibody to control for loading.

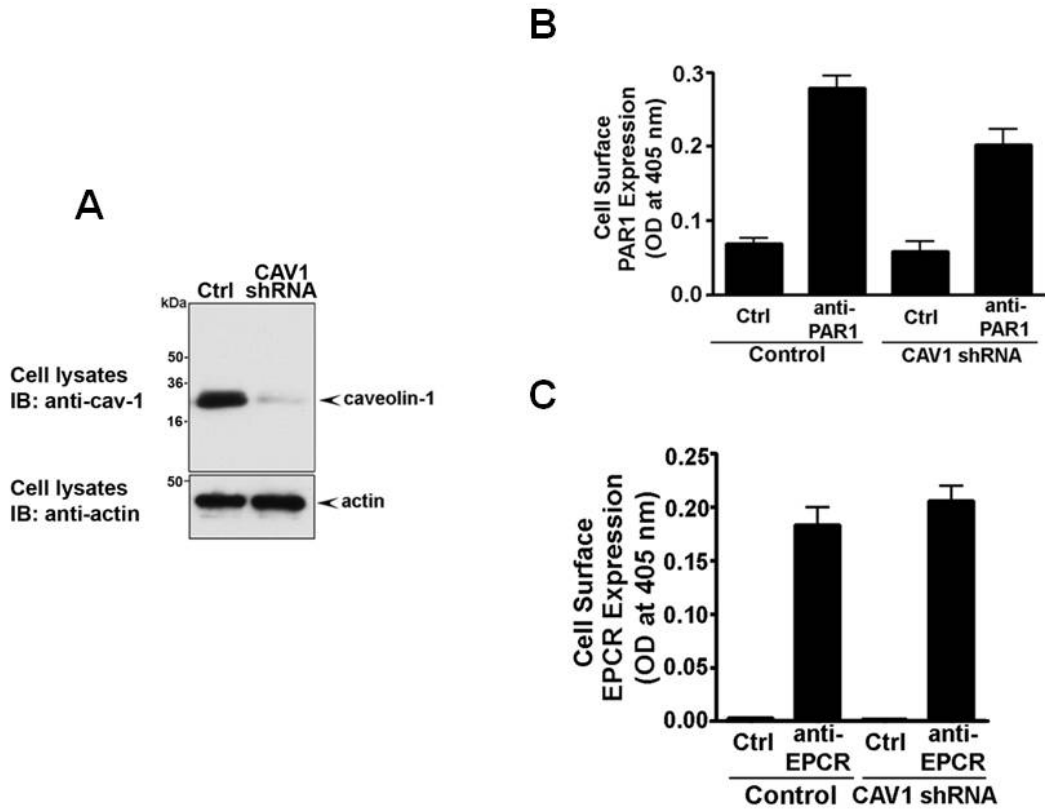


**Fig. 14. PAR1 is essential for thrombin and APC signaling in to ERK1,2 endothelial cells.** (A) and (B) Serum-deprived control and PAR1 shRNA-expressing EA.hy926 endothelial cells were incubated with thrombin or APC (0.5 U/ml hirudin) for 5 min at 37°C and ERK1,2 activity was determined by immunoblotting. These data are representative of three independent experiments.

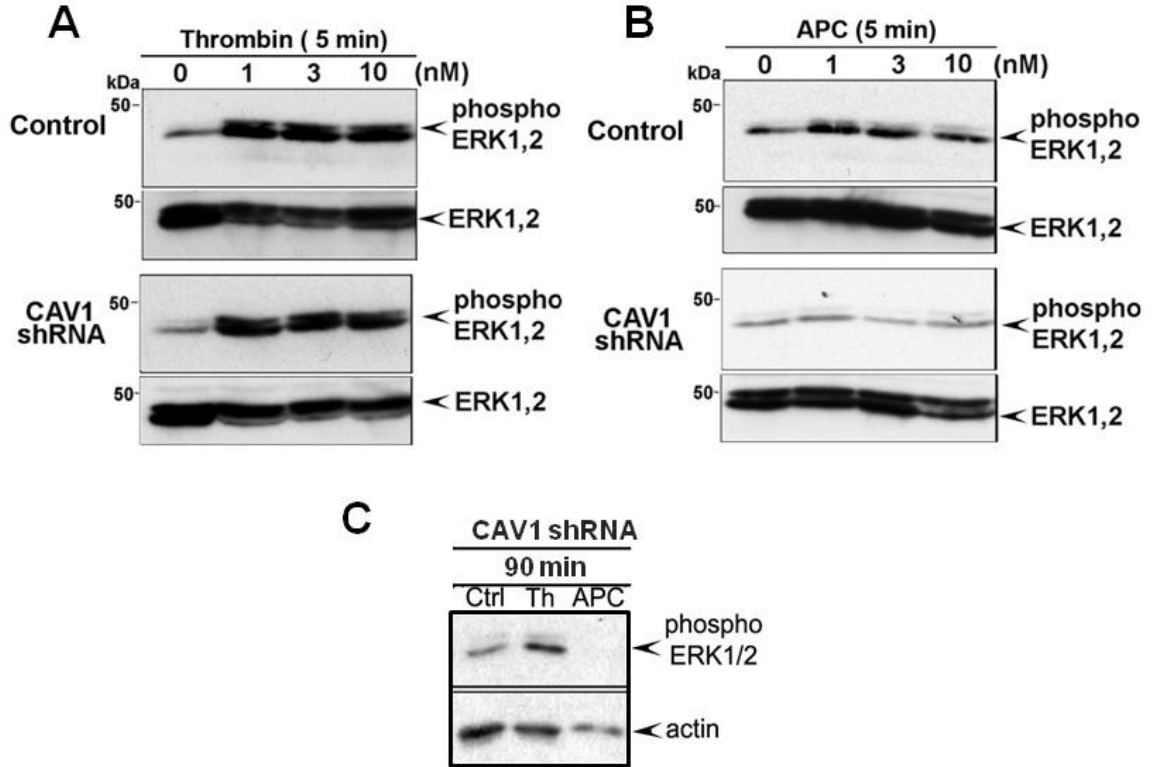


**Fig. 15. Thrombin and APC differentially activate endothelial permeability and RhoA and activation. (A)** Confluent EA.hy926 cells were preincubated with or without 10 nM APC for 3 h at 37°C in medium containing 0.5 U/ml hirudin and then treated with 10 nM APC or 10 nM thrombin (Th) for 20 min at 37°C and endothelial barrier permeability was performed. The data (mean  $\pm$  S.D.,  $n=3$ ) are representative of three independent experiments. **(B) and (C)** Cells were incubated with or without 10 nM thrombin (Th) or 10 nM APC (0.5 U/ml hirudin) at 37°C. Cells were lysed and activated RhoA and Rac1 were detected by immunoblotting. The data are representative of three separate experiments. **(D)** Control and PAR1 deficient endothelial cells were incubated with or without 10 nM APC for 5 min at 37°C and activation of Rac1 was determined. The data (mean  $\pm$  S.E.) are expressed as the fold-increase over untreated control and are the averages of three independent experiments. The difference between Rac1 activation induced by APC in control *versus* PAR1-deficient cells was significant (\*,  $P<0.05$ ).

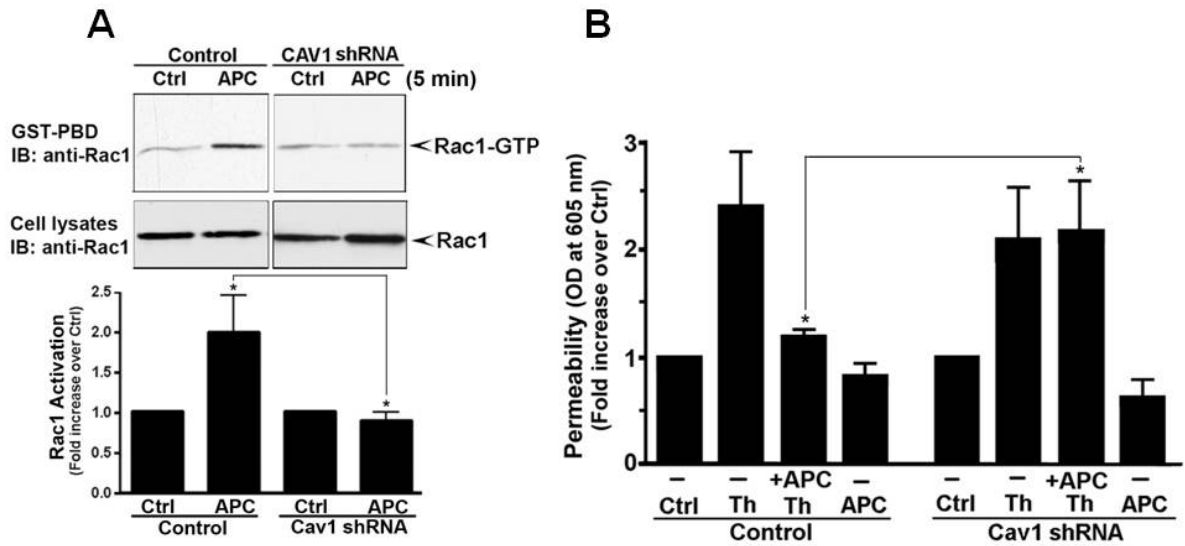




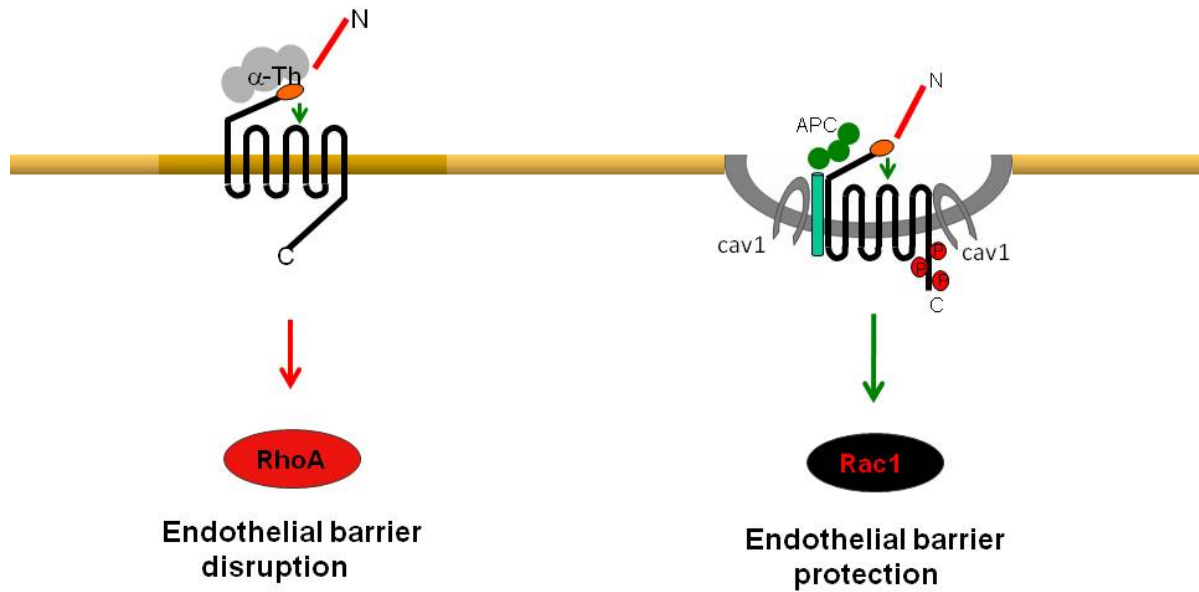
**Fig. 16. Caveolin-1 knock-down does not affect PAR1 and EPCR cell surface expression.** (A) Cells were lysed and protein extract was processed by SDS-PAGE and detected using antibodies. (B) and (C) Control and caveolin-1 (CAV1) deficient endothelial cells were fixed, and the amounts of cell surface PAR1 and EPCR at steady state were determined by ELISA. The data (mean  $\pm$  S.D.,  $n=3$ ) are representative of replicate experiments.



**Fig. 17. Caveolin-1 is required for APC but not thrombin-induced ERK1,2 activation.** Serum-deprived caveolin-1 shRNA expressing EA.hy926 endothelial cells were incubated with 10 nM thrombin (**A**) or 10 nM APC (0.5 U/ml hirudin) (**B**) for 5 min or 90 min (**C**) at 37°C. Cells were lysed and ERK1,2 activity was determined by immunoblotting. The membranes were stripped and reprobbed with an anti-actin antibody to control for loading. Similar results were observed in multiple independent experiments.



**Fig. 18. Caveolin-1 is essential for APC-induced Rac1 activation and endothelial barrier protection.** **(A)** Control and caveolin-1 (CAV-1) deficient cells were incubated with or without 10 nM APC at 37°C and Rac1 activation was determined. The data (mean  $\pm$  S.E.) are expressed as fold-increase over control and are the averages of three different experiments. The difference between Rac1 activation induced by APC in control *versus* CAV-1 deficient cells was significant (\*,  $P < 0.05$ ). **(B)** Control and CAV-1 deficient cells were treated with or without 10 nM APC for 3 h at 37°C and then incubated with 10 nM thrombin (Th) or 10 nM APC for 10 min at 37°C and permeability was monitored. The data (mean  $\pm$  S.E.) are the averages of three independent experiments performed in triplicate. The difference between thrombin-induced permeability in control *versus* CAV-1 deficient cells was significant (\*,  $P < 0.05$ ).



**Fig. 19. Schematic of caveole-dependent selective signaling of PAR1.** Thrombin-activated PAR1 mediates increases in permeability through RhoA activation. Conversely, APC-activated PAR1 leads to activation of Rac1 and protects endothelial barrier.

## **CHAPTER 3: APC PREVENTS THROMBIN-INDUCED ENDOTHELIAL BARRIER DISRUPTION BY DESENSITIZING PAR1 SIGNALLING RESPONSE TO THROMBIN**

### **3.1: Abstract**

Activated protein C (APC) is known to maintain endothelial barrier integrity and also to protect against thrombin induced disruption of endothelial barrier (Feistritzer C. 2005). However, the mechanism underlying APC inhibition of thrombin induced endothelial disruption is not known. Here, I investigated whether APC exerts its endothelial barrier protective function by desensitizing PAR1 to further stimulation by thrombin. I show that APC pretreatment inhibits thrombin-induced signaling to ERK1,2, p38 and RhoA activation. Phosphorylation of activated PAR1 is the initial event critical for uncoupling the receptor from G-protein signaling (Ishii K. 1995). In addition to phosphorylation, internalization and lysosomal degradation are also critical for the regulation of PAR1 signaling (Coughlin S.R. 1999). I show that APC promotes phosphorylation of endogenous PAR1 in human endothelial cells. However, in contrast to thrombin I demonstrated that APC does not affect PAR1 internalization or degradation suggesting a new molecular mechanism for APC regulation of PAR1 signaling.

### **3.2: Introduction**

Persistent activation of receptors results in the eventual loss of receptor-activated function or desensitization. Three general temporarily distinct mechanisms are associated with desensitization of G-protein coupled receptors. The first involve GPCR phosphorylation by GRKs or second messenger kinases that uncouple the receptor from G-proteins. Once phosphorylated, activated GPCRs are rapidly internalized, removing activated receptors from signaling effectors. Receptor internalization may involve different membrane trafficking pathways involving either caveolae, clathrin-coated, or non-coated vesicles. Internalized receptors can then either recycle back to the cell-surface or enter the endocytic pathway and eventually be degraded in lysosomes. Both receptor phosphorylation and internalization are associated with rapid receptor desensitization, which occurs within minutes. With prolonged agonist exposure, a slower phase (typically hours) of receptor down-regulation occurs in which the steady-state level of receptor protein is diminished. Mechanisms responsible for the agonist-induced reduction in receptor number involve changes in either receptor synthesis and/or receptor lysosomal degradation. Activated PAR1 is rapidly phosphorylated and sorted directly to lysosomes and degraded.

The ability of APC to protect endothelial barrier integrity also involves inhibition of thrombin-induced increases in permeability (Feistritz C. 2005). However, the molecular mechanisms through which APC inhibits thrombin-induced increases in permeability are not known. In the present study I examined whether exposure of endothelial cells to APC desensitizes PAR1 to thrombin signaling. Herein I show that APC promotes protective effects in endothelial cells by desensitizing cells to thrombin signaling without affecting thrombin-mediated internalization or degradation of PAR1, suggesting a novel mechanism of receptor regulation.

### **3.3: Results**

#### **3.3.1: APC desensitizes endothelial cells to thrombin signaling**

To determine how APC prevents thrombin from causing endothelial barrier dysfunction we examined whether APC desensitizes cells to thrombin signaling. Thrombin caused robust ERK1,2 activation in naïve cells (Fig. 20 A). By contrast, thrombin-stimulated ERK1,2 activation was markedly reduced in cells pretreated with APC (Fig. 20 A). Moreover, thrombin-induced RhoA activation and p38 kinase activation were considerably attenuated in cells pretreated with APC (Fig. 20 B and C), providing further evidence that APC desensitizes cells to thrombin signaling to promote endothelial barrier protection. Interestingly, inhibition of thrombin-induced ERK1,2 activation by APC required caveolin-1 expression (Fig. 21), consistent with a critical role for caveolae in APC signaling. Signaling by PAR2 agonist peptide, and UTP, an agonist for endogenous purinergic receptors, was unperturbed by APC pretreatment, indicating that endothelial cells are generally responsive to GPCR activation after APC pretreatment (Fig. 22).

#### **3.3.2: APC induces PAR1 phosphorylation and does not affect trafficking or degradation of the receptor**

We determined whether APC promotes phosphorylation of endogenous PAR1 in endothelial cells. Phosphorylated PAR1 was detected after thrombin incubation and migrated as a broad band at ~64 kDa (Fig. 23 A and C). Cells exposed to APC also showed an increase in PAR1 phosphorylation, which was detected as multiple high molecular weight species migrating at and above ~64 kDa (Fig. 23 A). Strikingly, in APC pretreated cells, thrombin and APC failed to induce phosphorylation of PAR1 (Fig. 23 A). These data

suggest the APC regulates thrombin signaling at the level of the receptor to promote endothelial barrier protection.

In addition to phosphorylation, receptor internalization and lysosomal degradation also regulate PAR1 signaling (Trejo J. 1999; Trejo J H. S. 1998). We therefore examined whether APC affects PAR1 trafficking. Thrombin induced rapid and robust PAR1 internalization (Fig. 23 B). In contrast, APC failed to promote PAR1 internalization even at high concentrations (Fig. 23 B), consistent with retention of PAR1 on the cell surface (Schuepbach R.A. 2008). Remarkably, however, thrombin stimulated comparable increases in PAR1 internalization in both untreated and APC treated cells (Fig. 23 D), suggesting that APC exposure does not prevent thrombin-induced PAR1 internalization.

We further investigated whether thrombin promotes PAR1 degradation in cells exposed to APC. Thrombin caused a shift in mobility and a significant loss of PAR1 protein in control cells (Fig. 23 C), consistent with thrombin cleavage and degradation of activated PAR1. Activation of PAR1 with the peptide agonist TFLLRNPDNK also decreased receptor protein without altering receptor mobility, as expected (Fig. 23 C). To our surprise, prolonged exposure to APC failed to induce a significant change in PAR1 mobility or amount of receptor protein detected compared to control cells (Fig. 23 C). Moreover, the extent of PAR1 degradation induced by thrombin and peptide agonist was comparable in APC treated and untreated cells (Fig. 23 C). Together these studies suggest that APC desensitizes cells to thrombin signaling by inducing PAR1 phosphorylation, but causes limited receptor cleavage and negligible internalization and degradation.



### 3.4: Discussion

Our studies suggest that APC promotes protective effects by desensitizing endothelial cells to thrombin signaling (Fig. 24). We found that APC stimulates PAR1 phosphorylation and inhibits thrombin signaling but causes limited receptor cleavage, and negligible internalization and degradation. The molecular mechanism by which APC distinctly activates PAR1 signaling remains unclear. PAR1 is essential for APC signaling, but whether APC induces an active PAR1 conformation similar to thrombin is not known. Previous studies have shown that APC has the capacity to cleave PAR1, albeit with considerably less efficiency than thrombin (Riewald M. 2002; Ludeman M.J. 2005). However, whether APC cleavage of PAR1 is the only critical determinant that facilitates PAR1 activation of barrier protective signaling is not known. Thrombin cleaves the majority of PAR1, causing a shift in receptor mobility and induces receptor degradation, whereas the peptide agonist TFLLRNPNDK promotes PAR1 degradation but not cleavage and hence, does not alter receptor mobility. We show here that APC induces PAR1 signaling and phosphorylation but causes a minimal change in receptor mobility. Interestingly, we also show that prolonged APC incubation does not prevent thrombin-induced PAR1 cleavage, internalization or degradation. Thus, in endothelial cells exposed to APC the majority of PAR1 is retained on the cell surface and susceptible to thrombin cleavage. Our findings raise the intriguing possibility that APC activates a sub-population of PAR1 compartmentalized in caveolae and stabilizes an active receptor conformation that may be distinct from non-caveolar localized activated PAR1.

How can activation of the same receptor by two different proteases elicit distinct cellular responses? If APC activates PAR1 through cleavage and unmasking of the tethered ligand like thrombin a similar active receptor conformation would be induced.

However, the extent of PAR1 activation by APC would be different from thrombin, which efficiently cleaves the receptor (Ludeman M.J. 2005). In this case, we would expect to observe quantitative not qualitative differences in signaling. We previously showed that PAR1 trafficking is essential for the fidelity of thrombin signaling (Trejo J. 1998; Paing M.M. 2006). However, in contrast to thrombin, our findings here suggest that APC imparts a novel mechanism for regulation of PAR1 signaling that involves receptor phosphorylation but not internalization or degradation. Thus, in future pursuits it will be important to determine the mechanism(s) by which endothelial cells desensitize and resensitize to APC signaling.

This study has sought to investigate the mechanism by which APC inhibits thrombin-mediated PAR1 increase permeability. We asked the question whether APC is able to desensitize PAR1 to thrombin signaling. The molecular mechanisms responsible for GPCR desensitization have been widely studied for the  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) (Lefkowitz R.J. 1998; Pitcher J.A. 1998). Briefly, GPCRs are initially desensitized by rapid phosphorylation of the receptor in the active form by G protein-coupled kinases (GRKs). The phosphorylated receptor then binds arrestin, which inhibits interaction with G proteins. Arrestin also facilitates GPCR internalization by interacting with clathrin and the adaptor protein complex-2 (AP-2), components of the endocytic machinery. Once internalized into endosomes, receptor dissociates from the ligand, becomes dephosphorylated, and is then recycled back to the plasma membrane ready for activation again. Phosphorylation of activated PAR1 initiates rapid desensitization and internalization from the plasma membrane. Activated PAR1 is internalized through a dynamin- and clathrin-dependent pathway, like many recycling receptors (Trejo J. 2000; Hoxie J.A. 1993). Once internalized, PAR1 is sorted away from recycling receptors and targeted to lysosomes for degradation,

an event critical for termination of receptor signaling (Trejo J. 1998). Our studies suggest that APC promotes protective effects by desensitizing endothelial cells to thrombin signaling. Furthermore, we show that APC inhibits thrombin-induced PAR1 phosphorylation but not internalization or degradation. Our laboratory demonstrated that, in the absence of  $\beta$ -arrestins, rapid desensitization of PAR1 signaling is markedly impaired while internalization remains intact. Thus, internalization is not required for rapid desensitization of PAR1 signaling by  $\beta$ -arrestins. This may suggest a role for  $\beta$ -arrestins in APC-mediated PAR1 desensitization.  $\beta$ -arrestin indeed is required for rapid desensitization of PAR1 signaling, whereas internalization and lysosomal sorting appear to contribute to termination of PAR1 signaling observed at later times. Furthermore, most activated GPCRs require phosphorylation for  $\beta$ -arrestin binding and consequent receptor desensitization. In contrast,  $\beta$ -arrestins bind to activated PAR1 independent of phosphorylation to promote uncoupling from G protein signaling.

### **3.5: Material and methods**

#### **Reagents and Antibodies**

Human  $\alpha$ -thrombin was purchased from Enzyme Research Laboratories. Human APC was from Hematologic Technologies (Essex Junction, VT). The peptide agonists, TFLLRNPNDK and SLIGKV were synthesized at the UNC Peptide Facility, Chapel Hill, NC. Hirudin and actin antibody were obtained from Sigma (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and -rabbit secondary antibodies were from Bio-Rad (Richmond, CA).

[*myo*-<sup>3</sup>H]inositol was purchased from American Radiolabelled Chemicals Inc. Monoclonal anti-phospho-p44/42 mitogen-activated protein kinase (MAPK); extracellular signal-

regulated kinase (ERK 1,2) antibody and polyclonal anti-p44/42 MAPK (ERK1,2) antibody were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-phospho-p38 and anti-p38 were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-actin antibody was purchased from Sigma-Aldrich. Anti-RhoA was from Santa Cruz Biotech. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies were from Bio-Rad (Richmond, CA).

### **Cell lines**

EA.hy926 cells were obtained from Coragene Edgell (UNC-Chapel Hill). Cells were maintained in DMEM plus 10 % FBS and passaged once a week.

### **EA.hy926 cells expressing PAR1 shRNA or CAV1 shRNA**

The short hairpin RNAi (shRNA) 5'- AGAUUAGUCUCCAUCAUA-3' targeting PAR1 and non-specific siRNA 5'-CUACGUCCAGGAGCGCACC-3' were subcloned into *pSilencer 5.1-U6 Retro* (Ambion, Austin, TX) as described (Coughlin S.R. 2005). The caveolin-1 shRNA (5'-AAGATGTGATTGCAGAACCAGA-3') construct in pRVH1-puro was obtained from K. Simons and scrambled shRNA 5' -GTAAATGCCATACCTTATA-3' of PAR1 siRNA sequence was inserted into pSUPER.retro.puro vector (Oligoengine, Seattle, WA). Retroviruses were generated using PA317 packaging cells and used to infect EA.hy926 cells. Mass populations of cells stably transduced with PAR1 shRNA, caveolin-1 shRNA, non-specific, scrambled shRNAs and vector control constructs were selected with 0.6 µg/ml of puromycin.

### **RhoA Activity Assay**

GST-rhotekin RBD was purified and assays were conducted as described below. EA.hy926 endothelial cells were plated in 6-well dishes at  $5 \times 10^5$  cells per well, grown for two days, deprived of serum and then treated with or without agonists for various times at 37°C. To assess RhoA activation, cells were lysed in buffer containing 50 mM Tris-HCL, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 20 mM  $MgCl_2$  with protease inhibitors. Endogenous RhoA activity was then measured in pull-down assays using a GST fusion of the Rho binding domain (RBD) of Rhotekin. The GST PAK-PBD fusion constructs were transformed into BL21 (DE3) *E. coli* and fusion proteins were induced and prepared using standard techniques. Rhotekin-RBD (90 mg) bound to glutathione-Sepharose beads were then incubated with cell lysates for 1 h at 4°C and washed. GTP-bound RhoA was eluted in 2X SDS-sample buffer containing 100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue, resolved by SDS-PAGE and transferred to membranes. The amount of endogenous activated RhoA was then detected by immunoblotting using a monoclonal anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblots were developed with enhanced chemiluminescence (ECL) (Amersham Biosciences, Inc., Arlington, IL), imaged by autoradiography, and quantitated using a Bio-Rad Fluor-S-Multimager (Richmond, CA).

### **ERK1,2 and p38 activation**

EA.hy926 cells were plated in 24-well dishes at a density of  $0.7 \times 10^5$  per well. Cells were serum-starved in 0.2% FBS over-night. The cells were pre-incubated in the absence of serum for 2 hrs and then treated with 10nM thrombin or 5 nM APC plus 0.5U/ml of Hirudin or left untreated. Then the cells were lysed in 2x SDS-gel loading buffer [100 mmol/L Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.02% bromophenol blue]. Cell lysates were resolved

by SDS-PAGE, transferred to membranes, and immunoblotted with an anti-phospho-p44/42 MAPK (ERK1,2) or anti-phospho p38 antibodies (Cell Signaling). To detect total p44/42 MAPK (ERK1,2), membranes were stripped and reprobed with an anti-p44/42 MAPK (ERK1,2) or anti p38 antibodies. Immunoblots were developed, imaged, and quantitated using a Bio-Rad Fluor-S Multimager.

### **Cell Surface ELISA**

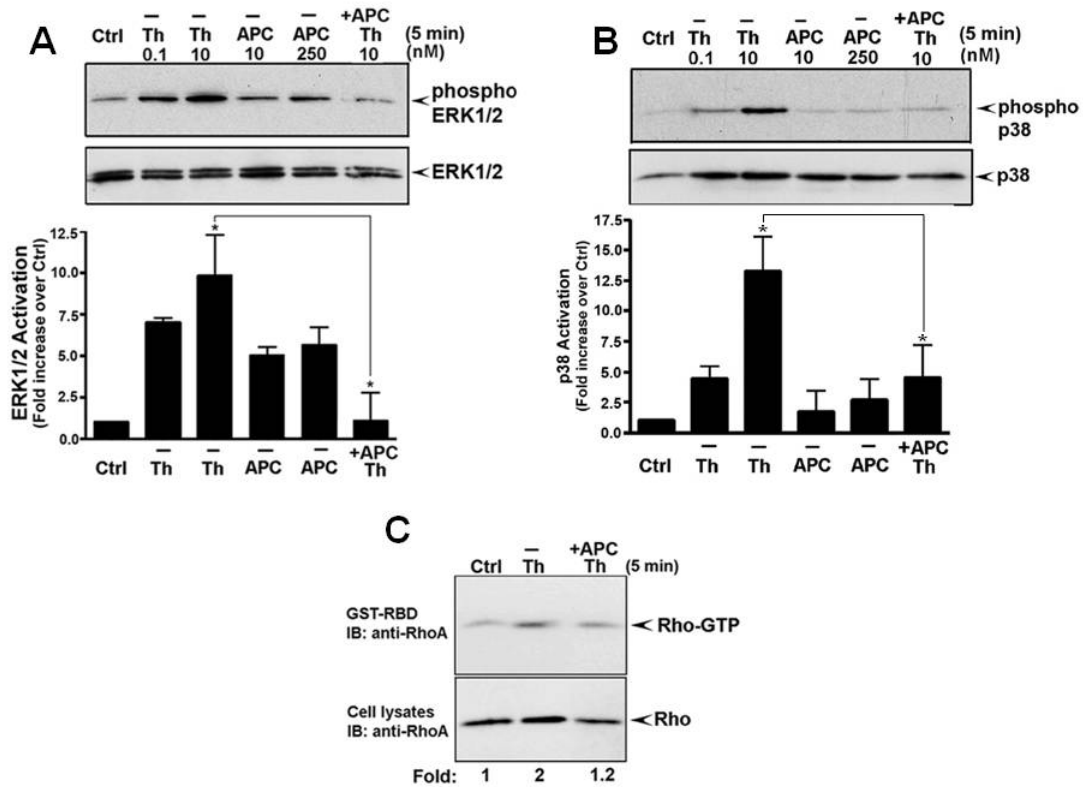
EA.hy926 cells were plated at  $0.7 \times 10^5$  cells per well in 24-well culture dishes. After incubations, cells were fixed and processed as previously described (Paing M.M., 2002). The amount of PAR1 remaining on the cell surface was detected using a rabbit polyclonal anti-PAR1 antibody generated against the hirudin-like domain as previously described (34, 38). The amount of EPCR on the cell surface was quantitated using a monoclonal anti-EPCR JRK 1500 antibody generously provided by C. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK).

### **PAR1 Phosphorylation and Degradation**

EA.hy926 cells plated at  $5 \times 10^5$  cells per well in 6-well dishes were grown overnight and PAR1 degradation was determined as described (Paing M.M., 2002). To assess PAR1 phosphorylation, EA.hy926 cells were labeled with  $200 \mu\text{Ci } [^{32}\text{P}]\text{orthophosphate}$  (Perkin-Elmer Inc., Boston, MA) in phosphate-free DMEM containing 1 mg/ml BSA for 3 h at 37°C. After cell treatments, PAR1 was immunoprecipitated with anti-PAR1 WEDE-15 monoclonal antibody, resolved by SDS-PAGE and transferred to membranes. Phosphorylated receptor was detected by autoradiography. The amount of PAR1 in immunoprecipitates was determined by immunoblotting with polyclonal anti-PAR1 antibody.

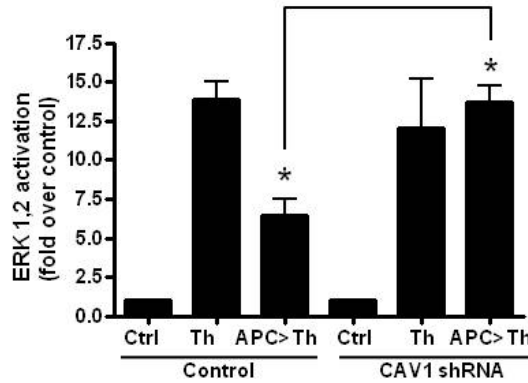
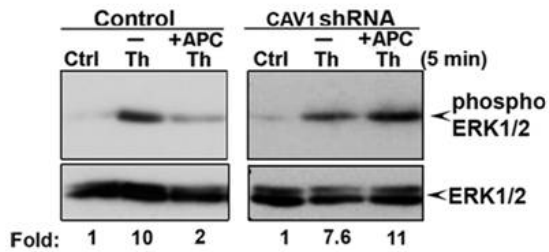
## **Data Analysis**

Data were analyzed using Prism 4.0 software (GraphPad), and statistical significance was determined using InStat 3.0 (GraphPad). Group comparisons were made using an unpaired t-test.

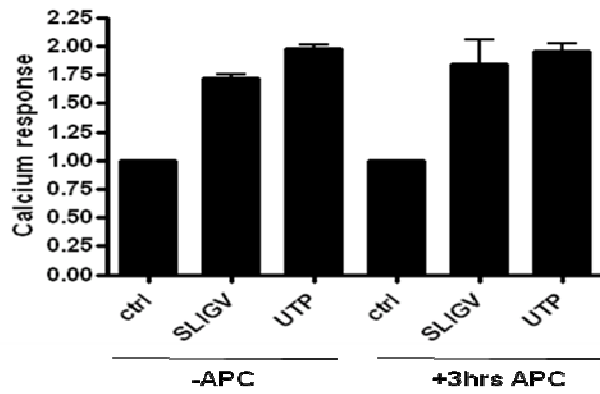


**Fig. 20. APC desensitizes PAR1 to thrombin signaling.** (A) EA.hy926 cells were preincubated with or without 10 nM APC for 1 h at 37°C and then stimulated with thrombin (Th) or APC at 37°C and ERK1,2 activation was examined by immunoblotting. The data (mean ± S.E.) are expressed as the fold-increase over untreated control and are the averages of three separate experiments. The difference between thrombin-induced ERK1,2 activation in untreated *versus* APC pretreated cells was significant (\*,  $P < 0.05$ ). (B) Serum-deprived endothelial cells were pretreated with 10 nM APC (with 0.5 U/ml hirudin) for 1 h at 37°C, washed and then incubated in the absence or presence of various concentrations of thrombin (Th) or APC (0.5 U/ml hirudin) for 5 min at 37°C. Cells were lysed and the activation of p38 kinase was determined using anti-phospho-p38 antibodies. Membranes were stripped and re-probed with anti-p38 antibody to control for loading. The data (mean ± S.E.) shown are expressed as the fold-increase over control and are the averages of three independent experiments. (C) Endothelial cells were preincubated with or without 10 nM APC for 1 h at 37°C and then stimulated with thrombin (Th) or APC at 37°C and activation of RhoA was determined. Similar findings were observed in two independent experiments.

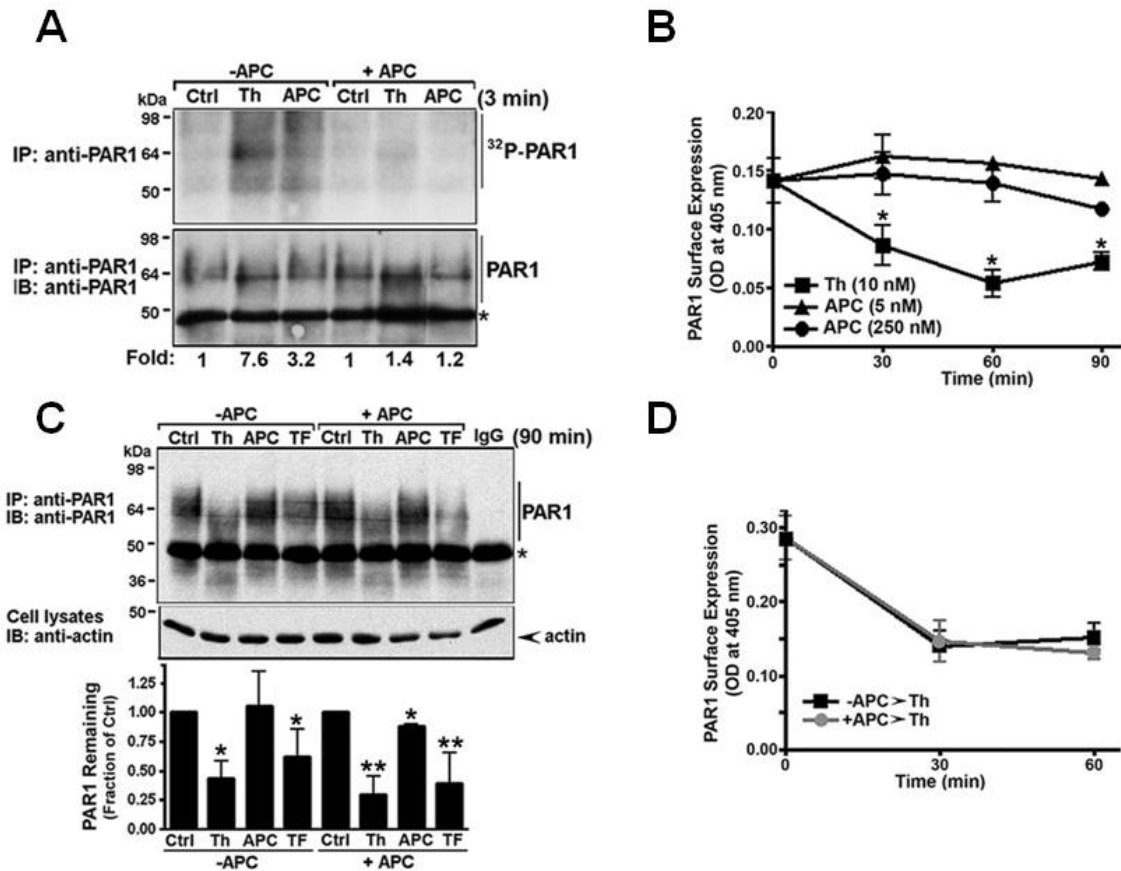




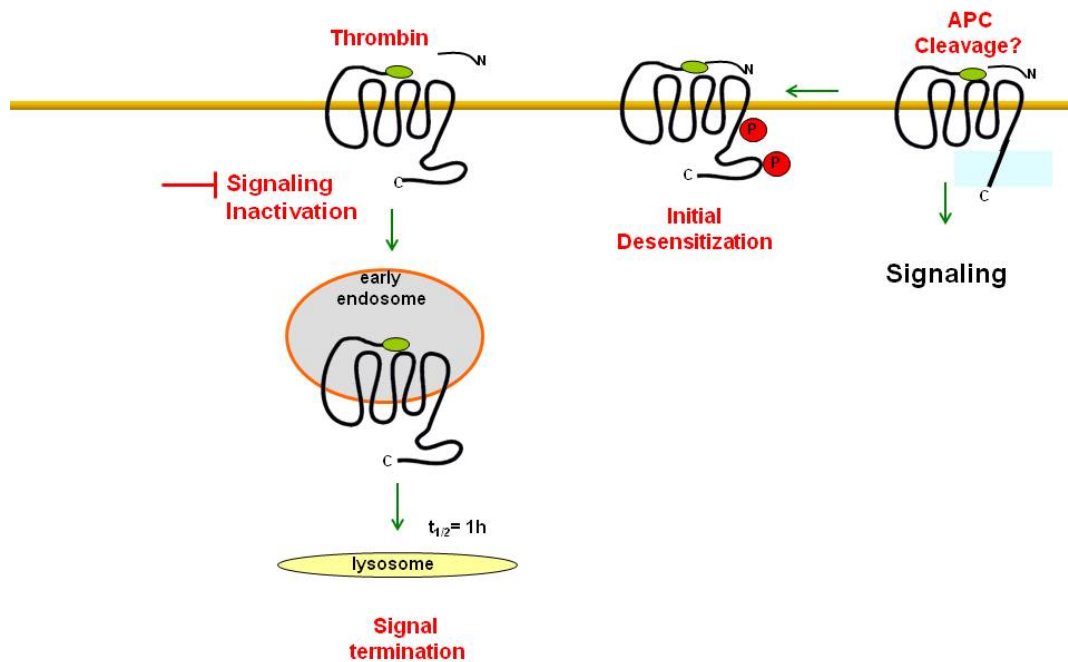
**Fig. 21. APC desensitization of PAR1 to thrombin signaling is caveolin-1 dependent.** Control and caveolin-1 (CAV1) deficient cells were preincubated with 10 nM APC for 1 h at 37°C and then stimulated with or without 10 nM thrombin (Th) at 37°C and ERK1,2 activation was determined. The difference between desensitization induced by APC in control *versus* Caveolin-1 deficient cells was significant (\*,  $P < 0.05$ ).



**Fig. 22. APC does not inhibit GPCRs activation through a general mechanism.** Pre-incubation with APC for 3 hrs does not inhibit PAR2 nor UTP signaling to calcium mobilization.



**Fig. 23. APC stimulates PAR1 phosphorylation but does not stimulate PAR1 internalization or degradation.** (A) EA.hy926 cells labeled with [ $^{32}\text{P}$ ]orthophosphate were preincubated with or without 10 nM APC for 3 h at 37°C and then stimulated with 10 nM Th or 10 nM APC for 3 min at 37°C. Immunoprecipitated  $^{32}\text{P}$ -labeled PAR1 was detected as described (21). Similar results were observed in three independent experiments. (B) Cells were incubated in the absence or presence of thrombin (Th) or APC for various times at 37°C and the amount of PAR1 remaining on the cell surface was quantitated by ELISA. The data (mean  $\pm$  S.D.,  $n=3$ ) are representative of three independent experiments. The difference between thrombin and untreated control at various times was significant (\*,  $P<0.05$ ). (C) Cells pretreated with or without 10 nM APC for 3 h at 37°C, and then incubated with 10 nM thrombin (Th), 10 nM APC or 100  $\mu\text{M}$  TFLLRNPNDK for 90 min at 37°C and the amount of PAR1 was determined as described. The asterisk indicates detection of the heavy and light chains of the immunoprecipitating antibodies. Data (mean  $\pm$  S.E.) are expressed as the fraction of PAR1 protein remaining compared to untreated control and are the averages of three independent experiments. A significant difference (\*,  $P<0.05$  or \*\*,  $P<0.01$ ) was detected between agonist-treated versus untreated control in some cases. (D) Cells were preincubated with or without 10 nM APC for 3 h at 37°C, and processed as in B). The data (mean  $\pm$  S.D.,  $n=3$ ) are from one representative experiment.



**Fig. 24. Model of APC-induced PAR1 desensitization.** When activated by thrombin PAR1 is rapidly desensitized and later the signal is terminated by internalization and degradation. Activated by APC, PAR1 is desensitized but not internalized nor degraded.

## **CHAPTER4: RATIONALLY DESIGNERED ARTIFICIAL TRANSCRIPTION FACTORS ARE ABLE TO REPROGRAM METASTATIC CANCER CELLS**

### **4.1: Abstract**

The serine protease inhibitor maspin is an atypical member of the family of serine protease inhibitors. Maspin inhibits the serine-protease urokinase plasminogen activator (uPA) *in vitro* and *in vivo*. Maspin has potent tumor suppression functions *in vitro* and *in vivo*. Interestingly, the maspin promoter is silenced by epigenetic and transcriptional mechanisms in cancer cells. In the third chapter of my dissertation I developed an inducible system to express artificial transcription factors (ATFs) that are able to re-activate maspin promoter in a mouse model. I show that re-expression of maspin by ATFs leads to reduction of tumor growth and metastasis formation in an *in vivo* xenograft animal model.

### **4.2: Introduction**

Metastasis is the leading cause of deaths of breast cancer patients. The metastatic process involves several steps including invasion, intravasation, extravasation and growth at a secondary site. Mammary serine protease inhibitor (maspin) is a tumor suppressor affecting multiple processes involved in neoplastic progression. Importantly, maspin is silenced in aggressive epithelial tumors by epigenetic and transcriptional mechanisms (Futscher B.W. 2004). Ectopic expression of maspin cDNA is associated with primary tumor growth reduction (Zhou Z. 1994), decreased angiogenesis (Zhang M. 2000), and reduction of tumor invasion and metastasis (Sheng S. 2004; (Shaefer J.S. 2003). Our laboratory has

previously characterized artificial transcription factors (ATFs) able to re-activate maspin in invasive breast cancer cell lines (Beltran A. 2007). ATFs are composed of sequence-specific zinc finger (ZF) domains to specifically target endogenous genes. The ZF domains are linked to the VP64 activator domain, which mediates up-regulation of the target genes. Among all the ATFs constructed, ATF-126 was the most efficient in up-regulating the maspin promoter in invasive breast cancer cells. Furthermore, ATF-126-mediated reactivation of maspin has been shown to inhibit primary tumor formation (Beltran A. 2007). In this study I address the therapeutic potential of ATF-126 to inhibit tumor growth and metastatic colonization using a xenograft animal model. The reactivation of maspin causes a concomitant induction of selective tumor-cell apoptosis, which complicates the study of the effect of ATF on pre-existing tumors. Therefore, I generated an inducible Tet-on retroviral expression system in order to control the expression of the ATFs *in vivo*. We show that the ATF-126 is able to reduce tumor growth and metastasis formation in an *in vivo* xenograft mouse model. Fig. 25 illustrates the overall strategy of this study.

### **4.3: Results**

#### **4.3.1: Inducible expression of the artificial transcription factor ATF-126 results in apoptosis of invasive breast cancer cell lines.**

A human invasive MDA-MB-231 breast cancer cell line stably expressing a Tet On-regulated ATF-126 was generated. Briefly, the ATF-126 cloned in the retroviral expression vector p-RetroX-Tight-Pur under the control of a tetracycline operator sequence (*tetO*) and vector controls were retroviral transduced in MDA-MD-231 cell line together with the Retro-Tet-on-Advance vector which constitutively expresses the tetracycline-controlled trans-

activator. The trans-activator binds *tetO* in the presence of doxycycline (Dox) and drives the transcription of the gene of interest.

The inducible tet-on-regulated was first validated in MDA-MB-231 cells *in vitro*. The expression of ATF and maspin mRNA were assessed in the presence or absence of doxycycline by real-time polymerase chain reaction (RT-PCR). The ATF and maspin were highly expressed in the presence of doxycycline (Fig. 26 A and B). Protein levels of both, the ATF and maspin have been assessed by immunoprecipitation followed by immunoblot (Fig. 27 A and B) using an HA antibody for the detection of ATF or maspin specific antibody for the detection of maspin. The ATF and maspin mRNA and protein expression was detected only in presence of Dox. We also have assessed maspin mRNA expression after treatment with doxycycline and subsequent removal of the drug. We have shown that after removal of Dox the level of maspin returns to normal levels suggesting that the ATF action is reversible (Fig. 26 C).

Second, we evaluated the capability of the ATF to induce early apoptosis by performing annexin staining in presence and absence of Dox. We found that only ATF-126 and not inactive variants of this ATF such as NOVP64 (which lacks the activator domain) effectively induced apoptosis in the breast cancer cells upon Dox treatment (Fig. 28 A and C). To assess whether the apoptotic phenotype was due to ATF's effect on maspin rather than off targets we further transduced MDA-MB-231 expressing the ATF-126 under control of doxycycline with shRNA targeting maspin or scramble shRNA. I first validated that the presence of maspin shRNA results in loss of maspin mRNA (Fig. 28 B). With this approach I demonstrated that in the presence of shRNA targeting maspin the apoptotic phenotype due to ATF-126 was rescued (Fig. 28D). This suggests that maspin up-regulation is responsible for the apoptotic phenotype.

#### **4.3.2: To study the capability of ATFs to reduce tumor growth and metastasis in an immunodeficient mouse model (SCID).**

To assess the effect of ATF-126 on pre-existing tumor and metastasis formation we transduced MDA-MB-231 cells stably expressing the Tet-on system for the ATF-126 with a luciferase retroviral vector. This luciferase vector allowed us to track tumor cells in vivo by monitoring luciferase activity by bioluminescence imaging (BLI). I implanted the MDA-MB-231 cells stably expressing both the Tet-on system for the ATF-126 and the luciferase retroviral vector subcutaneously in the flank of SCID mice. The MDA-MB-231 cells stably expressing the Tet-on system empty vector were used as control. Primary tumor formation over time was then followed by BLI. Tumor growth was also followed by caliper measurements and represented as tumor volume (mm<sup>3</sup>). When the tumors reached 0.5 cm of diameter the mice were fed with doxycycline and primary tumors volume were measured over time. In mice injected with MDA-MB-231-ATF-126-Luc the tumor volume was significantly reduced upon treatment with doxycycline (Fig. 29 C and D) compared to control (Fig. A and B). Hematoxyllin-eosin staining of the tumors extracted from the mice also showed that induction of ATF-126 results in reduction of blood vessel number (Fig. 30 A). The data were quantified by counting the number of vessels from 2 different animals using light microscopy (Fig. 30 B). To assess whether ATF-126 expression could also block metastasis formation, mice were injected via tail vein with MDA-MB-231 cells stably expressing the inducible ATF-126 or the control empty vector plus a luciferase retroviral vector. Tumor cell metastasis to the lungs was then assessed by BLI (Fig. 31). I show that induction of ATF-126 expression following Dox administration totally abolishes metastatic colonization.



#### **4.4 Discussion**

In this study I used artificial transcription factors to up-regulate the tumor suppressor maspin *in vivo*. I developed an inducible retroviral vector system for the controlled expression of ATF-126. I validated this approach by assessing up-regulation of maspin and ATF-126 re-expression in a doxycycline dependent manner. I demonstrated that maspin up-regulation is responsible for the apoptotic phenotype in breast cancer cells. We also have shown that after doxycycline removal the level of maspin returns to normal suggesting that the ATF action is reversible. We have shown that subcutaneous implantation of MDA-MB-231 breast cancer cells stably expressing ATF-126 under Dox control and also expressing luciferase, leads to tumor volume reduction and inhibition of metastasis formation. These results prove that ATF-126 has a potent therapeutic potential for breast cancer.

#### **4.5: Materials and Methods**

##### **Development of a Double Stable Tet-On Advanced Cell Line for ATF-126**

The Artificial Transcription Factors (ATFs) was delivered into the cells by retroviral transduction. Briefly, gagpol packaging cells ( $3 \times 10^5$  cells/ml) were seeded on 10 cm plates coated with 100  $\mu$ g/ml Poly-D-Lysine solution. Gagpol cells were then incubated at 37  $^{\circ}$ C, 5% CO<sub>2</sub> overnight. After 16 hrs the gagpol cells were transfected with pMDG.1 (VSV G-envelope-expressing plasmid) and the retrovirus vector pTight maspin specific ATFs or empty using Lipofectamin system. Another set of gagpols were transfected with pMDG.1 and the transactivator vector pAdvance. Gagpol cells were incubated for 3-hr at 37  $^{\circ}$ C, 5% CO<sub>2</sub>. After 3hrs, the transfection media was substituted with DMEM supplemented with 10% FBS. The plates were then incubated for 48-hr at 37  $^{\circ}$ C, 5% CO<sub>2</sub>. The host cells were

seeded 24 hr before harvesting the retrovirus at a seeding density of  $1 \times 10^5$  cells/plate in a 10 cm plate. 48hrs after transfection the gagpol supernatant containing the retrovirus was collected and filtered with a 0.22- $\mu$ m filter unit to eliminate any cell debris (residual packaging cells). The pAdvaced retrovirus and the pTight-ATF retrovirus were combined in a 1:1 ratio. 10  $\mu$ l/ml of Polybrene was added to the virus-containing media to a final concentration of 8  $\mu$ g/ml. Double retrovirus-containing media was added to each host cell plate (e.g. MDA-MB-231) and incubated for 6-8 hr at 37 °C, 5% CO<sub>2</sub>. Fresh media was added to the gagpol cells to allow more virus production. The retrovirus collected was added to the host cells every 8 hr for a total of four times. The day after the end of the last infection the retrovirus-containing media was removed from the host cells and fresh media was added to the host cells.

### **Immunoprecipitation and immunoblot**

Cells were plated at  $3.5 \times 10^5$  cells in 10 cm plates and 3 plates for condition were used. The cells were plated in the absence or presence of 100ng/ml Dox and incubated for 72 hrs. The cells were then lysed on ice using RIPA buffer (Pierce) plus protease inhibitors. After all cells were lysed, solubilization continued by rotating tubes for 1 hr at 4°C. The cells were spinned at 14,000 rpm for 20 min at 4°C. Protein concentration was determined by Bradford protein assay. Primary antibody (anti-maspin T50, Cell signaling or anti-HA from Covance) was added to the cell lysates incubate with gentle rocking overnight at 4°C. Protein A/G plus beads (Pierce) (20  $\mu$ l of 50% bead slurry) were added and incubated with gentle rocking for 2 hours at 4°C. Lysated were then microcentrifuged for 30 seconds at 4°C at 2000 g and pellets washed 3 times with 500  $\mu$ l of 1X cell lysis buffer and 1x with PBS. The pellets were resuspended with 20  $\mu$ l 3X SDS sample buffer and heated to 95–

100°C for 3 minutes, then microcentrifuged for 1 minute at 14,000 X g and loaded on SDS-PAGE gel (pre-cast gels 10% from Invitrogen). The proteins were transferred on pvdf membrane and analysed by Western blotting using anti-maspin antibody (BD) or anti-HA antibody.

### **Real-time quantitative PCR**

RNA was isolated from ATF-transduced and control cells using a commercial RNA extraction system (Qiagen's RNeasy Plus Mini kit, cat. no. 74134), following the manufacturers' instructions precisely. cDNA was then synthesized from RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) and following the manufacturers' instructions. RT-PCR reaction was performed using TaqMan system from Applied Biosystems. 3 PCR replicates for each cDNA sample were performed.

Briefly; 10 µl of 2X Fast Taqman, 1 µl of Primers/Probe mix 20X and 4 µl of Nuclease-free Water were used per reaction. 100 ng of cDNA in 5 µl were placed into 0.2 ml PCR tube. 15 µl of master mix were added into each tube.

The plate was plated into the real-time PCR machine. The PCR conditions were: 10 min at 95 °C, and 30 sec at 65 °C for 40 cycles. The data were analyzed using comparative C<sub>T</sub> method ( $2^{-\Delta\Delta C_T}$  method).

### **Analysis of apoptosis by Annexin V staining**

During early stages of apoptosis the phospholipid phosphatidylserine (PS) is exposed to the external cellular environment. Annexin V is a phospholipid-binding protein that has a high affinity for PS, and binds extracellular PS. The Annexin V protein conjugated with Phycoerythrin (PE) is used to quantify the early apoptotic effect of maspin-specific

ATFs in cancer cells. In addition, the 7-Amino-actinomycin D (7-ADD) is used to discriminate between live and dead cells. 7-ADD intercalates into double-stranded nucleic acids of dead or dying cells, but is excluded by viable cells. Briefly, cells were washed twice with cold PBS and resuspended in 1X Binding Buffer at a concentration of  $1 \times 10^6$  cells/ml. 100  $\mu$ l of the cell suspension ( $1 \times 10^5$  cells) were transferred into a 5 ml culture tube. 5  $\mu$ l of PE-Annexin V and/or 5  $\mu$ l of 7-ADD were added. The cells were gently vortexed and incubated for 15 min at RT (25 °C) in the dark. 400  $\mu$ l of 1X binding buffer was added into each tube. Apoptosis was then quantified by flow cytometry within one hour. The percentage of apoptosis is measured by flow cytometry using a FACS Calibur and CellQuest software.

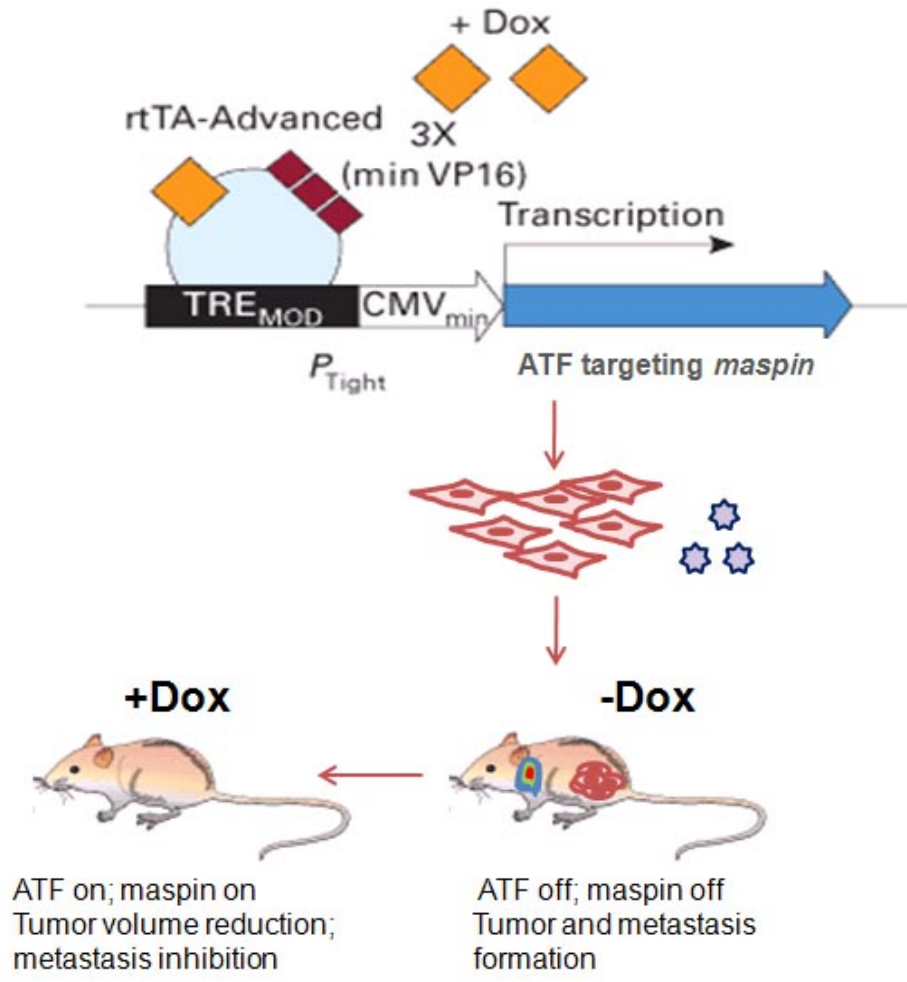
### **Subcutaneous Injections**

Cancer cells expressing the inducible ATF-126 or the control empty vector will be transduced with retrovirus expressing a Luciferase marker (pLXN-Luci). Approximately  $10^6$  cells were collected and re-suspended with matrigel (BD bioscience) 1:1 volume ratio in a total volume of 200  $\mu$ l. The cells-Matrigel mixture, kept on ice, will be injected into the mouse flank subcutaneously using syringe with a needle of size 21-25G. Tumor growth has been monitored by caliper twice a week. When the tumor reached a size of approximately 0.5 cm doxycycline was administered to mice to induce the expression of the ATF. Doxycycline was given in form of green food pellet with a concentration of 200 mg of doxycycline per Kg of mice chow for a period of 15-30 days. During the entire experiment the mice weight was monitored to verify whether toxicity occurred. Tumor volume was monitored by caliper and Bioluminescence imaging (BLI). Approximately days after doxycycline treatment the mice were sacrificed by CO<sub>2</sub> plus cervical dislocation. After death

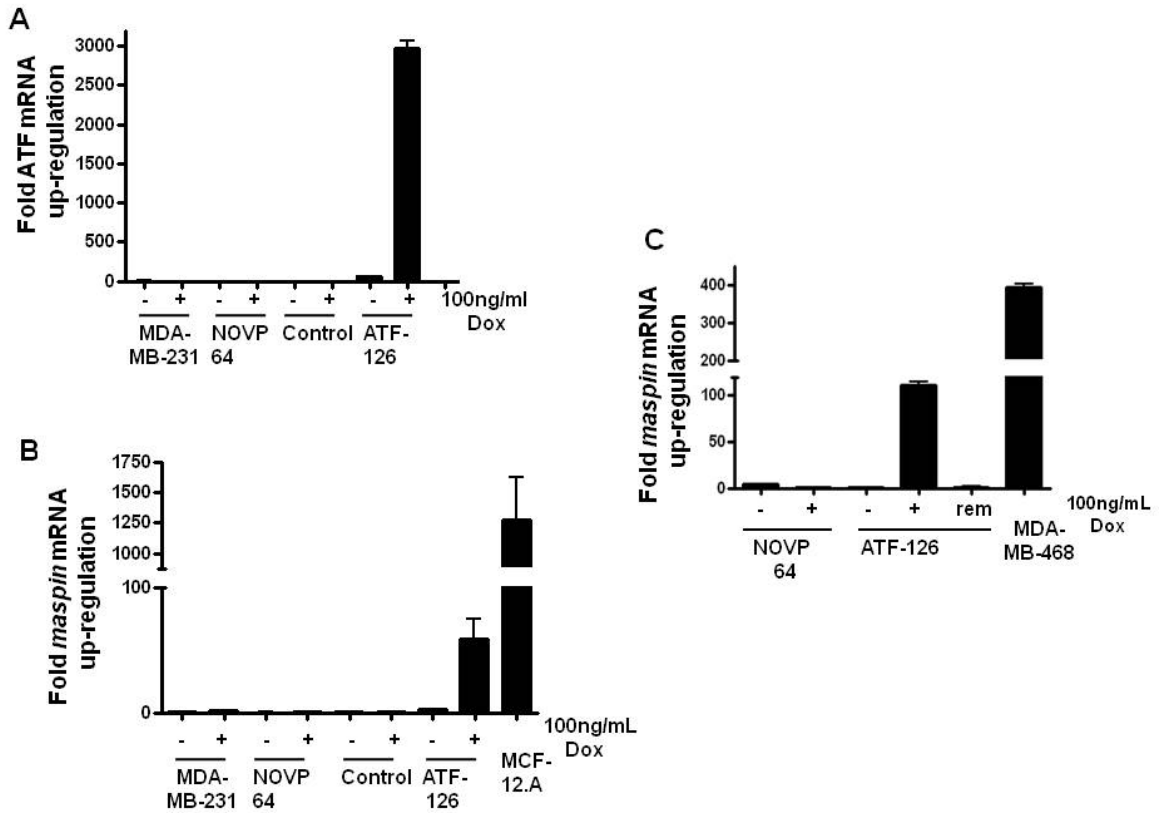
the tumors were extracted from control and ATF-induced animals and fixed in 10% buffered formalin for immunohistochemistry analysis.

### **Tail-vein**

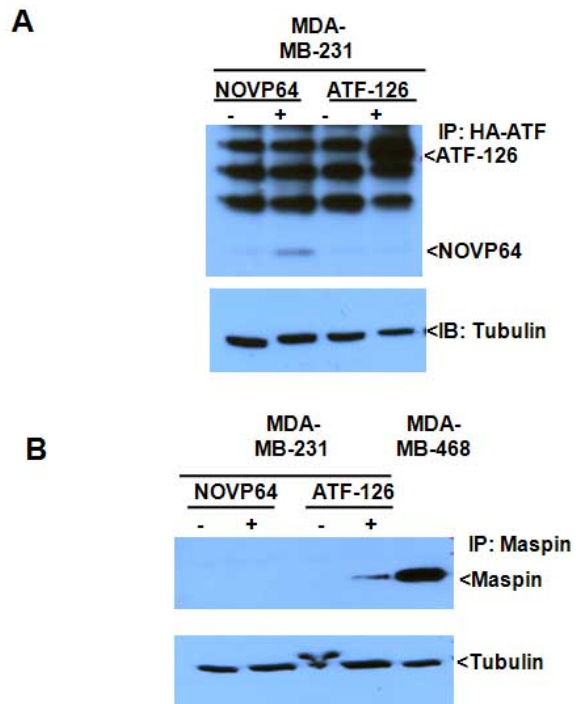
Three days before the injections the mice are kept in normal or doxycycline diet. Right before injections the animal is placed into a plastic mouse restraint so that the animal is not freely mobile, but its tail is able to be handled. Its tail is placed into warm water so that the tail vein is more visible. Alternatively a heat lamp may be used. Once the animal's tail is warmed to adequate temperature then it is ready for the procedure. The tail is wiped with alcohol to sterilize the area to be injected.  $1 \times 10^6$  double stable cell lines for ATF-126 or empty vector are injected per animal in a total volume of 200ul using a 27 gauge needle syringe. The animal is monitored during the procedure by observing its respiratory rate. After the procedure is done the animal is monitored to make sure it is not stressed and returns back to its normal respiratory rate, if elevated. The cells injected also express luciferase that allows monitoring metastasis formation into the lung by Bioluminescence imaging (BLI) once a week for 20 days. The mice were then sacrificed by CO<sub>2</sub> plus cervical dislocation. After death the lungs were extracted from control and ATF-induced animals and fixed in 10% buffered formalin for quantification of metastasis.



**Fig. 25 Strategy for inducing ATF expression in tumor cells and implantation in SCID mice.** The ATF was cloned into pRetroX-Tight inducible system. MDA-MB-231-Luc cells were retrovirally transduced with the clone. The ATF-transduced MDA-MB-231-Luc cells were implanted in SCID mice.

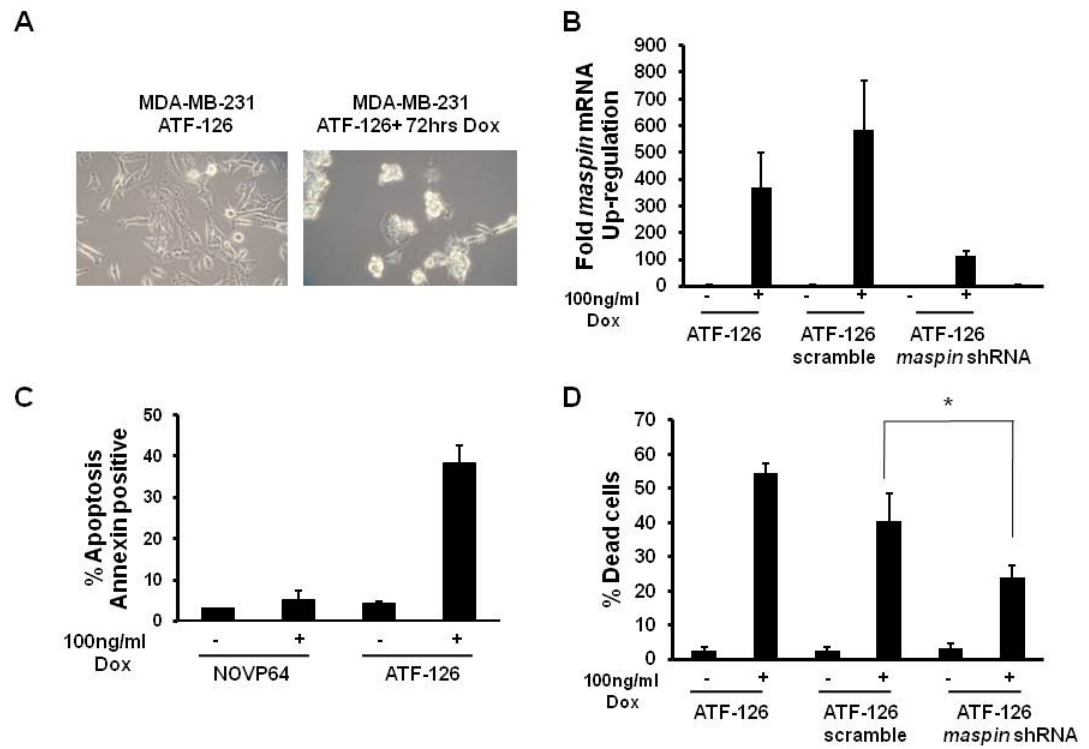


**Fig. 26. ATF and maspin mRNA are expressed in the presence of doxycycline (Dox).** (A) ATF mRNA up-regulation was assessed by RT-PCR using primers targeting the activator domain with and without Dox 100ng/ml for 72hrs in MDA-MB-231 non infected cell line, MDA-MB-231 plus NOVP64 which is the 6 zinc-fingers without the activator domain, the control empty vector and MDA-MB-231 plus ATF-126. (B) Maspin mRNA up-regulation was assessed by RT-PCR with and without Dox 100ng/ml for 72hrs. (C) Maspin mRNA up-regulation was assessed by RT-PCR with and without Dox 100ng/ml for 72hrs and after removal of Dox and recovery of the cells.

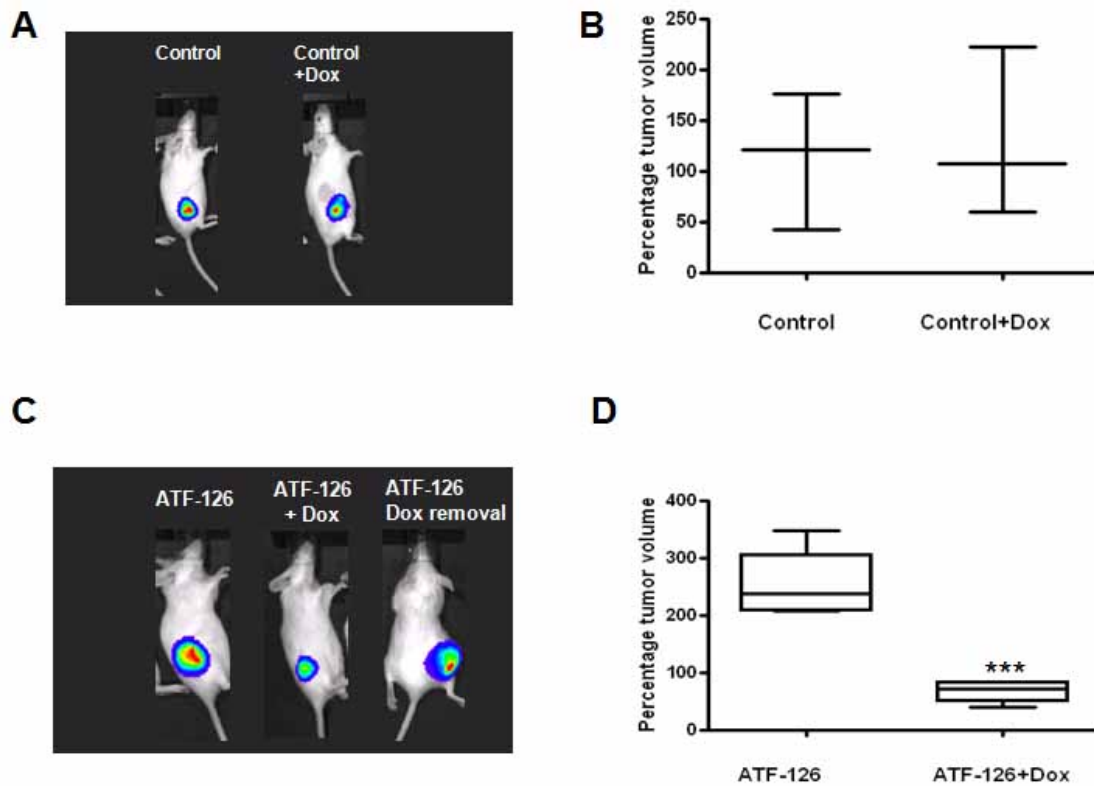


**Fig. 27. Expression of ATF and maspin protein. (A).** ATF protein up-regulation was measured by immunoprecipitation of the ATF using an anti-HA antibody followed by immunoblot. **(B).** Maspin protein up-regulation was measured by immunoprecipitation of maspin from total lysates using an anti-maspin antibody followed by immunoblot with maspin antibody.



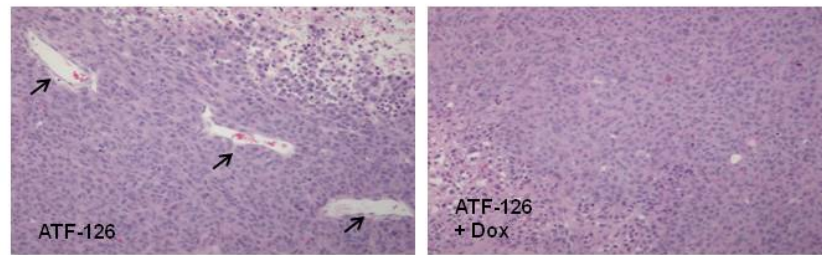


**Fig. 28. Expression of ATF-126 induces apoptosis and maspin shRNA rescues the apoptotic phenotype. (A).** MDA-MB-231 treated with Dox (100ng/ml) for 72 hrs present cell death. **(B)** Loss of expression of maspin mRNA in the presence of maspin shRNA compared to scrambled. **(C).** Quantification of ATF-126-induced apoptosis by Annexin V staining after 72 hrs of Dox (100ng/ml). **(D).** Rescue of apoptotic phenotype in the presence of maspin shRNA compared to scrambled.

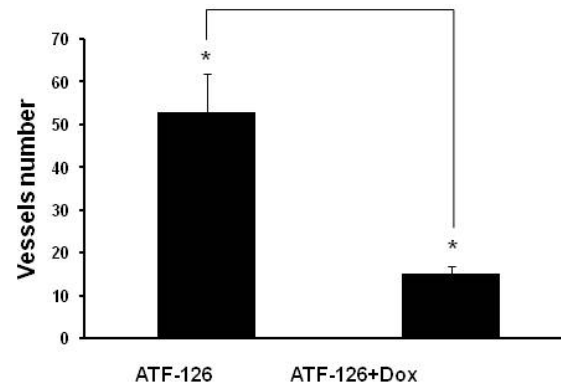


**Fig. 29. ATF-126 expression reduces primary tumor volume.** (A). Bioluminescence imaging of mice injected with control empty vector +/- Dox. (B). Quantification of tumor volume measurement by caliper of n=3 mice per group. (C). Bioluminescence imaging of mice injected with ATF-126 +/- Dox. The figure also shows a mouse that has been removed from Dox diet and put on normal diet. (D). Quantification of tumor volume measurement by caliper of n=4 mice injected with ATF-126 and n=4 mice injected with ATF-126 and treated with Dox (there is no quantification for the Dox removal condition).

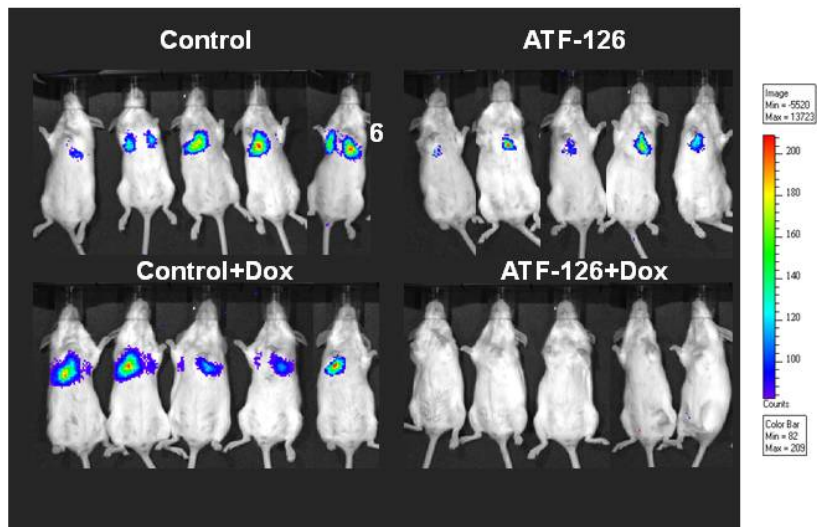
**A**



**B**



**Fig. 30. ATF-126 expression reduces blood vessels number. (A).** Hematoxylin-eosin staining of sections of tumors extracted from ATF-126 and ATF-126 + Dox. **(B).** Quantification of blood vessels number normalized by area in ATF-126 and ATF-126 + Dox treated mice.



**Fig. 31. ATF-126 expression blocks metastasis formation. (A).** Bioluminescence imaging of mice injected via tail vein with control empty vector +/- Dox or ATF-126 +/-Dox to assess metastasis to the lungs.

## **CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS**

The work presented in this dissertation has demonstrated that different proteases can signal through the same receptor to elicit differential cellular responses. I also showed that a protease inhibitor implicated in tumor progression can be regulated by an inducible artificial transcription factor (ATF) indicating a potential use of ATFs as a therapeutic agents.

In chapter 2, I show that endogenous PAR1 is required for thrombin and APC signaling in endothelial cells. I further demonstrate that caveolin-1 is essential for activation of PAR1 signaling by APC but not thrombin, indicating that caveolae are critical for protease-selective signaling by PAR1. Specifically, to investigate how PAR1 signals when activated by APC in endothelial cells I assessed activation of the small GTPases RhoA and Rac1. Thrombin cleaves PAR1 and in turn, PAR1 activates RhoA. Conversely, I show that APC activates PAR1 to stimulate Rac1 but not RhoA signaling. I further show that caveolin-1 is essential for APC induced Rac1 activation. To determine whether caveolin-1 is also required to promote APC-mediated endothelial barrier protective effects we assessed endothelial permeability in the presence or absence of shRNA targeting caveolin-1. We show that caveolin-1 knock-down abolished APC-mediated protective effects on endothelial permeability.

Evidence presented here suggests that compartmentalization of PAR1 in caveolae facilitates selective endothelial barrier protective signaling. The molecular determinants that

specify the targeting of PAR1 and signaling components to caveolae have not been established but may involve post-translational modifications. A large number of GPCRs appear to be modified by palmitoylation, which occurs through the covalent attachment of a C16 fatty-acid chain to cysteine residues localized within the cytoplasmic tail of the receptor. PAR1 has cytoplasmic cysteine residues that could serve as sites for palmitoylation but this has not been investigated. Previous studies have shown that palmitoylation of tissue factor (TF) facilitates its localization to caveolae and prevents protein kinase C dependent phosphorylation, a process that controls tissue factor pro-coagulant activity (Dorfleutner A. and Ruf W. 2003). Thus, the modulation of TF with palmitoylation may facilitate localization of TF-FVIIa and Xa with PAR2 in caveolar microdomains to promote cellular signaling. Interestingly, several studies suggest that protein palmitoylation protects proteins from ubiquitination and subsequent degradation (Valdez-Taubas J. 2005; Abrami L. 2006). Modification of proteins with ubiquitin occurs through the covalent attachment of ubiquitin, a 76-amino acid protein, to lysine residues on the target protein. PAR1 is ubiquitinated (Wolfe B.L. 2007; Jacob C. 2005), but whether PAR1 ubiquitination facilitates receptor palmitoylation and/or targeting of proteins to caveolae has not been examined. To address this possibility a mutant form of PAR1 can be generated where the cytoplasmic cysteine residues are replaced with alanine residues. Endothelial cells expressing shRNA targeting PAR1 and therefore PAR1 deficient can then be transfected with the mutant or the wild-type forms of PAR1 and then Rac1 activation by APC can be assessed to verify whether APC signaling to caveolae depends on PAR1 palmitoylation sites. Furthermore, these mutants can be used to assess PAR1 localization into caveolae by performing a subcellular fractionation in a sucrose gradient. We expect that the expression of the palmitoylation

deficient PAR1 will abolish APC signaling to Rac1 and also will affect PAR1 localization to caveolae.

By bioinformatic analysis we have also found that both EPCR and PAR1 contain caveolin-1 binding motifs. The caveolin-1 binding motifs, reported in the literature, are  $\Phi X \Phi X X X X \Phi$  or  $\Phi X X X X \Phi X X \Phi$  or the combined motif  $\Phi X \Phi X X X X \Phi X X \Phi$  where  $\Phi$  is any aromatic amino acid (tryptophan/W, phenylalanine/F or tyrosine/Y) (Couet J. 1997; Liu P. 2002). EPCR contains the following caveolin-1 binding motif: **Y<sup>35</sup>-F-R-D-P-Y-H-V-W<sup>43</sup>** whereas PAR1 contains two caveolin-1 binding motifs: **Y<sup>161</sup>-Y-F-S-G-S-D-W<sup>168</sup>** and **Y<sup>267</sup>-A-Y-Y-F-S-A-F<sup>274</sup>**. The latter motif is more likely to bind caveolin-1 since is situated in the region of fifth transmembrane domain whereas the other motif, which is extracellular probably does not. Indeed, it has been reported in the literature that the hedgehog receptor patched interacts with caveolin-1 through a caveolin-1-binding motif located in the region of its seventh transmembrane domain (Karpen H.E. 2001). To investigate caveolin-1 interactions with EPCR and/or PAR1, the caveolin-1 binding motifs on EPCR and/or PAR1 can be mutated by replacing the aromatic amino acids with alanines and localization of PAR1 and EPCR into caveolae can be assessed by subcellular fractionation in a sucrose gradient.

Endothelial permeability is induced by invasive cancer cells which secrete VEGF and other factors to increase the leakiness of blood vessels. However, whether APC inhibits thrombin induced PAR1-mediated cancer progression has not been examined. Recently Bezuhly M et al. demonstrated that APC inhibits tumor cell metastasis (Bezuhly M. 2009). However, whether APC prevents thrombin ability to promote tumor progression has not been determined. APC also inhibits VEGF-induced increase in endothelial cell permeability (Feistritz C. 2005) which suggests that APC may play an important role in inhibiting

tumor-induced vascular leakiness. To test this hypothesis a trans-endothelial migration assay can be performed in a double chamber system. Endothelial cells would be seeded in the upper chamber and checked for monolayer formation. The endothelial cells will be first treated with thrombin and then invasive breast cancer cells loaded with a fluorescent dye will be added on top of the endothelial monolayer. Migration of the cancer cells through the endothelium can then be followed by fluorescent microscopy. Briefly the upper chamber will be cleaned and non-migrating cancer cells as well as endothelial cells were removed. Fluorescence at the bottom of the chamber will be assessed. I expect that thrombin treatment will enhance breast cancer cells migration through the monolayer. To test the hypothesis that APC inhibits thrombin induced breast cancer cells migration, the endothelial cells seeded in the upper chamber will be first pre-treated with APC and then treated with thrombin. The breast cancer cells will be plated on top of endothelial cells as described above and number of fluorescent cells migrated will be assessed by microscopy and quantified. I expect that pre-treatment with APC will block thrombin-induced cancer cells migration through the endothelial monolayer.

In chapter 3 I determined that APC desensitizes PAR1 to thrombin signaling by inhibiting thrombin-induced activation of ERK1/2, p38 and RhoA signaling. Interestingly, I also found that APC stimulates PAR1 phosphorylation but causes limited receptor internalization and degradation. Strikingly, APC does not prevent thrombin-induced PAR1 cleavage, internalization or degradation. Thus, in endothelial cells exposed to APC the majority of PAR1 is retained on the cell surface and susceptible to thrombin cleavage. Our findings here suggest that APC imparts a novel mechanism for regulation of PAR1 signaling that involves receptor phosphorylation but not internalization or degradation. The desensitization of PAR1 signaling is controlled by agonist-induced PAR1 phosphorylation



and the interaction with  $\beta$ -arrestin. PAR1 internalization and lysosomal degradation are also critical for termination of receptor signaling (Paing M.M. 2002). Interestingly, activated PAR1 internalization occurs independently of arrestins (Chen C.H. 2004). Here I found that APC induces desensitization but does not affect internalization or degradation of the receptor suggesting that  $\beta$ -arrestin may be involved in this process. This possibility can be evaluated using siRNA technology and assessing APC-induced PAR1 desensitization in  $\beta$ -arrestin knock-down background.

In chapter 4 I developed an inducible retroviral vector system for the controlled expression of ATF-126. I validated this approach by assessing up-regulation of maspin and ATF-126 re-expression in a doxycycline dependent manner. I demonstrated that maspin up-regulation is responsible for the apoptotic phenotype in breast cancer cells. I also have shown that after doxycycline removal the level of maspin returns to normal levels suggesting that the ATF action is reversible. I have shown that subcutaneous implantation of MDA-MB-231 breast carcinoma cells stably expressing ATF-126 under Dox control leads to tumor volume reduction and inhibition of metastasis formation. Further experiments will elucidate the mechanism through which ATF-126 exerts its anti-cancer action. This can be addressed through genome wide array technology. The genome array will allow identification of genes regulated by ATF-126. Preliminary data I have obtained show that genes responsible for mesenchymal to epithelial transition (MET) such as E-cadherin and claudins are up-regulated, suggesting that this cancer cells are re-programmed toward a more epithelial-cell like phenotype. Genes involved in apoptosis and senescence are also found up-regulated, whereas genes involved in mitosis are down-regulated. This may suggest a mechanism by which ATF-126 may exert an apoptotic effect in cancer cells but not in normal cells (Beltran A. 2007 ). A detailed kinetic analysis also will be performed to

assess which genes are early regulated by ATF-126 expression. This will help to uncover the overall signaling pathway regulated by ATF-126. Furthermore we can use shRNA targeting maspin to address whether the genes regulated require maspin expression or whether they are a result of ATF-126 function and its potential off-target effects. Another important method to fully understand the ATF-126 function is to determine the ATF-126 binding sites within genomic DNA. To address ATF-126 specificity a Chromatin Immunoprecipitation-Sequencing (ChIP-Seq) assay will be performed. It has previously been shown in our lab that ATFs synergies with chromatin remodeling drugs enhancing the ATF-effects *in vitro*. Therefore, we sought to validate *in vivo* that the effect of ATFs can be potentiated using chromatin remodeling drugs in concomitance. For this purpose a xenograft model can be established in which mice will be kept in the absence of Dox and MDA-MB-231-luc transduced with ATF-126 under Dox control will be implanted subcutaneously. After the tumor reaches a visible size Dox will be administered in the presence or absence of chromatin remodeling drugs and tumor growth assessed by BLI and caliper measurements. Further studies will be oriented towards the development of advanced delivery system for ATFs *in vivo*. Current studies in our lab are taking advantage of non toxic nanoparticles for the delivery of ATF-126 specifically in tumor cells. Specifically, the Nan particles will have a ligand for CD44 which is only expressed in cancer cells. Briefly, tumors will be established in the mice by implanting MDA-MB-231 expressing luciferase subcutaneously. Then, the nano-particles containing ATF-126 will be injected via tail vein and the tumors will be monitored by bioluminescence imaging (BLI) over time to assess eventual tumor reduction.

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