Overexpression of CD109 in the epidermis reduces skin fibrosis and inflammation

Joshua Vorstenbosch

Doctor of Philosophy

Department of Surgery

Division of Experimental Surgery

McGill University

Montreal, Quebec, Canada.

December 7, 2012

A thesis submitted to the Faculty of Graduate Studies and Research at McGill University in partial fulfillment of the requirements of the degree of *Doctor of Philosophy in Experimental Surgery*.

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Acknowledgments:

I would like to begin by thanking Dr. Anie Philip for the opportunity to conduct research in her lab, and for fostering my curiosity and encouraging me to further pursue the project contained herein. Additionally, I would like to thank Dr. Philip for all of her support throughout the years, through the ups and downs I have endured and for her unwavering encouragement without which this degree would not have been possible. I would also like to thank my thesis advisory committee, comprised of Dr. Maria Petropavlovskaia, Dr. Stephane Roy, Dr. Fernando Congote, and Dr. John Hanrahan for their advice, direction, and for providing me with the focus I required to move forward throughout my doctorate studies.

I am also exceptionally grateful for the camaraderie, scientific input, teaching and learning, and altogether good times shared with members of the Philip Lab over the years. I would like to thank Dr. Ken Finnson, Anne Marcoux, and Ying Wang for welcoming me to Dr. Philip's lab, and I would also like to thank Ken for his jokes, Clodhoppers, sports chat, and expertise and input to help in advancing the work described here. Dr. Albane Bizet, Carter Li, and Yoon Chi are also owed an infinite amount of gratitude from me, for their friendship and support to help me keep graduate school in perspective, and for their reminders if I stay the course that I will one day graduate. Not to mention all of the scientific support that Albane, Carter, and Yoon have offered throughout the years. Albane, you have been a guiding light, showing me that people do get PhDs! I also would like to thank Alissa Trzeciak, whose dedication to science and friendship was an

inspiration and "tricked me" me into working even harder to complete experiments. Anshuman, or "Shu-Dawg", master orderer and lab manager, thank you for putting up with my outrageous demands, and turning them into reality. I didn't think that you could turn around most of those requests as quickly as you did, but you did it, and I am both impressed and grateful. Dr. Hasan Al-Ajmi and Dr. Sebastian Winocour, thank you. Thank you for being great collaborators, and providing me with the inspiration to pursue medicine in addition to research, and helping me realize that my true passion in life revolves around translating basic science knowledge into clinical cures. With your help and support I am now in a position to straddle the boundary between medicine and science, and I look forward to continue being in this position for the remainder of my career. Melani Lighter, Jean-Philippe Lacroix, and Mihiran Karunanayake, thank you! You have all been fantastic colleagues both scientifically and medically, and I look forward to continue working with you all into the future. Thank you also to Aya Siblini for keeping my seat warm and for respecting my OCD as it relates to my lab desk, and to Dr. Irvens Fanelus and Dr. Hari Babu for their camaraderie during my random work hours after I started medical school.

I would also like to thank the other members of the Montreal General Hospital C9 community for all of the great experiences. Dr. Chiu for your wisdom and guidance, Minh Duong for your assistance with histology, Dr. Maria Petropavlovskaia for input on nearly every technique I performed, and especially to Dr. Stephen Hanley, who offered outstanding guidance, resources and support, both personally and scientifically, throughout my graduate studies. I would also

like to thank Dr. Emily Austin and Mr. Jason Patapas, who have been great friends and lab-neighbours during graduate school and beyond.

Our collaborators also deserve recognition for all of the knowledge they conveyed, the support they offered, and general friendship. Dr. Stephane Roy has been an instrumental figure in my scientific development, always offering advice on a moments notice since my first week of graduate school. I would also like to thank his graduate students, Mathieu Levesque, Jean-Charles Guimond, Eric Villiard, Jean-Francois Denis, who have been great teachers and friends in and out of the lab. Dr. Fernando Congote, thank you for all of your lessons, assistance, and guidance as I attempted to navigate through the world of insect cells and protein purification. Dr. Thomas Mustoe, thank you so much for allowing me to learn so much at your lab, and to Dr. Corrie Gallant-Behm and Dr. Rob Fang for teaching me the finer details of how to conduct wound healing studies. Janice Penney and Michelle Read, our list of collaborators would not be complete without a huge thank you to you two – for the guidance that you've offered since day 1 of graduate school, and for your infinite knowledge of transgenic mice. I don't know what I would have done without you!

Dr. Lessard, I would like to thank you for your encouragement and opportunities to present my research to the plastic surgery community. These opportunities have reinforced my career goals, and I would like to thank you for this and also for your push for me to sprint across the grad school finish line.

Family and friends, thank you too. A lot. Dimitry Ofengeim, I suppose I owe you a huge thank you for the conversation we had walking through Central

Park one sunny summer day, when you convinced me that getting a PhD was the way to go. It wasn't easy, but it was certainly rewarding. Kyle Graham, whether you were living in Montreal or London, thank you for being there, forcing me to keep my bearings on the important aspects of life. To my sister Valerie, thank you for pushing me to work harder and achieve more. To my mom and dad, thank you. Thank you for always encouraging me to be the best I can be while staying true to myself. This thesis is dedicated to you.

Abstract:

Transforming growth factor-beta (TGF-ß) is a multifunctional growth factor involved in a variety of cellular processes including wound healing, extracellular matrix deposition, inflammation, and fibrosis. Excessive TGF-ß signaling during wound healing causes sustained inflammation and elevated expression of extracellular matrix proteins, which have both been associated with fibrosis and scarring. Therefore, inhibition of TGF-ß during wound healing is an attractive target to reduce fibrotic skin disorders. Our lab is interested in CD109, a 150 kDa GPI-anchored protein which has been shown to bind TGF-ß in its soluble form and to inhibit TGF-ß signaling in keratinocytes and fibroblasts *in vitro*. The TGF-ß antagonist properties of CD109 suggest that modulation of its expression *in vivo* might ameliorate fibrosis and scarring, perhaps via differential activation of keratinocytes and fibroblasts. To investigate the role of CD109 in the skin, we generated transgenic mice overexpressing CD109 in the epidermis.

To explore the role of CD109 during wound healing, I conducted incisional and excisional wound healing studies using CD109 transgenic mice and wild-type littermate controls. These studies demonstrate improved scarring parameters including reduced myofibroblast differentiation, reduced granulation tissue, and improved extracellular matrix architecture in the CD109 transgenic mice. Additionally, the data in this thesis show that overexpression of CD109 in the epidermis inhibits immune cell recruitment, and is associated with a reduction in expression of the proinflammatory cytokines IL-1 and MCP-1. I correlated these data with immunohistochemical analysis of Smad2/3 phosphorylation, and

show that CD109 overexpression is associated with a reduction in TGF-ß signaling. Collectively, these data suggest that overexpression of CD109 in the epidermis inhibits fibrosis during wound healing in a TGF-ß dependent manner.

To better understand the role of CD109 in fibrosis, I employed a murine bleomycin-induced model of fibrosis, which exhibits similarities to scleroderma, in CD109 transgenic mice and wild-type littermates. This study shows that CD109 transgenic mice express reduced levels of the extracellular matrix proteins fibronectin and collagen I, as well as Smad2/3 phosphorylation, and also display improved extracellular matrix architecture and reduced dermal thickness. Collectively, these data suggest that CD109 resists bleomycin-induced fibrosis, and could thus be an attractive target for therapeutic treatment of fibrotic skin disorders.

To better understand how CD109 modulates TGF-ß *in vivo*, I analyzed the TGF-ß signaling molecules in skin, primary keratinocytes, and primary fibroblasts harvested from CD109 transgenic mice and wild-type littermates. Here, I show that CD109 transgenic mice express less collagen I and fibronectin, and display elevated ALK1 expression and signaling through the Smad1/5/8 pathway while wild-type mice express elevated ALK5 and preferentially signal through the Smad2/3 pathway. Investigation of cultured keratinocytes showed a similar expression pattern, with the only difference existing in the expression of ALK1 and ALK5 which were both increased in the CD109 transgenic keratinocytes, and fibroblasts from both genotypes showed similar expression patterns. In both cultured keratinocytes and whole skin extracts, the CD109 transgenic tissue

expressed less TGF-ß than their wild-type counterparts. The differential TGF-ß signaling described here could underscore our previous observations indicating that overexpression of CD109 inhibits scarring and fibrosis *in vivo*.

Collectively, the data presented here demonstrate that CD109 potently alters physiological processes in the skin *in vivo*. The capacity for CD109 to reduce scarring, fibrosis, and inflammation *in vivo* makes it an attractive target for treating fibrotic skin disorders.

Résumé:

Le facteur de croissance transformant bêta (TGF-ß), est un facteur de croissance multifonctionnel impliqué dans une multitude de processus cellulaires tel que la cicatrisation, la déposition de matrice extracellulaire, l'inflammation et la fibrose. Ainsi, l'inhibition de TGF-ß semble être une cible de choix pour réduire les désordres fibrotiques de la peau. Notre laboratoire a identifié CD109, une proteine de 180 kDa ancrée à un glycosylphosphatidylinositol agissant comme co-récepteur cellulaire de TGF-ß. Ce nouveau récepteur, auquel TGF- ß s'ancre avec très haute affinité, aurait pour propriété d'inhiber la signalisation intra-cellulaire de TGF-ß. Les propriétés antagonistes de CD109 suggèrent que la modulation de son expression *in vivo* dans la peau pourrait améliorer la cicatrisation et la fibrose cutanée. Pour investiguer le role de CD109 dans la peau, nous avons généré une souris transgénique en clonant CD109 en aval du promoteur de keratin-14, limitant ainsi la surexpression de CD109 à l'épiderme.

Afin d'explorer le rôle de CD109 au sein de la guérison des plaies, nous avons conduit des études de ce processus où nous avons pu observer une amélioration des paramètres de cicatrisation tels que la réduction de la différentiation des myofibroblastes, la réduction du tissu de granulation et une amélioration de l'architecture de la matrice extracellulaire chez les souris transgénique CD109 comparées au souris de génotype sauvage pour la même portée. De plus, la surexpression de CD109 dans l'épiderme inhibe le recrutement de cellules immunitaires au site de la plaie et est associé avec une réduction de la signalisation de TGF-ß ainsi que l'expression des cytokines pro-inflammatoire IL-

1 et MCP-1. Toutes ces données, suggèrent que la surexpression de CD109 dans l'épiderme inhibe la fibrose cutanée lors de la guérison des plaies.

Nous avons ensuite examiné le rôle de CD109 lors de la fibrose en utilisant un model de sclérodermie murine induit par bleomycine. La souris transgénique CD109 exprime des niveaux réduits des protéines fibronectine et collagène 1 dans la matrice extracellulaire, ainsi que des niveaux réduits de phosphorylation de Smad2/3. Elle démontre aussi une meilleure architecture de la matrice extracellulaire et une réduction de l'épaisseur de la couche dermique. Toutes ces données suggèrent que CD109 résiste à la fibrose induite par bleomycine. Cela pourrait donc en faire une cible attrayante pour traitement thérapeutique des désordres fibrotiques de la peau.

Pour mieux comprendre comment CD109 modifie l'action de TGF-ß *in vivo*, nous avons analysé les molécules de signalisation de TGF-ß dans la peau, les keratinocytes primaires et les fibroblastes primaires récoltés des souris transgéniques et de leur sœurs de portée du génotype sauvage. Les souris transgéniques CD109 expriment substantiellement moins de collagène I, de fibronectine et démontre une expression élevée de ALK1 et une activation élevée de la voie Smad1/5/8. Les études utilisant des keratinocytes cultivés démontrent une phosphorylation de Smad et une expression des gènes de la matrice extracellulaire similaire; l'expression de ALK1 et ALK5 étant toutefois plus élevée dans les keratinocytes transgéniques CD109 comparé à ceux du génotype sauvage. Les fibroblastes des deux génotypes démontrent des profils d'expression similaires. La signalisation différentielle de TGF-ß pourrait rehausser nos

observations précédente et ainsi renforcer l'idée que la surexpression de CD109 inhibe la cicatrisation et la fibrose cutanée *in vivo*.

Ainsi, les données présentée démontrent que CD109 est un puissant altérateur des processus physiologiques de guérison des plaies, cicatrisation, fibrose et inflammation dans la peau *in vivo*. La capacité pour CD109 de réduire la cicatrisation, la fibrose et l'inflammation dans la peau *in vivo* en font une cible attrayante pour traiter les désordres fibrotiques de la peau.

Contributions to Original Knowledge:

This thesis has been prepared and written in accordance with the guidelines stipulated by the Faculty of Graduate and Postdoctoral Studies of McGill University. I attest that this thesis, entitled "Overexpression of CD109 in the epidermis reduces skin fibrosis and inflammation", has been written by myself, Joshua Vorstenbosch. This thesis has been prepared in the "manuscript-based" format, and consists of five referenced chapters entitled: (1) Introduction; (2) Transgenic Mice Overexpressing CD109 in the Epidermis Display Decreased Inflammation and Granulation Tissue and Improved Collagen Architecture During Wound Healing; (3) CD109 overexpression ameliorates skin fibrosis in a bleomycin-induced mouse model of scleroderma; (4) Overexpression of CD109 in the Epidermis Differentially Regulates ALK1 vs ALK5 Signaling and Modulates Exrtracellular Matrix Synthesis; and (5) Conclusions & Summary.

I assert that I, Joshua Vorstenbosch, 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 generated a mouse model to study CD109 by producing three transgenic mouse lines overexpressing CD109 in the epidermis of the skin under the control of a keratin-14 promoter

2) I have shown that transgenic mice overexpressing CD109 in the epidermis display improved extracellular matrix organization as compared to wild-type littermate controls using excisional and incisional wound models
3) I have shown that overexpression of CD109 in the epidermis promotes epidermal thickening, delays dermal thickening, and reduces granulation tissue volume in excisional wounds
4) I have shown that CD109 overexpression in the epidermis inhibits Smad2 and Smad3 phosphorylation *in vivo* in excisional wounds and unwounded skin and in response to a bleomycin-induced model of skin fibrosis
5) I have shown that CD109 transgenic mice display improved dermal architecture during incisional wound healing compared to wild-type controls
6) I have shown that overexpression of CD109 in the epidermis reduces macrophage and neutrophil recruitment in excisional wounds
7) I have shown that overexpression of CD109 in the epidermis reduces IL-1, MCP-1, Collagen I and Fibronectin expression in excisional wounds
8) I have shown that over expression of CD109 in the epidermis improves extracellular matrix architecture and decreases dermal thickening in a bleomycin-induced skin fibrosis in a murine model of scleroderma
9) I have shown that overexpression of CD109 in the epidermis reduces Smad2/3 phosphorylation and extracellular matrix gene expression in a bleomycin-induced murine model of scleroderma

10) I have shown that overexpression of CD109 in the epidermis results in increased ALK1 expression and Smad1/5/8 phosphorylation *in vivo*, with reduced ALK5 levels and Smad2/3 phosphoyrlation compared to wild-type littermates

11) I have shown that cultured primary keratinocytes from CD109 transgenic and wild-type mice display different expression patterns of ALK1 and ALK5 *in vitro* compared to levels observed in mouse skin extracts *in vivo*

The data and texts from Chapters 2, 3, and 4 comprise the following manuscripts:

Vorstenbosch J, Gallant-Behm C, Trzeciak A, Roy S, Mustoe T, and Philip A. Transgenic Mice Overexpressing CD109 in the Epidermis Display Decreased Inflammation and Granulation Tissue and Improved Collagen Architecture During Wound Healing. *Accepted for publication by Wound Repair & Regeneration, November 27, 2012.*

Vorstenbosch J, Al-Ajmi H, Winocour S, Trzeciak A, Lessard L, and Philip A. CD109 overexpression ameliorates skin fibrosis in a bleomycin-induced mouse model of scleroderma. *Revisions submitted to Arthritis & Rheumatism.*

Vorstenbosch J, Trzeciak A, Bizet A, and Philip A. Overexpression of CD109 in the Epidermis Differentially Regulates ALK1 vs ALK5 Signaling and Modulates Exrtracellular Matrix Synthesis. *In preparation.*

All of the data presented herein are the work of Joshua Vorstenbosch with the following exceptions:

1) Wound healing experiments were conducted by Joshua Vorstenbosch in collaboration with Corrie Gallant-Behm and Rob Fang in the laboratory of Dr. Thomas Mustoe

2) Immunohistochemistry to identify neutrophil and macrophage recruitment to excisional wounds in CD109 transgenic and wild-type littermate mice, as well as quantification of cells per high power field, was conducted by Alissa Trzeciak in Dr. Anie Philip's lab
3) Bleomycin and PBS injections were performed in collaboration with Hasan Al-Ajmi, Alissa Trzeciak, and Sebastian Winocour in Dr. Anie Philip's lab
4) Immunoblots of pSmad2/3, Fibronectin, Collagen in skin tissue harvested from bleomycin-induced fibrotic skin were conducted by Alissa Trzeciak, Sebastian Winocour and Hasan Al-Ajmi in Dr. Anie Philip's lab
5) Histological staining and dermal thickness measurement of mouse skin treated with bleomycin was conducted by Alissa Trzeciak in Dr. Anie Philip's lab

Abbreviations:

5-FU	5-Fluoro-Uracil
ADP	Adenosine Diphosphate
ALK1	Activin-Like Receptor 1
ALK5	Activin-Like Receptor 5
α-sma	alpha-smooth muscle actin
ATP	Adenosine Triphosphate
bFGF	basic fibroblast growth factor
C3	Complement 3
C4	Complement 4
C5	Complement 5
CBP	CREB Binding Protein
CCN2	CYR61/CTGF/NOV
CIHR	Canadian Institutes of Health Research
CTGF	Connective Tissue Growth Factor
DAB	3,3-Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FGF-2	Fibroblast Growth Factor 2
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPI	Glycophosphatidylinositol
HHT	Hereditary Hemorrhagic Telangiectasia
HLA-15	Human Leukocyte Antigen 15
IFN-α	Interferon Alpha
IFN-α2b	Interferon Alpha-2b
IL-1	Interleukin 1
IL-13	Interleukin 13
IL-1R	Interleukin 1 Receptor
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
K14	Keratin 14
kDa	kilodalton
KGF-1	Keratinocyte Growth Factor 1
KGF-2	Keratinocyte Growth Factor 2

LAP	Latency Associated Peptide
LTBP	Latent TGF-beta Binding Protein
M6P	Mannose-6-Phosphate
MCP-1	Monocyte Chemotactic Protein 1
MIP-1α	Macrophase Inflammatory Protein alpha
MIP-2	Macrophage Inflammatory Protein 2
MMLV	Moloney Murine Leukemia Virus
MMP	Matrix Metalloproteinase
MT1-MMP	Membrane Type 1 Matrix Metalloprotease
NGS	Normal Goat Serum
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PMSF	Phenylmethanesulfonylfluoride
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SERM	Selective Estrogen Receptor Modulator
siRNA	Short Interfering Ribonucleic Acid
SSc	Systemic Sclerosis
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween
TG	Transgenic
TGF-ß	Transforming Growth Factor Beta
TGF-ßRI	Transforming Growth Factor Beta Receptor Type 1
TGF-ßRII	Transforming Growth Factor Beta Receptor Type 2
Th2	T-Helper 2
TIMP	Tissue Inhibitor of Metalloproteinase
TNF-α	Tumour Necrosis Factor alpha
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
WT	Wild-Type

Chapter 1: Introduction

Wound Healing

Wound healing is a complex process involving a diverse population of cells, cytokines, and growth factors. A wide variety of wound types can arise from a wealth of different injuries to the skin, ranging from abrasions to burns to trauma. Effective wound healing with tight coordination of the many events restoring integrity of the compromised skin is important to prevent infection and loss of blood from the site of injury. A variety of conditions exist that can impair proper wound healing, including diabetes, steroid treatment, and compromised immunity (Guo and Dipietro, 2010). Additionally, complications can arise during wound healing due to severe infections or repeated injuries that can progress to excessive scarring (Wolfram *et al.*, 2009). One major growth factor that has been implicated in the regulation of wound healing is TGF-ß, and dysregulation of its signaling has been associated with many inflammatory and fibrotic wound healing disorders (Beanes *et al.*, 2003). Due to the current paucity of pharmacologic treatment strategies for impaired wound healing, the morbidity associated with impaired wound healing warrants significant scientific attention in an attempt to develop treatments to correct these pathological aberrations.

Phases of Wound Healing

Wound healing consists of three distinct yet overlapping phases, which include the inflammatory phase, proliferative phase, and remodeling phase

(Singer and Clark, 1999). Each of these phases involve various cell types and growth factors that function to progress from hemostasis, to inflammation, tissue proliferation, angiogenesis, reepithelialization, fibroblast differentiation, extracellular matrix deposition, and extracellular matrix remodeling.

Inflammatory Phase

The inflammatory phase of wound healing begins immediately following tissue injury and progresses through hemostasis, platelet degranulation, and chemotaxis for immune cells that phagocytose foreign material present in the wound and release a host of growth factors to progress into the proliferation phase. TGF-ß plays an important role throughout the inflammatory phase, which will also be discussed here.

Hemostasis

Hemostasis occurs in response to tissue injury via vasoconstriction, coagulation, clot formation, and platelet aggregation. Immediately following injury to the skin, the injured epithelium and local reduction in oxygen tension induce vasoconstriction to reduce blood flow to the damaged vessel (Nurden, 2011). Concurrently, tissue factor is exposed on the damaged epithelium activating the extrinsic clotting pathway, which works in concert with the intrinsic clotting pathway to activate thrombin (Teller and White, 2009). Activated thrombin then converts fibrinogen to fibrin to form a meshwork into which platelets, also activated by thrombin, aggregate to form a clot (Furie and Furie, 2008). Adhesive proteins including Von Willebrond Factor (vWF) and fibronectin accumulate in the fibrin meshwork and facilitate platelet aggregation

by binding to receptors on activated platelets (Nesbitt *et al.*, 2009). The fibrin clot prevails throughout the inflammatory phase, acting as a provisional matrix scaffolding upon which other cells including neutrophils, monocytes, fibroblasts, and endothelial cells use for migration to the wound (Broughton *et al.*, 2006; Tsirogianni *et al.*, 2006). Vasoconstriction, clot formation, and platelet aggregation occlude the injured vessel to minimize subsequent blood loss, and allowing platelets aggregated at the clot to release a wealth of growth factors to propagate the inflammatory response (Laurens *et al.*, 2006).

Platelet Degranulation

Upon binding to adhesive extracellular matrix components such as collagen, fibronectin, and thrombin at the site of the clot, activated platelets secrete alpha granules and dense granules containing many cytokines and growth factors (Nurden, 2011). Alpha granules store the majority of proteins released by platelets, and release additional adhesive proteins including fibronectin,

vitronectin, and thrombospondin-1, which bind cell surface receptors on platelets and further growth of the thrombus (Nurden *et al.*, 2008).

Some of the growth factors that play an important role in wound healing released from alpha granules include platelet derived growth factor (PDGF), transforming growth factor beta (TGF-ß), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) (Nurden *et al.*, 2008). These growth factors are important during the wound healing process to support progression of the inflammatory phase. Notably, PDGF plays a major role in immune cell recruitment to the wound and is

mitogenic for neighbouring dermal fibroblasts (Li *et al.*, 2007). TGF-ß is associated with recruitment of inflammatory cells to the wound and is also involved in the proliferation and remodelling phases of wound healing (Brunner and Blakytny, 2004).

Alpha granules also release a variety of cytokines to the wound to direct the inflammatory response including MIP-1α, MIP-2, MCP-1, RANTES, IL-1, IL-8, and TGF-ß (Nurden, 2011). Collectively, these molecules modulate inflammation, and are instrumental in the recruitment of neutrophils and macrophages to the wound to initiate an innate immune response (Nurden, 2011).

Dense granules, while less in number than alpha granules, release ADP/ATP, calcium, and serotonin, which all contribute to hemostasis. ADP and ATP are important co-factors facilitating platelet aggregation (Grenegard *et al.*, 2008; Kahner *et al.*, 2006). Calcium is required for fibrin formation, and release from dense granules reinforces formation of the fibrin clot (Lansdown, 2002). Serotonin release induces vasoconstriction of the compromised blood vessels at the wound, contributing to hemostasis (Steed, 1997).

Immune Cell Recruitment/Chemotaxis

The growth factors, cytokines, and other molecules released upon platelet degranulation support wound healing by causing an influx of inflammatory cells and in turn initiate inflammation. Once hemostasis has been achieved at the wound, prostaglandins are released increasing vascular permeability (Broughton *et al.*, 2006). The resulting increase in vascular permeability and release of

cytokines including IL-1 and TGF-ß at the wound site promotes infiltration of neutrophils and monocytes across the vessel wall.

Increased vascular permeability is associated with expression of neutrophil intracellular adhesion molecules in response to IL-1 and TGF-ß (Pohlman *et al.*, 1986; Witte and Barbul, 1997). This allows neutrophils to adhere to endothelial cells and traverse the vessel wall, and neutrophils then adhere to ECM proteins via integrin receptors (Werr *et al.*, 2000). Once neutrophils bind to ECM, they migrate through the wound to eliminate cell debris and bacteria by producing oxygen free-radicals in addition to the proteases elastase and matrix metalloproteinase (MMP) (Yager and Nwomeh, 1999). Neutrophils eventually become senescent, and they then undergo apoptosis and are cleared via phagocytosis by macrophages (Newman *et al.*, 1982; Simpson and Ross, 1972).

Following neutrophil infiltration, monocytes are similarly recruited to the wound site by TGF-ß, PDGF, and IL-1. Monocytes crossing the vessel wall into the extravascular space transform into macrophages in response to fibronectin, complement, thrombin, and TGF-ß (Fukai *et al.*, 1991). Fibroblasts also assist in macrophage activation by secreting interferon-gamma (IFN-gamma) (Broughton *et al.*, 2006). Activated macrophages then debride the wound of bacteria and foreign material via phagocytosis (Newman *et al.*, 1982). In addition to clearing the wound, macrophages also produce substantial quantities of growth factors and cytokines including PDGF, TNF-α, IL-6, and GM-CSF, which facilitate further macrophage and fibroblast recruitment, and participate in initiation of the proliferation phase (Broughton *et al.*, 2006; Monaco and Lawrence, 2003).

Macrophages persist in the wound for approximately seven days, and their numbers slowly decline as the proliferation phase progresses (Martin and Leibovich, 2005).

Proliferation Phase

During the proliferative phase of wound healing, the inflammatory cells populating the fibrin clot are gradually replaced by granulation tissue consisting of declining macrophages, provisional extracellular matrix, fibroblasts, and endothelial cells. As the proliferative phase progresses, fibroblasts will differentiate into myofibroblasts and deposit a provisional extracellular matrix, upon which keratinocytes will migrate to reepithelialize the skin, and endothelial cells form new vasculature.

Fibroblast Proliferation and Differentiation

Upon wounding, dermal tissue is damaged and consequently resident fibroblasts are activated to begin repairing the defect. Fibroblasts are recruited to the wound site by induction of fibroblast migration from healthy dermis adjacent to the wound under the control of growth factors released by macrophages including TGF-ß, PDGF, EGF, and fibronectin (Broughton *et al.*, 2006; Monaco and Lawrence, 2003). Growth factor activation of fibroblasts induces integrin expression, which allows fibroblasts to bind fibronectin on the provisional matrix and stimulate cell migration into the wound bed (Carter, 1970; Grzesiak and Pierschbacher, 1995; Reed *et al.*, 1993). In addition to migration, TGF-ß and PDGF also induce fibroblast proliferation, thereby increasing the fibroblast population at the wound (Seppa *et al.*, 1982).

Fibroblasts in the wound bed elaborate the provisional matrix by depositing collagen type III and fibronectin in response to PDGF (Pierce *et al.*, 1991). TGF-ß levels rise as the proliferation phase progresses, resulting in stabilization of the matrix and further elevation of ECM proteins (Broughton *et al.*, 2006). TGF-ß induces collagen type I expression, a component of a more permanent ECM, and also reduces MMP expression while increasing tissue inhibitor of matrix metalloproteinases (TIMP) to further stabilize the ECM by reducing catabolic proteolytic activity (Goldman, 2004).

TGF-ß expression at the wound also induces fibroblast differentiation to myofibroblasts (Hinz, 2007). Myofibroblasts are characterized by α-smooth muscle actin expression, conferring a contractile phenotype to facilitate wound contraction (Eyden, 2005). Myofibroblasts further contribute to ECM deposition by continuing to produce collagen type I as their fibroblast precursors did as well (Faouzi *et al.*, 1999).

Reepithelialization

Reepithelialization is a crucial component of wound healing that serves to restore the cutaneous barrier protecting the skin. Within a day following wounding, basal keratinocytes at the wound margin and in the skin appendages, such as sweat glands and hair follicles, begin to migrate towards the injured tissue (Nanney *et al.*, 1984; Tsirogianni *et al.*, 2006). Integrins on keratinocytes recognize provisional matrix components including collagen I, fibronectin, and vitronectin to promote migration (Henry and Garner, 2003). As the keratinocytes migrate across the provisional matrix, they re-establish a basement membrane by

secreting tenascin, vitronectin, collagen I, and collagen IV (Mackie *et al.*, 1988). TGF-ß produced by keratinocytes and macrophages promotes keratinocyte migration, and when keratinocytes from each side of the wound meet each other, they experience contact inhibition to inhibit further cell migration and promote keratinocyte proliferation (Monaco and Lawrence, 2003). Keratinocyte migration and proliferation is under control of KGF-1, KGF-2, and IL-6, which are secreted by fibroblasts in response to IL-1 and TNF-α at the wound bed (Smola *et al.*, 1993). The result of the keratinocyte proliferation and migration, as well as production of basement membrane, is restoration of the cutaneous layer and division of the epidermis and dermis.

Angiogenesis

Angiogenesis is crucial to wound healing. In order to provide oxygen and nutrients to the proliferating cells in the wound, neovascularization must occur (Monaco and Lawrence, 2003). Various factors at the wound site contribute to initiation of angiogenesis, including low pH, high lactate levels, and reduced oxygen tension (Witte and Barbul, 1997). Keratinocytes, and to a lesser extent fibroblasts and macrophages, secrete VEGF in response to local tissue hypoxia, EGF and TGF-ß (Detmar *et al.*, 1995). Additonally, macrophages secrete bFGF which also regulates endothelial cell function, directing cells in capillaries at the wound margin migrate towards the wound bed in response to VEGF and bFGF (Gospodarowicz *et al.*, 1989). As the endothelial cells migrate, they express MMPs to breakdown ECM to facilitate movement throughout the wound bed (Cornelius *et al.*, 1995). Newly formed capillaries facilitate granulation tissue

development, and once new granulation tissue has been deposited at the wound, many of the newly formed blood vessels undergo apoptosis (Ilan *et al.*, 1998).

Remodelling Phase

Many of the cellular activities in the inflammatory phase set the stage for the remodeling phase, which begins and overlaps with the inflammatory and proliferation phases as cells residing in the granulation tissue undergo apoptosis (Gurtner *et al.*, 2008). Fibroblasts and myofibroblasts play a particularly important role during the remodeling phase by further depositing ECM proteins and propagating wound contraction, respectively (Broughton *et al.*, 2006). As the wound contracts and more ECM is deposited, a delicate balance of proteases and protease inhibitors contributes to the remodeling of the ECM, perhaps under the control of paracrine signaling between the epidermis and dermis, for approximately a year after tissue injury to restore integrity of the skin as much as possible (Gill and Parks, 2008).

Wound Contraction

Wound contraction begins as fibroblasts differentiate into myofibroblast. α-smooth muscle actin fibers expressed in myofibroblasts confer contractility to the wound, enhancing wound closure (Hinz, 2007). Wound contraction begins in the proliferative phase and ends in the remodelling phase, and is promoted by TGF-ß and fibronectin interaction with myofibroblasts (Gabbiani, 2003). Wound contracture is stabilized by deposition of extracelluar matrix proteins from both fibroblasts and myofibroblasts (Gabbiani, 2003; Tomasek *et al.*, 2002). Other molecules such as MMP-3 and MMP-13 are secreted by fibroblasts and have also

been implicated in wound contraction, with MMP-3 and MMP-13 knockout mice displaying impaired wound contraction (Bullard *et al.*, 1999; Hattori *et al.*, 2009). Myofibroblasts continue to contract around the extracellular matrix until epithelialization has completed, at which point ECM remodeling continues to reinforce the healed wound.

Extracellular Matrix Remodeling

Immediately after wound healing, hemostasis occurs when platelets bind to a fibrin meshwork, which forms the initial foundation of the provisional matrix (Singer and Clark, 1999). This temporary matrix serves as a scaffold for infiltrating fibroblasts, endothelial cells, and inflammatory cells, and is largely composed of fibrin and fibronectin (Nurden, 2011). As fibroblasts infiltrate into the provisional matrix, they begin to deposit collagen III and collagen I into the wound bed, while secreting proteinases such as MMP-1, MMP3, and MMP-9 that catabolize the existing matrix as collagen molecules are deposited (Barrientos *et al.*, 2008).

As wound healing progresses, fibroblasts and myofibroblasts deposit increasingly more collagen I, and secrete a balance of proteinases and inhibitors of proteinases, such as TIMPs, to control ECM deposition and catabolism (Hinz, 2007; Toriseva and Kahari, 2009). ECM remodeling is mediated by a host of various growth factors, with deposition being driven by CTGF, TGF-ß, FGF-2 and PDGF while degradation is induced by IL-1 and TNF-α (Toriseva and Kahari, 2009; Werner and Grose, 2003). In addition to promoting ECM degradation,

MMPs also play a significant role in liberation of growth factors from the ECM as well as their activation (Gill and Parks, 2008).

Extracellular matrix remodeling continues for approximately one year after the initial injury, and after about 21 days, the synthesis and degradation of ECM proteins reaches an equilibrium (Broughton *et al.*, 2006). As ECM remodeling progresses, the majority of cells residing in the once highly active wound undergo apoptosis, with relatively few fibroblasts with low metabolic activity remaining in an avascular scar composed largely of dense collagen fibrils (Singer and Clark, 1999). Once healed, the maximum resulting tensile strength of the collagen fibers reaches only 80% of the original skin (Gurtner *et al.*, 2008).

Role of TGF-ß Signaling in Wound Healing

Since its discovery in 1981, a wealth of data has been collected about the role of TGF-ß in wound healing (Beanes *et al.*, 2003). Classic reports from the late 1980's uncovered the capacity of TGF-ß to accelerate wound healing, modulate inflammation, and enhance extracellular matrix synthesis (Mustoe *et al.*, 1987; Roberts *et al.*, 1992; Wahl *et al.*, 1989). It was also found that TGF-ß is expressed by or released from macrophages, fibroblasts, keratinocytes, and platelets, further suggesting a significant role for TGF-ß during wound healing in light of its potent *in vitro* and *in vivo* effects on skin cells (Eppley *et al.*, 2004; Lee *et al.*, 1997; Wu *et al.*, 1997).

TGF-ß is released in large amounts to the wound via the expulsion of alpha-granules from degranulating platelets during hemostasis in the inflammatory phase of wound healing (Nurden, 2011). At this site, TGF-ß has

potent chemotactic activity, recruiting neutrophils and macrophages to the wound (Pohlman *et al.*, 1986; Wahl *et al.*, 1987). In addition to recruiting macrophages, TGF-ß also activates macrophages to further produce more TGF-ß, acting in a feed-forward mechanism to sustain macrophage activity at the wound (Lawrence and Diegelmann, 1994). TGF-ß also enhances the inflammatory response by upregulating expression of pro-inflammatory growth factors IL-1, TNF-α, PDGF, and FGF-2 (Wahl *et al.*, 1987). These observations are explained in part by Ashcroft et al, who demonstrate that mice deficient in the TGF-ß intracellular second messenger, Smad3, display an impaired inflammatory response (Ashcroft *et al.*, 1999). As the inflammatory phase declines, TGF-ß prepares the wound for the proliferative phase by inducing fibroblast proliferation and chemotaxis from the dermis surrounding the wound to the wound site (Beanes *et al.*, 2003).

The infiltrating fibroblasts are the major players during the proliferation phase, and are largely regulated by TGF-ß signaling. TGF-ß promotes expression of various integrin proteins on the extracellular surface of the fibroblasts, thereby promoting adhesion of fibroblasts to the provisional matrix (Margadant and Sonnenberg, 2010). One of the early functions of TGF-ß identified in the skin was the ability of TGF-ß to induce ECM proteins in fibroblasts (Roberts *et al.*, 1992). Under control of TGF-ß, during the proliferative phase, fibroblasts deposit significant amounts of collagen I and collagen III to replace the provisional matrix of the granulation tissue (Singer and Clark, 1999). TGF-ß reinforces ECM deposition by upregulating tissue inhibitors of matrix metalloproteinases (TIMPs),

thereby inhibiting proteinase activity at the wound and reducing the degradation of collagen (Goldman, 2004).

While the regulation of fibroblasts by TGF-ß is well defined, its role in angiogenesis and its effects on endothelial cells *in vivo* remains controversial. Studies administering TGF-ß subcutaneously in mice demonstrate improved angiogenesis, but *in vitro* studies have yielded contradictory results (Collo and Pepper, 1999; Roberts *et al.*, 1986). Another study investigating the interplay between TGF-ß and VEGF, however, suggests that TGF-ß does in fact induce angiogenesis both *in vitro* and *in vivo* in concert with VEGF, via a series of inter-related apoptotic events (Ferrari *et al.*, 2009).

The regulatory function of TGF-ß on epithelialization is complex, but the net effect of TGF-ß signaling enhances re-epithelializiation during wound healing. TGF-ß induces expression of $\alpha_5ß_1$, $\alpha_5ß_5$, and $\alpha_5ß_6$ integrins on the extracellular surface of keratinocytes, facilitating keratinocyte migration across the wound bed (Margadant and Sonnenberg, 2010). However, TGF-ß signaling also inhibits keratinocyte proliferation, which suggests an inhibitory contribution to re-epithelialization (Yang *et al.*, 1996). However, *in vivo* studies investigating cutaneous wound healing in Smad3 null mice and in mice over expressing Smad2 in the epidermis demonstrate accelerated and impaired reepithelialization, respectively, indicating that the balance between TGF-ß effects on migration and proliferation shifts in favour of migration during wound healing (Ashcroft *et al.*, 1999; Hosokawa *et al.*, 2005).

Toward the end of the proliferation phase through the beginning of the remodeling phase, TGF-ß plays an important role in fibroblast differentiation to myofibroblasts and subsequent wound contraction. The hallmark of myofibroblast differentiation is the induction of α-smooth muscle actin expression, which is largely induced via TGF-ß signaling, coupled by mechanotransduction of cellular stress forces (Hinz, 2007). Thus, by facilitating the differentiation from fibroblast to myofibroblast, TGF-ß promotes wound contraction and in turn enhances wound closure.

During the remodeling phase, TGF-ß signaling promotes ECM deposition by enhancing collagen deposition, and reduces degradation by reducing MMP-1, MMP-3, and MMP-9 expression and enhancing expression of TIMPs (Barrientos *et al.*, 2008). While the action of TGF-ß to promote ECM deposition can increase the strength of the wound, dysregulation of TGF-ß signaling can contribute to the progression of fibrotic wound healing and scarring. Shah and Ferguson showed that addition of TGF-ß to incisional wound in mice results in more extensive scarring, while addition of neutralizing antibodies to TGF-ß isoforms 1 and 2 inhibits wound fibrosis (Shah *et al.*, 1992, 1995). Synergistic signaling between TGF-ß and CTGF also contributes to scarring (Colwell *et al.*, 2005). Collectively, these reports reinforce the capacity of TGF-ß to induce ECM expression, and demonstrate the importance of tightly regulated TGF-ß signaling during wound healing to prevent scarring and fibrosis.

Another interesting aspect of TGF-ß signaling occurs during fetal wound healing. Until the third trimester, fetal wounds do not scar (Larson *et al.*, 2010).

Unlike adult wounds and fetal wounds during the third trimester, there is an elevated ratio of the isoform TGF-ß3 to the isoforms TGF-ß1 and TGF-ß2 (Krummel *et al.*, 1988; Nath *et al.*, 1994). This is consistent with previous studies conducted by Shah and Ferguson, where they demonstrated that neutralizing TGF-ß1 and TGF-ß2 resulted in reduced scarring (Shah *et al.*, 1992, 1995). These data further support the integral role that TGF-ß signaling plays in deposition of extracellular matrix during wound healing, and its association with scar formation.

Epidermal-Dermal Interactions in Wound Remodeling

While the effects of isolated cell types have been studied extensively in wound healing models, recently there has been an increased focus on the effects of signaling between various cell types on the wound healing response. Keratinocytes in the epidermis and fibroblasts in the dermis secrete a wealth of growth factors throughout the wound healing response to regulate cellular responses in adjacent layers of the skin in a paracrine manner. Fibroblast-keratinocyte co-culture studies conducted *in vitro* have demonstrated that keratinocyte derived IL-1 can induce fibroblast expression of a host of growth factors including KGF and IL-6 (Smola *et al.*, 1993; Waelti *et al.*, 1992; Werner *et al.*, 2007). Similar growth factor profiles are also observed during wound healing, as IL-1 is one of the first cytokines released by keratinocytes in response to tissue injury (Freedberg *et al.*, 2001). Also consistent with the co-culture data is the observation that fibroblasts express elevated levels of KGF that target keratinocytes during early stages of wound healing, further supporting the notion

of paracrine signaling between keratinocytes and fibroblasts during wound healing (Maas-Szabowski and Fusenig, 1996; Werner and Smola, 2001).

Recent studies employing selective knockout of the TGF-ß receptor type II in dermal fibroblasts have provided further insight into the role of TGF-ß action on epidermal-dermal paracrine signaling. A study by Denton et al investigating wound healing in mice deficient in TGF-ß receptor II in the dermis demonstrated that the knockout mice display impaired dermal healing (Denton *et al.*, 2009). Additionally, the reduction in dermal TGF-ß signaling was accompanied by enhanced keratinocyte proliferation and reduced fibroblast differentiation to myofibroblasts (Denton *et al.*, 2009). Martinez-Ferrer et al conducted a wound healing study in a similar wound healing model, corroborating the results reported by Denton et al by showing accelerated re-epithelialization, reduced extracellular matrix deposition, and impaired myofibroblast differentiation with reduced wound contraction (Martinez-Ferrer *et al.*, 2010). Interestingly, this study also showed a reduction in macrophage recruitment to the wound, highlighting the importance of the dermal contribution to inflammation (Martinez-Ferrer *et al.*, 2010). Collectively, these two studies highlight the importance of cellular interactions during wound healing, and the complex roles played by TGF-ß on multiple cell types during the wound healing response.

Investigations into the role of the epidermis in scleroderma have indicated that keratinocyte signaling also plays an important role in the interplay between epidermal-dermal interactions. Aden et al report in an *in vitro* model of scleroderma that activated keratinocytes release growth factors affecting dermal

cellular physiology (Aden *et al.*, 2008). Notably, in a co-culture system they showed that IL-1 released from scleroderma keratinocytes induce dermal TGF-ß production and in turn, a fibrotic phenotype (Aden *et al.*, 2010). These studies highlight that aberrations in both keratinocytes and fibroblasts can significantly affect the neighbouring layer of skin tissue to induce fibrosis.

The transmission of growth factors from epidermis to dermis has been proposed to be mediated via exosomal secretion by keratinocytes. Recent studies have demonstrated that stratifin and 14-3-3 proteins modulate fibroblast MMP-1 stimulatory effect, and were shown to be released from keratinocytes by exosomes (Chavez-Munoz *et al.*, 2009; Chavez-Munoz *et al.*, 2008). While exosomes provide an interesting mechanism underlying paracrine signaling, further investigation into the molecular mechanisms underlying transmission of growth factors between the epidermis and dermis could provide insight into how the many different cell types participating in the wound healing process interact with each other.

Wound Healing Disorders

Wound healing is a dynamic and highly regulated process. Dysregulation of signaling at any phase of wound healing in any one of the many cell types involved can result in a series of pathologies. Certain conditions and treatments such as obesity, diabetes, and steroid therapy, can lead to impaired wound healing. Fibrosis is a common manifestation of aberrant wound healing, and can cause significant morbidity and mortality among patients affected by such conditions as hypertrophic scarring, keloids, and scleroderma.

Conditions Contributing to Impaired Wound Healing

There are various physiological and iatrogenic contributors to impaired wound healing. Obesity, an increasingly common cause of morbidity and mortality, is associated with impaired wound healing. The wound healing response in obese individuals has been associated with a higher rate of infection in surgical wounds (Guo and Dipietro, 2010). Furthermore, the increased adiposity in obese patients puts additional pressure on the injured skin causing mechanical tension on the wound, which in addition to the decreased vascularity and oxygenation at the wound site impairs the wound healing response (Anaya and Dellinger, 2006; Wilson and Clark, 2004). Consistent with the negative impact of obesity on wound healing, individuals who have lost weight have been shown to demonstrate improved wound healing (de Mello *et al.*, 2008; Nieman *et al.*, 1999).

Diabetes is another pathophysiological condition that has been associated with impaired wound healing. Chronic wounds develop in up to 15% of the diabetic population, which are associated with significant rates of infection due to impaired neutrophil chemotaxis and phagocytosis at the wound site (Brem and Tomic-Canic, 2007; Franz *et al.*, 2007). The well-described vasculopathies associated with diabetes contribute to a hypoxic wound environment, with further exacerbates wound healing in diabetic patients (Tandara and Mustoe, 2004). Other factors leading to impaired wound healing in diabetics are a reduced fibroblast response to growth factors, and decreased release of cytokines by macrophages, particularly VEGF, with further contributes to the relative hypoxia

in diabetic wound compared to non-diabetic wounds (Falanga, 2005). Collectively, the considerations mentioned here impair wound healing in diabetics, and ultimately contribute to a chronic wound healing state, which can have dire outcomes for the affected individual.

Individuals treated with systemic steroids also demonstrate a compromised wound healing response. Steroids are anti-inflammatory agents, which inhibit cellular responses in many different resident cells at the wound site, leading to increased rates of wound infection. Specifically, macrophage signaling is inhibited which leads to reduced VEGF expression, and in turn, less angiogenesis at the wound, and fibroblast activation and differentiation are also reduced which leads to diminished wound contraction (Hunt, 1980; Stephens *et al.*, 1971). There is also markedly less hydroxyproline, a key component of collagen, in the wounds of individuals treated with steroids, which also impedes wound repair (Gupta *et al.*, 1999). Thus, steroid therapy, while possessing significant value in the treatment of various diseases, should be taken into consideration when this therapy is used in individuals with healing wounds.

Hypertrophic Scaring

Hypertrophic scars present as a raised, red, scar comprised of excessive extracellular matrix within the margins of the original injury and can cause significant functional impairment, and are commonly associated with deep burns and traumatic injury to the skin (van der Veer *et al.*, 2009). Although the exact mechanism underlying the pathogenesis of hypertrophic scars is unknown, there are a variety of different factors that can contribute to the formation of

hypertrophic scars. With an exaggerated inflammatory response during wound healing, an excess of growth factors including PDGF and TGF-ß are present at the wound and contribute to fibroblast activation, and in turn, excessive extracellular matrix deposition (Niessen *et al.*, 1999). Circulating lymphocytes may also contribute to hypertrophic scarring in the case of excess Th2 inflammatory response. The Th2 response is characterized by the presence of cytokines IL-4, IL-13, and TGF-ß, which promote further cytokine production as well as activate fibroblasts to produce collagen and fibronectin (Armour *et al.*, 2007; Doucet *et al.*, 1998; Fertin *et al.*, 1991; Postlethwaite *et al.*, 1992). Sustained activation of keratinocytes may also induce hypertrophic scarring (Andriessen *et al.*, 1998; Machesney *et al.*, 1998). During wound healing, activated keratinocytes produce significant amounts of IL-1, which also stimulates fibroblast production of ECM proteins (Niessen *et al.*, 2001). Hypertrophic scars are also characterized by increased myofibroblast density at the wound site, further participating in the production of excessive amounts of ECM proteins including collagen I and III (Armour *et al.*, 2007; Nedelec *et al.*, 2001; Su *et al.*, 1998). The resulting collagen fibers in hypertrophic scars have a characteristic thin, nodular pattern, and accumulate in part due to reduced levels of MMP and increased levels of TIMP (Ehrlich *et al.*, 1994; Lee *et al.*, 2004; Verhaegen *et al.*, 2009). Furthermore, as the hypertrophic scar matures, fibroblast apoptosis is reduced compared to normal wound healing, sustaining the pro-fibrotic fibroblastic environment in the dermis (Aarabi *et al.*, 2007).

Keloids

While superficially keloids resemble hypertrophic scars, biochemically there are many distinct features. Notably, unlike hypertrophic scars, keloids extend beyond the margin of the original wounds, are more rare, and have a greater genetic component (Bran *et al.*, 2009). Histologically, keloid scars lack a nodular pattern display thicker collagen fibres than hypertrophic scars, and keloid scars also contain far fewer myofibroblasts (Ehrlich *et al.*, 1994; Kischer and Brody, 1981; Verhaegen *et al.*, 2009). Four histological characteristics deemed pathognomonic for keloids by Butler et al include keloidal hyalinized collagen, a tongue-like advancing edge under normal epidermal and papillary dermal tissue, horizontal fibrous bands composed of cells in the reticular dermis, and fibrous bands resembling fascia (Butler *et al.*, 2008). However, there are still many similarities between keloids and hypertrophic scars. Keloid scars express significantly higher levels of TGF-ß than normal, which contributes to an auto-inductive pattern in fibroblasts whereby TGF-ß signaling induces further TGF-ß expression (Lee *et al.*, 1999; Van Obberghen-Schilling *et al.*, 1988). The TGF-ß signaling in the dermis results in elevated levels of ECM proteins such as collagen and fibronectin, as well as increased fibroblast proliferation (Babu *et al.*, 1989; Bran *et al.*, 2009; Friedman *et al.*, 1993; Kischer and Hendrix, 1983). Keratinocytes may also play a role in keloid fibrosis, as cultured keratinocytes from keloid scars cultured with normal fibroblasts induced a fibrotic phenotype in the fibroblasts, characterized by increased levels of TGF-ß and collagen expression (Funayama *et al.*, 2003; Lim *et al.*, 2002; Lim *et al.*, 2001). As in

hypertrophic scars, the sustained activation of dermal fibroblasts in keloids results in stabilization of ECM proteins by a reduction in MMP expression and an increase in TIMP expression, which is further supported by a decrease in fibroblast apoptosis (Chodon *et al.*, 2000; Ravanti and Kahari, 2000).

Scleroderma

Scleroderma is yet another important fibrotic skin disease causing significant morbidity. It is characterized by a thickened dermis that results from accumulation of excessive ECM proteins (Trojanowska *et al.*, 1998; Uitto and Kouba, 2000). The precise mechanism resulting in scleroderma remains unknown, however there are a series of proposed causative factors including inflammation and autoimmunity (Abraham and Varga, 2005). Like hypertrophic scars, there appears to be a link between fibrosis in scleroderma and a Th2 immune response as indicated by increased release of IL-4, IL-13, and TGF-ß (Jinnin *et al.*, 2004; Oriente *et al.*, 2000; Salmon-Ehr *et al.*, 1996). Activated keratinocytes in scleroderma also express significant amounts of IL-1, which contribute to fibrosis by inducing ECM production in fibroblasts (Aden *et al.*, 2008). The dermis in skin affected by scleroderma contains elevated levels of TGF-ß, and like keloids, there exists autocrine expression of TGF-ß via signaling through fibroblasts and further TGF-ß activation via increased expression of integrins (Ihn, 2008). Specifically, TGF-ß expression was found to be increased at the leading edge of forming fibrotic lesions and sites of inflammation in the dermis of scleroderma skin (Querfeld *et al.*, 1999). TGF-ß signaling appears to be the main regulator of excessive collagen I production, with collagen expression

decreasing upon addition of anti-TGF-ß antibody to scleroderma (Ihn *et al.*, 2001). However, not only is TGF-ß ligand expression increased in scleroderma, its receptors are also upregulated in dermal fibrosis to reinforce the pro-fibrotic effects of TGF-ß (Ihn *et al.*, 2001; Kawakami *et al.*, 1998). The ratio of Type I to Type II TGF-ß signaling receptors has also been shown to contribute to fibrosis in scleroderma, as Pannu et al have demonstrated that the ratio of TGF-ßR-I:TGF-ßRII is increased in scleroderma fibroblasts and contributes to increased collagen I expression (Pannu *et al.*, 2004). Collectively, these factors contribute to the formation of excessive ECM production in the dermis of the affected area of skin, resulting in fibrosis.

Current Treatment Strategies for Fibrotic Skin Pathologies

Despite increasing understanding about the pathogenesis of fibrotic skin disorders, there currently lacks a truly effective therapy. Surgical excision of keloids and hypertrophic scars had yielded some success, but there remains significant recurrence in keloids (Mofikoya *et al.*, 2007; Wolfram *et al.*, 2009). Hydrocoritsone steroid injections can be effective in some instances for keloids and hypertrophic scars, but recurrence occurs in up to 50% of patients, and steroid application has not proven to be useful in scleroderma (Berman and Bieley, 1995; Leask, 2012). Applying pressure to the fibrotic area of skin has been used for quite some time, and is thought to work via reducing blood flow to deprive fibrotic fibroblasts of the nutrients they require to produce ECM proteins (Urioste *et al.*, 1999). However, pressure does not work uniformly across all fibrotic conditions (Mustoe *et al.*, 2002). Laser ablation and radiation treatment have

been proposed to function by reducing fibroblast proliferation and induce apoptosis, but fibrosis is also a potential side effect of both of these treatments (Donkor, 2007; Kuo *et al.*, 2004; Kuo *et al.*, 2005). While these techniques may alleviate fibrosis to a certain extent, they are invasive and inconvenient to patients receiving the therapy. Pharmacologic agents provide a more convenient means of treatment, and would result in increased patient compliance. To address this, various agents are currently being assessed for the treatment of fibrotic skin disorders.

There exists a handful of pharmacological treatment strategies for skin fibrosis in development that function by reducing TGF-β levels. Tamoxifen citrate, a known selective estrogen receptor modulator, and antihistamines have shown some therapeutic potential *in vitro* by reducing TGF-β levels and in turn, decreasing fibroblast proliferation and collagen production in cells cultured from keloid scars (Chau *et al.*, 1998; Gragnani *et al.*, 2010; Topol *et al.*, 1981). 1,4-Diaminobutane and Mannose-6-Phosphate (M6P) reduce TGF-β locally at the site of application by inhibiting TGF-β activation from its latent form (Gary-Bobo *et al.*, 2007; Telci *et al.*, 2009). Application of neutralizing antibodies to TGF-β have demonstrated a strong capacity to reduce scarring in rats (Ferguson, 1994). Despite the promise that these therapies present, they have not yet been optimized for use in human subjects.

Treatment strategies for fibrotic skin disorders are also in development that target TGF-β signaling, aiming to inhibit transmission of the TGF-β signal and subsequent target gene transcription. Retinoic acid offers mild relief and

improvement of scarring in keloids and hypertrophic by inhibiting TGF-β induced collagen production (Daly and Weston, 1986; Janssen de Limpens, 1980). Quercetin is a flavonoid found in many different topical scar creams that appears to inhibit TGF-β receptor expression, but its exact mechanism and actual efficacy remains obscure (Armour *et al.*, 2007; Phan *et al.*, 2003). Imatinib inhibits TGF-β signaling by blocking the non-canonical TGF-β signaling pathways, and while it has been suggested to have some utility in scleroderma, it has not yet been investigated in keloids and hypertrophic scars (Bhattacharyya *et al.*, 2009; Distler *et al.*, 2007). These inhibitors of TGF-β signaling all present some potential for treatment of fibrotic skin disorders, and as such warrant further investigation.

Other attempts to inhibit formation of fibrotic skin disorders pharmacologically target fibroblast profliration and action. Imiquimod and resiquimod are both toll-like receptor 7 and 8 agonists that inhibit fibroblast proliferation, but application to keloid scars has yielded variable results (Prado *et al.*, 2005; Sauder *et al.*, 2003). 5-Flurouracil (5-FU) has been shown to decrease fibroblast proliferation by incorporating itself preferentially into the DNA of the dividing fibroblasts and inducing apoptosis, but is highly associated with keloid recurrent and side effects such as ulceration, burning and pain (Al-Attar *et al.*, 2006; Huang *et al.*, 2010; Wang *et al.*, 2009). Interferon-α 2b (IFN-α2b) possesses antiproliferative properties, and has been shown to reduce fibroblast proliferation in keloids, and while it provides some promise, it is accompanied by significant pain and flu-like symptoms and is also very expensive (Gauglitz *et al.*, 2011; Lee *et al.*, 2008a). While targeting fibroblasts could yield significant

improvements in the treatment of fibrotic skin disorders, further research into management of side effects, recurrence and cost need to be assessed before they can receive widespread approval.

Propagation and Regulation of TGF-ß Signaling

TGF-ß is a multifunctional growth factor that plays a major role in a variety of different biological processes including wound healing, angiogenesis, carcinogenesis, development, and reproduction. TGF-ß is expressed by several different cell types, and its widespread physiological functions requires tight regulation of its signaling at the ligand, receptor, intracellular, and nuclear levels. Briefly, TGF-ß transmits its signal through the cell upon activation of latent-TGF-ß to active-TGF-ß, and then binds the TGF-ß Receptor Type II (TGF-ß-RII), which then transphophorylates TGF-ß Receptor Type I (TGF-ß-RI). The activated TGF-ß-RI then activates intracellular second messengers which direct transcription in the nucleus in tandem with a series of transcriptional co-activators and co-repressors. Further modulating TGF-ß signaling are other accessory and co-receptors including CD109, endoglin, and ß glycan, as well as intracellular regulators including Smad7 and Smurf.

TGF-ß Isoforms

TGF-ß exists as three isoforms referred to as TGF-ß1, TGF-ß2, and TGF-ß3. Of the three isoforms, TGF-ß1 is the most highly expressed in adult tissues, with TGF-ß2 and TGF-ß3 being expressed at much lower levels. However, despite similar effects of each of the isoforms in *in vitro* studies, *in vivo* studies in knockout mice show that each isoform has unique contributions to development

(Coker *et al.*, 1997). Knockout mice deficient in TGF-ß1 develop normally, but approximately 20 days after birth, perish due to a wasting syndrome characterized by a mixed inflammatory response and tissue necrosis that leads to multi-system organ failure (Shull *et al.*, 1992). TGF-ß2 knockout mice die perinatally and display developmental defects in the cardiovascular system, skeletal malformations, eye and ear abnormalities, and a defective urogenital system (Sanford *et al.*, 1997). TGF-ß3 knockout mice are characterized by cleft palate, and also die within 24 hours of birth (Proetzel *et al.*, 1995; Taya *et al.*, 1999). The redundancy of *in vitro* signaling, but distinct effects *in vivo*, highlight the complex roles of the three different TGF-ß isoforms.

Activation of Latent TGF-ß

TGF-ß is secreted from the cell as an inactive precursor that is unable to bind its cell surface receptors to initiate signal transduction (Sporn *et al.*, 1987). When synthesized, the TGF-ß propeptide is cleaved by furin, yielding the mature TGF-ß protein and its latency associated peptide (LAP), which remain bound in a non-covalent manner during the secretory process (Gentry *et al.*, 1988; Gentry and Nash, 1990). The small latent complex can covalently bind to latent-TGF-ß binding protein (LTBP), forming the large latent complex (Rifkin, 2005). The LTBP is expressed separately, and can form a covalent bond with the LAP to further stabilize the LAP-TGF-ß complex and promote secretion of the LTBP-LAP-TGF-ß complex, as well as to render the TGF-ß in an inactive state (Miyazono *et al.*, 1991; Taipale *et al.*, 1994). When TGF-ß is secreted as the TGF-ß-LAP-LTBP complex, known as large latent complex (LLC), it interacts

with extracellular matrix proteins such as fibronectin to stabilize the inactive TGF-ß in the extracellular milieu (Rifkin, 2005; Wipff and Hinz, 2008). In order to exert its functions, latent-TGF-ß must be acted upon by factors such as serine proteases, matrix metalloproteinases, integrins, and thrombospondin-1 to become active and bind its cell surface receptors. Due to the excess of TGF-ß secreted into the extracellular environment, tight spatiotemporal regulation of its activation is crucial in order to ensure proper biological activity (Annes *et al.*, 2003).

Integrins

The expression of integrins on the extracellular surface of cells plays an important role in the activation of latent-TGF-ß (Worthington *et al.*, 2011). The $\alpha_V ß_5$, $\alpha_V ß_6$, and $\alpha_V ß_8$ integrins have all been shown to be involved in the activation of latent TGF-ß (Wipff and Hinz, 2008). One possible mechanism underlying integrin-mediated activation of TGF-ß involves integrins acting as a docking site for proteases and TGF-ß. $\alpha_V ß_3$ integrin can interact with MMP2 and MMP9 to activate TGF-ß (Brooks *et al.*, 1996; Rolli *et al.*, 2003). Another mechanism by which integrins could activate TGF-ß involves transmission of cell traction forces to the large latent complex. Studies in myofibroblasts have shown that contractile activity facilitates TGF-ß activation, and that $\alpha_V ß_5$ integrin can induce TGF-ß activation in the absence of proteases (Wipff *et al.*, 2007). This raises the possibility that integrins can transmit the contractile force produced by myofibroblasts to activate TGF-ß alone, as well as in tandem with other proteases.

Serine Proteases

Several serine proteases including plasmin, thrombin, and neutrophil elastase have been shown to activate TGF-ß by dissociating it from its latency-associate peptide. Plasmin is activated by plasminogen activator and inhibited by plasminogen-activator inhibitor, and activates TGF-ß by proteolytically cleaving LAP, thereby activating TGF-ß (Jenkins, 2008). The main role of thrombin is to convert fibrinogen to fibrin during the clotting cascade, however its proteolytic effect on latent-TGF-ß appears to be similar to that of plasmin, and dissociates TGF-ß from its LAP (Taipale *et al.*, 1992). Neutrophil elastase is another serine protease that contributes to TGF-ß activity, but rather than directly liberating TGF-ß from the LAP and LTBP, it instead releases the LAP-TGF-ß complex from the ECM where it can be further modified by additional proteases (Taipale *et al.*, 1995).

Matrix Metalloproteinases (MMPs)

The matrix metalloproteinases are a family proteolytic enzymes with a variety of potential substrates. Of all the MMPs, MMP2, MMP9, MMP13, and MMP14 have been shown to have the ability to activate TGF-ß (Jenkins, 2008). MMP2 and MMP9 are also known as Gelatinase-A and Gelatinase-B, respectively, and have been shown to cleave LAP, and activate TGF-ß (Ge and Greenspan, 2006; Yu and Stamenkovic, 2000). MMP13 has also been associated with TGF-ß activation. MMP13 is released along with latent-TGF-ß, and reduction of MMP13 activity results in concurrent reduction in TGF-ß activity (Jenkins, 2008). MMP14 is a transmembrane MMP, also called MT1-MMP, and

colocalizes with $\alpha_V ß_8$ integrin on the cell surface to participate in TGF-ß activation (Jenkins, 2008).

Thrombospondin-1

The thrombospondins are a family of glycoproteins consisting of five members, of which thrombospondin-1 functions as an activator of latent-TGF-ß both *in vitro* and *in vivo* (Adams, 1997; Ahamed *et al.*, 2009). Thrombospondin-1 can activate latent-TGF-ß indirectly by facilitating the conversion of plasminogen to plasmin, but also directly by inducing a conformational change in the latent-TGF-ß complex to expose the receptor binding site of TGF-ß, allowing it to interact with its cell surface receptor TGF-ß-RII (Jenkins, 2008; Moser *et al.*, 1995).

TGF-ß Receptor Signaling

Activated TGF-ß transmits its signal into the cell by interacting with its TGF-ß Type I receptor (TGF-ß-RI) and TGF-ß type II receptor (TGF-ß-RII). In the absence of TGF-ß ligand, both the TGF-ß-RII and TGF-ß-RI exist as monomers on the cell surface, but that addition of ligand induces dimerization of both receptors (Zhang *et al.*, 2009; Zhang *et al.*, 2010). When the TGF-ß ligand approaches the cell surface, it first binds the TGF-ß-RII to induce dimerization and subsequently complex formation between the TGF-ß ligand, TGF-ß-RII dimer, and TGF-ß-RI dimer (Zhang *et al.*, 2009; Zhang *et al.*, 2010). TGF-ß-RII has a constitutively active kinase domain, and upon ligand binding and receptor complex formation, it transphosphorylates TGF-ß-RI (Moustakas and Heldin, 2009). Phosphorylation of TGF-ß-RI activates its intracellular kinase domain,

which then serves as an activating dock for intracellular second messenger proteins known as receptor Smads (rSmad) which propagate the TGF-ß signal to the nucleus (de Caestecker, 2004).

TGF-ß receptor signaling is regulated by intracellular proteins that block downstream phosphorylation and direct internalization of the TGF-ß receptors. Smad7 inhibits TGF-ß receptor signaling by blocking interactions between the activated TGF-ß receptor complex and rSmads, and by also by acting as a docking protein for the ubiquitin ligase Smurf1/2, which targets the activated receptor complex for degradation (Ebisawa *et al.*, 2001; Massague, 2008; Zhu *et al.*, 1999). Additionally, sumoylation and receptor internalization also function to decrease TGF-ß receptor signaling (Kang *et al.*, 2009).

Genetic analysis of the TGF-ß superfamily receptors has shown that in humans and other mammals, there exist five type II and seven type I TGF-ß superfamily receptors. The TGF-ß superfamily consists of at least 35 members divided into several subgroups: TGF-ß, bone morphogenic proteins (BMP), growth and differentiation factor (GDF), Activin/Inhibin, Nodal, Mullerian Inhibiting Substance (MIS), and other members as well (Knight and Glister, 2006). The type I receptors are also known as 'activin receptor-like kinase' (ALK), with various ligands in the TGF-ß superfamily preferentially activating various ALKs, with the ALK5 receptor defined as "TGF-ß receptor type I" (ten Dijke and Hill, 2004). The type II receptors are named regarding their natural ligand, with TGF-ß signaling predominantly through the TGF-ß type II receptor. While TGF-ß signals preferentially through its TGF-ß-RII and ALK5 receptors, it

can also signal through receptor complexes formed of TGF-ß-RII and ALK1 (Finnson *et al.*, 2008; Goumans *et al.*, 2003b; Goumans *et al.*, 2002). BMP can also activate ALK1, and the activation of receptor complexes comprised of ALK1 compared to ALK5 results in different downstream transcriptional activity, and thus has significant cellular consequences.

SMAD Dependent Signaling

The canonical pathway for TGF-ß superfamily members involves signaling through intracellular second messenger proteins named Smads. Upon binding of TGF-ß ligand to its receptors, receptor-activated-Smads (rSmad) are phosphorylated by the activated TGF-ß-RI (Shi and Massague, 2003). Members of the rSmad family include Smad1, Smad2, Smad3, Smad5, and Smad8 (Shi and Massague, 2003). Of note in TGF-ß signaling, upon activation ALK5 signals preferentially through the Smad2 and Smad3 while ALK1 preferentially phosphorylates Smad1, Smad5, and Smad8 (ten Dijke and Hill, 2004). Upon phosphorylation, rSmads heteromerize with a co-Smad, Smad4, which then translocates to the nucleus. Heteromerization studied in the context of ALK5 signaling has shown that complexes consisting of Smad2/Smad2/Smad4, Smad2/Smad3/Smad4, and Smad3/Smad3/Smad4 exist in the cell (Heldin and Moustakas, 2012). However, upon concurrent activation of ALK1 and ALK5, dimers consisting of Smads downstream of ALK1 (Smad1/Smad5/Smad8) and ALK5 (Smad2/Smad3) can form and ultimately bind Smad4, resulting in antagonism of ALK5 signaling by ALK1 (Daly *et al.*, 2008; Goumans *et al.*, 2003a; Goumans *et al.*, 2003b). Upon translocation to the nucleus, the resulting

rSmad/coSmad complex functions as a transcription factor, inducing expression of TGF-ß target genes. Transcriptional regulation of the Smad complex is affected positively by co-activators p300 and CBP, while Ski and SnoN negatively affect Smad-mediated transcription by disrupting complex formation and preventing co-activators from binding the transcription factor complex (Akiyoshi *et al.*, 1999; Luo *et al.*, 1999; Stroschein *et al.*, 1999; Wu *et al.*, 2002).

TGF-ß Signaling Through ALK1 versus ALK5 Type I Receptors

Although traditionally TGF-ß had been thought to signal through ALK5-Smad2/3, emerging evidence particularly in endothelial cells and chondrocytes has demonstrated that TGF-ß also signals through ALK1-Smad1/5/8 (Blaney Davidson *et al.*, 2009; Finnson *et al.*, 2008; Goumans *et al.*, 2003b; Goumans *et al.*, 2002). The role of ALK1 in TGF-ß signaling was first proposed in endothelial cells, where it was shown that the ALK1 response was both opposite and antagonistic to the ALK5 response (Goumans *et al.*, 2002). Additionally, despite its contrasting biological signal, ALK5 recruits ALK1 to the TGF-ß receptor complex, and ALK1 requires ALK5 kinase activity in order to effectively propagate its signal into the cell (Goumans *et al.*, 2003b).

In chondrocytes, activation of ALK1 induces Smad1/5/8 phosphorylation to inhibit a fibrotic phenotype. ALK1 signaling has been shown to inhibit, while ALK5 signaling has been shown to enhance expression of PAI-1, fibronectin, and collagen type II in chondrocytes (Finnson *et al.*, 2008). Dermal fibroblasts cultured from patients affected by scleroderma display increased ALK1-Smad1 signaling, which has been demonstrated to result in elevated expression of CTGF

and collagen type I (Morris *et al.*, 2011; Pannu *et al.*, 2007). However, these results are also associated with elevated endoglin expression, raising the possibility that the fibrotic phenotype observed in these studies may require endoglin overexpression in order to induce fibrosis associated increased ALK1 signaling (Finnson *et al.*, 2008; Morris *et al.*, 2011).

The ratio of ALK1:ALK5 at the cell surface also affects the signaling response to TGF-ß. An elevated ALK1:ALK5 ratio is observed in cultured osteoarthritic chondrocytes and is associated with increased expression of MMP-13, which has been shown to also activate TGF-ß (Blaney Davidson *et al.*, 2009). Changes in the ALK1:ALK5 ratio have also been shown to direct TGF-ß signaling in endothelial cells to promote either proliferation or differentiation (Goumans *et al.*, 2003a). Recent studies suggest a link between ALK1-Smad1/5/8 signaling and fibrosis in scleroderma, similar to previous report describing renal and hepatic fibrosis (Matsubara *et al.*, 2006; Pannu *et al.*, 2008; Wiercinska *et al.*, 2006). However, there also exists a large volume of literature describing the pro-fibrotic effects of ALK5-Smad2/3 signaling in the skin (Dong *et al.*, 2002; Wei *et al.*, 2011). The observation that endoglin, as noted above, is a major determinant of the pro-fibrotic phenotype associated with ALK1-Smad1/5/8 signaling, could therefore imply that expression of TGF-ß co-receptors play an important role in the physiological outcome of TGF-ß signaling in the context of the ratio ALK1:ALK5 expression and fibrosis.

Co-Receptors in TGF-ß Signaling

CD109

CD109 is a GPI-anchored protein that was first identified on hematopoetic stem cells, and is expressed on megakaryoplastic precursor cells, but then its expression levels decline on mature blood cells but return on activated CD4 and CD8 T-cells (Furley *et al.*, 1986; Murray *et al.*, 1999; Sutherland *et al.*, 1991). Further studies have shown that CD109 is also expressed under normal conditions on activated platelets, keratinocytes, chondrocytes, and testis, and in pathological conditions such as glioblastoma, basal-like breast cancer and certain adenocarcinomas and sarcomas (Finnson *et al.*, 2006; Hasegawa *et al.*, 2008; Hashimoto *et al.*, 2004; Smith *et al.*, 1995). CD109 functions to inhibit TGF-ß signaling, via promotion of TGF-ß receptor degradation (Bizet *et al.*, 2011). CD109 can bind TGF-ß ligand with high affinity in its membrane-bound form, and can also be cleaved from the membrane at its GPI anchor rendering it a soluble protein with the ability to bind TGF-ß ligand, potentially acting as a sink to further inhibit TGF-ß signaling (Tam *et al.*, 2001).

Betaglycan

Betaglycan was originally characterized as an accessory receptor lacking a signaling domain but functions by presenting the TGF-ß ligand to the TGF-ß-RII, thus earning the name "TGF-ß-RIII" (Lopez-Casillas *et al.*, 1991). Further investigations of betaglycan have demonstrated that its expression is variable across different cell types and cell tissues, playing a major role in biological processes including reproduction and fetal development (Sarraj *et al.*, 2007; Sarraj *et al.*, 2010; Stenvers *et al.*, 2003). Despite original reports suggesting that

betaglycan promotes TGF-ß signaling, more recent evidence has shown that it can either positively or negatively contribute to the TGF-ß response, largely depending on the cell context (Eickelberg *et al.*, 2002; Lopez-Casillas *et al.*, 1994). Betaglycan expression is increased in dermal fibroblasts cultured from scleroderma patients and is associated with increased CTGF expression, and evidence suggests it might be associated with tumor suppression as well (Bernabeu *et al.*, 2009; Holmes *et al.*, 2011). The capacity of betaglycan to be cleaved from the membrane via ectoderm shedding, as well as its effects on receptor trafficking also highlight its complex and cell dependent role in modulating TGF-ß signaling (Bilandzic and Stenvers, 2011).

Endoglin

Endoglin, also referred to as CD105, expressed predominantly on endothelial cells, but also on immune cells, chondrocytes, fibroblasts and basal keratinocytes (Cheifetz *et al.*, 1992; Diez-Marques *et al.*, 2002; Parker *et al.*, 2003; Rodriguez-Pena *et al.*, 2002). Endoglin plays a major role in blood vessel wall integrity, and deficiency of endoglin results in hereditary hemmhoragic telangiectasia (HHT), characterized by excessive bleeding and vascular insufficiency (McAllister *et al.*, 1994; ten Dijke *et al.*, 2008). Increased endoglin expression in dermal fibroblasts has also been noted in scleroderma, leading to increased signaling through the ALK1 compared to ALK5 pathways (Leask *et al.*, 2002; Morris *et al.*, 2011; Pannu *et al.*, 2007). This is similar to reports in endothelial cells and chondrocytes which describe how endoglin preferentially directs TGF-ß signaling through the ALK1 rather than ALK5 TGF-ß receptor

(Dallas *et al.*, 2008; Finnson *et al.*, 2010). Endoglin has been shown to bind TGF-ß1 and TGF-ß3 in the presence of the TGF-ß type II receptor, and can also modulate the phosphorylation states of ALK1 and ALK5 receptors via interaction with the cytoplasmic domain of endoglin independently the TGF-ß ligand (Morris *et al.*, 2011).

CD109 as a Regulator of TGF-ß Signaling

Discovery and Characterization of CD109

CD109 was originally discovered as an unidentified antigen of mass 180 kDa, 150 kDa, and 120 kDa in 1988 on activated platelets and T-cells (Brashem-Stein *et al.*, 1988). It was later suggested that rather than detecting three distinct isoforms, the antibody used to detect CD109 actually recognized a native 180 kDa form as well as 150 kDa and 120 kDa products of proteolytic modification (Smith *et al.*, 1995; Sutherland *et al.*, 1991). Further studies with additional antibodies produced against the same antigen showed that in addition to the activated macrophages and T-cells, this antigen was anchored to the membrane by a GPI-anchor and highly expressed on endothelial cells, megakaryoblastic and myelo-erythroid progenitors, as well as in a variety of different tumour cells (Haregewoin *et al.*, 1994; Murray *et al.*, 1999). Smith et al characterized CD109 further upon mapping the Gov alloantigen involved in immune reactions to platelet transfusions to CD109 (Kelton *et al.*, 1998; Smith *et al.*, 1995). In 1998, our lab published a study reporting the presence of a 180 kDa TGF-ß binding protein on the cell surface of fibroblasts (Tam *et al.*, 1998). Following this, our group also demonstrated that keratinocytes express a 150 kDa GPI-anchored

TGF-ß binding protein, referred to as r150, that when cleaved from the membrane retains its ability to bind TGF-ß1 in the absence of other TGF-ß receptors (Tam *et al.*, 2001), and later showed using microsequence analysis of r150 that it is in fact CD109 (Finnson *et al.*, 2006).

CD109 as a Member of the α-2-Macroglobulin Family

Bioinformatic analysis of the CD109 primary protein sequence has identified CD109 as a member of the α-2-macroglobulin/C3,C4,C5 family of proteins that contain a thioester (Lin *et al.*, 2002). Further *in vitro* characterization verified the presence of a thioester in CD109, and subsequent analyses also showed shared motifs including a putative bait region, a furin site composed of four amino acids, and a thioester-reactivity defining hexapeptide (Finnson *et al.*, 2006; Lin *et al.*, 2002; Solomon *et al.*, 2004). The capacity for both CD109 and α-2-macroglobulin to bind TGF-ß also highlight a similarity between these two proteins (Finnson *et al.*, 2006; Tam *et al.*, 2001). Whether there exist other functional similarities between CD109 and the α-2-macroglobulin/complement family remains to be determined. It has been suggested that there might be functional overlap at the level of covalent-cross linking between the thioester site and proximal molecules or proteases (Lin *et al.*, 2002).

CD109 as a TGF-ß Binding Protein

Our group serendipitously began investigating CD109, which was referred to as r150 at the time, upon identification of a GPI-anchored protein in the TGF-ß receptor complex possessing TGF-ß binding capabilities (Tam *et al.*,

1998; Tam *et al.*, 2001). In these studies, our group demonstrates that radiolabelled TGF-ß binds to CD109, with highest affinity for the isoform TGF-ß1, less affinity for TGF-ß3, and virtually no affinity for TGF-ß2 (Tam *et al.*, 1998; Tam *et al.*, 2001). Endogenous CD109 can be released from the cell surface, and using affinity labeling studies, our group showed that CD109 can bind TGF-ß when bound to the membrane and in its soluble form *in vitro* (Finnson *et al.*, 2006; Tam *et al.*, 2001).

CD109 as a TGF-ß Co-Receptor That Regulates TGF-ß Signaling

In addition to possessing TGF-ß ligand binding properties, our lab has also shown that CD109 regulates TGF-ß signaling through participation in the TGF-ß receptor complex as a TGF-ß co-receptor. Our group reported the presence of a 150 kDa GPI-anchored protein in the TGF-ß signaling complex, which we later identified as CD109 (Tam *et al.*, 1998). Further studies showing that GPI-mutant keratinocytes display enhanced TGF-ß induced Smad2 phosphorylation suggest that CD109 might negatively regulate TGF-ß signaling in keratinocytes (Tam *et al.*, 2003). Finnson et al expanded our understanding of the role of CD109 in the TGF-ß signaling complex by showing that CD109 binds TGF-ß signaling receptors both in the presence and absence of the TGF-ß ligand (Finnson *et al.*, 2006). The mechanism underlying CD109 mediated inhibition of TGF-ß signaling was described by Bizet et al, who demonstrated that CD109 promotes internalization and degradation of TGF-ß receptors in a manner dependent on Smad7 and Smurf2 (Bizet *et al.*, 2011; Bizet *et al.*, 2012). The capacity for

CD109 to modulate TGF-ß receptor signaling may also depend on its activation, mediated via processing by furin (Hagiwara *et al.*, 2010).

CD109 in Disease

CD109 was originally identified as a marker of activated T-cells and activated platelets, but during these studies it was also found to be overexpressed in cells cultured from a variety of different tumour lines (Haregewoin *et al.*, 1994). Since these observations, relationships between CD109 and several different cancers, platelet disorders, and skin diseases have also been documented.

Hashimoto et al were among the first to describe the relationship between CD109 and human cancers. In their study, they demonstrate that CD109 expression is upregulated in squamous cell carcinoma of the lung and esophagus, as well as glioblastoma but not in neuroblastoma, small-cell carcinoma of the lung, and adenocarcinoma of the lung (Hashimoto *et al.*, 2004). Subsequent investigations further showed that CD109 is upregulated in squamous cell carcinoma of the uterine cervix, squamous cell lung carcinoma, breast and prostate carcinoma, and squamous cell carcinoma of the oral cavity (Hagiwara *et al.*, 2008; Hasegawa *et al.*, 2007; Hasegawa *et al.*, 2008; Sato *et al.*, 2007; Zhang *et al.*, 2005). The strong relationship between CD109 upregulation and squamous cell carcinomas suggests that it might serve as an important diagnostic marker of these cancers.

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. The Gov antigen on CD109, also referred to as HLA-15, when expressed on activated platelets can be immunogenic and result in an immune reaction targeting platelets for destruction (Ertel *et al.*, 2005). Thus, CD109 expression on activated platelets harbours a strong relationship with the progression of immune reactions against platelets that can manifest as thrombocytopenia and result in significant clotting disorders.

CD109 has also recently been implicated skin pathologies involving aberrant TGF-ß signaling. Litvinov et al have recently shown that CD109 might play a TGF-ß dependent role in psoriasis, and given the strong relationship between excessive TGF-ß signaling and fibrosis, CD109 might play a role in the propagation or prevention of fibrotic skin disorders as well (Litvinov *et al.*, 2011; Man *et al.*, 2012).

Rationale for Study

TGF-ß is a multifunctional growth factor involved in many different cellular processes including extracellular matrix deposition, angiogenesis, immune cell recruitment, and cell migration. Dysregulation of TGF-ß signaling is a hallmark of fibrotic skin disorders such as keloids, hypertrophic scars, and scleroderma, and thus represents an attractive therapeutic target for treatment of these diseases. There are several different proposed mechanisms by which TGF-ß contributes to fibrosis, including an augmented inflammatory response, dermal fibroblast proliferation, extracellular matrix deposition, and impaired epidermal-dermal interactions. At present, truly effective pharmacologic therapies for fibrotic skin disorders are lacking. Investigating novel approaches to modulation

of TGF-ß signaling using *in vivo* models could therefore provide a better understanding of the mechanisms underlying skin fibrosis, and in turn, build a foundation upon which therapeutic strategies for treating fibrotic skin disorders can be developed.

Our lab has identified CD109, a novel GPI-anchored TGF-ß co-receptor. It has the capacity to bind TGF-ß, heteromerize with TGF-ß signaling receptors, and inhibit TGF-ß signaling by promoting receptor degradation. We have also shown that overexpression of CD109 in human keratinoctyes *in vitro* inhibits TGF-ß induced expression of the extracellular matrix proteins fibronectin and collagen I. Whether increasing the level of CD109 expression in the skin *in vivo* could potentially reduce fibrosis by decreasing the level of TGF-ß signaling or by altering epidermal-dermal interactions to reduce the expression of pro-fibrotic growth factors remains to be determined.

The specific objectives of this thesis include (1) to determine the role of CD109 *in vivo* in the skin during wound healing using transgenic mice overexpressing CD109 in the epidermis; (2) to investigate the role of CD109 *in vivo* in a bleomycin-induced model of skin fibrosis; (3) and to characterize the effect of overexpression of CD109 in the epidermis on TGF-ß signaling pathways in whole skin, cultured keratinocytes and fibroblasts harvested from CD109 transgenic and wild-type mice.

Global Hypothesis:

I hypothesize that CD109, when overexpressed in the epidermis of transgenic mice, will improve wound healing by reducing fibrosis and inflammation; will inhibit bleomycin-induced skin fibrosis; and inhibit TGF-beta signaling both *in vivo* and in primary cultured skin cells isolated from these mice and cultured *in vitro*.

Chapter 2: Transgenic Mice Overexpressing CD109 in the Epidermis Display Decreased Inflammation and Granulation Tissue and Improved Collagen Architecture During Wound Healing

Joshua Vorstenbosch BSc[1], Corrie Gallant-Behm PhD[2], Alissa Trzeciak BSc[1], Stéphane Roy PhD[3], Thomas Mustoe MD[2], and Anie Philip PhD[1].

1: Department of Surgery, Division of Plastic Surgery, McGill University, Montréal, Québec, Canada.

2: Division of Plastic and Reconstructive Surgery, Northwestern University, Chicago, Illinois.

3: Faculté de Médecine Dentaire, Université de Montréal, Montréal, Québec, Canada.

Accepted for publication in: Wound Repair & Regeneration, November 27, 2012

Abstract:

Transforming Growth Factor-ß is a multifunctional growth factor involved in all aspects of wound healing. TGF-ß accelerates wound healing, but an excess of its presence at the wound site has been implicated in pathological scar formation. Our group has recently identified CD109, a glycophosphatidylinositol-anchored protein, as a novel TGF-ß co-receptor and inhibitor of TGF-ß signaling *in vitro*. To determine the effects of CD109 *in vivo* on wound healing, we generated transgenic mice overexpressing CD109 in the epidermis. In excisional wounds, we show that CD109 transgenic mice display markedly reduced macrophage and neutrophil recruitment, granulation tissue area, decreased Smad2 and Smad3 phosphorylation, while wound closure remains unaffected as compared to wild-type littermates. Futhermore, we demonstrate that the expression of the pro-inflammatory cytokines IL-1α and MCP-1, and ECM components is markedly decreased during wound healing in CD109 transgenic mice. In incisional wounds, CD109 transgenic mice show improved dermal architecture while the tensile

strength of the wound remains unchanged. Taken together, our findings demonstrate that CD109 overexpression in the epidermis reduces inflammation and granulation tissue area and improves collagen organization *in vivo*.

Introduction:

Transforming growth factor-ß (TGF-ß) is a multifunctional growth factor that plays a major role in wound healing by promoting immune cell recruitment, angiogenesis, fibroblast proliferation, and extracellular matrix (ECM) production. In response to tissue injury, degranulating platelets release chemotactic factors and cytokines including TGF-ß and platelet derived growth factor to recruit neutrophils and macrophages to the wound site which in turn release a wide array of growth factors leading to reepithelialization and granulation tissue formation (Gurtner *et al.*, 2008; Singer and Clark, 1999). In addition to promoting matrix accumulation and re-epithelialization (Hinz, 2007; Nolte *et al.*, 2008) TGF-ß regulates granulation tissue resolution via apoptosis of the cells within the granulation tissue, resulting in a relatively acellular scar (Desmouliere *et al.*, 1995; Lucas *et al.*, 2010). TGF-ß also contributes to the differentiation of fibroblasts to myofibroblasts, which facilitate wound contraction and transition to the remodeling phase (Hinz, 2007; Nolte *et al.*, 2008).

Wound healing is a tightly regulated process, and dysregulation of wound healing results in many disorders including hypertrophic scarring and keloids. Deregulation of TGF-ß action has been shown to contribute to the progression of these diseases by promoting excessive ECM production by fibroblasts and keratinocytes (Armour *et al.*, 2007). There are three mammalian TGF-ß isoforms-

TGF-ß1, TGF-ß2 and TGF-ß3. While the TGF-ß1 and TGF-ß2 isoforms have been shown to exhibit proscarring properties, the TGF-ß3 display anti-scarring properties (Shah *et al.*, 1995; Singer and Clark, 1999). Fetal wounds heal without scars and exhibit low levels of TGF-ß1 and high levels of TGF-ß3 production, providing support for the notion that excess TGF-ß contributes to the progression of fibrosis in the skin (Lorenz, 2001).

TGF-ß signaling is transduced by two transmembrane serine-threonine kinases known as type I and type II receptors. The TGF-ß ligand binds to the type II receptor (TGF-ßRII), which then transphosphorylates the TGF-ß type I receptor (TGF-ßRI). The activated type I receptor then phosphorylates Smad2 and Smad3, allowing these two Smads to complex with Smad 4 (Shi and Massague, 2003). The Smad2/3-Smad4 complex then translocates into the nucleus where it interacts with various co-activators and co-repressors to direct transcription of TGF-ß target genes (Shi and Massague, 2003).

Previous studies on the significance of TGF-ß signaling during wound healing have demonstrated that neutralizing TGF-ß1 and TGF-ß2 activity in incisional wounds significantly reduces scarring, macrophage recruitment, and ECM deposition (Shah *et al.*, 1995). In addition, mice lacking Smad3 show improved wound healing as detected by reduced inflammation and improved reepithelialization (Ashcroft *et al.*, 1999). Conversely, mice overexpressing Smad2 in basal keratinocytes show impaired wound healing due to inhibition of keratinocyte migration across the wound bed (Hosokawa *et al.*, 2005). Also, mice harbouring a TGF-ßRII knockout or dominant-negative TGF-ßRII in

keratinocytes display enhanced reepithelialization and accelerated keratinocyte migration (Amendt *et al.*, 2002; Guasch *et al.*, 2007). More recently, mice lacking the TGF-ßRII gene in the dermis have been reported to display accelerated reepithelialization, a reduction in inflammation and attenuated scar formation (Denton *et al.*, 2009; Martinez-Ferrer *et al.*, 2010). The correlation between decreased TGF-ß signaling and improved wound healing suggests that TGF-ß antagonists may be of therapeutic value for the treatment of aberrant wound healing.

Our group has recently identified CD109, a glycophosphatidylinositol (GPI) anchored protein, as a novel TGF-ß co-receptor and a component of the TGF-ß receptor system (Finnson *et al.*, 2006). We have shown that CD109 inhibits TGF-ß signaling and ECM production in skin cells *in vitro* (Bizet *et al.*, 2011; Finnson *et al.*, 2006). In the present study, to determine whether CD109 plays a role regulating TGF-ß action during wound healing, we generated transgenic mice overexpressing CD109 in the epidermis. Our results show that CD109 transgenic mice display reduced inflammation and granulation tissue area and improved collagen architecture in a manner consistent with inhibition of TGF-ß action.

Materials and Methods:

Generation of Transgenic Mice Overexpressing CD109 in the Epidermis

Transgene encoding human CD109 downstream of the human keratin 14 promoter was prepared by subcloning a Gateway Cassette (Invitrogen, Carlsbad, CA) into BamHI blunted sites of the pGEM-3Z-K14 vector to yield pGEM3Z-

K14-Gateway. pCMV-Sport6-CD109 was then reacted with pGEM3Z-K14-Gateway to produce pGEM3Z-K14-CD109. The final construct (consisting of the human keratin 14 promoter, ß-globin intron, human CD109, and the keratin 14 polyadenylation signal) was excised using AseI and PvuI, and isolated by agarose gel electrophoresis. The resulting construct was purified using the QIAquick gel extraction kit (Qiagen, Mississauga, Canada), and submitted to the McGill University Transgenic Core Facility for generation of transgenic FVB mice. Founder mice were identified by Southern Blot and presence of the transgene was confirmed by PCR (Forward Primer: 5'-GGCGAATACGATCACAAGTT-3', Reverse Primer: 5'-TCCTGGGTACGTCCGGTTACA-3').

Measurement of CD109 Expression in Mouse Skin

Total RNA was prepared from mouse skin by homogenization in Trizol (Invitrogen, Carlsbad, CA) followed by purification using an RNEasy Kit (Qiagen, Mississauga, Canada). Reverse transcription was performed using MMLV Reverse Transcriptase and an oligo-dT primer (Invitrogen). PCR was performed using *Taq* DNA Polymerase (New England Biolabs) and primers specific to the transgenic transcript (Forward Primer: 5'-CAGCCTATGCACTGCTCTCA-3'; Reverse Primer: 5'-CAGGACTTGGTGAGCTAGGC-3').

Mouse skin was homogenized using RIPA buffer (Tris 50mM; NaCl 150 mM; SDS 0.1 %; Na.Deoxycholate 0.5 %; Triton X 100 or NP40 1%) containing complete, EDTA-free protease inhibitors (Roche, Mississauga, Canada), 1mM PMSF, and 1mM sodium fluoride and CD109 expression was visualized by

immunoblot using a novel mouse monoclonal anti-CD109 antibody (diluted 1:1000) generously donated by R&D Systems (R&D, Burlington, ON, Canada) as previously described (Finnson *et al.*, 2006).

Wounding

Male K14-CD109 transgenic mice and wild-type littermates (10-12 weeks old) were anesthetized by isofluorane, shaved and depilated using Nair (Church & Dwight, York, PA). Four full thickness 5mm excisional wounds were created on the cranial aspect of the back of the mice and a 2cm full thickness incisional wound was created on the caudal aspect of the back of the mouse, and sutured using 4-0 nylon sutures (Ethicon, Johnson & Johnson, Somerville, NJ). 6 wild-type and 6 transgenic were sacrificed on days 3, 7, and 14 post-wounding. Two excisional wounds were collected for histology and the other two excisional wounds were snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis. Incisional wounds were cut into three sections: one for histology, and the other two for tensile strength measurements. All animal experiments were approved by the McGill University Animal Care Committee, the Montreal General Hospital Animal Care Committee, and the Animal Care and Use Committee of Northwestern University.

Histology, Immunochistochemistry, and Image Analysis

Immediately after sacrificing the mice, wounds were photographed with a ruler in the field of view. Wound areas were measured (n=6) using ImageJ Software (NIH, Bethesda, MD). Wound skin was harvested and fixed overnight in 10% formalin, and embedded in paraffin. 7 μm sections were stained with

Masson Trichrome or Picrosirius Red, and then evaluated using ImageProPlus6 Software (MediaCybernetics, Bethesda, MD). Epidermal thickness was measured as the distance from the basement membrane to the superficial aspect of the epidermis at the epithelial tongue. Epidermal gap was determined as the distance between the leading edge of the wound margins in the excisional wounds. Dermal thickness was determined as the distance from the superficial aspect of the hypodermis to the epidermal-dermal junction at the edge of the wound. Granulation tissue area was measured in mm^2 on Masson Trichrome stained slides. For each mouse, each of these measurements was made 10 times, and the average values for each mouse were determined. Data are presented as the mean of the averages ± SEM calculated from six mice per group. For immunohistochemistry, macrophages and neutrophils were visualized by using anti-F4/80 and Ly-6G(Gr-1) antibodies (both diluted 1:50) respectively (ebioscience, San Diego, CA) as previously described (Wang *et al.*, 2008). Antibodies against α-smooth muscle actin and CD31 (diluted 1:500 and 1:200, respectively) (Abcam, Cambridge, MA) were used to visualized myofibroblasts and angiogenesis/endothelial cells. Anti-phospho-Smad2 and anti-phospho-Smad3 antibodies (Cell Signaling, Boston, MA) were used to visualize phosphorylated Smad2/3. Briefly, paraffin embedded sections were deparaffinized and dehydrated using xylene and graded ethanol washes. Antigen retrieval was performed by boiling slides in sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 10 minutes. Slides were washed and blocked using 5% NGS, and then incubated at the aforementioned dilutions in 5%

NGS overnight at 4°C overnight. The following day, slides were treated with biotinylated secondary antibody, followed ABC reagent (Vector Labs) and then developed using ImmPACT DAB (Vector Labs).

Immunohistomorphometry was performed as previously described (Bodo *et al.*, 2009; Ramot *et al.*, 2010). Briefly, using ImageJ software (NIH, Bethesda, MD), the intensity of staining within the area of the granulation tissue was measured, normalized to granulation tissue area, and then averaged to quantify the amount of Smad2 and Smad3 phosphorylation within the wound *(n=6)*.

Tensile Strength Measurement

Tensile strength was measured using a tensometer (Tensometer 10; Monsanto, St. Louis, MO). Briefly, 1cm fresh skin samples harvested from 2cm incisional wounds that were sutured closed was placed in the tensometer, which applied tension to the wound. The breaking strength of the WT and TG incisional wounds was measured and plotted. Unwounded skin posterior to the incisional wound was also tested.

Quantitative Real-Time PCR

Total RNA was extracted from skin by homogenization in Trizol (Invitrogen, Carlsbad, CA) and purified using an RNEasy Kit following manufacturer's protocol (Qiagen, Mississauga, Ontario, Canada). Reverse transcription was performed using MMLV Reverse Transcriptase (Invitrogen). 1 ug of RNA was reverse transcribed with oligo dT primer in a final volume of 20 uL. qPCR was performed to amplify Collagen Type I (Forward Primer: 5'-CACCCTCAAGAGCCTGAGTC-3', Reverse Primer: 5'-

GTTCGGGCTGATGTACCAGT-3'), Fibronectin (Forward Primer: 5'-GAAGCAACGTGCTATGACGA-3', Reverse Primer: 5'-ATCTAGCGGCATGAAGCACT-3'), TGF-ß1 (Forward Primer: 5'-CTGCTGCTTTCTCCCTCAAC-3', Reverse Primer: 5'-GACTGGCGAGCCTTAGTTTG-3'), IL-1α (Forward Primer: 5'-TCGGGAGGAGACGACTCTAA-3', Reverse Primer: 5'-GTATCATATGTCGGGGTGGC-3'), and MCP1 (Forward Primer: 5'-CATGCTTCTGGGCCTGCTGTTC-3', Reverse Primer: 5'-CATGCTTCTGGGCCTGCTGTTC-3') with iQ SYBR Green Supermix on a CFX96 Thermocycler and normalized to a GAPDH (Forward Primer: 5'-GGCGTCTTCACCACCATGGAG-3', Reverse Primer: 5'-AAGTTGTCATGGATGACCTTGGC-3') housekeeping control (Bio-Rad, Hercules, CA).

Statistical Analysis

All values are expressed as average±SEM. For each measured criteria, samples were taken from WT (n=6) and TG (n=6) mice at days 3, 7, and 14 and tested for statistical significance using the unpaired, two-tailed, Student's *t*-Test (significant if $p<0.05$).

Results:

Overexpression of CD109 in Mouse Epidermis

In previous studies, we have shown that CD109 potently inhibits TGF-ß signaling in keratinocytes *in vitro* (Finnson *et al.*, 2006). To determine whether

CD109 affects TGF-ß action *in vivo* and to study the effects of CD109 in wound healing, we generated transgenic mice overexpressing CD109 in the epidermis. We cloned CD109 downstream of the K14 promoter (Figure 2.1a) to spatially restrict overexpression to basal keratinocytes. We confirmed overexpression of CD109 in skin homogenates by western blot (Figure 2.1b). Elevated expression of CD109 in basal keratinocytes in the epidermis and hair follicles in transgenic animals compared to wild-type littermates was also demonstrated by immunohistochemistry (Figure 2.1c). These results are consistent with previous reports demonstrating that the K14 promoter directs transgene overexpression to the epidermis (Guo *et al.*, 1993). The modest increase in CD109 levels in the dermis may represent exosomal secretion of CD109 from keratinocytes reaching the dermal compartment or may involve some other mechanism by which CD109 overexpression in epidermal keratinocytes affecting dermal fibroblast function (Chavez-Munoz *et al.*, 2009).

Overexpression of CD109 in the Epidermis Leads to Decreased Granulation Tissue Area, Improved Collagen Organization, With No Change in Rate of Wound Closure

To study the effects of CD109 during wound healing, we generated 5mm full-thickness wounds on the back of 10-12 week old CD109 transgenic (TG) and wild-type littermate (WT) mice. Histological analysis of wound tissue showed alterations in several wound healing parameters in CD109 transgenic mice when compared to wild-type littermates. The transgenic mice display reduced scarring response as evidenced by improved ECM organization (Figure 2.2). At day 3

post-wounding, transgenic mice display increased thickening of the epithelial tongue at the edge of the wound (Figure 2.2 – Day 3). At day 7 post-wounding, thickening at the epithelial tongue was evident in both transgenic and wild-type mice. However there was decreased cellularity in the wound bed in transgenic mice compared to wild-type controls (Figure 2.2 – Day 7). On day 14 post-wounding, transgenic mice display improved scarring parameters when compared to wild-type littermates, as indicated by more organized collagen architecture and decreased granulation tissue area (Figure 2.2 – Day 14). Additionally, we measured gross wound closure by photographing wounds 7 and 14 days post-wounding and calculating wound area using ImageJ software. Gross analysis of wound closure showed no difference between wild-type and transgenic mice (Figure 2.3a). Taken together, our data suggest that CD109 overexpression decreases granulation tissue area and enhances collagen architecture without affecting wound closure.

Overexpression of CD109 in the Epidermis Alters Epidermal Thickness, Dermal Thickness, and Granulation Tissue Area in an Excisional Wound Healing Model

To determine the effects of CD109 in wound healing, we quantified epidermal thickness, epidermal gap, dermal thickness, and granulation tissue area histologically. We measured the thickness of the epithelial tongue at the wound edge, and found that epidermal overexpression of CD109 results in a significant increase in epidermal thickness on day 3 ($p<0.05$) and day 7 ($p<0.02$) post-wounding (Figure 2.3b). However, there was no difference in the epidermal gap

between wild-type and transgenic mice, as measured by the distance between the leading edges of the epithelial tongues (Figure 2.3c). Thus, elevated levels of CD109 in the epidermis appear to increase epidermal thickness without impairing keratinocyte migration during wound healing.

We also explored the effects of epidermal overexpression of CD109 on dermal thickness during wound healing. Dermal thickness, measured as the distance between the basement membrane and hypodermis at the wound margin, was decreased in CD109 transgenic mice on day 7 post-wounding ($p<0.05$). No significant difference was noted on day 3 and 14 (Figure 2.3d).

We next analyzed the effect of CD109 on granulation tissue area and α-smooth muscle actin expression in the dermis. We show that overexpression of CD109 does not affect the granulation tissue area on day 3 post-wounding. In contrast, CD109 significantly reduces the amount of granulation tissue area present on day 7 and day 14 post-wounding ($p<0.05$) (Figure 2.3e). We also found that the number of α-smooth muscle actin positive cells are reduced at day 7 and day 14 post-wounding in the CD109 transgenic mice when compared to wild-type littermates, suggesting that CD109 overexpression decreases myofibroblast differentiation (Figure 2.3f,g). Taken together, these findings demonstrate that epidermal overexpression of CD109 leads to a decrease in dermal thickening on day 7 post-wounding while reducing granulation tissue as noted on days 7 and 14 post-wounding. These findings corroborate our results illustrating improved dermal architecture during wound healing in mice overexpressing CD109 in the epidermis.

CD109 Overexpression in the Epidermis Inhibits Smad2 and Smad3 Phosphorylation During Wound Healing

We measured the effect of CD109 overexpression in the epidermis on TGF- ß signaling by determining phosphorylation of Smad2 and Smad3 in excisional wounds. We found that overexpression of CD109 in the epidermis causes a marked decrease in Smad2 and Smad3 phosphorylation in the wounded skin on days 3, 7, and 14 post-wounding compared to wild-type controls in both the epidermis and the dermis (Figure 2.4). Quantification of the intensity of staining within the granulation tissue shows a statistically significant $(p<0.05)$ reduction in phosphorylation on days 3, 7, and 14 in the CD109 transgenic mice compared to wild-type littermates (Figure 2.4b,c). These data are consistent with our previous work demonstrating that CD109 inhibits TGF-ß signaling in keratinocytes *in vitro* through the Smad2/3 pathway, and illustrate that CD109 negatively regulates TGF-ß signaling during wound healing.

CD109 Overexpression in the Epidermis Results in Improved Dermal Architecture Without Altering the Tensile Strength of the Wound During Incisional Wound Healing

We next examined the effect of CD109 overexpression on wound healing and tensile strength in an incisional wound model. A single 2 cm incision was made on the back of CD109 transgenic mice and wild-type littermates as described in the methods. Wounds were harvested 3, 7, and 14 days post-wounding and subjected to histological and tensile strength analyses. Transgenic mice overexpressing CD109 in the epidermis show improved wound healing as

detected by accelerated closure and increased approximation of the incised skin on day 3 and day 7 post-wounding (Figure 2.5a). Additionally, transgenic mice display markedly improved collagen assembly and less granulation tissue on day 7 and day 14 post-wounding by Picrosirius Red and Masson Trichrome staining, respectively, consistent with our results in excisional wounds shown in Figure 2.2. We also show that epidermal overexpression of CD109 does not affect the tensile strength of incisional wounds or unwounded skin (Figure 2.5b). Taken together, these results suggest that CD109 reduces scarring parameters without compromising tissue tensile strength.

CD109 Overexpression in the Epidermis Inhibits Immune Cell Recruitment in Excisional Wounds

Next, we assessed the effect of CD109 overexpression on inflammation by measuring neutrophil and macrophage recruitment in excisional wounds 3, 7, and 14 days post-wounding. To identify neutrophils and macrophages, we stained wound sections with anti-Ly-6Gr and anti-F4/80 antibodies, respectively, and counted the number of positive cells per high-power field (Figure 2.6a,b) (Wang *et al.*, 2008). Our results show that CD109 transgenic mice display a significant reduction in the number of neutrophils present at days 3, 7, and 14 ($p<0.05$) and the number of macrophages present on day 3 ($p<0.05$) and day 7 ($p<0.01$) post-wounding (Figure 2.6c,d). These results suggest that CD109 exhibits anti-inflammatory properties leading to decreased scarring parameters such as less granulation tissue and improved collagen assembly in CD109 transgenic mice.

CD109 Overexpression in the Epidermis Decreases The Expression of Proinflammatory Cytokines and ECM Components in Excisional Wounds

We next examined proinflammatory cytokine expression in CD109 transgenic mice and wild-type littermates by real-time PCR on day 0 and day 3 post-wounding, as these were the only time points that we analyzed during the inflammatory phase of wound healing (Figure 2.7a,b). CD109 transgenic mice display reduced levels of IL-1α and MCP1 compared to WT littermates in both unwounded skin and in excisional wounds on day 3 post-wounding ($p<0.05$). By contrast, CD109 transgenic mice express higher levels of the TGF-ß on day 3 post-wounding while levels are similar in unwounded skin. Reduced expression of IL-1α and MCP1 in CD109 transgenic mice is in agreement with our results in Figure 2.5 demonstrating that elevated epidermal expression of CD109 inhibits immune cell recruitment in excisional wounds. The reduced expression levels of IL-1α and MCP1 despite the elevated TGF-ß levels on day 3 post-wounding is consistent with our immunohistochemical data demonstrating decreased phosphorylation of Smad2/3 in excisional wounds in CD109 transgenic mice.

To determine the effect of CD109 on ECM gene expression, we evaluated collagen I and fibronectin levels in unwounded skin and excisional wounds on day 3, 7, and 14 post-wounding in wild-type and CD109 transgenic mice using real-time PCR (Figure 2.7c,d). Our results show that CD109 transgenic mice express less collagen I ($p<0.01$) on day 7 post-wounding, and less fibronectin on day 3 ($p<0.05$) and on day 7 ($p<0.01$) post-wounding. There was no difference on days 0, 3, and 14 after wounding for collagen I expression, and no difference at 0

and 14 days after wounding for fibronectin expression. The observed reduction in ECM gene expression during the inflammatory (day 3) and proliferative (day 7) phases of wound healing is in agreement with our data (Figure 2.2) illustrating decreased granulation tissue area and improved collagen assembly in CD109 transgenic mice compared to wild-type littermates.

Discussion:

We have recently identified CD109 as a novel TGF-ß co-receptor, which inhibits TGF-ß signaling and responses in skin cells *in vitro* (Finnson *et al.*, 2006; Tam *et al.*, 2003). To examine whether CD109 regulates wound healing *in vivo* in the skin, we generated transgenic mice overexpressing CD109 in the epidermis. Using excisional wounds in these mice, we show that overexpression of CD109 in the epidermis results in decreased phosphorylation of Smad2/3, reduced immune cell recruitment, decreased expression of proinflammatory cytokines and ECM components and improved scarring parameters, in a manner consistent with inhibition of TGF-ß action, while wound closure remains unchanged. In addition, using incisional wounds in these mice we demonstrate that CD109 overexpression leads to improved ECM organization and a smoother epidermis in the healed wound, while the tensile strength of the wound remains unaffected. Taken together, our findings demonstrate that CD109 overexpression in the epidermis reduces inflammation and granulation tissue area while improving collagen architecture *in vivo*.

Our results demonstrating that overexpression of CD109 in the epidermis leads to an increase in epidermal thickness at the wound edge 3 days after

wounding without affecting epidermal gap in excisional wounds, suggest that CD109 may promote keratinocyte proliferation without affecting keratinocyte migration. The early increase in epidermal thickness in the transgenic mice may reflect an accelerated proliferative response in the keratinocytes of the CD109 transgenic mice compared to wild-type littermates as a result of wounding. TGF-ß has been shown to inhibit keratinocyte proliferation during wound healing, but also to promote a migratory phenotype through induction of integrin and fibronectin expression (Amendt *et al.*, 2002; Margadant and Sonnenberg, 2010). Overexpression of Smad2 in basal keratinoctyes has been correlated with impaired re-epithelialization, while Smad3 knockout mice and dominant-negative TGF-ß Receptor II mice display enhanced wound re-epithelialization (Amendt *et al.*, 2002; Ashcroft *et al.*, 1999; Hosokawa *et al.*, 2005). These reports, together with our previous data indicating that CD109 inhibits TGF-ß signaling and responses *in vitro*, support our finding in the current study that CD109 inhibits the anti-proliferative effects of TGF-ß on keratinocytes during wound healing (Finnson *et al.*, 2006). Our results showing that CD109 has no effect on epidermal gap suggests that CD109 may not alter the effects of TGF-ß on keratinocyte migration during wound healing. Thus, CD109 may differentially regulate the growth inhibitory and pro-migratory effects of TGF-ß in keratinocytes.

Our results showing that CD109 overexpression in the epidermis reduces dermal thickening on day 7 post-wounding suggests that CD109 reduces dermal fibrosis during wound healing. Excessive and persistent provisional ECM has

been linked to elevated TGF-ß signaling and fibrotic wound healing disorders (Armour *et al.*, 2007). These data, together with our histological observations showing improved ECM organization in wounds from the CD109 transgenic mice, suggest that CD109 contributes to improved dermal architecture by dampening TGF-ß-induced ECM deposition.

The reduction of granulation tissue on day 14 post-wounding and reduced numbers of α-smooth muscle actin positive fibroblasts in excisional wounds in CD109 transgenic mice, observed in the current study is likely related to inhibition of TGF-ß signaling by CD109. Sustained activation of myofibroblasts in the wound has been reported to lead to excessive ECM deposition characteristic of fibrotic wound healing disorders (Armour *et al.*, 2007). In addition, excessive TGF-ß signaling has been shown to sustain myofibroblast activation and immune cell activity, to alter normal ECM deposition and to prolong the presence of granulation tissue in the wound (Owens *et al.*, 2010). Thus, CD109 might promote granulation tissue resolution by impeding myofibroblast differentiation via inhibition of TGF-ß signaling. However, a decrease in the formation of granulation tissue in the CD109 transgenic mice cannot be ruled out.

We also found that the level of Smad2 and Smad3 phosphorylation is reduced in CD109 transgenic mice compared to wild-type littermates. Excessive TGF-ß signaling has been associated with impaired wound healing, and is linked with pathological scarring such as keloids and hypertrophic scars (Armour *et al.*, 2007). The wound healing parameters altered by the overexpression of CD109, including reduced dermal thickness, reduced α-smooth muscle actin expression,

reduced inflammation, and reduced granulation tissue area are consistent with a reduction in TGF-ß signaling. The significant reduction of Smad2 and Smad3 phosphorylation at the wound site provides further support that CD109 inhibits TGF-ß signaling to decrease inflammation and improve collagen architecture.

Our finding that overexpression of CD109 in the epidermis improves incisional wound healing with a smoother epidermis, better dermal collagen architecture and less granulation tissue, is in agreement with our data obtained in excisional wounds. Also, our data are consistent with the report of Shah et al who have shown that inhibition of TGF-ß signaling using neutralizing anti-TGF-ß antibodies in incisional wounds results in decreased scarring in rats (Shah *et al.*, 1995). Thus our results showing that the transgenic mice overexpressing CD109 in the epidermis exhibit reduced granulation tissue area and improved collagen organization while the tensile strength of the wound remains unchanged suggest that CD109 may reduce scarring parameters.

Inflammation plays an important role during wound healing. However, sustained inflammation has been suggested as a cause for aberrant wound remodeling and increased fibrosis, and is associated with increased TGF-ß action at the wound site (Owens *et al.*, 2010). Neutrophils and macrophages are recruited to the wound site by chemotactic agents released by degranulating platelets upon clotting, and are known to persist until the resolution of the granulation tissue (Singer and Clark, 1999). Our finding that CD109 overexpression in the epidermis impedes neutrophil and macrophage recruitment

to the wound, suggests that CD109 has anti-inflammatory properties which may mediate its anti-fibrotic effect during wound healing.

Our analysis of the expression of proinflammatory cytokines in excisional wounds demonstrates significant decreases in IL-1α and MCP-1 levels in unwounded skin and on day 3 post-wounding in CD109 transgenic mice as compared to their wild type littermates. As these proinflammatory cytokines are known to be chemotactic for neutrophils and macrophages, the reduction in their expression may account for, at least in part, for the decrease in immune cell recruitment to the wound site in the CD109 transgenic mice. Mice deficient in the interleukin-1 receptor (IL-1R) have been reported to show reduced inflammatory cell infiltration and a reduction in fibrosis during wound healing with no significant alterations in the wound tensile strength or rate of wound closure (Thomay *et al.*, 2009). Similarly, MCP-1 knockout mice have been shown to exhibit reduced macrophage and neutrophil infiltration and improved collagen organization as compared to wild type controls, during bleomycin-induced fibrosis (Ferreira *et al.*, 2006). Thus, since inflammation is linked to aberrant wound healing and increased fibrosis, the decreased proinflammatory cytokine production and immune cell recruitment at the wound site in the CD109 transgenic mice in the current study may at least partly, mediate the improved healing parameters observed in these mice.

Additionally, we found that there is no difference in TGF-ß expression in unwounded skin, however TGF-ß expression was elevated in CD109 transgenic mice compared to wild-type littermates 3 days after wounding. Although

increased TGF-ß levels are associated with increased immune cell recruitment to the wound and elevated expression of IL-1α and MCP-1, we observe reduced expression of these proinflammatory cytokines and less immune cell recruitment in CD109 transgenic mice despite increased TGF-ß expression on day 3 post-wounding compared to wild-type littermates. Importantly, our data showing that TGF-ß signaling through the Smad2/3 pathway is decreased during wound healing in CD109 transgenic mice offers an explanation for the reduced expression of the proinflammatory cytokines IL-1α and MCP-1 in the presence of elevated TGF-ß expression during the inflammatory phase of wound healing.

It is well documented that TGF-ß promotes fibronectin and collagen type I production, and that elevated levels of these ECM proteins contribute to scar formation (Singer and Clark, 1999). Our results demonstrating that the CD109 transgenic mice express less collagen I (on day 7) and fibronectin (on day 3 and day 7) suggest that CD109 inhibits ECM production *in vivo* during wound healing. These results confirm our findings *in vitro* in keratinocytes showing that CD109 is an inhibitor of TGF-ß signaling and TGF-ß induced ECM synthesis (Finnson *et al.*, 2006). The decrease in ECM synthesis by CD109 *in vivo* may involve both direct inhibition of TGF-ß signaling in keratinocytes and/or alterations in keratinocyte-fibroblast interactions to modify fibroblast proliferation and function. In addition, as mentioned above, CD109 may also reduce ECM production indirectly by decreasing immune cell recruitment. The precise mechanisms by which epidermal overexpression of CD109 decrease ECM synthesis and fibrotic responses during wound healing remain to be determined.

In conclusion, our results show that overexpression of CD109 in the epidermis reduces inflammation, decreases granulation tissue area and improves collagen architecture without affecting wound closure in excisional wounds or tensile strength in incisional wounds. This is consistent with our previous finding that CD109 acts as a TGF-ß antagonist *in vitro* (Finnson *et al.*, 2006). It is generally held that the expression of TGF-ß during wound healing occurs at levels higher than that required for optimal wound healing. CD109 by dampening the TGF-ß effects may improve aspects of wound healing such as inflammation and excessive ECM deposition without compromising the quality of the healed wound. The ability of CD109 to regulate wound healing parameters such as epidermal and dermal thickness, granulation tissue area and immune cell infiltration during wound healing suggests that CD109 may represent a potential target for therapeutic intervention for wound healing disorders such as hypertrophic scarring.

Conflict of Interest:

The authors state no conflict of interest.

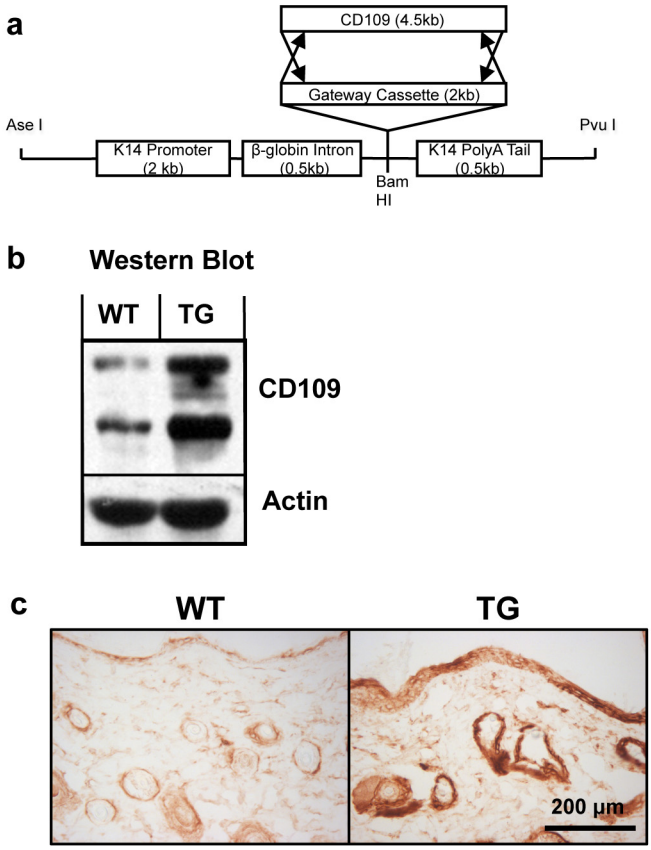
Acknowledgements:

We thank Dr. Kenneth Finnson and Dr. Albane Bizet for their assistance in the editing of this manuscript. Additionally, we would like to thank Dr. Rob Fang for his assistance with the surgeries, as well as Janice Penney and Michelle Read for support with animal care. We thank R&D Systems for generously donating the anti-CD109 antibody (clone ID: 496910.111) which was used in this study. This

work was supported by a CIHR Operating Grant to A.P. (FRN13732) and a CIHR Banting & Best Doctoral Research Award to J.V.

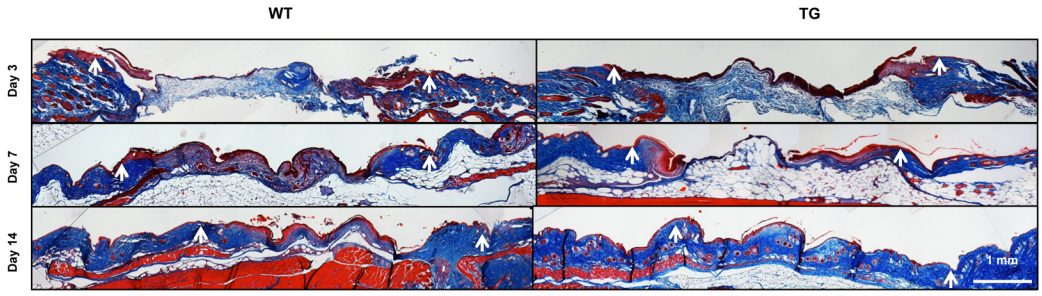
Figures:

Figure 2.1: Generation of Transgenic Mice Overexpressing CD109 in the Epidermis



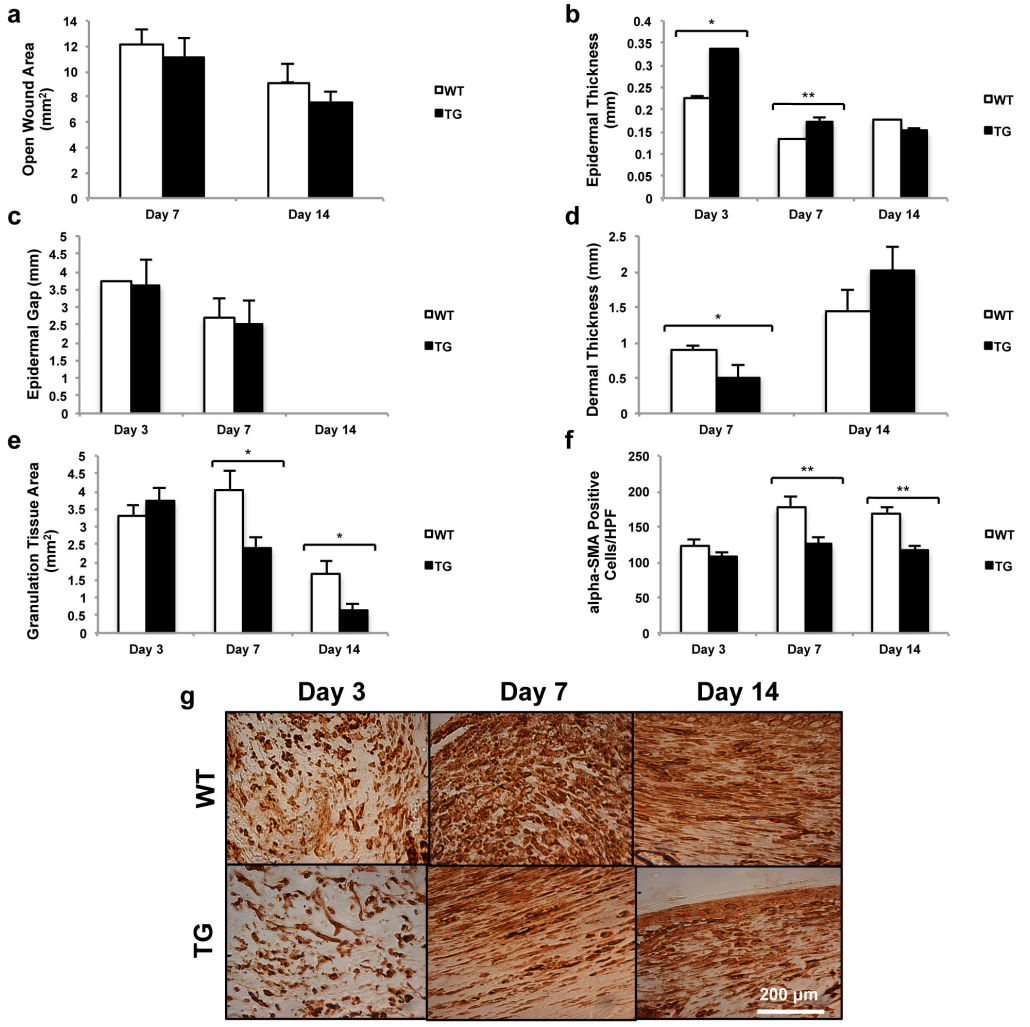
a) Human CD109 was cloned downstream of the K14 promoter and ß-globin intron, and upstream of the K14 Poly-A tail using the Gateway system from Invitrogen, and excised by digestion with AseI and PvuI restriction enzymes. *b)* Expression of CD109 protein in mouse skin homogenates harvested from transgenic mice overexpressing CD109 (TG) and wild-type littermates (WT). *c)* Immunolocalization of CD109 in WT and TG mouse skin. *Original Magnification: 20x*

Figure 2.2: CD109 Overexpression in the Epidermis Improves Tissue Architecture During Excisisonal Wound Healing



Representative images of wound sections stained with Masson Trichrome. Wounds were harvested from wild-type (WT) and transgenic mice (TG) overexpressing CD109 in the epidermis at 3, 7, and, 14 days after wounding. At day 3, we note increased cellularity and epidermal thickness in TG wounds compared to WT. At day 7, we observe reduced dermal thickness and increased epidermal thickness in TG wounds compared to WT. At day 14, we observe improved tissue architecture and reduced granulation tissue area in TG wounds compared to WT. Arrows represent wound margins. *(Original magnification 4x, n=6)*

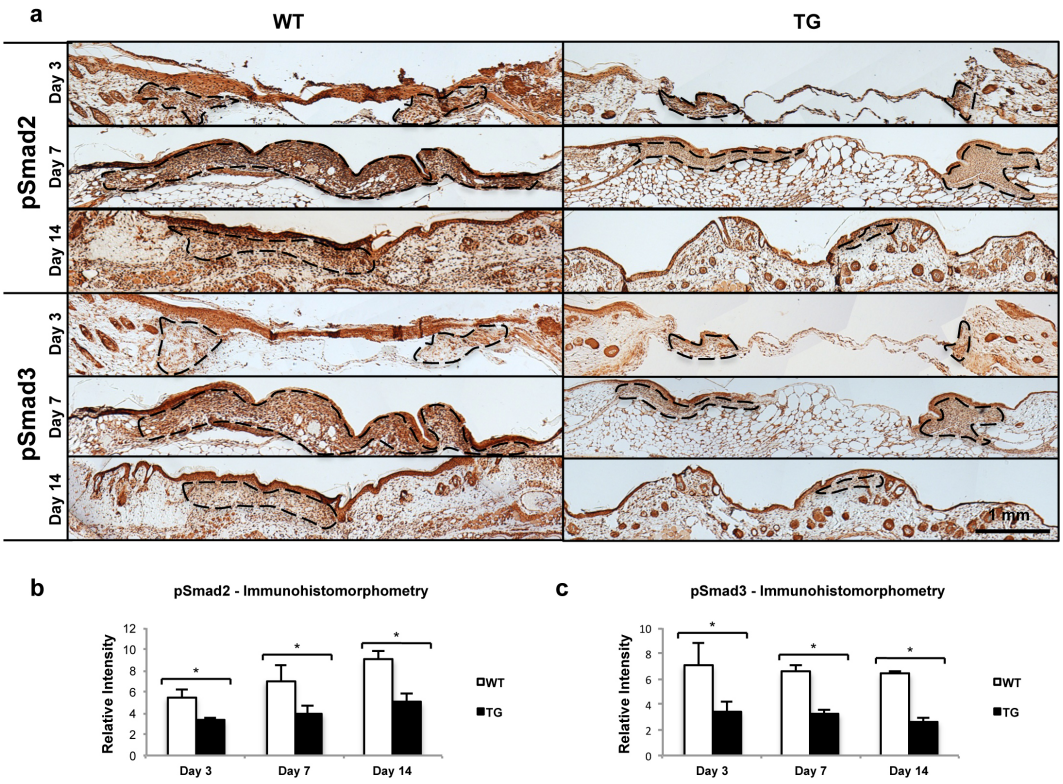
Figure 2.3: Overexpression of CD109 in the Epidermis Promotes Epidermal Thickening, Delays Dermal Thickening, and Reduces Granulation Tissue volume in Excisional Wounds



a) There was no statistical difference in wound area between WT and TG mice at either day 7 or day 14 post-wounding, suggesting that wound closure was not affected. b) CD109 transgenic mice (TG) show significantly thicker epidermis at the wound edge at 3 and 7 days post-wounding compared to wild-type (WT) littermates. c) TG mice show no difference in epidermal gap compared to WT controls. d) TG mice show delayed dermal thickness compared to WT controls,

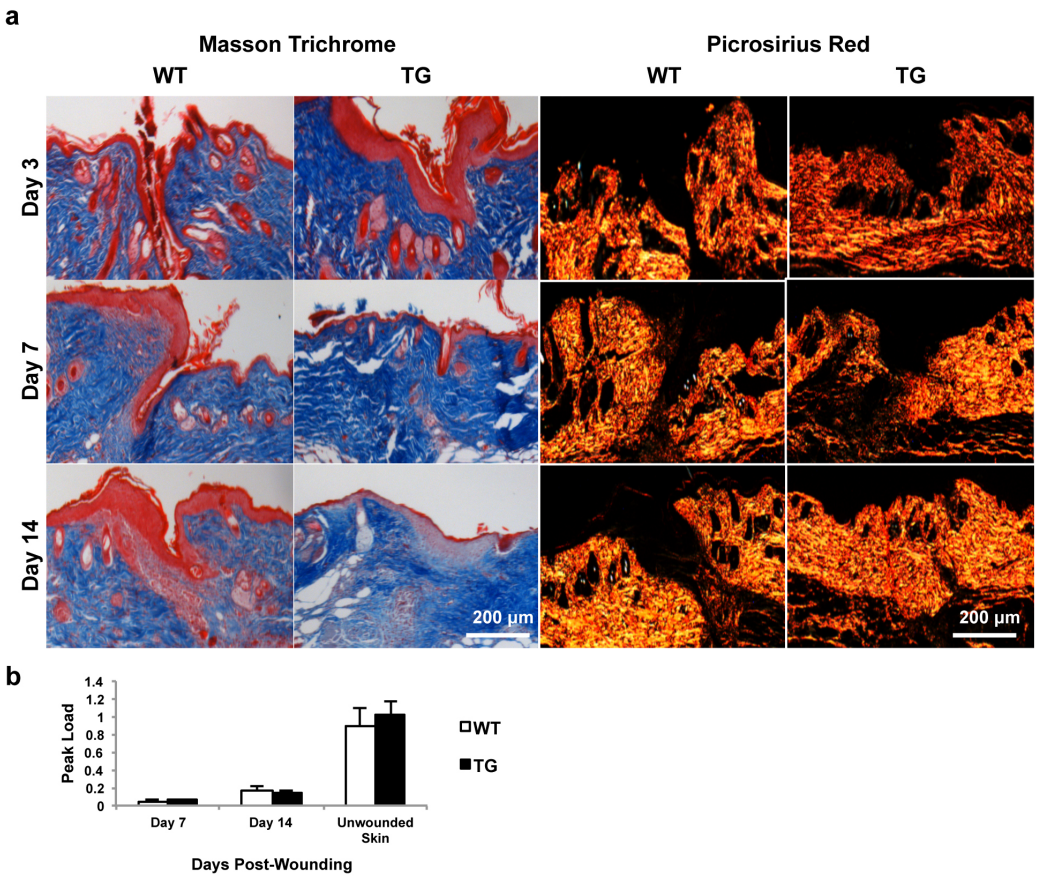
exhibiting a significantly thinner dermis at 7 days post-wounding. e) TG mice show decreased granulation tissue area on 7 and 14 days after wounding compared to WT controls. f) TG mice display reduced α-smooth muscle actin positive cells in the healing dermis 7 and 14 days after wounding. g) Representative immunohistochemistry photos of α-smooth muscle actin positive cells in mouse wound granulation tissue *(Original magnification: 40x, *$p<0.05$, **$p<0.01$, $n=6$)*

Figure 2.4: CD109 Overexpression in the Epidermis Inhibits Smad2 and Smad3 Phosphorylation



a) Representative wound sections immunostained with anti-phospho-Smad2 and anti-phospho-Smad3 antibodies. CD109 transgenic mice display reduced Smad2 and Smad3 phosphorylation compared to wild-type littermate controls 3, 7, and 14 days after wounding. Immunohistomorphometry of b) phospho-Smad2 and c) phospho-Smad3 to detect the amount of staining within the wound site. Dotted lines on immunohistochemical stained sections outlines area of granulation tissue. *(Original Magnification: 4x, *p<0.05, n=6)*

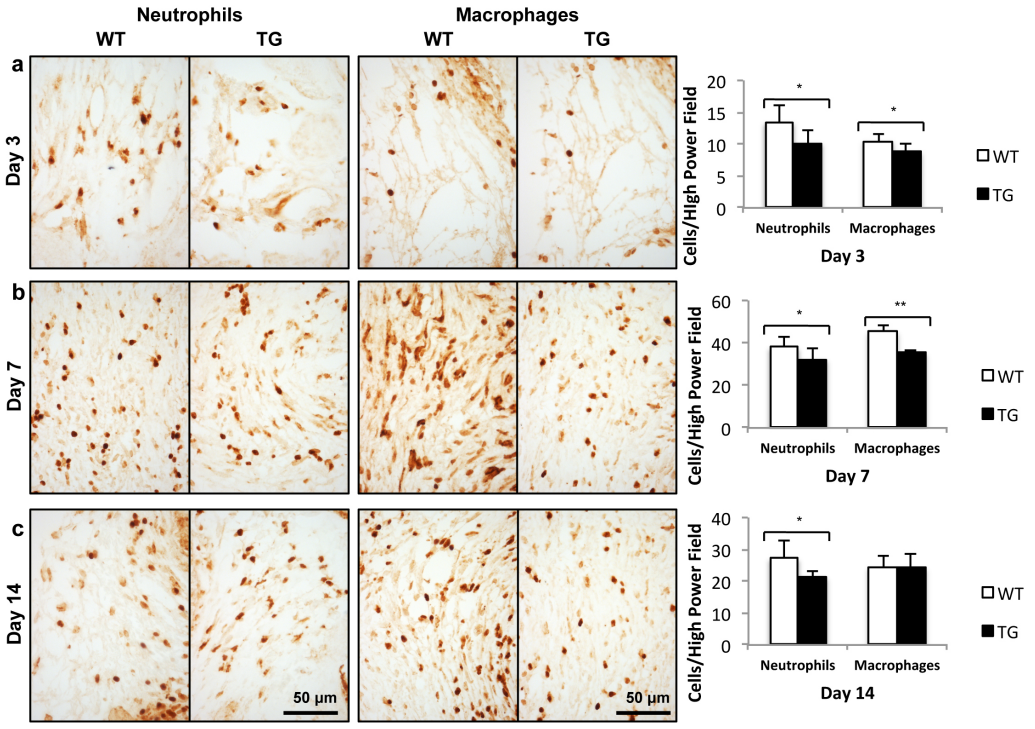
Figure 2.5: CD109 Overexpression in the Epidermis Leads to Improved Collagen Architecture and Smoother Epidermis Without Altering Tensile Strength of the Wound During Incisional Wound Healing



a) Histological assessment by Masson Trichrome and Picrosirius Red staining of incisional wounds from transgenic mice overexpressing CD109 in the epidermis and wild-type littermates *(n=6 per WT and TG mice per time point)* 3, 7, and 14 days post-wounding with a 2 cm incisional wound sutured closed shows improved dermal architecture and smoother epidermis in CD109 transgenic mice, evidenced by a wavy, basket-weave collagen orientation in the CD109 transgenic mouse wounds compared to the whorl-like appearance of the collagen fibres in the wild-type littermate control wounds. *b)* Tensiometric analysis of breaking strength of

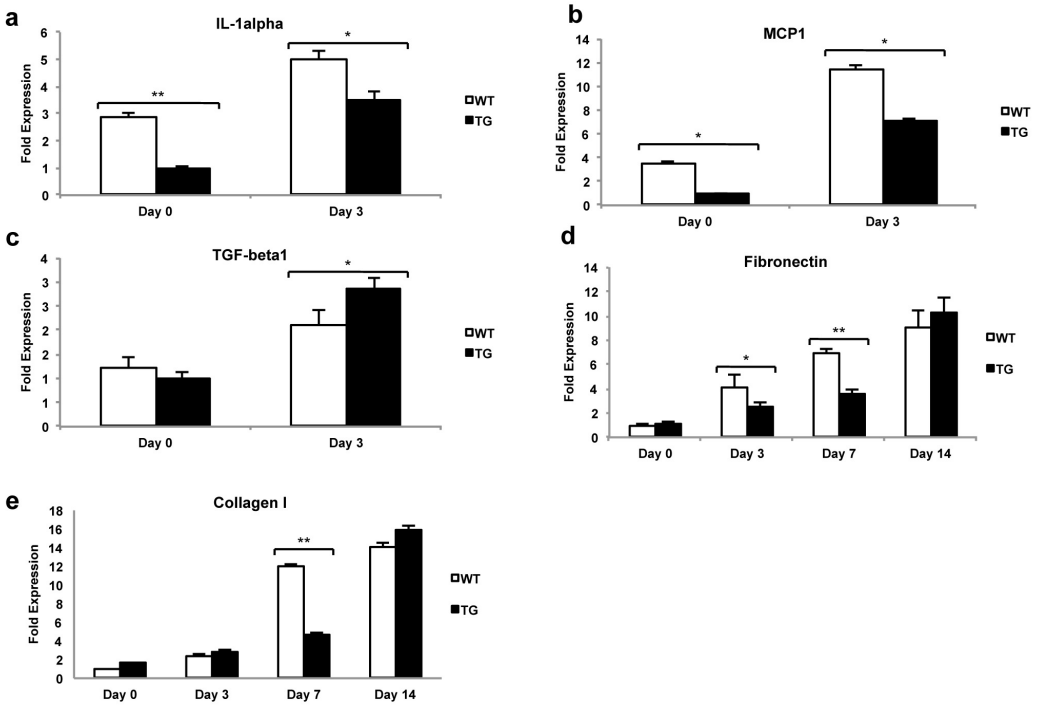
incisional wounds from TG mice and WT showed no significant difference. *(Original Magnification 10x, n=6)*

Figure 2.6: CD109 Overexpression in the Epidermis Inhibits Immune Cell Recruitment in Excisional Wounds



Immunohistochemical analysis of excisional wounds shows significantly reduced recruitment of neutrophils and macrophages using Ly6-Gr and F4/80 antibodies, respectively, to the granulation tissue of the wound at day 3 *(a)*, day 7 *(b)*, and day 14 *(c)* post-wounding by quantification of cells per high power field in TG mice compared to WT littermate controls. *(Original Magnification 40x, *p<0.05, **p<0.01, n=6)*

Figure 2.7: CD109 Overexpression in the Epidermis Decreases Inflammatory Cytokine and ECM Component Gene Expression in Excisional Wounds



RNA extracted from wound tissue was analyzed by quantitative real-time PCR and showed decreased expression of IL1-α *(a)* and MCP-1 *(b)* at 0 and 3 days after wounding in TG mice. TGF-ß levels were similar between WT and TG mice in unwounded skin, however TGF-ß expression was elevated in TG mice 3 days after wounding compared to wild-type controls *(c)*. Fibronectin levels (*d)* were similar in unwounded skin and 14 days after wounding, but decreased in TG mice at days 3 and 7. Collagen I levels *(e)* were decreased in CD109 transgenic mice at day 7, but similar to controls at 0, 3, and 14 days. *(*p<0.05,**p<0.01, n=6)*

Bridging Statement Between Manuscripts

In the previous manuscript, we describe the development of a transgenic mouse overexpressing CD109 in the epidermis under the control of a Keratin-14 promoter. The wound healing response in CD109 transgenic mice displayed a reduction in inflammation and fibrosis as compared to wild-type littermates. The CD109 transgenic mice also displayed reduced Smad2/3 phosphorylation, suggesting a reduction in TGF-ß signaling. The reduction in fibrosis and TGF-ß signaling observed in the previous manuscript prompted us to further explore the role of CD109 in fibrosis.

To better understand how CD109 might affect the progression of fibrosis, we employed the K14-CD109 transgenic mouse line and wild-type littermates in a bleomycin-induced model of fibrosis. This model has been validated as a good murine model of scleroderma, a fibrotic skin disorder that causes significant morbidity for individuals affected by it. The data presented in the following manuscript were collected with the goal of understanding how CD109 overexpression in the epidermis affects progression of fibrosis.

Chapter 3: CD109 overexpression ameliorates skin fibrosis in a bleomycin-induced mouse model of scleroderma

Authors:
Joshua Vorstenbosch, Hasan Al-Ajmi, Sebastian Winocour, Alissa Trzeciak, Lucie Lessard and Anie Philip.
Department of Surgery, Division of Plastic Surgery, McGill University, Montreal, Quebec, Canada.

Submitted to: Arthritis & Rheumatism *(revisions submitted)*

Abstract:

Objective: TGF-ß is a profibrotic cytokine and its aberrant function is implicated in several types of fibrotic pathologies including scleroderma (systemic sclerosis [SSc]). Multiple lines of evidence show that increased TGF-ß signaling contributes to progressive fibrosis in SSc by promoting fibroblast activation, excessive extracellular matrix deposition and dermal thickening. We have previously identified CD109 as a TGF-ß co-receptor and have shown that it antagonizes TGF-ß signaling and TGF-ß induced extracellular matrix expression *in vitro* in skin cells. The aim of the present study was to examine the ability of CD109 to prevent skin fibrosis in a bleomycin-induced mouse model of SSc.

Methods: Transgenic mice overexpressing CD109 in the epidermis and their wild-type littermates were injected with bleomycin or PBS every other day for 28 days. Dermal thickness and collagen deposition were determined histologically using Masson's Trichrome and picrosirius red staining. In addition, collagen and fibronectin content was analyzed by Western blot and activation of TGF-ß signaling was examined by determining phospho-Smad2 and phospho-Smad3 levels by Western blot and immunohistochemistry.

Results: Transgenic mice overexpressing CD109 in the epidermis showed resistance to bleomycin-induced skin fibrosis, as compared to their wild-type littermates, suggesting that CD109 regulates epidermal-dermal interactions. The CD109 transgenic mice displayed a significant decrease in dermal thickness, collagen cross-linking, collagen and fibronectin content and phospho-Smad2 and phospho-Smad3 levels, after belomycin treatment, as compared to their wild-type littermates.

Conclusion: Our findings suggest that CD109 inhibits TGF-ß signaling and fibrotic responses in experimental murine scleroderma, and reveal CD109 as a potential molecular target for therapeutic intervention in scleroderma.

Introduction:

Accumulating evidence indicates that the profibrotic cytokine, transforming growth factor-ß (TGF-ß) is a key factor driving the fibrotic process in scleroderma (systemic sclerosis [SSc]). A large number of studies have demonstrated that TGF-ß signaling is dysregulated in scleroderma (Varga, 2002). Cultured SSc fibroblasts display aberrant activation of autocrine TGF-ß signaling and increased TGF-ß receptor levels, leading to elevated extracellular matrix (ECM) synthesis (Ihn, 2008). Despite the lack of success with several approaches to develop effective anti- TGF-ß therapies to ameliorate fibrosis, strategies to block the production, activation and intracellular signaling of TGF-ß are increasingly being explored. TGF-ß exacerbates fibrosis through deposition and accumulation of extracellular matrix (ECM) not only by promoting the expression of ECM proteins such as collagen type I and fibronectin (Leask and Abraham, 2004), but also by reducing ECM degradation through decreased synthesis of matrix metalloproteinases and increased expression of protease inhibitors (Ferreira *et al.*, 2006).

TGF-ß signaling is transduced by a pair of transmembrane serine/threonine kinases known as the type I and type II TGF-ß receptors, which propagate the signal by phosphorylating intracellular Smad2 and Smad3 (Thatcher, 2010). The Smad2 and Smad3 then complex with Smad4 and translocate to the nucleus where they regulate the expression of TGF-ß target genes such as type I collagen, fibronectin and CCN2 (Leask, 2008; Rosenbloom *et al.*, 2010). We have recently identified CD109 as a TGF-ß co-receptor and

inhibitor of TGF-ß signaling and TGF-ß-induced fibrotic responses in human keratinocytes and mouse fibroblasts *in vitro* (Finnson *et al.*, 2006). We found that as a component of the TGF-ß receptor complex, CD109 inhibits TGF-ß signaling and fibrotic responses by promoting TGF-ß receptor degradation (Bizet *et al.*, 2011). Together, these results suggested that CD109 may have anti-fibrotic effects in vivo. In the current study, we examined whether CD109 inhibits skin fibrosis *in vivo* by generating transgenic mice overexpressing CD109 in the epidermis and analyzing the development of bleomycin-induced scleroderma in these mice.

Materials and Methods:

Generation of CD109 Transgenic Mice

Transgenic mice overexpressing CD109 in basal keratinocytes were generated by cloning the CD109 gene downstream of the keratin-14 promoter and ß-globin intron and upstream of a poly-A tail, using Gateway cloning technology (Invitrogen, Carlsbad, CA). The transgene was then excised using Ase I and Pvu I restriction enzymes, and the purified transgene was injected into fertilized oocytes and implanted into pseudopregant female FVB mice at the McGill University Transgenic Core Facility. Founder mice were identified by southern blot and the presence of the transgene was confirmed by PCR of genomic DNA (Forward Primer: 5'-GGCGAATACGATCACAAGTT-3'; Reverse Primer: 5'-TCCTGGGTACGTCCGGTTACA-3'). PCR reactions were performed using 1 uL cDNA for 35 cycles: denaturation (94°C, 30 seconds), annealing (55°C, 30 seconds), and elongation (72°C, 30 seconds), and resolved on 2% agarose gels in

Tris/Acetate/EDTA buffer. Transcriptional expression of the K14-CD109 transgene was confirmed by RT-PCR. Total RNA was extracted by homogenizing skin in Trizol (Invitrogen). 1ug of RNA was reverse transcribed using MMLV Reverse Transcriptase (Invitrogen), and 1uL of cDNA was amplified using the conditions and primers listed above (specific for the K14-CD109 mRNA sequence).

Bleomycin Treatment

K14-CD109 transgenic and wild-type littermate male mice aged 6-8 weeks were anesthetized with isofluorane and their backs shaved and depilated using Nair (Church & Dwight, York, PA). Mice were injected with 50uL (5ug) of filter-sterilized bleomycin (Mayne Pharma, Montreal, QC) in PBS or PBS alone, intradermally into a single location on the shaved backs every other day for 21 days or 28 days (8 groups with n=6 in each group). Mice were sacrificed by carbon dioxide asphyxiation and injected skin tissue was harvested, bisected, and either snap-frozen in liquid nitrogen for biochemical analysis or fixed in 10% neutral-buffered formalin (Sigma-Aldrich, Oakville, ON) for histological analysis. All animal experiments were approved by the McGill University and Montreal General Hospital Animal Care Committees.

Histology and Immunohistochemistry

The formalin fixed skin tissue was embedded in paraffin and 7um sections were stained using Masson's Trichrome or Picrosirius Red for microscopic evaluation by ImageProPlus6 Software (MediaCybernetics, Bethesda, MD). Dermal thickness was determined as the distance from the basement membrane to

the hypodermis, and evaluated from five different high power fields per section in two different sections from six different animals per group. Immunohistochemical analysis involved antigen retrieval with 10mM citrate buffer and blocking of endogenous peroxidases with 3% hydrogen peroxide for 10 minutes, and of non-specific binding with 5% normal goat serum (Vector Labs, Burlington, ON) for 1 hour at room temperature. The levels of phospho-Smad2 and phospho-Smad3 were determined by incubating with anti-phospho-Smad2 and anti-phospho-Smad3 antibodies (CS-3101, CS-9520; Cell Signaling, Pickering, ON, Canada), respectively; CD109 (Clone 496910.111, R&D Systems, Minneapolis, MN); TGF-ß1 (SC-146, Santa Cruz Biotechnology, Santa Cruz, CA); or negative control IgG (Santa Cruz) at 4°C overnight. Incubation with a biotin-conjugated anti-rabbit or anti-mouse secondary antibody (Vector Labs) was performed for 1 hour at room temperature, followed by treatment with ABC Reagent (Vector Labs) and visualization using ImmPACT DAB (Vector Labs). pSmad2 and pSmad3 positive cells were quantified by calculating the mean number of immunostained nuclei per high power field at injection site in PBS and bleomycin treated wild-type and transgenic mice *(n=5 each)* treated with PBS or bleomycin for 28 days.

Immunoblotting and Densitometry

Skin tissue (1cm x 1cm) was homogenized using RIPA buffer containing EDTA-free protease inhibitors (Roche, Mississaugua, ON), 1mM PMSF, and 1mM sodium ortho-vanadate. Tissue homogenates were clarified at 12,000*g* for 10 minutes and supernatants were normalized for protein concentration using Bio-

Rad D_C Protein Assay (Bio-Rad, Hercules, CA). Samples were resolved by SDS-PAGE (7.5%) under reducing conditions, transferred to nitrocellulose membranes (Whatman Protran, Whatman GmbH, Germany), and blocked using 5% non-fat dried milk in tris-buffered saline-tween (50 mM tris, 150 mM NaCl, 1% Tween-20). Proteins of interest were detected using anti-collagen type I (AB34710, Abcam, Cambridge, MA), anti-fibronectin (610078, BD Biosciences, Mississauga, ON), anti-phospho-Smad2/3 (SC-11769-R), anti-Smad2/3 (SC-8332), CD109 (496110.111, R&D) and anti-ß-actin (SC-47778, Santa Cruz) antibodies, followed by treatment with an anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Cell Signaling, Pickering, ON) diluted 1:5000 and visualized using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford IL) and densitometry (ImageJ, NIH, Bethesda, MD). Student's t-Test was used to determine statistical significance.

Results:

Generation of transgenic mice overexpressing CD109 in the epidermis

Transgenic mice were generated by cloning CD109 downstream of the human keratin-14 (K14) promoter to direct transgene overexpression to basal keratinocytes of the epidermis and hair follicles as described in methods *(Vorstenbosch et al, manuscript submitted).* CD109 overexpression in transgenic mice skin was confirmed by reverse transcriptase-PCR, using K14-CD109 transgene-specific primers to amplify cDNA prepared using mice skin (Figure

3.1a), by immunohistochemistry, and by Western blot analyses of mice skin extracts using an anti-CD109 antibody (Figure 3.4 - Supplemental Figure 1a).

Reduced dermal thickness, collagen deposition and collagen cross-linking in CD109 transgenic mice during bleomycin-induced skin fibrosis

To determine whether CD109 overexpression confer resistance to bleomycin induced skin fibrosis, histological examination of skin sections with Masson's Trichrome was performed to visualize collagen deposition and organization (Figure 3.1b). By day 28 of initial bleomycin injection (the time required for full induction of fibrosis using this regimen (Yamamoto *et al.*, 1999)), a fibrotic phenotype is apparent in both the wild-type and transgenic mice as indicated by more intense blue staining, when compared to PBS controls. Importantly, the bleomycin-treated CD109 transgenic mice display improved collagen organization with a loose, basket-weave pattern consistent with normal dermal collagen architecture, as compared to bleomycin-treated wild-type littermates which display densely packed collagen fibrils characteristic of fibrosis.

Quantification of the dermal thickness in the stained sections demonstrates that at 28 days after initial bleomycin injection, dermal thickness is significantly decreased in transgenic mice, as compared to wild-type littermates ($p<0.0005$) (Figure 3.1c). The decrease is not statistically significant at 21 days after bleomycin injection, consistent with the previous reports that in the murine model of bleomycin-induced fibrosis, full fibrosis is achieved after 4 weeks of bleomycin injections (Yamamoto *et al.*, 1999).

We next evaluated the effect of CD109 over expression on collagen organization and maturation by staining skin sections with picrosirius red and determining birefringence in polarized light (Constantine and Mowry, 1968). As shown in Figure 1d, bleomycin treatment leads to an increase in birefringence in both CD109 transgenic mice and wild-type littermates, as compared to PBS controls. Importantly, the bleomycin-induced increase in birefringence is markedly reduced in transgenic mice, as compared to wild-type littermates. We observed that the intensity of picrosirius red staining is decreased in transgenic mice as compared to wild type littermates, after bleomycin treatment (Figure 3.1d). Together, these results suggest that CD109 confers resistance to bleomycin-induced collagen cross-linking and maturation.

Reduced expression of extracellular matrix proteins in CD109 transgenic mice during bleomycin-induced skin fibrosis

We next determined the effect of CD109 overexpression on the ECM deposition during bleomycin-induced skin fibrosis by analyzing the expression of fibronectin and collagen type I using immunoblot followed by densitometry (Figure 3.2). We found that CD109 transgenic mice show a statistically significant reduction ($p<0.05$) in both fibronectin and collagen type I expression after 28 days of bleomycin injections, as compared to wild-type littermates, with no detectable difference after 21 days of injections. Taken together, these data suggest that reduction in bleomycin-induced fibrosis by CD109 involves inhibition of collagen type I and fibronectin expression.

Decreased Smad2 and Smad3 phosphorylation in CD109 transgenic mice during bleomycin-induced skin fibrosis

Next, we examined whether CD109 overexpression inhibits TGF-b signaling during bleomycin induced skin fibrosis, by determining levels of phospho-Smad2 and phospho-Smad3 by immunoblot and immunohistochemistry. Immunoblot followed by quantification with densitometry demonstrates that Smad2 and Smad3 phosphorylation (normalized to total Smad2 or total Smad3) are reduced in CD109 transgenic mice on both 21 ($p<0.005$) and 28 ($p<0.05$) days after both bleomycin and PBS injection, as compared to wild-type controls (Figure 3.3a,b). Immunohistochemical analysis of phosho-Smad2 and phospho-Smad3 shows that their levels are decreased in the both the epidermis and dermis in the CD109 transgenic mice as compared to wild-type controls (Figure 3.3c, Figure 3.4 - Supplemental Figure 1b). Bleomycin injection increases Smad2 and Smad3 phosphorylation in both wild-type and transgenic mice, as compared to PBS injection. Importantly, however, this increase is less pronounced in both the epidermis and the dermis in the CD109 transgenic mice, as compared to wild-type controls. Interestingly, CD109 transgenic mice express less TGF-ß1 when compared to their wild-type littermates on both 21 and 28 days after bleomycin or PBS injections as determined by immunohistochemistry. Also, bleomycin injection increased TGF-ß1 expression in both wild-type and transgenic groups (Figure 3.4 - Supplemental Figure 1c). Taken together, these results suggest that CD109 inhibits TGF-ß1 expression and TGF-ß-Smad2/3 signaling during bleomycin-induced fibrosis.

Discussion:

The critical role of TGF-b in the initiation and progression of fibrosis is well documented, and strategies to inhibit TGF-b signaling are thought to represent promising avenues for anti-fibrotic therapy (Ihn, 2008). We have recently identified CD109 as a novel TGF-b co-receptor and regulator of TGF-b signaling, inhibiting both Smad2/3 signaling and TGF-b-induced ECM synthesis, in skin cells *in vitro* (Finnson *et al.*, 2006). In the current study we examined whether CD109 regulates TGF-b signaling and fibrosis in the skin *in vivo* by generating transgenic mice overexpressing CD109 in the epidermis and analyzing bleomycin-induced skin fibrosis. Our results indicate that in response to bleomycin treatment, CD109 transgenic mice exhibit reduced dermal thickening and more organized collagen architecture as compared to wild-type littermates. Furthermore, this is associated with a decrease in TGF-ß1 expression, Smad2/3 phosphorylation and expression of type I collagen and fibronectin in the transgenic mice. Together, our data suggest that transgenic mice overexpressing CD109 in the epidermis display decreased TGF-b signaling and responses and resistance to bleomycin-induced skin fibrosis.

The bleomycin-induced mouse model of skin fibrosis mimics human scleroderma in that the fibrotic lesions in both are characterized by densely packed collagen fibrils, extensively cross-linked collagen and increased dermal thickening (Brinckmann *et al.*, 1999; Constantine and Mowry, 1968). Our results demonstrating that over expression of CD109 in the skin in this mouse model results in a striking decrease in bleomycin-induced elevation of collagen

deposition, collagen cross-linking, collagen fiber disorganization and dermal thickening, suggest that CD109 may exert similar effects in reducing fibrotic progression in human scleroderma and thus may have clinical relevance.

Our finding that transgenic mice overexpressing CD109 display decreased Smad2/3 phosphorylation and ECM protein expression in the fibrotic lesion is consistent with our previous report that CD109 inhibits Smad2/3 signaling and ECM synthesis in human keratinocytes and mouse embryonic fibroblasts *in vitro* (Finnson *et al.*, 2006), and suggests that CD109 may inhibit skin fibrosis in these mice by inhibiting TGF-ß-Smad2/3 signaling. The increased Smad2/3 phosphorylation observed in the both transgenic and wild type mice after bleomycin treatment is consistent with the previous reports showing that bleomycin treatment activates the TGF-b-Smad2/3 pathway (Leask and Abraham, 2004; Varga, 2002). Importantly, our immunohistochemistry data showing that overexpression of epidermal CD109 decreases both epidermal and dermal Smad2/3 phosphorylation suggest that the decreased ECM deposition detected in the transgenic mice may involve inhibition of TGF-ß signaling in both keratinocytes and fibroblasts.

The precise mechanisms by which epidermal overexpression of CD109 decrease Smad2/3 phosphorylation and ECM synthesis in the dermis and leads to decreased dermal thickness during bleomycin-induced fibrosis is not known. It is possible that CD109 overexpression in keratinocytes results in alterations in keratinocyte-fibroblast interactions to modify fibroblast proliferation and function. The decreased TGF-ß1 expression associated with CD109

overexpression detected in our study may mediate the decreased bleomycin-induced dermal fibrosis observed in the CD109 transgenic mice. We have previously shown that CD109 can be released from the cell surface and that the released CD109 can also bind TGF-ß1 (Tam *et al.*, 2001). Exosomal release of proteins from keratinocytes have also been shown to modulate dermal fibroblast function, raising the possibility that CD109 released from keratinocytes may reach the dermis and modulate dermal function by trapping dermal TGF-ß or by interacting with the TGF-ß receptor complex on fibroblasts (Chavez-Munoz *et al.*, 2009). In addition, we have found that CD109 transgenic mice express decreased levels of IL-1α during wound healing *(Vorstenbosch et al, submitted)*. Recent evidence suggests that SSc skin exhibits an activated epidermal phenotype, leading to altered epidermal-dermal crosstalk associated with elevated IL-1α expression in keratinocytes, leading to TGF-ß expression in fibroblasts and fibroblast activation (Aden *et al.*, 2010; Aden *et al.*, 2008). Whether the reduction in dermal fibrosis and TGF-ß signaling exhibited by CD109 transgenic mice involves changes in the keratinocyte release of CD109, TGF-ß or other factors remains to be determined.

Despite many efforts, there still lacks an effective treatment strategy for scleroderma and other cutaneous fibrotic disorders. Our finding in the current study showing that transgenic mice overexpressing CD109 in the epidermis display resistance to bleomycin-induced skin fibrosis suggests that CD109 represents a potential target for therapeutic intervention in SSc.

List of Abbreviations:

ALK5: activin-like kinase 5; CTGF: connective tissue growth factor; ECL: electrochemiluminescence; ECM: extracellular matrix; EDTA: ethylenediaminetetraacetic acid; HRP: horseradish peroxidase; LTBP: latent TGF-beta binding protein; PBS: phosphate buffered saline; PCR: polymerase chain reaction; PMSF: phenylmethanesulfonylfluoride; RIPA: radioimmunoprecipitation assay; SDS-PAGE: sodium-dodecyl-sulfate polyacrylamide gel electrophoresis; SSc: systemic sclerosis; TBS: tris buffered saline; TG: transgenic; TGF-ß: transforming growth factor-beta; TIMP: tissue inhibitor of metalloproteinases; WT: wild-type.

Competing Interests:

JV, HA, & AP have filed a patent concerning the use of CD109 in the skin.

Authors' Contributions:

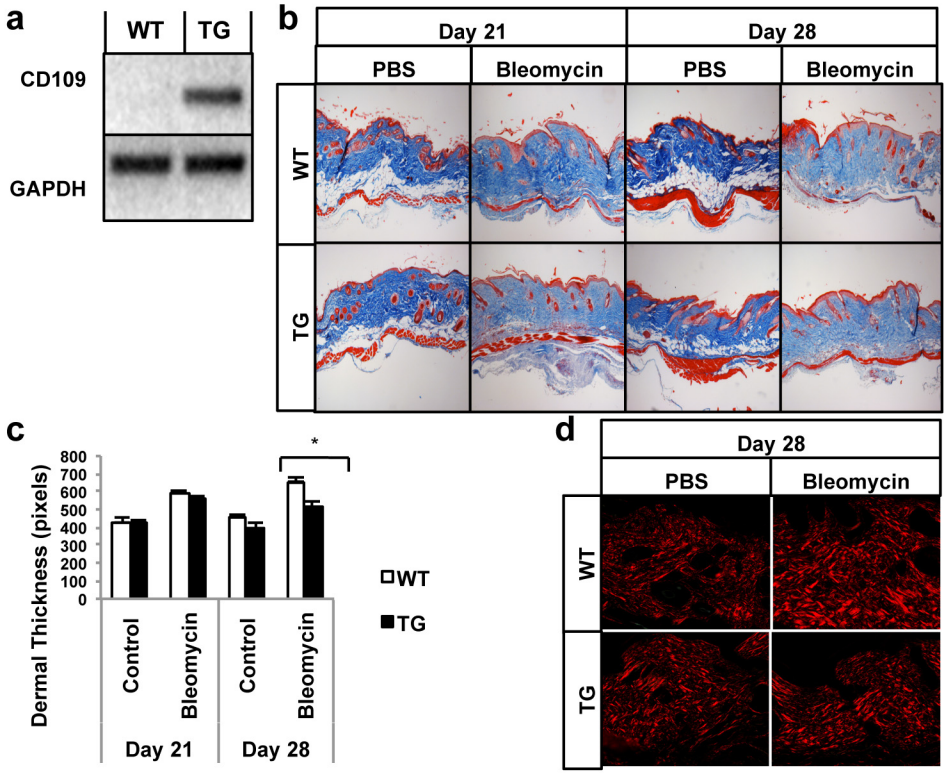
JV participated in the design of the study, animal experimentation, western blot procedure and analysis, immunohistochemistry, densitometry, and drafted the manuscript. HA carried out animal experimentation, western blot procedures, and Masson Trichrome staining. SW carried out animal experimentation, western blot procedures, and Masson Trichrome staining. AT participated in the animal experimentation, histological processing and analysis, and dermal thickness measurements. LL participated in animal experimentation. AP conceived the study, participated in the design, and helped to draft the manuscript. All authors have read and approve of the final manuscript.

Acknowledgements:

We would like to thank Dr. Kenneth Finnson his assistance in the editing of this manuscript. Additionally, we would also like to thank Dr. Stéphane Roy and Éric Villiard for their assistance using a polarized microscope, as well as Janice Penney and Michelle Read for support with animal care. This work was supported by a CIHR Operating Grant to A.P. (FRN13732) and a CIHR Banting & Best Doctoral Research Award to J.V.

Figures:

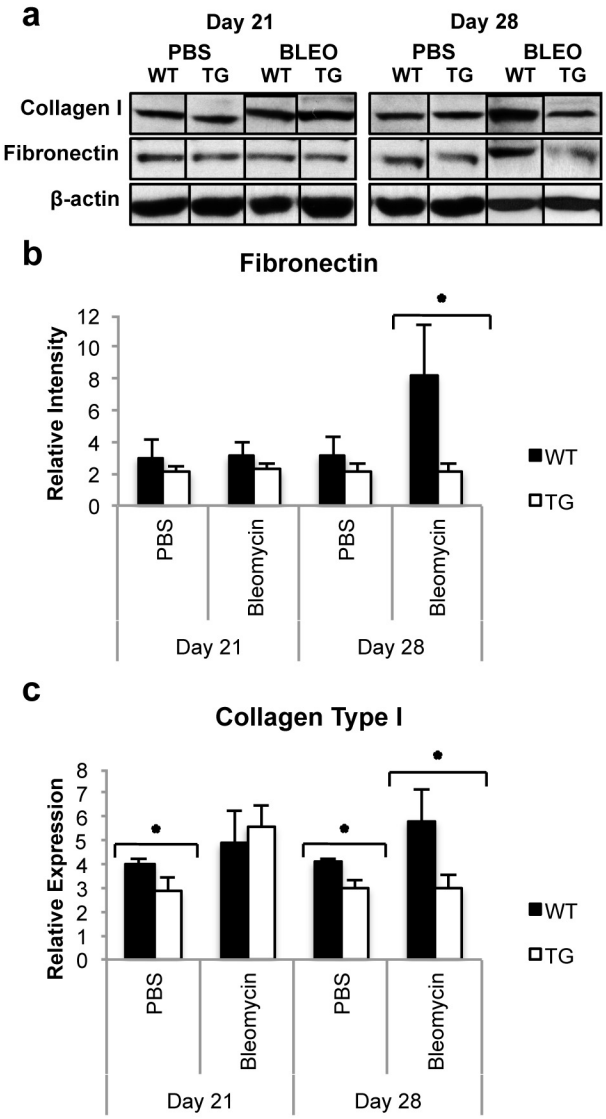
Figure 3.1: Overexpression of Epidermal CD109 Improves Dermal Architecture in Bleomycin-Induced skin Fibrosis



(A) Confirmation of transgene expression by reverse-transcriptase PCR using primers specific to the K14-CD109 transgene construct demonstrates expression of transgene RNA. *(B)* CD109 transgenic mice and wild-type littermates received intradermal injections of bleomycin or PBS every other day for 21 or 28 days. Skin tissue from injected site was fixed in formalin, embedded in paraffin and 7um sections were stained using Masson's Trichrome. *Original Magnification 4x (C)* Masson's Trichrome stained tissue sections were analyzed by ImageProPlus6 Software (MediaCybernetics, Bethesda, MD). Dermal thickness was determined

as the distance from the basement membrane to the hypodermis, and evaluated from five different high power fields per section in two different sections from each mouse *(n=6 per group)*. Results are expressed as mean±SEM ($p<0.005$). *(D)* Skin tissue from injected site was collected on day 28 of bleomycin injection, fixed in formalin, embedded in paraffin and 7um sections were stained with Picrosirius red. *Original Magnification 10x.*

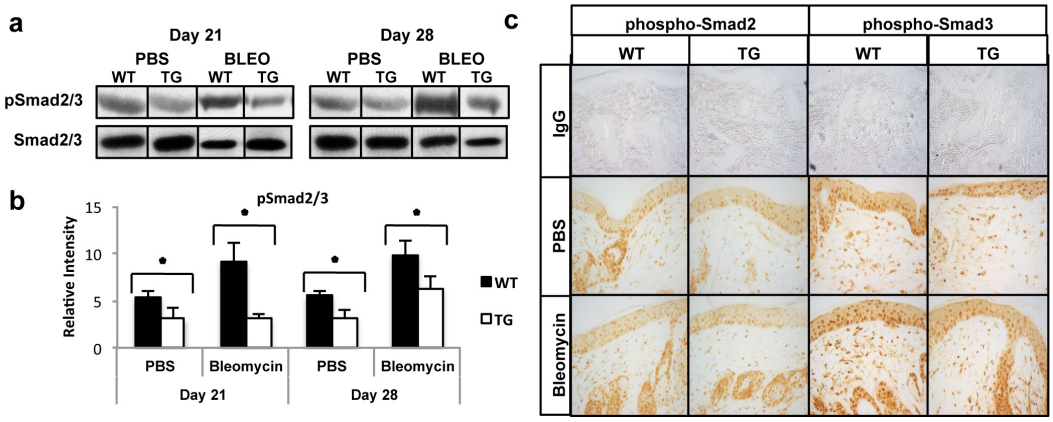
Figure 3.2: Reduced ECM Production in Bleomycin-Induced skin Fibrotic lesions of Transgenic Mice Overexpressing CD109 in the Epidermis



(A) Western blot analysis of skin tissue from PBS and bleomycin injected sites was performed for the detection of collagen type I and fibronectin using anti-collagen type I and anti-fibronectin antibodies, respectively, as described in Methods. Detection of ß-actin using anti-ß-actin antibody was done to ensure equal protein loading. Representative immunoblots of skin from CD109

transgenic and wild-type littermates on day 21 and day 28 of bleomycin treatment are shown. *(B)* Fibronectin and *(C)* Collagen type I levels above were quantified by densitometry, and levels normalized to actin expression are expressed as mean±SEM. Student's t-Test was used to determine statistical significance. *($p<0.005$). The CD109 transgenic and wild-type samples were analyzed on the same gel, and samples harvested from six different mice per time point *($n=6$)* and treatment group were analyzed for densitometry.

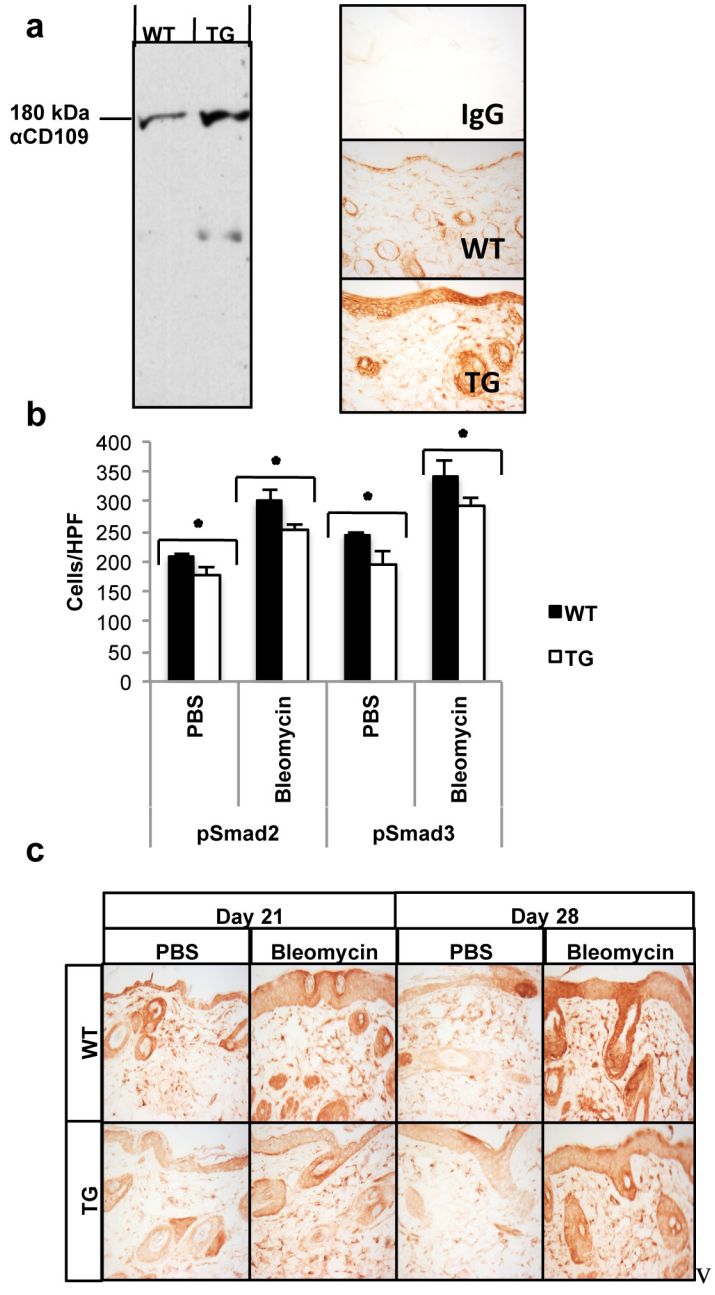
Figure 3.3: Reduced Smad2/3 Phosphorylation in Bleomycin-Induced skin Fibrotic lesions of Transgenic Mice Overexpressing CD109 in the Epidermis



Immunoblot analysis of skin tissue was performed to detect phospho-Smad2/3 and total Smad2/3 levels as described in Methods. Representative immunoblots of skin sections derived from PBS and bleomycin injected sites of CD109 transgenic and wild-type littermates on day 21 and day 28 of injection are shown. *(B)* phospho-Smad2/3 levels above were quantified by densitometry, and levels normalized to total Smad2/3 are expressed as mean±SEM. Student's t-Test was used to determine statistical significance. *($p<0.05$), **($p<0.005$). The CD109 transgenic and wild-type samples were analyzed on the same gel. Samples from six different mice per time point *($n=6$)* and treatment group were analyzed for densitometry. *(C)* Immunohistochemical analysis was performed for the detection of phospho-Smad2 and phospho-Smad3 levels as described in Methods. Representative immunohistograms of sections of skin derived from CD109 transgenic and wild-type littermates on day 28 of bleomycin or PBS treatment are shown. *Original Magnification 20x.* Specificity of immunostaining was confirmed by using control IgG for staining. Samples from CD109 transgenic and

wild-type mice were analyzed on the same slide. A total of six different mice per treatment group *(n=6)* were analyzed.

Figure 3.4: Supplementary Figure 1. Confirmation of CD109 Overexpression in the Skin, Quantification of Smad Phosphorylation in Immunohistochemistry, and TGF-ß1 Expression at Injection Site



(A) Anti-CD109 antibody specificity was confirmed by immunoblot analysis of CD109 transgenic and wild-type mouse skin homogenates, and by

immunohistochemical analysis of CD109 transgenic and wild-type mouse skin sections and an IgG negative control. *(B)* Five stained sections *(n=5)* for each CD109 transgenic and wild-type mouse which both received PBS and bleomycin treatment for 28 days were quantified by taking the mean of the number of positive nuclei per high-power field *($p<0.05$)*. *(C)* CD109 transgenic and wild-type mice treated with PBS and bleomycin for 21 or 28 days were immunostained with an anti-body against TGF-ß1 *(n=6 per group/treatment/time-point)*. Representative images shown. *Original Magnification: 20x.*

Bridging Statement Between Manuscripts

The previous two manuscripts demonstrate that overexpression of CD109 in the epidermis inhibits fibrosis. In both models employed, a wound healing model and a bleomycin-induced model of fibrosis, the CD109 transgenic mice display improved extracellular matrix architecture, reduced extracellular matrix deposition, and decreased TGF-ß signaling through the Smad2/3 pathway compared to wild-type littermate controls.

To better understand how overexpression of CD109 in the epidermis affects normal skin physiology, we characterized tissue samples from CD109 transgenic and wild-type mice. We also explored the individual contributions of keratinocytes and fibroblasts cultured from CD109 transgenic and wild-type mice in light of evidence that keratinocyte-fibroblast interactions are important for normal skin homeostasis. In the following manuscript, we explore how CD109 overexpression in the epidermis affects TGF-ß signaling and receptor expression both *in vivo* and *in vitro*.

Chapter 4: Overexpression of CD109 in the Epidermis Differentially Regulates ALK1 vs ALK5 Signaling and Modulates Extracellular Matrix Synthesis

Joshua Vorstenbosch, Alissa Trzeciak, Albane Bizet, and Anie Philip
Department of Surgery, Division of Plastic Surgery, McGill University Health Centre, Montreal General Hospital, Montreal, Quebec

Manuscript in preparation

Abstract:

Transforming growth factor-beta (TGF-ß) is a multifunctional growth factor involved in many physiological processes including wound healing, angiogenesis, and inflammation. Excessive TGF-ß signaling in the skin has been implicated in the progression of fibrotic skin disorders including keloids and scleroderma. We have previously identified CD109 as a TGF-ß co-receptor which binds TGF-ß with high affinity, inhibits TGF-ß signaling, and have shown that transgenic mice overexpressing CD109 in the epidermis display decreased scarring parameters and reduced bleomycin-induced skin fibrosis. TGF-ß signals through two type I receptors, ALK1 and ALK5, which in association with the TGF-ß type II receptor activate distinct intracellular Smad signaling pathways. The ALK1 receptor signals by activating the Smad1/5/8 pathway, while ALK5 activates the Smad2/3 pathway. To determine whether CD109 differentially regulates TGF-ß signaling through the ALK1 and ALK5 pathways to modulate fibrotic responses, we compared ALK1 and ALK5 expression and downstream TGF-ß signaling in skin harvested from CD109 transgenic mice and wild-type littermates, as well as in primary keratinocytes and skin fibroblasts cultured from these mice. We demonstrate here that overexpression of CD109 in the epidermis

is associated with increased ALK1-Smad1/5/8 signaling but decreased ALK5-Smad2/3 signaling and decreased extracellular matrix production in the skin. In cultured keratinocytes, CD109 overexpression is associated with increased expression of ALK1 and ALK5, increased Smad1/5/8 phosphorylation but decreased Smad2/3 phosphorylation and decreased extracellular matrix production. In both CD109 transgenic mouse skin and cultured keratinocytes overexpressing CD109 we report a reduction in TGF-ß1 expression. Taken together, our data demonstrate that CD109 differentially regulates TGF-ß signaling through the ALK1-Smad1/5/8 pathway and the ALK5-Smad2/3 pathway *in vivo*, which is associated with a reduction in extracellular matrix production and TGF-ß1 expression. These findings suggest that CD109 may have clinical utility in the treatment of fibrotic skin disorders.

Introduction:

We have recently identified CD109 as a 150 kDa, glycophosphatidylinositol-anchored transforming growth factor-beta (TGF-ß) co-receptor that binds TGF-ß with high affinity, associates with TGF-ß signaling receptors, and inhibits TGF-ß signaling (Bizet *et al.*, 2011; Finnson *et al.*, 2006). We have also shown that mice overexpressing CD109 in the epidermis display improved scarring parameters, reduced inflammation, and diminished TGF-ß signaling through the Smad2/3 pathway when challenged with wounding or when treated with bleomycin to induce fibrosis *(Vorstenbosch et al, submitted; Vorstenbosch et al, in preparation)*. Collectively, these results suggest that CD109 might mediate biological processes *in vivo* by altering TGF-ß signaling.

TGF-ß signaling is propagated by the binding of the TGF-ß ligand to its type-II receptor, which then heteromerizes with its type-I receptor to activate intracellular Smad signaling(Shi and Massague, 2003). Although ALK5 is the type I receptor widely accepted to be enganged in the canonical TGF-ß signaling pathway, TGF-ß also signals through another type I receptor, ALK1, which phosphorylates the intracellular proteins Smad1/5/8 while ALK5 phosphorylates Smad2/3 (Santibanez *et al.*, 2011). In both cases, these phosphorylated Smad proteins heteromerize with Smad4, and the resulting complexes translocate into the nucleus where they regulate gene transcription with the phosphorylated Smad1/5/8-Smad4 complex affecting gene transcription differently than the phosphorylated Smad2/3-Smad4 complex (Goumans and Mummery, 2000).

Differences in TGF-ß signaling through the ALK1 versus ALK5 receptors have been reported in various physiological circumstances. For example, signaling through ALK1 as opposed to ALK5 has been demonstrated to have a significant impact in the promotion of angiogenesis (Oh *et al.*, 2000). TGF-ß signaling through ALK1-Smad1 induces endothelial cell proliferation and migration, which promotes angiogenesis, while TGF-ß signaling through ALK5-Smad2/3, inhibits these processes (Ruiz-Ortega *et al.*, 2007). Moreover, increased ALK1-Smad1 signaling has been correlated with TGF-ß dependent fibrogenesis in scleroderma (Pannu *et al.*, 2007). On the other hand, ten Dijke et al in endothelial cells and Finnson et al in chondrocytes have shown that ALK1 expression opposes ALK5 signaling, and that ALK1 signaling downregulates fibrogenic TGF-ß target genes including PAI-1, collagen II, and fibronectin in chondrocytes (Finnson *et al.*, 2008; Goumans *et al.*, 2003a). In addition, expression of the TGF-ß co-receptor endoglin has been shown to promote TGF-ß signaling through the ALK1 pathway over the ALK5 pathway (Lebrin *et al.*, 2004). Collectively, these reports suggest that an alteration in the regulation of ALK1 versus ALK5 signaling pathways could contribute to fibrosis and angiogenesis, and could be dependent on the expression of TGF-ß accessory receptors including endoglin and CD109.

We have previously demonstrated that transgenic mice overexpressing CD109 in the epidermis display reduced inflammation, improved scarring parameters during wound healing, reduced bleomycin-induced skin fibrosis, and decreased TGF-ß signaling through the Smad2/3 pathway *in vivo (Vorstenbosch*

et al, submitted; Vorstenbosch et al, submitted). In addition, our group has shown that CD109 interacts directly with ALK5 and promotes ALK5 internalization and degradation, leading to termination of TGF-ß (Bizet *et al.*, 2011; Finnson *et al.*, 2006). These data together with the reports demonstrating the role of the ALK5/ALK1 pathway in regulating fibrotic responses promted us to examine whether CD109 differentially regulates these pathways *in vivo* and modulates extracellular matrix production.

Materials & Methods:

Histology & Immunohistochemistry

Wild-type and CD109 transgenic mouse skin was harvested and fixed overnight in 10% formalin, and embedded in paraffin. 7 µm sections were stained with both hematoxylin & eosin and Masson's Trichrome, and then evaluated using ImageProPlus6 Software (MediaCybernetics, Bethesda, MD). For immunohistochemistry, paraffin embedded sections were deparaffinized and dehydrated using xylene and graded ethanol washes. Antigen retrieval was performed by boiling slides in sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 10 minutes. Slides were washed in TBST and blocked using 5% NGS/TBST, and then treated with antibodies against ALK1 (R&D Systems, Minneapolis, MN), ALK5, TGF-ß1 (Santa Cruz Biotechnologies, Santa Cruz, CA), phospho-Smad1/5/8, phospho-Smad2, and phospho-Smad3 (Cell Signaling Technology, Boston, MA) diluted in 5% NGS/TBST overnight at 4°C overnight. The following day, slides were treated with biotinylated secondary antibody, followed ABC reagent (Vector Labs) and then developed

using ImmPACT DAB (Vector Labs). Images were captured using ImageProPlus6 software.

Cell Culture

Primary mouse keratinocytes prepared from CD109 transgenic mice and wild-type littermates were grown to confluence in collagen-coated flasks in keratinocyte media as previously described(Hodivala-Dilke, 2002). Fibroblasts prepared from CD109 transgenic mice and wild-type littermates were grown in standard flasks in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 50 µg/ml streptomycin as described previously (Finnson *et al.*, 2006).

Immunoblotting and Densitometry

Mouse skin was homogenized and cultured cells were lysed using RIPA buffer containing complete, EDTA-free protease inhibitors (Roche, Mississaugua, ON, Canada), 1mM PMSF, and 1mM sodium ortho-vanadate. Lysates were clarified at 12,000*g* for 10 minutes and supernatants were normalized for protein concentration using the Bio-Rad D_C Protein Assay (Bio-Rad, Hercules, CA). Samples were resolved by SDS-PAGE (7.5%) under reducing conditions, transferred to nitrocellulose membranes (Whatman Protran, Whatman GmbH, Dassel, Germany), and blocked for non-specific binding using 5% non-fat dried milk in TBS containing 1% Tween-20. Membranes were incubated overnight at 4°C with primary antibodies against CD109, ALK1 (R&D Systems, Minneapolis, MN), ALK5, phospho-Smad2/3, Smad2/3, Smad1, Actin (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Smad1/5/8 (Cell Signaling Technology, Boston, MA), Fibronectin (BD Biosciences, Mississauga, ON), and

Collagen I (Abcam, Cambridge, MA) and subsequently treated with HRP-conjugated secondary antibody (Cell Signaling Technology, Boston, MA) and visualized using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford IL) on photographic film. Immunoblot data was further analyzed by normalizing ALK1, ALK5, Fibronectin, Collagen I, and CD109 expression to ß-Actin levels, phospho-Smad1/5/8 phosphorylation to Smad1 expression, and phospho-Smad2/3 phosphorylation to Smad2/3 expression (ImageJ Software, Bethesda, MD). Each immunoblot experiment was performed three times, and data from each sample from each experiment was averaged in the densitometry analysis. Statistical significance was determined using Students T-Test.

Quantitative Real-Time PCR

Total RNA was extracted from wild-type and CD109 transgenic cultured mouse keratinocytes and from skin tissue using the RNEasy Kit (Qiagen, Mississauga, ON) according to manufacturer's protocol. Reverse transcription was performed using MMLV Reverse Transcriptase (Invitrogen). 1 µg of RNA was reverse transcribed with oligo dT primer in a final volume of 20 µL. qPCR was performed to amplify TGF-ßI (Forward Primer: 5'-CTGCTGCTTTCTCCCTCAAC-3', Reverse Primer: 5'-GACTGGCGAGCCTTAGTTTG-3'), with iQ SYBR Green Supermix on a CFX96 Thermocycler and normalized to a GAPDH (Forward Primer: 5'-GGCGTCTTCACCACCATGGAG-3', Reverse Primer: 5'-AAGTTGTCATGGATGACCTTGGC-3') housekeeping control (Bio-Rad, Hercules, CA). Six transgenic and six wild-type samples were processed for both

keratinocyte and whole skin analysis. Statistical significance was determined using Student's T-Test.

Results:

Skin Tissue Architecture in Transgenic Mice Overexpressing CD109 in the Epidermis is Similar to that of Wild-Type Littermates

In previous studies, we have shown that CD109 is a potent inhibitor of TGF-ß signaling and that transgenic mice overexpressing CD109 in the epidermis display reduced scarring parameters during wound healing and also decreased bleomycin-induced skin fibrosis *(Vorstenbosch et al, submitted; Vorstenbosch et al, submitted)*. To assess whether CD109 affects skin architecture in unchallenged skin, we compared CD109 transgenic mouse skin sections to that of wild-type littermate controls stained with both hematoxylin & eosin and Masson's Trichrome (Figure 4.1). We report no difference between the CD109 transgenic and wild-type littermate skin, with both showing similar morphology, epidermal and dermal thickness, and organization of collagen fibrils.

Overexpression of CD109 in the Epidermis Alters ALK5 versus ALK1 Expression and Favors Signaling Through the Smad1/5/8 Pathway Over the Smad2/3 Pathway In the Skin

We evaluated the effect of CD109 overexpression on the TGF-ß signaling pathway by immunostaining for TGF-ß type I receptors and phosphorylated receptor-Smad second-messenger proteins (Figure 4.2). We report here that overexpression of CD109 in the epidermis promotes a marked increase in ALK1

expression throughout the epidermis and in the hair follicles with a moderate increase in the dermis when compared to wild-type littermates. On the other hand, we found that the expression of ALK5 is significantly *($p<0.05$, Figure 4.7-Supplemental Figure 1)* decreased in the CD109 transgenic mice when compared to wild-type littermates. Consistent with these findings, levels of Smad1/5/8 phosphorylation are increased in the CD109 transgenic mice primarily in the epidermis and hair follicles, while the levels of Smad2 and Smad3 phosphorylation are decreased in both the epidermis and hair follicles in CD109 transgenic mice when compared to wild-type littermates. Taken together, these data suggest that overexpression of CD109 in the skin *in vivo* leads to a decrease in ALK5 expression and Smad2/3 phosphorylation, while promoting ALK1 expression and Smad1/5/8 phosphorylation.

To confirm our immunohistochemical results, we compared the ALK1 and ALK5 protein expression and Smad phosphorylation by immunoblot analysis in mouse skin homogenates harvested from CD109 transgenic mice and wild-type littermates. Consistent with our findings by immunohistochemistry, we demonstrate that CD109 transgenic mouse skin displays reduced ALK5 but elevated ALK1 (Figure 4.3a) expression compared to wild-type controls. Additionally, tissue homogenates of skin harvested from CD109 transgenic mice show reduced Smad2/3 phosphorylation and elevated Smad1/5/8 phosphorylation (Figure 4.3b) compared to wild-type littermate mouse skin extracts. Importantly, fibronectin and collagen type I expression are reduced in CD109 transgenic mouse skin (Figure 4.3c) when compared to that of wild-type controls.

Collectively, these data support our findings above that CD109 overexpression in the epidermis enhances ALK1 expression and Smad1/5/8 phosphorylation while reducing ALK5 expression and Smad2/3 phophorylation in the skin, and demonstrates that this is associated with a decrease in the expression of the extracellular matrix proteins collagen type I and fibronectin.

Primary Cultures of Keratinocytes Overexpressing CD109 Isolated From CD109 Transgenic Mice Display Enhanced Smad1/5/8 Phosphorylation, Decreased Smad2/3 Phosphorylation, and Decreased Extracellular Matrix Production

To determine the effect of CD109 on the epidermis in the absence of dermal influence, we cultured primary keratinocytes from neonatal CD109 transgenic mice and wild-type littermates. Interestingly, immunoblot analysis of TGF-ß type I receptors showed that both ALK1 and ALK5 are increased in primary transgenic mouse keratinocytes *in vitro* compared to wild-type controls (Figure 4.4a). This is in contrast to our results *in vivo*, which showed that ALK1 was increased while ALK5 was decreased in the CD109 transgenic mice. However, evaluation of Smad phosphorylation showed that the primary mouse keratinocytes exhibit the same pattern observed *in vivo*, with the CD109 transgenic primary mouse keratinocytes displaying increased Smad1/5/8 phosphorylation and decreased Smad2/3 phosphorylation compared to wild-type controls in response to 15 pM TGF-ß treatment for 30, and 60 minutes while basal levels of phosphorylation measured at time 0 are similar (Figure 4.4b). We next examined the production of extracellular matrix proteins by CD109 transgenic

and wild-type littermate keratinocytes to increasing levels of (0, 15, 50 pM) TGF-ß treatment for 24. Consistent with our results obtained *in vivo*, transgenic keratinocytes display minimal collagen type I expression while the wild-type keratinocytes show a dose-dependent increase in collagen I expression in response to TGF-ß1 treatment (Figure 4.4c). Both the transgenic and wild-type keratinocytes exhibit a dose-dependent increase in fibronectin expression in response to TGF-ß, however the CD109 transgenic keratinocytes express significantly less compared to wild-type controls. We also confirmed that the transgenic keratinocytes overexpress CD109 independently of TGF-ß treatment, and that there is no increase in CD109 expression in wild-type keratinocytes in response to TGF-ß.

Because dermal fibroblasts can regulate keratinocyte function and may account for the discrepancies we observed between our results in the skin *in vivo* and in keratinocytes *in vitro*, we determined whether the ALK1-Smad1/5/8 versus ALK5-Smad2/3 pathways are altered in cultured primary mouse fibroblasts isolated from wild-type and CD109 transgenic neonatal mouse skin. As shown in Figure 4.5a, no difference in the expression of ALK5 between wild-type and transgenic dermal fibroblasts was observed, and ALK1 expression was barely detectable in both wild-type and transgenic fibroblasts (Figure 4.5a). Consistent with the lack of difference in ALK5 and ALK1 expression, Smad1/5/8 phosphorylation and Smad2/3 phosphorylation were also similar in wild-type and transgenic fibroblasts under basal signaling conditions and in response to 15 pM TGF-ß treatment (Figure 4.5b). In response to TGF-ß treatment for 24 hours at

increasing doses (0, 15, 50 pM), we observe no differences in CD109, collagen type I, or fibronectin expression between the wild-type and CD109 transgenic dermal fibroblasts (Figure 4.5c).

Transgenic Mice Overexpressing CD109 in the Epidermis Exhibit Decreased TGF-ß1 Expression in the Skin and in Keratinocytes Isolated From Their Skin

We next assessed whether regulation of TGF-ß signaling by CD109 involves alterations in TGF-ß expression levels. Using immunohistochemistry, we show that TGF-ß1 expression is decreased in CD109 transgenic mouse skin when compared to wild-type littermate skin. TGF-ß1 staining is markedly decreased in the dermis and at basal keratinocytes that reside at the deep layer of the epidermis as well as in hair follicles when compared to wild-type littermate skin (Figure 4.6a). Further comparison by quantitative real-time PCR analysis of TGF-ß1 transcript levels showed a nearly 2-fold reduction in TGF-ß1 expression in CD109 transgenic keratinocytes compared to wild-type controls (Figure 4.6b). There was no statistically significant difference between TGF-ß1 transcript levels between CD109 transgenic mouse skin homogenates and wild-type controls.

Discussion:

Differential TGF-ß signaling through ALK1 versus ALK5 receptors has been well documented in several physiological processes including angiogenesis and fibrogenesis, and the dominance of one signaling pathway over another has profound physiological outcomes (Oh *et al.*, 2000; Pannu *et al.*, 2007). Our group has previously identified CD109 as a novel TGF-ß co-receptor that reduces

scarring during wound healing, reduces bleomycin-induced skin fibrosis, reduces extracellular matrix production, and inhibits Smad2/3 phosphorylation overexpressed in the skin *in vivo (Vorstenbosch et al, submitted; Vorstenbosch et al, submitted)*. However, to date much is unknown about the mechanism by which CD109 inhibits TGF-ß in the skin. We demonstrate here that overexpression of CD109 in the epidermis of mouse skin promotes signaling through the ALK1-Smad1/5/8 pathway rather than the ALK5-Smad2/3 pathway and is associated with a reduction in extracellular matrix protein expression. However, in cultured keratinocytes overexpressing CD109, we report an associated increase in both ALK1 and ALK5 expression compared to wild-type controls. In whole mouse skin, we also report a reduction in TGF-ß1 expression in the CD109 transgenic samples compared to wild-type controls.

Our results showing that CD109 transgenic mice express more ALK1 and less ALK5 than wild-type littermates in the skin suggests that CD109 modifies TGF-ß signaling through changes in receptor expression. Previous studies have shown that endoglin, another TGF-ß co-receptor, promotes ALK1 signaling over ALK5 signaling (Lebrin *et al.*, 2004). Subsequent studies into endoglin function, which like CD109 is classified as a TGF-ß co-receptor, show that interactions between endoglin, intracellular scaffolds, and TGF-ß receptors contribute to its capacity to augment TGF-ß signaling (Lee *et al.*, 2008b; Ray *et al.*, 2010). While CD109 is a GPI-anchored protein and thus only has a limited intracellular domain, our group has shown that CD109 associates with ALK5 and promotes its internalization, which could contribute to the observed reduction in ALK5 protein

expression in transgenic mouse skin (Bizet *et al.*, 2011). The elevated ALK1 and reduced ALK5 expression observed in CD109 transgenic mouse skin compared to wild-type littermates is consistent with our observations showing elevated Smad1/5/8 phosphorylation and reduced Smad2/3 phosphorylation in CD109 transgenic mouse skin (Goumans *et al.*, 2003a). Taken together, our data demonstrate that CD109 overexpression in the epidermis reduces ALK5 levels and Smad2/3 phosphorylation while promoting ALK1 expression and Smad1/5/8 phorphorylation in the skin.

We also found that transgenic mice overexpressing CD109 in the epidermis display reduced extracellular matrix protein expression in the skin compared to wild-type littermate controls. Activation of Smad2/3 phosphorylation through TGF-ß signaling has been shown to be intimately associated with elevated extracellular matrix protein expression, and Smad3-null mice have also been shown to resist radiation and bleomycin-induced fibrosis (Flanders, 2004; Zhao *et al.*, 2002). These findings are consistent with our observations demonstrating the reduction in Smad2/3 phosphorylation and decreased fibronectin and collagen type I expression in CD109 transgenic mouse skin. However, several studies have recently reported an association between ALK1-Smad1 signaling and fibrogenesis (Morris *et al.*, 2011; Pannu *et al.*, 2008; Pannu *et al.*, 2007). These studies correlate elevated endoglin expression with increased ALK1-Smad1 signaling, which they claim leads to elevated collagen and CTGF expression in scleroderma fibroblasts. In contrast, Finnson et al show that in chondrocytes that ALK1 inhibits while ALK5 promotes expression of

collagen type II and fibronectin (Finnson *et al.*, 2008). The results obtained in the current study are in accordance with those of Finnson et al, as we demonstrate reduced fibronectin and collagen type I expression despite elevated Smad1/5/8 phosphorylation (Finnson *et al.*, 2008). It should be noted that the studies reporting elevated fibrogenesis in association with increased Smad1/5/8 phosphorylation were investigating systems with elevated endoglin levels, which could explain the apparent discrepancy. Thus, our data suggest that overexpression of CD109 in the epidermis inhibits extracellular matrix protein production by reducing Smad2/3 phosphorylation.

Interestingly, when we cultured primary keratinocytes from CD109 transgenic mice and wild-type littermates we found that the transgenic keratinocytes display both elevated ALK1 and elevated ALK5 levels compared to wild-type controls. This is in contrast to what we observed *in vivo*, where the CD109 transgenic mouse skin show higher ALK1 levels but lower ALK5 levels. There have been several reports illustrating the importance of paracrine signaling in the skin between the epidermis and the dermis, which highlight the complex interactions between keratinocytes and fibroblasts *in vivo* (Maas-Szabowski *et al.*, 1999; Werner *et al.*, 2007; Werner and Smola, 2001). The discrepancy that we report here between the cultured keratinocytes and whole skin could arise from the lack of paracrine signaling from the dermis to the epidermis. Fibroblasts cultured from the dermis of CD109 transgenic mice and wild-type littermate skin were cultured to determine whether there were any differences in fibroblast signaling in response to TGF-ß that might account for the discrepancy between

our *in vitro* results in cultured keratinocytes and *in vivo* results from mouse skin homogenates. We observed no differences in the fibroblast signaling between CD109 transgenic and wild-type cells under basal conditions and in response to TGF-ß treatment. It should be noted though that the cultured fibroblasts examined in this study were not under the influence of keratinocyte signaling, suggesting that these *in vitro* results might not mirror fibroblast signaling *in vivo.* However, many other cell types reside in the skin including endothelial cells, monocytes, lymphocytes, and a host of other immune cells that could also contribute to TGF-ß signaling in the skin. The presence of these additional cell types could further contribute to the interaction between keratinocytes and fibroblasts, thereby further modulating *in vivo* signaling, and potentially explain the difference in ALK5 expression observed between our *in vitro* and *in vivo* models.

The increased ALK1 and ALK5 expression observed in the CD109 transgenic keratinocytes *in vitro* was associated with elevated Smad1/5/8 phosphorylation and reduced Smad2/3 phosphorylation in response to TGF-ß treatment when compared to wild-type controls. This is consistent with our observations in the skin *in vivo* in transgenic mice versus wild-type littermates. The sustained reduction in Smad2/3 phosphorylation in response to TGF-ß treatment despite the increased ALK5 levels could be mediated via elevated ALK5 degradation, as our lab has previously shown that CD109 inhibits TGF-ß signaling by promoting ALK5 internalization into caveloae for degradation (Bizet *et al.*, 2011; Finnson *et al.*, 2006). Consistent with the reduction in Smad2/3

phosphorylation, in CD109 transgenic mouse keratinocytes we also observe inhibition of the extracellular matrix proteins collagen type I and fibronectin when treated with TGF-ß, further reinforcing that CD109 reduces TGF-ß signaling.

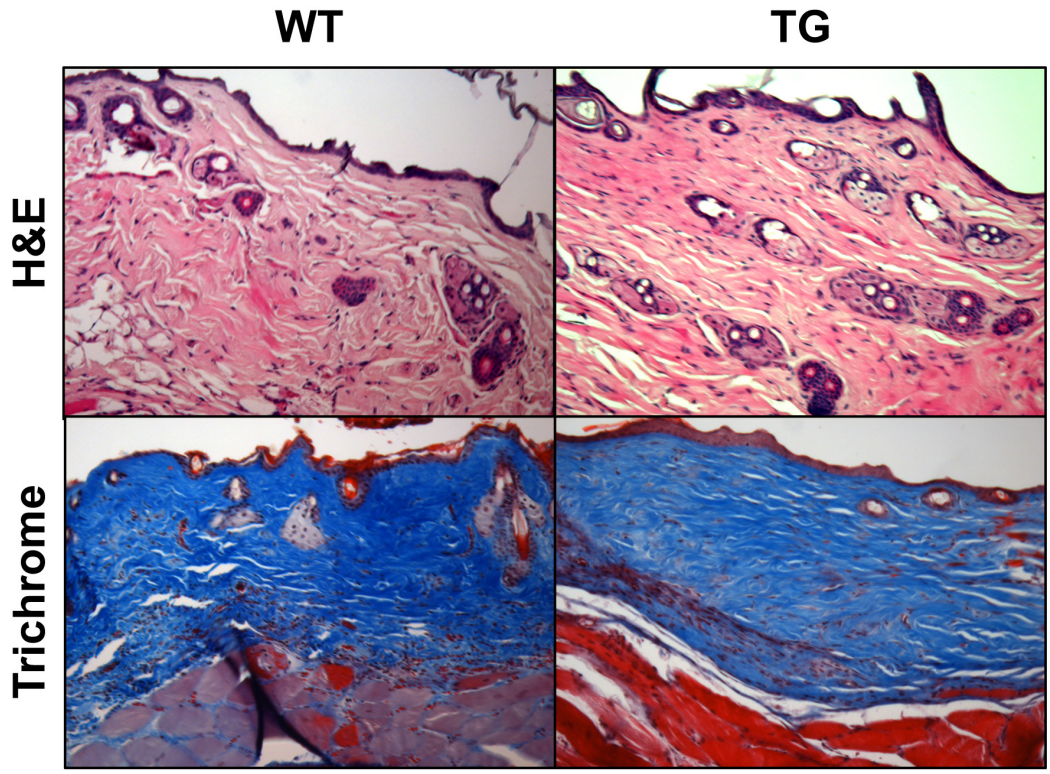
Reduced TGF-ß1 expression associated with overexpression of CD109 in the epidermis could also be involved with the observed reduction in Smad2/3 phosphorylation and extracellular matrix protein expression in CD109 transgenic mouse skin compared to wild-type littermates. TGF-ß treatment of cultured fibroblasts has been shown to increase TGF-ß mRNA and protein expression *in vitro* (Flanders *et al.*, 1995). A possible explanation for the decreased TGF-ß1 levels observed *in vivo* by immunohistochemistry, as well as *in vitro* in cultured keratinocytes from CD109 transgenic mice could be due to the inhibition of TGF-ß signaling by CD109, which in turn would further reduce TGF-ß expression. Importantly, our *in vitro* data showing that CD109 transgenic keratinocytes treated with TGF-ß demonstrate a robust inhibition of TGF-ß induced extracellular matrix protein expression and Smad2/3 phosphorylation compared to wild-type controls suggests that our *in vivo* observations are likely due to the overexpression of CD109 rather than reduced TGF-ß1 levels. Although CD109 may further reduce TGF-ß signaling by causing a decrease in the expression of TGF-ß ligand, under controlled TGF-ß treatment levels we observe that CD109 transgenic keratinocytes demonstrate a decrease in TGF-ß signaling through the Smad2/3 pathway compared to wild-type littermates, indicating a reduced TGF-ß response associated with overexpression of CD109. Collectively, we show here that in addition to reducing Smad2/3 phosphorylation and extracellular matrix

protein production, overexpression of CD109 also reduces expression of the TGF-ß1 ligand.

In conclusion, we show here that overexpression of CD109 in the epidermis *in vivo* directs TGF-ß signaling through the ALK1-Smad1/5/8 pathway rather than the ALK5-Smad2/3 pathway, increases ALK1 while reducing ALK5 expression, and reduces extracellular matrix protein production. These data presented are consistent with our previous findings demonstrating the capacity for CD109 to improve wound healing and reduce fibrosis, and suggest that increased phosphorylation of Smad1/5/8 when accompanied by decreased phosphorylation of Smad2/3 could be an important target for reduction of extracellular matrix. Further investigation into the mechanism by which CD109 reduces extracellular matrix protein expression, the key component of fibrosis, could provide avenues by which CD109 might become an important therapeutic agent for the treatment of fibrotic skin disorders such as scleroderma.

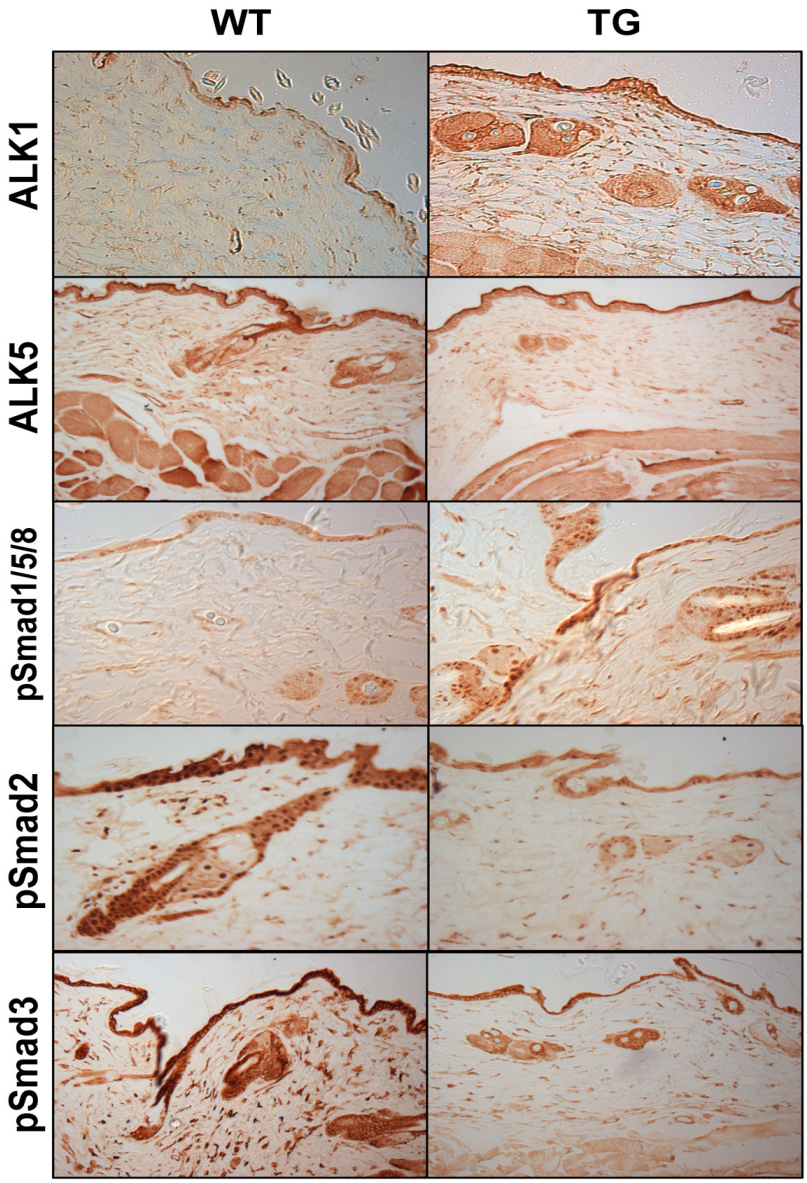
Figures:

Figure 4.1: Transgenic Mice Overexpressing CD109 in the Epidermis Exhibit Similar Skin Architecture Compared to Wild-Type Littermate Controls



Skin was harvested from wild-type and transgenic mice (n=6, each) as described in methods and stained with hematoxylin & eosin and Masson's Trichrome. Five different fields of view from two different sections from each mouse were assessed for differences in tissue architecture. The wild-type and CD109 transgenic mouse skin display similar extracellular matrix assembly, architecture, and overall appearance of the dermis and epidermis.

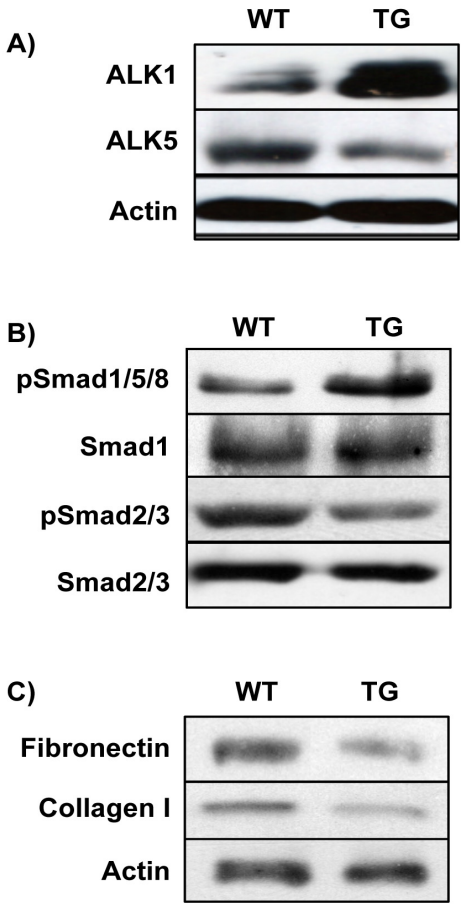
Figure 4.2: Overexpression of CD109 in the Epidermis Alters ALK5 vs ALK1 Expression and Favors Signaling Through the Smad1/5/8 Pathway Over the Smad2/3 Pathway



Skin was harvested from wild-type and transgenic mice (n=6, each), prepared for histology as described in methods and analyzed by immunohistochemistry. Five different fields of view from two different sections from each mouse were assessed for differences in protein expression. CD109 transgenic mice show

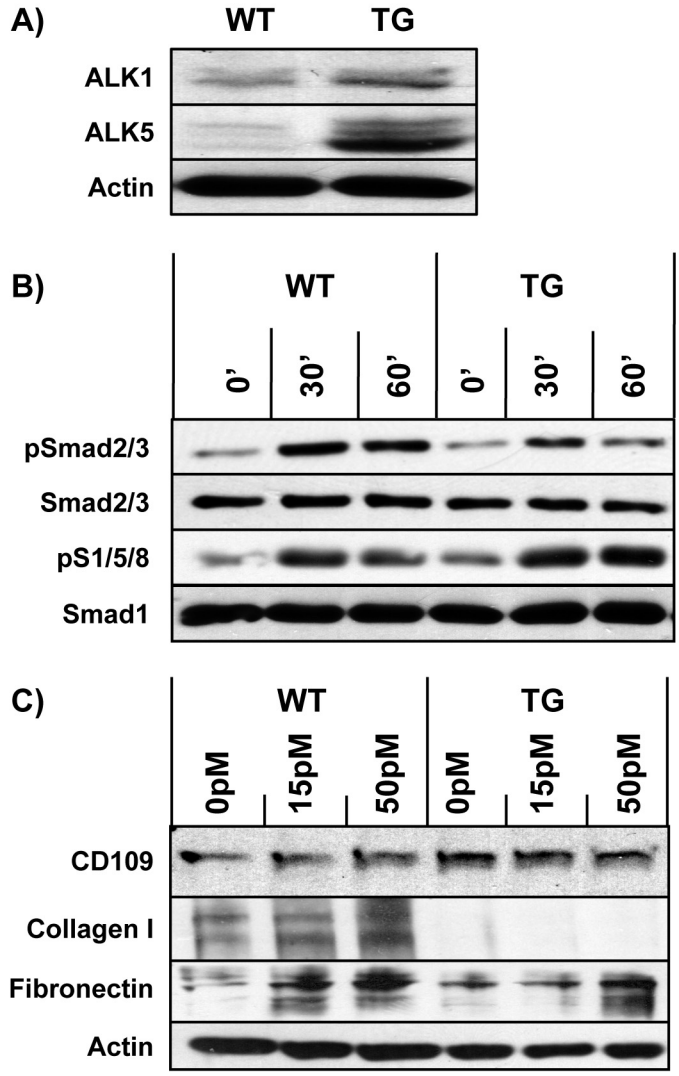
increased ALK1 expression, decreased ALK5 expression, increased phosphosphorylation ofSmad1/5/8, and decreased phosphorylation of Smad2 and Smad3 when compared to wild-type littermates.

Figure 4.3: Overexpression of CD109 in the Epidermis Increases ALK1 Expression, Reduces ALK5 Expression, Increases pSmad1/5/8, Decreases pSmad2/3, and Reduces ECM Protein Expression



Representative immunoblots of mouse skin homogenates demonstrate increased ALK1 *(A)* expression, reduced ALK5 *(A)*, fibronectin *(C)*, and collagen type I *(C)* expression, increased Smad1/5/8 phosphorylation *(B)*, and decreased Smad2/3 phosphorylation *(B)* in CD109 transgenic mice skin compared to skin from wild-type littermate controls. All samples for the same panel were run on the same gel and repeated three times. **See Supplemental Figure 1 (Figure 4.7) for densitometric analysis of immunoblots.*

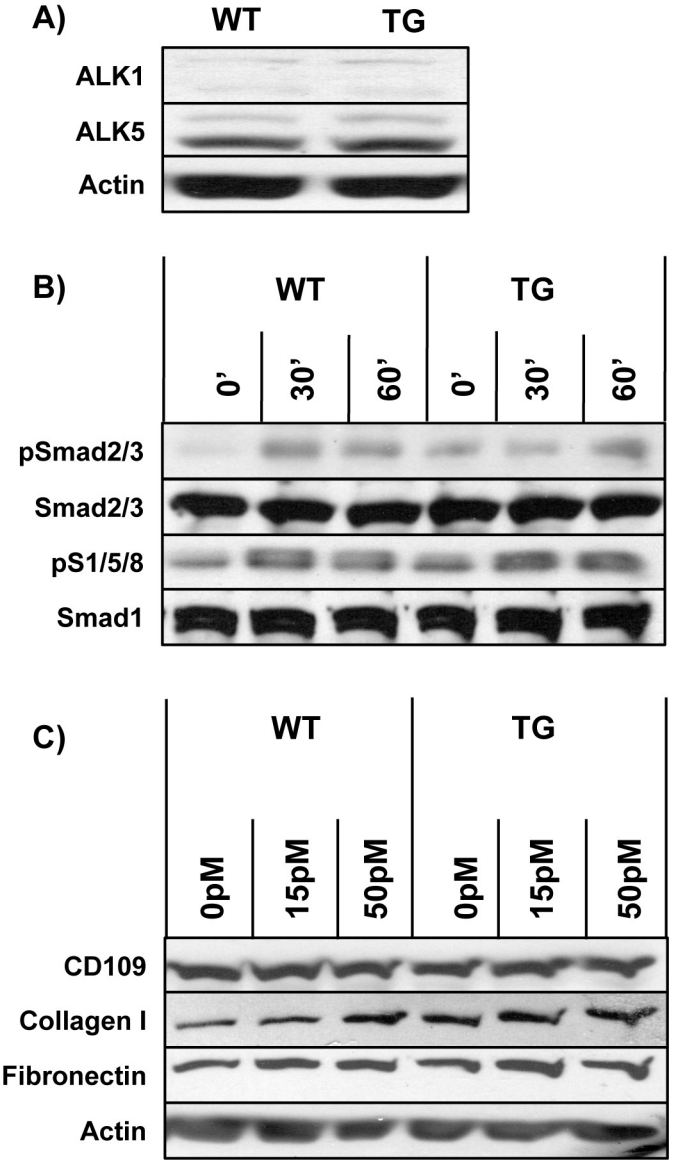
Figure 4.4: Keratinocytes Cultured From Transgenic and Wild-Type Mice Differentially Express ALK5 Compared to Intact Skin



Representative immunoblots of primary mouse keratinocytes show that transgenic keratinocytes express elevated levels of ALK1 and ALK5 *(A)* compared to wild-type controls. Transgenic keratinocytes in response to 15 pM TGF-ß treatment for 0, 30, and 60 minutes show increased Smad1/5/8 phosphorylation and decreased Smad2/3 phosphorylation *(B)* compared to wild-type primary keratinocytes. CD109 transgenic keratinocytes overexpress CD109 independently

of TGF-ß treatment compared to wild-type controls *(C)*, and also display reduced expression of collagen type I and fibronectin in response to increasing doses (0, 15, 50 pM) of TGF-ß treatments for 24 hours. All experiments were repeated a minimum of three times, and all blots from each panel were run on the same gel. **See Supplemental Figure 2 (Figure 4.8) for densitometric analysis of immunoblots.*

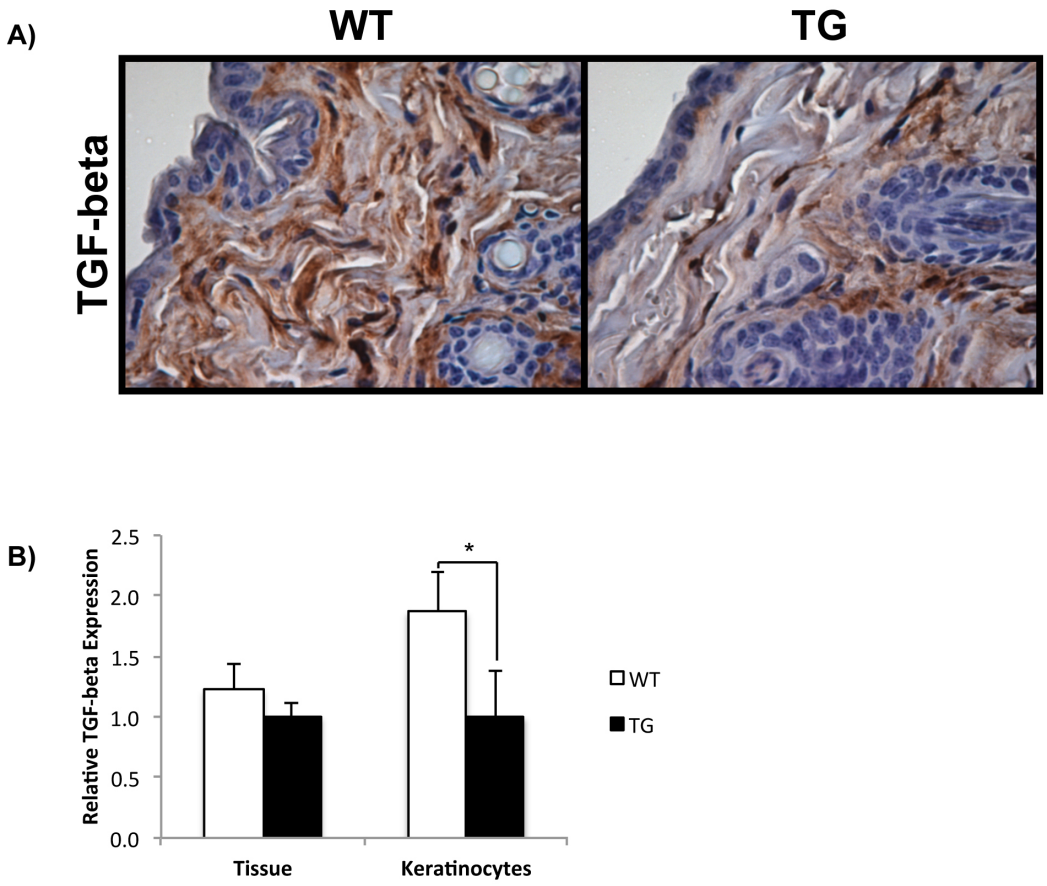
Figure 4.5: Similar Phenotypes Observed in Primary Dermal Fibroblasts Cultured from Wild-Type and CD109 Transgenic Mice



Representative immunoblots of primary mouse fibroblasts show that transgenic and wild-type fibroblasts express similar levels of ALK1 and ALK5 *(A)*. In response to 15 pM TGF-ß treatment for 0, 30, and 60 minutes, similar results were observed for Smad1/5/8 and Smad2/3 phosphorylation between wild-type and CD109 transgenic fibroblasts *(B)*. In response to 24 hour TGF-ß treatment at

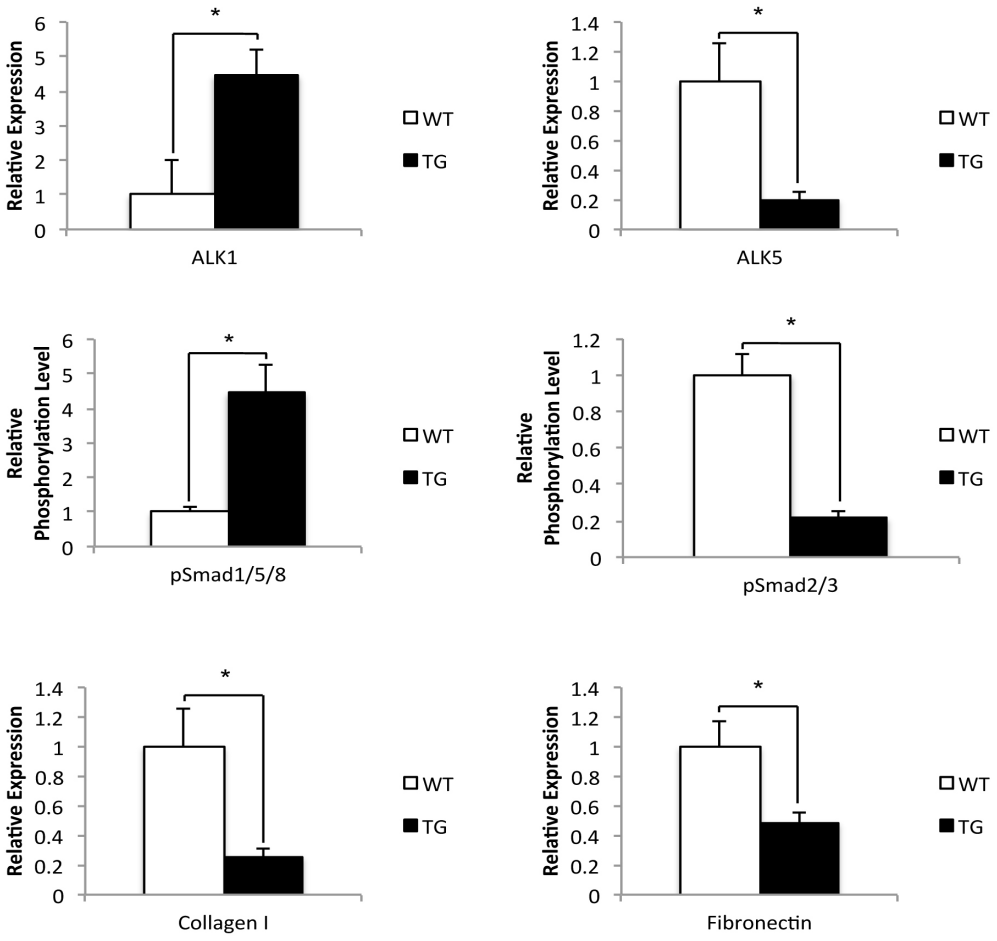
increasing doses (0, 15, 50 pM), no difference in CD109 expression was observed between the wild-type and transgenic fibroblasts *(C)*. A similar but dose-dependent response to TGF-ß was observed for fibronectin expression between wild-type and transgenic fibroblasts. Collagen type I expression was slightly increased in the CD109 transgenic fibroblasts compared to wild-type controls, and both cell lines showed a dose-dependent response to TGF-ß. All experiments were repeated a minimum of three times, and all blots from each panel were run on the same gel. **See Supplemental Figure 3 (Figure 4.9) for densitometric analysis of immunoblots.*

Figure 4.6: TGF-ß Expression is Reduced In CD109 Transgenic Mouse Skin and Cultured Keratinocytes Compared to Wild-Type Littermate Controls



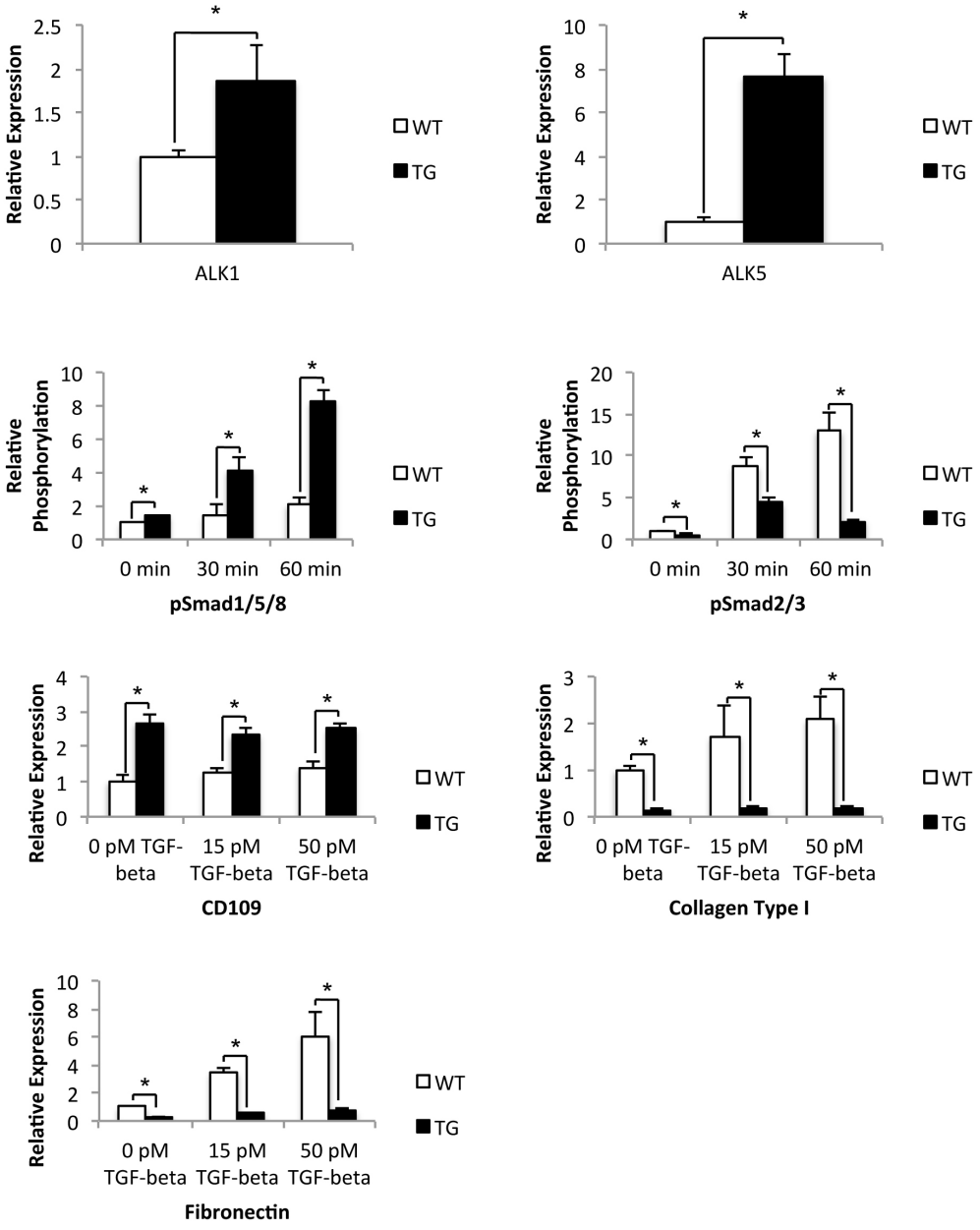
Reduced TGF-ß1 levels are observed in CD109 transgenic mouse skin compared to wild-type littermate controls *(A)*. Quantitative real-time PCR analysis demonstrates reduced TGF-ß1 expression in cultured CD109 transgenic mouse keratinocytes compared to cultured wild-type littermate control keratinocytes *(B)*. *$p<0.05$

Figure 4.7 (Supplemental Figure 1): Densitometric Analysis of *"Overexpression of CD109 in the Epidermis Increases ALK1 Expression, Reduces ALK5 Expression, Increases pSmad1/5/8, Decreases pSmad2/3, and Reduces ECM Protein Expression"*



Densitometric analysis of Figure 3 performed as described in methods demonstrating a statistically significant reduction in ALK1 expression, increase in ALK5 expression, increase in Smad1/5/8 phosphorylation, reduction in Smad2/3 phosphorylation, decrease in Collagen I expression, and decrease in Fibronectin expression in CD109 transgenic mice compared to wild-type littermates.

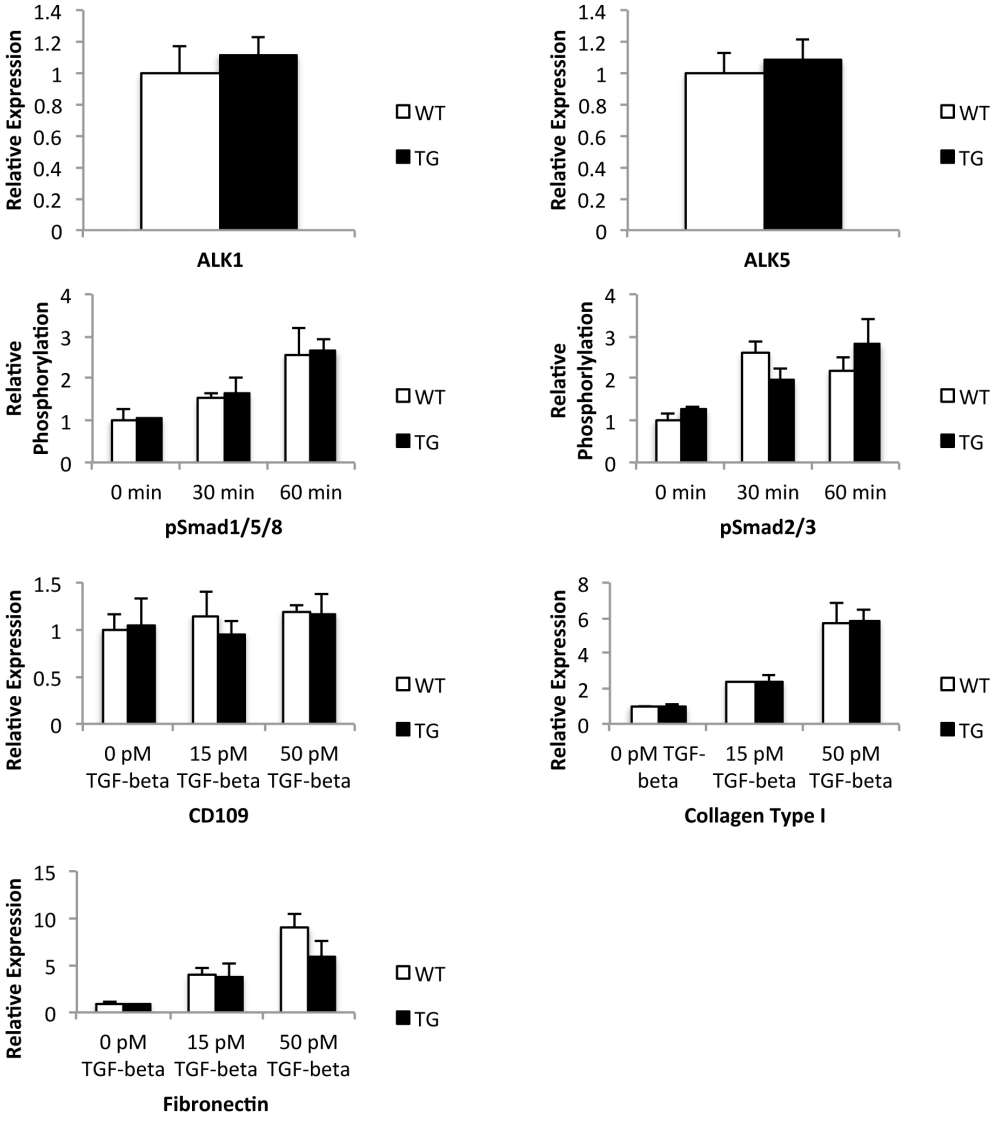
Figure 4.8 (Supplemental Figure 2): Densitometric Analysis of *"Keratinocytes Cultured From Transgenic and Wild-Type Mice Differentially Express ALK5 Compared to Intact Skin"*



Densitometric analysis of Figure 4 as described in methods demonstrating statistically significantly reduced ALK1 and ALK5 expression, increased

Smad1/5/8 phosphorylation, decreased Smad2/3 phosphorylation, increased CD109 expression, decreased Collagen I expression, and decreased Fibronectin expression in keratinocytes cultured from CD109 transgenic mice compared to keratinocytes cultured from wild-type littermate controls.

Figure 4.9 (Supplemental Figure 3): Densitometric Analysis of *"Similar Phenotypes Observed in Primary Dermal Fibroblasts Cultured from Wild-Type and CD109 Transgenic Mice"*



Densitometric analysis of Figure 5 demonstrating that there is no difference in ALK1 expression, ALK5 expression, Smad1/5/8 phosphorylation, Smad2/3 phosphorylation, CD109 expression, Collagen Type I expression, and Fibronectin expression between primary dermal fibroblasts cultured from transgenic mice

overexpressing CD109 in the epidermis compared to dermal fibroblasts cultured from wild-type littermate controls.

Chapter 5: Conclusion & Summary

TGF-ß is a multifunctional growth factor involved in a multitude of cellular and biological processes including cellular proliferation, cellular differentiation, inflammation, wound healing, and fibrosis. Dysregulation of TGF-ß signaling in the skin can result in a variety of different pathologies, such as hypertrophic scarring, keloids, and scleroderma. Our group is interested in how CD109, which has recently identified as a novel TGF-ß co-receptor in our lab, regulates TGF-ß signaling in the skin. Previous studies from our laboratory have demonstrated that CD109 negatively regulates TGF-ß signaling in human keratinocytes *in vitro*, as indicated by reduction in Smad2/3 phosphorylation and decreased extracellular matrix protein expression (Finnson *et al.*, 2006), and that CD109 inhibits TGF-ß signaling by facilitating receptor internalization and degradation (Bizet *et al.*, 2011; Tam *et al.*, 2001).

In this thesis, I tested the hypothesis that overexpression of CD109 in the epidermis could inhibit TGF-ß dependent processes such as fibrosis and inflammation. To better understand this relationship, I developed transgenic mice overexpressing CD109 in the epidermis under the control of the Keratin-14 promoter (K14), which restricts overexpression to basal keratinocytes of the skin. The data presented in this thesis explore the link between CD109 function in the skin and fibrosis, and assess the effect of CD109 overexpression on wound healing and fibrosis *in vivo* using the aforementioned transgenic mice, as well as the contribution of CD109 overexpression on TGF-ß signaling in cultured primary mouse keratinocytes and fibroblasts from K14-CD109 transgenic mice and wild-

type littermates. Using the K14-CD109 transgenic mouse model in wound healing studies and in a bleomycin-induced model of fibrosis, I demonstrate that CD109 improves extracellular matrix architecture in response to profibrotic stimuli, and also reduces inflammation and extracellular matrix protein expression while decreasing TGF-ß signaling through the Smad2/3 pathway. In primary keratinocyte and fibroblast cultures prepared from CD109 transgenic and wild-type littermate mice, I show that CD109 overexpression also alters TGF-ß receptor expression and signaling by increasing the ratio of ALK1 to ALK5 expressed in the skin. Collectively, these data demonstrate that CD109 reduces inflammation and fibrosis *in vivo*, and also modulate TGF-ß signaling by altering receptor expression profiles *in vitro*.

The decision to overexpress CD109 under control of the K14 promoter was made after several careful considerations. The significant developmental defects present in TGF-ß knockout mice, in conjunction with our previous data demonstrating the capacity of CD109 to inhibit TGF-ß signaling *in vitro*, suggested that transgenic mice overexpressing CD109 could exhibit developmental defects. Therefore, I elected to spatially-restrict overexpression of CD109 to the skin. At the time I was developing the CD109 transgenic mice, the K14 promoter had been used in a handful of studies to successfully demonstrate the effects of overexpression of certain genes in the epidermis on wound healing, including thrombospondin-1, TGF-ß, and Smad2 (Chan *et al.*, 2002; Hosokawa *et al.*, 2005; Streit *et al.*, 2000). In a manner similar to these other transgenic mice generated using transgenes under the control of the keratin-14 promoter, the K14-

CD109 transgenic mice have been a helpful tool to investigate the effect of CD109 in the epidermis during wound healing.

Using these K14-CD109 transgenic mice, I demonstrate here that CD109, when overexpressed in the epidermis, significantly inhibits scarring parameters during wound healing and bleomycin-induced skin fibrosis. Specifically, the data in this thesis show that CD109 enhances keratinocyte proliferation without affecting keratinocyte migration, that CD109 inhibits fibroblast differentiation into myofibroblasts, and that CD109 overexpression is associated with decreased granulation tissue area during wound healing. Histomorphometric analysis of Masson Trichrome stained excisional wounds shows increased epidermal thickness at the wound margin, but unchanged epidermal gap measurements in CD109 transgenic mice compared to wild-type littermate controls. TGF-ß signaling has been shown to decrease keratinocyte proliferation while inducing integrin and fibronectin expression, which are required for keratinocyte migration across the wound bed (Amendt *et al.*, 2002; Margadant and Sonnenberg, 2010), and contribute to accelerated re-epithelialization associated with increased TGF-ß signaling (Amendt *et al.*, 2002; Ashcroft *et al.*, 1999; Hosokawa *et al.*, 2005). In this thesis, I show that although TGF-ß signaling through the Smad2/3 pathway is reduced in wounds harvested from CD109 transgenic mice, re-epithelialization is unaffected. Fibrosis is associated with excessive extracellular matrix expression (Armour *et al.*, 2007), which I demonstrate is decreased in CD109 transgenic mice in response to excisional wounding and bleomycin-induced fibrosis as indicated by a reduction in dermal thickness when compared to wild-type

littermate controls. Additionally, TGF-ß signaling also induces myofibroblast differentiation, which is also associated with the progression of fibrosis, and is decreased in CD109 transgenic mice as indicated by fewer of cells expressing α-smooth muscle actin at the wound site. Collectively, the data in this thesis describing reduced Smad2/3 phosphorylation, reduced deposition of extracellular matrix, decreased myofibroblast differentiation, decreased dermal thickness, and the decrease in granulation tissue at the wound site in CD109 transgenic mice is consistent with recent literature highlighting the link between TGF-ß signaling and persistence of granulation tissue (Owens *et al.*, 2010), and suggest that CD109 reduces fibroblast activation and differentiation without compromising re-epithelialization of the wound to improve scarring parameters and reduce bleomycin-induced fibrosis *in vivo*.

Although the data in this thesis demonstrate the significance of CD109 in the inhibition of bleomycin-induced fibrosis and improvement of scarring parameters during wound healing, additional studies could further our understanding of how CD109 regulates these processes. For example, wound healing studies with transgenic or knockout mice engineered to alter CD109 expression in other cell types could provide further information on the role of CD109 in the skin and wound healing. Based on the results presented in this thesis, our lab proposes to study transgenic mice overexpressing CD109 in dermal fibroblasts under the control of the fibroblast-specific Col1a2 promoter, as well as in CD109 knockout mice. We could also explore these genetic modifications under the control of inducible promoters, as constitutively active transgenic or

knockout mice demonstrate many adaptive and compensatory mechanisms, which could obscure the true effect of acutely altering levels of a particular protein in a system such as wound healing.

While this thesis was under review, Mii et al published a paper describing the phenotype of CD109 knockout mice, and showed that these mice displayed hair follicle abnormalities, epidermal hyperplasia, with no effect on wound healing but increased Stat3 signaling (Mii *et al.*, 2012). In light of these findings, it should be noted that the authors only evaluated the rate of wound closure, which we also found to be unaffected during wound healing. Further analysis of other parameters, such as cytokine expression and re-epithelialization are important to establish whether they are significantly affected given the increased Stat3 signaling and the epidermal hyperplasia Mii et al describe.

Moreover, while excisional wounds in mice typically heal within 14 days, scar remodeling occurs for approximately one year after the initial injury, and while bleomycin-induced fibrosis is completely induced after 28 days of injection, further modification of the fibrotic skin continues to occur after the skin becomes fibrotic. Future experiments analyzing wound repair and remodeling of fibrotic skin using the K14-CD109 transgenic mice beyond the time points described in this thesis could provide insight into whether long-term improvements of fibrosis and scarring are associated with overexpression of CD109 in the epidermis. Specifically, our group aims to investigate the extent of extracellular matrix turnover as the wound matures and matrix metalloproteinase activity to assess the role of epidermal CD109 in wound remodeling. While CD109 transgenic mice

have increased proliferation of the epidermis at the wound margin as assessed by epidermal thickness, corroboration of this finding by a proliferation marker, such as Ki-67 or PCNA staining, would reinforce these data. Furthermore, we show that there is less granulation tissue in the CD109 transgenic mouse wounds compared to wild-type littermate controls, however, the mechanism underlying this observation is unclear. It is well documented that granulation tissue resolves via apoptosis, thus subsequent experiments investigating apoptotic events within the granulation tissue could distinguish whether there was less granulation tissue formation in the transgenic mouse wounds or whether it resolved faster.

In addition to reduced scarring parameters during wound healing and bleomycin-induced fibrosis, the data in this thesis demonstrates a reduction in extracellular matrix deposition at the site of wounding and fibrosis, respectively. During wound healing, much of the extracelluar matrix deposition occurs during the remodeling phase, which is characterized by a balance of extracellular matrix deposition and degradation, as well as apoptosis of cells in the healed wound. TGF-ß participates in the remodeling phase by promoting expression of tissue inhibitors of metalloproteinases, inhibition of MMP expression, and sustains fibroblast activation thereby inhibiting apoptosis (Singer and Clark, 1999). We report that in both excisional wounds and bleomycin-induced fibrosis, CD109 transgenic mice display improved extracellular matrix organization, reduced myofibroblasts at the wound, and decreased granulation tissue area compared to wild-type littermate controls. The reduction in α-smooth muscle actin positive myofibroblasts in the wounds of CD109 transgenic mice is consistent with our

observed reduction in Smad2/3 phosphorylation, as the Smad2/3 complex has been shown to directly activate α-smooth muscle actin expression and myofibroblast differentiation (Hinz, 2007). An alternate explanation for the reduction in α-smooth muscle actin positive cells in the CD109 transgenic mice could be due to sustained activation of the myofibroblasts in the wild-type mice (Zhang and Phan, 1999), as there is more TGF-ß available to maintain cellular activation and inhibit apoptosis. However, it should be noted that a recent manuscript published by Tomasek et al shows that α-smooth muscle actin knockout mice still demonstrate myofibroblast differentiation, suggesting that compensatory mechanisms, such as redundancy between α- and γ-smooth muscle actin, exist which could compromise the previously accepted definition of α-smooth muscle actin as a definitive marker of myofibroblasts (Tomasek *et al.*, 2013).

Another important role of the myofibroblast during wound healing is regulation of wound contraction. While we saw no difference in wound closure between the wild-type and CD109 transgenic mice, one of the major differences between murine and human wound healing is the amount of contraction in the mouse excisional wound model due to the presence of the panniculus carnosus muscle in addition to the resident myofibroblasts of the wound. To control for this major difference, many investigators fasten a silicone splint around the excisional wound to diminish the contractile elements of the panniculus carnosus, and restrict the contractile activity to myofibroblasts. Although we compared CD109 transgenic mice to wild-type littermate controls using this splinted

excisional wound healing model, due to variability, presumably resulting from experimental human error, we excluded the data from our study.

In both the wound healing and bleomycin model, we also observe improved extracellular matrix organization by both Masson Trichrome and Picrosirius Red analysis in CD109 transgenic mice. Disorganized ECM is a hallmark of fibrotic skin pathologies including scleroderma and keloids, and is typically associated with a decrease in MMP activity (Goldman, 2004). This observation could be explained by reduced inhibition of MMP activity resulting from CD109 induced TGF-ß antagonism in the CD109 transgenic mouse skin. Thus, CD109 promotes improved extracellular matrix organization under profibrotic conditions, and could be associated with either augmented MMP activity or reduced TGF-ß signaling through the Smad2/3 pathway as suggested by the data presented in the current thesis.

These data provide evidence that overexpression of CD109 in the epidermis inhibits extracellular matrix production, however additional lines of evidence could offer a more comprehensive description of the mechanism underlying this observation. We show that there is a link between CD109 overexpression in the epidermis and reduced ECM production in the skin, *in vivo*, but it is unclear which specific cell types are involved. Recent evidence shows the importance of paracrine signaling between keratinocytes and fibroblasts in the regulation and expression of cytokines and extracellular matrix proteins (Werner *et al.*, 2007). Therefore, overexpression of CD109 in the epidermis may alter keratinocyte intracellular signaling, and consequently the keratinocyte secretory

profile, and thus change the signaling patterns of neighbouring fibroblasts. Additionally, while we report that there is less extracellular matrix produced in CD109 transgenic mice, we did not evaluate ECM catabolism. Our lab is currently performing studies to better understand the role of CD109 in regulating MMP activity, which will help to identify the role of CD109 in ECM turnover.

Through wound healing studies in transgenic mice overexpressing CD109 in the epidermis, I have demonstrated that CD109 results in reduced inflammation as measured by reduced neutrophil and macrophage recruitment to the wound and less proinflammatory cytokine expression during the inflammatory phase of wound healing. There are many cell types that contribute to inflammation during the inflammatory phase of wound healing including platelets, neutrophils, macrophages, and keratinocytes. Immediately following administration of a wound, hemostasis is initiated which involves an accumulation of fibrin and fibronectin at the site of injury, which forms a tight meshwork upon which platelets aggregate. CD109 transgenic mice express lower levels of fibronectin basally, in response to wounding, and in response to bleomycin-induced fibrosis. Fibronectin is involved in initiation of inflammation by facilitating platelet accumulation at the wound site, which precedes platelet degranulation and subsequent release of growth factors to the wound (Nesbitt *et al.*, 2009). Furthermore, fibronectin also promotes monocyte differentiation into macrophages, which also release growth factors to promote inflammation (Fukai *et al.*, 1991). It follows that the decrease in fibronectin expression in the skin of CD109 transgenic mice could participate in the reduction of inflammation by

reducing the amount of growth factors released by platelets and macrophages to minimize the inflammatory response at the wound. The reduced TGF-ß signaling at the wound site in CD109 transgenic mice, as indicated by decreased levels of Smad2/3 phosphorylation, could also explain the reduced inflammatory response. TGF-ß signaling has been linked to sustained inflammation, and induces expression of several proinflammatory cytokines including IL-1, TNF-α, PDGF, and MCP-1 (Owens *et al.*, 2010; Wahl *et al.*, 1987). The data presented here show that TGF-ß signaling, IL-1 expression, MCP-1 expression, and immune cell recruitment are decreased in wounds administered to CD109 transgenic mice when compared to wild-type littermates, suggesting a link between a reduction in TGF-ß signaling, reduced cytokine expression, reduced immune cell recruitment, and overexpression of CD109 during wound healing.

While the data presented in this thesis show that CD109, when overexpressed in the skin, inhibits immune cell recruitment and pro-inflammatory cytokine expression during wound healing, other parameters could be evaluated with respect to the role of CD109 in inflammation, including analysis of other models of fibrosis, assessment of expression profiles of other inflammatory cytokines, and evaluation of intracellular signaling mechanisms. Our lab is currently conducting experiments to investigate the role of CD109 on inflammation in a bleomycin-induced skin fibrosis model, and investigating the mechanism by which CD109 might alter cytokine production/secretion. From our preliminary results, it appears that CD109 has a similar effect on inflammation in

the bleomycin-induced model of fibrosis, and that the NF-κβ signaling pathway might be augmented when CD109 is overexpressed.

TGF-ß is a key regulator of during wound healing, fibrosis, and inflammation, which are altered in transgenic mice overexpressing CD109 in the epidermis. Furthermore, the data presented in this thesis show that the Smad2/3 signaling in CD109 transgenic mice is markedly reduced basally, during wound healing, and in bleomycin-induced fibrosis. Thus, we demonstrate CD109 inhibits TGF-ß signaling *in vivo*. The role of TGF-ß during wound healing and fibrotic skin disorders including scleroderma has been well documented, and this pathway is a potential target of many therapeutic strategies aimed at decreasing fibrosis in the skin. CD109 transgenic mice display inhibition of various TGF-ß dependent processes including inflammation and fibrosis, and these data raise the possibility for several different mechanisms by which CD109 may modulate TGF-ß signaling. I show that TGF-ß expression is decreased both basally and in a bleomycin-induced model of fibrosis, and that Smad2/3 phosphorylation is inhibited basally, during wound healing, and after bleomycin treatment in CD109 transgenic mice. TGF-ß has been shown to induce its own expression in fibroblasts (Flanders *et al.*, 1995), and thus, CD109 overexpression could inhibit TGF-ß signaling by preventing further TGF-ß expression. In conjunction with previous data, demonstrating that CD109 inhibits TGF-ß signaling *in vitro*, this observation could suggest an amplified inhibitory effect of CD109 on TGF-ß signaling *in vivo* (Finnson *et al.*, 2006). Although CD109 transgenic mice demonstrate an inhibitory effect on fibrosis and TGF-ß signaling through the

Smad2/3 pathway observed during wound healing and bleomycin-induced fibrosis, overexpression of CD109 in untreated skin induces an increase in Smad1/5/8 phosphorylation, increased ALK1, and decreased ALK5 expression observed by immunohistochemistry and immunoblot analysis. Elevated ALK1:ALK5 expression is associated with angiogenesis and fibrogenesis (Goumans *et al.*, 2003a; Pannu *et al.*, 2007), which is in contrast to our *in vivo* data in CD109 transgenic mice showing decreased ECM expression in the skin both basally and when challenged with pro-fibrotic stimuli. It is possible that CD109 mediates this effect by augmenting epidermal-dermal signaling, as the observed phenotype in keratinocytes cultured from CD109 transgenic mice shows increased ALK1 and ALK5 expression compared to keratinocytes cultured from wild-type littermate controls. Despite the increased expression of both the ALK1 and ALK5 TGF-ß Type I receptors in the CD109 transgenic keratinocytes, treatment with TGF-ß showed sustained inhibition of Smad2/3 phosphorylation, increased Smad1/5/8 phosphorylation, and reduced collagen I and fibronectin expression when compared to wild-type control keratinocytes. These observations, coupled with our data demonstrating that cultured primary fibroblasts from the CD109 trasngenic and wild-type littermate mice have identical phenotypes, suggest that epidermal-dermal interactions might be involved with the differential expression of ALK1:ALK5 *in vitro* in keratinocytes and *in vivo* in the skin, as the effect of dermal fibroblast signaling is absent in the kerationcyte monocultures used in the experiments presented here. It is also possible that CD109 could modulate TGF-ß receptor signaling preferentially

through the Smad1/5/8 pathway. Endoglin, which like CD109 is classified as a TGF-ß accessory receptor, also promotes ALK1 over ALK5 signaling (Lebrin *et al.*, 2005).

Further experiments investigating keratinocyte-fibroblast interactions in co-culture will provide more insight into the effect of CD109 overexpression in the epidermis in the skin. Our lab is currently conducting co-culture experiments using primary keratinocytes and fibroblasts harvested from CD109 transgenic mice and wild-type littermate controls. We aim to assess the growth factors released by each cell type when grown independently and when cultured together. We hypothesize that the discrepancy between ALK1 and ALK5 expression in keratinocytes and whole skin may be due to paracrine signaling between keratinocytes and fibroblasts and further investigation will offer an explanation for this difference. It would also be interesting to assess the different contributions from Smad1/5/8 and Smad2/3 signaling to the expression of extracellular matrix proteins and profibrotic cytokines and growth factors. Previous studies have suggested that Smad1/5/8 might contribute to fibrosis in scleroderma, thus by selective knockdown of Smad1/5/8 compared to knockdown of Smad2/3, we can assess whether these signaling pathways differentially affect extracellular matrix protein expression in CD109 transgenic mouse skin.

Given the many cell types present in the skin, it is important to consider interactions between different cell types in both the epidermis and dermis, of the skin. Previous studies have shown that keratinocyte-fibroblast interactions *in vitro* affect the expression of various growth factors and cytokines in the skin

including IL-1, IL-6, and KGF (Smola *et al.*, 1993; Waelti *et al.*, 1992). Additionally, activated keratinocytes in scleroderma express additional levels of IL-1 to promote a fibrotic phenotype in the dermis (Aden *et al.*, 2010; Aden *et al.*, 2008). In our model, we have overexpressed CD109 in the epidermis, and observe decreased IL-1 and MCP-1 expression during wound healing along with improved ECM organization. The congruency between our observations in CD109 transgenic mice and the scleroderma model described by Aden et al suggests an important link between IL-1 produced in the epidermis and dermal fibrosis.

Collectively, the data presented herein suggest an important role for CD109, *in vivo,* during wound healing and fibrosis, and that CD109 can modulate TGF-ß signaling by regulating receptor expression and defined signaling pathways. Manipulation of CD109 levels in skin presents an interesting therapeutic target, as it can reduce inflammation and fibrosis, which are hallmarks of scleroderma, keloids, and hypertrophic scars. Further investigation into the mechanism of CD109 action during wound healing and fibrosis, particularly how it affects extracellular matrix remodeling, will provide a more comprehensive understanding of its therapeutic applicability. Moreover, investigation into the effects of CD109 on epidermal-dermal interactions and on ALK1-Smad1/5/8 versus ALK5-Smad2/3 signaling during wound healing could provide further insight into the mechanism by which overexpression of CD109 in the epidermis contributes to a reduction in fibrosis and inflammation.

In summary, I have developed a transgenic mouse model overexpressing CD109 in the epidermis to investigate the role of CD109 in the skin. I have demonstrated that during wound healing, CD109 reduces immune cell recruitment and proinflammatory cytokine production, as well as inhibits fibrosis, myofibroblast differentiation, and extracellular matrix deposition in the dermis while improving extracelluar matrix architecture at the wound site. Furthermore, in a bleomycin-induced model of fibrosis, I have shown that CD109 overexpression reduces extracellular matrix production, improves extracellular matrix architecture, and reduces TGF-ß expression at the site of fibrosis. Additionally, I also show that overexpression of CD109 can modulate TGF-ß receptor expression *in vitro* and *in vivo*, and regulate signaling through the ALK1-Smad1/5/8 pathway over the ALK5-Smad2/3 pathway while reducing ECM expression. Collectively, these *in vivo* data illustrate the capacity of CD109 to inhibit the progression of inflammation and fibrosis, making it an interesting target for therapeutic intervention of fibrotic skin disorders such as scleroderma, keloids, and hypertrophic scars.

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Appendix:

Acceptance of: "Transgenic Mice Overexpressing CD109 in the Epidermis Display Decreased Inflammation and Granulation Tissue and Improved Collagen Architecture During Wound Healing" for publication by *Wound Repair and Regeneration*

Preview

From: wrreditor@woundheal.org

To: anie.philip@mcgill.ca

CC: editorialoffice@woundheal.org

Subject: Acceptance of Manuscript in WRR (WRR-12-01-0025.R2)

Body: Dear Anie,

WRR-12-01-0025.R2

I am pleased to inform you that your manuscript, " Transgenic Mice Overexpressing CD109 in the Epidermis Display Decreased Inflammation and Granulation Tissue and Improved Collagen Architecture During Wound Healing", has been accepted for publication in Wound Repair and Regeneration and will appear in an upcoming issue.

Please make certain that you sign the "Assignment of Copyright" and "Color Charges" forms which are attached. Once you have completed and signed these forms, fax these documents to our editorial office at (+1) 781-338-8491. You may also email them to the editorial office as pdf files. Returning these forms quickly is critical to ensure timely publication. Your manuscript will not enter the production stream until both forms have been received.

Thank you for your contribution to Wound Repair and Regeneration.

Sincerely,

Pat

Patricia A. Hebda, Ph.D.
Editor-in-Chief
Wound Repair and Regeneration

Date Sent: 27-Nov-2012

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