CD109 Deficiency Promotes Skin Fibrosis in a Murine Model

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ABSTRACT

Objectives

Fibrotic disorders like hypertrophic scarring are debilitating pathologies characterized by excessive extracellular matrix (ECM) deposition, resulting in skin thickening and stiffness. Transforming growth factor-beta (TGF-β) plays a critical role in skin homeostasis, and its aberrant signaling is implicated in pathological skin fibrosis. Hence, targeting this pathway represents a promising strategy for disease prevention and treatment. CD109 has been identified as a TGF-β co-receptor that antagonizes its signaling action and inhibits ECM production in vitro. The current study was aimed at investigating the role of CD109 deficiency on murine dermal fibroblasts' function, as well as the skin's response to bleomycin-induced fibrosis in a mouse model.

Hypothesis

In line with earlier studies on CD109 inhibition resulting in increased TGF-β signaling with consequences on cellular functions, we propose that CD109 deficiency leads to unopposed TGF-β activation. As a result, we hypothesize that CD109-deficient mice have increased TGF-β action, leading to enhanced dermal fibroblasts proliferation, migration, and differentiation, as well as increased skin fibrosis in response to bleomycin stimulation. This would translate in significant effects on skin fibrosis and scarring.

Methods

Fibroblasts were isolated and cultured from CD109 knockout (CD109 KO) and wild type (WT) mice, and were treated with or without TGF-β. Migration, proliferation, and contractile properties of the cells were then analysed using an in vitro wound healing (migration) assay, cell count, and a collagen gel contraction assay, respectively.

To study skin fibrosis in vivo, CD109 KO and WT mice received intradermal injections of bleomycin in phosphate-buffered saline (PBS) or PBS alone on alternating days over 28 days. The dermal architecture and collagen structure were examined histologically using hematoxylin and

eosin, Masson's trichrome, and Picrosirius Red staining. The ECM production in skin was investigated by examining the expression of alpha-smooth muscle actin (α-SMA), fibronectin, type 1 collagen, and connective tissue growth factor (CTGF/CCN2) using Western blot and immunohistochemistry. TGF-β downstream signaling was determined by quantification of phospho-Smad1 (pSmad1), phospho-Smad2 (pSmad2) and phospho-Smad3 (pSmad3) levels by immunohistochemistry.

Results

CD109 KO fibroblasts showed enhanced proliferation, migration, and increased collagen gel contraction in vitro, compared to WT fibroblasts. Moreover, in vivo, when compared to their wild type littermates, CD109 KO mice displayed stronger dermal fibrotic responses to bleomycin injections, as evidenced by significantly increased collagen deposition ($p<0.05$), fibronectin ($p<0.05$), and CCN2 ($p<0.01$) as detected by Western blot and immunohistochemistry. α-SMA was also significantly increased in the skin of CD109 KO mice at baseline ($p<0.0005$) and with bleomycin treatments ($p<0.01$). Furthermore, bleomycin-treated CD109 KO mice skin displayed increased levels of pSmad1, pSmad2 and pSmad3 compared to WT skin as detected by immunohistochemistry.

Conclusion

Our results demonstrate that CD109 deficiency promotes fibroblast proliferation, migration and wound contraction in response to TGF-β in vitro. In addition, our findings indicate that CD109 deficiency in vivo leads to increased TGF-β signaling pathway activation and increased production of ECM proteins in the skin in a mouse model of bleomycin-induced skin fibrosis. Understanding of the mechanisms by which CD109 regulates TGF-β signaling may lead to therapeutic strategies targeting the TGF-β pathway to reduce or reverse skin fibrosis and scarring.

RÉSUMÉ

Objectifs

Caractérisées par une accumulation excessive de la matrice extracellulaire, les pathologies cutanées liées à la fibrose tissulaire entraînent des conséquences fonctionnelles débilitantes. Le facteur de croissance transformant-β (TGF-β) joue un rôle essentiel dans l'homéostasie de la peau. Sa signalisation aberrante est impliquée dans la pathogénèse des cicatrices hypertrophiques et kéloïdiennes. Ainsi, cibler la signalisation du TGF-β représente une stratégie prometteuse pour la prévention, voire le traitement de ces maladies. Le CD109, un co-récepteur du TGF-β, est un inhibiteur efficace de sa signalisation et de la production de la matrice extracellulaire in vitro. L'étude présente vise à investiguer l'impact de la déficience de CD109 sur la fonction in vitro des fibroblastes *in vitro*, ainsi que la fibrose cutanée induite par les injections de bléomycine chez une souris déficiente en CD109.

Hypothèse

Nous proposons que l'absence du CD109 entraîne un effet d'activation du TGF-β accru. Donc, les souris déficientes en CD109 pourraient démontrer une augmentation de la prolifération, migration et la différenciation des fibroblastes, ainsi qu'une réponse fibrotique exagérée induite par l'administration de bléomycine.

Méthodes

Les fibroblastes des souris CD109 knock-out (CD109 KO) et des contrôles sont isolés, cultivés, et traités avec ou sans TGF-β. La migration, prolifération, et contraction cellulaire sont analysées par le biais d'un essai de migration, un décompte cellulaire, et d'un essai de contraction du collagène in vitro.

Afin d'étudier la fibrose cutanée in vivo, les souris CD109 KO et les contrôles reçoivent des injections de solution de bléomycine ou salines sous-cutanées en alternance à chaque jour pendant 28 jours. La structure dermique est examinée par coloration à l'hématoxyline et à l'éosine, le trichrome de Masson, ainsi que le rouge Picrosirius. L'expression de l'actine alpha 2,

fibronectine, le collagène de type 1, le facteur de croissance du tissu conjonctif (CCN2) est analysée par immunobuvardage de type Western et immunohistochimie. La signalisation du TGF-β est déterminée par la quantification de l'activation des protéines Smad, soient phospho-Smad1, phospho-Smad2, et phospho-Smad3 par immunohistochimie.

Résultats

Comparés aux contrôles, les fibroblastes CD109 KO démontrent une vitesse de prolifération et migration accrue et une augmentation de la contraction du gel de collagène.

De plus, in vivo, la fibrose cutanée est plus prononcée chez les souris CD109 KO injectées avec bléomycine. Ceci est supporté par l'augmentation de la détection du collagène de type 1 ($p<0.05$), de la fibronectine ($p<0.05$) et de CCN2 ($p<0.01$) à l'immunobuvardage de western et à l'immunohistochimie. L'expression de l'α-SMA est également augmentée de façon significative chez les souris CD109 KO traitées avec des injections de salin ($p<0.0005$) ou de bléomycine ($p<0.01$). De surcroît, un niveau élevé de pSmad1, pSmad2 et pSmad3 est détecté à l'immunohistochimie chez les souris CD109 KO ayant reçu des injections de bléomycine.

Conclusion

Nos résultats démontrent que la déficience en CD109 favorise la prolifération, la migration et la contraction des fibroblastes en réponse au TGF-β in vitro. De surcroît, les résultats in vivo indiquent que la déficience en CD109 résulte en l'augmentation de la signalisation par le TGF-β, et une production accrue des protéines de la matrice extracellulaire dans la peau d'un modèle de souris de fibrose cutanée induite par la bléomycine. L'étude des mécanismes par lesquels CD109 interagit avec la signalisation du TGF-β pourrait contribuer au développement de stratégies thérapeutiques ciblant le TGF-β afin de réduire ou de reverser les états pathologiques de fibrose cutanée.

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PREFACE

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1) Postgraduate Experimental Surgery Research Day, Department of Surgery, McGill University, Montreal, Quebec, Canada, October 2014.

2) Skin Research Group Annual Meeting, Quebec, Quebec, Canada, May 2015.

3) Postgraduate Experimental Surgery Research Day, Department of Surgery, McGill University, Montreal, Quebec, Canada, January 2016.

4) Association des Spécialistes en Chirurgie Plastique et Esthétique du Québec, Annual Meeting, Montebello, Quebec, Canada, February 2016.

5) International Wound Healing Society Annual Meeting, Atlanta, Georgia, USA, April 2016.

6) Fraser N. Gurd Day, Department of Surgery, McGill University, Montreal, Quebec, Canada, June 2016.

7) Canadian Association of Plastic Surgery Annual Meeting, Ottawa, Ontario, Canada, June 2016.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

This thesis has been written in accordance with the McGill University Faculty of Graduate and Postdoctoral Studies guidelines. I attest that this thesis entitled "CD109 Deficiency Promotes Skin Fibrosis in a Murine Model" has been written by me, Liqin Xu.

This thesis is submitted in the manuscript-based format, consisting of three referenced chapters including: 1) General introduction; 2) CD109 Deficiency Promotes Skin Fibrosis in a Murine Model (manuscript); 3) General Discussion and Conclusion.

I, Liqin Xu, have designed the experiments, collected and analyzed the data, and created the framework of my study in context of currently available literature unless otherwise stated. My contributions to original knowledge in this thesis are as follows:

1) I have expanded a CD109 wild type and knockout mouse colony from CD109 heterozygotic parents
2) I have genotyped the offspring from the CD109 mouse colony
3) I have harvested and cultured skin fibroblasts from CD109 wild type and knockout mice
4) I have shown that CD109 KO mouse fibroblasts have increased collagen type I, connective tissue growth factor, fibronectin expression in response to TGF-β stimulation
5) I have shown that CD109 KO results in increased mouse fibroblasts migration, proliferation, and wound contraction *in vitro* via cell count, the scratch assay and collagen matrix contraction assay
6) I have shown that CD109 KO mice display stronger cutaneous fibrotic response to intradermal bleomycin injections compared to their WT littermates in a bleomycin-induced skin fibrosis model
7) I have shown that CD109 KO increases Smad1, Smad2, and Smad 3 phosphorylation in a murine bleomycin-induced skin fibrosis model.

All the data presented herein are the work of Liqin Xu except for:

1) Immunohistochemistry to determine the levels of markers of fibrosis (α-SMA, CTGF, collagen type I, and fibronectin) and TGF-β downstream signaling (pSmad1, pSmad2, and pSmad3) are conducted by Meryem Blati in Dr. Anie Philip's lab.

LIST OF ABBREVIATIONS

ALK 1	Activin-like receptor 1
ALK 5	Activin-like receptor 5
α-SMA	Alpha-smooth muscle actin
CTGF/ CCN2	Connective tissue growth factor
DMEM	Dulbeco's Modified Eagle Medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
GPI	Glycosylphosphatidylinositol
IL-1	Interleukin-1
IL-4	Interleukin-4
IL-13	Interleukin-13
KO	Knockout
LAP	Latency-associated peptide
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
pSmad2/3	Phospho-Smad2/3
pSmad1/5/8	Phospho-Smad1/5/8
TGF-β	Transforming growth factor-beta

TGF-β-RI	Transforming growth factor-beta receptor I
TGF-β-RII	Transforming growth factor-beta receptor II
TGF-β-RIII	Transforming growth factor-beta receptor III
TNF-α	Tumor necrosis factor-alpha
WT	Wild type

GENERAL INTRODUCTION

Skin Anatomy

The skin is the largest organ in the human body. Composed of two distinct layers, it protects the organism by acting as a physical barrier to the external environment. The epidermis, the outermost layer, consists of the stratum corneum, stratum granulosum, stratum spinosum, and the stratum basale. Keratinocytes, pigmented melanocytes, antigen-presenting Langerhans cells, and sensory Merkel cells are found within the epidermis. In addition to providing protection against physical trauma, the stratum corneum, a superficial layer of keratinized epithelium devoid of nuclei, limits water loss, and shields against micro-organismal invasion.[1,2] The dermis, distinguished by its superficial papillary layer and the deep reticular layer, contains blood vessels, lymphatics, and is populated by fibroblasts, mast cells, histiocytes, and immune cells. Collagen, its main extracellular component, provides the skin with its robust strength, while elastin, present in lower quantity, accounts for its elasticity.[3] Growing evidence demonstrates that papillary and reticular fibroblasts differ both in structure and function. While resident fibroblasts in the papillary dermis show increased gene expression of complement pathway components, indicating an important role in immune response, the more differentiated reticular myofibroblasts partake in structural arrangement.[4,5] The subcutaneous tissue, or hypodermis, as its name implies, lies immediately deep to the dermis. Its main constituent, fat lobules, plays a major role in thermoregulation. Moreover, hair follicles, nerve endings, sebaceous and sweat glands are found within this structure.

Normal Wound Healing

Wound healing is an intricate balance between tissue turnover and deposition. Homeostasis is maintained when the production rate of new skin cells and appendages matches that of cellular loss from regular turnover or trauma. The body's response to injury can be divided into three distinctive stages of healing: the inflammatory, proliferative, and remodeling phases. The first step, starting from the time of trauma to the following 3 to 5 days, is characterized by transient vasoconstriction, the formation of a hemostatic platelet plug, as well as vasodilation for inflammatory cell infiltration and activation.[6] Following tissue damage and vessel disruption, platelets aggregate to the exposed dermal elements and vessel intima to

minimize blood loss; their degranulation results in the release of platelet-derived growth factors (PDGF), platelet factor IV, and TGF-β.[7] The coagulation cascade ends with the formation of a fibrin meshwork, a temporary platform for the attachment of subsequently recruited inflammatory cells. Neutrophils are the first responders to chemoattractants such as bacterial wall lipopolysaccharides, TGF-β, or tumor necrosis factor-α (TNF-α) released at the site of injury.[8] They actively participate in the debridement of devitalized tissue and the elimination of infectious organisms. Polymorphonuclear cells, mast cells and monocytes also invade the open wound and play an immunological role in the clearance of debris and contamination. Monocytes, in particular, transform into macrophages within 24 to 48 hours and participate in wound debridement as well as the secretion of numerous growth factors.[6] Under normal circumstances, the inflammatory response is rapid and transient; prolonged excessive inflammation, however, is detrimental to wound healing as it opens a potential pathway to aberrant scarring.[2] Recent data suggest that unopposed overactive M2 macrophage subtype contributes to the development of hypertrophic scars.[9]

Overlapping with the inflammatory phase, the migration of perivascular fibroblasts into the wound site around day four post injury marks the beginning of the proliferative phase. Wound re-epithelialization heavily relies on the translocation of epithelial cells from the wound edges and intact dermal appendages, such as hair follicles and sweat glands.[6] Secreted into the environment by platelets in response to cellular and vascular damage, TGF-β plays a key role in intercellular signaling by promoting inflammatory cell chemotaxis, and by inducing the production of glycosaminoglycans, hyaluronic acid, and other extracellular matrix (ECM) components by thus recruited and activated fibroblasts.[10] Collagen type III fibrils, synthesized rapidly in abundance by activated fibroblasts onto this amorphous gel-like ground substance, accumulate over the next 2-3 weeks; its replacement by type I collagen, a more robust structural protein, accounts for the increasing wound tensile strength. Fibroblasts are key players during this stage, as their synthesis activity allows for rapid and significant wound contraction. Their numbers begin to decrease towards the end of the proliferative stage, which persists for approximately 3 weeks. Simultaneously, vascular proliferation and ingrowth into the wounded area is mediated by an amalgamation of growth factors including vascular endothelial growth factor, fibroblast growth factor, etc. secreted by platelets and macrophages.[2] Owing to its newly

formed rich capillary network, the proliferative scar appears elevated, erythematous and indurated.

The remodeling phase, the final phase in wound healing, spans from week three until one to two years post trauma. In this step, collagen deposition and degradation reach a balanced state, with no net change in the total number of fibrils.[1] Collagen fiber architecture in a final scar differs from its unwounded counterpart by a dense, packed appearance, as opposed to the native reticular dermal pattern.[2] The scar strength promptly increases over the first 8 weeks following injury, and plateaus at a maximum of approximately 80% of the skin's original tensile strength. Moreover, during this stage, the scar's true pigmentation is revealed as a major part of the temporary vascular network regresses. Hence, the remodeling and mature scars are distinguished from their surrounding uninjured skin by their color, the lack of epidermal appendages, decreased elasticity, as well as a decreased tensile strength.[1,2]

Fibroproliferative Skin Disorders

In states of aberrant wound healing, as seen in systemic sclerosis, hypertrophic scarring, and keloid formation, fibroproliferative skin disorders invariably produce a phenotype of skin thickening, ECM deposition, and loss of elasticity at a focal or wide-spread level.

Hypertrophic scars are erythematous, raised, and confined within their original borders. They can form following deep dermal injury, severe burns or trauma. The exact mechanisms by which these scars are formed are not fully elucidated, but growing evidence suggests that factors such as a prolonged or exaggerated inflammatory phase of wound healing and excessive wound tension may contribute to their development. In inflammatory states, the presence of abundant growth factors such as TGF-β results in fibroblasts activation and exaggerated ECM production.[9,11,12] In addition, the prolonged presence of inflammatory lymphocytes in healing wounds may contribute to aberrant scarring through the autocrine and paracrine secretion of cytokines. Macrophages, lymphocytes, fibroblasts, and endothelial cells all produce the pro-inflammatory peptides interleukin-1 (IL-1) and interleukin-8, which induce chemotaxis and activation of polymorphonuclear cells, fibroblasts, and keratinocytes. Activated keratinocytes in turn stimulate fibroblasts action via IL-1 secretion.[13] Moreover, T helper cells 2, secrete

interleukin-4 (IL-4), interleukin-13 (IL-13), and TGF-β, which activate fibroblasts' ECM proteins synthesis action.[14] When a hypertrophic scar matures and its proliferation finally halts, resident fibroblasts' number is reduced as they undergo apoptosis, although they remain more abundant than normal.[15]

Keloids resemble hypertrophic scars in that they are also elevated and pigmented. However, they phenotypically differ in their extension beyond the original wound edges. Keloids and hypertrophic scars share many similarities at the molecular level. TGF-β is expressed in abundance in keloids, and auto-induces fibroblasts-mediated fibronectin and collagen production.[16]

Patients afflicted with systemic sclerosis have thickened, leather-like skin due to ECM accumulation. Sustained inflammation and autoimmunity resulting in skin fibrosis appear to contribute to its etiology. As in hypertrophic scars, T helper lymphocytes have increased IL-4, IL-13, and TGF-β production, and keratinocytes IL-1 overexpression.[17,18]

TGF-β Signaling and Regulation

TGF-β is synthesized as an inactive precursor. Once cleaved by furin, the mature TGF-β and its latency associated peptide (LAP) remain bound and secreted together as a small latency complex. This complex can then covalently bind to the latent TGF-β binding protein, forming a large latent complex, facilitating its secretion and conferring stability to the TGF-β-LAP complex. TGF-β is inactive in this bound state. The secreted large latent complex interacts with ECM proteins, which stabilizes the dormant TGF-β in the extracellular environment. Upon signaling activation, the large latent complex is released from the ECM, and proteolytic cleavage of the latent associated peptide releases the TGF-β ligand, enabling its binding to cell receptors. TGF-β signal transduction occurs via a pair of transmembrane serine-threonine kinase receptors, type I and II TGF-β receptors (TGF-β-RI, TGF-β-RII), that complexes into a heterodimer.[19]

Upon ligand binding, TGF-β-RII first forms a homodimer. This is followed by recruitment of the TGF-β-RI homodimer to complex into a tetramer. Once in proximity, the TGF-β-RII intracellular serine-threonine kinase phosphorylates TGF-β-RI, allowing for downstream signaling activation. The canonical pathway involves phosphorylation of

intracellular receptor Smad proteins by the activated TGF-βR-I into phospho-Smad2/3 (pSmad2/3), or phospho-Smad1/5/8 (pSmad1/5/8), which in turns pairs with Smad4, prior to co-translocating into the nucleus to regulate gene transcription.[19]

Intracellular TGF-β signaling is negatively regulated by proteins that block downstream Smad phosphorylation or that affect TGF-β receptor internalization. Smad7, a TGF-β signaling inhibitor, halts signal transduction in two ways: it blocks the interaction between activated TGF-β receptor and receptor Smads, and serves as a docking protein for Smurf1/2, a ubiquitin ligase, thereby targeting the activated receptor for degradation.[20]

TGF-β Co-Receptors

Betaglycan, endoglin, and CD109 are accessory receptors in TGF-β signaling. Although they lack intrinsic signaling or enzymatic activity, they interact with TGF-β receptors and modulate their activities.[21]

Betaglycan, or type III TGF-β receptor (TGF-βR-III), is a third TGF-β transmembrane receptor identified as a TGF-β-RI and TGF-β-RII co-receptor.[22] Betaglycan plays a dual role in the modulation of TGF-β signaling.[23] As an promoter of TGF-β signaling activation, betaglycan presents the ligand to the TGF-β receptor, increases the affinity of TGF-β binding to TGF-β-RI and TGF-β-RII, and enhances their response.[24,25] Moreover, the presence of betaglycan increases TGF-β receptors' responsiveness different isoforms of TGF-β ligand, eliminating the difference in their efficacy.[26] When the membrane-anchored protein undergoes proteolytic cleavage, it produces a soluble secreted ectodomain that can enhance or inhibit TGF-β signaling depending on the concentration of TGF-β.[27,28] Most often, soluble betaglycan acts as an antagonist to TGF-β signaling through ligand sequestration.[29]

As a TGF-β-RII co-receptor, endoglin interacts with TGF-β1 and TGF-β3.[30,31] Expressed primarily in proliferating endothelial cells, endoglin can also be found in immune cells, hematopoietic cells, chondrocytes, skin fibroblasts, and plays a role in wound healing through both hypoxia and TGF-β pathways.[32-35] Soluble endoglin binds to circulating TGFβ1, thus preventing ligand interaction at the membrane level.[36]

CD109

Prior to the identification of CD109, several groups have reported the existence of a cell surface glycosylphosphatidylinositol (GPI)-anchored protein that interacted with TGF-β, including its presence on megakaryotes.[37-39] Although its function was not yet defined, CD109 was cloned as a novel member of the α2-macroglobulin/complement gene family.[40,41] This GPI-anchored protein is expressed at various levels in different structures in the human body: brain, skin, testis, prostate, as well as salivary, mammary, and lacrimal glands under normal conditions.[39,42-44] Our laboratory was the first to report that CD109 is a TGF-β co-receptor and a potent TGF-β antagonist.[45,46] Previous work from our group has also shown that CD109 is released from the cell surface and can exist in a soluble form.[47] Present both in the membrane-bound and the secreted forms, there is evidence to support its roles in enhancing TGF-β receptor degradation, sequestering TGF-β ligand, and inhibiting its binding to TGF-β receptors.[45,48] Recent studies demonstrate that an elevated CD109 level is implicated in enhanced wound healing, decreased inflammation, and improved collagen structure in skin.[49,50] In human fibroblasts, CD109 decreases TGF-β signaling and promotes TGF-β receptor degradation in vitro.[45] In animal studies, our group has demonstrated that in contrast to WT mice, transgenic mice overexpressing epidermal CD109 demonstrate decreased inflammatory cells infiltration, less granulation tissue formation, and improved collagen fibers organization during the healing of incisional wounds, without negatively affecting the rate of wound closure.[50] In addition, the skin's fibrotic response to bleomycin injections is attenuated in these CD109 transgenic mice as documented by their resistance to epidermal thickening, improved collagen architecture, decreased ECM proteins synthesis, decreased TGF-β1 expression, and increased ALK1-Smad1/5/8 signaling, but decreased ALK5-Smad2/3 TGF-β downstream signaling compared to WT mice.[49,51] Furthermore, CD109 transgenic mice keratinocytes show decreased TGF-β1 expression. In response to exogenous TGF-β stimulation, transgenic mice keratinocytes showed decreased collagen type I compared to WT keratinocytes, while there was no difference in collagen I and fibronectin levels of expression between transgenic and WT fibroblasts.[51]

Molecular Mediators of Fibrosis

Released by macrophages, platelets, fibroblasts and keratinocytes in response to injury and during tissue repair, TGF-β is a 25 kDa heterodimer that plays central roles in wound healing, inflammation, and ECM synthesis. TGF-β is abundantly expressed in fibrotic skin, and acts as the most profibrotic stimulus to fibroblasts where it favors fibronectin and collagen production.[52-56]

Found in skeletal tissues, skin, and endothelium, CCN2, a matricellular protein, plays important roles in the development of epithelial tissues, and is involved in wound healing, including cell adhesion, migration, and proliferation.[57,58] Identified as a hallmark of tissue fibrosis, CCN2 overexpression and activation can act independently or mediate TGF-β-induced fibroblasts proliferation and sustained skin fibrosis.[59-61] In the skin, it is present in the epidermis and dermis, and its overproduction is shown to promote collagen synthesis and deposition in keloid scars and systemic sclerosis.[57,61]

PDGF, released during platelet degranulation in sites of injury, mediates inflammatory cells chemotaxis and stimulation of cells involved in wound repair. Under basal condition, PDGF receptors are expressed in low levels on fibroblasts and smooth muscles. In the setting of acute injury and inflammation, however, these receptors are upregulated, promoting tissue deposition and wound contraction.[62] Dysregulated PDGF signaling is well documented in atherosclerosis and organ fibrosis. In systemic sclerosis, PDGF and its receptor are upregulated in skin and lung tissues in a TGF-β and IL-1-dependent autocrine fashion.[63]

Objectives of Current Study

The main objectives of this thesis are: 1) to study the effect of CD109 deficiency on wound healing *in vitro*; 2) to elucidate the role of CD109 in murine bleomycin-induced skin fibrosis; 3) to verify the effect of CD109 on TGF-β signaling.

MANUSCRIPT:

CD109 Deficiency Promotes Skin Fibrosis in a Murine Model

BACKGROUND

Excessive skin fibrosis, manifested locally by the development of hypertrophic scars following deep dermal injury, or by widespread disease in patients afflicted by systemic sclerosis, is a debilitating pathology with limited treatment options.[12]

Dysregulated TGF-β signaling is proven to play a substantial role in fibrotic skin diseases.[11,52] Its aberrant activation and receptor upregulation result in over-production of ECM proteins such as collagen I and fibronectin.[10] Additionally, ECM degradation is slowed through upregulated expression of protease inhibitors and decreased matrix metalloproteinases. The net effect is an imbalance between ECM production and turnover.

The transforming growth factor beta (TGF-β) canonical signaling pathway through the activin-receptor-like kinase 5 (ALK5) is mediated by a pair of transmembrane serine-threonine kinase receptors, where type I receptor is trans-phosphorylated upon ligand binding to type II receptor. ALK5 activation favours signal transduction via phosphorylation of downstream intracellular Smad2 and Smad3, which complexes with Smad4 upon activation, and co-translocate into the nucleus to regulate the transcription of target genes.[64,65] An alternate TGF-β signaling pathway, mediated by ALK1 activation, preferentially signals through Smad1, Smad5, and Smad8 phosphorylation and nuclear co-translocation with Smad4. While the ALK5/Smad2/3 pathway activation's role is well documented in fibrotic skin phenotypes, ALK1/Smad1/5/8 signaling has also been implicated in skin fibrosis in scleroderma patients.[66-68]

Our group has previously shown CD109, a TGF-β co-receptor, to be a potent TGF-β antagonist.[45,46] In human fibroblasts, CD109 decreases TGF-β signaling and promotes TGF-β receptor degradation in vitro.[45] Furthermore, transgenic mice overexpressing epidermal CD109 demonstrate decreased fibrotic response to bleomycin injections.[49,50] The present investigation aims to validate the anti-fibrotic role of CD109 by studying CD109-deficient mice.

MATERIALS AND METHODS

Generation of Mouse Colony

Frozen CD109 heterozygotic C57BL/6 mouse embryos were obtained from the group of Mii et al. who generated the mice, and did the initial characterization.[42] The colony was expanded using CD109 heterozygote male and female mice. Sixteen wild type (WT) and 16 knockout (CD109 KO) mice were identified by genotyping and selected from the third-generation offspring for the experiment. Mice were selected based on genotype (WT or KO), age (4-6 weeks), and gender (male) for the in vivo experiments.

Genotyping of CD109 Mice

Mouse tail samples obtained at the time of weaning were prepared for genomic DNA extraction using the REDExtract-N-Amp^{TM} Tissue PCR Kit (Sigma). 100 µl of extraction buffer and 25µl of preparation buffer were added to each tail sample. The mixture was then vortexed and incubated for 12 minutes at room temperature, followed by a four-minute incubation at 95-100^{o}C. The reaction was halted by adding 100 µl of neutralization buffer and the solution is stored at -20^{o}C.

Polymerase chain reaction (PCR) of genomic DNA used the WT forward and reverse primers 5'-GTCCCGCTTTCTGGTGACAG-3' and 5'- GTGTGACTGTTAGACAGTGCAG-3', and the CD109 KO forward and reverse primers 5'-CCATCGCCATCTGCTGCACG-3' and 5'-ACGATCCTGAGACTTCCACAC-3' respectively. The reaction was initiated by adding 10 µl of Taq polymerase (REDExtract-N-Amp^{TM} Tissue PCR Kit, Sigma) to 2 µl of the genomic DNA mixture, for a total of 32 two-minute cycles at 96^{o}C, followed by 4^{o}C until the samples were resolved on a 3% agarose gel. The expected size for the WT allele was 205 base pairs, and 603 base pairs for the CD109 KO allele; if both bands were present, the mouse is a CD109 heterozygote.[42]

In Vivo Bleomycin Treatment

Sixteen WT and 16 CD109 KO littermates were assigned to either phosphate buffered saline (PBS) or bleomycin in PBS treatment group (Fig. 1). Under isofluorane anesthesia, the

dorsal skin was shaved and depilated using Nair (Church & Dwight). A 1 x 1 cm injection site was marked on the shaved back with a non-toxic permanent marker (Sharpie, Sanford Manufacturing Company). Mice were either treated on alternate days with 100 µl subcutaneous single-site injection of bleomycin sulfate (15 µg, Wisent Bioproducts) dissolved in PBS or 100 µl of PBS alone, for a total of 28 days. On day 29, mice were euthanized by CO_2 asphyxiation and cervical dislocation. The marked treatment area was harvested, bisected, and either snap-frozen in liquid nitrogen or embedded overnight in 10% formalin (Fisher Scientific) for histological analysis.

Histology and Immunohistochemistry

Paraffinized formalin-fixed samples were cut into 7 µm sections for Hematoxylin and Eosin, Masson's trichrome, or Picrosirius Red staining. Dermal thickness was measured from the basement membrane to the hypodermis in five high power fields per section, in two different sections per animal, and then analyzed using ImageProPlus6 Software (MediaCybernetics, Bethesda, MD).

Immunohistochemistry was performed at 4^{o}C overnight using specific antibodies to assesses the level of proteins markers of fibrosis α-smooth muscle actin (α-SMA), type I collagen, connective tissue growth factor (CCN2), or fibronectin against negative controls IgG. This was followed by incubation with biotinylated link and streptavidin-horseradish peroxidase. The expression of activated TGF-β downstream signaling proteins phospho-Smad 1 (pSmad1), phospho-Smad 2 (pSmad2), and phospho-Smad 3 (pSmad3) was detected similarly. Quantification was performed by measuring the calibrated images' optical density using Fiji ImageJ.

Western Blot and Densitometry

Full thickness skin tissue (0.5 x 1cm) was minced and homogenized in RIPA buffer, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonylfluoride (PMSF), and ethylenediaminetetraacetate (EDTA)-free protease inhibitors (Roche, Mississauga, ON, Canada) for protein extraction. Tissue homogenate was clarified at 12,000 x g for 10 minutes. The

supernatant was collected for protein quantification using the Bio-Rad Lowry Protein Assay (Bio-Rad, Hercules, CA). Samples were resolved by 7.5% or 10% SDS-PAGE under reducing and denaturing conditions. Measurement of markers of fibrosis (α-SMA, fibronectin, CTGF, collagen I) was performed using the primary and secondary antibodies as listed. Each experiment was performed at least in quadruplicates, and densitometry of immunoblots was measured using ImageJ software (NIH). The Students T-test is used for the calculation of statistical significance. At least three independent experiments were used.

Isolation of Dermal Fibroblasts

CD109 KO mice and WT littermates aged between 0 to 5 days of life were euthanized by decapitation. The newborn mouse's entire body was then cleaned using 10% Poviodine-Iodine solution (Laboratoire Atlas Inc., Montreal, Quebec) and rinsed in sterile water. Under the sterile hood, the pup's skin was harvested in entirety, minced, placed into a 50 ml conical flask containing 20 ml of supplemented medium and 10 ml of 0.1% collagenase (C9891, Sigma) in DMEM, and placed in a 37ºC rotating incubator. The samples were stirred vigorously every 15 minutes until quasi-complete dissolution of dermal pieces or for a maximum of 2 hours. Samples were centrifuged at 180 x g for 10 minutes and its supernatant is discarded. The cell pellet was then suspended in 30 ml of supplemented medium and centrifuged at 180 x g for 10 minutes; its supernatant was discarded. Finally, the cell pellet was re-suspended in 15 ml of supplemented medium (DMEM), transferred entirely to culture flaks, and incubated at 37ºC with 5% CO_2. The cell culture medium was changed initially at 24h, and then as needed.

Fibroblasts Cell Count and Proliferation

A suspension approximately 1 x 10^6 cells/ml of mouse fibroblasts was prepared. The cell suspension was mixed with 0.4% trypan blue solution at 1:1 ratio, and was left to stand for 5 minutes at room temperature. 20µl of cell suspension and trypan blue mixture was applied to a hemocytometer. The total number of cells (viable and non-viable) in each quadrant was recorded. The average of the four readings was multiplied by 10^4 and then by 2 (to account for sample dilution by trypan blue) to calculate the sample's number of cells per ml. The resulting

number was multiplied by the original sample dilution to determine the total cell number. Similarly, the number of viable cells (stained) was calculated.

0.3 x 10^5 KO and WT fibroblasts were plated onto a six well plate and cultured under the above conditions (refer to "Isolation of Dermal Fibroblasts"). When the first plate of cells reached confluence, all fibroblasts were detached and counted using the described method, and the number of viable KO and WT cells was recorded for comparison.

In Vitro Wound Healing Scratch Assay

Third passage WT and CD109 KO mouse fibroblasts were counted and plated onto six-well plates and grown to confluence. Cells were serum starved for 6.5 hours. The fibroblasts were then washed with serum-free medium; mitomycin C and 0 pM, 25 pM, or 100 pM of TGF-β1 (Sanofi Genzyme) were added respectively. A linear scratch was performed on the fibroblasts monolayer using a 200 µl pipette tip and serial photograph were obtained at 0, 12, and 24 hours. Wound healing (migration) is determined by the percent gap measured with ImageJ (NIH).

Collagen Contraction Assay

10mg of rat tail collagen (11179179001, Sigma) was dissolved in 2.5ml of 0.2% mM acetic acid solution at 4ºC according to the manufacturer's protocol for a final concentration of 4 mg/ml. The rat tail collagen solution, 400 µl of DMEM containing 10% fetal bovine serum and 400,000 third passage WT and CD109 KO mouse fibroblasts at 36ºC, and 10 µl of 1M NaOH were mixed into a uniform solution. 500 µl of this mixture was transferred into a 24 well plate, and the gel solidified at room temperature for 30 minutes. 800 µl of supplemented medium was then added to each well, and the plate was incubated at 37ºC, 5% CO_2 overnight. The collagen gel was then gently freed from the walls with a 10 µl pipette tip, and the fibroblasts were serum starved for 6-8 hours, followed by incubation with 0 pM or 100 pM of TGF-β1 (Sanofi Genzyme). The wells were photographed at 0 and 24 hours. Collagen contraction was calculated as a percentage of the original gel size using ImageJ (NIH).

RESULTS

Genotyping of CD109 Mouse Colony

PCR of mouse tail DNA was performed to identify the genotype for 16 WT (205 base pairs) and 16 CD109 KO (603 base pairs) mouse offspring (Supplementary Fig.1). Heterozygote mice (205 base pairs and 603 base pairs both present) were not selected for this study. The results were verified by Western blot detection of CD109 protein. The detection of CD109 protein expression was confirmed in skin samples of WT mice, while it was absent in CD109 KO mice (Fig.2a); the presence of both bands represented CD109 heterozygotic mice.

Dermal Collagen Deposition and Density is Increased in CD109 KO Mice in Response to Bleomycin Injections

To characterize the effect of bleomycin intradermal injections on collagen content and structure in WT and CD109 KO mice, the treated skin areas were harvested, and were either homogenized for protein extraction or embedded in 10% formalin for histological staining. Immunoblotting showed that collagen I content in WT and CD109 KO mice was not significantly different under basal conditions, but its level was significantly increased in CD109 KO mouse skin ($p<0.05$) injected with bleomycin (Fig.2a). These results were also confirmed by immunohistochemistry (Fig.2b), where bleomycin-treated skin sections of CD109 KO mice consistently demonstrate markedly increased staining for collagen I. Additionally, congruent with the above findings, type I collagen bundles appeared more densely packed in CD109 KO mice skin sections on Masson's Trichrome (Fig.2c) and Picrosirius Red (Fig.2d) staining. These results indicated that CD109KO mice exhibited increased collagen I production and deposition contributing to enhanced fibrosis in CD109KO mice treated with bleomycin.

Dermal Fibronectin and CCN2 Production in Response to Bleomycin is Increased in CD109 KO Mice

We next examined whether the expression of fibronectin and CCN2 is altered in CD109 KO mice in response to bleomycin injections. Our results showed that the CD109 KO displayed significantly increased expression of fibronectin ($p<0.05$) and CCN2 ($p<0.01$), as detected by

western blot analysis (Fig.3a). These results were confirmed by immunohistochemical analysis of fibronectin and CCN2 (Fig.3b).

Altogether, these results indicated that in comparison to WT mice, CD109-deficient mice displayed a stronger dermal fibrotic response in response to bleomycin treatment, as evidenced by greater production and accumulation of ECM proteins.

CD109 KO Mice Display Enhanced α-SMA Expression

Fibroblasts differentiation into myofibroblasts is a hallmark of excessive ECM synthesis and fibrosis. α-SMA is a well-known marker of myofibroblast phenotype.[69] We therefore examined whether the increased collagen I, fibronectin and CCN2 expression observed in CD109 KO mouse skin was associated with enhanced α-SMA expression.

To evaluate the degree of dermal fibroblast to myofibroblast differentiation in mice in response to bleomycin intradermal injections, 7 μm skin sections were probed for the myofibroblast marker α-SMA. With bleomycin injections, α-SMA levels were consistently more elevated in CD109 KO compared to WT mouse skin as determined by immunohistochemistry staining ($p<0.01$). These findings indicated that CD109 deficiency led to a greater degree of myofibroblast differentiation (Fig.3b) in mouse skin treated with bleomycin as measured by α-SMA expression.

Bleomycin-Treated CD109 KO Mice Display Increased Phosphorylation of Smad1, Smad2, and Smad3

Next, we determined whether CD109 deficiency affected TGF-β/Alk5 or TGF-β/Alk1 signaling in the context of bleomycin-induced skin fibrosis. Immunohistochemistry was performed using anti-phospho-Smad1, anti-phospho-Smad2, and anti-phospho-Smad3 antibodies. Representative histological sections showed an increase in pSmad1 ($p<0.05$), pSmad2 ($p<0.05$) and pSmad3 ($p<0.005$) expression levels in dermal fibroblasts of CD109 KO mice compared to WT controls on day 29 following bleomycin injections (Fig.4).

CD109 KO Fibroblasts Display Accelerated Proliferation, Migration (Wound Closure), and Collagen Matrix Contraction

Fibroblast proliferation and migration play an important role in wound healing and fibrosis.

To study proliferation, an equal number of CD109 WT or CD109 KO fibroblasts were plated, cultured, and counted when the first plate of cells reached confluence. Within 11 days of cell culture, second passage CD109 WT fibroblasts increased from 0.3 x 10^5 to 0.85 x 10^5 in number and CD109 KO fibroblasts increased to 1.7 x 10^5, as detected by cell counting using hemocytometer (Fig.5a, lower panel). This represented an average of 2.0 folds increase in CD109 KO fibroblasts number in comparison to WT fibroblasts ($p<0.0005$). These results suggested that CD109 KO fibroblasts proliferated at a faster rate than WT fibroblasts.

To examine whether fibroblast migration was altered, an in vitro wound healing (scratch assay) was performed on WT and CD109 KO mouse fibroblasts. Our results demonstrated that WT and CD109 KO fibroblasts migration occurred at a similar rate under basal conditions at 12h ($p=0.20$) and 24h ($p=0.42$). When stimulated with TGF-β1 at a concentration of 25 pM (Fig.5b), the wound gap in WT fibroblasts was 90.37 ± 3.9% compared to 75.12 ± 1.2% in CD109 KO fibroblasts at 12 hours ($p<0.05$), and 77.52 ± 2.8% and 58.60 ± 2.3% respectively at 24 hours ($p<0.01$). With the addition of 100 pM of TGF-β1, WT fibroblasts displayed a wound gap of 83.06 ± 1.7% compared to 68.54 ± 3.6% in CD109 KO fibroblasts at 12 hours ($p<0.05$), and 77.37 ± 2.5% compared to 43.69 ± 11.5% respectively at 24 hours ($p<0.05$, data not shown).

Myofibroblast-mediated wound contraction plays a major role in rapidly decreasing a wound's size following dermal injury.[70] Therefore, we next evaluated whether CD109 deficiency alters wound contraction by a collagen matrix contraction assay using WT and CD109 KO fibroblasts. The collagen scaffold's change in size was measured at 0 and 24 hours following TGF-β1 treatment, and results were expressed as a percentage of the gel's original size. By 24 hours following treatment with 100 pM of TGF-β1 (known to induce myofibroblast differentiation),[71] the measured collagen matrix area decreased to 13.22 ± 1.1% of its original size in WT fibroblasts, compared to 9.96 ± 0.4% ($p<0.05$) for CD109 KO fibroblasts (Fig.5c). This demonstrated that CD109 deficiency led to a significant increase in myofibroblast collagen gel contraction.

Together, our results indicated that CD109 KO dermal fibroblasts exhibited accelerated proliferation, migration, and collagen matrix contraction, indicative of myofibroblast activity compared to their WT counterparts in response to TGF-β1, corresponding to a faster wound closure rate on scratch assay and increased collagen gel contraction.

DISCUSSION

TGF-β plays a critical role at multiple levels in the pathogenesis of fibrotic skin disorders like hypertrophic scars, keloids and scleroderma.[12,52,72-74] Produced in abundance in the setting of tissue injury, it promotes inflammatory cells chemotaxis.[10] As the most pro-fibrotic stimulus for dermal fibroblasts, it stimulates their proliferation, induces fibroblasts differentiation into contractile myofibroblasts, favours the deposition of ECM proteins like collagen, fibronectin, and proteoglycans, and inhibits the synthesis of proteases that degrade ECM.[9,52] For these reasons, targeting the TGF-β signaling pathway represents a promising strategy for disease prevention and treatment. Previous results from our laboratory show that CD109, a TGF-β co-receptor, is a potent inhibitor of TGF-β signaling and ECM production.[45,46,48] CD109 is available in both membrane-anchored and soluble forms; the former enhances TGF-β receptor degradation, whereas the latter sequesters the TGF-β ligand and inhibits its binding to TGF-β receptors.[45] Our laboratory has recently shown the protective effect of CD109 overexpression in a transgenic mouse model, and has demonstrated that increased epidermal CD109 expression is associated with decreased inflammation, improved collagen architecture and wound healing.[49,50] The present study utilizes a CD109 KO mouse to elucidate the effects of CD109 deficiency on dermal fibroblasts' ability to migrate, proliferate, as well as their contractile property and α-SMA expression (myofibroblast differentiation). Furthermore, CD109's regulatory role on TGF-β signaling in vivo is examined by analyzing the levels of phosphor-Smad1, 2, and 3 in CD109 KO versus WT mice in response to bleomycin injections.

CD109 KO mice injected with bleomycin exhibit increased expression of ECM proteins, namely the expression of type I collagen and fibronectin. Other fibrosis markers, like CCN2 and α-SMA are also upregulated. In addition, CD109 KO mice treated with bleomycin display denser collagen organization on Masson's Trichrome and Picrosirius Red staining. These findings are consistent with our group's previous report that epidermal CD109 overexpression in transgenic

mice leads to less collagen and ECM production compared to WT mice when injected with bleomycin.[49]

Furthermore, compared to WT mice, CD109 KO mice injected with bleomycin display significantly increased Smad1, Smad2, and Smad3 phosphorylation. These findings complement results in our previous study, where CD109 overexpression is associated with decreased Smad2/3 activation.[49,50] Previous reports from our laboratory have demonstrated the inhibitory effect of CD109 on Smad2/3 phosphorylation and ECM deposition in human keratinocytes and fibroblasts.[45,46] Enhanced pSmad1 immunohistochemical staining in bleomycin-treated CD109 KO mice suggests the potential involvement of the ALK1 signaling pathway in modulating skin fibrosis, as seen in patients with scleroderma.[66-68] In contrast, we have also reported that compared to WT controls, transgenic mice overexpressing epidermal CD109 favour ALK1 pathway activation, have decreased ALK5 signaling in cultured keratinocytes, and show no difference in dermal fibroblasts ALK1 and ALK5 signaling.[51] In the same study, fibroblasts co-cultured with CD109 transgenic epidermal explants or transgenic keratinocytes conditioned medium show significantly decreased ECM proteins production, suggesting a paracrine signaling effect. As a global knockout, the lack of inhibitory effect of CD109 on TGF-β signaling likely affects both epidermal keratinocytes and dermal fibroblasts in CD109 KO mice whereby an overall increase in TGF-β activation through ALK1 and ALK5 is observed. Evidence of increased TGF-β activation and its effect on skin fibrosis in CD109 KO mice is not limited to the ALK1 and ALK5 pathways. The STAT3 pathway, also known for its implication in angiogenesis and tissue fibrosis, is enhanced in CD109 KO mice.[42] The mechanism by which the absence of CD109 alters keratinocyte-fibroblast function and interaction remains to be elucidated.

The rate of skin healing relies on properties such as fibroblasts proliferation, migration, and wound contraction. TGF-β influences fibroblasts migration through the induction of fibronectin and integrin expression.[75] Our results demonstrate that KO fibroblasts proliferate at a faster rate compared to WT fibroblast, *in vitro*. The scratch assay is commonly used *in vitro* to assess the ability for cells at the edge of the wound to migrate to fill a gap.[76] Our results indicate that in response to TGF-β, CD109 KO dermal fibroblasts exhibit enhanced gap closure rate, and increased gel contraction in the collagen gel assay. Myofibroblasts are thought to be mainly responsible for wound contraction, and their differentiation from fibroblasts is a well-

documented response to TGF-β stimulation.[77,78] However, in fibrotic skin disorders, excessive TGF-β signaling activation is reported to promote dysregulated myofibroblast activity with ECM protein accumulation.[14] Taken together, our *in vitro* studies have shown that CD109 KO mouse fibroblasts have a greater potential to proliferate, migrate and differentiate into myofibroblasts. It is possible that CD109 deficiency and the resulting unopposed TGF-β signaling contributes to the enhanced fibrotic responses observed.

To our knowledge, this is the first study to demonstrate that CD109 deficiency leads to increased fibrotic responses in mouse skin fibroblasts *in vitro* and enhanced skin fibrosis *in vivo* in a bleomycin-induced mouse model of fibrosis. Our findings form a basis for future studies to validate the potential of CD109 as a molecular target for the treatment of fibrotic conditions of the skin.

GENERAL DISCUSSION & CONCLUSION

Identified as the most pro-fibrotic cytokine, TGF-β has long been an important focus of study in tissue fibrosis. As a multi-functional growth factor, TGF-β is largely implicated in different cellular processes including proliferation, differentiation, healing, and cancer, and its dysregulated signaling can result in debilitating consequences. Several cutaneous fibrotic disorders, such as systemic sclerosis, hypertrophic scars, and keloids, have been linked to TGF-β over activity. Our laboratory has identified CD109 as a TGF-β co-receptor which antagonizes TGF-β action by facilitating the internalization and degradation of TGF-β receptors.[45,48] In addition, our group has shown that CD109 overexpression in the transgenic mouse epidermis reduces inflammation, and improves healing in a bleomycin-induced skin fibrosis model.[49]

Complementary to the above study using transgenic mice overexpressing epidermal CD109, the main objective in the current thesis is to determine whether knocking out CD109 leads to decreased inhibition of TGF-β-dependent signaling, and results in increased skin fibrosis. To examine this, I used CD109 global KO mice subjected to subcutaneous injections of bleomycin to induce skin fibrosis.

Compared to the model of transgenic mice overexpressing CD109 in the epidermis, the advantages of choosing a global CD109 KO mouse include the opportunity to study an animal with CD109 deficiency involving dermis and epidermis. Results from the bleomycin-induced skin fibrosis model in CD109 transgenic mice, which showed that CD109 overexpression leads to decreased skin fibrosis in mice, are consistent with the results for the KO model, demonstrating that CD109 deficiency leads to excessive fibrosis.[49] However, several limitations exist for the CD109 KO mouse model used in the current study. Firstly, the CD109 KO mouse is produced through the knock-in of a lacZ vector replacing a portion of the CD109 exon 1 and the entire exon 2.[42] As a result, a fragment of the CD109 gene remains present in the CD109 KO mouse. Whether this small portion of CD109 yields a functional protein containing the amino acid sequence that at least partially interacts with the TGF-β receptor CD109 binding site cannot be ruled out. However, compared to their WT littermates, the baseline phenotypical differences (transiently impaired hair growth, sebaceous and epidermal hyperplasia) observed in CD109 KO mice's skin and appendages and the more pronounced fibrotic response to bleomycin treatment

support the effects of the genetic deletion.[42] Other confounding factors to be considered in using global KO mouse include the existence of compensatory pathways and mechanisms, and possible developmental anomalies.[79] CD109 is expressed in most tissues, and its dysfunction has been reported to result in skin appendage abnormalities and epidermal hyperplasia in CD109 KO mice.[42,44] Lacrimal gland inflammation, sebaceous glands hyperplasia, as well as alopecia as a result of hair shaft kinking and difficulty penetrating the thickened epidermis have all been previously observed in CD109 KO mice.[42] Hence, the enhanced growth pattern observed in CD109 KO fibroblasts is consistent with these skin findings. An ideal alternative to the CD109 KO mouse model employed in the current study would be the creation of a skin-specific conditional knock-out of CD109 gene using the Cre/loxP or Flp-FRT technology.[80] This would permit a specific gene deletion in the skin at a pre-programmed time point to minimize the consequences of CD109 deletion on the animal's growth and development.[80] Despite the strengths and weaknesses of various manipulations that alter segments of the genetic code, these animal models can only assist in the understanding of the complex signaling pathways' interactions and their associated biological responses. While they represent different approaches to study complex *in vivo* interactions between whole proteins, they cannot totally recapitulate the *in vivo* situation or the profound changes in a protein's function that result from mutations that alter specific amino acids.[81]

The effect of CD109 deficiency on TGF-β canonical pathway signaling activation in dermal fibroblasts has been demonstrated in this study. Its action on non-canonical TGF-β signaling has, however, not been elucidated. In addition, as TGF-β cross-talks with numerous signaling pathways, the action of CD109 is unlikely to be solely restricted to the canonical TGF-β signaling pathway. Reports suggest that CD109 plays an influential role in Jak-Stat3 and EGF signaling pathways.[42,82,83] Although Jak-Stat3 or EGF signaling is not directly implicated in the pathogenesis of skin fibrosis, these pathways can possibly exert effects via cross-talk with the TGF-β pathway. Therefore, there is potential for additional investigations aimed at studying the role of CD109 in regulating alternative TGF-β signaling pathways, as well as major pathways that cross-talk with the TGF-β pathway to modulate skin fibrosis.

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells gradually lose their original properties and acquire mesenchymal characteristics.[84] Also known as

molecular exaptation, this transformation represents an economical way for cells to recycle known physiologic processes into newly reprogrammed functions.[85] By altering the cells' pre-programmed fate, EMT enables embryogenesis and is an important feature for fibrosis and the behaviour of cancer cells.[86] Recent studies uncovered evidence of EMT in the development of organ fibrosis in the liver, kidneys, lungs, and skin.[84,85] In hypertrophic scars, TNF-α and TGF-β are both involved in the induction of EMT in keratinocytes, while TNF-α facilitates EMT in wound healing through activation of bone morphogenetic protein-2 signaling.[87] In the immunohistological analysis of keloid scars, decreased epithelial markers and elevated levels of mesenchymal markers in the epidermis match the location of enhanced TGF-β/Smad3 signaling, suggesting a possible correlation between the TGF-β canonical signaling pathway activation and EMT.[88] Moreover, keratinocytes cultured in the presence of inflammatory cytokines like TNF-α and TGF-β undergo EMT, as evidenced by increased number of cells expressing the mesenchymal markers vimentin and fibroblast-specific protein 1.[89] In light of new understandings of the pathogenesis of skin fibrosis, investigating whether CD109 KO mice's cutaneous response to bleomycin injections involves the process of EMT as a critical component contributing to the enhanced fibrotic responses observed would be a logical next step.

In summary, I have shown that, CD109 KO mice display increased skin fibrosis and TGF-β-dependent signaling pathways activation *in vivo* in a bleomycin-induced skin fibrosis model. I have demonstrated that CD109 global KO in mice results in increased fibroblasts migration, myofibroblast differentiation, as well as enhanced *in vitro* wound contraction. In combination with previously published reports of CD109's ability to inhibit excessive inflammation and fibrotic parameters, my findings emphasize the potential of CD109 as a promising therapeutic target for the treatment of excessive skin fibrosis.

CD109 plays an important role in the regulation of TGF-β downstream signaling pathways. CD109 global KO mice exhibit a stronger fibrotic phenotype during bleomycin-induced skin fibrosis, as well as increased fibroblast migration and contraction. Altogether, these findings suggest that CD109 is a promising therapeutic target for the treatment of fibrotic skin conditions.

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FIGURES

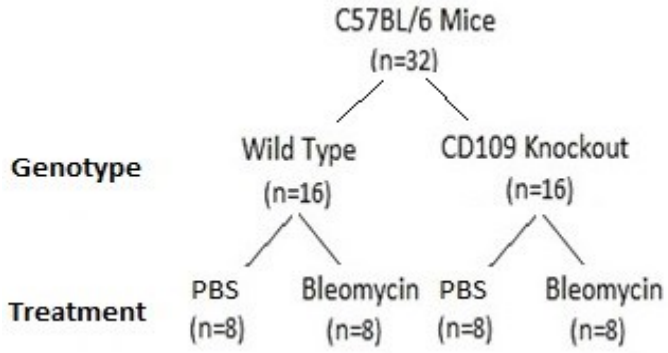


Fig. 1 Treatment Assignment for Bleomycin Injections in Mice.

After genotype confirmation with PCR, sixteen WT and CD109 KO littermates were randomly assigned to two treatment groups for intradermal injection of either 100 µl of phosphate buffered saline (PBS) or 100 µl of bleomycin sulfate solution (15 µg of bleomycin per injection) within the marked, shaved areas on their back, on alternate days over 28 days.

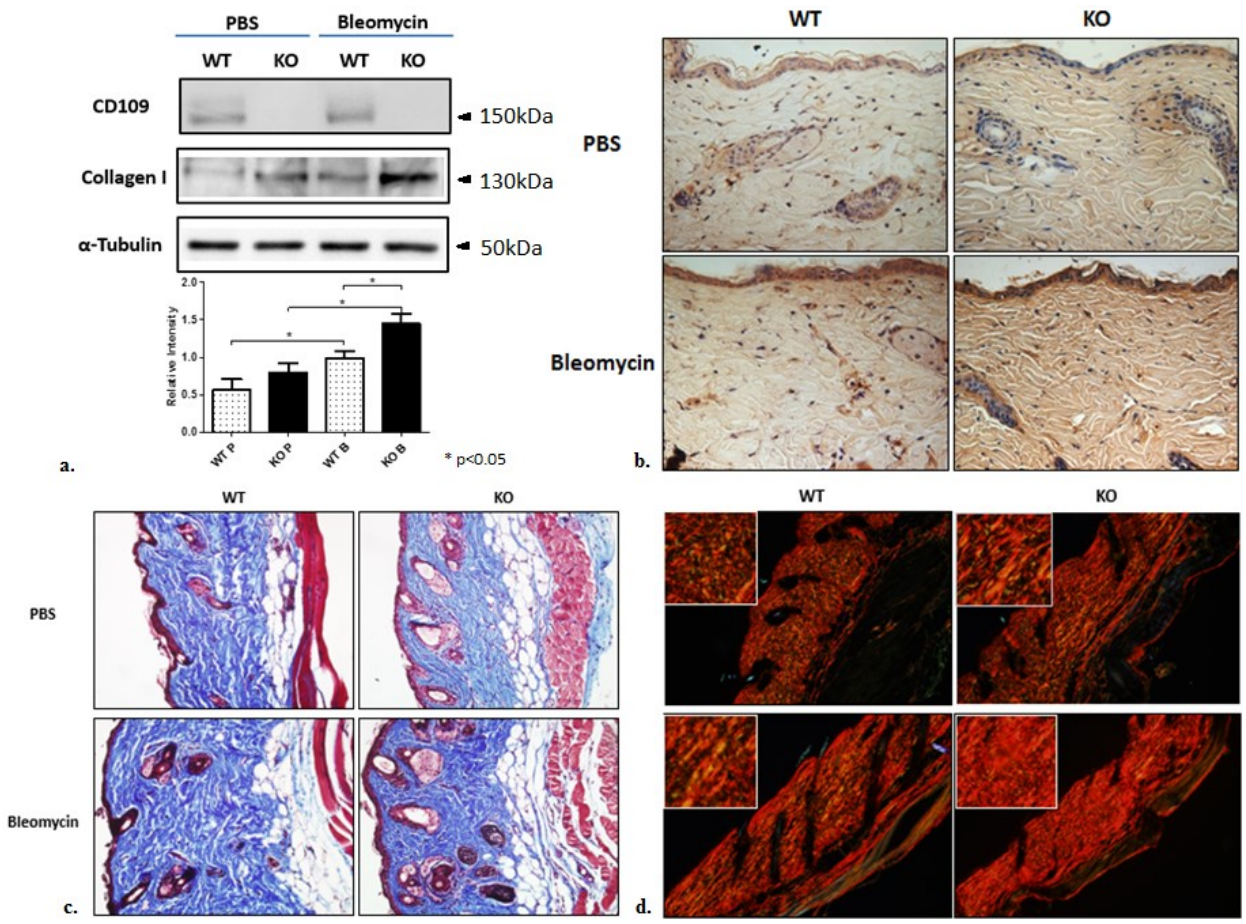


Fig.2 Increased Dermal Type I Collagen Production, Deposition, and Density in CD109 KO Mice Skin Following Bleomycin Injections.

WT and CD109 KO mice were randomized to receive either intradermal PBS or bleomycin injections on alternating days over 28 days. The treated skin areas were harvested for protein extraction and histological staining. **a)** Protein extracts from mice skin were probed for collagen I by Western blot analysis (n=4). While the collagen I content in the skin extracts of WT and KO mice was similar in the PBS injected groups (p=0.27), CD109 KO mice skin extracts showed significantly higher collagen I content after bleomycin injected groups. **b)** Representative mice skin sections stained for collagen I by immunohistochemistry (brown staining) showed enhanced collagen I expression in CD109 KO mice injected with bleomycin compared to WT mice. **c)** Total collagen content using Masson's Trichrome (collagen stains blue) and **d)** Picrosirius Red (collagen I stains red and orange) showed higher dermal collagen content and fiber density. *(Original magnification: 20x)*

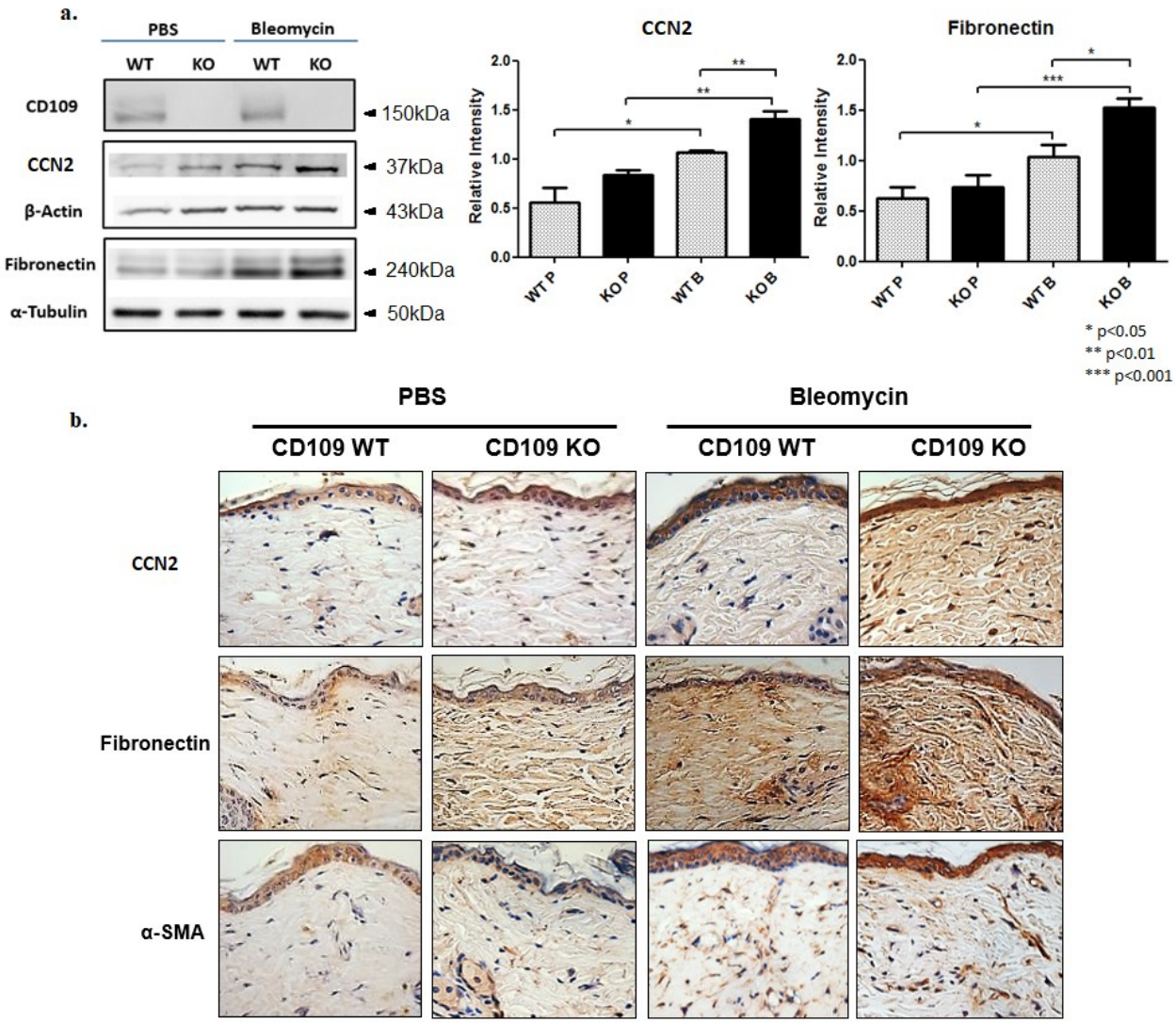


Fig.3 Bleomycin-injected CD109 KO mice display increased ECM production and myofibroblast differentiation.

WT and CD109 KO mice were treated with PBS or bleomycin injections on alternating days over 28 days. The treated skin areas were harvested for protein extraction and for immunohistochemistry. **a)** Western blots and densitometric analysis for CCN2 (n=4) and fibronectin (n=5) content of skin protein extracts demonstrated increased CCN2 ($p<0.01$) and fibronectin ($p<0.01$) expression in CD109 KO mouse skin injected with bleomycin compared to WT. **b)** Immunohistochemistry of skin section for CCN2 (extracellular), fibronectin (extracellular), and α-SMA (intracellular marker of myofibroblasts) showed increased production

in CD109 KO mice injected with bleomycin compared to WT littermates. *(Original magnification: 20x)*

Note: in order to show homogenous skin sections (to avoid the uneven distribution of hair follicles, glands), the original images were cropped to demonstrate dermal staining.

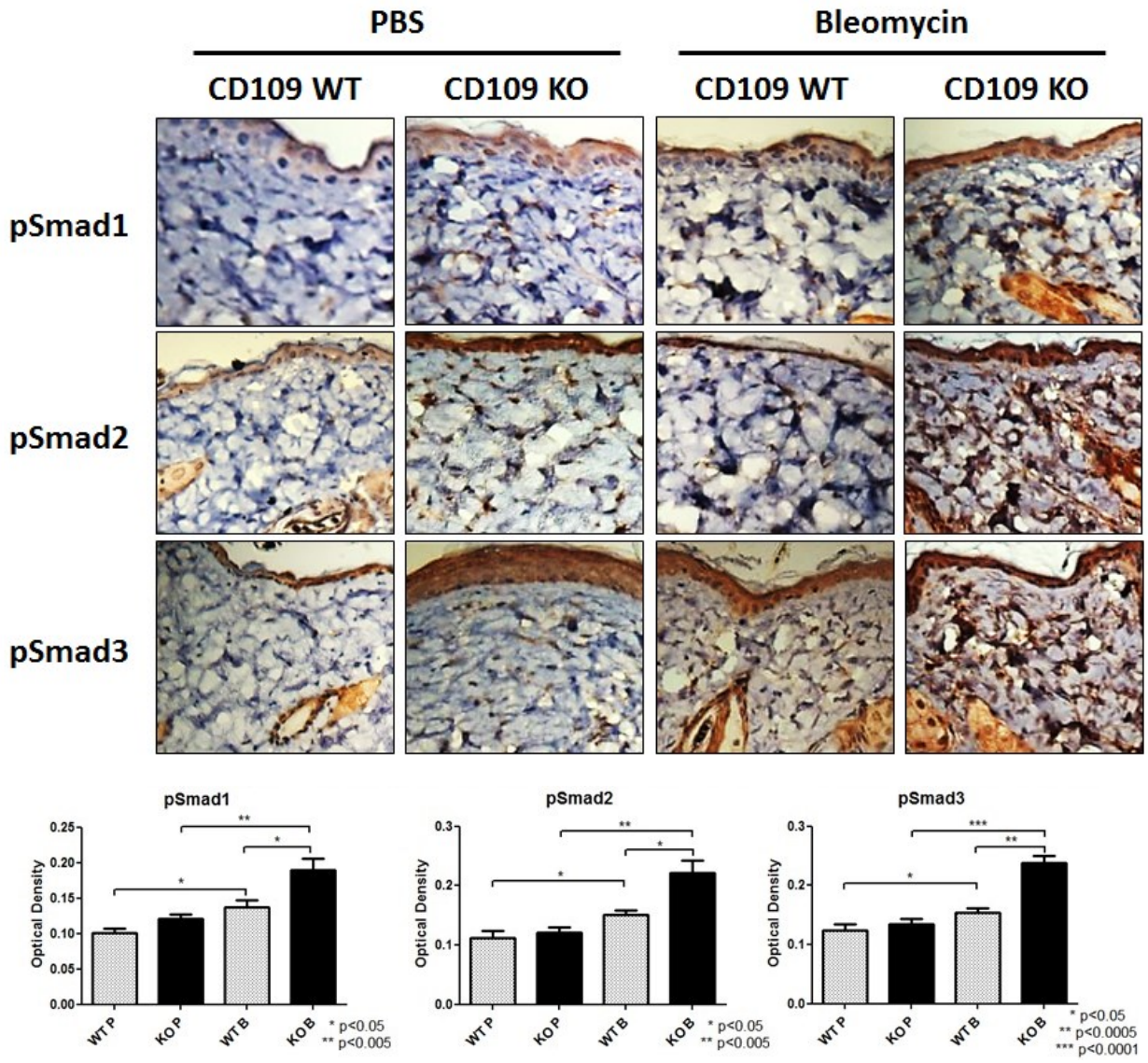


Fig.4 Increased Smad1, Smad2, and Smad3 Phosphorylation in CD109 KO Mice Skin Following Intradermal Bleomycin Injections.

WT and CD109 KO mice were treated with PBS or bleomycin injections on alternating days over 28 days. The treated skin areas were harvested for histological staining. Representative mouse skin sections immunostained (brown staining) with anti-phospho-Smad1 (n=4), anti-phospho-Smad2 (n=4), and anti-phospho-Smad3 (n=4) antibodies showed increased Smad1, Smad2 and Smad3 phosphorylation in CD109 KO mice treated with bleomycin. *(Original magnification 20x)*

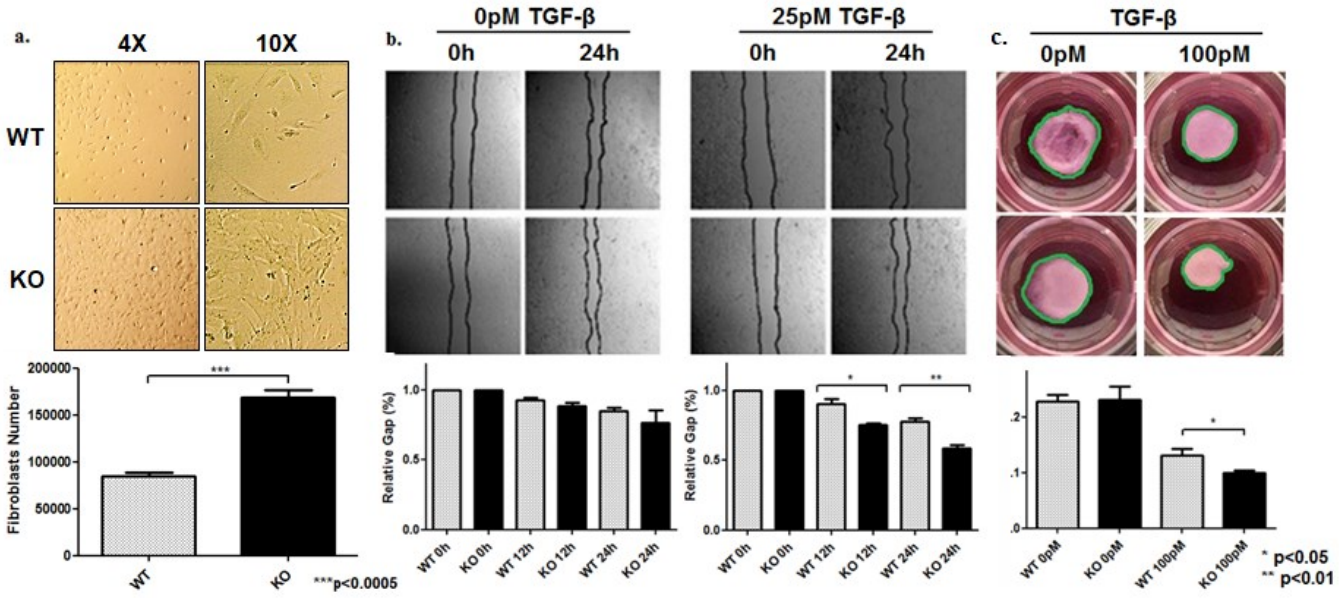


Fig.5 Cell Proliferation, and TGF-β-Induced Wound Closure and Contraction Rates are Significantly Increased in CD109 KO Mouse Fibroblasts, *in vitro*.

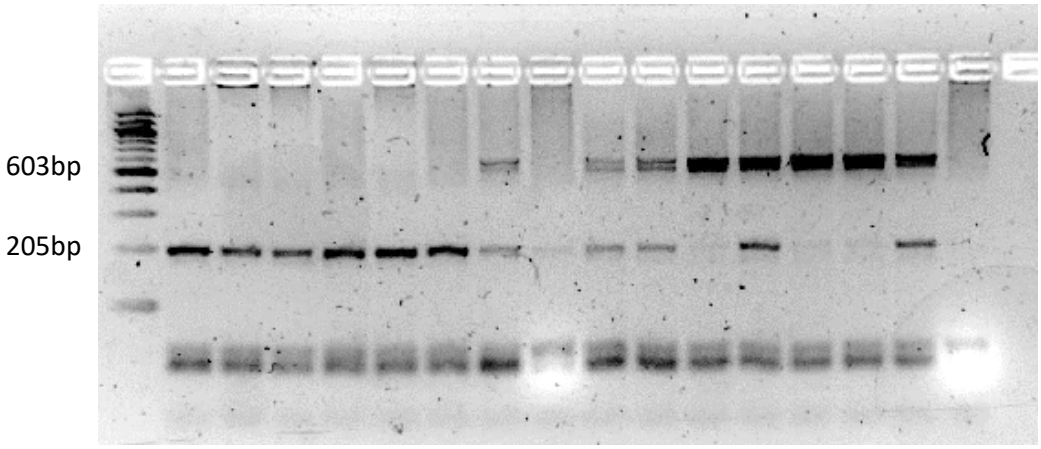
Fibroblasts were isolated from WT and CD109 KO mouse skin and cultured. **a)** Under basal conditions, CD109 KO fibroblasts doubled in number at a more rapid rate compared to WT fibroblasts (n=3, $p<0.0005$). **b)** Fibroblasts were plated and cultured with the addition of mitomycin, with or without the addition of 25pM of TGF-β1. In vitro scratch assay demonstrating the rate of wound closure in CD109 KO mouse fibroblasts compared to WT 24 hours post wounding showed significantly faster migration rate in CD109 KO compared to WT fibroblasts in the presence of 25 pm of TGF-β1 (n=3) at 12 hours ($p<0.05$) and 24 hours ($p<0.01$). **c)** CD109 KO and WT mice fibroblasts were casted onto a floating collagen gel. The rate of collagen matrix contraction was documented photographically (n=3). At 24 hours, the collagen gel size was significantly contracted in CD109 KO compared to WT fibroblasts ($p<0.05$).

TABLES

Table 1. Antibodies for Protein Detection

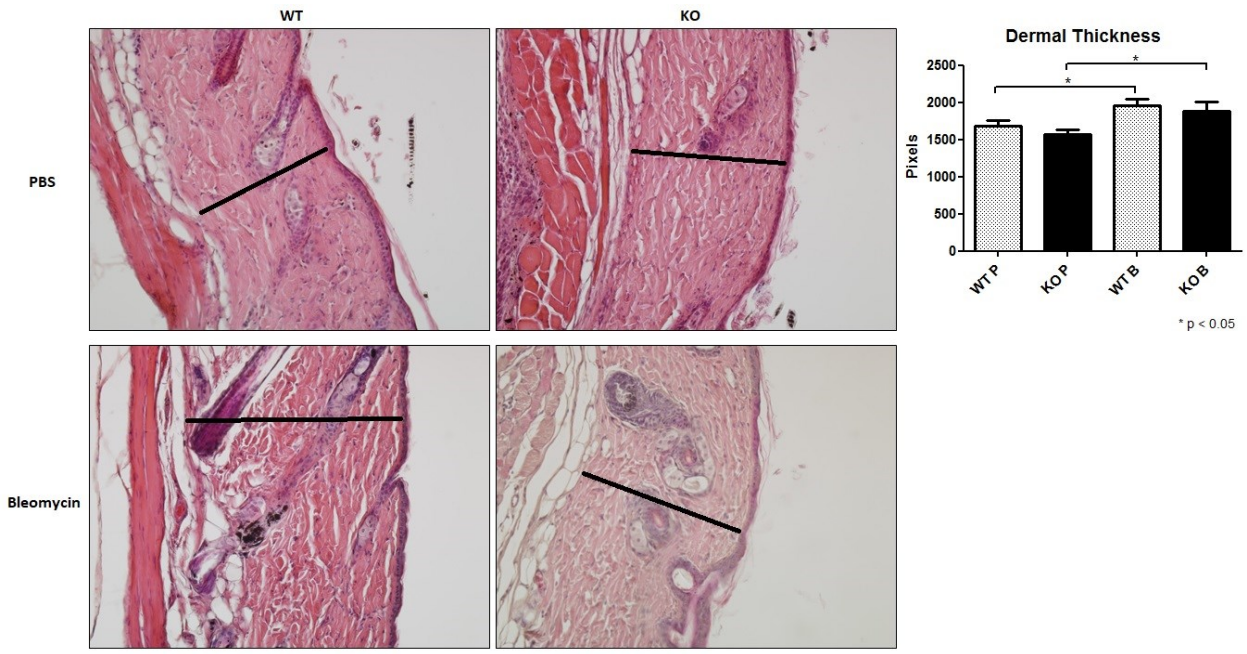
Antibodies	Company	Concentration	Molecular Weight
Western Blot			
α-SMA	Ab5694, Abcam	1:1000	42 kDa
α-Tubulin	Ab7291, Abcam	1:5000	50 kDa
β-Actin	C4, Santa Cruz	1:1000	43 kDa
CD109	C9, Santa Cruz	1:1000	150-180 kDa
Collagen I	Ab6308, Abcam	1:1000	130 kDa
CTGF	Ab6992, Abcam	1:5000	36 kDa
Fibronectin	610078, BD biosciences	1:1000	240 kDa
Immunohistochemistry			
α-SMA	MA5-11547, Life Technologies	1:400	
Collagen I	NB 600-408, Novus Bio	1:500	
CTGF	Ab6992, Abcam	1:300	
Fibronectin	Ab2413, Abcam	1:300	
pSmad1	Ab73211, Abcam	1:100	
pSmad2	Ab188334, Abcam	1:100	
pSmad3	9520S, Cell Signaling	1:100	

APPENDIX



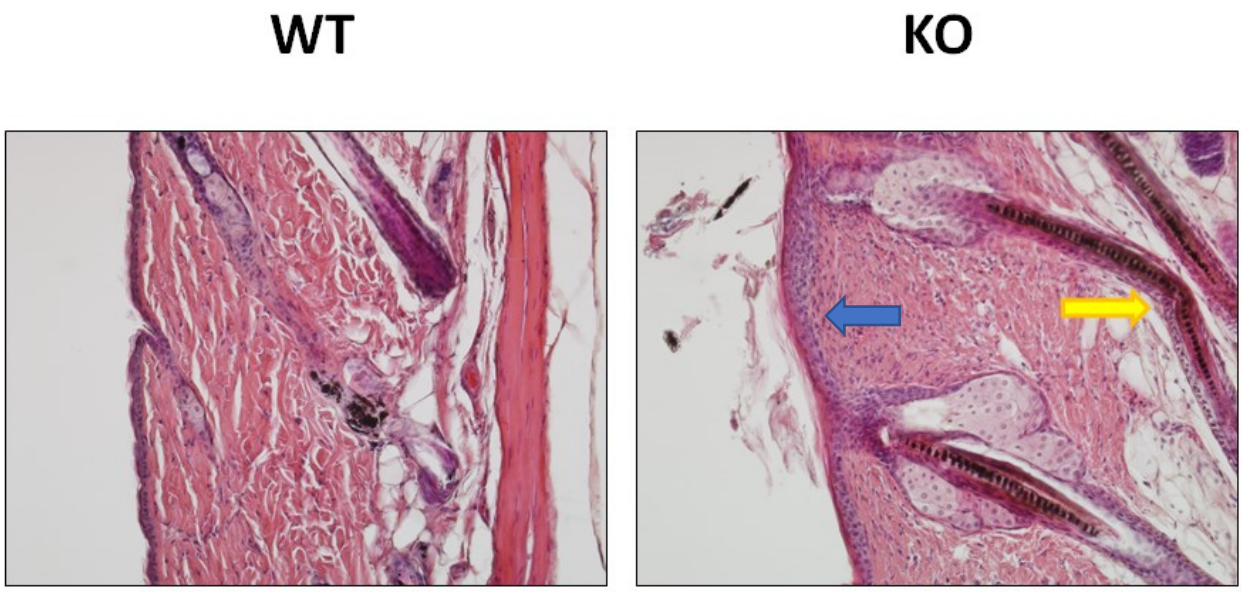
Supplementary Fig. 1 Genotyping of CD109 mouse colony.

Mouse tail samples obtained at the time of weaning were prepared for genomic DNA extraction and PCR. The expected size for the WT allele was 205 base pairs, and 603 base pairs for the CD109 KO allele, while heterozygotic mice displayed bands at both 205 and 603 base pairs. Representative PCR gel.



Supplementary Fig. 2 Bleomycin injections increase dermal thickness in CD109 WT and KO mice.

WT and CD109 KO mice were treated with PBS or bleomycin injections on alternating days over 28 days. The treated skin areas were harvested for protein extraction and for histological staining. Dermal thickness was measured from the basement membrane to the hypodermis in five high power fields per section, in two different sections per animal, and then analyzed using ImageProPlus6 Software. Dermal thickness was increased both in WT and KO mice treated with bleomycin intradermal injections. The increase in dermal thickness was not significantly different between the WT and KO groups.



Supplementary Fig. 3 CD109 KO mice display epidermal hyperplasia and abnormal epidermal appendages.

Skin sections from WT and CD109 KO mice treated with PBS intradermal injections were stained with hematoxylin and eosin to analyze tissue architecture. The findings of epidermal hyperplasia, sebaceous gland hyperplasia, abnormal kinking of hair shafts observed in CD109 KO by Mii et al. was confirmed.[42] Yellow arrow indicates kinked hair shaft. Blue arrow indicates area of epidermal hyperplasia.