STRUCTURE-FUNCTION STUDIES OF A NOVEL TGF-β CO-RECEPTOR, CD109

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In loving memory of my father, Dr. Sheng Li – McGill Ph.D. (1957–2013)

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Abstract

Transforming Growth Factor β (TGF-β) is a multifunctional growth factor involved in a wide variety of cellular processes. Expressed in a majority of mammalian cells, TGF-β has been shown to play pivotal roles in immunoregulation, cellular homeostasis, apoptosis, and embryonic development. Perturbations in the TGF-β signaling pathway results in extensive vascular, neural, and osteal developmental defects, and massive autoimmune inflammatory responses. TGF-β signals through a pair of transmembrane serine/threonine kinases known as type I (TβRI) and type II TGF-β (TβRII) signaling receptors. The TGF-β ligand binds to TβRII, which transphosphorylates the TβRI receptor, initiating an intracellular phosphorylation cascade where the activated TβRI phosphorylates receptor-regulated Smads (R-Smads) which subsequently bind common-mediator Smads (Co-Smads) forming R-Smad/Co-Smad complexes. The R-Smad/Co-Smad complexes then translocate into the nucleus to regulate gene expression through interactions with transcription factors, co-activators and co-repressors.

In addition to the TβRI and TβRII signaling receptors, many cell types express TGF-β co-receptors known as betaglycan and endoglin that bind TGF-β and modulate TGF-β signaling in a cell-specific manner. Our research group has previously identified CD109, a GPI-anchored protein, as a novel TGF-β co-receptor. CD109 binds TGF-β to form a heteromeric complex with the TGF-β signaling receptors and inhibits TGF-β signaling through caveolae-mediated TGF-β receptor internalization and degradation. Furthermore, our group has

demonstrated that endogenous CD109 can be released from the cell surface by endogenous PIPLC enzymes and subsequently bind TGF-β with high affinity.

Aberrant TGF-β signaling plays a central role in a variety of pathologies. Strategies for regulating aberrant TGF-β action by diminishing TGF-β access to its receptors include development of neutralizing anti-TGF-β antibodies and TGF-β ligand traps. Characterizations of receptor-ligand interactions of soluble type II TGF-β receptor, soluble betaglycan, and alpha-2-macroglobulin have demonstrated the potential of receptor ectodomain-based ligand traps to sequester TGF-β and inhibit TGF-β signaling. As CD109 and alpha-2-macroglobulin (α2m) belong to the same family of thioester containing proteins, I set out to determine if soluble CD109 can act as a TGF-β antagonist, by investigating the ability of recombinant soluble CD109 protein to bind all three mammalian TGF-β isoforms and negatively modulate TGF-β signaling and TGF-β-induced cellular responses. As CD109 and α2m share similar structural homologies, I set out to determine the putative TGF-β binding domain of CD109.

In my findings, I established that soluble CD109 acts as a TGF-β antagonist, by demonstrating the ability of recombinant soluble CD109 protein to bind all three mammalian TGF-β isoforms and negatively modulate TGF-β signaling and TGF-β-induced cellular responses. Furthermore, based on my sequence homology analysis, I predict that the putative TGF-β binding domain of CD109 to encompass amino acids 687-711, which includes a WIW hydrophobic sequence and acidic

residues thought to confer TGF-β binding functionality similar to that of TβRII and α2m. Moreover, I have generated CD109 derived peptides based on the putative binding domain of the CD109 protein and CD109 site-directed mutants to determine the TGF-β binding domain of CD109. My results indicate that peptides derived from the putative binding domain of TGF-β (CD109 amino acid 687-711) can bind all three mammalian TGF-β isoforms, modulate TGF-β signaling and TGF-β-induced cellular responses, and that CD109 site-directed mutants can abrogate CD109 inhibition of TGF-β induced transcriptional activity.

Collectively, these findings suggest that soluble CD109 plays an important role in negatively regulating TGF-β signaling and that a putative TGF-β putative binding region of CD109 is at least partially responsible for binding TGF-β and antagonizing TGF-β signaling and responses. In addition to unravelling a potential mechanism by which TGF-β action is regulated by CD109 *in vivo*, the above findings have important implications in diseases such as cancer and organ fibrosis, where aberrant TGF-β action is known to play a pathophysiological role.

Résumé

Le TGF-β (facteur de croissance transformant β) est une cytokine multifonctionnelle impliquée dans une grande variété de processus cellulaires. Exprimé dans la majorité de cellules de mammifères, le TGF-β joue un rôle essentiel dans l'immunorégulation, l'homéostasie cellulaire, l'apoptose, et le développement embryonnaire. Des perturbations dans la signalisation de TGF-β pourraient engendrer des défauts vasculaires, nerveux, osteal, et auto-immune. TGF-β transmet son signal grâce aux récepteurs transmembranaires connus sous le nom de type I (TβRI) et de type II (TβRII) du TGF-β. Le ligand TGF-β se lie à TβRII qui phosphoryle TβRI, qui initie une cascade de signalisation intracellulaire où le TβRI activé phosphoryle les Smad2 and Smad3 (R-Smad) qui se lient Smad4 en formant des complexes de Smad2/3/4. Les complexes de Smad2/3/4 vont dans le noyau pour régler l'expression de gènes par des interactions en liaison avec des facteurs de transcription, co-activateurs et co-répresseurs.

Outre les récepteurs TβRI et TβRII, de nombreux types de cellules expriment des co-récepteurs du TGF-β appelés le betaglycan et l'endogline qui se lient au TGF-β et modulent la signalisation du TGF-β d'une manière spécifique à la cellule. La protéine CD109 a été identifiée précédemment dans notre laboratoire; elle est capable de se lier au TGF-β et de former un complexe hétéromèrique avec les récepteurs du TGF-β. Le complexe hétéromèrique inhibe la signalisation des récepteurs par l'internalisation et la dégradation par la voie des cavéoles. En outre, notre groupe a démontré que CD109 endogène peut être libéré de la surface

cellulaire par des enzymes endogènes PIPLC et que CD109 peut se lier TGF-β1 avec une affinité élevée.

La signalisation aberrante du TGF-β joue un rôle central dans une grande variété de pathologies. La caractérisation des interactions récepteur-ligand de TβRII soluble du TGF-β, du betaglycan soluble, et de l'alpha-2-macroglobuline a démontré le potentiel des récepteurs de séquestrer le TGF-β et d'inhiber la signalisation du TGF-β. Comme CD109 et l'alpha-2-macroglobuline (α2m) appartiennent à la même famille des protéines, j'ai voulu déterminer si CD109 soluble peut agir comme un antagoniste du TGF-β, en étudiant la capacité de la protéine CD109 soluble recombinante, à se lier au trois isoformes du TGF-β et ainsi moduler les réponses cellulaires du TGF-β. Comme CD109 et α2m sont des homologues, j'ai voulu identifier le domaine putatif de liaison du TGF-β dans la protéine CD109.

J'ai établi que sCD109 agit comme un antagoniste du TGF-β, en démontrant la capacité de la protéine CD109 soluble recombinante à lier tous les trois isoformes TGF-β et d'inhiber la signalisation TGF-β et les réponses cellulaires induites par le TGF-β. En outre, d'après mon analyse de séquences d'homologie, je prédis que le domaine de liaison de TGF-β avec CD109 est autour d'acides aminés 687 à 711, qui comprend une séquence hydrophobe WIW et les résidus acides considérées comme offrant une fonctionnalité similaire à celle de liant TGF-β TβRII et α2m. En outre, j'ai généré des peptides dérivés basés sur le domaine putatif de liaison de

la protéine CD109 et des mutants dirigée site-CD109 pour déterminer le domaine de liaison de TGF-β de CD109. Mes résultats indiquent que les peptides dérivés du domaine de liaison putatif de TGF-β (CD109 de l'acide aminé 687 à 711) peuvent se lier ensemble des trois isoformes de TGF-β de mammifères et moduler la signalisation et les réponses cellulaires du TGF-β induites par le TGF-β.

En résumé, ces résultats suggèrent que CD109 soluble joue un rôle important dans la régulation négative de la signalisation du TGF-β et que la région de liaison putative du TGF-β dans la protéine CD109 est au moins partiellement responsable de la liaison du TGF-β et de l'inhibition de la signalisation du TGF-β. En plus d'avoir un mécanisme potentiel par lequel l'action du TGF-β est régulée par CD109 *in vivo*, les résultats ci-dessus ont des implications importantes dans les maladies où l'action aberrante du TGF-β est connue pour jouer un rôle physiopathologique, y compris dans le cancer et la fibrose.

Contributions to Original Knowledge

This thesis has been prepared in accordance with the guidelines specified by the Faculty of Graduate and Postdoctoral Studies of McGill University. This thesis, entitled "Structure-function studies of a novel TGF-β co-receptor, CD109" has been prepared in the manuscript-based format. This thesis consists of an Abstract and its French translation (Résumé); five chapters entitled Introduction, Soluble CD109 binds TGF-β and antagonizes TGF-β signaling and responses, Mapping of the TGF-β binding domain of CD109, Discussion and Perspectives, Conclusion and Summary; and an Appendix containing all the literature referenced.

I, Carter Li, under the direct supervision of Dr. Anie Philip, have designed the experiments, collected and analyzed the data, and framed these studies in the context of previously published literature unless otherwise stated. My contributions to original knowledge described in this thesis are as follows:

1) I have created a mammalian cell based protein production system for soluble CD109 protein expression and purification using the HEK293T cell line in producing soluble CD109-1268 and CD109-1420 recombinant proteins

2) I have demonstrated that soluble CD109 proteins can endogenously exist as 25 kDa, 60 kDa, 120 kDa, 150 kDa, 180 kDa and 205 kDa proteolytic fragments

3) I have demonstrated that soluble recombinant CD109 protein (sCD109-1268 and sCD109-1420) can bind all three mammalian TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) with high binding affinity and slow association/dissociation

4) I have demonstrated that soluble CD109 peptides (Peptides A, B, X, Y, Z) derived from the putative TGF-β binding domain of CD109 can bind all three mammalian TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3)

5) I have demonstrated that soluble recombinant CD109 and CD109 derived from on the putative TGF-β binding domain (Peptides A, B, Y, Z) can inhibit Smad signaling and TGF-β-induced growth inhibition

6) I have demonstrated that overexpression of soluble CD109 in the human keratinocytes can inhibit TGF-β-induced cell migration

7) I have demonstrated that soluble recombinant CD109 proteins and CD109 derived peptides (Peptides A, B, X, Y, Z) based on the putative TGF-β binding domain of CD109 can inhibit TGF-β-induced cell migration

8) I have demonstrated that regions encompassing the putative TGF-β binding domain of CD109 are at least partially responsible for mediating CD109-TGF-β binding and interactions

The data and texts from Chapters 2 and 3 comprise the following manuscripts:

Li C, Hancock M, Reinhardt DP, Seghal P, Zhou SF, and Philip A. Soluble CD109 binds TGF-β and antagonizes TGF-β signaling and responses. *Accepted pending revisions, Biochemical Journal.*

Li C, Hancock M, and Philip A. Mapping of the TGF-β binding domain of CD109. *To be submitted to Cytokine Journal.*

All of the data presented herein are the work of Carter Li with the following exceptions:

1) Surface plasmon resonance experiments were conducted by Mark Hancock in collaboration with Carter Li and Dr. Anie Philip at the McGill SPR Facility

2) CD109 GST peptide experiments were partially conducted by Dr. Hahn Soe-Lin in collaboration with Carter Li and Dr. Anie Philip at the laboratory of Dr. Anie Philip

3) CD109 site-directed mutagenesis experiments were conducted by Dr. Elsy Edouard in collaboration with Carter Li and Dr. Anie Philip at the laboratory of Dr. Anie Philip

Abbreviations

α2m	α-2-Macrogloblin
ActRII	Activin Type II Receptor
ADP	Adenosine Diphosphate
AKT	Protein kinase B (PKB)
ALK	Activin Receptor-like Kinase
AIP	Atrophin 1-interacting Protein
AMH	Anti Müllerian Hormone
AP2	Adaptor Protein 2
α-sma	Alpha-smooth Muscle Actin
ATP	Adenosine Triphosphate
bFGF	Basic Fibroblast Growth Factor
BAMBI	BMP and Activin Membrane-bound Inhibitor
BMP	Bone Morphogenetic Protein
BMPRII	BMP Type II Receptor
BS^3	Bissulfosuccinimidyl Suberate
BSA	Bovine Serum Albumin
C3	Complement 3
C4	Complement 4
C5	Complement 5
CBP	CREB Binding Protein
CCN2	CYR61/CTGF/NOV
CD	Cluster of Differentiation
CDC42	Cell division control protein 42 homolog
CDK	Cyclin Dependent Kinse
CIHR	Canadian Institutes of Health Research
CNV	Copy Number Variation
Co-Smad	Common-mediator-Smad
CSC	Cancer Stem Cell
CTGF	Connective Tissue Growth Factor
cPML	Cytosolic Form of Promyelocytic Leukemia Protein
DAB	3,3-Diaminobenzidine
DAXX	Death-associated protein 6
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EEA1	Early Endosome Antigen 1
EGF	Epidermal Growth Factor

ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
ERK	Extracellular Signal-Regulated Kinase
FBS	Fetal Bovine Serum
FGF-2	Fibroblast Growth Factor 2
GAG	Glycosaminoglycan
GEB	Glutathione Elution Buffer
GIPC	GAIP-Interacting Protein, C-terminus
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPI	Glycophosphatidylinositol
Grb2	Growth Factor Receptor Binding Protein 2
HDAC	Histone Deacetylase
HHT	Hereditary Hemorrhagic Telangiectasia
HIF	Hypoxia Inducible Factor
HLA	Human Leukocyte Antigen
HPA	Human Platelet Antigen
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
I-Smad	Inhibitory Smad
IFN	Interferon
IL	Interleukin
K14	Keratin 14
Kb	Kilobases
kDa	Kilodalton
KGF-1	Keratinocyte Growth Factor 1
KGF-2	Keratinocyte Growth Factor 2
LAP	Latency Associated Peptide
LLC	Large Latent Complex
LTBP	Latent TGF-beta Binding Protein
M6P	Mannose-6-Phosphate
MAD	Mothers Against Decapentalegic
MAP3K	MAP Kinase Kinase Kinase
MAPK	Mitogen Activate Protein Kinase
MCP	Monocyte Chemotactic Protein
MIP	Macrophase Inflammatory Protein
miRNA	MicroRNA
MIS	Müllerian Inhibitory Substance
MKK	MAP Kinase Kinase
MMLV	Moloney Murine Leukemia Virus
MMP	Matrix Metalloproteinase
mTOR	Mammalian Target of Rapamycin

Mv1Lu	Mink Lunk Epithelial Cells
NES	Nuclear Export Sequence
NF-κB	Nuclear Factor-κB
NICD	Notch Intracellular Domain
NGS	Normal Goat Serum
PAK	P21-activated Kinase
PAR	Protease Activated Receptor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PIAS	Protein Inhibitor of Activated STAT
PMSF	Phenylmethanesulfonylfluoride
pRb	Hypophosphorylated Retinoblastoma Protein
R-Smad	Receptor-regulated-Smad
RhoA	Ras Homolog Gene Family, Member A
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
SARA	Smad Anchor for Receptor Activation
SCC	Squamous Cell Carcinoma
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Deviation of the Mean
SERM	Selective Estrogen Receptor Modulator
ShcA	Src homology 2 domain containing protein A
siRNA	Short Interfering Ribonucleic Acid
SLC	Small Latent Complex
Smurf	Smad Ubiquitylation Regulatory Factor
SUMO	Small Ubiquitin-like Modifier
SnoN	Ski-novel Gene N
Sos	Son of Sevenless
SSc	Systemic Sclerosis
STAT	Signal Transducer and Activator of Transcription
STRAP	Serine-threonine Kinase Receptor-associated Protein
TAK	Transforming Growth Factor Beta Activated Kinase
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween
TG	Transgenic
TGF-β	Transforming Growth Factor Beta
TβRI	Transforming Growth Factor Beta Receptor Type 1

TβRII	Transforming Growth Factor Beta Receptor Type 2
TH2	T-Helper 2
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll-like Receptor
TNF-α	Tumour Necrosis Factor alpha
TSP-1	Thrombospondin-1
uPA	Urokinase-type Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
WT	Wild-Type

1 Introduction

1.1 TGF-beta superfamily

1.1.1 TGF-β superfamily members and their functions

The Transforming-Growth Factor-β (TGF-β) superfamily of secreted proteins consist of over 30 members in mammals and over 60 members in all multicellular organisms including frogs, fish, flies, and worms. The TGF-β superfamily can be subdivided into two major subfamilies, one comprised of TGF-β, activin, nodal, lefty and their respective isoforms and the other consisting of bone morphogenetic protein (BMP), growth and differentiation factor (GDF), and the Müellerian inhibiting substance (MIS) (Weiss and Attisano, 2013). The TGF-β superfamily is involved in a wide array of cellular processes that regulate development and homeostasis. Deregulation of its processes is at the basis of many diseases (Blobe *et al.*, 2000). The majority of the TGF-β proteins regulate cellular proliferation and differentiation. BMPs, GDFs and activins are known for their roles in cell and tissue differentiation and morphogenesis. Activins are involved in hormonal control and homeostasis. TGF-β regulates differentiation and function of hematopoietic and immune cells (Xu *et al.*, 2012). Deregulation of TGF-β can lead to cancer and fibrotic pathologies whereas deregulation of BMP signaling can lead to developmental disorders (Gordon and Blobe, 2008). The wide variety of diseases associated with the deregulation of the TGF-β superfamily demonstrates the important role it plays in various biological processes.

1.1.2 TGF-β Isoforms

Three isoforms of TGF-β exist in mammals, TGF-β1, β2, and β3 which share approximately 70-80% structural homology and have similar *in vitro* but distinct *in vivo* functions. The most abundantly expressed and physiologically relevant TGF-β isoform in humans is TGF-β1. TGF-β2, though important in specific developmental contexts, exhibits relatively weak endogenous affinity for its receptor. TGF-β3 is most prevalent in keratinocytes and fibroblasts but is expressed in low levels in adults (Moses and Roberts, 2008). Previous immunolocalization and in situ hybridization studies in developing mouse and human embryos have shown complex and distinct patterns of distribution of the three TGF-β isoforms. Despite similar effects in *in vitro* studies, *in vivo* knockout mice studies illustrate the distinct functions the three TGF-β isoforms *in vivo*. Specifically, *Tgf-b1* null mice die within 21 days of birth due to inflammation (Shull *et al.*, 1992). *Tgf-b2* null mice die in the perinatal period due to heart and pulmonary insufficiency (Sanford *et al.*, 1997), while *Tgf-b3* null mice, characterized by a cleft palate, die within 24 hours after birth (Proetzel *et al.*, 1995). Together, these studies suggest differential regulation of the three TGF-β isoforms, suggesting that they play unique roles during biological development.

1.1.3 Synthesis of latent TGF-β

The TGF-β isoforms are transcribed as 390–412 amino acid precursor proteins (Gentry and Nash, 1990; Wrana, 1998), consisting of a 20-30 amino acid N-terminal signal peptide region, a pro-peptide region and a 112-114 amino acid C-

terminal mature peptide region. The TGF-β proteins undergo a number of processing steps intracellularly, prior to their secretion by a cell. During processing in the Golgi apparatus, the precursor protein is proteolytically cleaved by a furin-type enzyme at amino acids 278 and 279 (Blanchette *et al.*, 1997; Dubois *et al.*, 1995). The proteolysis results in two products consisting of a 65-75 kDa homodimer from the N-terminal pro-peptide region, called the latency-associated peptide (LAP) and a 25 kDa homodimer from the C-terminal mature peptide region called the mature TGF-β protein (Lawrence, 1996; Munger *et al.*, 1997). The LAP sequences differ for each TGF-β isoform and are named in accordance (LAP-β1, -β2 and -β3) with its associated isoform. Following cleavage, the TGF-β propeptide homodimer retains a strong affinity for the mature TGF-β homodimer and is consequently secreted in association with the mature TGF-β as part of a non-covalent tetrameric complex (Derynck and Miyazono, 2008). The association of the mature TGF-β with LAP renders TGF-β latent and this latent non-covalent complex is referred to as the small latent complex (SLC). The SLC complex is stabilized by an intermolecular disulfide bond of the mature TGF-β homodimers at Cysteine-77 (Schlunegger and Grutter, 1992) and by two disulfide bonds of the TGF-β propeptide homodimers at Cysteine-223 and Cysteine-225 (Brunner *et al.*, 1989). The SLC then covalently binds to latent-TGF-β binding protein (LTBP), forming the large latent complex (LLC) (Rifkin, 2005). There are 4 LTBP isoforms: LTBP-1, -2, -3, and -4 (Koli *et al.*, 2004; Olofsson *et al.*, 1995). LTBP 1, 3, and 4 forms covalent complexes with the SLC whereas LTBP-2 does not. Additionally, LTBP-1 and -3 bind the SLC of all three TGF-β isoforms, whereas LTBP-4 binds only

TGF-β1 SLC and with less affinity than LTBP-1 and -3 (Saharinen and Keski-Oja, 2000). LTBP has also been shown to facilitate secretion of the SLC, and target it to the ECM (Miyazono *et al.*, 1993). When depositited to the ECM, LTBP is able to covalently bind ECM proteins such as heparin and fibronectin to stabilize the LLC in the ECM. By sequestering the TGF-β ligand away from its receptor and by controlling the release of the ligand, LTBP is able to determine the localization and distribution of TGF-β and play an important role in regulating TGF-β ligand bioavailability (Annes *et al.*, 2003).

1.1.4 Activation of latent TGF-β

As TGF-β released into the ECM is in the latent form, the activation of latent TGF-β plays an important role in regulating TGF-β signaling and responses. Activation of latent TGF-β has been demonstrated through a variety of processes, including heat, extreme pH, reactive oxygen species, proteolysis, thrombospondin-1, and integrin activation (Worthington *et al.*, 2011).

While treatments with alkali and acidic agents have shown to dissociate the LLC and release the active form of TGF-β (Brown *et al.*, 1990), need for extreme pH conditions to activate TGF-β makes it an unlikely source for TGF-β activation *in vivo*. However, low-pH environments formed by bone-degrading osteoclasts may in specific cases facilitate acid-mediated activation of TGF-β (Bonewald *et al.*, 1997).

Reactive oxygen species (ROS) can activate the latent TGF-β complex through the redox-mediated process (Ehrhart *et al.*, 1997; Vodovotz *et al.*, 1999). The redox-mediated activation mechanism characteristically involves the oxidation of cysteine or methionine residues in the propeptide region of the SLC, which may be the common method for TGF-β activation as macrophages and neutrophils both produce ROS when properly induced (Dabovic and Rifkin, 2008).

Proteolysis plays a vital role in the activation of the mature TGF-β protein. Serine proteases including plasmin, thrombin, neutrophil elastase, chymase and tryptase have been implicated in TGF-β activation in a cell-type specific manner (Jenkins, 2008). Plasmin is formed by proteolysis of plasminogen by the urokinase type plasminogen activator (uPA) and the tissue type plasminogen activator (tPA). Plasmin activates TGF-β by proteolytically cleaving matrix-bound LLC, which liberates a remnant of the LLC, which then binds to the mannose-6 phosphate receptor (M6P). Once bound to the M6P receptor, LAP is cleaved by a second plasmin proteolytic cleavage, which destabilizes the SLC and liberates the mature TGF-β to bind to its receptors, thereby activating TGF-β (Nunes *et al.*, 1998). Thrombin works similarly to plasmin in its ability to directly dissociate TGF- β from its LAP (Taipale *et al.*, 1992) but it can also induce Ras homolog gene family, member A (RhoA) expression through the Protease Activated Receptor-1 (PAR1) pathway, which can promote αvβ6 integrin activation of TGF-β (Jenkins *et al.*, 2006). Neutrophil elastase is another serine protease that contributes to the activation of TGF-β but instead of directly liberating TGF-β from the LAP and

LTBP, it releases the SLC complex from the ECM where it can be further modified by additional proteases (Taipale *et al.*, 1995). Chymase is a neutral protease stored in mast cell granules along with latent TGF-β. Upon mast cell degranulation the latent TGF-β is released and activated by mast cell chymase in a protease dependent manner (Lindstedt *et al.*, 2001). Tryptase is a product of mast cell secretory granules and it can activate TGF-β via a Protease Activated Receptor-2 (PAR-2) dependent mechanism (Fiorucci and Ascoli, 2004).

Four matrix metalloproteinases (MMPs) have been implicated in TGF-β activation, including MMP2 and MMP9, also known as gelatinase A and B (Ge and Greenspan, 2006; Yu and Stamenkovic, 2000), MMP13, also known as collagenase (D'Angelo *et al.*, 2001), and MMP14, also known as MT1-MMP (Jenkins, 2008). MMP2 and MMP9 are both able to cleave the LAP of TGF-β directly, releasing active TGF-β (Ge and Greenspan, 2006; Yu and Stamenkovic, 2000). MMP13 is released along with latent TGF-β, and reduction of MMP13 activity results in concurrent reduction in TGF-β activity (Jenkins, 2008). MMP14 is responsible for αvβ8 integrin mediated activation of TGF-β in a cell-dependent manner (Jenkins, 2008).

The matricellular protein thrombospondin-1 (TSP-1) functions as an activator of latent TGF-β (Adams, 1997; Ahamed *et al.*, 2009). TSP-1 can activate latent TGF-β indirectly by facilitating the conversion of plasminogen to plasmin, but also by binding the amino-terminal region of the LAP, which induces a conformational

change in the LLC complex and thereby liberating the mature TGF-β from the LLC (Jenkins, 2008; Moser *et al.*, 1995)). The activation of latent TGF-β by TSP-1 may require additional accessory molecules as breast carcinoma cells treated with tamoxifen require the TSP-1 receptors αvβ3 and integrin-associated protein (IAP) for activation (Harpel *et al.*, 2001). The need for accessory molecules may explain why TGF-β remains latent in platelets even though TSP-1 and latent TGF-β are both located in the same granule (Abdelouahed *et al.*, 2000).

Recent evidence has shown that in many physiological situations that the key activators of TGF-β are integrins (Worthington *et al.*, 2011) . Integrins are a large family of cell adhesion and signaling receptors, consisting of α and β subunits that combine to form a family of 24 transmembrane receptors in mammals. Of the 24 integrin receptors, 6 members, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and α8β1 have been reported to bind latent TGFβ *in vitro* via the RGD integrin-binding motif of LAP-1 and LAP-3 (Munger *et al.*, 1998). Of the 6 LAP binding integrins, only 3 members, αvβ3 (Asano *et al.*, 2005a), αvβ5 (Asano *et al.*, 2005b), αvβ6 (Munger *et al.*, 1999) and αvβ8 (Mu *et al.*, 2002) have demonstrated the ability to activate TGFβ.

Previous studies have reported that integrins can activate TGF-β through (i) mechanical induction and (ii) protease-mediated induction. Studies have shown that activation of the latent TGF-β complex by integrins αvβ5 (Wipff *et al.*, 2007) and αvβ6 (Munger *et al.*, 1999) is not prevented by protease inhibitors, indicating that proteolysis is not required for αvβ5 and αvβ6-mediated activation. Evidence now

points to the crucial role of cell contraction in the activation of TGFβ. As an intact actin cytoskeleton is required for TGFβ activation by cell contraction, activation can be greatly reduced by treatment of cells with the actin polymerization inhibitor cytochalasin D (Wipff *et al.*, 2007). LTBP-1 is another crucial element for mechanical induction-mediated TGFβ activation (Annes *et al.*, 2004), as the LTBP-1 hinge region is able to target the latent complex to the ECM and bind to matricellular proteins. Taken together, activation of TGF-β via mechanical induction requires (i) binding of LAP by integrin and generation of a contractile force by the actin cytoskeleton connected to the cytoplasmic domain of the integrin and (ii) binding of LTBP-1 to mechano-resistant ECM which creates an opposing holding force. Applying both forces to the LLC leads to a conformational change in the complex that liberates and activates TGF-β. (Worthington *et al.*, 2011).

Whereas mechanically-induced activation of TGF-β is spatially restricted, this does not appear to be the case for protease-induced activation of TGF-β. The cytoplasmic domain of integrin αvβ8 does not connect to the actin cytoskeleton, and is not required for αvβ8-mediated TGFβ activation (Mu *et al.*, 2002). Instead, αvβ8 causes the release of active TGFβ from the latent complex, and this process is dependent on the proteolytic cleavage of LAP by membrane type 1-matrix metalloproteinase (MTI-MMP) in αvβ8-expressing cells (Mu *et al.*, 2002). Thus, MT1-MMP may serve as the actual activator and αvβ8 as a localizer of the latent TGF-β substrate (Dabovic and Rifkin, 2008). Taken together, integrins facilitate protease-induced activation of TGF-β by functioning as docking proteins for latent TGF-β and

proteases, leading to the enzymatic cleavage of the latent complex and activation of TGF-β (Wipff and Hinz, 2008).

1.1.5 Structure of mature TGF-β

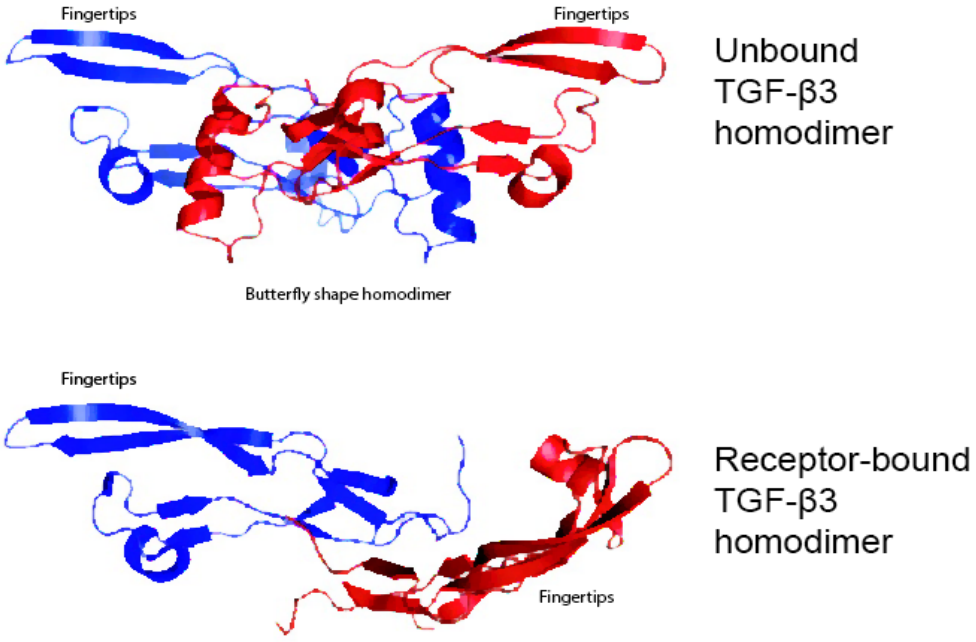
The structural and sequence features of the mature TGF-β protein are highly conserved throughout its family members. The main elements differentiating the TGF-β subfamilies are the number and location of the cysteine residues, whose spacing and conservation are the distinguishing features of the TGF-β family. The TGF-β1, -β2, -β3 family have nine conserved cysteine residues. Of the nine cysteines in each monomer, eight cysteines form four intrachain disulfide bonds, and one cysteine forms an intermolecular disulfide bond with its homolog at Cysteine-77. The four intramolecular disulfide bonds are between Cysteine-7-Cysteine-16, Cysteine-15-Cysteine-78, Cysteine-44-Cysteine-109, and Cysteine-48-Cysteine-111. The latter three cysteine pairs, Cysteine-15-Cysteine-78, Cysteine-44-Cysteine-109, and Cysteine-48-Cysteine-111, define a topological cysteine knot where Cysteine-15- Cysteine-78 passes through a ring bounded by Cysteine-44-Cysteine-109 and Cysteine-48- Cysteine-111 disulfides together with the connecting polypeptide backbone at residues 44-48 and 109-111 (Sun and Davies, 1995).

The rest of the mature TGF-β protein family members share the structural and sequence features as TGF-β1, -β2, -β3 but vary between 7-9 cysteine residues that form intramolecular cysteine knots along with an intermolecular disulfide bond that

facilitates homodimerization (Hinck *et al.*, 1996). There are 5 unique TGF-β family members that have only six cysteine residues instead of the uneven seven-cysteine or nine-cysteine sequences that facilitate intermolecular disulfide bonds required for dimer formation. Specifically, lefty A, lefty B, BMP-15, GDF-9, and GDF-3 have six cysteine residues and lack the fourth cysteine needed for intermolecular disulfide-mediated dimerization (Derynck and Miyazono, 2008).

The crystal structure of TGF-β monomer subunit consists of two antiparallel β sheets and a few helices, the longest of which, named H3, runs perpendicular to the β strands. The subunit structure is often described as a hand, consisting of the wrist region, centered along the long helix; the knuckle region, located on the convex side of the β sheets; and the fingers, each formed by a β strand. The dimeric interaction is mediated mostly by the H3 helice of one subunit and the β-sheet core of the adjacent subunit and is further stabilized by an intersubunit disulfide bond, forming a butterfly shape homodimers (Figure 1.1) (Shi and Massague, 2003).

Figure 1.1 Schematic representation of TGF-β homodimer structure



1.2 TGF-beta superfamily receptors

1.2.1 TGF-β superfamily type I and type II receptors

Five type II TGF-β (TβRII) and seven type I TGF-β (TβRI) receptors exist in mammals. TGF-β receptors are characterized by a short N-terminal extracellular ligand binding domain, a single transmembrane domain, and a C-terminal cytoplasmic kinase domain that has strong serine/threonine kinase activity along with some weak tyrosine kinase activity (Ten Dijke and Heldin, 2006). Many of the TGF-β receptors were named on the basis of the first reports of ligand binding properties and were categorized into one of two groups based on their ability to propagate the signal to either the TGF-β-like or BMP-like Smads (Weiss and Attisano, 2013). However, it is now known that this handful of receptors pair up in various combinations to mediate signals for the over 30 TGF-β superfamily

members. To address the ligand-receptor promiscuity nomenclature challenge, type I TGF-β receptors are now also known as activin receptor-like kinases (ALKs) for all seven type I receptors (ALK1-7) (Moustakas and Heldin, 2009).

1.2.2 TGF-β ligand-receptor binding and signaling

TGF-β signaling is initiated by binding of the mature TGF-β homodimers ligands to the TGF-β receptors to form a heteromeric complex (Feng and Derynck, 2005; Schmierer and Hill, 2007). Previous studies have shown that TβRII exists as a monomer at low expression level and that TβRII dimerizes at high receptor concentration or following TGF-β activation (Zhang *et al.*, 2009). TβRII does not undergo significant structural rearrangement upon binding and no contact is made between the two TGF-β-bound TβRII extracellular domains, as found in crystallography studies (Hart *et al.*, 2002). After TβRII-TGF-β binding, the TβRII-TGF-β complex recruits TβRI, which exists as a monomer at basal state and ligand addition induces its dimerization (Zhang *et al.*, 2010) and interaction with TβRII to form the TGF-β heteromeric complex.

Three residues are critical for TGF-β1 and -β3 ligand binding to TβRII: TGF-β-Valine-92 contacts TβRII-Isoleucine-53, TGF-β-Arginine-25 binds to TβRII-Glutamic acid-119 and TGF-β- Arginine-94 interacts with TβRII Aspartic acid-32, Glutamic acid-55 and Glutamic acid-75. These three residues are substituted in TGF-β2 by Lysine-25, Isoleucine-92 and Lysine-94. The substitution in TGF-β2 is responsible for the lower affinity of this subtype for TβRII as compared to TGF-β1 and -β3 (De Crescenzo *et al.*, 2006).

Formation of the heteromeric complex appears universal across the TGF-β family (Massague, 2008; Wrana *et al.*, 1994) but there are different mechanisms whereby this assembly is achieved. In the case of TGF-β, ligand binding to TβRII creates a cleft between TGF-β and TβRII that accommodates a five-residue loop from TβRI to yield a TGF-β binding complex of high specificity and cooperativity (Groppe *et al.*, 2008). In the case of BMPs, the ligands can first interact with either type II receptors or with BMP type I receptors, which lack the five-residue loop found in TβRII and ACVRIB, yielding a receptor binding interface with greater flexibility (Groppe *et al.*, 2008). Regardless of the mechanism, formation of the active receptor complex allows the type II receptor kinase, which is constitutively active, to phosphorylate the Glycine-Serine (GS) region of the type I receptor (Feng and Derynck, 1997).

This GS region is a 30 amino acids sequence located immediately upstream the kinase domain of TβRI and is conserved among all type I receptors of the TGF-β superfamily. The phosphorylation of the many serine and threonine residues of the GS region by TβRII is required for the downstream type I kinase to adopt a high activity state (Wieser *et al.*, 1995). In the absence of a ligand, the 12 kDa FK506-binding protein (FKBP12) binds to the GS region of the type I receptor and prevents its inadvertent phosphorylation (Chen *et al.*, 1997b). FKBP12, by pressing against the active center of TβRI kinase, induces a catalytically inactive conformation of TβRI (Huse *et al.*, 1999). The phosphorylation of the GS region

by TβRII eliminates the binding site for FKBP12, while creating a new binding site for its substrates, the Receptor-SMADs (R-Smads) (Wrighton *et al.*, 2009).

The serine-threonine kinase receptor family is the only known signaling receptors for the TGF-β family members making it a unique ligand-receptor system. Despite the limited number of receptor types, these serine/threonine kinase receptors are able to mediate the biological activity of more than 30 TGF-β ligands by using its promiscuous receptors to signal through a complex system of combinatorial ligand-receptor interactions (Wrana *et al.*, 2008). One exception to the promiscuous and combinatorial role of the TGF-β receptor family is the MIS and its type II receptor MISRII, where studies have shown that MIS is the only ligand for MISRII (Josso *et al.*, 2005).

1.2.3 TGF-β superfamily receptor combinations

The TGF-β receptors mediate biological activity through combinatorial interactions allowing diverse responses. TGF-β ligands bind to a specific combination of type I and type II receptors, which activate specific groups of R-Smads (Moustakas and Heldin, 2009). TGF-β type I receptors commonly cross the boundaries among classes of TGF-β members while TGF-β type II receptors tends not to cross the boundaries (Wrana *et al.*, 2008). Accordingly, ligands that have primary transcriptional responses that are BMP-like, interact with ALK-1 (ACVRL1), -2 (ACVRI), -3 (BMPRIA) and -6 (BMPRIB); whereas ligands that induce TGF-β-like transcriptional responses interact with ALK-5 (TβRI), -4 (ACVRIB), and -7

(ACVRIC) (Massague, 2012). The specificity of the TGF-β type I receptors in transcriptional response is generally mediated via the high conserved kinase domains of the type I receptors; ALK-1 is an exception to this rule, as it can assemble in heteromeric complexes that mediate both TGF-β and BMP-like responses (Goumans *et al.*, 2003). ALK-2 and ALK-3 can also mediate both TGF-β and BMP-like responses, albeit to a lesser capacity (Wrana *et al.*, 2008).

TGF-β receptor specificity is dependent on the type I receptor that is involved in the complex. Studies on R-Smad interactions demonstrate that specificity is achieved by complementarity between the L45 loop of the type I receptor, located just downstream of the GS motif, and the L3 loop of the R-Smads (Chen *et al.*, 1998c). In the classical model, TGF-β signals via the type II receptor TGFBR2 and the type I receptor ALK5 (Figure 1.2) (Feng and Derynck, 2005). However, TGF-β is able to activate alternative receptor combinations in a cell-specific manner. In endothelial cells and chondrocytes, TGF-β can signal via a mixed TGFBR2/ALK-5/ALK1 complex and activate SMAD1/5 (Figure 1.2) (Finnson *et al.*, 2008; Goumans *et al.*, 2003) Moreover, another study has suggested that the L45 loop of ALK5 alone could mediate SMAD1/5 recruitment in tumorigenic epithelial cells but not in immortalized epithelial cells (Liu *et al.*, 2009b). Furthermore, while TGFBR2 cannot bind to TGF-β2 on its own, some cells express a splice variant of TGFBR2, named as TGFBRII-B. TGFBR2-B contains a 26 amino acids insert after Serine-31, replacing TGFBR2-Valine32, a critical residue for interaction with TGF-β (De Crescenzo *et al.*, 2006) and can thus interact with TGF-β2 in the

presence of TGFBR1 (Parker *et al.*, 2007). These studies illustrate the ability of TGF-β receptors to induce diverse responses via different combinations of type I and type II receptors.

Figure 1.2 Schematic representation of TGF-β ALK5/1 signaling pathways

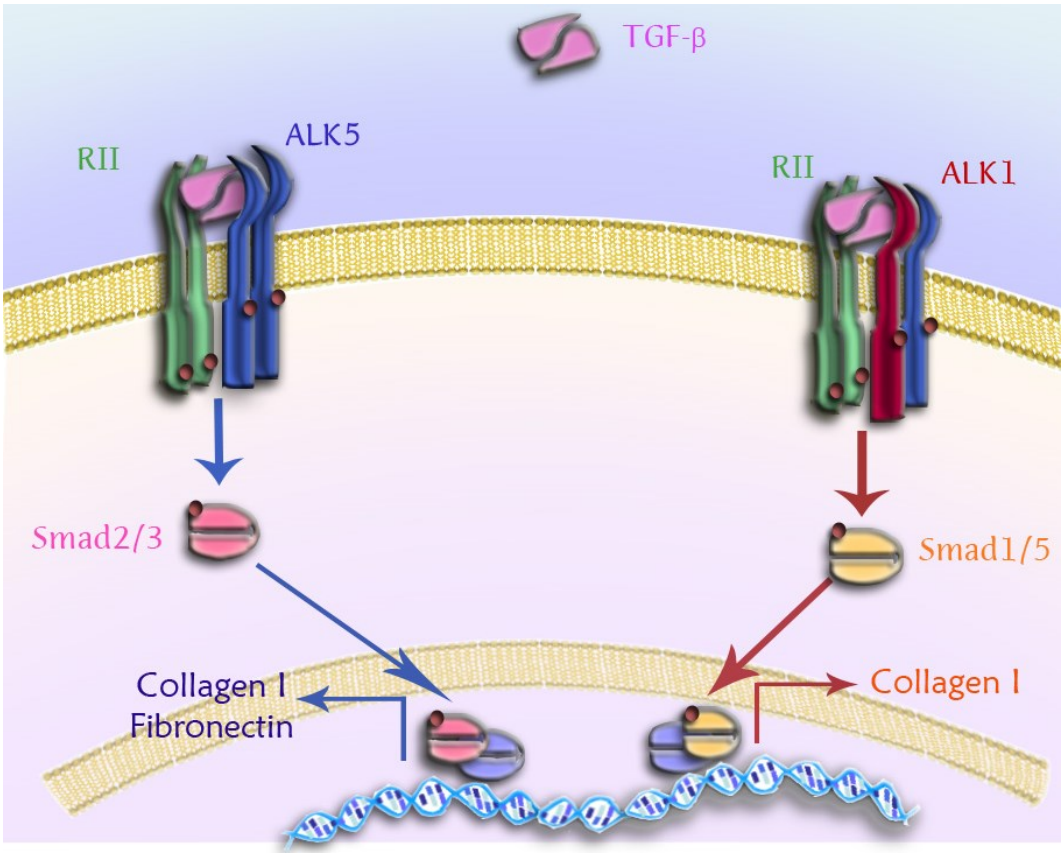


Table 1.1: TGF-β superfamily receptor combinations

	Ligand	Type II receptor	Type I receptor	Receptor Smad
Canonical model	TGF-β	TβRII	ALK5 (TβRI) (Feng and Derynck, 2005)	SMAD2 SMAD3
	Activin & Nodal	ACVRII ACVRIIB	ALK4 (ACVRIB) (Feng and Derynck, 2005) ALK7 (ACVRIC) (Reissmann *et al.*, 2001)	

	BMP	BMPRII ACVRII	ALK1 ALK2 (ACVRI) ALK3 (BMPRIA) ALK6 (BMPRIB) (Goumans *et al.*, 2003)	SMAD1 SMAD5 SMAD8
Non-canonical model in epithelial cells	TGF-β	TβRII	ALK5 + ALK2 ALK5 + ALK3 (Daly *et al.*, 2008)	SMAD1 SMAD5
Non-canonical model in chondrocytes and endothelial cells	TGF-β	TβRII	ALK5 + ALK1 (Goumans *et al.*, 2002; Parker *et al.*, 2007)	SMAD1 SMAD5

1.3 TGF-beta receptor trafficking

1.3.1 Endocytosis

Endocytosis is an important mechanism in regulating TGF-β signaling. Endocytosis enables the internalization of TGF-β receptor molecules from the cell surface into various vesicles and internal cellular compartments such as the Golgi apparatus, endoplasmic reticulum, and endosomes (Le Roy and Wrana, 2005). Two major pathways of endocytosis have been described for TGF-β receptors: (i) the non-lipid-raft, clathrin-dependent pathway and (ii) the lipid-raft, clathrin-independent pathways (Wrana *et al.*, 2008).

The non-lipid-raft, clathrin pathway is characterized by coated clathrin vesicle pits that are formed after recognition of TGF-β receptor molecules by an adaptor protein, such as AP2 or β-arrestin and then subsequently assembled by clathrin-coated vesicles around the cargo molecule (Benmerah and Lamaze, 2007). The vesicle is then internalized from the clathrin-coated pits into Early Endosome

Antigen 1(EEA1)-positive early endosomes, which are then subsequently degraded or recycled in various endosomes (Le Roy and Wrana, 2005).

The lipid-raft, clathrin-independent pathway involves the assembly of lipids such as glycosphingolipids and cholesterol with the TGF-β receptor molecule into an ordered domain within the plasma membrane bilayer and then subsequently sorted to caveolin-positive compartments where they can interact with signaling complexes and mediate receptor complex degradation (Wrana *et al.*, 2008).

1.3.2 TGF-β receptor internalization

TGF-β receptor internalization via the clathrin pathway leads TGF-β receptors to the early endosomes membrane through SARA (Smad anchor for receptor activation), which contains a FYVE domain that targets SARA to the membrane of the early endosomes and then binds to early endosome enriched protein, PI3P (Tsukazaki *et al.*, 1998). In the clathrin pathway, SARA binds Smad2 and Smad3 through a 45-amino acid Smad-binding-domain (SBD) and then presents the Smads to the internalized receptors for phosphorylation (Wu *et al.*, 2000). Clathrin-mediated TGF-β receptor internalization is further facilitated by Cytosolic Form of Promyelocytic Leukemia Protein (cPML), which promotes Smad-SARA binding and recruitment of the TGF-β receptor complexes to early endosomes (Lin *et al.*, 2004). Thus, internalization of TGF-β receptor via the clathrin pathway is generally thought to promote TGF-β signaling (Wrana *et al.*, 2008).

TGF-β receptors can also be internalized via the lipid-raft-mediated pathway into caveolin-1-positive vesicles enriched in Smad7/Smurf2 (Di Guglielmo *et al.*, 2003). Smad7 serves as an adaptor protein for Smurf2, which ubiquitylates the type I receptor through the Smad7-Smuf2 ubiquitin ligase complex, resulting in lysosomal and proteasomal degradation of the type I receptor (Galbiati *et al.*, 2001). Thus, internalization of TGF-β receptors via the caveolae/lipid-raft-mediated pathway is generally thought to hinder TGF-β signaling (Bizet *et al.*, 2011; Bizet *et al.*, 2012; Wrana *et al.*, 2008).

As TGF-β receptor internalization is a constitutive process but TGF-β receptor degradation is ligand-induced, changes in the internalization and recycling rates or in the ratio of constitutive to ligand-induced degradation rate is predicted to influence the strength and duration of TGF-β signaling (Vilar *et al.*, 2006). Therefore, molecules that regulate TGF-β receptor trafficking and compartmentalization are thought to play an important role in mediating TGF-β signaling (Wrana *et al.*, 2008).

1.4 TGF-beta Smad canonical signaling pathway

1.4.1 Smad classes and structure

Unlike most signaling cascades and besides cytokines, the TGF-β pathway can directly transduce extracellular signals from the cell-surface transmembrane receptors to the nucleus through intracellular mediators, known as Smads. Smads are a well conserved family of signal transcription factors and were named after the homologs SMA and Mad in *C. elegans* and *Drosophila* (Patterson and Padgett,

2000). In mammals, there are eight Smads, as compared to six in the *C. elegans* and four in *Drosophila (Huminiecki et al., 2009)*.

Smads can be divided into three subfamilies, receptor-regulated Smads (R-Smads) composed of Smad1/2/3/5/8; common-mediator Smads (Co-Smads) composed of Smad4; and inhibitory Smads (I-Smads) composed of Smad6/7 (Derynck *et al.*, 1996). Smads consist of three structural domains including an N-terminal Mad-homology 1 (MH1) DNA-binding domain; a middle linker domain that interacts with ubiquitin ligases and is enriched in prolines; and a C-terminal MH2 domain that binds to type I receptors and mediates Smad oligomerization (Ten Dijke and Heldin, 2006).

The R-Smad structure consist of the conserved MH1 and MH2 domains, the less conserved connecting linker region, and the C-terminal SSXS (Ser-Ser-X-Ser) kinase motif. The co-Smad structure consists of the MH1, MH2, and linker regions similar to R-Smad, but does not have the SSXS motif. The I-Smad structure shares the same MH2 domain as R-Smad and co-Smad, but does not have the MH1 domain, nor the SSXS motif of R-Smad (Heldin, 2008).

R-Smads and co-Smads bind DNA through the MH1 domain with the exception of Smad2. MH1 DNA binding is accomplished through its β-hairpin structure, which contacts the major groove of DNA. Additionally, the MH1 domain participates in interactions with various DNA-binding proteins to establish DNA binding complexes (Lin *et al.*, 2008), as well as facilitates nucleocytoplasmic shuttling

through its nuclear localization signals (NLSs). The MH2 domain is known for mediating Smad oligomerization and establishing signal specificity. The MH2 domain mediates specific interactions with the type I receptor through its L3 loop which interacts with L45 loop of the type I receptor (Chen and Massague, 1999). Additionally, MH2 mediates Smad oligomerization by facilitating interactions with SARA and other proteins including CBP and Ski (Lin *et al.*, 2008). The linker region is known for regulating cross-talk with other signaling cascades. As the linker region is rich in serines and threonines which promote phosphorylation, as well as prolines which promote interactions with diverse regulatory proteins such as ubiquitin ligases, it plays a vital role in the cross-talk between various signaling pathways (Weiss and Attisano, 2013).

1.4.2 Smad activation

Direct phosphorylation of the C-terminal SXSS motif of R-Smads by type I receptors results in the activation of R-Smads. Upon activation of type I receptor by TGF-β stimulation, the L45 loop of an activated type I receptor interacts with the L3 loop of a R-Smad (Chen *et al.*, 1998c), and the phosphoserines and phosphothreonines in the active GS region of the type I receptor are able to provide a target for the positively charged pocket in the L3 loop of R-Smads (Wu *et al.*, 2001). Bringing the L3 loop of R-Smads close to the active GS region of the type I receptor facilitates the phosphorylation of the SXSS motif of the R-Smads (Abdollah *et al.*, 1997). The phosphorylation of the R-Smad SXSS motif occurs on two of its most C-terminal serine residues, which consequently removes the

autoinhibitory interaction between its MH1 and MH2 domains (Hata *et al.*, 1997) and promotes Smad oligomerization with Smad4. The Smad2/3/4 oligomer formation is stabilized by extensive protein-protein interactions between the phosphorylated R-Smad SXSS motif and the L3 loop of the other R-Smad subunit (Chacko *et al.*, 2001). Phosphorylation of the Smad SXSS motif also destabilizes R-Smad interaction with SARA by interfering with its binding to the R-Smad MH2 domain. As a result, R-Smads dissociate from SARA and become available for oligomerization with Smad4 (Wu *et al.*, 2000). Dissociation of R-Smads from SARA subsequently exposes a nuclear import region of the R-Smad MH2 domain for Smad translocation to the nucleus (Xu *et al.*, 2000).

Adaptor proteins Hgs, Dab-2, Filamin-1, ELF β-spectrin, and Axin have also been reported to mediate Smad activation and signaling (Shi and Massague, 2003). Hgs, a member of the FYVE domain family is similar to SARA both structurally and functionally, as it enables the presentation of Smads to the TGF-β receptors by working with SARA in a synergistic manner (Miura *et al.*, 2000). Dab-2 facilitates receptor activation of Smads by binding concurrently both R-Smad and TGF-β receptors (Hocevar *et al.*, 2001). As R-Smads have been shown to attach cytoskeletal elements in the cell, it has been hypothesized that R-Smads can interact with tubulin, filamin and spectrin proteins. Studies on microtubule-destabilizing agent nocodazole support this hypothesis as it is able to prevent Smad3 binding to microtubules and is able to enhance Smad2 phosphorylation and nuclear accumulation (Dong *et al.*, 2000). Similarly, in filamin-1-deficient cells, Smad2

cannot be phosphorylated in response to TGF-β stimulation (Sasaki *et al.*, 2001). Analogously, TGF-β signaling is also found to be defective in ELF-β-spectrin-deficient mice (Tang *et al.*, 2003). In addition, cytoplasmic protein Axin has also been shown to facilitate TGF-β signaling by supporting Smad3 binding and activation (Furuhashi *et al.*, 2001) and by promoting Smad7 ubiquitylation and degradation (Liu *et al.*, 2006). With such a variety of proteins involved in Smad activation, TGF-β signaling is regulated by a complex system of regulatory proteins that can illicit their effects in a cell-dependent and context-specific manner.

1.4.3 Inhibitory Smad Interactions

Inhibitory Smads (I-Smads), which include Smad6 (Imamura *et al.*, 1997) and Smad7 (Hayashi *et al.*, 1997; Nakao *et al.*, 1997) in vertebrates and Dad in *Drosophila* (Tsuneizumi *et al.*, 1997) play an important role in the negative regulation of TGF-β signaling. I-Smads regulate TGF-*β* family signaling primarily by interfering with the receptor mediated activation of R-Smads. Similar to the R-Smads, I-Smads interact with the type I receptor via their L3 loop of its MH2 domain. Unlike R-Smads, I-Smads do not have an MH1 domain or a SSXS motif in their MH2 domain. Thus, binding of I-Smads to the type I receptor does not result in phosphorylation of the I-Smads but blocks the access of the R-Smads to the activated receptor (Massague *et al.*, 2005).

Smad6 expression is induced by Smad1/5 while Smad7 expression is induced by Smad3 (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997).

Accordingly, BMP signaling induces an inhibitory feedback loop through Smad6 expression while TGF-*β* induces an inhibitory feedback loop through Smad7 expression. Interestingly, Smad7 inhibits both TGF-β and BMP pathway whereas Smad6 is more specific for the BMP pathway (Miyazono, 2000). Additionally, Smad6 has been shown to interact with phospho-Smad1 and prevent Smad1/4 complex formation (Hata *et al.*, 1998) while Smad7 can interact with Smad binding elements in the nucleus and compete with R-Smads for binding (Zhang *et al.*, 2007). Intriguingly, overexpression of either Smad6 or Smad7 has shown to inhibit both TGF-β and BMP signaling, blurring the distinctions in Smad6 and Smad7 function (Feng and Derynck, 2005). Furthermore, inflammatory cytokines including IL-1, IFN-γ and TNF-α as well as EGF, ultraviolet radiation and fluid shear stress (Afrakhte *et al.*, 1998; Topper *et al.*, 1997; Yan *et al.*, 2009b) can also induce I-Smad expression in a cell-type-dependent manner. The induced expression of inhibitory Smads consequently decreases receptor-mediated Smad activation and cell responsiveness to TGF-*β* ligands (Feng and Derynck, 2005).

Along with its ability to interfere with the receptor mediated activation of R-Smads, I-Smads can also inhibit TGF-*β* family signaling by interacting directly with Smurf E3 ubiquitin ligases and mobilizing these E3 ubiquitin ligases to the type I receptors, leading to proteasomal degradation of the receptors (Ebisawa *et al.*, 2001; Kavsak *et al.*, 2000; Murakami *et al.*, 2003).

1.4.4 SMAD nucleocytoplasmic shuttling

R-Smads and Co-Smads were initially thought to have been located exclusively in the cytoplasm in the absence of TGF-β signaling. Upon signaling, the R-Smads and Co-Smads were thought to translocate into the nucleus to regulate gene transcription and be degraded (Lo and Massague, 1999). Recent studies on Smad4 (Pierreux *et al.*, 2000; Watanabe *et al.*, 2000), in the absence of a TGF-β ligand, have demonstrated that Smad4 is equally distributed between the nucleus and the cytoplasm, suggesting that Smads are continuously shuttled between the cytoplasm and the nucleus (Hill, 2009). Fluorescence perturbation and photoactivation experiments with GFP fusions of Smad2 have provided additional evidence of continuous exchange between the nuclear and the cytoplasmic pools of Smads (Batut *et al.*, 2007).

Nucleocytoplasmic transport occurs through the nuclear pore complex (NPC), which forms a hydrophobic channel through the nuclear envelope. In mammalian cells, NPCs are made up of complexes of 30 nucleoporins (Hetzer *et al.*, 2005). Interaction of transport substrates with the NPC and their subsequent translocation are frequently mediated by transport receptors known as karyopherins (Gorlich and Kutay, 1999). Depending on the transport direction, karyopherins can be categorized as importins or exportins (Gorlich and Kutay, 1999).

The nuclear import of Smads is mediated by either karyopherin-dependent pathways or by direct interaction with nucleoporins, depending on the Smad type and on the state of oligomerization (Chapnick and Liu, 2010). Phosphorylation of

R-Smads increases their interaction with karyopherins and their nuclear import and accumulation, as compared to unphosphorylated R-Smads (Kurisaki *et al.*, 2001). In the context of Smad complexes after ligand stimulation, all of them require karyopherins to import them into the nucleus (Hill, 2009).

Nuclear export of Smad complexes follows dephosphorylation of the SSXS motif of the R-Smad-Co-Smad complex. As the association between Co-Smads and R-Smads blocks interaction between the nuclear export sequence (NES), dephosphorylation is thus a prerequisite for Smad4 nuclear export (Chen *et al.*, 2005). Once the complex is dissociated, Smad4 is exported via Exportin 1, Smad2 and Smad3 are exported via Exportin 4 (Kurisaki *et al.*, 2006) or via RANBP3, which recognizes the unphosphorylated forms of Smad2 and Smad3 (Dai *et al.*, 2009). Nuclear phosphatase PPM1A has been shown to dephosphorylate R-Smads, thereby increasing nuclear export of Smads (Lin *et al.*, 2006). After nuclear export, if the receptors are still active, the Smads will be rephosphorylated and reimported back into the nucleus. If the receptors are no longer active, the Smads will reaccumulate in the cytoplasm (Inman *et al.*, 2002).

Nucleocytoplasmic distribution of Smads is determined by the rates of nuclear import, nuclear export, and by factors that cause nuclear retention (Hill, 2009). Recent data suggest that neither increased import nor release from cytoplasmic retention play a predominant role in TGF-β-induced Smad nuclear accumulation but rather from selective trapping of active Smad complexes in the nucleus

(Schmierer and Hill, 2005). Transcriptional regulator TAZ has recently been shown to be involved in retaining Smad complexes in the nucleus. When TAZ is knocked down Smad2/3/4 complexes form, but fail to accumulate in the nucleus (Varelas *et al.*, 2008). Whether binding to TAZ in the nucleus is sufficient to account for the measured decrease in nuclear export rate of Smad complexes relative to monomeric Smads remains to be investigated. Overall, Smad nucleocytoplasmic shuttling provides a mechanism where the intracellular transducers of the signal can continuously monitor receptor activity.

1.4.5 Smad transcriptional regulation

Smads play a vital role in regulating DNA transcriptional activity. Smads regulate transcriptional activity by associating with sequence-specific DNA binding transcriptional factors, specific DNA response elements, and non DNA-binding transcription coactivators or corepressors that interact with the basal transcription machinery and/or modify the DNA chromatin structure (Feng and Derynck, 2005; Massague *et al.*, 2005).

Smads interact directly with defined DNA sequences containing two copies of the Smad-binding-element (SBE), GTCT and its reverse complement AGAC in the opposite DNA strand, which is the optimal DNA sequence for Smad3 and Smad4 binding (Zawel *et al.*, 1998). Tandem repeats of the SBE sequence confer TGF-β-inducible transcriptional activation (Johnson *et al.*, 1999). Crystal structure studies have shown that the Smad3 MH1 domain interacts through an 11-amino acid β-hairpin with the major groove of the DNA at the half-site GTCT and its reverse

complement AGAC of the SBE (Shi *et al.*, 1998). SBE sequences, or close variants including the CAGAC sequence, are needed for the transcription of many TGF-β-responsive genes including *PAI-1* (Dennler *et al.*, 1998), *JunB* (Jonk *et al.*, 1998), *collagenase I* (Zhang *et al.*, 1998) and $P15^{INK4B}$ (Feng *et al.*, 2000).

Smads can bind to non-SBE DNA sequences including GC-rich sequences. Smad1 and its *Drosophila* homolog Mad bind to GCCG sequences with higher affinity than to SBE sequences, which confers BMP responsiveness (Korchynskyi and ten Dijke, 2002). Smad4 and its *Drosophila* homolog Medea have been shown to bind to GC-rich sequences and also confer BMP responsiveness (Ishida *et al.*, 2000). Smad3 binds a GGCGG sequence in the c-myc promoter, which is responsible for the transcriptional repression of c-myc by TGF-β signaling (Frederick *et al.*, 2004). Interestingly, Smad2 is unable to directly bind SBE and GC-rich DNA sequences, which is like a result of a sequence inserted immediately adjacent to the 11-amino acid β-hairpin, which may interfere with DNA recognition (Shi *et al.*, 1998). Interestingly, a splicing variant of Smad2 with a deletion of this insert has similar DNA-binding properties as Smad3 (Yagi *et al.*, 1999). Although Smad2 cannot directly bind SBE or GC-rich DNA sequences directly, it is thought that Smad2/4 complexes bind DNA through Smad4 and interacting transcription factors (Lin *et al.*, 2008).

Smad binding to DNA is not very selective and is marked by minimal sequences requirements and low binding affinity. The specific DNA-binding activity of the

Smad complex may be through Smad-interacting transcription factors that have a higher affinity and specificity to bind specific DNA sequences as observed for Smad complex interactions with FoxHI/FAST-1 (Chen *et al.*, 1997a; Labbe *et al.*, 1998; Zhou *et al.*, 1998). Thus, the high affinity and specificity of the Smad complex with the regulatory transcriptional sequences are provided via direct interactions of Smads with DNA and with sequence-specific transcription factors (Massague *et al.*, 2005).

Smads and DNA-sequence binding transcription factors cooperate through a variety of physical interactions that are mediated by the MH1 or MH2 domains of the Smads (Miyazono, 2008). The diversity of interactions between transcription factors and their associated binding sequences, along with the minimal sequence requirements for Smad binding, explains the complexity of the transcription regulation and why there are no consensus TGF-β responsive promoter sequences for their target genes (Feng and Derynck, 2005). The specificity of Smad and DNA-sequence binding transcription factor cooperation depends largely on spatial and temporal conditions.

Smads recruit a variety of coactivators and corepressors into the transcription machinery. These coactivators and corepressors interact with Smads to regulate the magnitude of Smad-mediated transcriptional activity (Derynck and Zhang, 2003). Transcription coactivators, such as CBP/p300 and the Mediator complex, promote gene activation by bringing the DNA sequence-specific transcription factors into

close physical proximity with the RNA polymerase II complex and/or by relaxing the chromatin structure through their histone acetyltransferase (HAT) activity to the target gene promoter (Feng *et al.*, 1998; Simonsson *et al.*, 2006). Transcription corepressors, such as c-Myc, c-Ski/SnoN, and Evi-1, inhibit gene activation by disrupting the formation of Smad coactivators complexes and/or by tightening the chromatin structure through their histone deacetylase (HDAC) activity to the target gene promoter (Alliston *et al.*, 2005; Feng *et al.*, 2002; Wu *et al.*, 2002).

In addition to its roles in interfering with receptor-mediated activation of R-Smads, studies have shown that I-Smads can also act as transcription regulators in the nucleus (Miyazono, 2008). Smad7 can interact with transcriptional coactivator p300, which confers acetylation of Smad7 on Lysine-64 and Lysine-70 in the nucleus (Gronroos *et al.*, 2002). Conversely, Smad7 can also interact with HDACs, which deacetylate Smad7 (Simonsson *et al.*, 2005). Smad6 can inhibit transcription activation by directly recruiting corepressors CtBP to the *Id1* promoter (Lin *et al.*, 2003c). Smad6 can also prevent transcription activity by interacting with homeobox transcription factors Hoxc-8 and Hoxc-9 upon BMP stimulation and inhibit transcription of the *osteopontin* gene (Bai *et al.*, 2000). Smad6 can also interact directly with HDACs, including HDAC1 and HDAC3, through its MH2 domain. Additionally, Smad6 binds to DNA through its N domain and recruits HDACs to DNA (Bai and Cao, 2002).

Altogether, Smads recruit not only DNA-binding transcription factors, but also coactivators and corepressors to regulate the magnitude of the transcriptional activation. A balance between corepressors and coactivators determines Smad transcriptional responses that affect numerous aspects of regular physiological and pathological processes (Lin *et al.*, 2008).

1.4.6 Smad transcriptional crosstalk

Essential to the mechanism of Smad-mediated transcription is the cooperation with, and dependence on, a variety of non-TGF-β Smad signaling regulated DNA binding transcription factors that define the target regulatory DNA sequences of Smad cooperation (Luo, 2008). Consequently, these transcription complexes serve as excellent platforms for functional crosstalk between the TGF-β Smad signaling pathway and other signaling pathways.

TGF-β Smad signaling extensively cooperates with Wnt signaling, and crosstalk occurs at multiple levels, including induction of TGF-β ligand expression (Guo and Wang, 2009), and interactions of TGF-β receptors or Smads with Axin or GSK3 (Furuhashi *et al.*, 2001; Guo *et al.*, 2008; Liu *et al.*, 2006). In nucleoprotein transcription complexes, activated Smad3 and Smad4 associate and cooperate with Tcf and Lef1, transcription factors that serve as effectors, to regulate Wnt-induced transcriptional activity (Labbe *et al.*, 2000; Nishita *et al.*, 2000). Smad also form transcription complexes with β-catenin and their association is hypothesized to be stabilized by p300 and CBP coactivators (Lei *et al.*, 2004). In mesenchymal stem

cells, TGF-β induces a fast co-translocation of Smad3 and β-catenin into the nucleus, to then cooperatively regulate a set of genes that cannot be recognized by either Smads or β-catenin alone. The cooperative regulation is mediated by the association between Smads and TCF/LEF in the nucleus, and results in the synergistic activation of specific target genes (Jian *et al.*, 2006).

TGF-β Smad signaling extensively also cooperates with Notch signaling. TGF-β-induced Smad3 complexes associate with the Notch intracellular domain (NICD) and the DNA-binding transcription factor CSL to regulate transcription of Hes, Hey and Herp2 Notch signaling target genes (Blokzijl *et al.*, 2003; Dahlqvist *et al.*, 2003; Takizawa *et al.*, 2003). Similarly, activated Smad3 also associates with NICD to cooperatively activate the Foxp3 expression in murine regulatory T cells (Samon *et al.*, 2008). Interestingly, NICD also promotes cell growth and cancer development by suppressing the growth inhibitory effects of TGF-β by sequestering coactivator p300 from activated Smad3 (Masuda *et al.*, 2005).

The MAPK signaling pathway interacts with Smads at multiple levels. In addition to activating the expression of TGF-β ligands and the inhibitory Smad7, MAPKs also phosphorylate R-Smads and C-Smads at multiple sites (Guo and Wang, 2009). At the level of transcriptional regulation by Smads, MAPKs target various transcription factors that associate with Smads and enable Smads to cooperatively coordinate gene expression responses including c-Jun, Jun-B, c-Fos and AP-1 transcription factors (Javelaud and Mauviel, 2005; Shaulian and Karin, 2002;

Zhang *et al.*, 1998). Furthermore, TGF-β directly induces, through Smad3/4, the expression of transcription factor ATF3, which then cooperates with Smad3 in ATF3-mediated secondary gene expression responses. As target of both TGF-β signaling and p38 MAPK stress signaling, ATF3 integrates both pathways in the response of epithelial cells to stress and injury (Zhang, 2009).

Smad-mediated transcription through crosstalk interactions with transcription factors provides extensive complexity in the transcriptional regulation of target genes. Cross-talk between pathways can be direct or indirect, unidirectional or bidirectional, and often occurs as part of a feed-back loop. Thus, further mechanistic studies are necessary to determine the causality and to identify the convergence points of the pathways (Luo, 2008).

1.5 TGF-beta non-canonical signaling pathways

At the cell surface, signaling functions of the TGF-β family is mediated by the TβRI and TβRII serine-threonine kinase receptor complex. Binding of TGF-β ligands to the TGF-β receptor complex leads to phosphorylation and activation of TβRI by TβRII (Shi and Massague, 2003). Downstream from the TGF-β receptor complex, the uniformity ends, as the TGF-β receptor complex is able to relay signals to a variety of intracellular pathways. In addition to the canonical Smad-mediated signaling, TGF-β family proteins also signal through non canonical pathways including those mediated by mitogen-activated protein kinases (MAPK), Rho-like GTPases, and phosphatidylinositol-3 kinases (PI3K) (Derynck and Zhang, 2003;

Moustakas and Heldin, 2005; Yi *et al.*, 2005). Subsequent studies have shown these non-Smad pathways can regulate TGF-β-induced cellular responses independently or in conjunction with the Smad signaling pathway (Zhang, 2008).

1.5.1 TGF-β-induced MAPK signaling pathways

Previous studies on TGF-β non-canonical signaling pathways have shown that TGF-β is capable of activating three different MAP kinase pathways: the ERK MAPK (Frey and Mulder, 1997), JNK MAPK (Hocevar *et al.*, 1999), and the p38 MAPK (Yu *et al.*, 2002) signaling pathways.

1.5.1.1 TGF-β-induced ERK signaling pathway

The kinetics of TGF-β-induced ERK phosphorylation is highly contextual, in terms of cell type and cell condition (Zhang, 2008). TGF-β is able to activate ERK signaling in epithelial cells (Hartsough and Mulder, 1995), breast cancer cells (Frey and Mulder, 1997) and fibroblasts (Mucsi *et al.*, 1996) within 5-10 minutes of TGF-β stimulation. In contrast, other cell lines showed a delayed response in TGF-β-induced ERK signaling, with peak ERK phosphorylation occurring hours after initial TGF-β stimulation, suggesting an indirect response requiring protein translation (Simeone *et al.*, 2001). Smad-dependent transcriptional mechanism can partially explain for the delayed activation of ERK by TGF-β but it does not explain the rapid activation of ERK by TGF-β.

TGF-β receptors are well-defined serine-threonine kinases although they can also be phosphorylated on tyrosine kinases, albeit at a lower level than on serine and threonine residues (Lawler *et al.*, 1997). Tyrosine residues can serve as docking sites for many signaling molecules that contain the Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, including the SH2 domain containing protein A (ShcA) and growth factor receptor binding protein 2 (Grb2) (Lee *et al.*, 2007b). Grb2 is an adaptor protein that is bound to the son of sevenless (Sos) protein in the cytoplasm in the absence of ligand stimulation. Generally, upon phosphorylation of the receptor tyrosine residues, the Grb2/Sos complex gets recruited to the plasma membrane, where Sos activates Ras by catalyzing the exchange of GDP for GTP. In its GTP bound state, Ras can bind and activate Raf, leading to downstream activation of MEK and ERK (Lee *et al.*, 2007b).

TβRII can undergo autophosphorylation on three tyrosine residues: Tyrosine-259, Tyrosine-336, and Tyrosine-424. TβRII can also be tyrosine phosphorylated by Src, on Y284, which serves as a docking site for the recruitment of Grb2/Sos and ShcA proteins (Galliher and Schiemann, 2007). Although it is unclear whether the tyrosine phosphorylation of the TβRI comes from autophosphorylation or transphosphorylation by TβRII, activated TβRI can directly bind and phosphorylate ShcA on tyrosine and serine residues and encourage the formation of a ShcA/Grb2/Sos complex and leading to ERK activation (Lee *et al.*, 2007b). Although ShcA can be phosphorylated by either TβRI or TβRII *in vitro*, ShcA interacts with TβRI with higher affinity than TβRII. Overexpression of a ShcA

mutant lacking either the PTB or the SH2 domain diminishes TGF-β-induced ShcA tyrosine phosphorylation, leading to decreased ERK activation by TGF-β (Zhang, 2009). In summary, ShcA plays a vital role in TGF-β-induced ERK activation, and receptor tyrosine residue phosphorylation is an important mechanism for MAPK cascade activation.

1.5.1.2 TGF-β-induced JNK and p38 signaling pathway

Studies on Smad3/4-deficient cells (Engel *et al.*, 1999; Hocevar *et al.*, 1999) as well as on TβRI receptors with an altered L45 loop (Itoh *et al.*, 2003; Yu *et al.*, 2002) have demonstrated that TGF-β activates JNK and p38 signaling in a Smad-independent manner. Previous studies have shown that JNK is activated by MKK4 and p38 is activated by MKK3/6 in a variety of cell lines (Bhowmick *et al.*, 2001b; Hanafusa *et al.*, 1999; Sano *et al.*, 1999) and that MKK4 and MKK3/6 are activated upstream by TGF-β activated kinase 1 (TAK1) (Yamaguchi *et al.*, 1995). Additional studies have also demonstrated that TAK1 is required for TGF-β-induced activation of JNK and p38 signaling (Shim *et al.*, 2005) and that TAK1 directly interacts with TβRII (Watkins *et al.*, 2006). What remained unresolved was how TGF-β activated TAK1. Recent studies on TRAF6, which is vital in the activation of TAK1 in interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR) mediated pathways, have shown it be also vital for TGF-β-induced activation of the TAK1-JNK/p38 pathways (Sorrentino *et al.*, 2008; Yamashita *et al.*, 2008).

TRAF6 contains a highly conserved C-terminal TRAF domain and less conversed N-terminal E3 ligase RING finger domain (Haglund and Dikic, 2005). TRAF6 associates with IL-1R and TLR complexes through its C-terminal TRAF domain, which leads to activation of the RING finger E3 ligase and Lysine-63 linked polyubiquitylation of TRAF6. Lysine-63-linked polyubiquitin chains function as scaffolds to assemble protein complexes and facilitate their activation. Predictably, TRAF6 also associates with the TGF-β type I and type II receptors through the C-terminal TRAF domain (Yamashita *et al.*, 2008). The association of TRAF6 with the TGF-β receptor complex induces K63-linked polyubiquitylation of TRAF6, and promotes the association between TRAF6 and TAK1 and the subsequent polyubiquitylation and activation of TAK1. Interestingly, MAP3Ks ML3 and MEKK1 have also been implicated in TGF-β-mediated activation of the JNK and p38 pathways via MKK4 and MKK3/6, although the mechanism of activation remains unclear (Kim *et al.*, 2004; Zhang *et al.*, 2003).

Although TGF-β induces JNK/p38 activation independently of Smad activation, the TRAF6-TAK1-JNK/p38 signaling cascade works in union with the canonical TGF-β Smad-signaling pathway to regulate downstream cellular responses to TGF-β. Overexpression studies on TAK1 and knockdown studies on TRAF6 both showed that the TRAF6-TAK1-JNK/p38 pathway plays a cooperative role with the canonical TGF-β Smad signaling pathway in TGF-β-induced apoptosis (Liao *et al.*, 2001). Additionally, inhibition experiments on JNK (Yamashita *et al.*, 2008) and

p38 (Bakin *et al.*, 2002) revealed that TRAF6-TAK1-JNK/p38 pathway activation is necessary for TGF-β-induced EMT.

1.5.2 TGF-β-mediated regulation of Rho-like GTPases

Rho-like GTPases, including RhoA and Cdc42 play important roles in regulating dynamic cytoskeletal organization, cell motility, and gene expression through a variety of effectors (Jaffe and Hall, 2005). Studies have shown that Rho-like GTPases are able to regulate TGF-β-induced EMT and cell cycle arrest in a cell-dependent manner.

Previous studies have demonstrated that TGF-β rapidly activates RhoA-dependent pathways to induce stress fiber formation and mesenchymal characteristics in epithelial cells and primary keratinocytes, providing the framework for TGF-β-induced EMT progression (Bhowmick *et al.*, 2001a; Edlund *et al.*, 2002). The rapid activation of RhoA induced by TGF-β is likely to be independent of Smad2/3, as suggested by the inability of dominant-negative Smad3 mutants to block RhoA activities in epithelial cells. Interestingly, TGF-β is able to also downregulate RhoA-dependent activities at tight junctions (Ozdamar *et al.*, 2005). Par6, a protein that regulates cell polarity, interacts constitutively with TβRI at tight junctions of epithelial cells (Gao *et al.*, 2002). Following TGF-β stimulation, TβRII associates with TβRI-Par6 and phosphorylates Par6 at Serine-345, leading to the recruitment of Smurf1 at tight junctions. The Par6-Smurf1 complex then mediates localized RhoA ubiquitylation by Smurf1 at Lysine-6 and Lysine-7, as well as turnover of

RhoA at cellular protrusions, which enables TGF-β-dependent dissolution of tight junctions (Ozdamar *et al.*, 2005). Thus, through regulating RhoA degradation, TGF-β promotes dissolution of tight junctions and rearrangement of the actin cytoskeleton. It is possible that TGF-β regulates RhoA activity through two different cellular mechanisms: 1) TGF-β induces a rapid activation of RhoA during the early phase of stimulation to induce stress fiber formation and mesenchymal characteristics in epithelial cells, and 2) TGF-β then downregulates the level of RhoA protein at later stages to facilitate the dissolution of tight junctions and rearrangement of the actin cytoskeleton; both of these mechanisms appear to be essential for TGF-β-induced EMT (Zhang, 2009).

Additionally, Cdc42 GTPase can promote the reorganization of actin filaments and membrane ruffling in a Smad2/3-independent manner (Wilkes *et al.*, 2003). This process involves Smad7, which acts as a scaffold protein for Cdc42 (Edlund *et al.*, 2004). In fibroblasts, Cdc42, Rac1 and/or PI3K can activate p21-Activated kinase 2 (PAK2) in response to TGF-β stimulation, resulting in activation of mitogenic responses which promotes the dissolution of tight junctions and EMT. In contrast, in most epithelial cells, PAK2 is not activated. This difference could explain why in fibroblasts, TGF-β promotes cell proliferation whereas in most epithelial cells, TGF-β inhibits cell growth (Wilkes *et al.*, 2009).

1.5.3 TGF-β-induced PI3K/AKT signaling pathways

The PI3K/AKT pathway is another non-canonical TGF-β signaling pathway that plays a role in TGF-β-mediated EMT and transcription regulation (Bakin *et al.*, 2000). PI3K interacts with the TGF-β receptors indirectly through its p85 regulatory subunit, and upon ligand stimulation, TβRI activates PI3K, independently of Smad2/3 (Yi *et al.*, 2005).

The PI3K/AKT pathway plays an important role in TGF-β-mediated EMT partly because of its downstream effector, mammalian target of Rapamycin (mTOR), a promoter of protein synthesis. TGF-β-induced activation of mTOR has been observed in several different cell types, including murine NMUMG mammary gland epithelial cells and human HaCAT keratinocytes, both of which undergo EMT in response to TGF-β (Lamouille and Derynck, 2007). Treating these cells with rapamycin, an inhibitor of mTOR, inhibits the enhanced migration and invasive behavior associated with TGF-β-induced EMT (Lamouille and Derynck, 2007). Thus, the TGF-β-induced PI3K/AKT/mTOR pathway may complement the transcription pathway mediated by Smads in TGF-β-induced EMT.

In addition to regulating EMT progression, PI3K plays a role in TGF-β-mediated fibroblast proliferation and morphological transformation (Wilkes *et al.*, 2005). Tyrosine kinase c-Abl acts downstream of PI3K and is partly responsible for the TGF-β-mediated fibroblast response. Inhibition of the c-Abl kinase prevents TGF-β-mediated morphological alterations, expression of ECM genes, and cell proliferation in fibroblasts (Daniels *et al.*, 2004; Wang *et al.*, 2005).

Interestingly, the PI3K/ AKT pathway can also antagonize TGF-β-mediated effects. Studies have shown that activation of PI3K or Akt can protect cells from TGF-β-induced apoptosis and growth inhibition (Chen *et al.*, 1998b; Shin *et al.*, 2001; Song *et al.*, 2006). AKT, which relays signals downstream of PI3K, can directly associate with Smad3. The interaction between Smad3 and AKT is thought to prevent TβRI-mediated phosphorylation and nuclear localization of Smad3, thereby resulting in inhibition of Smad3-mediated transcription (Conery *et al.*, 2004; Remy *et al.*, 2004). Besides Akt, the forkhead transcription factor, FoxO, also plays a role in the antagonizing effect of PI3K/Akt on Smad-mediated transcription (Seoane *et al.*, 2004). Since FoxO proteins are targets of the PI3K/AKT pathway, AKT can inhibit nuclear localization of FoxO proteins by phosphorylating the FoxO proteins and consequently barring them from their target genes (Gomis *et al.*, 2006).

1.6 Posttranslational regulation of TGF-beta signaling

As we elucidate the complexity and contextuality of the TGF-β responses, we have come to understand the important role of posttranslational regulation in providing specificity and versatility in TGF-β signaling (Feng and Derynck, 2005). Posttranslational regulation of the TGF-β receptor and Smad complexes signaling define receptor and Smad stability, provide negative feedback mechanisms, and help define their functions, providing insight into the exquisite regulation of the complex responses (Xu *et al.*, 2012).

1.6.1 Phosphorylation and dephosphorylation

1.6.1.1 TGF-β receptor phosphorylation and dephosphorylation

Receptor phosphorylation provides the basis for signal transduction in response to TGF-β activation. TGF-β ligand binding stimulates TβRII-TβRI interactions, enabling TβRII to phosphorylate the GS domain of TβRI on serine and threonine residues (Huse *et al.*, 2001). TGF-β binding to the TβRII-TβRI receptor complex also induces TβRI phosphorylation outside its GS domain, likely by TβRII and through autophosphorylation upon TβRI activation. The phosphorylation status of TβRII also remains largely uncharacterized, and TβRII phosphorylation may similarly combine autophosphorylation and transphosphorylation by TβRI (Kang *et al.*, 2009).

As mentioned in Section 1.5.1, the TβRI and TβRII receptors are phosphorylated on tyrosine residues as TβRI and TβRII are dual specificity kinases, characterized by strong Serine and Threonine but weaker Tyrosine phosphorylation capacities (Hanks and Hunter, 1995). TβRII phosphorylation on Tyrosine residues may result from autophosphorylation (Lawler *et al.*, 1997), although TβRII can also be transphosphorylated by the Tyrosine kinase Src (Galliher and Schiemann, 2007). TGF-β can also induce Tyrosine phosphorylation of TβRI, resulting from autophosphorylation or transphosphorylation by TβRII (Lee *et al.*, 2007b).

The activity of TGF-β receptors can also be regulated by dephosphorylation (Wrighton *et al.*, 2009). Smad7 was shown to interact with GADD34, a regulatory

subunit of the protein phosphatase 1 (PP1) holoenzyme, and thus recruit the catalytic subunit of PP1 (PP1c) to TβRI, resulting in TβRI dephosphorylation (Shi *et al.*, 2004), which inhibits TGF-β-induced cell cycle arrest, making it an effective mechanism for governing negative feedback in TGF-β signaling. Additionally, regulatory subunits of protein phosphatase 2A (PP2A) also associate with TGF-β receptors and regulate their activity. Specifically, the Bα subunit of PP2A enhances the growth inhibitory effect of TGF-β, while the Bδ is thought to negatively modulate the growth inhibitory effect by restricting the receptor activity (Batut *et al.*, 2008).

1.6.1.2 Smad phosphorylation and dephosphorylation

Smad activation typically results from TGF-β-induced phosphorylation of the C-terminal serine residues of the R-Smad SSXS motif by TβRI. Interestingly, the R-Smad linker region can also be phosphorylated on serine or threonine residues by kinases including ERK MAPK and CDK kinases (Derynck and Zhang, 2003). TGF-β induces phosphorylation at three linker region sites in R-Smads. Smad linker phosphorylation is either mediated by GSK3β, a downstream effector of Wnt signaling, or by CDK8/9 (Alarcon *et al.*, 2009; Millet *et al.*, 2009; Wang *et al.*, 2009), which tags activated R-Smads for proteasomal destruction. The E3 ubiquitin ligase Nedd4L then recognizes the phospho-Threonine/Proline/Tyrosine motif in the linker region, leading to R-Smad polyubiquitylation and degradation (Gao *et al.*, 2009). CDK8/9 can also phosphorylate the linker region of Smad1, resulting in recruitment of YAP, an effector of the Hippo pathway of cell size control. This

interaction initially enhances Smad-mediated transcription, but the phosphorylated linker is eventually recognized by E3 ubiquitin ligase Smurf1, leading to Smad degradation (Alarcon *et al.*, 2009). Recently, studies have demonstrated that the functional switch from initial Smad activation to Smad degradation results from a switch in recognition of Smad phospho-Serines by WW domains of transcription factors and E3 ubiquitin ligases (Aragon *et al.*, 2011).

GRK2, a kinase involved in desensitizing G protein-coupled receptors (GPCR), also inhibit TGF-β responses (Ho *et al.*, 2005). Phosphorylation of R-Smad linker regions by GRK2 inhibits TGF-β-induced Smad phosphorylation, nuclear translocation, and target gene expression, providing a negative feedback to control TGF-β responses. Non-activated Smad3 can also be phosphorylated in its MH1 domain by GSK3-β in association with the scaffolding protein Axin which results in Smad3 ubiquitylation and degradation (Guo *et al.*, 2008).

Studies have shown that C-terminal SSXS motif of R-Smads can be targeted by kinases that are distinct from the type I TGF-β family of receptors. Mps1, a kinase that regulates mitotic progression, can phosphorylate R-Smads (Zhu *et al.*, 2007). Additionally, the WNK family kinase WNK1 (Lee *et al.*, 2007a) and MPK38 kinase (Seong *et al.*, 2010) can also phosphorylate R-Smads and promote TGF-β signaling.

Dephosphorylation of the C-terminal Serines regulates the termination of Smad signaling. Among many phosphatases evaluated, PPM1A/PP2Ca acts as Smad

phosphatase for Smad2 and Smad3. PPM1A removes the receptor-mediated phosphorylation at the SSXS motif, and promotes nuclear export of TGF-β-activated Smad2/3, thereby terminating TGF-β signaling (Lin *et al.*, 2006). The phosphatase PTEN, a negative regulator of the PI3K/AKT pathway, interestingly serves as co-factor of PPM1A, and helps terminate Smad2/3 phosphorylation by stabilizing PPM1A (Bu *et al.*, 2008). Moreover, the phosphatase MTMR4 was also found to C-terminally dephosphorylate the activated Smad2 and Smad3 in endosomes (Yu *et al.*, 2010).

SCP phosphatases can dephosphorylate Smad1 and thus attenuate BMP signaling (Knockaert *et al.*, 2006). Under hypoxic conditions, the phosphatase PP2A was shown to C-terminally dephosphorylate Smad3, but not Smad2, suggesting that hypoxia plays a role in regulating Smad signaling (Heikkinen *et al.*, 2010). Interestingly, the phosphatases SCP1, SCP2 and SCP3 can promote TGF-β signaling by removing linker phosphorylation at certain sites, without affecting C-terminal phosphorylation (Wrighton *et al.*, 2006).

1.6.2 Ubiquitylation and deubiquitylation

1.6.2.1 TGF-β receptor ubiquitylation and deubiquitylation

Ubiquitylation and sumoylation result from sequential actions of E1 ubiquitin-activating, E2-ubiquitin-conjugating and E3 ubiquitin-ligating enzymes. The factor that modulates protein ubiquitylation or sumoylation is the E3 ligase, which binds to the substrate protein and provides specificity to protein modifications (Lin 2008

book). Many E3 ubiquitin ligases are involved in TβRI ubiquitylation, including the HECT-type WW domain-containing E3 ligases Smurf1, Smurf2, WWP1, and NEDD4–2, which associate with E2-conjugating enzyme UbcH7 and with Smad7, leading to proteasomal degradation (Kavsak *et al.*, 2000; Komuro *et al.*, 2004; Kuratomi *et al.*, 2005; Ogunjimi *et al.*, 2005).

Deubiquitylating hydrolase USP15, on the other hand, was shown to associate with the Smad7–Smurf2 complex and to deubiquitylate TβRI, leading to enhanced TGF-β receptor stability and enhanced signaling in glioblastoma, breast and ovarian cancer (Eichhorn *et al.*, 2012). Additionally, deubiquitylating enzyme UCH37 forms a complex with Smad7 and deubiquitylates TβRI (Wicks *et al.*, 2005). Finally, the 90-kD heat shock protein HSP90 is also able to associate with TβRI and TβRII, and prevent Smurf2-mediated receptor ubiquitylation and degradation (Wrighton *et al.*, 2008).

1.6.2.2 Smad ubiquitylation and deubiquitylation

Various ubiquitin ligases have been implicated in R-Smad degradation, including Smurf1, Smurf2, Nedd4–2, WWP1, ROC1-SCF, and CHIP (Inoue and Imamura, 2008). For instance, TGF-β-induced Smad2 activation is followed by polyubiquitylation and proteasomal degradation in the nucleus. By targeting nuclear activated Smad2 for destruction, TGF-β ensures the irreversible termination of its own signaling function (Lo and Massague, 1999). Additionally, TGF-β induced Smad linker phosphorylation marks activated Smads for proteasomal

destruction by E3 ubiquitin ligase Nedd4L, which limits the half-life of TGF-β-activated Smads and restricts the intensity and duration of TGF-β signaling (Gao *et al.*, 2009). Furthermore, E3 ubiquitin ligase Arkadia, known to ubiquitylate Smad7 and SnoN (Levy *et al.*, 2007), can also ubiquitylate activated R-Smads, leading to their degradation (Mavrakis *et al.*, 2007). Finally, Smads were shown to associate with the estrogen receptor, facilitating estrogen dependent recruitment of Smurf1 for subsequent R-Smad receptor degradation (Ito *et al.*, 2010).

In contrast to polyubiquitylation, which leads to degradation, monoubiquitylation can regulate signaling through other disparate mechanisms (Moren *et al.*, 2005). For instance, the HECT-domain E3 ligase Itch/AIP4 can monoubiquitylate Smad2, which enhances the Smad2 interaction with TβRI, and consequently promotes Smad2 activation (Bai *et al.*, 2004). Smad3 can also be monoubiquitylated in its MH1 domain at multiple Lysines, which interferes with Smad3 binding to regulatory promoter sequences (Inui *et al.*, 2011). Interestingly, the deubiquitylating enzyme USP15 can oppose monoubiquitylation of the Smad3 MH1 domain, and rescue Smad3 signaling (Inui *et al.*, 2011), demonstrating a dynamic regulatory balance between monoubiquitylation and deubiquitylation in R-Smad signaling regulation. Similarly, abrogating USP15 expression eliminates the recruitment of TGF-β-activated Smad complexes to regulatory DNA transcription factors. Furthermore, Smad3 can be monoubiquitylated at four Lysine residues in the MH2 domain through the E3 ligase Smurf2 (Tang *et al.*, 2011). Smurf2-mediated monoubiquitylation interferes with the formation of functional

Smad3 complexes. This monoubiquitylation requires prior TGF-β-induced phosphorylation of Threonine-179 and adjacent Proline/Threonine motif in the linker region by CDKs. Finally, the deubiquitylase CYLD negatively regulates TGF-β signaling by AKT deubiquitylation, which releases AKT-mediated inhibition of GSK3β-CHIP-induced degradation of Smad3 (Lim *et al.*, 2012).

1.6.3 Sumoylation

1.6.3.1 TGF-β receptor sumoylation

Sumoylation covalently attaches a SUMO (Small Ubiquitin-like Modifier) polypeptide that resembles ubiquitin to its substrate. SUMOs use a conjugation system similar to that of ubiquitylation, with Aos1/Uba2 as the E1-activating enzyme and Ubc9 as the E2-conjugating enzyme (Hay, 2005; Johnson, 2004). Three different classes of proteins have identified as SUMO E3 ligase including nucleoporins RanBP2, polycomb protein Pc2, and PIAS proteins (Jackson, 2001; Kagey *et al.*, 2003; Pichler *et al.*, 2002). Sumoylation functions vary from protein degradation protection (Desterro *et al.*, 1998) to protein subcellular localization regulation (Zhong *et al.*, 2000) to biological activity modulation (Muller *et al.*, 2004). The TβRI receptor is sumoylated at one defined Lysine residue in response to TGF-β. TβRII sumoylation requires the kinase activities of TβRI and TβRII, indicating that phosphorylation and possibly a conformational change of TβRI are required for docking of the E3 ligase. Sumoylation of TβRI stabilizes the Smad2/3 binding to the TβRI receptor, leading to enhanced Smad activation. Accordingly,

lack of TβRI sumoylation decreases TGF-β-induced Smad2 and Smad3 activation, and TGF-β-induced transcription of target genes (Kang *et al.*, 2008).

1.6.3.2 Smad sumoylation

Smad4 can be sumoylated on Lysine-113 and Lysine-159 in its MH1 domain by the E3 ligase PIAS1 (Lee *et al.*, 2003; Lin *et al.*, 2003b; Long *et al.*, 2004). The two sumoylation sites of Smad4 are absent from other members of the Smad family, suggesting that sumoylation is specific for Smad4 (Lin *et al.*, 2008). The effects of sumoylation on Smad4 are multidimensional. Studies have shown that Smad4 sumoylation can prevent Smad4 ubiquitylation and proteasomal degradation (Lee *et al.*, 2003; Lin *et al.*, 2003a; Ohshima and Shimotohno, 2003). However, Smad4 sumoylation can also repress Smad-mediated transcription by recruiting Daxx to the sumoylated form of Smad4 on Lysine-159 by the recruitment of either histone deacetylases or silencing factors (Chang *et al.*, 2005).

Biochemical studies have suggested that Smad4 sumoylation requires the presence of a PIAS protein as an E3 SUMO ligase (Lee *et al.*, 2003; Liang *et al.*, 2004). The mammalian PIAS protein family consists of PIAS1, PIAS3, PIASxα, PIASβ, and PIASγ protein members. Structurally, PIAS protein contains a conserved SP-RING domain that binds to E2-conjugating enzyme Ubc9 (Kotaja *et al.*, 2002) and is vital for Smad4 sumoylation. PIAS1 and PIASxβ can sumoylate Smad4 at Lysine-159 and activate Smad-dependent transcription (Ohshima and Shimotohno, 2003). Furthermore, PIAS3 and PIASγ can bind the MH2 domain of Smad3 and regulate

transcriptional activity (Imoto *et al.*, 2003; Long *et al.*, 2003; Long *et al.*, 2004). PIAS3 can promote transcriptional activity through its interaction with TGF-β coactivators P300/CBP, while PIASγ can inhibit transcriptional activity through its interaction HDAC1 (Long *et al.*, 2004). Interestingly, sumoylation is absent in many cancer-derived mutants of Smad4, particularly those with missense mutations in the MH1 domain (Lin *et al.*, 2003a). These observations support the hypothesis that cancer-associated mutations in the MH2 domain can modify sumoylated Smad4 into ubiquitylated Smad4.

1.6.4 Acetylation and deacetylation

As mentioned earlier in section 1.4.5, acetylation can mediate Smad functions by relaxing the DNA chromatin structure through their histone acetyltransferase (HAT) activity to promote transcription (Feng *et al.*, 1998; Simonsson *et al.*, 2006) or by tightening the chromatin structure though their histone deacetylase (HDAC) activity to inhibit transcription (Alliston *et al.*, 2005; Feng *et al.*, 2002; Wu *et al.*, 2002).

Specifically, R-Smads can be acetylated by the coactivators CBP and p300 in response to TGF-β stimulation, resulting in enhanced Smad-mediated transcription (Inoue *et al.*, 2007; Tu and Luo, 2007). Conversely, R-Smads have shown to recruit HDACs, leading to histone deacetylation and transcription repression (Xu *et al.*, 2012). Additionally, the interaction of Smad7 with the transcription coactivator p300 leads to direct Smad7 acetylation. As the Smad7 acetylated residues are also

targeted by ubiquitylation, acetylation can therefore prevent ubiquitylation and subsequent degradation of Smad7 (Gronroos *et al.*, 2002). Conversely, HDACs are able to interact with Smad7 and reverse acetylation, thus enhancing ubiquitylation and proteasomal degradation of Smad7 (Simonsson *et al.*, 2005).

1.6.5 Ectodomain shedding

Ectodomain shedding is a highly regulated process by which the extracellular domain of membrane-anchored proteins is released from the cell surface as soluble proteins (Hayashida *et al.*, 2010). TACE, a transmembrane ADAM metalloproteinase also known as ADAM17, proteolytically cleaves the ectodomain of TβRI (Liu *et al.*, 2009a) by TRAF6-mediated TβRI ubiquitylation (Mu *et al.*, 2011). TACE-mediated ectodomain shedding is activated in response to ERK or p38 MAPK pathway signaling (Xu and Derynck, 2010). Activation of either MAPK pathways decreases the cell surface levels of TβRI, but not of TβRII. Thus, MAPK activation attenuates TGF-β-induced Smad3 activation without affecting TGF-β ligand binding to TβRII.

Conversely, inhibition of TACE increases the cell surface TβRI level, and consequently, Smad3 activation and TGF-β-induced EMT (Liu *et al.*, 2009a). Downregulation of TGF-β signaling through TACE provides an additional mechanism for autocrine tumor suppression by TGF-β, and for modulation of EMT in cancer progression. Interestingly, after proteolytic cleavage of the TβRI ectodomain, the TβRI cytoplasmic domain can translocates into the nucleus, and

then acts as a transcription factor in potentiating the expression of Snail and MMP2 (Mu *et al.*, 2011).

1.6.6 ADP-ribosylation

ADP-ribosylation is another posttranslational modification that can regulate TGF-β signaling (Corda and Di Girolamo, 2003). ADP-ribosylation mechanism consists of the addition of one or more ADP-ribose moieties to a protein. Smad3 can also be ADP-ribosylated in its MH1 domain through the action of poly (ADP-ribose) polymerase-1 (PARP-1). ADP-ribosylation of Smad3 in the nucleus results in the dissociation of Smad complexes from DNA, and, thus, inhibition of Smad-mediated TGF-β responses (Lonn *et al.*, 2010). Similarly, ADP-ribosylation of the DNA binding MH1 domain of Smad4 by PARP-1 interferes with the DNA binding of Smad4 in the nucleus, resulting in attenuated Smad-mediated transcription (Lonn *et al.*, 2010). ADP-ribosylation of Smad proteins may function as another important step in controlling the magnitude and duration of Smad-mediated transcription.

1.7 MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules (containing about 22 nucleotides) that specifically bind target mRNAs and inhibit mRNA translation or promote mRNA degradation (Ambros, 2004; Bartel, 2004). Signaling pathways are ideal candidates for miRNA-mediated regulation owing to the dose-sensitive nature of their effects. Indeed, emerging evidence suggests that miRNAs affect the responsiveness of cells to signaling molecules including TGF-β (Inui *et al.*, 2010).

Several miRNAs target mRNAs encoding TβRII, or the type II Nodal receptor, including the miR-302/367 cluster, miR-372, miR-520/373, miR-17–92 cluster, miR-15, and miR-16 (Keklikoglou *et al.*, 2012; Lipchina *et al.*, 2011; Martello *et al.*, 2007; Mestdagh *et al.*, 2010; Subramanyam *et al.*, 2011). As these miRNAs are able to inhibit TGF-β receptor expression, (Liu *et al.*, 2009a; Wu and Derynck, 2009), these miRNAs are also able to control the threshold for signaling activation, due to the fact that receptor levels correlate with TGF-β transcriptional activity. Interestingly, SMADs can also be targeted by miRNAs. The miR-23b cluster has been shown to target SMAD3, SMAD4 and SMAD5, subsequently inhibiting the anti-proliferative response mediated by TGF-β (Rogler *et al.*, 2009). Interestingly, Smads can also promote a rapid increase in the expression of mature miR-21 by associating with the Drosha complex. As a consequence, miR-21 mediates the TGF-β-induced differentiation of vascular smooth muscle cells into contractile cells (Davis *et al.*, 2008). How Smads can control the biogenesis of miRNAs remains to be elucidated.

1.8 TGF-beta co-receptors

TGF-β co-receptors provide another mechanism to regulate TGF-β signaling. Co-receptors are cell-surface proteins that bind the TGF-β ligand and allosterically regulate the signaling of TβRI and TβRII receptors, without necessarily binding to the type I or type II receptors.

1.8.1 Betaglycan

Betaglycan, also known as the TGF-β type III receptor, was originally identified as a non-signaling co-receptor for TGF-β, and its main function was to present the TGF-β ligand to its receptors (Lopez-Casillas *et al.*, 1991). Since then, many ligand-dependent and ligand-independent functions have also been identified for betaglycan. Specifically, betaglycan is an important regulator of reproduction (Glister *et al.*, 2010) and fetal development (Walker *et al.*, 2011) and is also a tumor suppressor in many cell types (Mythreye and Blobe, 2009). The current understanding indicates that betaglycan plays complex roles *in vivo*, affecting the activities and interactions of a number of TGF-β superfamily members and regulating diverse cellular processes.

Betaglycan is an 851 amino acid proteoglycan that associates with the TGF-β receptor complex (Lopez-Casillas *et al.*, 1994). Betaglycan exists on the cell surface as noncovalently linked homodimers (Mendoza *et al.*, 2009) and is comprised of a large extracellular domain modified by heparan and chondroitin sulfate glycosaminoglycan (GAG) side chains, a single-pass transmembrane region, and a short 42 amino acid cytoplasmic domain containing a PDZ motif (Bernabeu *et al.*, 2009). Structure-function studies have identified two non-overlapping ligand binding regions within the extracellular domain, an N-terminal binding region and a membrane-proximal binding region, joined together by 50 amino acid linker region (Mendoza *et al.*, 2009). The two TGF-β binding domains function independently to present ligand to TβRII (Esparza-Lopez *et al.*, 2001). Betaglycan

binds TGF-β1, -β2 and -β3 isoforms but has a higher specific affinity for TGF-β2. Betaglycan facilitates TGF-β binding to TβRII by presenting the ligand to the receptors. This ligand presentation by betaglycan is crucially important for TGF-β2 activation, as TβRII cannot bind TGF-β2 on its own. Thus, betaglycan plays a vital role in facilitating TGF-β2 isoform signaling (Lopez-Casillas *et al.*, 1993).

Interestingly, betaglycan can either promote or inhibit TGF-β signaling, depending on the cellular context. The dual role of betaglycan on TGF-β signaling can be partly explained by the regulation of ectodomain shedding and by the collective impact of the various membrane-bound and soluble forms of betaglycan.

Following proteolytic cleavage, betaglycan ectodomain is released from the cell surface (Velasco-Loyden *et al.*, 2004) where it can bind and sequester the TGF-β ligand away from the TGF-β receptors, thus inhibiting TGF-β signaling (Fukushima *et al.*, 1993). Additionally, the presence of large glycosaminoglycan (GAG) chains on betaglycan ectodomain can also impair the association between TβRI and TβRII, thus inhibiting TGF-β signaling. Interestingly, the presence of shorter GAG chains results in increased TGF-β signaling by betaglycan in certain cell types (Eickelberg *et al.*, 2002). At large, the betaglycan ectodomain plays an important role in determining whether betaglycan acts as a promoter or an inhibitor of TGF-β signaling.

Betaglycan's cytoplasmic domain is not required for ligand presentation and does not have kinase activity. Betaglycan's cytoplasmic domain does however play a

significant role in betaglycan function (Esparza-Lopez *et al.*, 2001). Specifically, the last three amino acids in the cytoplasmic tail containing a PDZ binding motif have been identified as sites that allow for adaptor protein binding and can associate with GAIP-interacting protein, C-terminus (GIPC) (Blobe *et al.*, 2001) GIPC-betaglycan interactions can stabilize membrane-bound betaglycan and increase TGF-β responsiveness in Mv1Lu and L6 myoblast cells. Conversely, GIPC-betaglycan interaction can also inhibit TGF-β-mediated Smad signaling in breast cancer cells (Lee *et al.*, 2010). The mechanisms by which the GIPC-betaglycan interactions mediate betaglycan responses are not well understood, although recent findings suggest that additional adaptor proteins may play significant roles in mediating GIPC-betaglycan function.

The cytoplasmic tail has been shown to be phosphorylated by TβRII at Threonine-841 (Chen *et al.*, 2003), which results in the association with β-arrestin2, and subsequent co-internalization with TβRII via a clathrin-independent pathway, and inhibition of TGF-β signaling (Finger *et al.*, 2008). As arrestins are known to scaffold receptors to particular signaling pathways (Kovacs *et al.*, 2009), the association of betaglycan with β-arrestin2 have been involved in regulating many signaling pathways including activation of Cdc42 as well as downregulation of NF-κB signaling pathways (Criswell and Arteaga, 2007; Mythreye and Blobe, 2009; You *et al.*, 2009). Furthermore, betaglycan is able to activate p38 MAPK signaling, via its association with TβRII, in myofibroblasts (Santander and Brandan, 2006).

Recent studies have identified betaglycan as a modulator of TGF-β superfamily ligands through its role in regulating receptor trafficking and turnover (Bilandzic and Stenvers, 2011). Similar to the TGF-β signaling receptors, betaglycan has been shown to be internalized via clathrin-dependent and clathrin-independent pathways (Finger *et al.*, 2008). Betaglycan can bind TGF-β superfamily receptors BMP-2, BMP-4, BMP-7, and GDF-5 ligands and enhance the binding of these ligands to ALK3 and ALK6 and promote BMP signaling (Kirkbride *et al.*, 2008). Intriguingly, betaglycan can differentially dictate the trafficking of the signaling receptors for its various ligands by either enhancing the internalization of ALK6 and Smad1 activation via its interaction with β-arrestin2 and by maintaining ALK3 at the cell surface, independently of β-arrestin2 (Lee *et al.*, 2009)(Lee *et al.*, 2009).

Finally, betaglycan can also associate with inhibin as an inhibin co-receptor and enhance inhibin affinity for ActRII, which results in the increased ability of inhibins to displace activin form ActRII (Wiater *et al.*, 2009). Interestingly, inhibin A-mediated betaglycan internalization resulted in the downregulation of cell surface betaglycan but not in total betaglycan levels, suggesting that betaglycan was recycled rather than degraded following inhibin binding (Looyenga *et al.*, 2010) These findings are significant because they provide a novel mechanism by which TGF-β function can be suppressed.

1.8.2 Endoglin

Endoglin, also known as CD105, is a 180 kDa glycoprotein that acts as an accessory co-receptor for TGF-β superfamily members (Guerrero-Esteo *et al.*, 2002). Endoglin is structurally related to betaglycan, with a short cytoplasmic domain containing a PDZ-binding motif, a single transmembrane domain, and a large extracellular domain (Cheifetz *et al.*, 1992). Unlike betaglycan, endoglin exists exclusively as disulfide-linked homodimers, it does not have GAG chains attached to its ectodomain, and it can associate with the receptors without the presence of ligand (Barbara *et al.*, 1999).

Endoglin is expressed primarily in endothelial cells (Cheifetz *et al.*, 1992), but is also expressed in hematopoietic cells (Rokhlin *et al.*, 1995), immune cells (Weber *et al.*, 2005), chondrocytes (Parker *et al.*, 2003), and keratinocytes (Quintanilla *et al.*, 2003). While endoglin is unable to directly bind the TGF-β ligand, it can however bind TGF-β1 and TGF-β3 ligands in the presence of TβRII (Letamendia *et al.*, 1998). Endoglin can also bind activin-A, BMP-2 and BMP-7, in the presence of their analogous receptors (Barbara *et al.*, 1999). Interestingly, endoglin can also associate with betaglycan to form a complex, suggesting a role in regulating TGF-β signaling (Wong *et al.*, 2000).

The function of endoglin in TGF-β family signaling has yet to be completely elucidated although endoglin is generally considered to be an inhibitor of TGF-β signaling (Li *et al.*, 2000). Inhibition of TGF-β signaling by endoglin is assumed to be mediated by its ability to regulate the balance between the TGF-β ALK1/ALK5

signaling pathways. In chondrocytes and endothelial cells, endoglin interacts with ALK1 to promote ALK1/Smad1/5/8 signaling and to inhibit ALK5/Smad2/3 signaling (Blanco *et al.*, 2005; Finnson *et al.*, 2010; Lebrin *et al.*, 2004). Interactions with TβRII, ALK1 and ALK5 result in the phosphorylation of the endoglin cytoplasmic domain, which in turn can mediate phosphorylation of type I and type II TGF-β receptors, and regulate TGF-β responses as either a promoter or inhibitor of TGF-β signaling depending on the cellular context (Koleva *et al.*, 2006; Ray *et al.*, 2010).

Endoglin can also interact with β-arrestin2, in a similar fashion to betaglycan, which leads to endoglin internalization and inhibition of TGF-β-induced MAPK ERK signaling (Lee and Blobe, 2007). Interestingly, endoglin has been found in cholesterol-rich caveolae membrane domains, where it forms a complex with endothelial NOS (eNOS) (Toporsian *et al.*, 2005). As caveolaer localization has been shown to enhance TGF-β/ALK1 signaling (Santibanez *et al.*, 2008), endoglin may modulate ALK1 localization in the caveolae. Furthermore, like betaglycan, endoglin ectodomain can be cleaved and the cleaved soluble endoglin is used a marker for preeclampsia (Venkatesha *et al.*, 2006). These findings suggest that endoglin plays key roles in controlling vascular homeostasis, especially as endoglin haploinsufficiency is known to cause hereditary hemorrhagic telangiectasia (HHT) (McAllister *et al.*, 1994).

1.8.3 Cripto

Cripto is a GPI-anchored protein that is part of the epidermal growth factor-Cripto, FRL-1, Criptic (EGF-CFC) family, which can act as a membrane-tethered co-receptor or a secreted co-ligand protein for nodal, activin, and TGF-β signaling (Wrana *et al.*, 2008). Cripto potentiates nodal signaling, by binding directly to nodal and then facilitating its interaction with ALK4, and ALK7 (Yeo and Whitman, 2001). Cripto brings the precursor of nodal to the lipid-raft compartment and recruit convertases, such as Furin and PACE4 to promote nodal activation (Blanchet *et al.*, 2008b). Cripto can also bring nodal to the limiting membrane of the endosome, in close proximity with Smad2, Smad3 and SARA, to encourage signaling (Blanchet *et al.*, 2008a). Cripto can also inhibit activin signaling, by forming a complex with activin and ActRII, which consequently prevents ALK4 from associating with the activin/ActRII receptor complex (Gray *et al.*, 2003). Additionally, Cripto can also inhibit TGF-β signaling by competing with TβRI for binding to TGF-β receptor complex (Gray *et al.*, 2006) leading to the inhibition of TGF-β tumor suppressive function during skin carcinogenesis (Shukla *et al.*, 2008).

1.8.4 RGM

Repulsive guidance molecule (RGM) is a family of GPI-anchored proteins that is composed of RGMa, RGMb, and RGMc isoforms. RGMb, also known as DRAGON, functions as co-receptor for BMP and enhance BMP signaling (Corradini *et al.*, 2009). RGMb can bind directly to BMP-2 and BMP-4 ligands, to ActRII and BMPRII type II receptors, as well as to ALK3 and ALK6 type I receptors (Samad *et al.*, 2005). RGMb is thought to allow BMP-2 and BMP-4

ligands, which typically signal through BMPRII, to also signal through ActRIIA and thus, enhance BMP signaling. Conversely, the soluble form of RGMb sequesters BMP and inhibits BMP signaling (Babitt *et al.*, 2006).

1.8.5 BAMBI

BMP and activin membrane-bound inhibitor (BAMBI) is a transmembrane glycoprotein that is structurally related to TβRI but lacks the cytoplasmic kinase domain (Villar *et al.*, 2013). BAMBI functions as a pseudo type I receptor that prevents the formation of active receptor complexes upon ligand binding, and thus inhibiting TGF-β signaling (Onichtchouk *et al.*, 1999). Recent studies have shown that BAMBI can also inhibit TGF-β through its association with Smad7 and ALK5, which consequently inhibits the interaction between ALK5 and Smad3, thus impairing Smad3 activation (Yan *et al.*, 2009a). Although the physiological functions regulated by BAMBI have yet to be elucidated, recent studies suggest that aberrant BAMBI expression plays an important role in the pathophysiology of inflammatory (Dromann *et al.*, 2010) and fibrotic processes (Seki *et al.*, 2007) and in cancer development (Fritzmann *et al.*, 2009).

1.9 TGF-beta in cancer and skin disease

1.9.1 Dual role of TGF-β as a tumor suppressor and promoter

TGF-β plays a crucial role in maintaining the balance between cell proliferation and apoptosis in a context-dependent manner. Perturbations to the expression of

proteins that mediate the balance between proliferative and apoptotic proteins can lead to cancer (Siegel and Massague, 2008).

1.9.1.1 TGF-β as a tumor suppressor

TGF-β is involved in growth inhibition in several cell types including, epithelial, endothelial, hematopoietic, and glial cells. In epithelial cells, TGF-β-induced growth inhibition is primarily determined by its activity to regulate the expression of CDK inhibitors $p15^{INK4b}$ and $p21^{Cip1}$ as well as the expression of growth-promoting transcription factors c-Myc, Id1, Id2, and Id3 (Siegel and Massague, 2003). In proliferating cells, c-Myc binds to $p15^{INK4b}$ and $p21^{Cip1}$, inhibiting transcription (Seoane *et al.*, 2001). Following TGF-β-activation, a complex consisting of Smad3/4, E2F4/5, and p107 represses *c-myc* transcription (Frederick *et al.*, 2004; Yagi *et al.*, 2002), which reduces the growth-promoting functions of c-Myc and reduces the inhibition of p15INK4b and p21Cip1 (Feng *et al.*, 2002; Moustakas and Kardassis, 1998). Upregulation of $p15^{INK4b}$ and $p21^{Cip1}$ leads to suppression of the cyclin-dependent kinases CDK2 and CDK4, accumulation of pRb and sequestration of E2F transcription factors, which consequently leads to G_1 to S-phase cell cycle arrest (Sardet *et al.*, 1995). Furthermore, TGF-β also antagonizes the expression of Id1, Id2, and Id3 proteins (Kang *et al.*, 2003a), which promote proliferation through the inhibition of basic helix-loop-helix transcription factors and interaction with pRb proteins (Ruzinova and Benezra, 2003).

TGF-β can also regulate growth control through inducing transcription factors that promote apoptosis. Although TGF-β can induce or suppress apoptosis, the common response is proapoptotic (Sanchez-Capelo, 2005). Caspases are generally involved in TGF-β stimulated apoptosis (Inman and Allday, 2000), although the mechanism is dependent on the cellular context and type. TGF-β has been recently shown to promote apoptosis by inducing specific proapoptotic transcription factors such as p53, in a p38 and Smad-dependent manner (Heldin *et al.*, 2009). Furthermore induction of TGF-β expression promotes apoptosis via Smad3-dependent transcription of the phosphatase MKP2, which enhances the proapoptotic effect of the Bcl-2 family member Bim (Ramesh *et al.*, 2008). As mentioned in section 1.5.3, the cross-talk between PI3K/AKT and TGF-β pathways is a key determinant in TGF-β-induced apoptosis. Activation of PI3K/AKT signaling can protect cells from TGF-β-induced apoptosis (Chen and Massague, 1999) whereas PI3K/AKT and its downstream protein mTOR have shown to suppress Smad3 phosphorylation and apoptosis (Song *et al.*, 2006). The contradictory apoptotic responses of TGF-β further supports the idea that TGF-β-induced apoptotic mechanisms are based on complex cellular processes and conditions.

Inactivation of TGF-β tumor suppressor activities results in malignant and invasive tumors (Bernabeu *et al.*, 2009; Padua and Massague, 2009). Cancer cells that have evaded TGF-β-induced growth inhibitory response produce more TGF-β as a compensatory mechanism (Blobe *et al.*, 2000; White *et al.*, 2010). This

overcompensation of TGF-β potentiates tumor invasion and metastases, by activating TGF-β tumor promoting activities (Lu *et al.*, 2006).

1.9.1.2 TGF-β as a tumor promoter

TGF-β was first described in the early 1980s as a transforming factor capable of stimulating growth of NRK cells in soft agar (Roberts *et al.*, 1980). TGF-β has since been implicated in a variety of cellular processes including, proliferation, adhesion, differentiation, motility, and apoptosis (Shi and Massague, 2003). Although the antiproliferative effect of TGF-β often overshadows the ability of TGF-β to promote cell proliferation, TGF-β has been shown to potentiate the proliferation of chondrocytes, osteoblasts, fibroblasts, endothelial cells (Lebrin *et al.*, 2004).

Recent studies have made significant progress in defining the TGF-β-induced mechanisms of tumor progression. Among the various pro-tumorigenic functions of TGF-β, immune suppression/evasion, angiogenesis, and epithelial-mesenchymal transition play important roles in enhancing tumor progression (Padua and Massague, 2009).

As tumor cells emerge, the body uses its arsenal of T lymphocytes and natural killer cells to identify and eliminate the tumor cells from the system. Tumor cells that have evaded the tumor-suppressive function of TGF-β can thus take advantage of the immunosuppressive functions of TGF-β to promote tumor progression (Padua and Massague, 2009). In transgenic mice studies, T lymphocytes been shown to be

negatively regulated by TGF-β (Gorelik and Flavell, 2001). TGF-β can also block T-cell activation by inhibiting the function of antigen-presenting cells including dendritic cells (Geissmann *et al.*, 1999). Additionally, TGF-β can also inhibit natural killer cell and neutrophil function (Arteaga *et al.*, 1993; Chen *et al.*, 1998a). Overall, TGF-β plays a critical role in enabling cancer progression through the suppression of the host immune system.

Tumor angiogenesis is another important method for TGF-β to elicit its pro-tumorigenic functions. By recruiting endothelial cells and vessels to the tumor, it provides the essential oxygen and nutrients needed for tumor progression. *In vitro* studies have shown that angiogenic mediators, vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) are directly mediated by TGF-β signaling (Kang *et al.*, 2003b). Furthermore, hypoxic conditions at the tumor cell core can induce increased VEGF expression through the activation of hypoxia-inducible factor 1 (HIF1) and Smad proteins in conjunction with TGF-β (Sanchez-Elsner *et al.*, 2001). Additionally, TGF-β can upregulate the expression of matrix metalloproteinases MMP-2 and MMP-9, and downregulate the expression of the protease inhibitor TIMP in the tumor cells (Derynck *et al.*, 2001). Through these metalloproteinase responses, TGF-β can enhance invasive properties of endothelial cells required for angiogenesis and tumor progression (Larsson *et al.*, 2001).

TGF-β can also enhance the migratory and invasive properties of cancer cells, by inducing a process known as epithelial-mesenchymal transition (EMT). EMT is a

normal physiological process that is integral to development but it is also a pathological feature associated with tumor progression and metastasis (Lee *et al.*, 2006). EMT begins with the disruption of epithelial cell–cell contacts and loss of apical–basal cell polarity (Kalluri and Weinberg, 2009). Afterwards, the expression of epithelial junction proteins and epithelial markers is downregulated, in parallel with increases in the expression of mesenchymal adhesion and marker proteins (Thiery and Sleeman, 2006). Additionally, the actin cytoskeleton is drastically reorganized, enabling the cells to acquire a front-rear polarity and motility. Finally, the cells acquire invasive properties through increased expression and secretion of MMPs, enabling the cells to synthesize components of the ECM to reconstitute their microenvironment following cell invasion (Nieto, 2011).

TGF-β promotes tumor growth by inducing EMT through a combination of Smad-dependent and Smad-independent effects (Massague, 2008). TGF-β cooperates with a variety of signaling pathways including integrin, Notch, Wnt, TNF-α, ERK and EGF to induce EMT (Miyazono, 2009). TGF-β signaling directly activates the expression of the Snail/Slug, ZEB1/ZEB2, and Twist transcription factors (Peinado *et al.*, 2007), which can repress the expression of epithelial junction proteins occludin and E-cadherin and disrupt cell junctions (Vincent *et al.*, 2009). TβRII-mediated phosphorylation of Par6 has also been shown to promote the dissolution of cell-junction complexes through its interaction Smurf1 (Ozdamar *et al.*, 2005). Thus, TGF-β-dependent EMT in cancer cells is mediated, in part, by the ability of TGF-β to induce the expression of cell-junction gene repressors as well as its ability

to alter the cell junctions (Padua and Massague, 2009). Additionally, TGF-β promotes the expression of MMPs such as MMP2 and MMP9, as well as components of the ECM such as fibronectin and collagens, likely through EMT associated transcription factors, to strengthen its invasive properties (Moustakas and Heldin, 2012). TGF-β can also promote the emergence of cancer stem cells (CSC) (Mani *et al.*, 2008), which in turn promotes metastasis not only by enabling primary carcinoma cells to invade and disseminate, but also by generating cells with both a mesenchymal and a stem-cell-like phenotype that then can give rise to secondary tumors with epithelial characteristics (Scheel and Weinberg, 2011). This concept has been proposed as a migrating CSC model, in which CSCs that undergo EMT drive clonal expansion at sites of dissemination (Brabletz *et al.*, 2005). Thus, TGF-β-induced EMT may enhance both cell invasion and cell self-renewal (Mani *et al.*, 2008).

1.9.2 TGF-β in skin development and regulation

The skin is formed by a stratified squamous epithelium composed of the epidermis of epithelial origin, and the underlying dermis and hypodermis of mesenchymal origin (Owens *et al.*, 2008). TGF-β plays a vital role in regulating skin homeostasis by mediating skin proliferation, apoptosis, differentiation, ECM production, migration, inflammation and angiogenesis (Bertolino *et al.*, 2005; Pardali *et al.*, 2005), Dysregulation of the TGF-β signaling pathway has been implicated in a variety of skin disorders including impaired wound healing (Cowin *et al.*, 2001), hypertrophic scarring (Armour *et al.*, 2007), psoriasis (Li *et al.*, 2004), scleroderma (Leask and Abraham, 2004) and skin cancer (Li *et al.*, 2005). As a whole, the TGF-

β signaling pathway plays a variety of important roles in skin development and homeostasis.

1.9.3 TGF-β in skin cancer

Skin cancers are divided into melanoma and non-melanoma types. Non-melanoma, which is about 20 times more common than melanoma includes basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) types (Shao *et al.*, 2012). BCCs and SCCs usually occur in sun exposed skin due to ultraviolet exposure. BCC is the most common type of skin cancer with a shiny, pearly nodule although it rarely metastasizes (Rubin *et al.*, 2005). SCC can form islands of squamous epithelium in the interfollicular epidermis and has a 3-10% rate of metastasis (Weinberg, 2007). Melanomas are cancers originating from melanocytes, primarily in the skin and are primarily caused by ultraviolet exposure (Autier and Dore, 1998). Although melanomas represent only approximately 4% of all cutaneous cancers, they are responsible for >80% casualties from skin-related cancers (Houghton and Polsky, 2002).

Recent studies have demonstrated a dual role for TGF-β during skin carcinogenesis. As TGF-β is a potent inhibitor of epithelial cell proliferation, it is considered a potent tumor suppressor during early stages of carcinogenesis (Elliott and Blobe, 2005). Conversely, as TGF-β can induce tumor proliferative activities at later stages of carcinogenesis leading to tumor growth, it is also considered a tumor promoter (Cui *et al.*, 1996).

Abnormalities in TGF-β signaling were observed in a large number of epithelial cancers (Bierie and Moses, 2006). In skin cancer, expression of Smad2 was lost in the majority of skin SCCs, suggesting that Smad2 plays an important tumour suppressive role (Han *et al.*, 2005). Similarly, knockout studies of Smad4 in murine tissues produced spontaneous skin carcinogenesis (Qiao *et al.*, 2006). Recently, studies have shown that an increase in copy number variations (CNVs) of Smad2 and Smad4 are associated with carcinogenesis (Ashktorab *et al.*, 2010; French *et al.*, 2009). Specifically, CNVs of Smad2 are associated with SCC and BCC, while CNVs of Smad4 are associated with SCC but not BCC (Shao *et al.*, 2012). The functional consequences of such CNV variation increases however remain to be further elucidated.

In melanoma cell lines, increased expression of the different TGF-β isoforms have been suggested to play a role in tumor progression (Krasagakis *et al.*, 1994; Rodeck *et al.*, 1994). Specifically, TGF-β1 is secreted by normal melanocytes and melanomas at various stages, whereas TGF-β2 and –β3 are not expressed in normal melanocytes but partially expressed in nevi and melanomas. TGF-β2 and –β3 seem to also appear in early melanoma tumors with their expression increasing in conjunction with tumor progression (Van Belle *et al.*, 1996). In addition, increased TGF-β1 and -β2 plasma levels were also observed at later stages of tumor development (Krasagakis *et al.*, 1998). Overall, studies suggest an increase in TGF-β expression enhances melanoma tumor progression.

1.9.4 TGF-β in wound healing

Wound healing is an intricate physiological response to injury which involves three main overlapping phases: the inflammatory phase, the proliferative phase, and the remodeling phase (Singer and Clark, 1999). Each of these wound healing phases involve various cell types, growth factors, and signaling pathways that facilitate wound healing progression from hemostasis, to inflammation, tissue proliferation, angiogenesis, reepithelialization, fibroblast differentiation, ECM deposition, and ECM remodeling (Schultz *et al.*, 2011). TGF-β plays a crucial role in regulating the cellular responses in all three phases of the wound healing process through its effects on cell proliferation and differentiation, ECM production, and immunomodulation (Finnson *et al.*, 2013).

Following injury, TGF-β is rapidly released into the wound site via the expulsion of alpha-granules from degranulating platelets in the inflammatory phase of wound healing (Nurden, 2011). This release of TGF-β is crucial for the inflammatory response, as TGF-β acts as a chemoattractant to recruit neutrophils, monocytes, and macrophages to the wound site to cleanse the wounded area of foreign particles, in order to prevent infection (Barrientos *et al.*, 2008). In addition to recruiting macrophages, TGF-β also activates macrophages to release additional TGF-β to stimulate capillary growth and initiate granulation tissue formation. (Lawrence and Diegelmann, 1994). Furthermore, TGF-β enhances the inflammatory response by upregulating expression of pro-inflammatory growth factors IL-1, TNF-α, PDGF,

and FGF-2 (Wahl *et al.*, 1987). As the inflammatory phase declines, TGF-β prepares the wound for the proliferative phase by inducing fibroblast proliferation and recruiting chemotaxis from the dermis surrounding the wound to the wound site (Beanes *et al.*, 2003).

TGF-β coordinates a variety of cellular responses during the proliferative phase, including fibroblast proliferation and differentiation, reepithelialization, and angiogenesis; leading to granulation tissue formation and wound contraction (Desmouliere *et al.*, 2005). TGF-β promotes fibroblast proliferation by increasing the expression of integrin proteins on the surface of fibroblasts, facilitating the adhesion of fibroblasts to the provisional matrix (Margadant and Sonnenberg, 2010). Under control of TGF-β, fibroblasts deposit substantial amounts of collagen I and III to replace the provisional matrix of the granulation tissue (Singer and Clark, 1999). TGF-β also reinforces ECM deposition by downregulating the expression and activity of matrix-degrading enzymes such as MMPs and increasing the expression of protease inhibitors such as tissue inhibitors of MMPs (TIMPs) (Goldman, 2004). TGF-β can also regulate wound angiogenesis by stimulating endothelial cell migration, differentiation, and capillary tubule formation (Li *et al.*, 2003). Additionally, TGF-β stimulates keratinocyte migration *in vitro*, potentially by regulating integrins (Gailit *et al.*, 1994), important for the dissolution of cell-cell junctions. Finally, TGF-β plays an important role in fibroblast differentiation to myofibroblasts which promotes wound contraction and in turn enhances wound closure (Hinz, 2007).

During the remodeling phase, TGF-β promotes ECM deposition by enhancing collagen and fibronectin expression, inhibiting MMP expression, and enhancing protease inhibitor expression (Schiller *et al.*, 2004). While TGF-β-induced ECM deposition can increase the strength of the wound, dysregulation of TGF-β signaling can contribute to the progression of fibrotic wound healing and scarring (Barrientos *et al.*, 2008). During the remodeling phase, TGF-β has also been shown to promote collagen crosslinking by upregulating lysyl oxidases (LOX) expression in many different cell types (Colwell *et al.*, 2005), which enhances wound tensile strength (Lau *et al.*, 2006). Furthermore, animal studies have shown that the addition of TGF-β into mice incisional wounds result in more extensive scarring, while addition of neutralizing antibodies to TGF-β1 and TGF-β2 inhibits wound fibrosis (Shah *et al.*, 1992, 1995). Collectively, these reports emphasize the capacity of TGF-β to induce ECM expression, and illustrate the importance of tightly regulated TGF-β signaling during the wound healing process.

1.10 CD109 as regulator of TGF-beta signaling

1.10.1 Discovery and characterization of CD109

CD109 was originally discovered as a marker of hematopoietic stem cells (Furley *et al.*, 1986) and as an unidentified antigen on activated platelets and T-cells (Sutherland *et al.*, 1991). CD109 was initially characterized as an antigen with three distinct isoforms of 180 kDa, 150 kDa, and 120 kDa in size (Brashem-Stein *et al.*, 1988). Later studies have demonstrated that CD109 occurs in a native 180 kDa

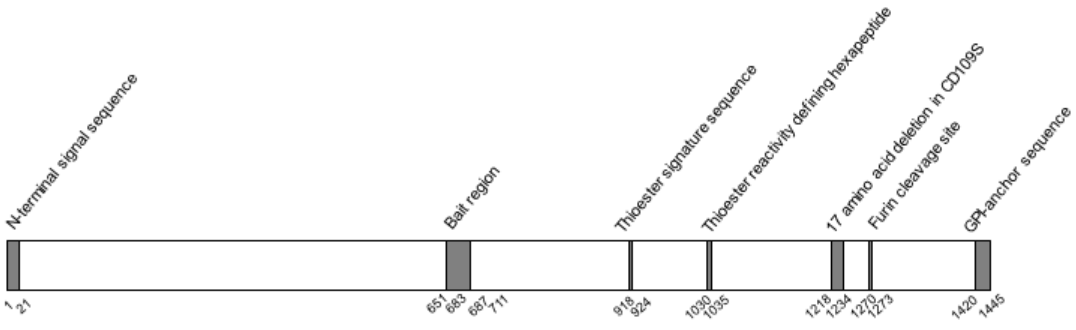
form with proteolytic modification and alternative splicing producing 150 kDa and 120 kDa products (Finnson *et al.*, 2006; Lin *et al.*, 2002; Smith *et al.*, 1995; Solomon *et al.*, 2004). Alternative splicing has demonstrated that CD109 can exist in various isoforms (Solomon *et al.*, 2004); for example, a shorter version of CD109 with a 17 amino acid deletion at position 1218-1234 (termed CD109S) is found in the placenta but not in keratinocytes (Finnson *et al.*, 2006). Interestingly, recent studies have suggested that CD109 may also exist in a 205 kDa glycosylated form, which is processed in the Golgi into 180 kDa N-terminal and 25 kDa C-terminal non-covalently linked parts by furin-mediated cleavage in the 1270-1273 region of CD109 (Hagiwara *et al.*, 2010). As the mechanisms that control CD109 isoform expression remain largely unidentified, further investigation is required to provide a better understanding of its underlying cause.

1.10.2 CD109 as a member of the α-2-macroglobulin protein family

Studies on the CD109 primary protein sequence have identified CD109 as a novel member of the α-2-macroglobulin/Complement family (C3, C4, C5) of thioester-containing proteins (Lin *et al.*, 2002). Further studies on the CD109 protein sequence have revealed that CD109 shares conserved α2m family motifs including a putative bait region, a furin cleavage site, and a thioester-reactivity defining hexapeptide (Figure 1.1) (Solomon *et al.*, 2004). Genomic sequence analysis by RT-PCR of the full-length human CD109 cDNA have showed that the CD109 gene, which consists of 33 exons spanning a 128 kb region located on human chromosome section 6q13, encodes a protein of 1445 amino acids in length and

possesses orthologs in mouse, rat, dog and cow (Solomon *et al.*, 2004). Evolutionary sequence analysis of the CD109 gene sequence suggests that CD109 is a distant ancestor of the α2-macroglobulin (α2M)/complement family as CD109 possesses a low sequence homology (<30%) but high structural functional region homology, especially among CD109 orthologs, suggesting an early divergence of CD109 and other α2m family members. Overall, bioinformatics analysis of the CD109 gene suggests that CD109 is not a paralog of any members of the α2m family but represents a separate α2m family branch (Solomon *et al.*, 2004).

Figure 1.1 Schematic representation of CD109



CD109 is unique among its α2m family members in that it exists as a monomer (Sutherland and Yeo, 1995) whereas most other α2m family proteins exist as oligomers of a 180 kDa subunit including α2m itself, which exists as a 720 kDa tetramer (Dodds and Law, 1998). Furthermore, CD109 is the only α2m family member to possess an N-terminal signaling peptide and a GPI-anchor cleavage site (Lin *et al.*, 2002). The N-terminal signal peptide allows for translocation of CD109 into the endoplasmic reticulum (ER) for protein processing. While in the ER

compartment, the GPI-attachment signal sequence of CD109 is recognized and processed through a phosphodiester bond to a preassembled GPI anchor by the action of a GPI transamidase (Lakhan *et al.*, 2009). After attachment of the GPI precursor, CD109 is transported into the Golgi apparatus, where fatty acid remodeling and association with lipid-raft occur. Subsequently, CD109 is transported to the cell surface and anchored onto the plasma membrane by its GPI anchor (Kinoshita *et al.*, 2008). Interestingly, studies have showed that GPI-anchored proteins can be released from the cell surface by endogenous phospholipases (Metz *et al.*, 1994; Movahedi and Hooper, 1997).

As CD109 is bound to the cell membrane by its GPI-anchored, accordingly, CD109 can exist endogenously as a soluble protein due to endogenous phospholipases activity (Tam *et al.*, 2001). Furthermore, mesotrypsin and furin-mediated cleavage has been shown to produce substantial amounts of soluble CD109, suggesting that secretion by cleavage of the GPI-anchor may not be the principal mechanism of CD109 release from the cell surface (Hagiwara *et al.*, 2010; Hockla *et al.*, 2009; Sakakura *et al.*, 2014).

α2m family members have been implicated in variety of immunoregulatory roles (Law and Dodds, 1997; O'Connor-McCourt and Wakefield, 1987). Specifically, α2m has been known to function as a protease inhibitor, a ligand for the low density lipoprotein receptor-related protein (LRP)-1 receptor, and a carrier for PDGF-BB and TGF-β1 (Philip and O'Connor-McCourt, 1991). Additionally, the C3/4/5

complement family has been involved in immune defense and inflammation (Law and Dodds, 1997). Interestingly, all α2m members, including CD109, share a common core of eight homologous macroglobulin (MG domains (Janssen *et al.*, 2005) and contain a putative bait region, a thioester signature sequence, and a hexapeptide motif that defines the chemical reactivity of the thioester. Proteolytic cleavage of the bait region by proteases for α2m or specific enzymes for C3/4/5 complement have been shown to induce conformational changes due to thioester becoming reactive toward nucleophiles, which promotes covalent binding (Dodds and Law, 1998). Studies have shown that α2m can trap proteases within its core structure causing a conformational change that reveals binding sites for LRP-1 and enhances the affinity of α2m for PDGF-BB and TGF-β1 (Webb *et al.*, 1994). CD109 also possesses an intact thioester bond and its activation has been suggested to induce proteolytic cleavage, resulting in smaller CD109 fragments (Finnson *et al.*, 2006; Lin *et al.*, 2002). Additionally, sequence alignment between CD109 and α2m indicates that the putative LRP-1 binding site is conserved in CD109, although the mechanism of interaction between CD109 and LRP-1 remains to be determined (Solomon *et al.*, 2004).

1.10.3 CD109 as a TGF-β co-receptor and inhibitor of TGF-β signaling

Previous studies from our laboratory identified the existence of a novel 150kDa TGF-β binding protein on the cell surface of human keratinocytes that can form complexes with TβRI and TβRII (Tam *et al.*, 1998). This novel TGF-β binding protein was referred to as r150, and possessed high affinity for the TGF-β1 isoform,

moderate affinity for the TGF-β3 isoform, and virtually no affinity for TGF-β2 isoform (Tam *et al.*, 2001). Additional studies showed r150 to be sensitive to PIPLC treatment, suggesting that it possessed a GPI anchor (Tam *et al.*, 1998). r150 treated with PIPLC was able to bind TGF-β and to prevent TGF-β binding with its signaling receptors (Tam *et al.*, 2001). To further elucidate the role of r150 and other GPI-anchored proteins in TGF-β signaling, HaCaT cells deficient in GPI anchor synthesis were used. Predictably, these GPI-anchor deficient cells demonstrated enhanced TGF-β-induced Smad2/3 phosphorylation and increased TGF-β signaling, suggesting that r150 and/or other GPI-anchored proteins negatively regulate TGF-β signaling (Tam *et al.*, 2003).

To determine the molecular identity of the r150 protein, our laboratory cloned r150 cDNA from r150 proteins that were affinity purified on a TGF-β1 column and microsequenced. Microsequencing results identified r150 as CD109, a GPI-anchored protein that is part of the α2m family of proteins (Finnson *et al.*, 2006). Additionally, our laboratory was able to confirm the ability of r150/CD109 to bind TGF-β in the presence and absence of the TGF-β ligand, to form a heteromeric complex with the TGF-β receptors, to directly interact with TβRI, and to inhibit TGF-β signaling (Finnson *et al.*, 2006).

Recent studies in our laboratory have demonstrated that CD109 can inhibit TGF-β signaling as a TGF-β co-receptor that facilitates TGF-β receptor internalization and degradation in a manner dependent on Smad7 and Smurf2 through the caveolae

pathway (Bizet *et al.*, 2011; Bizet *et al.*, 2012). Additionally, our laboratory have also demonstrated that PIPLC-released soluble CD109 can also bind TGF-β and regulate TGF-β signaling possibly by ligand sequestration and/or receptor interaction (Litvinov *et al.*, 2011; Tam *et al.*, 2001) Interestingly, other recent studies have suggested that CD109 can modulate TGF-β receptor signaling by furin-mediated cleavage of the CD109 protein, which appears to play an important role in CD109 and TβRI interaction (Hagiwara *et al.*, 2010).

Our laboratory has also demonstrated that CD109 can inhibit TGF-β signaling and responses *in vivo*, as studies on transgenic mice overexpressing CD109 in the epidermis showed decreased Smad2/3 signaling and ECM synthesis during wound healing as compared to wild-type littermates (Vorstenbosch *et al.*, 2013b). Additionally, transgenic mice overexpressing CD109 in the epidermis displayed reduced scarring parameters and inflammatory responses in the skin during normal wound healing (Vorstenbosch *et al.*, 2013b), in a bleomycin-induced model of scleroderma (Vorstenbosch *et al.*, 2013a), and in a hypoxic would healing model (Winocour *et al.*, 2014), as compared to wild-type littermates. Furthermore, analysis of the TGF-β signaling pathway has revealed that CD109 transgenic mouse skin exhibits diminished ALK5 expression and Smad2/3 phosphorylation and markedly increased ALK1 expression and Smad1/5 phosphorylation, with decreased ECM synthesis, as compared to wild-type skin (Vorstenbosch *et al.*, 2015). Similar results were obtained in keratinocytes cultured from the transgenic versus wild type littermate skin. Collectively, these findings suggest that

overexpression of CD109 in the epidermis decreases scarring and inhibits fibrosis during wound healing in a TGF-β dependent manner which may involve differential regulation of the ALK5-Smad2/3 and ALK1-Smad1/5 pathways.

Recent studies from our laboratory show that in addition to inhibiting the canonical TGF-β signaling, CD109 can also regulate TGF-β-induced MAP kinase signaling and consequently TGF-β-induced epithelial-to-mesenchymal transition (EMT) in keratinocytes (Bizet *et al.*, 2014). CD109 inhibits TGF-β-induced EMT responses including dynamic cytoskeleton remodeling (actin stress fiber formation), suppression of E-cadherin expression, induction of Slug, MMP-2 and MMP-7 expression, cell migration and invasion in human squamous cell carcinoma (A431 and SCC-13) cell lines (Bizet *et al.*, 2010). Inhibition of EMT by CD109 in SCC cells (A431 and SCC-13) has also been shown to involve both Smad-dependent and Smad-independent pathways (Bizet, 2011). CD109 inhibits TGF-β-induced ERK activation while facilitating TGF-β-induced p38 activation to block EMT in a caveolin-1 dependent manner, suggesting that CD109's regulation of TGF-β-induced MAP kinase activation involves the caveolae signaling pathway (Bizet *et al.*, 2014). Taken together, our recent laboratory findings demonstrate that CD109 is a novel TGF-β co-receptor that can negatively modulate TGF-β canonical and non-canonical signaling in a context dependent manner.

1.10.4 CD109 in Disease

CD109 has been reported to be broadly expressed in hematopoietic and mesenchymal progenitor cells, in activated T-cells and platelets, and in endothelial cells (Giesert *et al.*, 2003; Kelton *et al.*, 1990; Murray *et al.*, 1999). Subsequently, CD109 has also been reported to be expressed in myoepithelial cells of the breast, lacrimal, salivary, and bronchial glands, in basal cells of the bronchial and prostate epithelia, in basal to suprabasal layers of the epidermis, and in chondrocytes (Finnson *et al.*, 2006; Hasegawa *et al.*, 2007; Mii *et al.*, 2012; Stevens *et al.*, 2008). Additional studies have demonstrated that CD109 plays an active role in immunoregulation, cancer, and skin diseases (Ertel *et al.*, 2005; Hagiwara *et al.*, 2010; Litvinov *et al.*, 2011). Delineation of the molecular mechanisms by which CD109 differentially mediates its function in disease may lead to the development of novel treatments for diseases associated with CD109 including autoimmune disorders, cancer, and scarring.

1.10.4.1 CD109 in immunoregulation

Since the discovery of the Gov-alloantigen on the CD109 protein, much interest has been expressed in the relationship between CD109 expression and alloimmune thrombocytopenia, post-transfusion purpura, and platelet transfusion refractoriness (Lin *et al.*, 2002). Immunophenotyping studies first discovered platelet CD109 possessed the Gov alloantigen system, also known as HPA-15. The Gov antigen, when expressed on activated platelets can be immunogenic and result in an immune reaction targeting platelets for destruction (Ertel *et al.*, 2005). The Gov alloantigen system is caused by a gene polymorphism resulting in a Tyrosine-703 to Serine-

703 substitution in CD109 that yields the Gov(a) and Gov(b) alleles (Schuh *et al.*, 2002). Additionally, platelet CD109 can also be modified by glycosyltransferases so that platelet CD109 can carry blood group A and B determinants (Kelton *et al.*, 1998) further substantiating its possible role in alloimmune disease modulation.

1.10.4.2 CD109 in cancer

CD109 expression was first discovered to be dysregulated in human cancers about a decade ago, where CD109 expression was shown to be upregulated in squamous cell carcinoma (SCC) of the lung and esophagus as well as in glioblastoma (Hashimoto *et al.*, 2004). Subsequent studies have shown that CD109 is upregulated in SCC of the oral cavity, uterine, cervix and endometrium, head and neck (Hagiwara *et al.*, 2008; Hasegawa *et al.*, 2007; Hasegawa *et al.*, 2008; Ni *et al.*, 2012; Sato *et al.*, 2007; Zhang *et al.*, 2005). Interestingly, CD109 expression was shown not to be upregulated in cancers including neuroblastoma, small-cell carcinoma, leukemia, and lymphoma (Hashimoto *et al.*, 2004) or in normal squamous epithelia (Hagiwara *et al.*, 2008; Ni *et al.*, 2012). Whether CD109 protein upregulation in SCC is due to an adaptive response to TGF-β pro-metastatic effects or due to a causative role in disease progression is yet to be elucidated.

Several recent studies have demonstrated that CD109 expression is dysregulated in many other types of cancers including melanoma (Finnson *et al.*, 2006; Ohshima *et al.*, 2009), breast cancer (Geiger *et al.*, 2012; Hasegawa *et al.*, 2008), and colorectal cancer (Ashktorab *et al.*, 2013; Brim *et al.*, 2014; Sjoblom *et al.*, 2006).

Intriguingly, a recent study identified CD109 as a gene that possesses polymorphisms in its 3' untranslated region that are associated with colorectal cancer (Landi *et al.*, 2012), suggesting that dysregulation of CD109 expression can promote cancer development. Additionally, CD109 has been also recently identified as an exosome-associated protein that is preferentially secreted/released from cancer cell lines including SCC (Park *et al.*, 2010), thyroid cancer (Caccia *et al.*, 2011), and breast cancer cells (Hockla *et al.*, 2009). Furthermore, exosomes containing CD109 secreted from stromal cells have been shown to drive MDA-MB-231 cell invasive behavior (Luga *et al.*, 2012), providing another explanation of how CD109 can promote cancer progression. Conversely, recent results from our laboratory suggest that CD109 is a potent inhibitor TGF-β-induced EMT, cell migration and invasion in SCC cells (Bizet *et al.*, 2014). While the recent opposing findings may seem perplexing, it is possible that like TGF-β, CD109 may have a dual role in cancer progression with it promoting cell proliferation by inhibiting TGF-β-induced growth inhibition in early stages of cancer, and it suppressing TGF-β-mediated EMT and metastasis in later stages of cancer.

1.10.4.3 CD109 in skin

Recent results from our laboratory indicate that CD109 expression is also dysregulated in several non-cancer related skin diseases. In psoriasis, CD109 protein expression was found to be downregulated in lesional psoriatic skin as compared to normal skin (Litvinov *et al.*, 2011). In the psoriasis studies, when CD109 was released from the cell surface using PIPLC or when recombinant

soluble CD109 protein was exogenously added, psoriatic phenotypes appeared in keratinocytes, as confirmed by the upregulation of STAT3 and Bcl-2 protein expression, the downregulation of ALK5, as well as the enhancement of cellular growth and survival (Litvinov *et al.*, 2011). Additionally, in scleroderma, CD109 protein expression is found to be upregulated in SSc skin and in SSc fibroblasts as compared to normal skin or cultured normal fibroblasts, with CD109 siRNA promoting, and soluble recombinant CD109 inhibiting ECM production in SSc and normal skin fibroblasts (Man *et al.*, 2012). These findings suggest that CD109 plays a significant role in non-cancer related skin disease. Further research will be needed to understand how CD109 dysregulation can lead to disease progression.

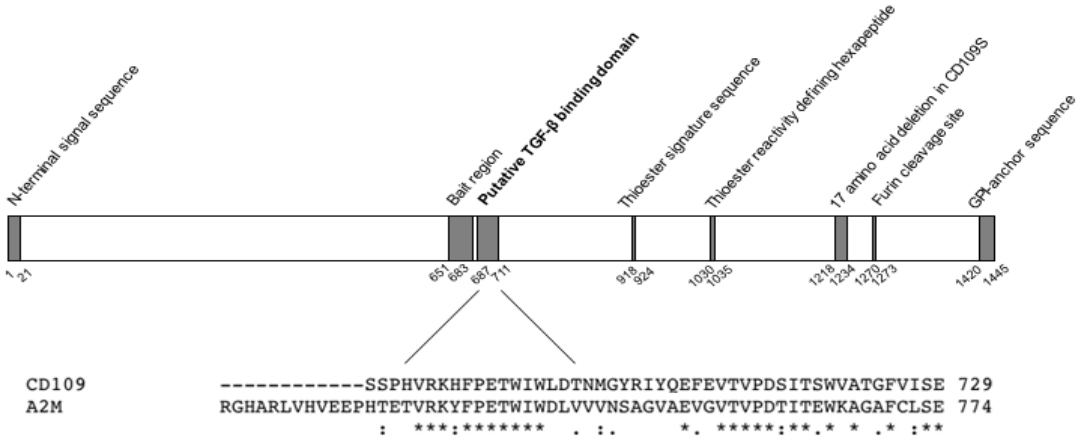
Rationale and objectives for study

As CD109 is as a member of the α2m/complement family of thioester-containing proteins with shared structural motifs (Lin *et al.*, 2002) and as α2m has been shown to possess a putative TGF-β binding domain (Arandjelovic *et al.*, 2006), sequence alignment between CD109 and α2m was done to determine the putative TGF-β binding domain of CD109. Specifically, sequence alignment between CD109 and α2m has allowed us to predict that the putative TGF-β1 binding domain of CD109 may include amino acids 687-711 (Soe-Lin, 2006). The putative TGF-β1 binding domain of CD109 contains the hydrophobic sequence WIW and the negative charged acidic residue Glu (Figure 1.2) thought to confer functionality similar to that of the TβRII and α2m TGF-β binding sites, which have a high density of

hydrophobic and negatively charged residues (Arandjelovic *et al.*, 2003; Webb *et al.*, 2000).

The specific objectives of this thesis were (1) to map the TGF-β binding domain(s) of CD109; (2) to investigate whether soluble CD109 negatively regulates TGF-β signaling and responses; and (3) to examine whether soluble CD109-based peptides negatively regulate TGF-β signaling and responses. Determining if soluble CD109 and soluble CD109-based peptides can bind TGF-β and negatively regulate TGF-β signaling and responses is important for the elucidation of the mechanisms by which CD109 may regulate TGF-β signaling and biological responses.

Figure 1.2 Schematic representation of CD109 with putative TGF-β binding domain



2 Soluble CD109 binds TGF-beta and antagonizes TGF-beta signaling and responses

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2.1 Abstract

Transforming growth factor-β (TGF-β) is a multifunctional cytokine implicated in many diseases, including tissue fibrosis and cancer. TGF-β mediates diverse biological responses by signaling through type I and II TGF-β receptors. We have previously identified CD109, a glycosylphosphatidylinositol-anchored protein, as a novel TGF-β co-receptor that negatively regulates TGF-β signaling and responses and demonstrated that membrane anchored CD109 promotes TGF-β receptor degradation via a SMAD7/Smurf2-mediated mechanism. To determine whether CD109 released from the cell surface (soluble CD109 or sCD109) also acts as a TGF-β antagonist, we determined the efficacy of recombinant sCD109 to interact with TGF-β and inhibit TGF-β signaling and responses. Our results demonstrate that sCD109 binds TGF-β with high affinity as determined by surface plasmon resonance (SPR) and cell-based radioligand binding and affinity labeling competition assays. SPR detected slow dissociation kinetics between sCD109 and TGF-β at low concentrations, indicating a stable and effective interaction. In addition, sCD109 antagonizes TGF-β-induced Smad2/3 phosphorylation, transcription and cell migration. Together, our results suggest that sCD109 can bind

TGF-β, inhibit TGF-β binding to its receptors and decrease TGF-β signaling and TGF-β-induced cellular responses.

2.2 Introduction

Transforming growth factor-β (TGF-β) is a multifunctional growth factor involved in cell growth and differentiation, ECM deposition, cell adhesion and immunomodulation (Massague *et al.*, 2000). The three mammalian TGF-β isoforms (TGF-β1, TGF-β2, TGF-β3) are approximately 70-80% homologous and exhibit similar functions *in vitro*. However, they play distinct roles *in vivo* as indicated by differential expression patterns during development and dissimilar phenotypes in null mice. Dysregulation of the TGF-β signaling pathway has been implicated in many physiological disorders including impaired wound healing, hypertrophic scarring, scleroderma, and cancer (Cowin *et al.*, 2001; Gordon and Blobe, 2008; Li *et al.*, 2004; Schmid *et al.*, 1993). TGF-β signaling is transduced by a pair of transmembrane serine/threonine kinases known as type I and type II TGF-β signaling receptors (TβRI and TβRII receptors, respectively) (Shi and Massague, 2003). Ligand binding of TGF-β to TβRII, results in the transphosphorylation of TβRI by TβRII on its GS domain (Wieser *et al.*, 1995). The activated TβRI constitutively active kinase then propagates the signal by phosphorylating its intracellular substrates, Smad2 and Smad3, on their C-terminal SSXS (Ser-Ser-Xaa-Ser) motif (Abdollah *et al.*, 1997; Macias-Silva *et al.*, 1996). The phosphorylated Smad2/3 then form heteromeric complexes with Smad4 (Feng and Derynck, 2005) and translocate into the nucleus to regulate gene expression through

interactions with transcription factors, co-activators and co-repressors (Schmierer and Hill, 2007). In addition to the TβRI and TβRII signaling receptors, many cell types express TGF-β co-receptors known as betaglycan and endoglin that bind TGF-β and modulate TGF-β signaling in a cell-specific manner (Bernabeu *et al.*, 2009).

Excessive TGF-β signaling is believed to play a central role in many diseases such as tissue fibrosis and cancer metastasis, and approaches to developing agents that inhibit TGF-β action are actively being pursued (Prud'homme, 2007). Promising strategies for blocking TGF-β action by diminishing TGF-β access to its receptors include development of neutralizing anti-TGF-β antibodies and TGF-β ligand traps (Arteaga, 2006; Mourskaia *et al.*, 2007; Verona *et al.*, 2008; Wojtowicz-Praga, 2003). The concept of blocking TGF-β signaling using anti-TGF-β neutralizing antibodies has been around since the early 1990s (Bush *et al.*, 2011; Shah *et al.*, 1994). TGF-β ligand traps represent another class of molecules in development for neutralizing excess TGF-β produced in pathological conditions. Characterizations of receptor-ligand interactions have demonstrated the potential of receptor ectodomain-based ligand traps to sequester TGF-β. Examples include the soluble type II TGF-β receptor (Avraham *et al.*, 2010) and soluble betaglycan (Santiago *et al.*, 2005) both of which have been shown to sequester TGF-β and block TGF-β signaling.

Our group has identified CD109, a 180-kDa glycosylphosphatidylinositol (GPI)-anchored protein which belongs to the α2-macroglobulin complement superfamily, as a novel TGF-β co-receptor (Lin *et al.*, 2002). We have also reported that membrane anchored CD109 binds TGF-β1 ligand with high affinity, forms a heteromeric complex with the TGF-β signaling receptors, and inhibits TGF-β signaling and responses through caveolae-mediated internalization of TGF-β receptors and receptor degradation via a SMAD7/Smurf2-mediated mechanism (Bizet *et al.*, 2011; Bizet *et al.*, 2012). In addition, our group has demonstrated that CD109 can be released from the cell surface by enzymes such as phosphatidylinositol phospholipase C that cleaves the GPI-anchor and that the released CD109 (soluble CD109 or sCD109) can bind TGF-β1 with high affinity (Tam *et al.*, 2001). In this current study, we determined the potential of sCD109 to act as a TGF-β antagonist, by demonstrating the ability of recombinant soluble CD109 protein to bind all three mammalian TGF-β isoforms and negatively modulate TGF-β signaling and TGF-β-induced cellular responses.

2.3 Materials and Methods

Cell lines

The human keratinocyte cell line HaCaT (kindly provided by P. Boukamp Heidelberg, Germany), human embryonic kidney 293 cells (HEK293, purchased from the American Type Culture), and HEK293T cells (kindly provided by M. Tremblay, Montreal, Canada) were cultured as described previously (Finnson *et al.*, 2006). HaCaT and HEK293T clones stably expressing sCD109 (or its empty vector,

EV) were selected and cultured in the presence of 0.5 mg/ml Geneticin (Invitrogen).

Soluble CD109

Human soluble CD109 protein (amino acids 22-1268) was purchased from R&D Systems (#4385-CD) and was designated as sCD109-1268. We have also recombinantly produced a soluble, 1420 amino acid form of CD109 based on the human CD109 sequence without the GPI-anchor region and adding a 6-histidine tag at the C-terminus and was designated as sCD109-1420 (Figure 9A). sCD109-1420 cDNA was cloned into a pcDNA3-Gateway vector for stable overexpression of the sCD109-1420 protein in HEK293T cells. Enriched cell medium from the HEK293-sCD109-1420 cells was collected, concentrated, and the sCD109-1420 protein was purified using nickel affinity chromatography followed by size exclusion chromatography as described previously (Kaur and Reinhardt, 2012) Homogeneity of the sCD109-1420 preparations were verified using SDS-PAGE (coomassie staining) (Figure 9B) and Western blot analysis using mouse monoclonal anti-CD109 (TEA 2/16, BD Biosciences) (Figure 9C)

Surface plasmon resonance assay

Binding of TGF-β isoforms (Humanzyme #HZ-1011 TGF-β1, #HZ-1092 TGF-β2, and #HZ-1090 TGF-β3) to BSA (Sigma #A8806), TGFβ-sRII (R&D Systems #241-R2/CF), and sCD109 (sCD109-1268 and CD109-1420) was examined using label-free, real-time BIACORE 3000 instrumentation (GE Healthcare). Experiments were performed on CM5 sensor chips at 25°C using filtered (0.2 μm) and degassed HBS-P running buffer (10 mM HEPES pH 7.4, 150 mM NaCl,

0.005% (v/v) Tween-20). Protein-grade detergents (Triton X-100, Empigen) were from Anatrace (Maumee, USA), Pierce Gentle Elution buffer was from Thermo Scientific (Illinois, USA), and low-retention microfuge tubes (preferred for TGFβ) were from Fisher Scientific (Ontario, Canada); all other chemicals were reagent grade quality. Resuspended TGF-β isoforms (100 μg/mL in 4 mM HCl) were diluted to 5 μg/mL (in 10 mM sodium acetate pH 4.0) for immobilization using the Biacore Amine Coupling Kit (~1200 RU final); corresponding reference surfaces were prepared in the absence of any ligand. BSA (negative control, 0 – 300 nM), TGFβ-sRII (positive control, 0 – 2000 nM), and sCD109 (0 – 626 nM) were injected over reference and isoform-immobilized surfaces at 5 μL/min (6 min association + 10 min dissociation; 2-fold dilution series). Between sample injections, sensor chip surfaces were regenerated at 50 μL/min using two 30-second pulses of Pierce Gentle Elution buffer containing 0.1% (v/v) Triton X-100 or Empigen. Apparent equilibrium dissociation constants (K_D) for the TGFβ-sRII titrations were determined by global fitting of the data to a steady-state affinity model and dissociation rate constants for the sCD109 titrations were estimated by global fitting the data using the fit separate k_a/k_d model in the BIA evaluation v4.1 software (Groppe *et al.*, 2008).

Radioligand binding assay

Carrier-free TGF-β1 (Genzyme) was radiolabelled with Iodine-125 (Perkin Elmer) as previously described (Philip and O'Connor-McCourt, 1991). Radioligand binding assays were performed as previously described (Christopoulos and

Kenakin, 2002). Briefly, HaCaT cells were incubated with [^{125}I]-TGF-β1 (10-200 pM) in the absence or presence of non-labelled TGF-β1 (100X concentration of [^{125}I]-TGF-β1) for 3 hours at 4°C; cell membranes were solubilized and analyzed using the non-linear fit model (global fitting of total and nonspecific) in GraphPad Prism 5 to determine the equilibrium binding constant (K_d) of [^{125}I]-TGF-β1 for its receptors. Afterwards, cells were incubated with 50 pM ^{I125}TGF-β1 in the presence or absence of sCD109-1268 (1-1000X concentration of [^{125}I]-TGF-β1) for 3 hours at 4°C; cell membranes were solubilized and analyzed using the alpha ternary complex model in GraphPad Prism 5 to determine the equilibrium dissociation constant for the allosteric modulator at the free receptor (K_b) and the ternary complex constant (α) of sCD109 on [^{125}I]-TGF-β1.

Affinity labeling assay

Affinity labeling assays were performed as previously described (Tam *et al.*, 2001). Briefly, HaCaT cells were incubated with 100 pM [^{125}I]-TGF-β1 in the absence or presence in the absence or presence of non-labelled TGF-β1 (10 nM), sCD109-1268 (5, 10, 50 nM) and GST (50 nM) for 3 hours at 4°C; sCD109-TGF-β1 complexes were formed using a non-permeable cross-linker BS^3; cell membranes were solubilized; membrane extracts were visualized and analyzed by SDS-PAGE/autoradiography; densitometry of SDS-PAGE/autoradiography was performed using the TotalLab data analysis software using the arbitrary densitometric intensity of TβRI (Nonlinear Dynamics).

Western blot analysis

HaCaT cells were treated with or without TGF-β1 (0, 10, 100 pM) in the absence or presence of sCD109-1268 (10 nM) and GST Control (10 nM). Cells were incubated with TGF-β1 for 45 min (to determine phosphoSmad3 levels) and for 18 h (to determine fibronectin and PAI-1 levels) under serum-free conditions as previously described (Finnson *et al.*, 2006). Cell lysates were analyzed by Western blot analysis using the following antibodies: anti-phosphoSmad3 (FL-425, Santa Cruz Biotechnology), anti-PAI-1 (BD Biosciences), anti-fibronectin (BD Biosciences), anti-β-actin (Santa Cruz Biotechnology), mouse monoclonal anti-CD109 (TEA 2/16, BD Biosciences) as previously described (Tam *et al.*, 2003).

$CAGA_{12}$-lux assay

HaCaT cells were co-transfected with $CAGA_{12}$-lux reporter constructs and β-galactosidase to monitor transfection efficiency as previously described (Dennler *et al.*, 1999; Tam *et al.*, 2003). Cells were allowed to recover for 24 h under serum free conditions after transfection and then incubated with or without TGF-β1 (100 pM) in the absence or presence of sCD109-1268 (10 nM) and GST Control (10 nM) for 18 h under serum-free conditions. Cell lysates were analyzed for luciferase activity, and the values were normalized to β-galactosidase activity.

Cell free ligand binding assay

Nickel-NTA agarose beads were coated with 1% BSA and incubated with or without recombinant His-tagged CD109 (10nM) for 4 h at 4°C. Beads were spun

down, washed and incubated with recombinant TGF-β1 (100 pM) in DMEM overnight. After spinning down the beads, the supernatant was collected and added to serum starved HEK293 cells stably expressing $CAGA_{12}$-lux reporter construct.

Cell migration assay

Cell migrations assays were performed as previously described (Wu *et al.*, 2012). Briefly, HaCaT cells stably overexpressing sCD109-1420 and EV were grown to confluency in 6-well tissue culture plates and then pre-incubated with serum-free medium for 24 h, to in order to inhibit cell proliferation (Boukamp *et al.*, 1988). The monolayer of HaCaT cells was scratched across the center with a sterile 200 μL pipette tip to create a cell-free line. Cell medium was aspirated and washed 3 times to remove cellular debris. The culture plates were replenished with serum free medium in the absence or presence of TGF-β1 (100, 200, 500 pM) or EGF (5 nM). Photographs were taken at 0 h, 24 h, and 48 h after scratch. Migration was quantified using TotalLab data analysis software (Nonlinear Dynamics) with data expressed as percentage of the scratch area filled by migrating cells at 24h and/or 48 h post scratch. Similarly, cell migration assays were also performed on HaCaT cells treated with or without TGF-β1 (100 pM) in the absence or presence of sCD109-1268 (10 nM) and GST control (10 nM) under serum free conditions.

Statistical analysis

Numerical results are represented as means of $n \geq 3$ independent experiments ± standard error of the mean (SEM). For statistical tests where only two data sets were

being compared, an unpaired Student's t test (two-tailed) was used where $P<0.05$ was deemed statistically significant. When more than two data sets were compared, one- or two-way analysis of variance (ANOVA) tests were used where $P<0.05$ was deemed statistically significant.

2.4 Results

Soluble CD109 binds to TGF-β1, TGF-β2, and TGF-β3 isoforms

The binding specificity and kinetics of sCD109 to all three TGF-β isoforms were determined using SPR (surface plasmon resonance), which allowed detailed analysis of the interaction between sCD109 (sCD109-1268 and -1420) and label-free TGF-β isoforms (TGF-β1, -β2, -β3) in real-time. In our initial studies, sCD109 was amine-coupled (to minimize sample consumption) to examine binding responses with TGF-β and BSA (negative control). Specific binding of TGF-β1 to immobilized sCD109 was detected at high flow rates (25–50 μL/min) and variable surface densities, but the isotherms were heterogeneous (i.e. biphasic association) and the dose-dependent binding deviated from a simple 1:1 kinetic model (Figure 2.10A and 2.10B). Similar results were obtained when sCD109-1420 was under similar SPR binding conditions (data not shown). Control experiments which examined the binding of TGF-β1 and TGF-β3 to amine coupled ectodomain of type II TGFβ receptor (TGFβ-sRII) also showed heterogeneous isotherms (data not shown), similar to what has been reported by others (De Crescenzo *et al.*, 2006).

SPR analysis of CD109-TGF-β interaction was prepared in the reverse orientation by amine-coupling the three TGF-β isoforms to SPR sensor chips, a strategy previously used by others (De Crescenzo *et al.*, 2001) for SPR analysis of TGF-β binding to proteins of interest. Titration of BSA (negative binding control) over the immobilized TGF-β surfaces revealed no significant binding responses as expected (Figure 2.1, left column). The titration of TGFβ-sRII (positive control) showed specific binding responses with TGF-β1 and TGF-β3, but not with TGF-β2 (Figure 2.1, middle column). As expected, sub-micromolar affinities for TGFβ-sRII were detected and the individual binding kinetics with TGF-β1 and TGF-β3 were characterized by fast-on, fast-off kinetics (Figure 2.1, middle column) as previously reported by others (De Crescenzo *et al.*, 2006; Groppe *et al.*, 2008). Importantly, analysis of CD109 binding to the TGF-β isoforms showed that, in contrast to TGFβ-sRII, sCD109 specifically bound to all three isoforms with distinctly different slow-on and slow-off kinetics (Figure 2.1, right column). Even in this reversed orientation, the sCD109 association phases were biphasic (i.e. kinetics that deviated from a simple 1:1 binding model) (Figure 2.1, right column), but the consistently slow dissociation rates suggest that sCD109 binding to each of the TGF-β isoforms is a stable, high affinity interaction, with estimated nM K_D values of CD109 that is similar to that of TGFβ-sRII. Collectively, the SPR results demonstrate that sCD109 can specifically bind to all TGF-β isoforms with high affinity and slow dissociation rates.

Soluble CD109 inhibits TGF-β1 binding to keratinocyte cell surface

To determine if sCD109 can inhibit TGF-β binding to the cell surface, radioligand binding studies were performed. Saturation analysis of [^{125}I]TGF-β1 specific binding to the cell surface of HaCaT cells (human keratinocyte cell line) yielded an equilibrium binding constant (K_d) value of 0.06 nM using the non-linear fit model in the GraphPad Prism 5 (Figure 2.2A and 2.2B). Since the labeled ^{I125}TGF-β1 and unlabeled sCD109 are acting via different receptor binding sites, the ternary complex model was used to determine sCD109 ability to modulate ^{I125}TGF-β1 binding to its receptors (Christopoulos and Kenakin, 2002) For the radioligand experiments HaCaT cells were incubated with [^{125}I]TGF-β1 and unlabeled sCD109-1268, Results shown in Figure 2.2C indicated that sCD109 inhibited [^{125}I]TGF-β1 binding to its receptors using the ternary complex model in GraphPad Prism 5. Statistical analysis of the ligand binding curve demonstrated substantial inhibition with an equilibrium dissociation constant (K_b) of 0.25 nM and a ternary complex constant (α) of 0.18 for the sCD109- [^{125}I]TGF-β1 interaction (Figure 2.2C and Table 2.1).

Soluble CD109 inhibits TGF-β1 binding to TGF-β signaling receptors on keratinocytes

To examine if sCD109 is able to block TGF-β1 binding to its signaling receptors on keratinocytes, affinity labeling assays were performed. Affinity labeling of keratinocytes with [^{125}I]TGF-β1 and increasing concentrations of sCD109-1268 show that sCD109 is able to inhibit [^{125}I]TGF-β1 binding to the type I and type II TGF-β signaling receptors, betaglycan and membrane anchored CD109 on the cell surface in a dose dependent manner (Figure 2.3A and 2.3B). Similar results were

obtained when sCD109-1420 was used (data not shown). Together, these results suggest that sCD109 can inhibit TGF-β1 away from its receptors through direct sequestration and allosteric inhibition and thus inhibit TGF-β signaling.

Soluble CD109 inhibits TGF-β1-induced signaling and transcriptional activity

Effect of sCD109 on TGF-β signaling and transcriptional activity was examined by determining TGF-β1-induced Smad3 phosphorylation and Smad3-driven transcriptional activity, respectively. Data shown in Figure 2.4A top panel demonstrate that TGF-β-induced phosphorylation of Smad3 is markedly decreased in sCD109 treated cells as compared to the untreated controls. The levels of total Smad3 remained unchanged under these conditions. Densitometric analysis of the Western blot data show that sCD109 significantly ($P<0.05$) decreases TGF-β-induced phosphoSmad3 levels (Figure 2.4A, bottom panel). The effect of CD109 on TGF-β1-induced transcriptional activity was determined by using TGF-β-responsive luciferase reporter construct $(CAGA)_{12}$-lux, which is a Smad3-specific synthetic construct containing 12 repeats of CAGA. Results shown in Figure 2.4C indicate that treatment with sCD109-1268 results in a significant reduction ($P<0.05$) in TGF-β1-induced stimulation of $(CAGA)_{12}$-lux activity in HaCaT cells when compared with cells treated with control GST protein. Similar results were obtained in a cell free assay when sCD109 was incubated with TGF-β1, as detected by TGF-β1-induced $(CAGA)_{12}$-lux transcriptional activity (Figure 2.12).

Soluble CD109 decreases TGF-β1-induced fibronectin and PAI-1 expression

As TGF-β1 is known to be a key regulator of ECM synthesis and breakdown, we examined whether sCD109 can regulate TGF-β1-induced PAI-1 and fibronectin expression. Results shown in Figure 2.5A demonstrate that while untreated HaCaT cells show a robust dose-dependent increase in PAI-1 and fibronectin protein expression in response to TGF-β1 treatment (Figure 2.5A, top and middle panel), treatment of HaCaT cells with sCD109 leads to a 2-fold decrease ($P<0.05$) in TGF-β1 (10 or 100 pM)-induced fibronectin levels (Figure 2.5B), and a 3-fold decrease ($P<0.05$) in TGF-β1 (100 pM)-induced PAI-1 levels (Figure 2.5C) as compared to untreated HaCaT cells. Determination of β-actin levels demonstrates that equivalent amounts of protein were loaded in each lane (Figure 2.5A, bottom panel).

Soluble CD109 inhibits TGF-β1-induced keratinocyte cell migration

As TGF-β is an enhancer of keratinocyte migration (Gailit *et al.*, 1994), we examined the effect of sCD109 on TGF-β1-induced wound closure in monolayers of HaCaT cells stably transfected with sCD109-1420 or EV. HaCaT cells stably overexpressing sCD109-1420 exhibited diminished ($P<0.05$) TGF-β1-induced wound closure at 24 h and 48 h post wounding when compared with EV stably transfected cells under basal and TGF-β1-induced conditions (Figure 2.6A, 2.6B Figure 2.13). EGF was used as a negative control to establish that this effect of sCD109 is specific to TGF-β induced migration of cells (Figure 2.13). We then confirmed the efficacy of sCD109 protein to decrease keratinocyte migration using exogenous sCD109-1268 protein. A significant decrease ($P<0.05$) in wound closure at 48 h post scratch was observed in HaCaT cells treated with exogenous sCD109-

1268 protein compared to untreated HaCaT cells, in the presence of TGF-β1 (Figure 2.7A and 2.7B). Cell proliferative effects of TGF-β1 were inhibited by 24 h pre-incubation under serum free conditions, rendering the cells quiescent (Boukamp *et al.*, 1988).

2.5 Discussion

We have previously reported that CD109, a GPI-anchored protein, binds TGF-β with high affinity, associates with TGF-β signaling receptors and is a novel co-receptor and component of the TGF-β signaling system (Finnson *et al.*, 2006). Since then, we have shown that CD109 downregulates TGF-β signaling by promoting localization of TGF-β signaling receptors into the lipid raft/caveolae compartment and enhancing TGF-β receptor degradation via the SMAD7/Smurf2-mediated mechanism (Bizet *et al.*, 2011; Bizet *et al.*, 2012). Furthermore, we have reported that CD109 can be released from cell surface by PIPLC treatment and that the released CD109 can bind TGF-β1 with high affinity and inhibit TGF-β signaling in a receptor independent manner (Tam *et al.*, 2001). Here, we demonstrate that recombinant sCD109 binds to all three mammalian TGF-β isoforms in a dose-dependent manner and that sCD109 is an antagonist of TGF-β induced signaling and transcriptional responses. Taken together, our results suggest that sCD109 may play an important role in TGF-β signal termination.

In our current study, we have used surface plasmon resonance (SPR), radioligand binding assays, and affinity labeling studies to provide direct evidence that

recombinant sCD109 specifically binds to all three mammalian TGF-β isoforms with binding affinities rivaling those observed with the TGFβ-sRII ectodomain (TGF-β1 and TGF-β3) suggesting that sCD109 is able to potentially inhibit TGF-β signaling through ligand sequestration. In previous studies, we demonstrated that cell membrane-anchored CD109 protein bound TGF-β in an isoform specific manner with TGF-β1 showing the highest affinity, TGF-β2 a modest affinity and TGF-β3 an intermediate level of affinity (Tam *et al.*, 1998). Given that these previous affinity labeling experiments were performed in a cellular environment where several TGF-β receptors, binding proteins and endogenous TGF-β are present, our current SPR studies are technically distinct due to the purified components involved including label-free TGF-β isoforms immobilized on separate sensor flow cells, as well as recombinant forms of soluble CD109 (sCD109-1268 and sCD109-1420). Our SPR results showing that recombinant sC109 interacts with all three TGF-β isoforms with similar affinity while TGFβ-sRII binds as expected TGF-β1 and TGF-β3, but not the TGF-β2 isoform validate the experimental protocol used. Also, our finding that sCD109 binds to the TGF-β isoforms with a slow-on, slow-off kinetics while TGFβ-sRII exhibits a distinctly different (rapid-on, rapid-off kinetics) for the TGF-β1 and TGF-β3 isoforms suggests that sCD109 with its more stable interaction at nM concentrations may represent a more potent TGF-β antagonist than TGFβ-sRII.

The above notion that sCD109 not only binds TGF-β isoforms with high affinity but can also efficiently antagonize TGF-β's cellular effects is strengthened by our

findings that sCD109 inhibits TGF-β-induced Smad3 signaling and transcriptional activity and decreases TGF-β-induced PAI-1 and fibronectin production and cell migration. While it is likely that the mechanism by which sCD109 inhibits TGF-β-induced responses involves ligand sequestration, the results presented in the current study do not allow us to rule out other possibilities. Whether sCD109 is able to form a heteromeric complex with the TGF-β signaling receptors, enhance receptor internalization or turnover, as has been shown for membrane-anchored CD109 (Bizet *et al.*, 2012),or induce a conformational change in the signaling receptors to disrupt signaling, remains to be determined. The potential mechanisms by which sCD109 and membrane-anchored CD109 may inhibit TGF-β signaling is shown in Figure 2.8.

Whether the release of CD109 from the cell surface represents a physiologically relevant control mechanism of CD109 action is not known. Similarly, there is little information on the nature and levels of sCD09 in the serum or tissues in vivo. Over the past decade, many groups have reported mutations in CD109 or deregulation of its expression in many cancers (Hagiwara *et al.*, 2008; Hasegawa *et al.*, 2007; Hasegawa *et al.*, 2008; Hashimoto *et al.*, 2004; Sato *et al.*, 2007; Sjoblom *et al.*, 2006; Zhang *et al.*, 2005). Similarly, potential dysregulation of CD109 release and proteolytic cleavage have been reported in breast cancer (Hockla *et al.*, 2009) and thyroid cancer cells (Caccia *et al.*, 2011).Thus, it is possible that alterations in CD109 release from the cell surface may contribute to aberrant TGF-β action

leading to human diseases such as cancer metastasis and tissue fibrosis where excessive TGF-β action is known to play a role.

Collectively, our results suggest that sCD109 binds TGF-β isoforms with high affinity and negatively regulates TGF-β/Smad3 signaling and cellular migration and ECM synthesis. The release of CD109 from the cell surface leading to the production of sCD109 may represent a physiologically important control mechanism for CD109 action and targeting CD109 release to manipulate sCD109 levels may have potential value for therapeutic intervention in diseases where excessive TGF-β signaling plays a pathological role.

2.6 Acknowledgements

We would like to thank the laboratory of Tom Thompson (University of Cincinnati) for insightful discussions and Nick Zhygan for sCD109 cell medium collection.

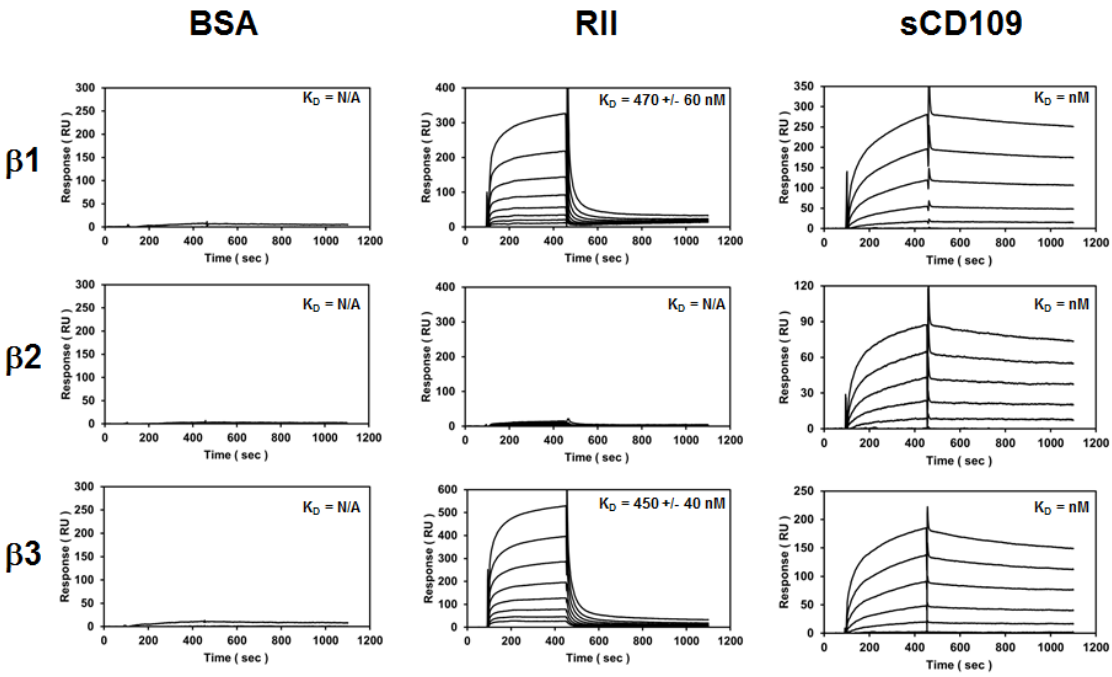
2.7 Author Contributions

Carter Li conceived, designed, and performed the research (except SPR), analyzed data, and co-wrote the paper; Mark Hancock performed the research (SPR only), analyzed data, and co-wrote the paper; Priyanka Sehgal performed the research (cell free binding assay) and analyzed data; Shufeng Zhou performed the research (migration assay) and analyzed data. Dieter Reinhardt performed the research

(sCD109 purification) and analyzed data; and Anie Philip conceived and directed the research and co-wrote the paper.

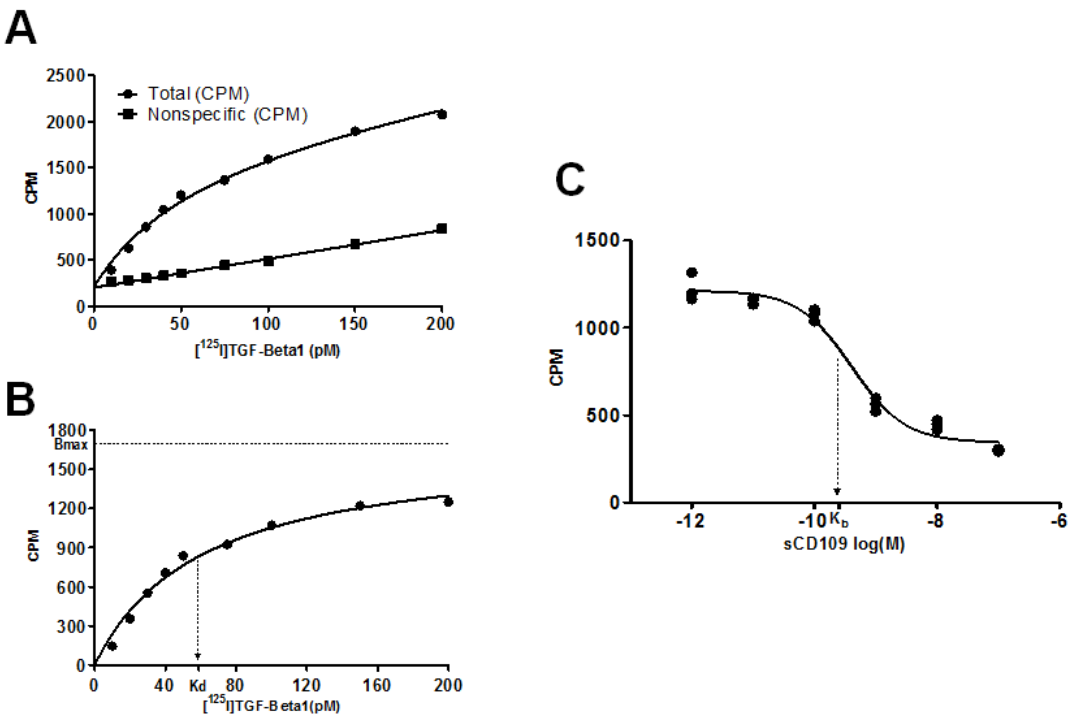
2.8 Figures

Figure 2.1: sCD109 binds TGF-β isoforms with high affinity



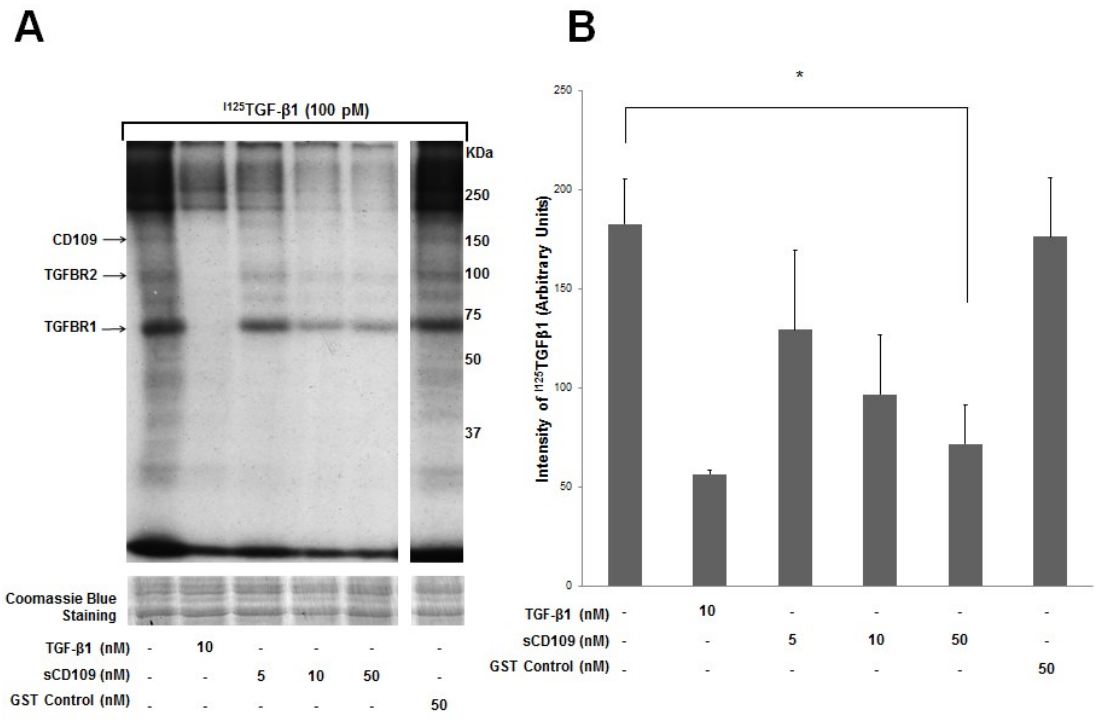
Representative SPR kinetics of BSA (0 – 300 nM), TGFβ-sRII (0 – 2000 nM), and CD109 (0 – 626 nM) binding to amine-coupled TGF-β1, TGF-β2, and TGF-β3 isoforms (~1200 RU each) at 5 µL/min (6 min association + 10 min dissociation; 2-fold dilution series). Apparent equilibrium dissociation constants (K_D) for the TGFβ-sRII titrations were determined by global fitting of the data to a steady-state affinity model and dissociation constants for the sCD109 titrations were estimated by global fitting the data using the fit separate k_a/k_d model in the BIA evaluation v4.1 software with values representing the average of n=3 independent experiments.

Figure 2.2 sCD109 is a negative modulator of TGF-β binding to keratinocyte cell surface



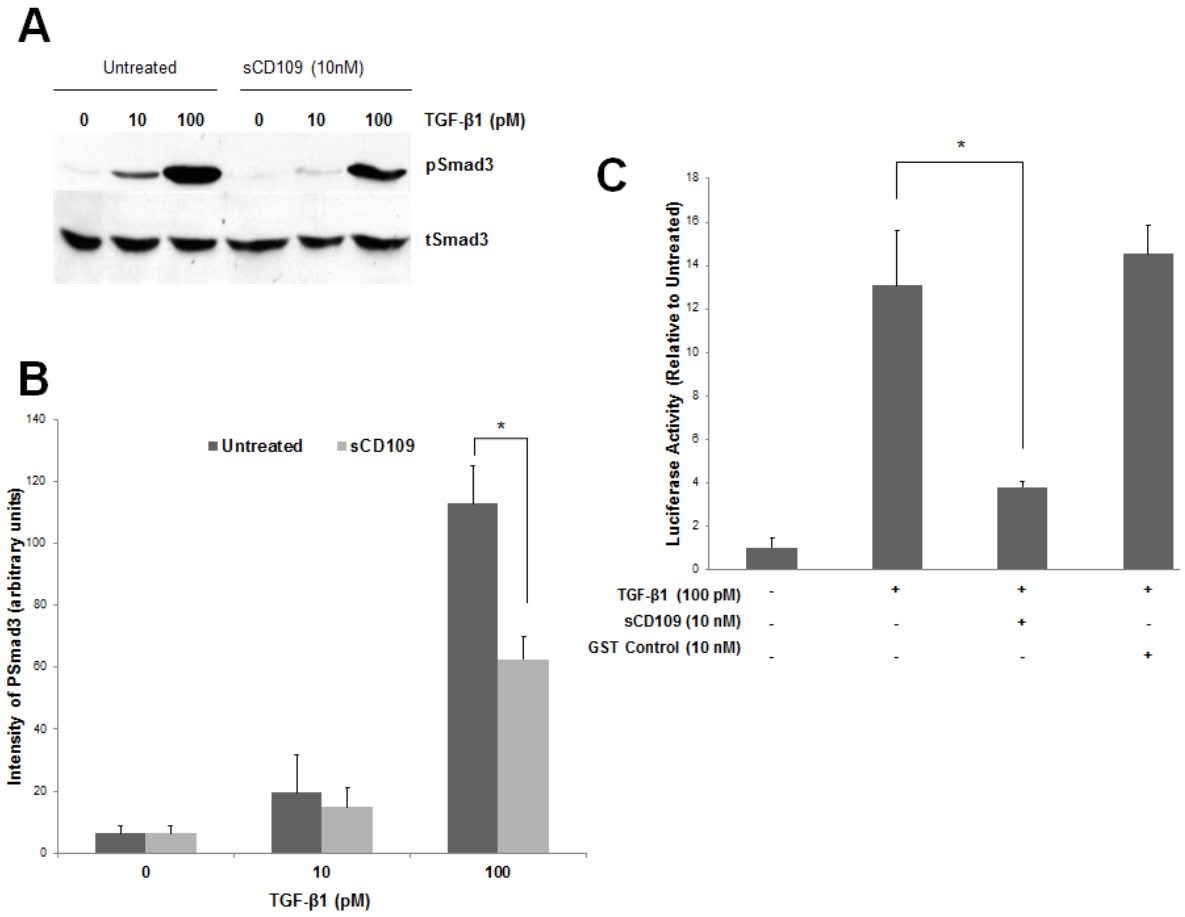
(**A**) HaCaT cells were radiolabelled at 4 °C with [^{125}I]TGF-β1(10-200 pM) in the absence or presence of non-radiolabelled TGF-β1 (100X of [^{125}I]TGF-β1) to determine its equilibrium binding constant (K_d). (**B**) Equilibrium binding constant (K_d) of [^{125}I]TGF-β1 was determined using the non-linear fit model (global fitting of total and nonspecific) in GraphPad Prism 5 with values representing n=3 independent experiments. (**C**) HaCaT cells were radiolabelled at 4 °C with 50pM [^{125}I]TGF-β1 in the absence or presence of CD109 (0.001-100 nM) to determine its equilibrium dissociation constant for the allosteric modulator at the free receptor (K_b) and the ternary complex constant (α) using the ternary complex model in GraphPad Prism 5 with values representing n=3 independent experiments.

Figure 2.3 sCD109 inhibits TGF-β binding to its signaling receptors



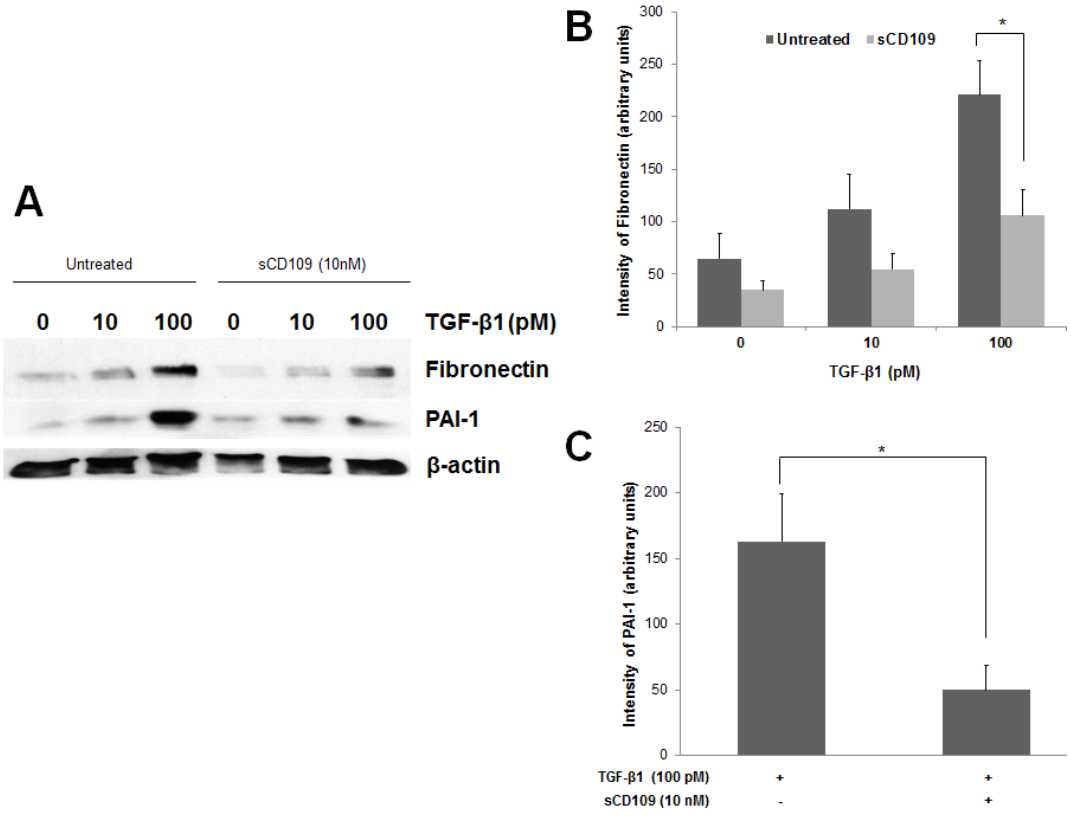
(**A**) HaCaT cells were affinity labeled at 4 °C with 100 pM [^{125}I]TGF-β1 in absence or presence sCD109, which was then covalently cross-linked to the cell surface receptors and solubilized. The solubilized membrane extracts were analyzed by SDS-PAGE and visualized by autoradiography with coomassie blue staining showing equal protein loading. (**B**) The amount of labeled TGFBR1 was quantified by densitometry based on the results shown in Figure 2.3A using TotalLab quantification software. The results are representative of n=3 independent experiments. *: $p < 0.05$.

Figure 2.4 sCD109 inhibits TGF-β1-induced phosphorylation of Smad3



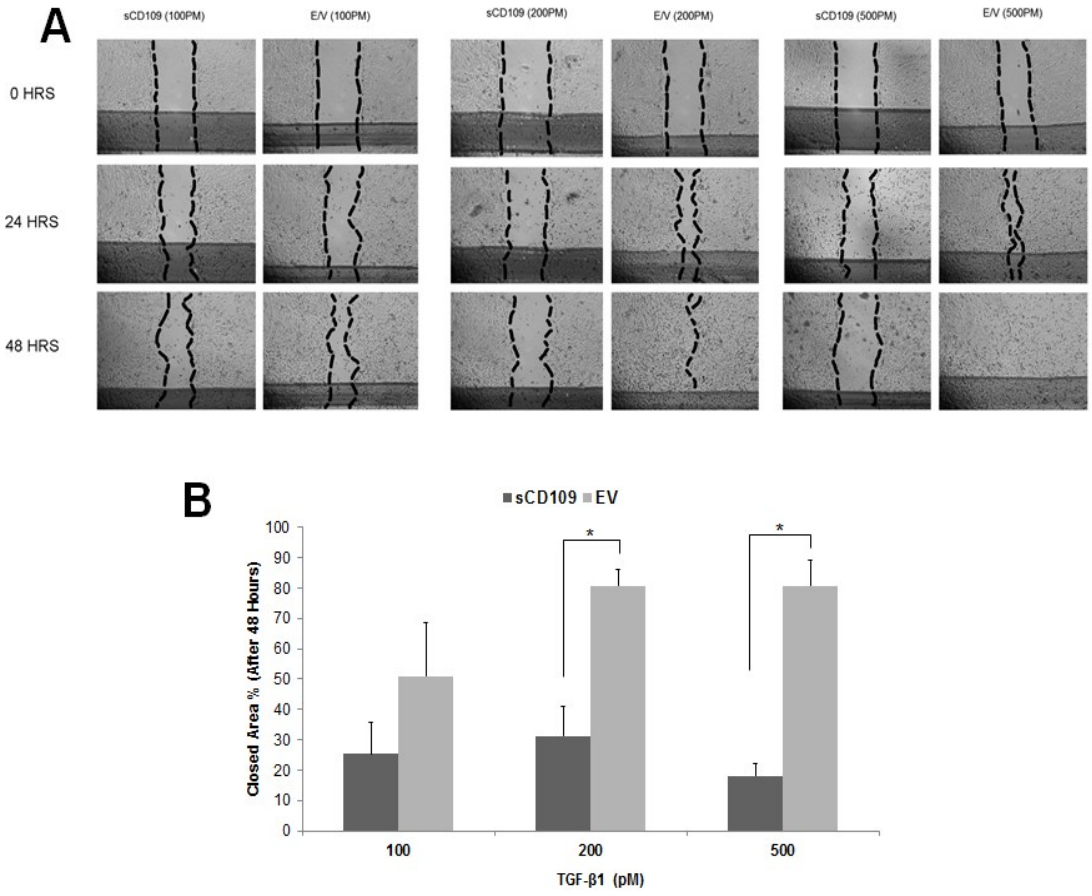
(**A**) HaCaT cells were affinity labeled at 4 °C with 100 pM [^{125}I]TGF-β1 in absence or presence sCD109, which was then covalently cross-linked to the cell surface receptors and solubilized. The solubilized membrane extracts were analyzed by SDS-PAGE and visualized by autoradiography with coomassie blue staining showing equal protein loading. (**B**) The amount of labeled TGFBR1 was quantified by densitometry based on the results shown in Figure 2.3A using TotalLab quantification software. The results are representative of n=3 independent experiments. *: $p < 0.05$. (**C**) HaCaT cells were co-transfected with $(CAGA)_{12}$-lux reporter andβ-galactosidase and treated with TGF-β1 in the absence or presence of sCD109 and GST negative control for 18 h under serum free conditions. Cell lysates were analyzed for luciferase activity and expressed as fold-stimulation ± SD (normalized to β-galactosidase) relative to activity of cells not treated with TGF-β1 with values representing the mean of n=3 independent experiments. *: $p < 0.05$.

Figure 2.5 sCD109 inhibits TGF-β1-induced PAI-1/fibronectin protein production



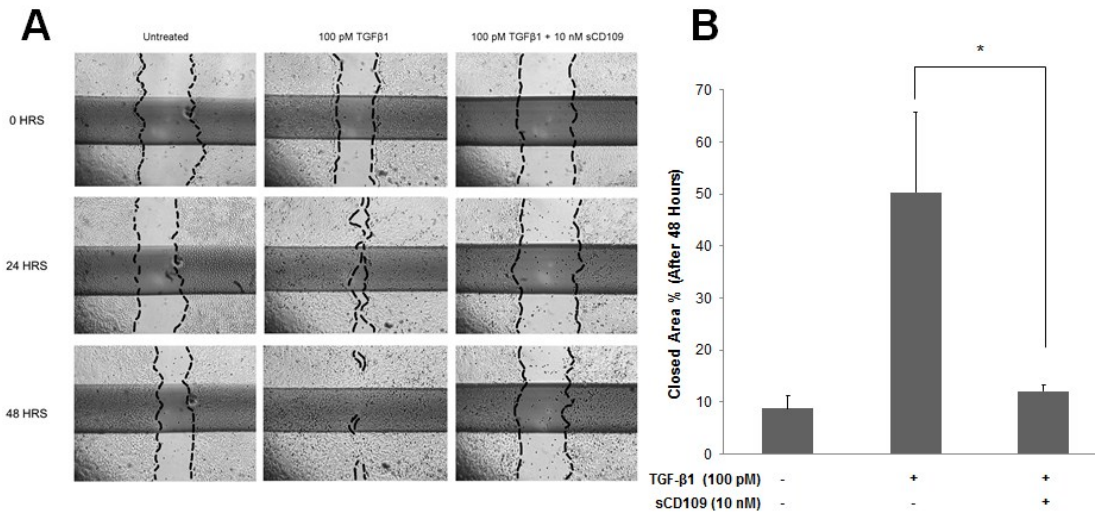
(**A**) HaCaT cells were treated with and without TGF-β1 in the presence and absence of sCD109 for 18 h under serum free conditions. Cell lysates were analyzed by western blotting using anti-fibronectin, anti-PAI-1, or anti-actin antibodies. (**B**) Densitometry of the fibronectin western blot band (expressed as mean of fibronectin band over actin band in arbitrary intensity units) was determined by TotalLab quantification software (mean of n=3 independent experiments, ± SEM, *: $p<0.05$). (**C**) Densitometry of the PAI-1 western blot film (expressed as mean of PAI-1 band over actin band in arbitrary intensity units) was determined by TotalLab quantification software (mean of n=3 independent experiments, ± SEM, *: $p<0.05$).

Figure 2.6 Cell released sCD109 inhibits TGF-β1-induced cell migration



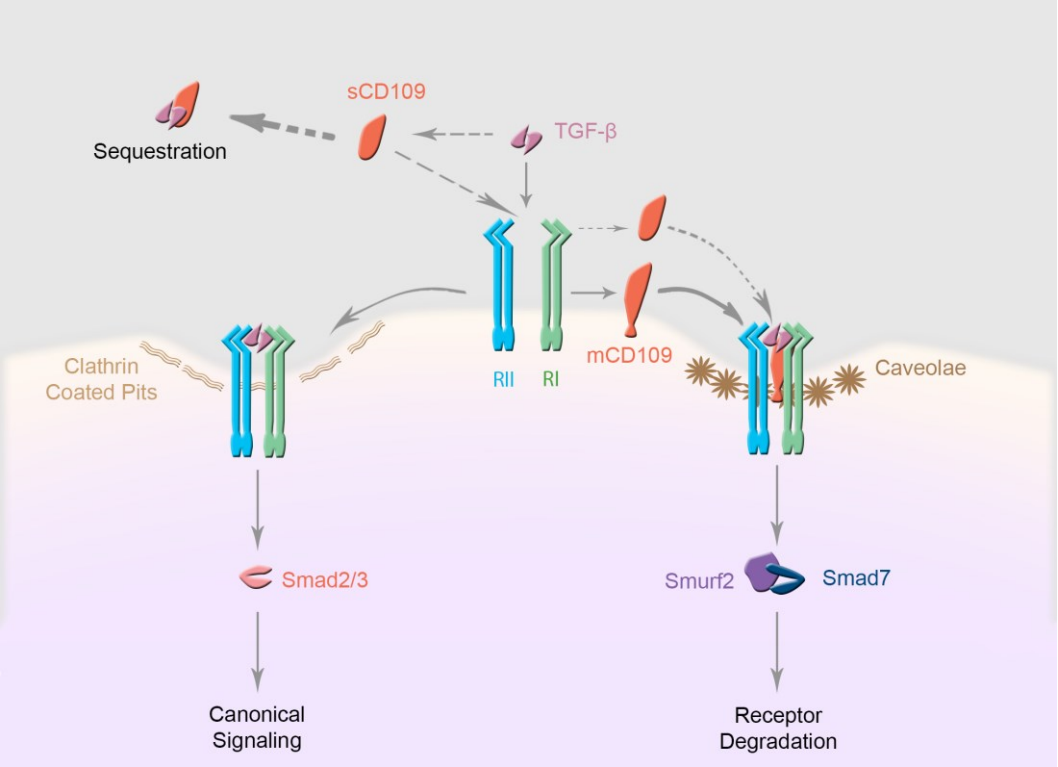
(**A**) HaCaT cells stably overexpressing sCD109 or EV were grown to confluence, serum starved for 24 h to inhibit proliferation and then treated with or without TGF-β1 under serum free conditions. Cells were wounded as described in methods and images were acquired 0 h, 24 h and 48 h post wounding. (**B**) Closed wound area % after 48 h of HaCaT sCD109 as compared to HaCaT EV cells, with values representing the mean of n=3 independent experiments, ± SEM, *: $p<0.05$.

Figure 2.7 Exogenous sCD109 inhibits TGF-β1-induced cell migration



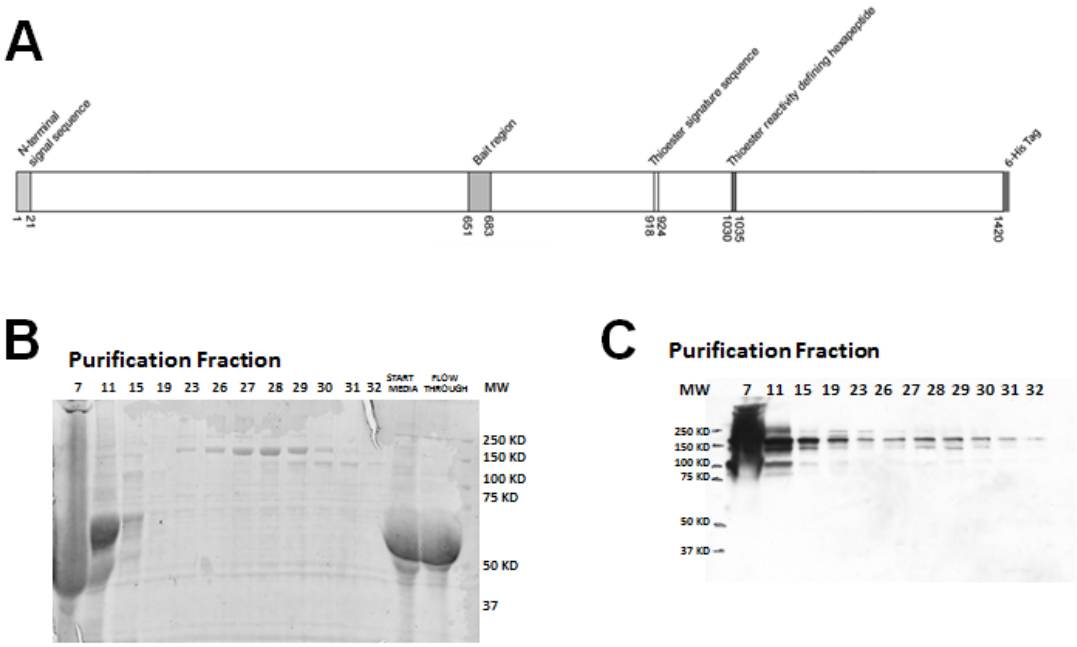
(**A**) HaCaT cells were grown to confluence, serum starved for 24 h to inhibit proliferation and then treated with TGF-β1 in the absence or presence of sCD109 and GST Control. Cells were wounded as described in methods and images were acquired 0 h, 24 h and 48 h post wounding. **(B)** Closed wound area % after 48 h of HaCaT cells treated with or without TGF-β1 in the absence or presence of sCD109-1268 and GST control, was determined by TotalLab quantification software (mean of n=3 independent experiments, ± SEM, *: $p<0.05$).

Figure 2.8 Schematic model of the potential mechanisms by which CD109 may regulate TGF-β signaling



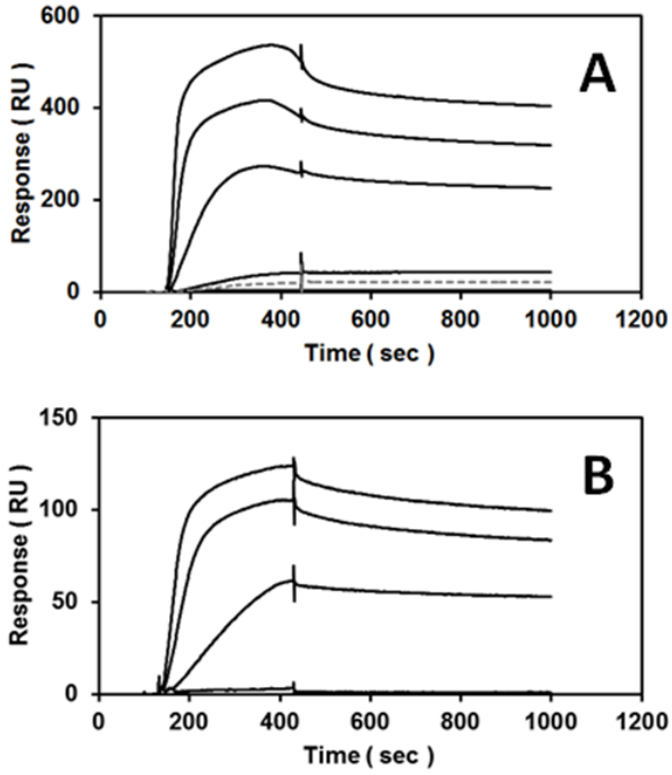
TGF-β receptors can internalize via the clathrin-coated pits or the caveolar pathway. Membrane CD109 promotes TGF-β binding to TGF-β receptors and promotes TGF-β receptor localization to the caveolae, enhancing proteasomal degradation of TβRI and downregulation of TGF-β signaling (Bizet *et al.*, 2011; Bizet *et al.*, 2012). Soluble CD109 antagonizes TGF-β signaling by direct sequestration of the TGF-β ligand away from its signaling receptors, by complexing with TβRI and inducing a conformational change within the TβRI receptor thus inactivating its function, or by promoting TGF-β receptor degradation from complexing with TβRI and TGF-β ligand to facilitate TGF-β receptor internalization and degradation via the caveolar pathway.

Figure 2.9 (Supplementary Fig. 1) Generation and expression of sCD109-1420 protein



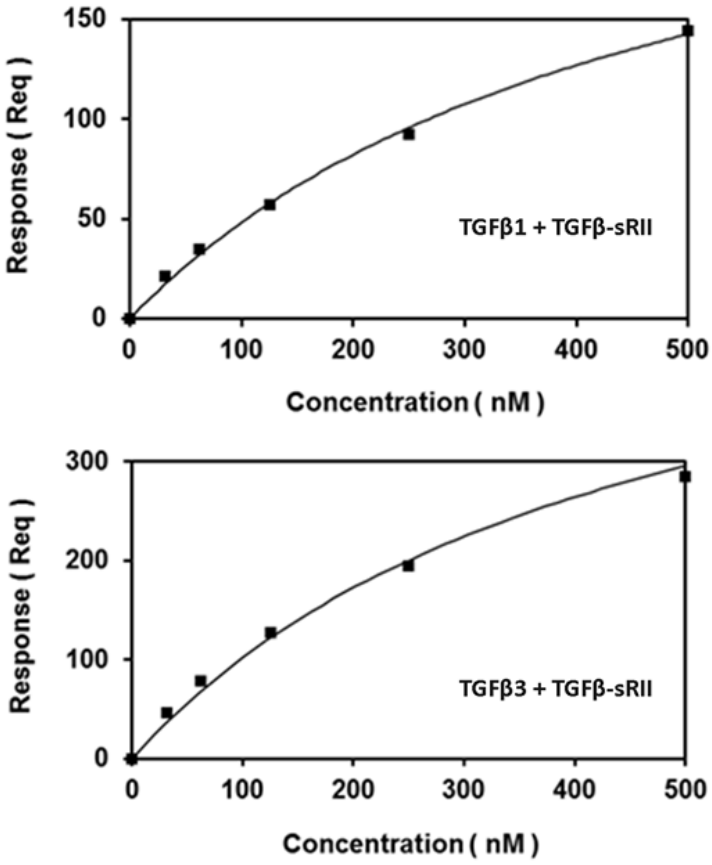
sCD109-1420 cDNA was cloned into a pcDNA3-Gateway vector for stable overexpression in HEK293T cells. Enriched sCD109-1420 cell medium from the HEK293-sCD109-1420 cells was collected, concentrated, and the protein was purified using nickel affinity chromatography followed by size exclusion chromatography. (**A**) Schematic illustration of sCD109-1420 shows CD109 sequence without GPI-anchor region (1-1420aa) and 6-his c-terminal tag to create sCD109-1420 (**B** and **C**) sCD109-1420 purification are passed through nickel affinity chromatography and elution fractions visualized by coomassie blue staining and western blot using anti-CD109 antibodies.

Figure 2.10 (Supplementary Fig. 2) TGF-β1 ligand binds amine-coupled sCD109 with high affinity



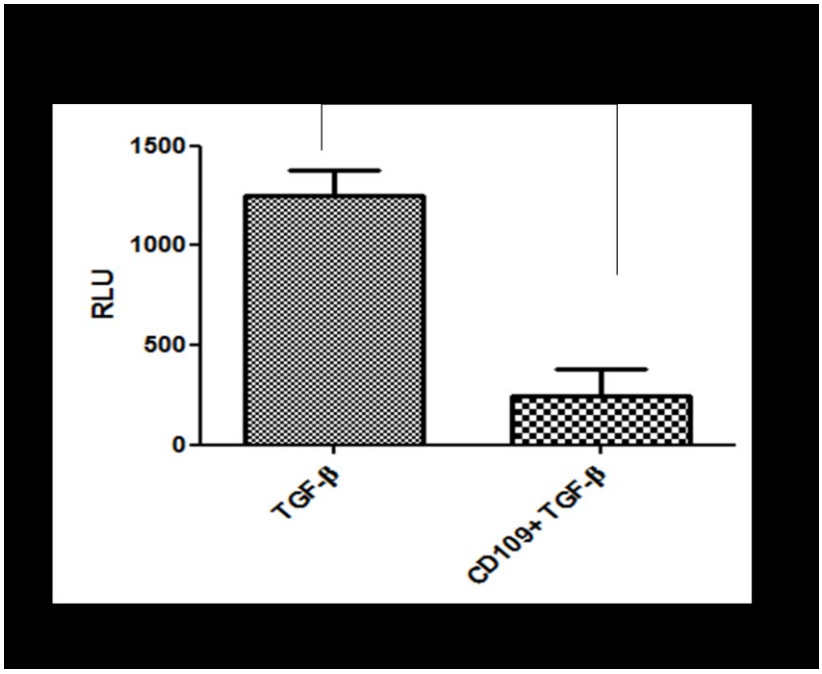
Representative SPR kinetics for amine-coupled sCD109 (**A**) Binding of TGF-β1 (0-100 nM, 2-fold dilution series; or 100 nM BSA, grey dashed line) to high-density, 1200 RU amine-coupled CD109 at 50 μL/min (5 min association + 10 min dissociation). (**B**) Binding of TGF-β1 (0-50 nM, 2-fold dilution series) to low-density, 200 RU amine-coupled CD109 at 25 μL/min (5 min association + 10 min dissociation).

Figure 2.11 (Supplementary Fig. 3) TGFβ-sRII exhibited similar affinities to previous published literature



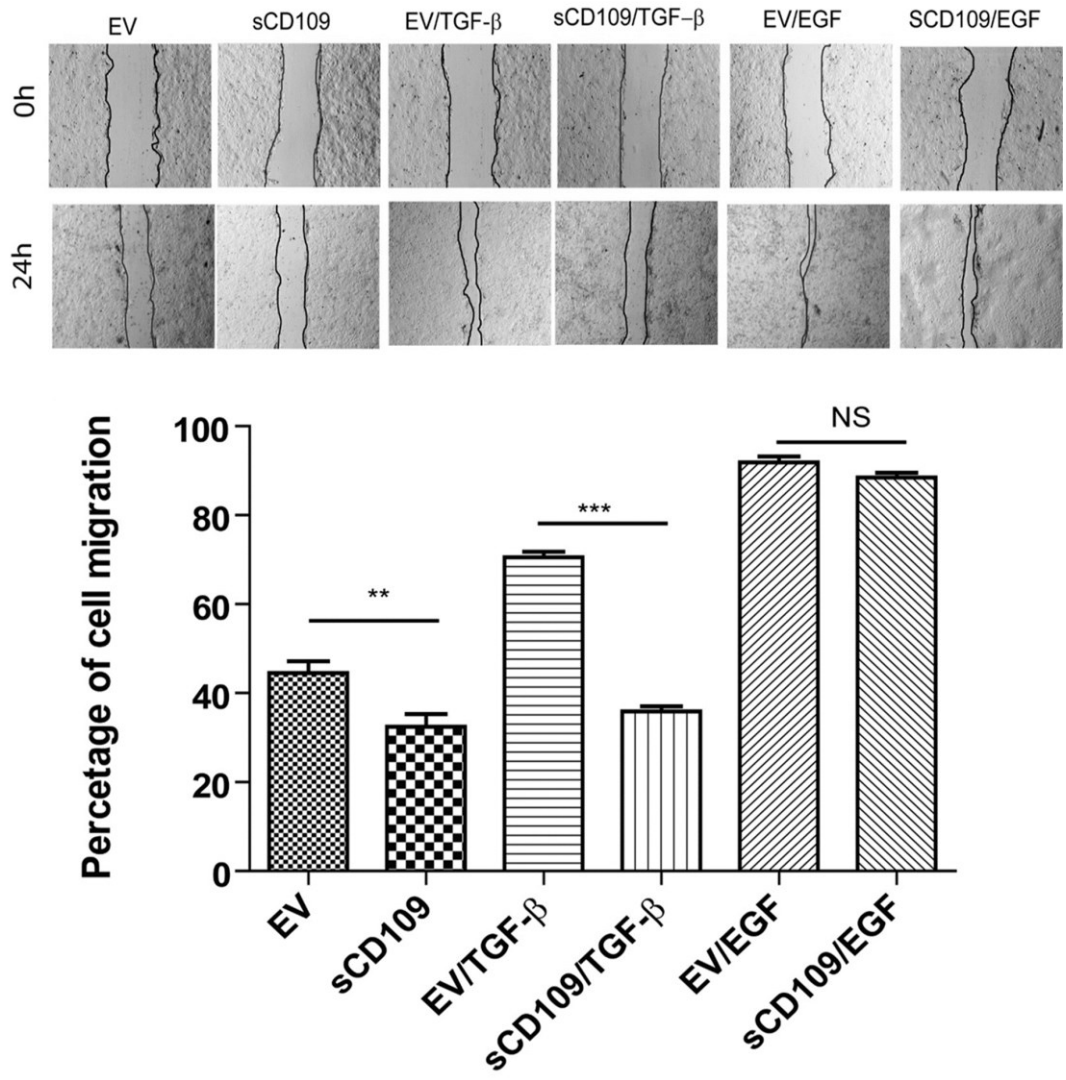
Representative SPR kinetics for TGFβ-sRII binding to amine-coupled TGF-β1 or TGF-β3 (symbols, equilibrium binding responses (Req) plotted as a function of TGFβ-sRII concentration; solid lines, fitting to "steady-state affinity" model in BIAevaluation v4.1 software). K_D values for TGF-β1 (470 +/- 60 nM; n = 3) and TGF-β3 (450 +/- 50 nM; n = 3).

Figure 2.12 (Supplementary Fig. 4) sCD109 binds TGF-β1 in a cell free assay



His-tagged CD109 and TGF-β1 were incubated in culture medium (DMEM) overnight. The CD109-TGF-β1 complex was pulled down using nickel beads and the supernatant containing the unbound TGF-β1 was added to HEK293 cells stably expressing $(CAGA)_{12}$-lux reporter construct. After 18 h, cell lysates were analyzed for luciferase activity and values were plotted. The data shown is representative of 3 independent experiments. *: $p < 0.05$.

Figure 2.13 (Supplementary Fig. 5) sCD109 inhibits TGF-β1-induced but not EGF-induced cell migration



(A) HaCaT cells overexpressing sCD109-1420 and EV were grown to confluence, serum starved for 24 h and then treated in the absence or presence of TGF-β1 (100 pM) or EGF (5 nM). Cells were wounded as described in methods and images were acquired 0 h and 24 h post wounding. **(B)** Closed wound area (%) was determined by TotalLab quantification software (mean of n=3 independent experiments, ± SEM, *: $p<0.05$).

Table 2.1 sCD109 is a negative modulator of [^{125}I]-TGF-β1

LOGK_b (M)	-9,597
LOGα	-0,7507
K_b (M)	2,531E-10
α	0,1776
Standard Error for LOGK_b	0,0853
Standard Error for LOGα	0,03737
95% Confidence Intervals for LOGKB	-9.778 to -9.415
95% Confidence Intervals for LOGα	-0.8303 to -0.6710
95% Confidence Intervals for Kb	1.665e-010 to 3.846e-010
95% Confidence Intervals for α	0.1478 to 0.2133

K_b = equilibrium dissociation constant for the allosteric modulator at the free receptor
α = ternary complex constant, modulator is an inhibitor (α<1) or promoter (α>1)
Values represent the mean of n=3 independent experiments.

Bridging statement between manuscripts

In the previous manuscript, we demonstrate that soluble CD109 inhibits TGF-β-induced signaling and responses potentially through ligand sequestration. These findings suggest that soluble endogenous CD109 is an antagonist of TGF-β signaling and that soluble exogenous recombinant CD109 can inhibit TGF-β signaling and responses.

To determine the specific domain of the soluble CD109 protein that is responsible for TGF-β binding and the potential mechanism by which CD109 negatively regulates TGF-β signaling, we set out to map the TGF-β binding domain of the soluble CD109 protein. We compared the sequences and structural features of CD109 and its homologue α2m, to initially predict the putative TGF-β binding domain of CD109. We measured the ability of overlapping regions of the CD109 protein, CD109 peptides based on the CD109 putative TGF-β binding domain, and site-directed sequence mutants of the CD109 putative TGF-β binding domain, to bind TGF-β. We also measured the ability of CD109 peptides and CD109 site-directed sequence mutants to negatively regulate TGF-β downstream signaling, in order to confirm the activity of the putative TGF-β binding region of the CD109 protein.

3 Mapping of the TGF-β binding domain of CD109

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3.1 Abstract

CD109, a GPI-anchored protein, is a novel TGF-β co-receptor that belongs to the α2m/complement family of proteins. Membrane-anchored CD109 negatively regulates TGF-β signaling and responses by promoting TGF-β receptor internalization and degradation. Soluble CD109 binds TGF-β and inhibits TGF-β signaling and responses in a receptor independent manner. Sequence alignment analysis of CD109 with its homologue α2m allowed us to predict the putative TGF-β binding domain of CD109 to contain amino acids 687-711, which include the WIW hydrophobic sequence and the Glu negative charged acidic residue thought to confer TGF-β binding functionality. To determine the ability of the putative TGF-β binding domain of CD109 to bind TGF-β, we generated CD109 peptides encompassing the TGF-β binding domain and CD109 site-directed mutants of the TGF-β binding domain. Here, we demonstrate that CD109 peptides spanning the putative TGF-β binding domain bind TGF-β as determined by surface plasmon resonance, cell-based radioligand binding, and affinity labeling competition assay. Moreover, we show that CD109 peptides spanning the putative TGF-β binding domain inhibit TGF-β-induced Smad phosphorylation, transcriptional activity, and cell migration. Furthermore, we demonstrate that site-directed mutations of the putative TGF-β binding domain abrogate inhibition of TGF-β-induced transcriptional activity. Overall, our results suggest that the CD109 putative binding

region is responsible for binding TGF-β and negatively regulating TGF-β signaling and responses.

3.2 Introduction

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine involved in a broad range of biological processes including cell growth and differentiation, ECM deposition, cell adhesion and immunomodulation (Massague, 2012). There are three mammalian TGF-β isoforms: TGF-β1, TGF-β2, and TGF-β3 isoforms which display similar functions *in vitro* but play distinct roles *in vivo* (Proetzel *et al.*, 1995; Sanford *et al.*, 1997; Shull *et al.*, 1992).

TGF-β signaling is transduced by a pair of transmembrane serine/threonine kinases known as type I (TβRI) and type II TGF-β (TβRII) signaling receptors. The TGF-β ligand binds to TβRII, which transphosphorylates the TβRI receptor, initiating an intracellular phosphorylation cascade where the activated TβRI phosphorylates receptor-regulated Smads (R-Smad – Smad2 and Smad3) which subsequently bind common-mediator Smads (Co-Smad – Smad4) forming R-Smad/Co-Smad complexes (Shi and Massague, 2003). The R-Smad/Co-Smad complexes (Smad 2/3/4) then translocate into the nucleus to regulate gene expression through interactions with transcription factors, co-activators and co-repressors (Gaarenstroom and Hill, 2014).

In addition to the TβRI and TβRII signaling receptors, many cell types express TGF-β co-receptors known as betaglycan and endoglin that bind TGF-β and modulate TGF-β signaling in a cell-specific manner (Bernabeu *et al.*, 2009). Our group has identified CD109, a 180-kDa glycosylphosphatidylinositol (GPI)-anchored protein which belongs to the α2-macroglobulin complement superfamily, as a novel TGF-β co-receptor (Lin *et al.*, 2002). We have also reported that membrane anchored CD109 binds TGF-β1 ligand with high affinity, forms a heteromeric complex with the TGF-β signaling receptors, and inhibits TGF-β signaling and responses through caveolae-mediated internalization of TGF-β receptors and receptor degradation via a SMAD7/Smurf2-mediated mechanism (Bizet *et al.*, 2011; Bizet *et al.*, 2012). Furthermore, our group has demonstrated that CD109 can be endogenously released from the cell surface by PIPLC enzymes, which can bind TGF-β1 with high affinity (Tam *et al.*, 2001). Most recently, our group has reported that exogenous soluble CD109 can bind all TGF-β isoforms and antagonize TGF-β signaling and responses (Li *et al.*, 2015).

Aberrant TGF-β signaling plays a central role in many pathologies including tissue fibrosis and cancer development and progression (Prud'homme, 2007). To determine the specific mechanisms by which soluble CD109 may regulate TGF-β signaling and to define the specific domain of soluble CD109 responsible for TGF-β binding, we sought to map the TGF-β binding domain of CD109. To this end, we compared CD109 sequence and structural features to alpha-2-macroglobulin (α2m), as both CD109 and α2m belong to the same family of thioester containing proteins

(Arandjelovic *et al.*, 2006). Based on homology alignment with the α2m protein-interaction-domain, we predicted that the putative TGF-β binding domain of CD109 to encompass amino acids 687-711 (Figure 3.1), which includes a WIW hydrophobic sequence and Glu acidic residue thought to confer TGF-β binding functionality similar to that of TβRII and α2m (Arandjelovic *et al.*, 2003).

In the current study, we generated CD109 derived peptides based on the putative binding domain of the CD109 protein and CD109 site-directed mutations to determine the TGF-β binding domain of CD109. Our results indicate that peptides spanning the putative binding domain of TGF-β can bind all three mammalian TGF-β isoforms and modulate TGF-β signaling and TGF-β-induced cellular responses. Moreover, our results also show that CD109 site-directed mutations of the putative binding domain of TGF-β can abrogate CD109 inhibition of TGF-β induced transcriptional activity. Together, our results indicate that the CD109 region encompassing amino acids 646-766 is at least partially responsible for binding TGF-β and inhibiting TGF-β signaling and responses.

3.3 Materials and Methods

Cell lines

The human keratinocyte cell line HaCaT (provided by P. Boukamp Heidelberg, Germany) and HEK293-$(CAGA)_{12}$ cells (provided by T. Thompson, Cincinnati, USA) were cultured as described previously (Finnson *et al.*, 2006).

CD109 protein and peptides

Human recombinant soluble CD109 proteins sCD109-1268 and sCD109-1420 were either purchased from R&D Systems (#4385-CD, CD109 amino acid sequence 21-1268) or produced using HEK293T mammalian protein production (sCD109-1268 and sCD109-1420) as previously described (Li *et al.*, 2015). GST-tag CD109 peptides overlapping the CD109 protein were created using a pDEST15-Gateway vector (Invitrogen, USA), bacterially expressed, and affinity purified (Smith and Johnson, 1988) (Figure 3.1). Bacterially expressed His-tag CD109 Peptide A (amino acid sequence 606-766), CD109 Peptide B (amino acid sequence 646-746), CD109 Peptide Y (amino acid sequence 606-725), and CD109 Peptide Z (amino acid sequence 646-766) were commercially generated by Genscript (Piscataway, USA) (Figure 3.1). Chemically synthesized CD109 Peptide X (amino acid sequence 680-719) was purchased from CanPeptide (Montreal, Canada) (Figure 3.1).

CD109 site-directed mutations

The full-length human CD109 cDNA in pCMVSport6 (Finnson *et al.*, 2006) with site-directed mutations at the putative TGF-β binding domain (CD109 WT amino acid sequence 687-711 5'-RKHFPETWIWLDTNMGYRIQEFEVTVPDSITS-3') were commercially generated by Mutagenex (Hillsborough, USA). The CD109 site-directed mutations were designed based on the CD109 polarity reversal method 5'-RKHFPRTWIWLRTNMGYRIYQRFRVTVPDSITS-3' (Arandjelovic *et al.*, 2006) (Table 3.1).

Surface plasmon resonance assay

Binding of TGF-β1 to CD109 peptides (CD109 Peptide A, B, X, Y, Z) were examined using label-free, real-time BIACORE 3000 instrumentation as previously described (Li *et al.*, 2015). Briefly, resuspended TGF-β1 were diluted to 5 μg/mL for immobilization using the Biacore Amine Coupling Kit (550 RU); corresponding reference surfaces were prepared in the absence of any ligand. For single-cycle kinetic analysis, CD109 peptides were injected over reference and isoform-immobilized CM5 sensor chip surfaces at 5 μL/min. For multiple-cycle kinetic analysis, CD109 peptides were injected over reference and isoform-immobilized CM5 sensor chip surfaces at 5 μL/min (6-min association; 10-min dissociation). Between sample injections, sensor chip surfaces were regenerated at 50 μL/min using Pierce Gentle Elution buffer containing Triton X-100. Apparent equilibrium dissociation constants (K_D) for the CD109 peptides were estimated by global fitting the data using the fit separate k_a/k_d model in the BIA evaluation v4.1 software (Groppe *et al.*, 2008).

Radioligand binding assay

Carrier-free TGF-β1 (Genzyme) was radiolabeled with Iodine-125 (Perkin Elmer) as previously described (Philip and O'Connor-McCourt, 1991). Radioligand binding assays were performed as previously described (Li *et al.*, 2015). Briefly, HaCaT cells were incubated with [^{125}I]-TGF-β1 (10-200 pM) in the absence or presence of non-labelled TGF-β1 (100X concentration of [^{125}I]-TGF-β1) for 3 h at 4°C; cell membranes were solubilized and analyzed using the non-linear fit model

in GraphPad Prism 5 to determine the equilibrium binding constant (K_d) of [^{125}I]-TGF-β1 for its receptors. Afterwards, cells were incubated with 50 pM ^{I125}TGF-β1 in the absence or presence of soluble CD109 protein (0.1-100 nM of [^{125}I]-TGF-β1) and CD109 peptides (Peptide A, B, X, Y, Z) (1-2000 nM of [^{125}I]-TGF-β1) for 3 h at 4°C; cell membranes were solubilized and analyzed using the alpha ternary complex model in GraphPad Prism 5 to determine the equilibrium dissociation constant for the allosteric modulator at the free receptor (K_b) of soluble CD109 protein and CD109 peptides on [^{125}I]-TGF-β1.

Affinity labeling assay

Affinity labeling assays were performed as previously described (Tam *et al.*, 2001). Briefly, HaCaT cells were incubated with 100 pM [^{125}I]-TGF-β1 in the absence or presence of non-labelled TGF-β1 (10 nM), soluble CD109 (10 nM) and CD109 Peptide A, B, X, Y, Z (10, 50, 100 nM) for 3 h at 4°C; ligand-[^{125}I]-TGF-β1 complexes were formed using a non-permeable cross-linker BS^3; cell membranes were solubilized; membrane extracts were visualized and analyzed by SDS-PAGE/autoradiography; densitometry of SDS-PAGE/autoradiography was performed using the TotalLab data analysis software using the arbitrary densitometric intensity of T*β*RI (Nonlinear Dynamics).

Western blot analysis

HaCaT cells were treated with or without TGF-β1 (0, 10, 100 pM) in the absence or presence of soluble CD109 (100 nM), CD109 Peptide A, B, X, Y, Z (100 nM)

and GST Control (100 nM). Cells were incubated with TGF-β1 for 45 min (to determine phosphoSmad3 levels) and for 18 h (to determine fibronectin and PAI-1 levels) under serum-free conditions as previously described (Finnson *et al.*, 2006). Cell lysates were analyzed by western blot analysis using the following antibodies: anti-phosphoSmad3 (FL-425, Santa Cruz Biotechnology), anti-PAI-1 (BD Biosciences), anti-fibronectin (BD Biosciences), anti-β-actin (Santa Cruz Biotechnology), mouse monoclonal anti-CD109 (TEA 2/16, BD Biosciences) (C9, Santa Cruz Biotechnology), as previously described (Tam *et al.*, 2003).

$CAGA_{12}$-luc assay

Luciferase reporter assays were performed with the HEK293-$(CAGA)_{12}$ cell line that stably expresses the $(CAGA)_{12}$-luciferase reporter gene as previously described (Cash *et al.*, 2012). Briefly, HEK293-$(CAGA)_{12}$ cells were grown in 24-well tissue culture plates for 24 h then incubated with or without TGF-β1 (10, 50, 100 pM) in the absence or presence of soluble CD109 protein (100 nM), CD109 Peptide A, B, X, Y, Z (100 nM) and GST Control (100 nM) for 24 h under serum-free conditions. Cell lysates were analyzed for luciferase activity as previously described (Finnson *et al.*, 2006). HEK293-$(CAGA)_{12}$ cells were also grown in 24-well tissue culture plates for 24 h then transfected with pCMVSport6 CD109 WT, pCMVSport6 CD109 Alanine Mutant, pCMVSport6CD109 Reverse Polarity, and pCMVSport6 EV constructs as previously described (Dennler *et al.*, 1999; Tam *et al.*, 2003). Cells were allowed to recover for 24 h under serum free conditions after transfection and then incubated with TGF-β1 (100 pM) for 24 h under serum-free conditions. Cell

lysates were analyzed for luciferase activity as previously described (Finnson *et al.*, 2006).

Cell migration assay

Cell migration assays were performed as previously described (Li *et al.*, 2015). Briefly, HaCaT cells were grown to confluency in 6-well tissue culture plates and then pre-incubated with serum-free medium for 24 h, to minimalize cell proliferation. The monolayer of HaCaT cells was scratched vertically across the center of the wells with a sterile 200 µL pipette tip to create a cell-free line. Cell medium was aspirated and washed 3 times to remove cellular debris. HaCaT cells were then treated with TGF-β1 (100, 200, 500 pM) in the absence or presence of soluble CD109 protein (100 nM), CD109 Peptide A, B, X, Y, Z (100 nM) under serum free conditions. Photographs were taken at 0 h, 24 h, and 48 h after scratch. Migration was quantified using TotalLab data analysis software (Nonlinear Dynamics) with data expressed as percentage of the scratch area filled by migrating cells at 48 h post scratch.

Statistical analysis

Numerical results are represented as means of $n \geq 3$ independent experiments ± standard error of the mean (SEM). For statistical tests where only two data sets were being compared, an unpaired Student's t test (two-tailed) was used where $P<0.05$ was deemed statistically significant. When more than two data sets were compared,

one- or two-way analysis of variance (ANOVA) tests were used where $P<0.05$ was deemed statistically significant.

3.4 Results

CD109 peptides inhibit TGF-β1 binding to TGF-β signaling receptors on keratinocytes

To determine if CD109 GST-tagged peptides spanning the CD109 protein can block TGF-β binding to its signaling receptors on keratinocytes, affinity labeling assays were performed. Affinity labeling of keratinocytes with [^{125}I]TGF-β1 and CD109 GST-tagged peptides demonstrate that peptides encompassing the putative TGF-β binding domain are able to inhibit [^{125}I]TGF-β1 binding to the TGF-β signaling receptors whereas CD109 GST peptides not encompassing the putative TGF-β binding domain are unable to inhibit [^{125}I]TGF-β1 binding to the TGF-β signaling receptors (Supplementary Figure 1). Furthermore, to identify if CD109 His-tagged peptides derived from the putative TGF-β binding domain can block TGF-β binding to its signaling receptors on keratinocytes, affinity labeling assays were performed. Affinity labeling of keratinocytes with [^{125}I]TGF-β1 and CD109 peptides (except Peptide X) indicate that peptides encompassing the putative TGF-β binding domain are able to significantly inhibit [^{125}I]TGF-β1 binding to the TGF-β signaling receptors ($p< 0.05$) (Figure 3.2A and 3.2B).

CD109 peptides inhibit TGF-β1 binding to keratinocyte cell surface

To ascertain if CD109 peptides can inhibit TGF-β binding to the cell surface, radioligand binding studies were performed. Saturation analysis of [^{125}I]TGF-β1 specific binding to the cell surface of HaCaT cells yielded an equilibrium binding constant (K_d) value of 0.096 nM using the non-linear fit model in the GraphPad Prism 5 (Supplementary Figure 2). As the labeled ^{I125}TGF-β1 and the nonlabeled CD109 peptides are acting via different receptor binding sites, the ternary complex model was used to determine ability of CD109 peptides to modulate [^{125}I]TGF-β1 binding to its receptors (Christopoulos and Kenakin, 2002) For the radioligand binding experiments, HaCaT cells were incubated with [^{125}I]TGF-β1 along with nonlabeled soluble CD109 (positive control) or nonlabeled CD109 Peptide A, B, X, Y, Z. Results shown in Figure 3.3 demonstrate that soluble CD109 and CD109 peptides can inhibit [^{125}I]TGF-β1 binding to its receptors (except Peptide X). Statistical analysis of the ligand binding curve demonstrate inhibition with an equilibrium dissociation constant (K_b) of 3 nM for soluble CD109, 152 nM for CD109 Peptide A, 808 nM for Peptide Z, 44 nM for Peptide Y, and 128 nM for Peptide B (Figure 3.3).

CD109 peptides bind to all TGF-β isoforms

To establish the binding specificity and kinetics of CD109 peptides to TGF-β, SPR single-cycle (Figure 3.4) and multiple-cycle (Supplementary Figure 3) kinetic analysis were performed. SPR allowed detailed analysis of the interaction between CD109 peptides (CD109 Peptide A, X, Y, Z) and label-free TGF-β (TGF-β1, -2, -3) in real-time. Specific binding of TGF-β1 to immobilized CD109 peptides were

detected for CD109 Peptide A (K_D = 3 uM), CD109 Peptide Y (K_D = 9 uM), CD109 Peptide Z (K_D = 12 uM), and CD109 Peptide X (K_D = 43 uM) using single-cycle kinetic analysis (Figure 3.4). Analysis of the larger CD109 Peptide A binding showed that it binds TGF-β1 with distinct slow-on and slow-off kinetics whereas analysis of the smaller CD109 Peptide X binding showed that it binds TGF-β1 with common fast-on and fast-off kinetics (Supplementary Figure 3). Titration of BSA (negative binding control) over the immobilized TGF-β surfaces revealed no significant binding responses as expected (data not shown).

CD109 peptides inhibit TGF-β1-induced signaling and transcriptional activity

Effect of CD109 peptides on TGF-β signaling and transcriptional activity was examined by determining TGF-β1-induced Smad3 phosphorylation and Smad3-driven transcriptional activity, respectively. Densitometric analysis of the western blot data (pSmad3 intensity normalized to tSmad3 intensity in arbitrary units) indicate that, similarly to soluble CD109 protein, CD109 peptides significantly ($P<0.05$) decreased TGF-β-induced phosphoSmad3 levels (with the exception of Peptide X) (Figure 3.5A-3.5F). The effect of CD109 peptides on TGF-β1-induced transcriptional activity was determined by using the HEK293-$(CAGA)_{12}$ cell line that stably expresses the $(CAGA)_{12}$-luciferase reporter gene, a Smad3-specific synthetic construct containing 12 repeats of CAGA. Results shown in Figure 3.6 indicate that treatment with CD109 peptides (except Peptide X) result in a significant reduction ($P<0.05$) in TGF-β1-induced stimulation of $(CAGA)_{12}$-luc

activity in HaCaT cells, and similarly to soluble CD109, when compared with cells treated with control GST protein.

CD109 peptides decrease TGF-β1-induced ECM protein production

To determine if CD109 peptides can inhibit TGF-β1-induced ECM synthesis, western blot analysis of fibronectin and PAI-1 expression were performed. Results shown in Figure 3.7 demonstrate that while untreated HaCaT cells show a robust dose-dependent increase in fibronectin and PAI-1 protein expression in response to TGF-β1 treatment, treatment of HaCaT cells with CD109 peptides (except Peptide X) leads to a significant decrease ($P<0.05$) in TGF-β1-induced ECM protein levels (Figure 3.7) as compared to untreated HaCaT cells. Densitometric analysis of the fibronectin western blot data (expressed as mean of fibronectin band over actin band in arbitrary intensity units) indicate that CD109 peptides significantly ($P<0.05$) decreased TGF-β-induced ECM levels (with the exception of Peptide X) (Figure 3.7).

Site-directed mutations to the predicated TGF-β binding domain of CD109 abrogate inhibition of TGF-β1-induced transcriptional activity

The effect of site-directed mutations to the predicted TGF-β binding domain of CD109 on TGF-β1-induced transcriptional activity was determined by $CAGA_{12}$-luc reporter assay. Results from the $CAGA_{12}$-luc assays indicate that HEK293-$(CAGA)_{12}$ cells transfected with CD109 reverse polarity mutant plasmid can abrogate TGF-β1-induced transcriptional activity as compared to CD109 wild type plasmid (Supplementary Figure 4).

CD109 peptides inhibit TGF-β1-induced keratinocyte cell migration

Because TGF-β is an potent enhancer of keratinocyte migration (Gailit *et al.*, 1994), we examined the effect of CD109 peptides on TGF-β1-induced wound closure in monolayers of HaCaT cells. A significant decrease ($P<0.05$) in wound closure at 24 h post scratch was observed in HaCaT cells treated with CD109 peptides (except Peptide X) compared to untreated HaCaT cells, in the presence of TGF-β1 (Figure 3.8). As cells were pre-incubated under serum free conditions for 24 h and the assay was performed under serum free conditions, effects due to cell proliferation were considered to be minimal (Boukamp *et al.*, 1988).

CD109 contains a protease cleavage site at a predicted furin-cleavage site

Furin-cleavage has been reported to play a role in CD109-mediated TGF-β signaling (Hagiwara *et al.*, 2010). Western blot analysis of purified recombinant CD109-1420 which has a furin cleavage site at CD109 amino acids 1270-1273 yielded a fragment with identical mobility as recombinant CD109-1268, the fragment predicted to form by furin cleavage of CD109-1420 and another band at 25 kDa as expected, consistent with the notion that CD109-1420 is cleaved by furin (Supplementary Figure 11).

3.5 Discussion

Our group has previously reported that membrane-anchored CD109, a GPI-anchored TGF-β co-receptor, binds TGF-β and downregulates TGF-β signaling by

promoting TGF-β receptor internalization and degradation (Bizet *et al.*, 2011; Bizet *et al.*, 2012; Finnson *et al.*, 2006). Our group has also reported that endogenous cell-released CD109 and exogenous soluble recombinant CD109 can bind TGF-β and inhibit TGF-β signaling and responses in a receptor independent manner (Li *et al.*, 2015; Tam *et al.*, 2001). However, the mechanism by which sCD109 binds and inhibits TGF-β signaling and responses has remained unclear. In our current study, we show that CD109 peptides spanning the putative TGF-β binding domain bind TGF-β and inhibit TGF-β signaling and responses. Furthermore, we demonstrate that site-directed mutation of the putative TGF-β binding domain abrogates CD109-mediated inhibition of TGF-β-induced transcriptional activity. Collectively, our results suggest that a sequence encompassing the smallest active peptide, CD109 Peptide B (CD109 amino acid sequence 646-746), is at least partially responsible for binding TGF-β and inhibiting TGF-β signaling and responses.

In our current study, we have used surface plasmon resonance (SPR) single-cycle and multiple-cycle kinetic analysis to demonstrate that CD109 peptides spanning the predicted TGF-β binding domain can bind TGF-β ligands with affinities comparable to the soluble CD109 protein. In our results, we have also demonstrated that the larger CD109 Peptide A exhibits association and dissociation kinetics similar to that of the sCD109 protein (slow-on/slow-off binding kinetics) (Li *et al.*, 2015), whereas the smaller CD109 Peptide X exhibits association and dissociation kinetics similar to that of the soluble type II receptor ectodomain (De Crescenzo *et al.*, 2001). The differences in binding kinetics and affinities between larger and

smaller CD109 peptides suggest that sequences surrounding the putative TGF-β binding domain may provide additional functionality and/or stability for TGF-β binding interactions. Furthermore, we have used radioligand binding assays and affinity labeling studies to demonstrate that CD109 peptides can not only bind TGF-β ligands in a specific, dose-dependent manner but also negatively regulate TGF-β binding to its signaling receptors by potential sequestration. Interestingly, CD109 Peptide X failed to significantly inhibit TGF-β binding to the cell surface despite having exhibited moderate but significant binding affinity for TGF-β in SPR assays.

Based on our current findings on TGF-β-induced transcriptional activity, we demonstrated that CD109 peptides can not only inhibit TGF-β ligand binding to its cell surface receptors but also negatively regulate TGF-β-induced downstream effects including Smad signaling, transcriptional activity, ECM protein production, and *in vitro* cell migration. CD109 Peptide X was again unable to negatively regulate TGF-β-induced downstream effects. Our findings on the inability of CD109 Peptide X to negatively regulate TGF-β signaling and responses suggest that there may be additional elements required to facilitate CD109-TGF-β interactions and/or that Peptide X may be subjected to degradation or aggregation due to its highly hydrophobic and acidic sequence structure. Furthermore, our findings from site-directed mutational analysis demonstrate that mutations to the putative TGF-β binding domain of CD109 can abrogate TGF-β1-induced transcriptional activity, which is consistent with previous site-directed mutagenesis studies of the CD109 homologue α2m (Arandjelovic *et al.*, 2006). Interestingly,

despite sharing similar sequence homologies, α2m and CD109 exhibit different TGF-β isoform specificity with α2m peptides corresponding to its protein-interaction-domain-1 (PID-1) binding only TGF-β1 and -β2 but not TGF-β3 (Arandjelovic *et al.*, 2003; Webb *et al.*, 1998) while CD109 peptides spanning the predicted TGF-β binding domain binding all three TGF-β isoforms. The TGF-β isoform specificity differences between α2m and CD109 peptides suggest that the sequence variation between the α2m PID-1 and the CD109 putative binding domain may determine TGF-β isoform specificity. Nevertheless, it is important to recognize that previous α2m peptide binding experiments were performed using cell-based assays whereas our CD109 peptide experiments were performed in a cell-free SPR environment without the presence of other binding components on the cell surface or extracellular milieu.

Recent findings have suggested that CD109 can modulate TGF-β signaling independent of TGF-β sequestration (Bizet *et al.*, 2012; Hagiwara *et al.*, 2010). Studies on furin-mediated processing of the CD109 protein fragments (Hagiwara *et al.*, 2010) and studies on CD109-mediated internalization and degradation of TβRI (Bizet *et al.*, 2012) have demonstrated that CD109 can regulate TGF-β signaling and responses independent of TGF-β ligand sequestration. While the mechanisms of how CD109 negatively regulates TGF-β signaling and responses through receptor turnover and degradation are clearly defined (Bizet *et al.*, 2011; Bizet *et al.*, 2012), the mechanism(s) of how furin-mediated processing of the CD109 protein into non-covalently linked fragments regulates TGF-β signaling and

responses remain unresolved. While findings from our current study support the possibility of non-covalently linked CD109 fragments as distinct CD109 fragments of ~20-30 kDa and ~150-180 kDa were detected using anti-6-His c-terminal antibody on sCD109-1420 and sCD109-1268 recombinant proteins, (Figure 3.19), further investigation is required to elucidate the specific mechanism(s) of how the non-covalently linked CD109 fragments are able negatively regulate TGF-β signaling and responses. These findings are of interest as studies have reported that furin cleavage and subsequent CD109 release from the cell surface may play a role in CD109-mediated inhibition of TGF-β signaling (Hagiwara *et al.*, 2010).

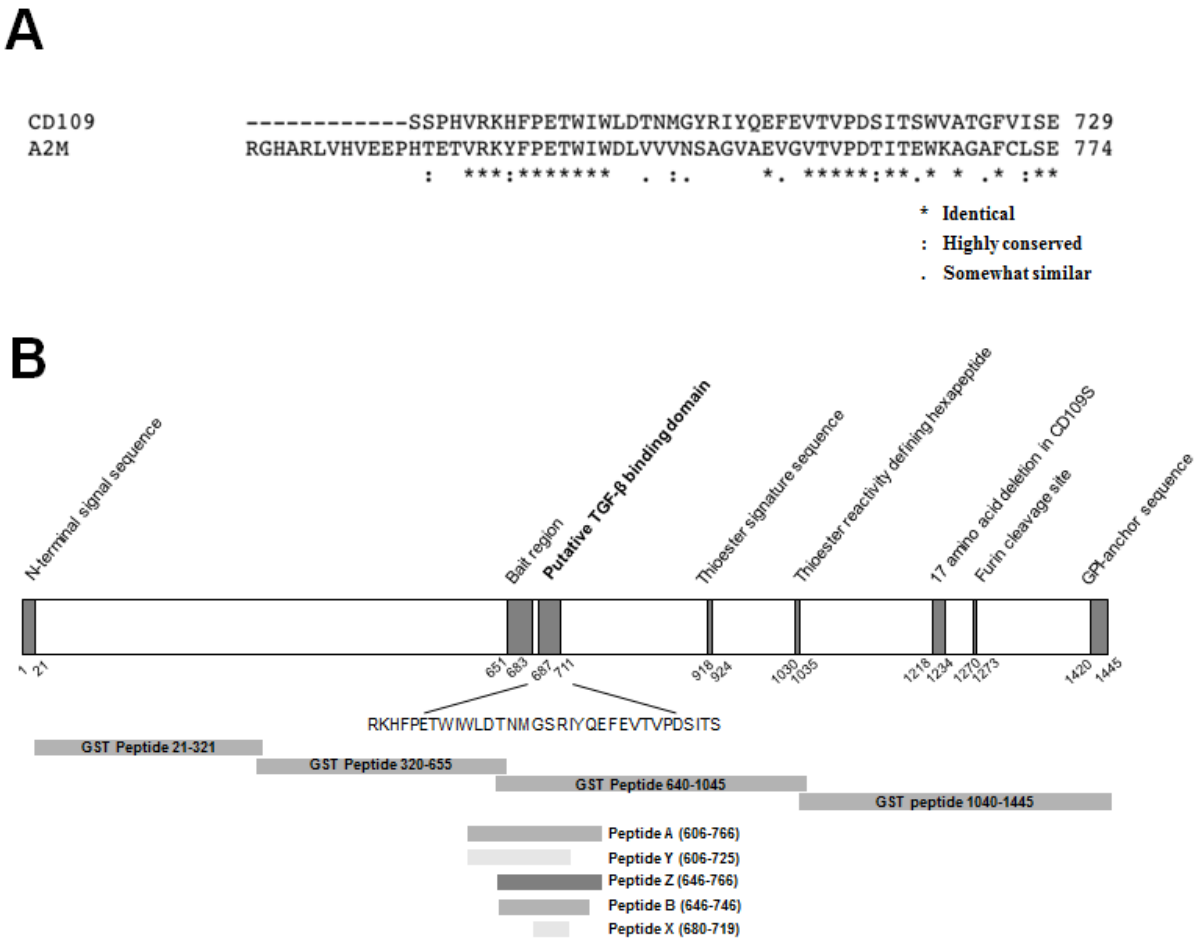
Previous studies have reported that CD109 plays an active role in immunoregulation, cancer, and skin diseases (Ertel *et al.*, 2005; Hagiwara *et al.*, 2010; Hockla *et al.*, 2009; Litvinov *et al.*, 2011). The loss of CD109's negative regulation of TGF-β signaling may contribute to aberrant TGF-β action. Our present findings suggest that the predicted TGF-β binding region of CD109 is at least partially responsible for binding TGF-β and may play a role negatively regulating TGF-β signaling and responses. The relative contribution of soluble CD109 mediated ligand sequestration vs membrane-anchored CD109-mediated receptor turnover in regulating TGF-β signaling and action remains to be determined. Nevertheless, the findings from the present study suggest that ligand sequestration by soluble CD109 may play an important role in regulating TGF-β signaling and that the predicted TGF-β binding domain of soluble CD109 is at least partially responsible for its action.

3.6 Acknowledgements

We would like to thank the laboratory of Tom Thompson (University of Cincinnati) and the laboratory of Dieter Reinhardt (McGill University) for helpful discussions. The study was supported by a Canadian Institutes of Health Research (CIHR) operating grant and a CIHR Proof of Principle grant to AP. The McGill SPR Facility thanks the Canada Foundation for Innovation for infrastructure support grant and the CIHR for research resource Grant to Sheldon Biotechnology Centre.

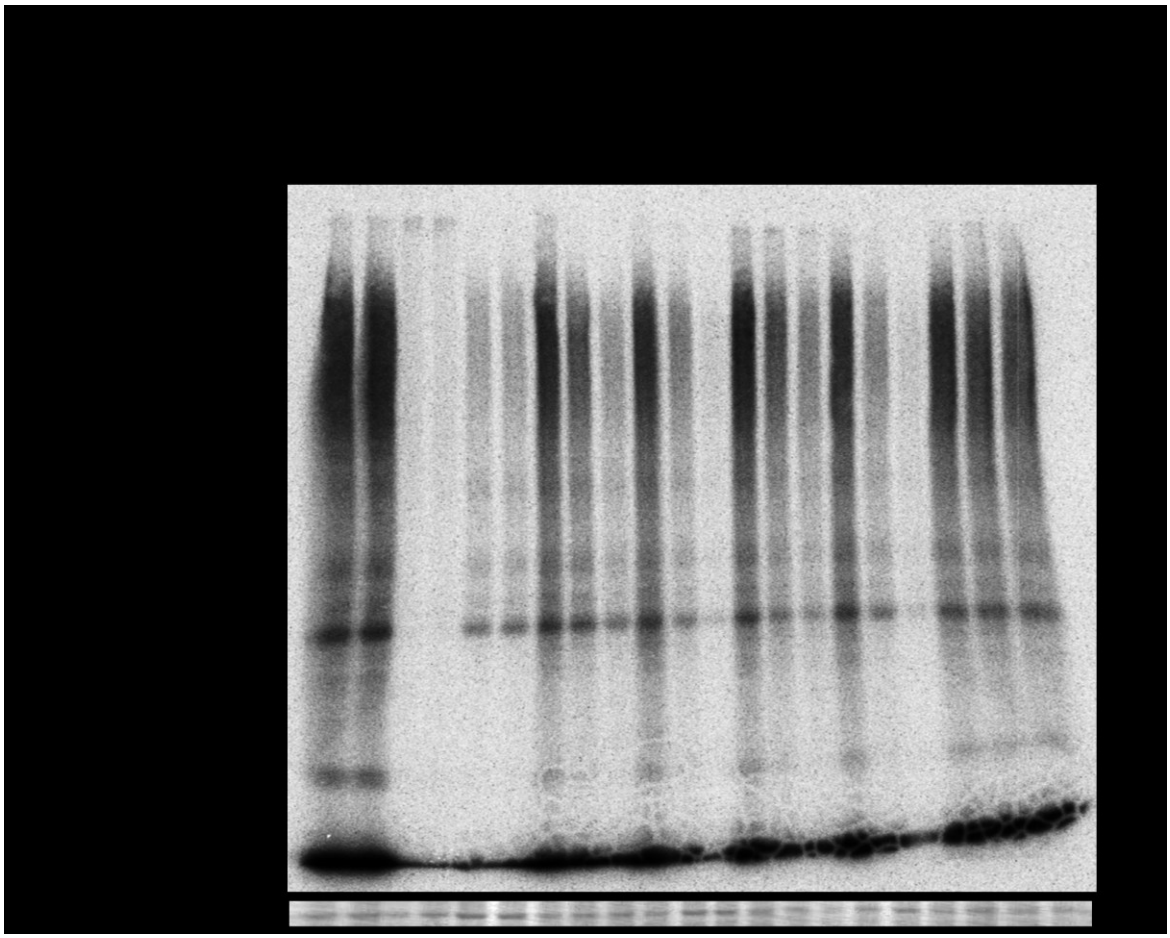
3.7 Figures

Figure 3.1 Schematic representation of the putative TGF-β binding domain of CD109



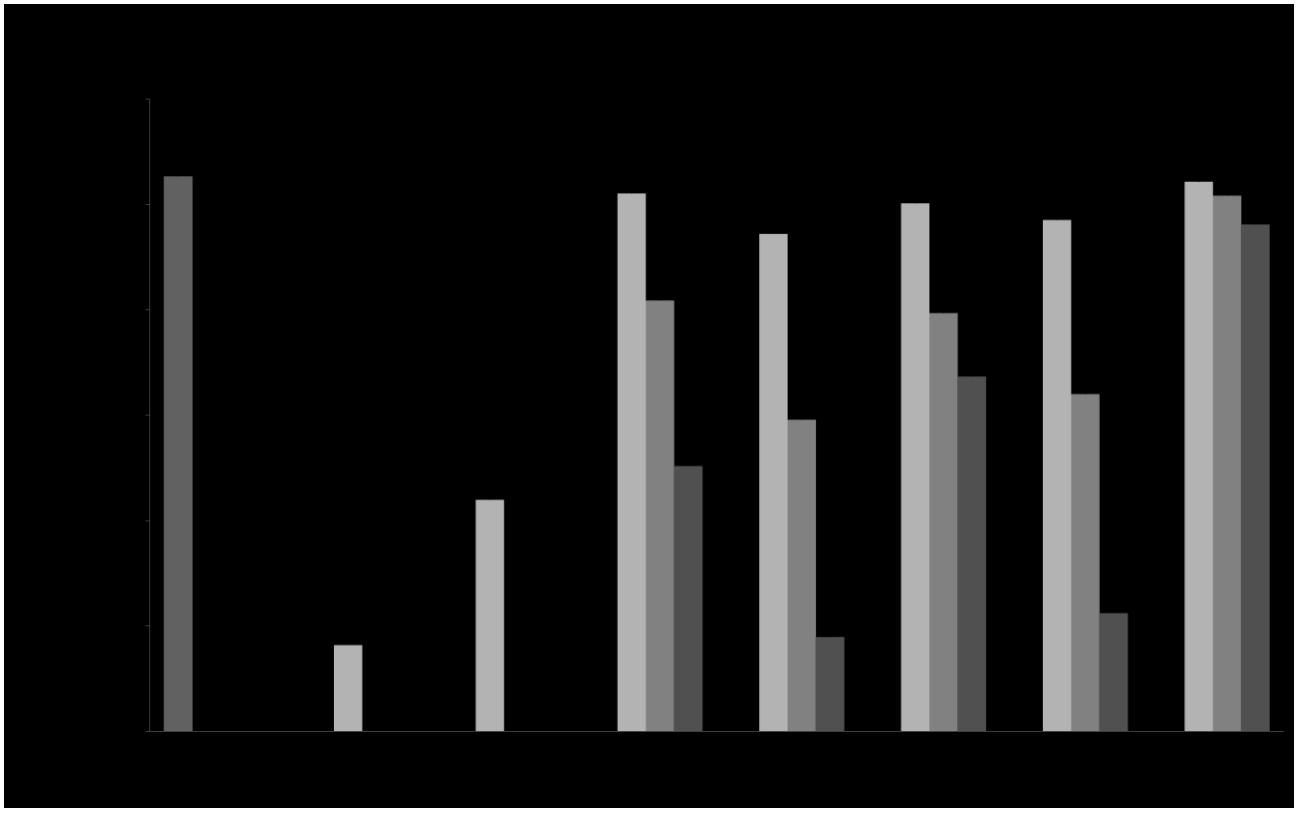
(**A**) Sequence alignment between CD109 and the α2m protein-interaction domain 1(PID-1) allows us to predict that the putative TGF-β binding domain of CD109 contains amino acids 687-711 (**B**) Schematic diagram of the bacterially generated CD109 peptides overlapping the entire CD109 sequence (GST Peptide 21-321, GST Peptide 320-655, GST Peptide 640-1045, GST Peptide 1040-1445) and the bacterially generated (Peptide A, Y, Z, B) and chemically synthesized (Peptide X) CD109 peptides based around the putative TGF-β binding domain of CD109

Figure 3.2 CD109 peptides inhibit TGF-β binding to its signaling receptors



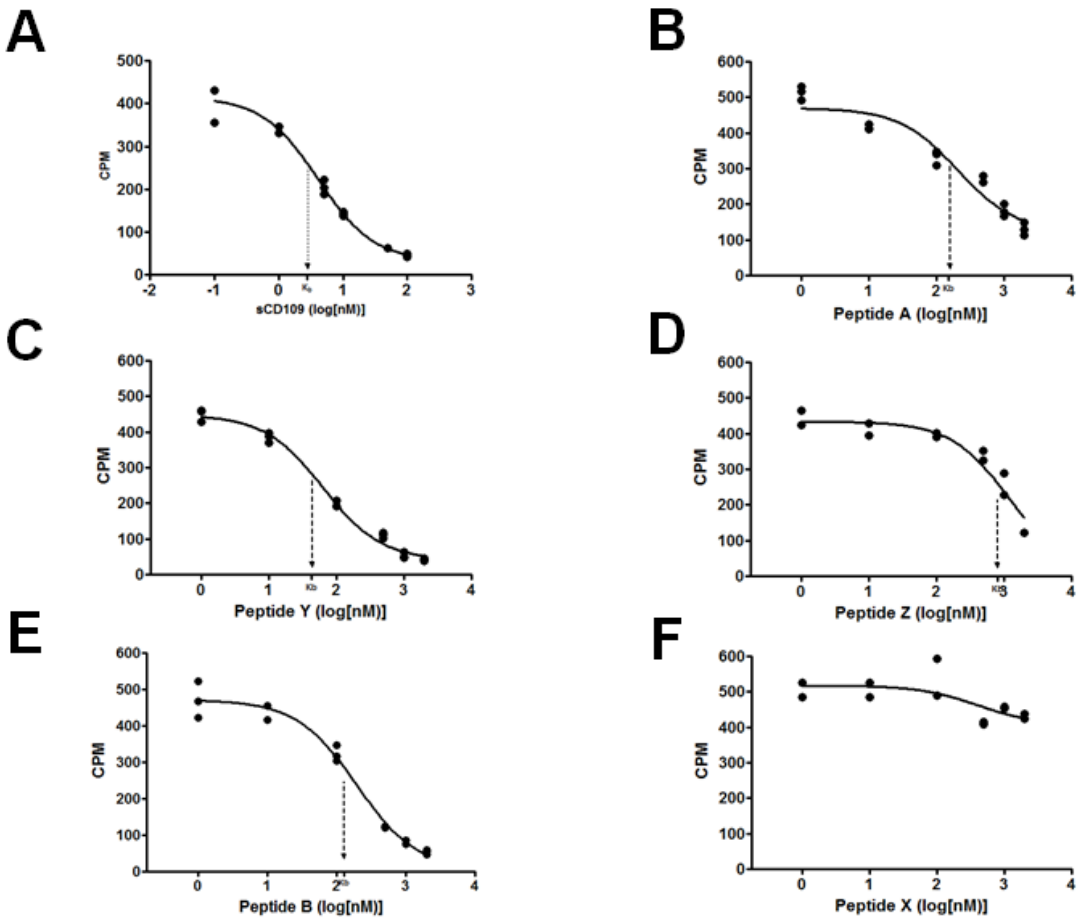
(**A**) HaCaT cells were affinity labeled at 4 °C with 100 pM [^{125}I]TGF-β1 in absence or presence of CD109 Peptide A, X, Y, Z, B, X (10, 50, 100 nM) which was then covalently cross-linked to the cell surface receptors and solubilized. The solubilized membrane extracts were analyzed by SDS-PAGE and visualized by autoradiography with coomassie blue staining showing equal protein loading.

Figure 3.2 CD109 peptides inhibit TGF-β binding to its signaling receptors



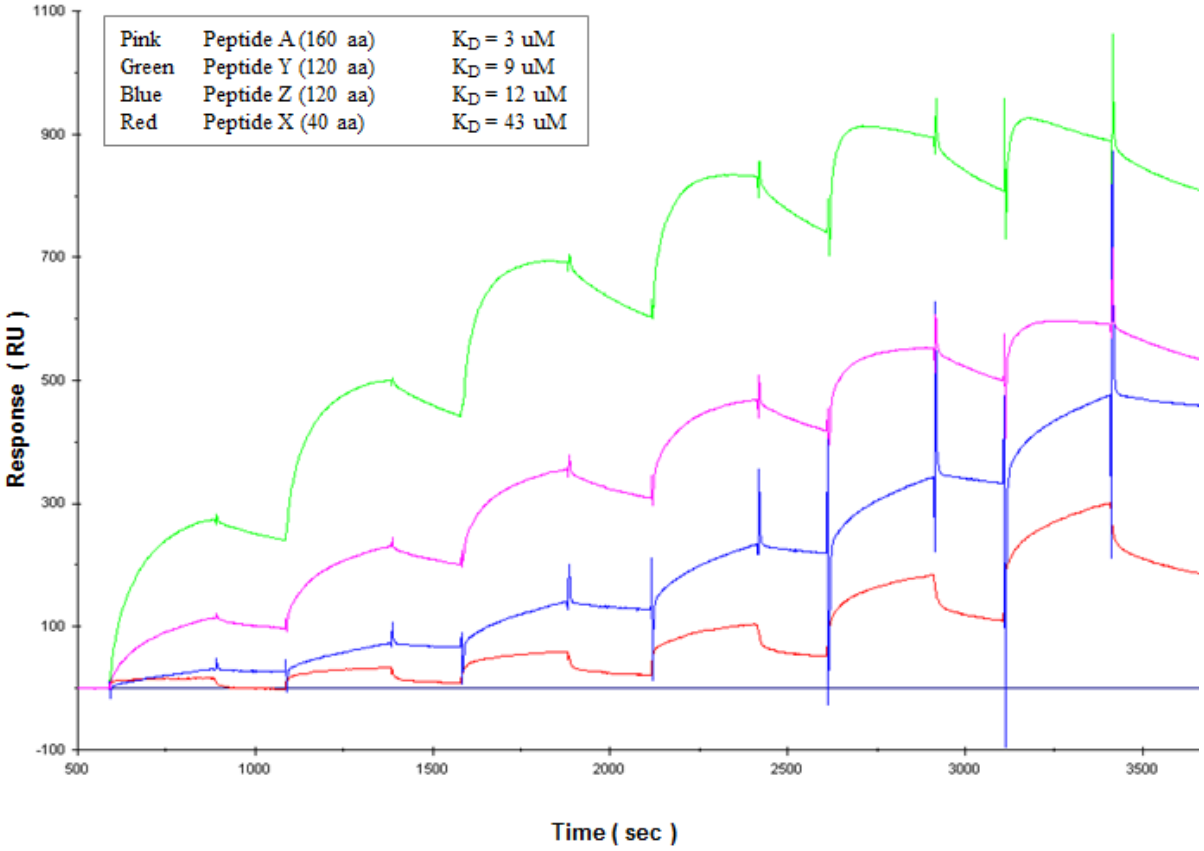
(**B**) The amount of labeled TβRI was quantified by densitometry based on the results shown in Figure 3A using TotalLab quantification software. The results are representative of n=3 independent experiments. *: $p < 0.05$.

Figure 3.3 CD109 peptides are negative modulators of TGF-β binding to keratinocyte cell surface



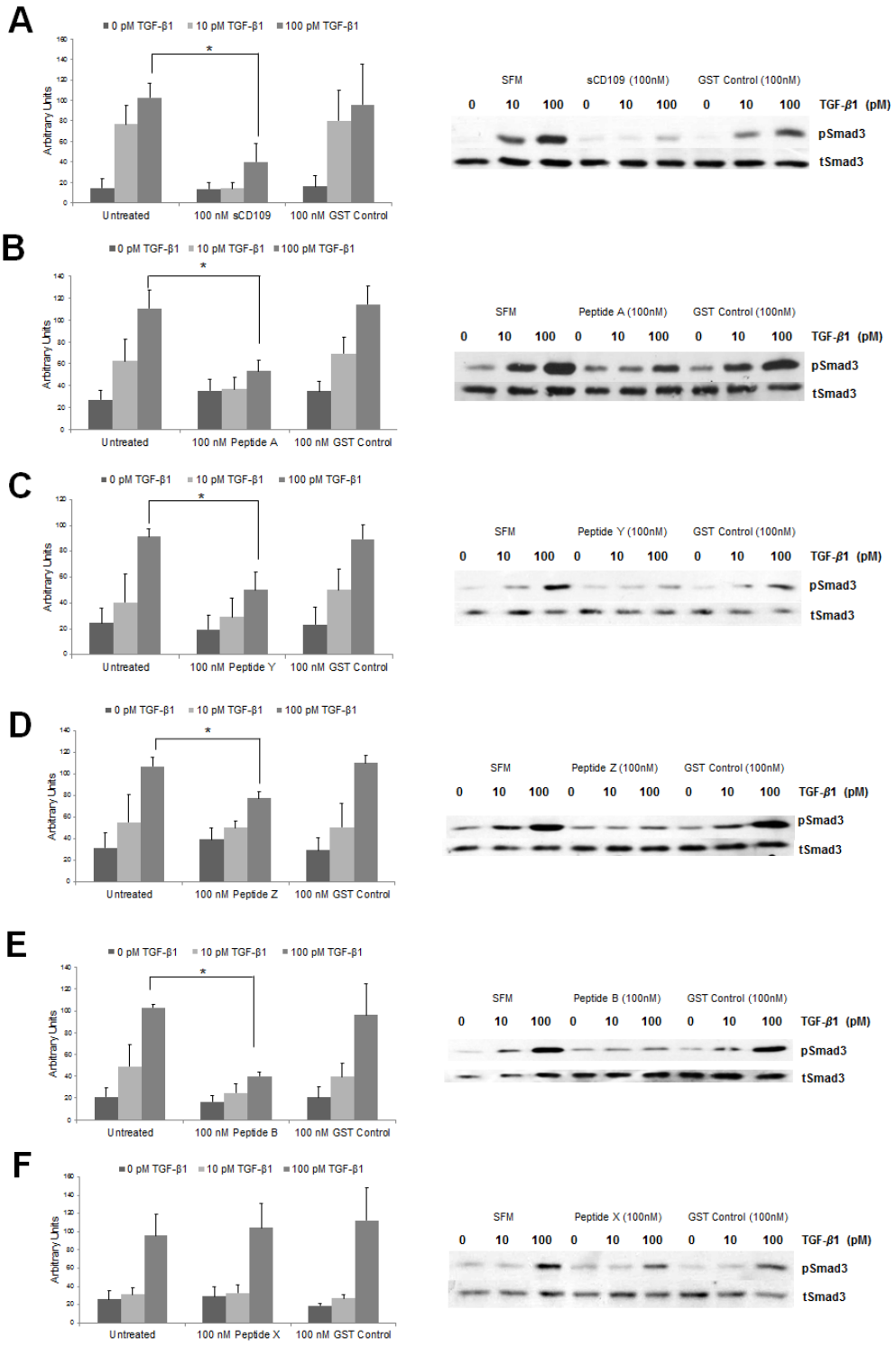
HaCaT cells were radiolabelled at 4 °C with 50pM [^{125}I]TGF-β1 in the absence or presence of (**A**) soluble CD109 protein (0.1-100 nM), (**B**) CD109 Peptide A (0.1-100 nM), (**C**) CD109 Peptide Y (0.1-100 nM), (**D**) CD109 Peptide Z (0.1-100 nM), (**E**) CD109 Peptide B (0.1-100 nM), and (**F**) CD109 Peptide X (0.1-100 nM), to determine its equilibrium dissociation constant for the allosteric modulator at the free receptor (K_b) and the ternary complex constant (α) using the ternary complex model in GraphPad Prism 5 with values representing the mean of n=3 independent experiments.

Figure 3.4 CD109 peptides bind TGF-β1 with moderate to high affinity



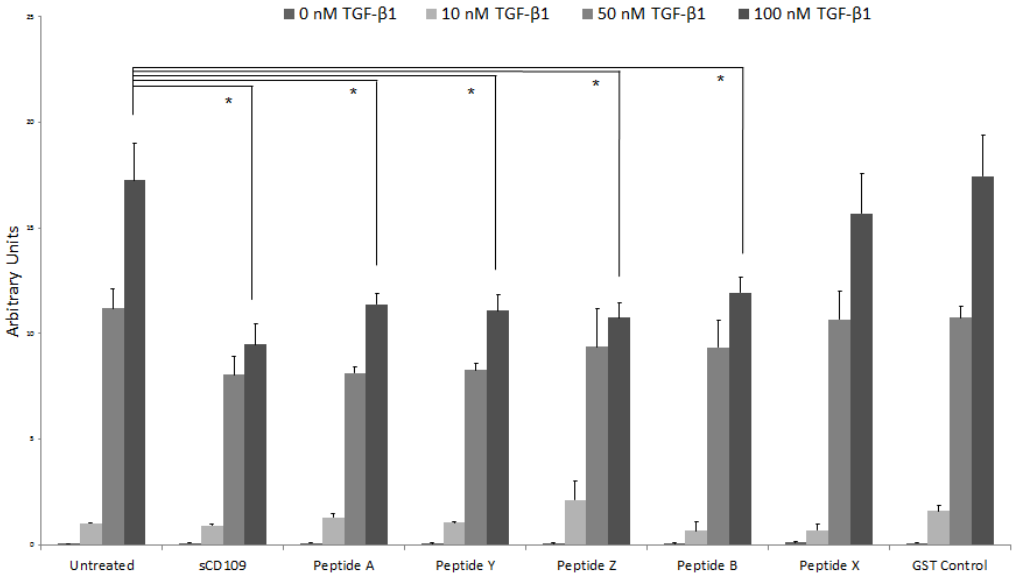
Representative SPR single-cycle kinetic analysis of CD109 Peptide A (0-1 uM), CD109 Peptide X (0-36 uM), CD109 Peptide Y (0-1 uM), and CD109 Peptide Z (0-3 uM) binding to amine-coupled TGF-β1 (550 RU) at 5 µL/min. Apparent equilibrium dissociation constants (K_D) for the CD109 peptide titrations were determined by global fitting of the data to a steady-state affinity model in the BIA evaluation v4.1 software with values representing the average of n=3 independent experiments.

Figure 3.5 CD109 peptides inhibit TGF-β1-induced phosphorylation of Smad3



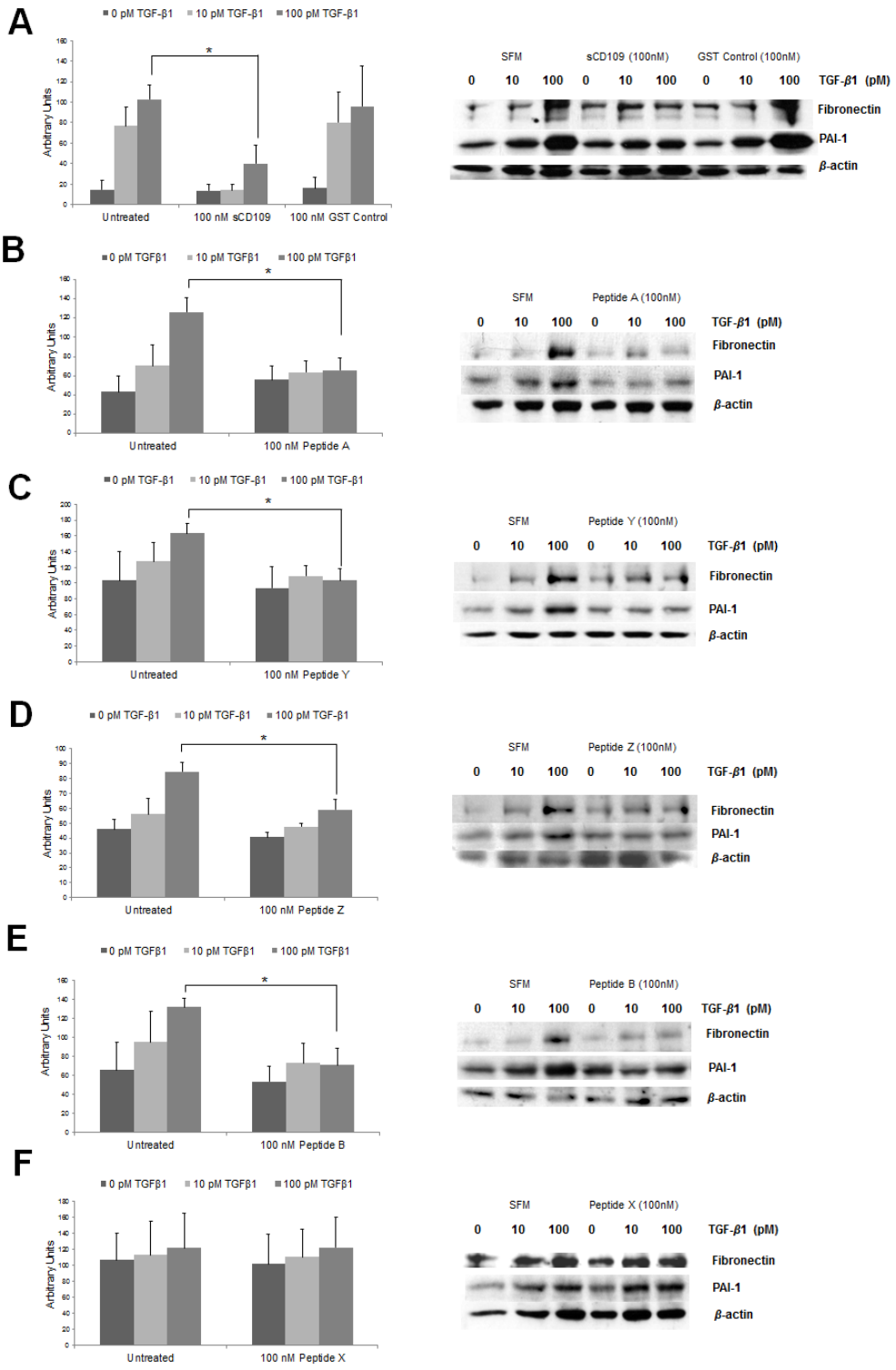
HaCaT cells were treated with and without TGF-β1 (0, 10, 100 pM) in the absence or presence of CD109 peptides (100 nM) for 45 min. Cell lysates were analyzed by western blot using anti-phosphoSmad3 and anti-totalSmad3 antibodies. Intensity of the phosphoSmad3 expression (normalized to tSmad3 intensity in arbitrary units) was quantified by densitometry for (**A**) soluble CD109 protein, (**B**) CD109 Peptide A, (**C**) CD109 Peptide Y, (**D**) CD109 Peptide Z, (**E**) CD109 Peptide B, and (**F**) CD109 Peptide X (normalized to tSmad3 intensity in arbitrary units) using TotalLab quantification software (mean of n=3 independent experiments, ± SEM), *P<0.05. GST control peptide was used as a negative control.

Figure 3.6 CD109 peptides inhibit TGF-β1-induced activation of Smad3 transcriptional activity



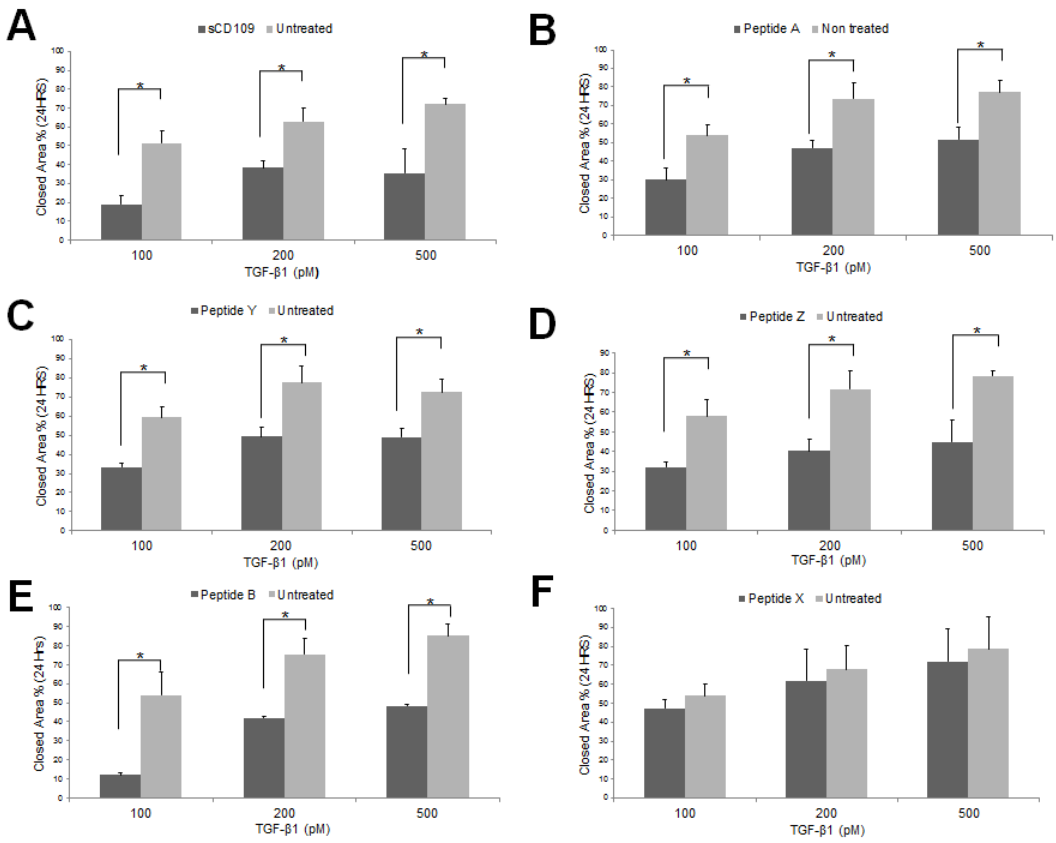
HEK293-$(CAGA)_{12}$ cells stably expressing the $(CAGA)_{12}$-luciferase reporter gene were treated with and without TGF-β1 (0, 10, 50, 100 pM) in the absence or presence of sCD109 (100 nM), CD109 Peptide A, B, X, Y, Z (100 nM) and GST control (100 nM) for 18 h under serum free conditions. Cell lysates were analyzed for luciferase activity and expressed as fold-stimulation ± SEM relative to activity of untreated cells + 10 pM TGF-β1 with values representing the mean of n=3 independent experiments. *: $p < 0.05$.

Figure 3.7 CD109 peptides inhibit TGF-β1-induced ECM protein production



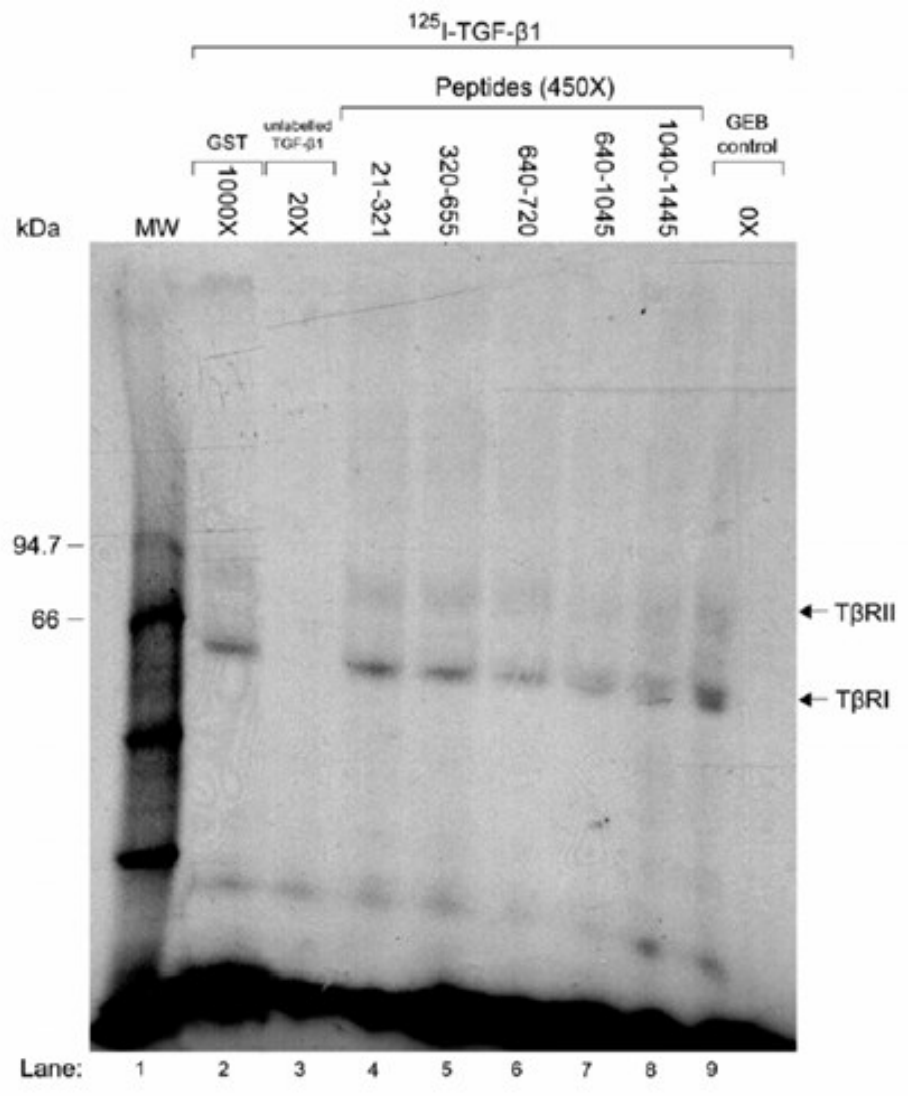
HaCaT cells were treated with and without TGF-β1 in the absence or presence of CD109 peptides for 18 h under serum free conditions. Cell lysates were analyzed by western blot using anti-fibronectin, anti-PAI-1, or anti-actin antibodies. Intensity of the fibronectin expression (expressed as mean of fibronectin band over actin band in arbitrary intensity units) was quantified by densitometry for (**A**) soluble CD109 protein, (**B**) CD109 Peptide A, (**C**) CD109 Peptide Y, (**D**) CD109 Peptide Z, (**E**) CD109 Peptide B, and (**F**) CD109 Peptide X (normalized to β-actin intensity in arbitrary units) using TotalLab quantification software (mean of n=3 independent experiments, ± SEM), *P<0.05. GST control peptide was used as a negative control.

Figure 3.8 CD109 peptides inhibit TGF-β1-induced cell migration



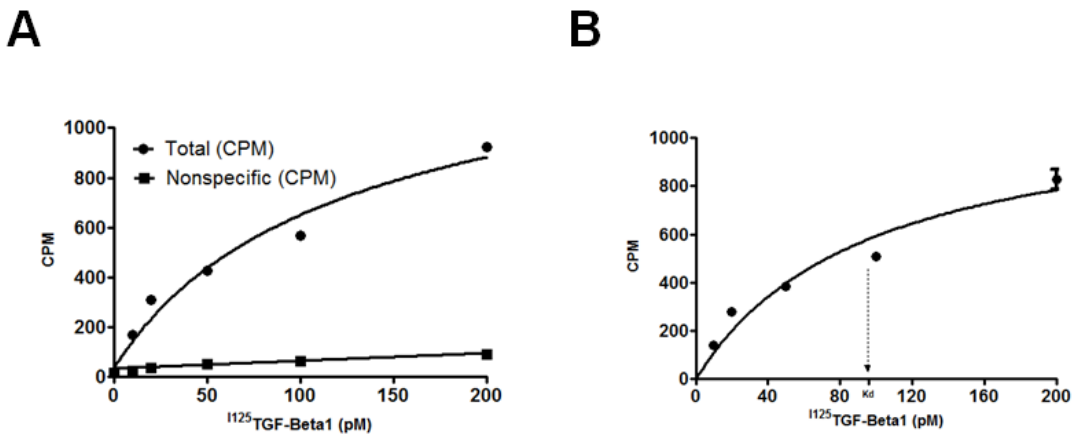
HaCaT cells were grown to confluence, serum starved for 24 h to inhibit proliferation and then treated with TGF-β1 (100, 200, 500 pM) in the absence or presence of sCD109 (100 nM) and CD109 Peptide A, B, X, Y, Z (100 nM). Cells were wounded as described in Methods section and images were acquired 0 h, 24 h and 48 h post wounding. Closed wound area % after 24 h of HaCaT cells treated with TGF-β1 in the absence or presence of **A**) soluble CD109 protein, (**B**) CD109 Peptide A, (**C**) CD109 Peptide Y, (**D**) CD109 Peptide Z, (**E**) CD109 Peptide B, and (**F**) CD109 Peptide X were determined by TotalLab quantification software (mean of n=3 independent experiments, ± SEM, *: $p<0.05$).

Figure 3.9 (Supplementary Figure 1) CD109 GST-fusion peptides encompassing the CD109 putative TGF-β binding domain inhibit TGF-β binding to its signaling receptors



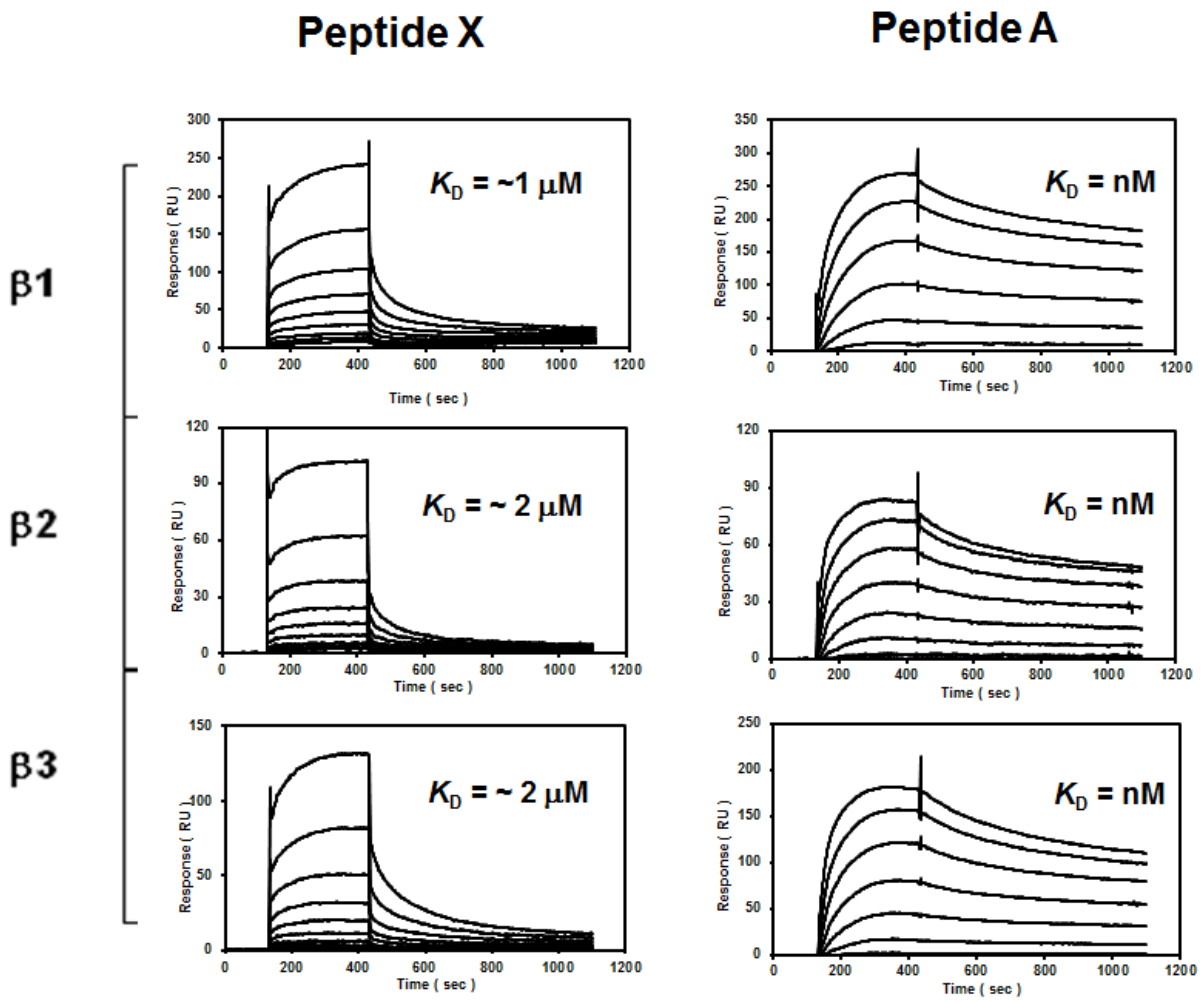
HaCaT cells were affinity labeled at 4 °C with 100 pM [^{125}I]TGF-β1 in absence or presence of CD109 GST Peptide 21-321, 320-655, 640-720, 640-1045, and 1040-1445 which was then covalently cross-linked to the cell surface receptors and solubilized. The solubilized membrane extracts were analyzed by SDS-PAGE and visualized by autoradiography. Glutathione elution buffer (GEB: 20mM reduced glutathione, 50mM Tris-HCL pH 8.5) was used as a negative control.

Figure 3.10 (Supplementary Figure 2) Equilibrium binding constant of [^{125}I]TGF-β1



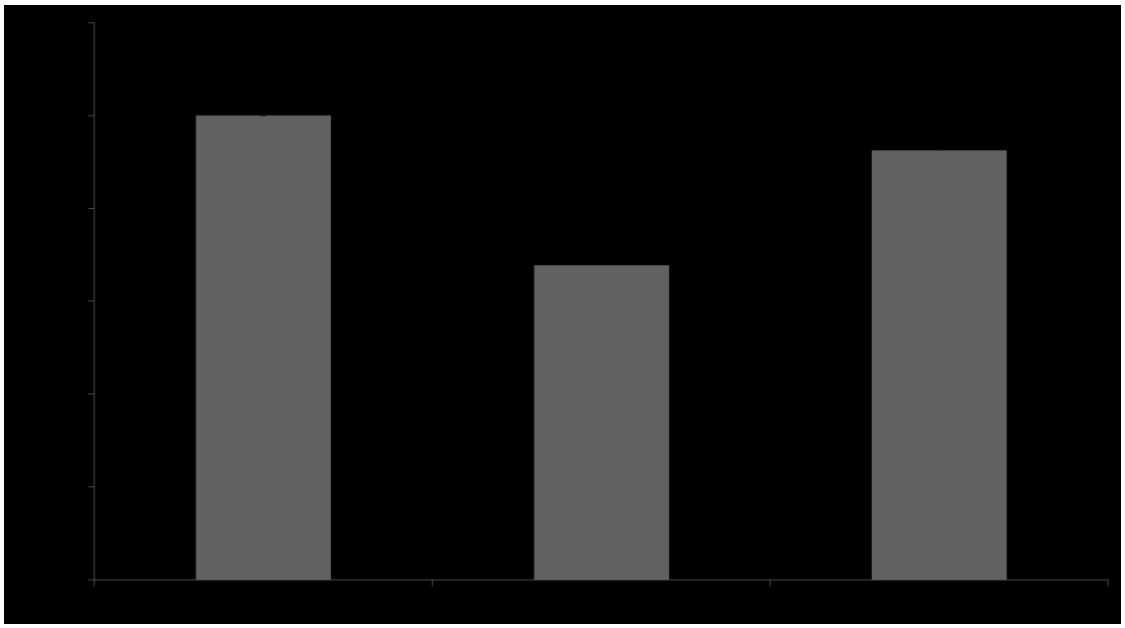
(**A**) HaCaT cells were radiolabelled at 4 °C with [^{125}I]TGF-β1(10-200 pM) in the absence or presence of non-radiolabelled TGF-β1 (100X of [^{125}I]TGF-β1) to determine its equilibrium binding constant (K_d). (**B**) Equilibrium binding constant (K_d) of [^{125}I]TGF-β1 was determined using the non-linear fit model (global fitting of total and nonspecific) in GraphPad Prism 5 with values representing n=3 independent experiments.

Figure 3.11 (Supplementary Figure 3) CD109 peptides bind TGF-β with differing kinetics



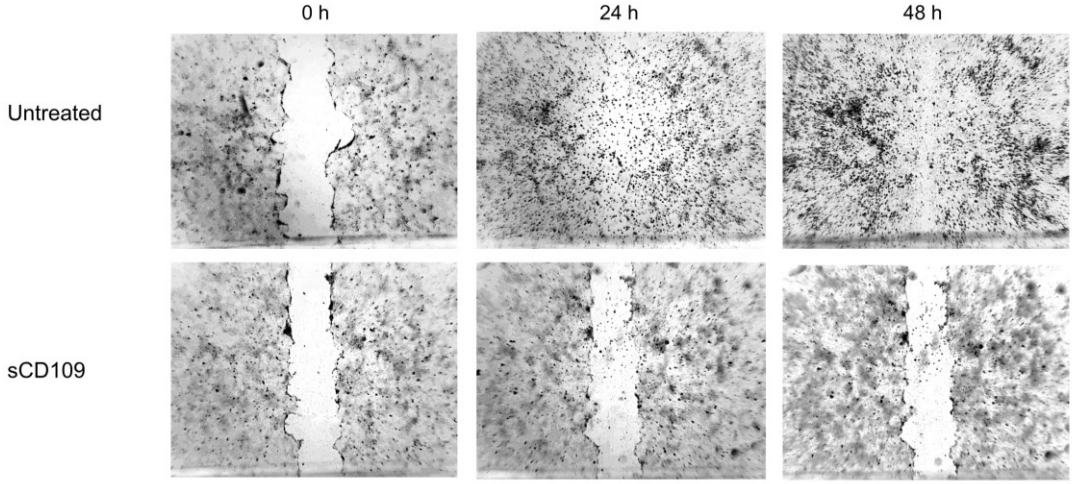
Representative SPR kinetics of CD109 Peptide A and CD109 Peptide X binding to amine-coupled TGF-β1 isoforms (550 RU) at 5 μL/min (6 min association + 10 min dissociation; 2-fold dilution series). Apparent equilibrium dissociation constants (K_D) for the CD109 Peptide A titrations were determined by global fitting of the data to a steady-state affinity model and dissociation constants for the CD109 Peptide X titrations were estimated by global fitting the data using the fit separate k_a/k_d model in the BIA evaluation v4.1 software with values representing the average of n=3 independent experiments.

Figure 3.12 (Supplementary Figure 4) CD109 site-directed mutants abrogate CD109-mediated inhibition of TGF-β1-induced transcriptional activity



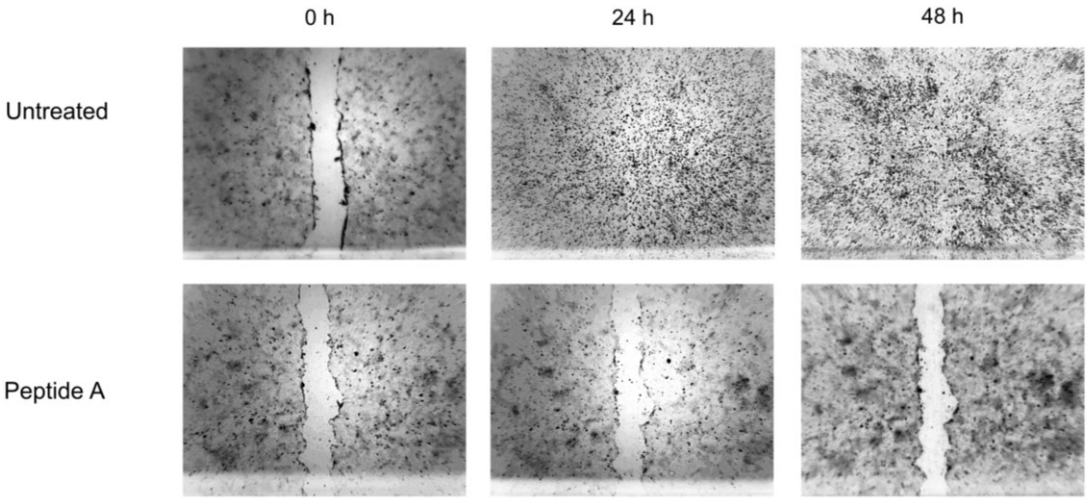
HEK293-$(CAGA)_{12}$ cells stably expressing the $(CAGA)_{12}$-luciferase reporter gene were transfected with pCMVSport6 CD109 Wild Type and CD109 Reverse Polarity Mutant plasmids then treated with TGF-β1. Cell lysates were analyzed for luciferase activity and expressed as fold-stimulation ± SD relative to activity of cells transfected with pCMVSport6 empty vector.

Figure 3.13 (Supplementary Figure 5) Soluble CD109 inhibits TGF-β1-induced cell migration



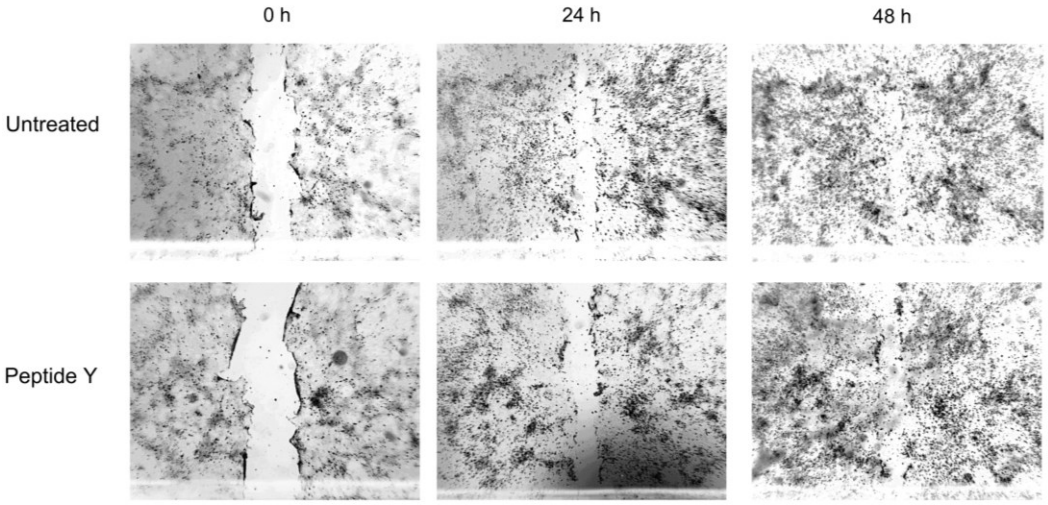
HaCaT cells were grown to confluence, serum starved for 24 h to minimalize proliferation effects, and then scratched vertically across the center of the wells with a sterile 200 µL pipette tip to create a cell-free line. HaCaT cells were then treated with TGF-β1 (500 pM) in the absence or presence soluble CD109 (100 nM) under serum free conditions and images were acquired 0 h, 24 h and 48 h post wounding. The results are representative of n=3 independent experiments.

Figure 3.14 (Supplementary Figure 6) CD109 Peptide A inhibits TGF-β1-induced cell migration



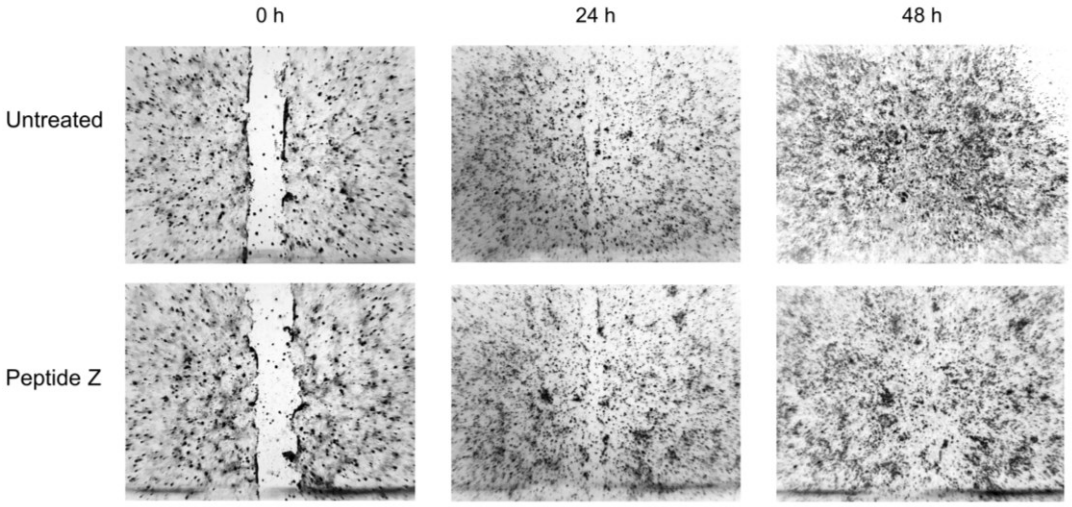
HaCaT cells were grown to confluence, serum starved for 24 h to minimalize proliferation effects, and then scratched vertically across the center of the wells with a sterile 200 µL pipette tip to create a cell-free line. HaCaT cells were then treated with TGF-β1 (500 pM) in the absence or presence CD109 Peptide A (100 nM) under serum free conditions and images were acquired 0 h, 24 h and 48 h post wounding. The results are representative of n=3 independent experiments.

Figure 3.15 (Supplementary Figure 7) CD109 Peptide Y inhibits TGF-β1-induced cell migration



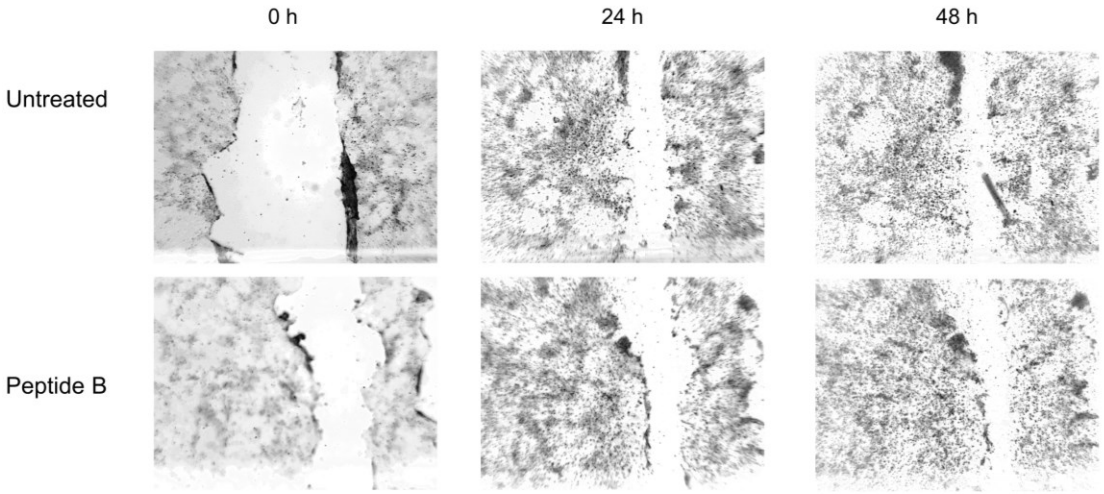
HaCaT cells were grown to confluence, serum starved for 24 h to minimalize proliferation effects, and then scratched vertically across the center of the wells with a sterile 200 µL pipette tip to create a cell-free line. HaCaT cells were then treated with TGF-β1 (500 pM) in the absence or presence CD109 Peptide Y (100 nM) under serum free conditions and images were acquired 0 h, 24 h and 48 h post wounding. The results are representative of n=3 independent experiments.

Figure 3.16 (Supplementary Figure 8) CD109 Peptide Z inhibits TGF-β1-induced cell migration



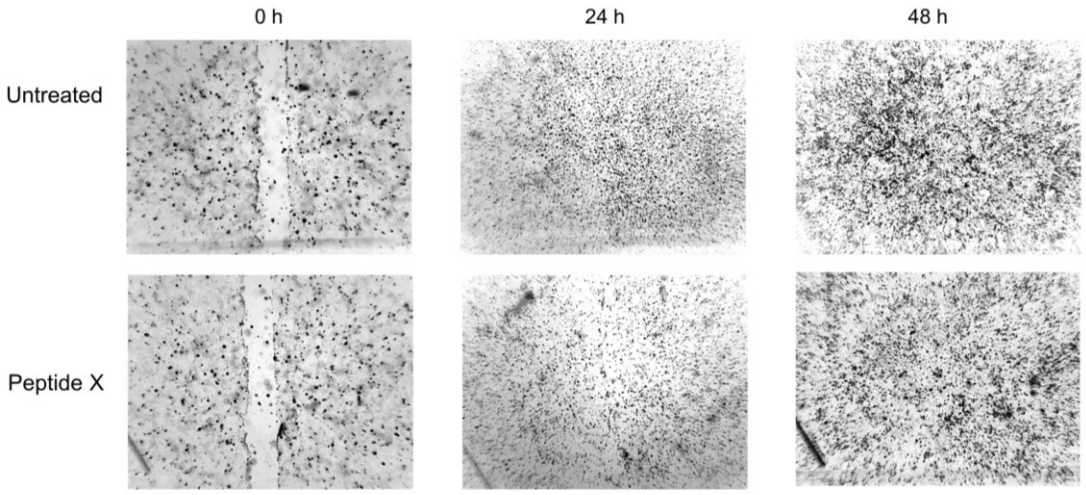
HaCaT cells were grown to confluence, serum starved for 24 h to minimalize proliferation effects, and then scratched vertically across the center of the wells with a sterile 200 µL pipette tip to create a cell-free line. HaCaT cells were then treated with TGF-β1 (500 pM) in the absence or presence CD109 Peptide Z (100 nM) under serum free conditions and images were acquired 0 h, 24 h and 48 h post wounding. The results are representative of n=3 independent experiments.

Figure 3.17 (Supplementary Figure 9) CD109 Peptide B inhibits TGF-β1-induced cell migration



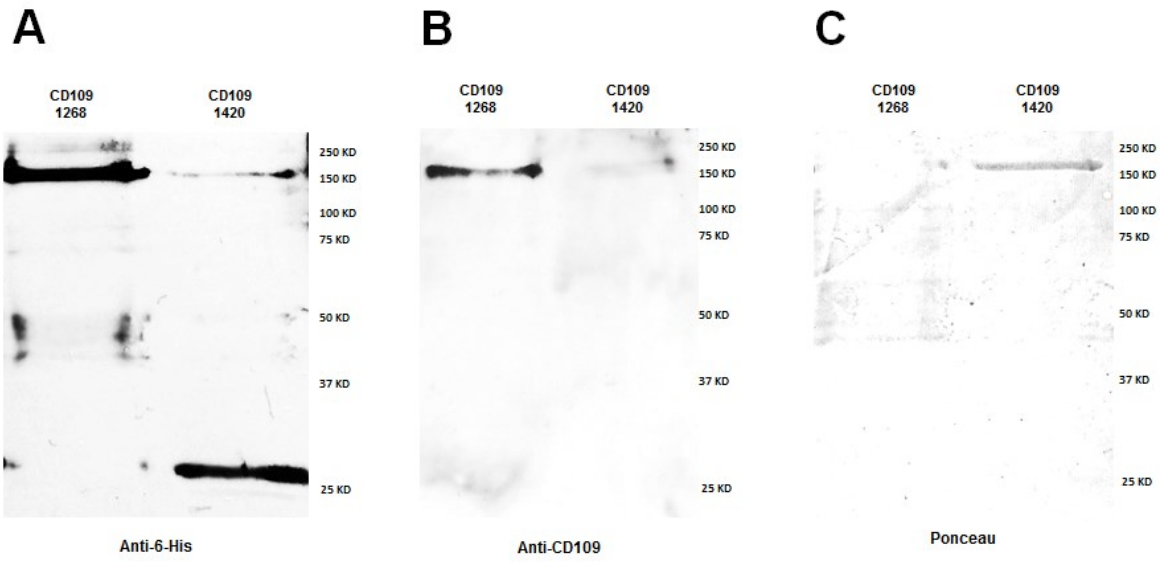
HaCaT cells were grown to confluence, serum starved for 24 h to minimalize proliferation effects, and then scratched vertically across the center of the wells with a sterile 200 μL pipette tip to create a cell-free line. HaCaT cells were then treated with TGF-β1 (500 pM) in the absence or presence CD109 Peptide B (100 nM) under serum free conditions and images were acquired 0 h, 24 h and 48 h post wounding. The results are representative of n=3 independent experiments.

Figure 3.18 (Supplementary Figure 10) CD109 Peptide X does not significantly inhibit TGF-β1-induced cell migration



HaCaT cells were grown to confluence, serum starved for 24 h to minimalize proliferation effects, and then scratched vertically across the center of the wells with a sterile 200 µL pipette tip to create a cell-free line. HaCaT cells were then treated with TGF-β1 (500 pM) in the absence or presence CD109 Peptide X (100 nM) under serum free conditions and images were acquired 0 h, 24 h and 48 h post wounding. The results are representative of n=3 independent experiments.

Figure 3.19 (Supplementary Figure 11) Soluble CD109-1268 and CD109-1420 fragments suggest that furin cleavage may occur at amino acids 1270-1273



Purified recombinant soluble CD109 1268 and soluble CD109 1420 protein were analyzed by western blot using (**A**) anti-His and (**B**) anti-CD109 antibodies. Purified recombinant soluble CD109 1268 and soluble CD109 1420 purified protein were visualized by (**C**) ponceau staining.

Table 3.1 Site-directed mutagenesis of the putative TGF-β binding domain

5' – 687	RKHFPETWIWLDTNMGYRIYQEFEVTVPDSITS	711 – 3'	CD109 WT
5' – 687	RKHFP**R**TWIWL**R**TNMGYRIYQ**R**F**R**VTVPDSITS	711 – 3'	CD109 Reverse Polarity Mutant

4 Discussion and Perspectives

Transforming Growth Factor β (TGF-β) is a pleiotropic cytokine involved in a wide variety of cellular processes. Expressed in a majority of mammalian cells, TGF-β has been shown to play pivotal roles in immunoregulation, cellular homeostasis, apoptosis, and embryonic development (Blobe *et al.*, 2000; Massague, 2012). Perturbations in the TGF-β signaling pathway results in extensive vascular (Bertolino *et al.*, 2005), neural (Seoane *et al.*, 2004), and osteal (Wu and Hill, 2009) developmental defects, and massive autoimmune inflammatory responses (Letterio and Roberts, 1998).

Previous studies in our laboratory on the regulation of TGF-β signaling in skin cells have yielded in the identification of a novel TGF-β1 binding protein, r150, which forms a heteromeric complex with the TGF-β signaling receptors (Tam *et al.*, 1998). Our lab has cloned r150 from keratinocytes by microsequencing and has identified r150 as CD109, a novel glycosylphosphatidylinositol (GPI)-anchored protein that is a member of the α2m complement superfamily (Finnson *et al.*, 2006). Subsequent studies in our lab have demonstrated that CD109 negatively regulates TGF-β signaling in human keratinocytes *in vitro* (Finnson *et al.*, 2006), and that CD109 inhibits TGF-β signaling by promoting receptor internalization and degradation (Bizet *et al.*, 2011; Bizet *et al.*, 2012). Moreover, our group has demonstrated that CD109 inhibits TGF-β signaling and responses *in vivo*, as overexpression of CD109 in murine epidermis reduces scarring and inhibits fibrosis in a TGF-β dependent manner (Vorstenbosch *et al.*, 2013a; Vorstenbosch *et al.*,

2013b). Additionally, our laboratory has demonstrated that PIPLC-released soluble CD109 can also bind TGF-β and regulate TGF-β signaling (Litvinov *et al.*, 2011; Tam *et al.*, 2001). These findings suggest that soluble CD109 has the potential to inhibit TGF-β signaling and responses independently of the TGF-β signaling receptors.

In this thesis, I have characterized the role of soluble CD109 in regulating TGF-β signaling and responses in greater detail and I have studied its structure and function. More specifically, I have shown that soluble CD109, like membrane-anchored CD109, is able to inhibit TGF-β-induced signaling and responses. These results strongly suggest that soluble CD109 is an antagonist of TGF-β action. Although it has been previously shown that released endogenous CD109 can sequester TGF-β1 away from the signaling receptors, my findings suggest that soluble exogenous recombinant CD109 is also able to inhibit TGF-β receptor activity through ligand sequestration and/or receptor interaction. To establish the region where CD109 interacts with TGF-β, I have provided evidence to suggest that the predicted TGF-β binding domain of CD109 to contain amino acids 687-711 based on sequence alignment with homologue α2m. Defining the putative TGF-β binding domain for CD109 raises the possibility of interfering with TGF-β availability and responses. Given the critical role of TGF-β in a diverse variety of cellular functions, inhibition of TGF-β signaling was expected to affect both the positive and negative aspects of TGF-β-induced responses. However, it has been demonstrated in animal studies that long term inhibition of TGF-β signaling using

a soluble TβRII does not significantly impact morbidity (Muraoka *et al.*, 2002; Yang *et al.*, 2002). Identification of TGF-β inhibitors may thus be of potential interest for the development of novel therapeutic strategies to treat disease with aberrant TGF-β signaling.

My results demonstrating that soluble CD109 can bind TGF-β and antagonize TGF-β signaling and responses together with previously findings of soluble CD109 inhibiting TGF-β signaling and responses in fibroblasts (Man *et al.*, 2012) and keratinocytes (Litvinov *et al.*, 2011) and with the previously findings of soluble CD109 existing endogenously in a variety of cell types (Caccia *et al.*, 2011; Hockla *et al.*, 2009; Tam *et al.*, 2001; Villarreal *et al.*, 2013) suggest that soluble CD109 may act as a TGF-β regulatory protein.

In my investigation on soluble CD109 structure and function, I have created a mammalian protein expression system to produce recombinant soluble CD109 proteins (CD109-1268 and CD109-1420) using HEK293T cells to express recombinant his-tagged soluble CD109 proteins, which were then subsequently purified using nickel affinity chromatography. I have used the purified CD109 proteins in surface plasmon resonance (SPR), radioligand binding, and affinity labeling assays to demonstrate that recombinant sCD109 specifically binds to all three mammalian TGF-β isoforms with binding affinities rivaling those observed with the TGFβ-sRII ectodomain (for TGF-β1 and TGF-β3), suggesting that sCD109 may potentially inhibit TGF-β signaling through ligand sequestration and/or protein

interaction. Previous published findings have suggested various mechanisms of CD109-mediated regulation of TGF-β signaling including CD109-mediated TGF-β receptor internalization and degradation via the caveolae pathway (Bizet *et al.*, 2011; Bizet *et al.*, 2012). In addition, furin-mediated cleavage and release of the CD109 protein appears to be play an important role in CD109 and TβRI interaction (Hagiwara *et al.*, 2010). Moreover, secretome studies have reported that soluble CD109 can be released by other proteases including PIPLC (Tam *et al.*, 2001) and mesotrypsin (Hockla *et al.*, 2009). These recent findings support the hypothesis that endogenous soluble CD109 released by endogenous proteases including furin and mesotrypsin may play an important role in regulating TGF-β signaling.

My findings on soluble CD109 structure and function support the notion of furin-mediated cleavage and release of soluble CD109 protein into the cell medium (Figure 3.19). Furin is a calcium-dependent serine endoprotease that functions in the trans-Golgi network and cleaves precursor proteins into their active forms (Nakayama, 1997; Thomas, 2002). Through my HEK293T mammalian protein expression system, I have produced recombinant soluble CD109 proteins with (sCD109-1420) and without (sCD109-1268) the predicted furin cleavage site, to investigate the prevalence of protease-mediated release of soluble CD109. Using anti-his and anti-CD109 antibodies in western blot analysis, I was able to detect distinctly different CD109 protein fragments between sCD109-1420 and sCD109-1268, which supports the hypothesis that a putative cleavage site located between amino acids 1270-1273 is the furin-cleavage site (Figure 3.19A). Furthermore, I

was able to detect distinct CD109 fragments of ~20-30 kDa and ~150-180 kDa using anti-6-His C-terminal antibody on sCD109-1420 and sCD109-1268 recombinant proteins, suggesting that CD109 may be expressed as two non-covalently linked protein fragments joined together after furin-mediated cleavage (Figure 3.19). These findings are of interest as studies have reported that furin cleavage and subsequent CD109 release from the cell surface may play a role in CD109-mediated inhibition of TGF-β signaling (Hagiwara *et al.*, 2010).

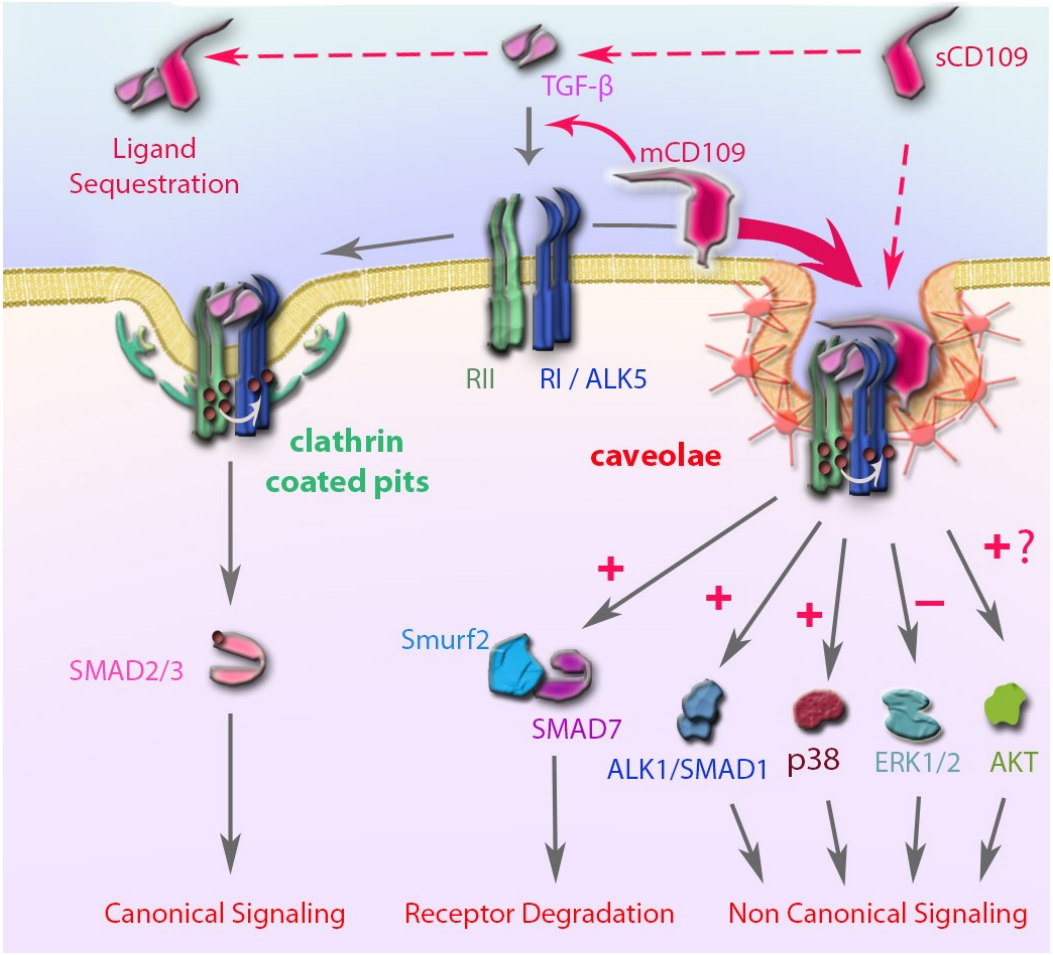
In my investigation of soluble CD109 and its TGF-β binding domain, I have generated recombinant CD109 peptides spanning the predicted TGF-β binding region of CD109 using a bacterial expression system to express recombinant GST-tagged and His-tagged CD109 peptides, which were then purified using affinity chromatography. Using the recombinant CD109-based peptides I used surface plasmon resonance to demonstrate that CD109 peptides can bind TGF-β ligands with affinities comparable to the soluble CD109 protein. Furthermore, I have demonstrated that the larger CD109 peptides exhibit association and dissociation kinetics similar to that of the sCD109 protein (slow-on/slow-off binding kinetics) (Li *et al.*, 2015), whereas the smaller CD109 peptides exhibit association and dissociation kinetics similar to that of the soluble type II receptor ectodomain (De Crescenzo *et al.*, 2001). The differences in binding kinetics and affinities between larger and smaller CD109 peptides suggest that sequence structure outside of the predicted TGF-β binding region may provide additional functionality and/or stability for TGF-β binding interactions.

Interestingly, in my surface plasmon resonance findings, I have demonstrated that soluble CD109 and α2m have different TGF-β isoform binding specificities despite sharing similar binding domain structures. Although the difference in TGF-β isoform specificity between α2m (binds TGF-β1 and -2) and soluble CD109 (binds TGF-β1, -β2 and -β3) could be due to differences in protein structure (e.g. CD109 exists as a monomer, α2m exists as a tetramer), the fact that CD109 exhibits TGF-β isoform specificity in cell-based assays but binds all TGF-β isoforms in SPR assays suggest that TGF-β receptor isoform preference may be an important factor to TGF-β isoform specificity. Moreover, my CD109 site-directed mutagenesis results demonstrate that mutations to the putative TGF-β binding domain of CD109 abrogates CD109-mediated inhibition of TGF-β signaling similarly to α2m mutants results (Arandjelovic *et al.*, 2006), which further supports the hypothesis that CD109 and α2m share functional similarity between their putative TGF-β binding regions. Collectively, my investigations into the putative TGF-β binding domain of CD109 demonstrate that the highly hydrophobic and negatively charged 687-711 region of the CD109 protein may play an important role in inhibiting TGF-β signaling and responses.

Although the putative TGF-β binding region of CD109 has shown to play an important role in inhibiting TGF-β signaling through ligand sequestration, recent studies have suggested that CD109 may regulate TGF-β signaling and responses through other additional mechanisms including the ALK1-Smad1/5 pathways

(Bizet *et al.*, 2014; Vorstenbosch *et al.*, 2015) (Figure 4.1). In addition to promoting TGF-β receptor internalization and degradation, CD109 may also regulate TGF-β-induced MAP kinase signaling by inhibiting TGF-β-induced ERK activation while promoting TGF-β-induced p38 activation (Bizet *et al.*, 2014). Furthermore, transgenic mice studies have shown that CD109 may also regulate TGF-β signaling by directing TGF-β signaling through the ALK1-Smad1/5/8 pathway rather than the ALK5-Smad2/3 pathway, thus increasing ALK1 receptor expression while reducing ALK5 receptor expression (Vorstenbosch *et al.*, 2015). These alternative mechanisms of TGF-β regulation suggest that there may other mechanisms on the CD109 protein that can directly interact with TGF-β ligand or TβRI to mediate TGF-β receptor degradation and TGF-β ALK5/ALK1 expression. Additionally, my surface plasmon resonance findings for CD109-TGF-β binding have demonstrated cooperative binding and non-linear stoichiometry, which supports the hypothesis that more than one TGF-β interaction domains may exist on the CD109 protein.

Figure 4.1: Schematic representation of the potential mechanism by which CD109 negatively regulates TGF-β responses



TGF-β receptors internalize via the clathrin-coated pits leading to ALK5-Smad2/3 signaling or via the caveolar pathway leading to TGF-β receptor degradation, ALK1-Smad1/5/8, and/or MAPK non-canonical signaling. Membrane CD109 inhibits TGF-β signaling by promoting TGF-β receptor localization to the caveolae, enhancing TβRI degradation, enhancing ALK1-Smad1/5/8 signaling and differentially regulating MAPK non-canonical signaling. Soluble CD109 inhibits TGF-β signaling by promoting sequestration of the TGF-β ligand. It is possible that sCD109 maybe also complex with TβRI and/or promote TGF-β receptor internalization and degradation via the caveolar pathway.

Intriguingly, recent transgenic mouse studies with CD109-deficient *(CD109 -/-)* mice showed no significant difference in levels of Smad2 phosphorylation in the epidermis between wild-type and CD109-/- mice (Mii *et al.*, 2012). Instead, STAT3 phosphorylation levels were found to be significantly elevated in the epidermis of *CD109*-/- mice when compared with wild-type littermates. These findings suggest that extended effects of CD109 deficiency on the TGF-β signaling pathway may be concealed by compensatory mechanisms and/or that CD109 may regulate keratinocyte differentiation through additional signaling pathways involving STAT3. As CD109 has been reported to regulate STAT3 activation in human keratinocytes (Litvinov *et al.*, 2011), the CD109-/- mice findings supports the prospect that CD109 may regulate STAT3 directly or indirectly via molecules upstream of STAT3 such as EGFR or IL6R.

Interestingly, in the reported STAT3 activation studies, CD109 has shown to inhibit pSmad2 signaling, suggesting that CD109-mediated STAT3 activation and pSmad3 inhibition are not mutually exclusive responses. Furthermore, due to the complex nature of CD109-mediated TGF-β regulation, there may be significantly different responses to TGF-β between CD109 deficient mice (Mii *et al.*, 2012) and CD109 overexpressing mice (Vorstenbosch *et al.*, 2013b) due to differences in epidermal-dermal paracrine signaling. As our knowledge of CD109 and TGF-β increases, we will be able to establish a better understanding of how CD109 interacts with TGF-β and we will be able to develop novel strategies to regulate TGF-β responses.

5 Conclusion and Summary

Recent advances in the understanding of TGF-β signaling have moved the field closer to resolving the context-dependent nature of TGF-β action. Despite the enormous progress made in the understanding of TGF-β signaling in recent years, the goal of reaching an unambiguous understanding of the context-dependent nature of TGFβ action remains elusive. It has been suggested that the diversity of TGF-β responses can be attributed to the differential activation of TGF-β receptors, and/or the differential regulation of TGF-β downstream components including the canonical and non-canonical signaling pathways and transcriptional complexes.

Depending on the cellular context, TGF-β signaling responses can include variations in proliferation, apoptosis, differentiation, or senescence. Thus, it is not surprising that TGF-β co-receptors can also regulate TGF-β signaling in a highly contextual manner. Determining the mechanisms of how TGF-β co-receptors regulate TGF-β action would have profound implications in understanding the pathological progression of diseases where TGF-β is implicated. Based on the characterization of TGF-β-α2m interaction and elucidation of the TGF-β interacting domain of α2m, I set out to map the TGF-β binding domain of soluble CD109.

In my findings, I established that soluble CD109 is an inhibitor of TGF-β action by demonstrating the ability of recombinant soluble CD109 protein to bind all three mammalian TGF-β isoforms and negatively modulate TGF-β signaling and TGF-β-induced cellular responses. Additionally, based on sequence homology analysis

between α2m and CD109, it was possible to predict that the putative TGF-β binding domain of CD109 contains amino acids 687-711, which includes a WIW hydrophobic sequence and acidic residues thought to confer TGF-β binding functionality similar to that of soluble TβRII and α2M. Furthermore, I have generated CD109-based peptides spanning the putative binding domain of the CD109 protein and CD109 site-directed mutations of that domain to define the region of CD109 responsible for TGF-β binding. My findings suggest that CD109 peptides that contain the TGF-β putative binding domain can bind all three mammalian TGF-β isoforms and modulate TGF-β signaling and TGF-β-induced cellular responses.

In summary, I have established that soluble CD109 is an antagonist of TGF-β signaling, by demonstrating the ability of recombinant soluble CD109 protein to bind TGF-β and negatively modulate TGF-β signaling. I have also established that the TGF-β putative binding region of CD109 is at least partially responsible for binding TGF-β ligand and antagonizing TGF-β signaling, by demonstrating that soluble CD109 directly interacts with TGF-β and inhibits TGF-β signaling and responses *in vitro* and by mapping the TGF-β binding domain.

Collectively, these findings suggest that soluble CD109 plays an important role in negatively regulating TGF-β signaling and that a putative TGF-β putative binding region of CD109 is at least partially responsible for binding TGF-β and antagonizing TGF-β signaling and responses. In addition to unravelling a potential

mechanism by which TGF-β action is regulated by CD109 *in vivo*, the above findings have important implications in diseases such as cancer and organ fibrosis, where aberrant TGF-β action is known to play a pathophysiological role.

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6 Appendix

Acceptance of: “Soluble CD109 binds TGF-β and antagonizes TGF-β signaling and responses” for publication by Biochemical Journal

Biochemical Journal Decision Letter (BJ2014/1488)

From: Editorial Office [editorial@biochemj.org]
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