Development of a novel co-culture technique to study dermal-epidermal interactions in the mouse skin

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TABLE OF CONTENTS

ABSTRACT

CD109, a negative regulator of TGF-β signaling, has been shown to improve several wound healing parameters and reduce the fibrotic response when overexpressed in the mouse epidermis. In fact, CD109 overexpression in the epidermis resulted in reduced dermal thickening at day 7 of wound healing, and caused a reduction in extracellular matrix production. We hypothesized that overexpression of CD109 in the epidermis can translate its effects to the dermis and alter dermal function. Our objective for the current study was to model the interaction between CD109 overexpressing epidermis and dermal fibroblasts *in vitro* using a novel co-culture method.

We co-cultured epidermal sheets isolated from newborn transgenic and wild-type mice with wild-type dermal fibroblasts, and analyzed fibroblast fibronectin expression in the conditioned media and cell-associated protein. We compared these results with those obtained from fibroblasts treated with condition media from epidermal explant culture. We then assessed the effect of TGF-β on fibroblast fibronectin expression when incubated in conditioned media from either transgenic or wild-type epidermal explants. Finally, we measured soluble collagen levels as a preliminary experiment to determine the effect of epidermal overexpression of CD109 on dermal collagen deposition.

A significant decrease in both cell-associated and soluble fibronectin expression by fibroblasts was observed when fibroblasts were co-cultured with transgenic epidermal explant (TgEE) as compared to fibroblasts co-cultured with wild-type epidermal explant (WtEE). A significant decrease in cell-associated fibronectin expression was also observed in fibroblasts treated with epidermal explant conditioned media alone. However, soluble fibronectin levels significantly increased using transgenic epidermal

explants (TgCM) as compared to wild-type epidermal explants (WtCM), suggesting an inhibitory effect by WtEE on soluble fibronectin levels. In the presence of TGF-β, no difference was observed in cell-associated fibronectin among all treatments. Control fibroblasts incubated in serum-free media treated with TGF-β exhibited an expected increase in soluble fibronectin levels. This increase was abolished in the presence of epidermal explant conditioned media. Fibroblasts treated with TGF-β in either WtCM or TgCM exhibited a significant decrease in soluble fibronectin compared to control. Lastly, collagen levels were significantly decreased using transgenic epidermal explants as compared to wild-type epidermal explants in both the co-culture and conditioned media study.

The decrease in soluble collagen and fibronectin levels by dermal fibroblasts co-cultured with TgEE compared to WtEE recapitulates the overall decrease in extracellular matrix production observed in CD109 transgenic mice *in vivo*. This implies that our co-culture study could be used as a novel *in vitro* model of dermal-epidermal interactions. On the other hand, the increase in soluble fibronectin levels by fibroblasts treated with TgCM compared to WtCM is opposite to the trend we see in co-culture, suggesting the involvement of a double paracrine loop with a differential cytokine profile between TgCM and WtCM. Cytokine analysis would also allow us to better understand the aforementioned observation as well as the novel observation with respect to the complete abolishment of TGF-β-stimulated soluble fibronectin levels in our conditioned media study. Thus, further research is required to draw further conclusions on the relationship between transgenic keratinocytes and wild-type fibroblasts.

RESUME

CD109, un régulateur négatif de la signalisation du TGF-β, améliore plusieurs paramètres de la cicatrisation et réduit la réponse fibrotique lorsqu'il est surexprimé dans l'épiderme de la souris. En effet, la surexpression de CD109 dans l'épiderme entraîne une diminution de l'épaisseur dermique au septième jour de la cicatrisation, et provoque une réduction de la production de matrice extracellulaire. Nous émettons l'hypothèse que la surexpression de CD109 dans l'épiderme peut traduire ses effets dans le derme et altérer la fonction dermique. Notre objectif pour la présente étude était de modéliser l'interaction entre l'epiderme surexprimant le CD109 et les fibroblastes dermiques in vitro en utilisant un procédé de co-culture.

Nous avons co-cultivé des explants épidermiques isolés à partir de souris transgéniques et de type sauvage nouveau-nés avec des fibroblastes dermiques de type sauvage, et nous avons analysé l'expression de la fibronectine par les fibroblastes dans les milieux conditionnés et les protéines associées aux cellules. Nous avons comparé ces résultats avec ceux obtenus à partir de fibroblastes traités dans des milieux conditionnés de culture des explants épidermiques. Ensuite, nous avons évalué l'effet de TGF-β sur l'expression de la fibronectine par les fibroblastes lorsqu'ils sont incubés dans des milieux conditionnés à partir des explants épidermiques transgéniques ou de type sauvage. Enfin, nous avons mesuré les niveaux de collagène soluble comme une expérience préliminaire pour déterminer l'effet de la surexpression de CD109 dans la l'épiderme sur le dépôt de collagène dermique.

Une diminution significative de l'expression à la fois de la fibronectine associée aux cellules et de la fibronectine soluble par des fibroblastes a été observée lorsque les

fibroblastes ont été co-cultivées avec des explants épidermiques transgéniques (TgEE) par rapport à des fibroblastes co-cultivés avec des explants épidermiques de type sauvage (WtEE). Une diminution significative de l'expression de la fibronectine associée aux cellules a également été observée dans des fibroblastes traités avec des milieux conditionnés d'explant épidermique seul. Cependant, les niveaux de fibronectine solubles ont augmenté de manière significative en utilisant des explants épidermiques transgéniques (TgCM) par rapport aux des explants épidermiques de type sauvage (WtCM), ce qui suggère un effet inhibiteur par de WtEE sur les niveaux de fibronectine solubles. En présence de TGF-β, aucune différence n'a été observée dans la fibronectine associée aux cellules parmi tous les traitements. Les fibroblastes de contrôle incubés dans un milieu sans sérum traités avec du TGF-β présentaient une augmentation attendue du niveau de la fibronectine soluble. Cette augmentation a été abolie en présence de milieu conditionné de l'explant épidermique. Fibroblastes traités avec TGF-β dans les deux WtCM ou TgCM présentaient une diminution significative de la fibronectine soluble par rapport au témoin. Enfin, les niveaux de collagène étaient significativement diminués en utilisant des explants épidermiques transgéniques par rapport aux explants épidermiques de type sauvage à la fois dans la co-culture, et les études de milieux conditionnés.

La diminution de collagène solubles et de fibronectine par des fibroblastes dermiques en co-culture avec TgEE rapport à WtEE, récapitule la diminution globale de la production de la matrice extracellulaire observé dans les souris transgéniques CD109 *in vivo*. Ceci implique que notre étude co-culture peut être utilisée en tant que nouveau modèle *in vitro* pour étudier les interactions derme-épiderme. D'autre part, l'augmentation des niveaux de fibronectine solubles par les fibroblastes traités avec

TgCM rapport à WtCM est opposée à la tendance que nous voyons en co-culture, ce qui suggère l'implication d' une double boucle paracrine avec un profil différentiel de cytokines entre TgCM et WtCM. Analyse des cytokines pourrait aussi nous permettre de mieux comprendre l'observation ci-dessus ainsi que la nouvelle observation de l'abolition complète des niveaux de fibronectine solubles stimulés par le TGF-β dans notre étude de milieu conditionné. Ainsi, davantage de recherche est nécessaire pour tirer de nouvelles conclusions sur la relation entre les kératinocytes transgeniques et des fibroblastes de type sauvage.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

1. I have designed and optimized a novel co-culture method to better understand dermal-epidermal interactions using mouse skin
2. I have demonstrated that epidermal explants co-cultured with dermal fibroblasts alters fibroblast protein expression by analyzing fibronectin and soluble collagen levels
3. I have provided evidence that transgenic CD109 epidermal explants decrease fibronectin and soluble collagen expression in co-cultured dermal fibroblasts compared to the wild-type control
4. I have shown that fibroblasts co-cultured with epidermal explants exhibit a differential fibronectin expression pattern than fibroblasts treated with conditioned media from epidermal explants

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LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ALK	Activin receptor-like kinase
ANOVA	Analysis of Variance
BMPs	Bone morphogenic proteins
CD109	Cluster of differentiation 109
DMEM	Dulbecco's modified eagle Medium
ECL	Enhanced chemiluminescent
ECM	Extracellular matrix
EMEM	Eagle's Minimal Essential Medium
EMT	Epithelial to mesenchymal transition
Erk	Extracellular signal-regulated kinases
EtOH	Ethanol
Fb	Fibroblast
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycophosphatidylinositol
IL-1α	Interleukin-1α
KGF	Keratinocyte growth factor
LAP	Latency associated peptide
LLC	Large latent complex
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinase
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PI-PLC	Phosphatidylinositol-specific phospholipase C
pM	Picomolar
SLC	Small latent complex
TBST	Tris-Buffered Saline and Tween
TG	Transgenic
TgCM	Transgenic Conditioned Media
TgEE	Transgenic Epidermal Explant
TGF-β1	Transforming growth factor beta1
TGF-βRI	TGF-β receptor type I
TGF-βRII	TGF-β receptor type II
VEGF	Vascular endothelial growth factor
WT	Wild-type
WtCM	Wild-type Conditioned Media
WtEE	Wild-type Epidermal Explant

CHAPTER 1 - LITERATURE REVIEW

1.1 STRUCTURE OF THE SKIN

The skin is the largest organ of the human body[1], and is the body's first line of defense against the external environment. Microscopically, skin is composed of two distinct layers: A topmost, stratified layer known as the epidermis, and a collagen-rich, underlying dermal layer [1]. The epidermis is predominantly composed of keratinocytes and is subdivided into five layers: the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum (Figure 1) [2]. The underlying basal layer is composed of stem cells, which divide and regenerate the topmost layers [3]. As keratinocytes move upwards, they begin to express an abundance of desmosomal junctions at the cell membrane thus contributing to their "spiny" histological appearance, and thereby forming what is known as the stratum spinosum [3]. As the name suggests, the keratinocytes in the stratum granulosum contain characteristic basophilic granules in their cytoplasm, which are abundant in intermediate keratin filaments and other proteins [3]. Keratinocytes of the stratum granulosum eventually undergo programmed cell death, and thus, a layer of keratinocytes lacking nuclei and other organelles make up the transitional layer known as the stratum lucidum [3]. Finally, the most superficial layer of the epidermis is the stratum corneum, which provides the skin its barrier function with the external environment. Unlike the epidermis, the underlying dermis is largely acellular, composed of a rich matrix of collagen, fibronectin, and stored growth factors. Fibroblasts, the primary cell type residing in the dermis, are involved in maintaining the integrity of the skin upon insult through extracellular matrix production and degradation. Separating the dermis and the epidermis is a basement membrane [4].

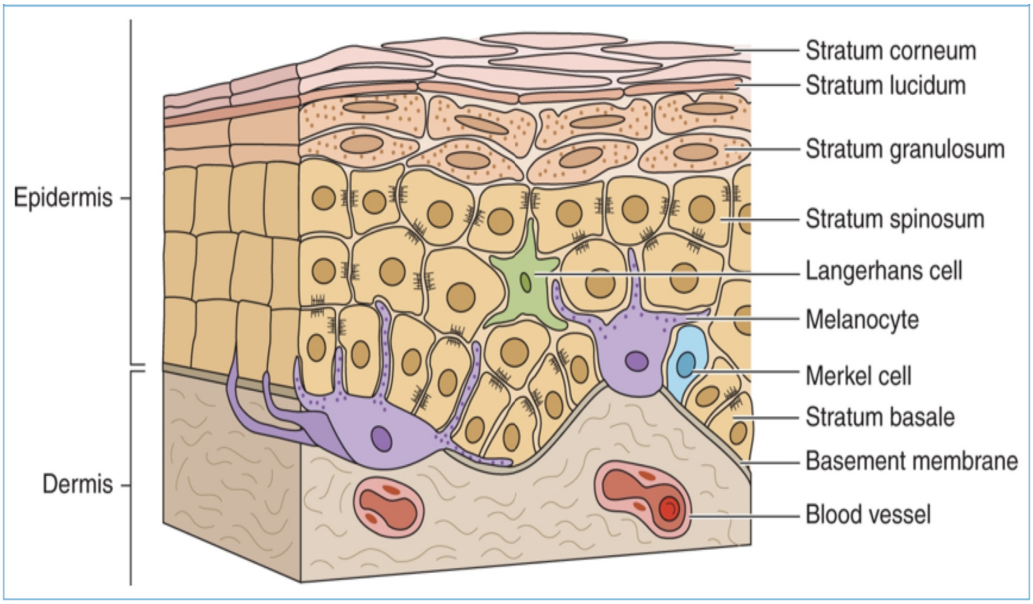

Figure 1: Schematic diagram of the epidermis and dermis. The topmost layer, known as the epidermis, is subdivided into five layers: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. The underlying dermis is largely acellular hosting multiple skin appendages such as blood vessels and nerve endings. Adapted from Gartner *et al*., 1998 [2]

1.2 WOUND HEALING

When skin is wounded, the body quickly initiates the wound healing response in order to restore homeostasis. Wound healing consists of three overlapping phases: inflammation, proliferation/ re-epithelialization, and remodeling, discussed below in more detail [5-8].

1.2.1 Inflammation phase

During the inflammatory phase, the coagulation process is activated in order to prevent further blood loss from damaged blood vessels[9]. Soluble substances released from injured blood vessels, first act to recruit platelets to the wound site, where they aggregate and promote clot formation. The platelets degranulate, releasing their alpha granules, packed with several adhesive proteins such as fibronectin and vitronectin, as well as an array of growth factors, including platelet-derived growth factor (PDGF) and transforming growth factor beta1 (TGF-β1)[10]. Activated platelets also express cell surface proteins that induce thrombin formation, a protease involved in converting plasma fibrinogen into insoluble strands of fibrin[10]. Cross-linked fibrin, fibronectin, and vitronectin form the clot scaffold in which embedded within are the aggregated platelets and their released cytokines[6].

In response to platelet-released chemotactic factors as well as bacterial degradation, a number of inflammatory cells are recruited to the wound site[7]. First to appear in large numbers are the neutrophils, which cleanse the wound from foreign particles and bacteria[7]. Neutrophil infiltration ceases after a few days and neutrophils are subsequently phagocytosed by macrophages[6]. Though long believed to play an

insignificant role in wound healing[11], recent studies have implicated neutrophils in retarding wound closure during normal healing[12].

Contrary to neutrophils, the critical role of macrophages has long been elucidated, dating back to the classic study of Ross and Leibovich on guinea pigs in the mid-70s[13]. In addition to neutrophil phagocytosis and wound debridement, macrophages were found to be critical in the progression of the fibrotic response; thus their effects extend beyond the inflammatory phase and into late stages of wound healing[14]. Macrophages are additional sources of a number of growth factors including TGF-β1, PDGF, and vascular endothelial growth factor (VEGF), therefore they have been found to promote fibroblast proliferation, angiogenesis, and extracellular matrix synthesis during the proliferation phase[13].

1.2.2 Proliferation Phase

Once the wound is rid of bacteria and debris, endothelial cells and tissue fibroblasts infiltrate the area and proliferate. Endothelial cells form new blood vessels, through a process known as angiogenesis, while fibroblasts deposit extracellular matrix proteins, particularly collagen type III. The resulting matrix, characterized by a high number of fibroblasts, neovascularization, and collagen type III, is known as granulation tissue. At the same time, the proliferation phase involves the restoration of the skin's barrier function through re-epithelialization and wound contraction by myofibroblasts.

Re-epithelialization, or epidermal renewal, occurs within hours of wounding, and involves migration and proliferation of epithelial cells over the wound[15]. Keratinocytes near the leading edge of the wound must first detach from the underlying

basement membrane by disrupting their hemidesmosomal interactions with laminin-322[4]. Following detachment, migrating keratinocytes change morphology and express new integrins to recognize and attach to the provisional wound extracellular matrix[4]. Specifically, fibronectin-specific α5β1 and αvβ6 integrins[16, 17], as well as vitronectin-specific αvβ5 integrins[18] are upregulated. At the same time, matrix metalloproteinase (MMP) expression is induced to degrade the wound matrix thereby allowing keratinocytes to migrate[4, 19]. MMPs are zinc-dependent enzymes that recognize and degrade extracellular matrix proteins including the collagens, fibronectin, and elastin. A study using a broad-spectrum MMP inhibitor (GM 6001) exemplifies the importance of MMPs in re-epithelialization, whereby topical application of GM 6001 on partial-thickness porcine wounds delayed wound closure[19]. Once a monolayer of keratinocytes covers the surface of the wound, epidermal cells proliferate and differentiate to re-establish the stratified epidermis, and a new basement membrane is laid down.

Concurrently, wound closure is facilitated by myofibroblasts, which act to bring together the edges of the open wound. Myofibroblasts in the wound were first identified by Gabbiani *et al*. in 1971, and described as modified fibroblasts with irregular nuclei, a striated cytoplasm, and surface attachment sites believed to transmit a mechanical pull to the surrounding extracellular matrix (ECM), so as to contract[20]. The origin of the myofibroblast remains unclear, but it is generally believed that they arise from various cell types including fibroblasts. Accordingly, fibroblasts stimulated with TGF-β display several characteristics similar to myofibroblasts such as α-smooth muscle expression, a marker for contractile cells, and extracellular matrix deposition[21]. Myofibroblasts

exert a local contraction of the wound matrix through intracellular stress fibers, which are connected to the surrounding granulation tissue through surface integrins[22]. At the same time, they are involved in depositing ECM, particularly collagen type I[23]. Myofibroblasts eventually undergo apoptosis and disappear from the granulation tissue, and this marks the transition of granulation tissue into a scar.

1.2.3 Remodeling Phase

Following re-epithelialization, the cellular density of the tissue decreases as cells either leave the wound or undergo apoptosis[24]. At this point, the remodeling phase is initiated, which can take up to a year as the dermis attempts to restore its tensile strength through collagen deposition and reorganization[25, 26]. Reorganization involves the action of numerous proteases, most prominent of which are the matrix metalloproteinases[26]. MMPs degrade the provisional wound matrix, not only to allow cells to migrate but also to deposit extracellular matrix and collagen. During the remodeling phase, the extracellular matrix gradually changes as collagen type III levels decrease from 30% in granulation tissue, to 10% in a mature scar[24, 25]. However, the collagen fibers of the healed scar never revert back to the organization of unwounded dermis[25]. Whereas collagen fibers in the normal dermis are arranged in a weave-like pattern, those of the healed scar are arranged parallel to the skin's surface[25].

1.3 ADULT VERSUS FETAL WOUND HEALING

Whereas adult skin leaves behind a scar, scarless healing is characteristic of fetal skin[27-30]. Wounded fetal skin heals rapidly and with complete restoration of normal

dermal architecture. After 24 weeks of gestation, scarring is observed in humans, whereas in mice, wounds scar at embryonic day 19[29]. The exact mechanisms underlying scarless fetal wound healing are still unknown, and thus the bulk of wound healing research focuses on elucidating these mechanisms and trying to minimize scar formation in adults. Various differences between fetal and adult skin attempt to explain scarless healing. For instance, the fetal extracellular matrix environment is rich in collagen, glycosaminoglycans (GAGs), and other adhesive proteins believed to facilitate cell migration and proliferation[29]. The finer collagen type III fibers are abundant in fetal skin, whereas collagen type I levels increase postnatally; the thicker fibers of collagen type I are believed to impede cell migration[29]. Furthermore, the fetal matrix is rich in the glycosaminoglycan known as hyaluronic acid, which retains water molecules due to its net negative charge[27, 29]. A more fluid environment enhances fibroblast migration into the wound, which correlates with early collagen deposition. In contrast, collagen deposition in the adult wound is attenuated while fibroblasts proliferate[31]. Additionally, there are virtually no myofibroblasts present in the fetal wound[27, 29, 30]. Thus, these differences in ECM composition and fibroblast behavior are believed to contribute to scarless healing in the fetus. In addition to a conducive ECM environment, a lack of an inflammatory response is characteristic of scarless healing in the fetus. All aforementioned characteristics of scarless fetal wound healing can be attributed to minute levels of TGF-β1 and TGF-β2 levels in the embryo[32]. On the other hand, exogenous addition of TGF-β3 on cutaneous excisional wounds results in reduced scarring in adult rats that is almost indistinguishable from embryonic scarless wound healing.

1.4 TRANSFORMING GROWTH FACTOR-β SUPERFAMILY

Transforming growth factors were first described as "sarcoma growth factors" in the late 1970s, when mouse fibroblast cells transformed by a sarcoma virus released factors into the media capable of inducing a phenotypic change in fibroblasts[33, 34]. Fibroblasts displayed uninhibited growth, a morphological change, and acquired the ability to grow on soft agar, a property only seen in malignant fibroblasts[34]. Since then, these factors have been purified and identified as TGF-α and TGF-β. The TGF-β superfamily of proteins consists of a highly conserved group of pleiotropic cytokines, including inhibins, activins, and bone morphogenic proteins (BMPs)[35]. Of particular interest are the TGF-betas, which are involved in various physiological processes in the body, and have diverse effects depending on cell type and cell context.

1.4.1 TGF-β subtypes

TGF-β exists in three isoforms (TGF-β1-3) that are structurally identical but encoded by three distinct genes[36]. All three isoforms play a critical role in development, as evidenced by knockout studies in mice. For instance, mice homozygous for the TGF-β1 allele die 20 days after birth due to wasting syndrome accompanied by immune and inflammatory response dysfunction[37]. On the other hand, TGF-β2 knockout mice show multiple developmental defects including craniofacial, heart, and eye development defects[38]. Finally, TGF-β3 knockout mice are born with a cleft palate due to unfused palatal shelves, implicating TGF-β3 in epithelial differentiation[39]. Structurally, however, all three isoforms exist as a homodimer with a molecular mass of 25 kDa[40]. The homodimer consists of monomers stabilized by three

disulfide bonds, and a free cysteine reside, which promotes dimerization of the protein[36].

1.4.2 Synthesis of TGF-β

TGF-β family members are synthesized as homo-dimeric pro-proteins that include a large pro-peptide and a C-terminal mature polypeptide. In the Golgi apparatus, furin-like enzymes cleave the propeptide, also known as Latency Associated Peptide (LAP) (75 kDa) from the TGF-β homodimer. The LAP has a high affinity for mature TGF-β (25 kDa) and remains attached to it by a non-covalent bond; this association renders the TGF-β inactive. In the secretory vessels of most cell types, the small latent complex can be covalently bound to an additional protein called the latent TGF-β binding protein (LTBP) (190 kDa) which forms another complex termed the large latent complex (LLC). There are four different LTBP isoforms, all of which have been shown to bind all three TGF-β isoforms, while LTBP-4 only binds to TGF-β1.

1.4.3 Post-transcriptional regulation of TGF-β

TGF-β is secreted into the extracellular matrix in an inactive complex consisting of the mature TGF-β non-covalently bound to LAP; this small latent complex (SLC) is in turn, associated with LTBP1-4 through disulfide bridges [35]. Release of the SLC from LTBP is not enough to activate TGF-β, rather, dissociation of mature TGF-β from LAP is necessary for its activity [35]. Once secreted, LTBP binds to components of the ECM such as heparin and fibronectin, which therefore determines the localization and distribution of TGF-β. Several molecules are involved in latent TGF-β activation

including plasmin, thrombospondin 1, and avβ6 integrin [35]. TGF-β can also be activated by acid, SDS, and urea [41].

1.5 TGF-β SIGNALING

1.5.1 Canonical TGF-β signaling

The TGF-β ligand induces its signal on the cell surface through transmembrane type I and type II serine-threonine receptors, termed TGF-βRI and TGF-βRII, respectively (Figure 2) [36, 42]. TGF-β first binds to TGF-βRII, which recruits TGF-βRI and promotes heterotetrameric receptor complex formation – consisting of two type I-receptors and two type II-receptors. There are seven known TGF-βRI receptors, termed activin receptor-like kinase (ALK1-7), among which the ALK5 receptor is constitutively expressed on most cell types[43]. On the other hand, there are five type-II receptors[44]. Receptor combinations transduce different signals, depending on cell type and cell context. In general, upon complex formation, TGF-βRII activates and phosphorylates a glycine and serine-rich domain, known as the GS-domain, on TGF-βRI[36]. The activated cytoplasmic serine/threonine kinase induces a downstream signal that is carried out by proteins known as Smads (Figure 2) [36]. The Smad family of proteins consists of eight members, subdivided into three groups: the receptor-associated Smads (R-Smad1, 2, 3, 5, 8), a co-Smad (Smad4) that associates with the receptor-Smads, and inhibitory Smads (Smad6, 7) that antagonize the signal. With respect to TGF-β signaling, ALK5 transduces its signal by phosphorylating R-Smad2 or R-Smad3, which then forms a heteromeric complex with Smad-4 [36, 45]. The complex then translocates to the nucleus and regulates the transcription of genes important to TGF-β signaling. R-Smad2

and R-Smad3 phosphorylation is transient, peaking at about 1 hour after TGF-β stimulation, before returning to basal levels by 2 hours[45].

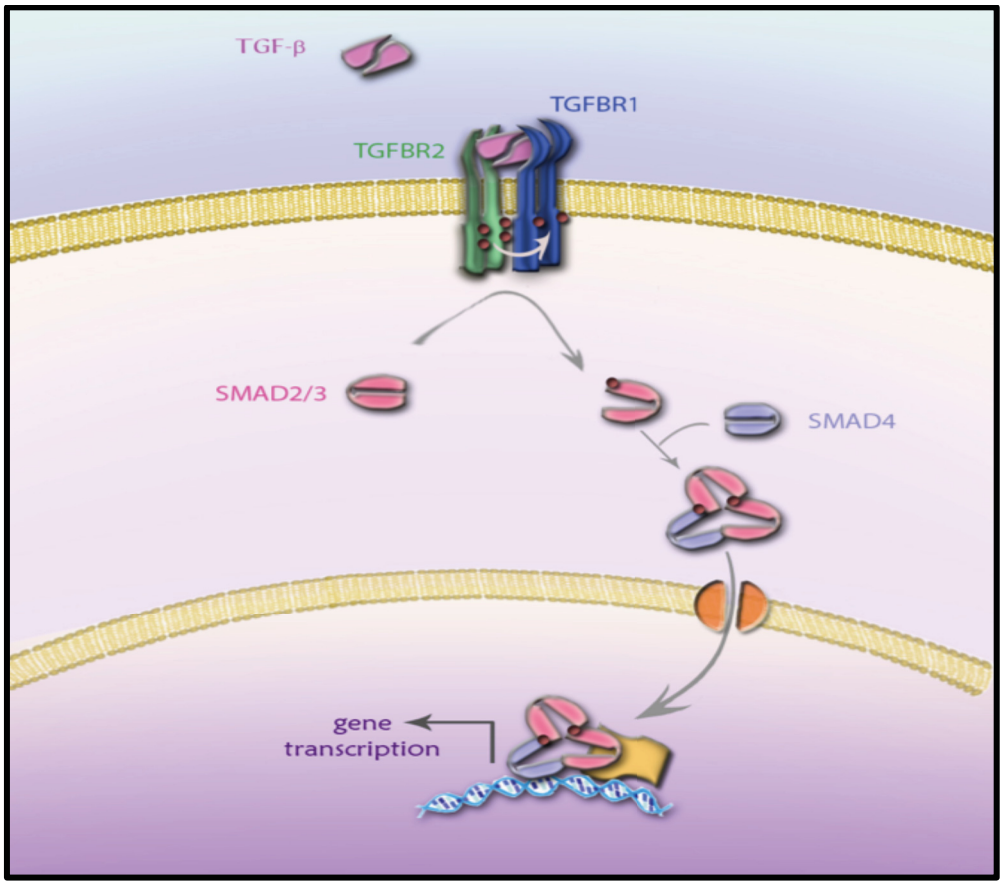

Figure 2: Schematic diagram of the canonical TGF-β signaling pathway. Upon TGF-β binding, the type II receptor recruits and activates the type I receptor, whilst forming a heterotetrameric receptor complex. The activated complex then phosphorylates receptor-associated Smads, Smad 2/3, which bind Smad 4 and translocate to the nucleus and induce gene transcription.

Adapted from Albane Bizet PhD thesis.

1.5.2 Non-canonical TGF-β signaling

Studies have shown that TGF-β can also signal through Smad-independent pathways, collectively termed non-canonical TGF-β signaling pathways. For example, TGF-β can signal through the Erk-MAPK pathway by activating p21 (Ras) protein[46]. Ras activation eventually leads to Erk activation through a number of mediators, which plays an important role in epithelial to mesenchymal transition (EMT). EMT is a normal physiological process in embryonic development, but can also present itself in pathological conditions such as tumor metastasis and fibrosis[46]. During EMT, TGF-β stimulation of epithelial cells results in a phenotypic change whereby cells gain mesenchymal characteristics, including downregulation of adherens junctions and increased MMP activity[46]. This leads to increased cell motility and invasiveness, and thus, could result in disease. TGF-β can signal through many other Smad-independent pathways, including the MAPK-JNK-p38 and PI3K/Akt pathway[46]. However, for the purposes of this thesis, we will focus on the canonical signaling pathway.

1.5.3 Negative regulation of TGF-β signaling

TGF-β receptors are constitutively internalized, independent of ligand binding. This is achieved through either the clathrin-dependent or caveolin-mediated pathway. With the clathrin dependent pathway, the TGF-β receptors are constitutively internalized in clathrin-coated pits and eventually recycled back to the cell surface[47]. On the other hand, the caveolin-mediated pathway involves receptor internalization through lipid rafts, followed by receptor degradation via ubiquitination[47].

1.6 TGF-β CO-RECEPTORS

In addition to the type II receptor, there are several co-receptors that are capable of binding to TGF-β with high affinity, including the type III receptor known as betaglycan[47], endoglin[48, 49], and Cluster of Differentiation 109 (CD109)[50]. Co-receptors are cell surface proteins that can bind ligands but are incapable of transducing the signal intracellularly[48]. All aforementioned co-receptors consist of a large extracellular domain that can be released upon proteolytic cleavage[51]. Furthermore, both the soluble and intact ectodomain can bind TGF-β, and either potentiate its signal or inhibit it by sequestration[51].

1.6.1 Endoglin and Betaglycan

The type III receptor, known as betaglycan, is a 200-300 kDA proteoglycan, which exists as a homodimer on the surface of many cell types[47]. It consists of a large extracellular domain with GAG attachment sites, a single-pass transmembrane region, and a short, highly conserved cytoplasmic tail[47]. Betaglycan binds all three TGF-β isoforms, and enhances TGF-β signaling by modulating access of the ligand to its receptors[52, 53]. In particular, it has been shown to modulate TGF-β2 signaling since, unlike the other two isoforms, TGF-β2 binds its receptors with low affinity[53].

Endoglin, also known as CD105, is a 180-kDa homodimeric transmembrane glycoprotein, predominantly expressed by endothelial cells[49]. It was first identified as a component of the TGF-β receptor system when sequencing studies of betaglycan revealed 70% homology between the two proteins[52]. Unlike betaglycan, endoglin cannot bind ligand on its own and requires co-expression of a ligand binding receptor to

induce its effects[52]. Furthermore, it is capable of binding TGF-β1 and TGF-β3, but not TGF-β2[53]. In terms of function, endoglin mediates TGF-β signaling through the ALK1/Smad1 pathway, while at the same time, inhibits ALK5/Smad3 signaling[54].

1.6.2 Cluster of Differentiation 109 - CD109

CD109 is a 150 – 170 kDA monomeric, glycophosphatidylinositol (GPI)–anchored membrane protein, first identified on various hematopoietic cells including, activated T cells, activated platelets and leukemic megakaryoblasts[55]. Since its discovery, it has been found to be highly expressed in several human tumor cell lines such as lung and skin squamous cell carcinomas, and stomach adenocarcinomas[56]. Structurally, it shares a thioester region, furin cleavage site and several conserved motifs with the α2-macroglobulin/complement gene family of proteins[57]. CD109 is first translated into a 155 kDA protein and then processed in the Golgi into a 205 kDa mature glycoprotein[58]. It is then further processed into a 180 kDa protein by an enzyme known as furinase, before localizing on the cell surface[58]. While at the surface, a soluble form of CD109 can be released upon cleavage by endogenous lipases such phosphatidylinositol-specific phospholipase C (PI-PLC)[59]. With respect to its function, CD109's role in the body is poorly understood. To date, it has been shown to negatively regulate TGF-β signaling, whereby its mechanism of action has only recently been understood[60].

1.6.2.1 Regulation of TGF-β signaling by CD109

Tam *et al*. first reported a novel TGF-β binding protein appearing at 150 kDA in 1998[61, 62]. This band was soon discovered to correspond to CD109, which was found to bind TGF-β with high affinity while negatively regulating TGF-β signaling[61-63]. In 2011, Bizet *et al*. published the first study elucidating CD109's mechanism of action using a human keratinocyte cell line (HaCaT)[60, 64]. CD109 was found to localize in caveolae pits, and to promote receptor internalization and degradation upon association with the type I receptor[60]. CD109 enhances TGF-β binding to its receptors, and promotes receptor internalization in a ligand-dependent manner (Figure 3) [60]. In fact, the presence of TGF-β was shown to increase CD109 localization into caveolae pits, thus facilitating receptor internalization[60]. At the same time, CD109 recruits the inhibitory Smad7 and the Smad ubiquitination regulatory factor, Smurf2, to the plasma membrane, which are involved in targeting caveolar comparments to the proteosome[64]. The Smad7/Smurf2 complex ubiquitinates receptors of the caveolar compartment and subsequently marks the TGF-β receptors for proteosomal degradation[64]. In addition to negatively regulating TGF-β signaling at the cell surface, soluble CD109 retains its TGF-β-binding capacity when released into the extracellular milieu, and can therefore decrease TGF-β signaling by sequestering TGF-β away from its receptors[59, 60].

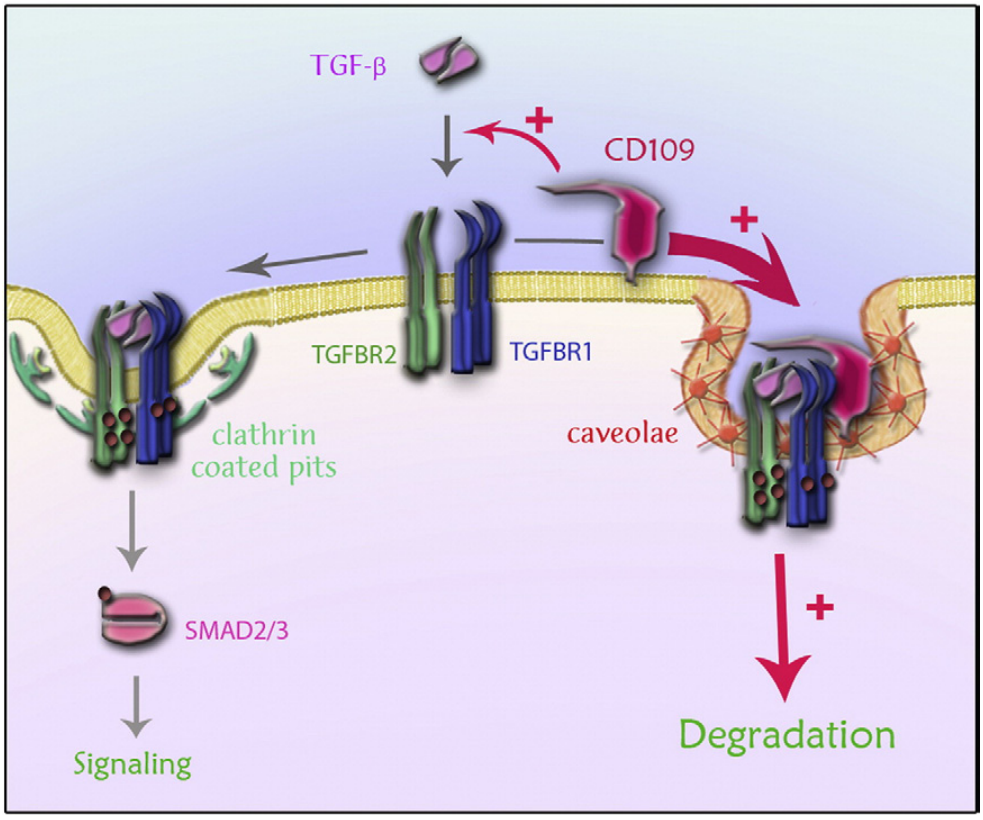

Figure 3: Schematic model of CD109 action on TGF-β signaling. CD109 binds to the type 1 receptor and promotes TGF-β receptor localization in caveolae pits, thereby promoting receptor internalization and degradation through the caveolae pathway. Downregulation of TGF-β signaling is believed to occur in a ligand dependent manner. Adapted from Bizet *et al*., 2011 [60]

1.7 ROLE OF TGF-β IN SKIN HOMEOSTASIS AND FIBROSIS

1.7.1 TGF-β in wound healing

TGF-β plays an important role in all stages of wound healing[65]. In fact, TGF-β1 is one of the first growth factors to be secreted after injury, whereby platelets, macrophages, and keratinocytes are of contributing sources[7]. It acts on multiple cell types at the wound site and induces different responses depending on the cell type and context. For instance, TGF-β1 acts as a chemoattractant to recruit inflammatory cells to the wound site, thus initiating the inflammatory response[66]. During the proliferation phase, it has been shown to stimulate the expression of fibronectin and vitronectin-specific integrins on keratinocytes thus promoting keratinocyte migration[62]. Interestingly, it can also inhibit keratinocyte proliferation, where studies have shown that the loss of epidermal TGF-β expression results in hyperproliferation and malignant tendency[62]. TGF-β also acts on fibroblasts and induces fibroblast to myofibroblast differentiation, while stimulating extracellular matrix production, particularly collagen type I and fibronectin expression[67-70]. Indeed, treatment of fibroblasts with TGF-β has been shown to induce a myofibroblast phenotype, with increased expression of intracellular stress fibers composed of cytoplasmic actins, and cellular fibronectin[22, 65].

1.7.2 Aberrant TGF-beta signaling and fibrosis

TGF-β has been the focus of much research due to its involvement in scar formation; a phenomenon that is largely due to TGF-β's stimulatory effects on ECM production and inhibitory effects on MMP expression in fibroblasts[71]. Aberrant TGF-β

signaling, particularly with relation to the aforementioned responses, can lead to the development of a multitude of skin pathologies, including keloid formation, hypertrophic scarring, and scleroderma. In keloid formation, an excessive deposition of collagen results in response to deregulated wound healing. At the cellular level, it has been shown that dermal fibroblasts isolated from keloid scars exhibit excessive levels of TGF-β1 compared to normal fibroblasts[72]. Similarly, in scleroderma, a condition characterized by dermal thickening and fibrosis of internal organs, excessive levels of receptor-associated Smad2 and Smad3 have been observed in fibroblasts isolated from scleroderma patients compared to normal fibroblasts [72]. As such, negative regulators of TGF-β signaling, such as CD109, hold therapeutic promise in modulating wound healing and downregulating TGF-β responses.

1.7.3 Role of CD109 in wound healing and fibrosis

Functional studies in mice have allowed us to better understand the role of CD109 *in vivo*. For instance, CD109 knockout mice showed no apparent abnormalities in all tissues but the skin[73]. Such abnormalities include epidermal and sebaceous gland hyperplasia, as well as transient impairment of normal hair growth[73]. Upon further analysis, it appeared that epidermal hyperplasia was the result of increased keratinocyte proliferation, particularly in the basal and suprabasal layers[73]. On the other hand, our group recently studied the effect of overexpressing CD109 in the mouse epidermis on several wound healing parameters and fibrosis. With respect to wound healing, CD109 transgenic mice exhibited a dampened inflammatory response compared to wild-type mice, showing a significant decrease in several pro-inflammatory cytokine levels as well

as impediment of inflammatory cell recruitment[74]. In addition, the overall collagen architecture of the wound improved in transgenic mice compared to wild-type mice[74]. Overexpression of CD109 in the epidermis also resulted in an increase in epidermal thickness 3 days post-wounding, an observation that conflicts with that of epidermal hyperplasia observed in the knockout mice. These contradicting results could be resolved by studying the effect of CD109 on keratinocyte proliferation using *in vitro* studies.

Two observations of particular importance to my thesis work were that transgenic mice displayed 1) a reduction in dermal thickness[75] and 2) a decrease in ECM synthesis (collagen and fibronectin) at day 7 post-wounding[74] (Figure 4). This suggests that overexpression of CD109 in the epidermis can extend its effects to the dermis and alter dermal fibroblast function. Vorstenbosch *et al*. studied the effect of epidermal overexpression of CD109 on the fibrotic response in a mouse model of wound healing. As previously mentioned, aberrant TGF-β signaling is involved in many skin pathologies such as scleroderma, psoriasis, and hypertrophic scarring. An increase in extracellular matrix production induced by TGF-β is characteristic of all conditions. Our group therefore studied the effect of epidermal overexpression of CD109 on the fibrotic response in a mouse model of wound healing. As seen with the wound healing study, transgenic mice exhibited reduced TGF-β signaling responses, such as Smad 2/3 phosphorylation and ECM production, when compared to wild-type mice[75].

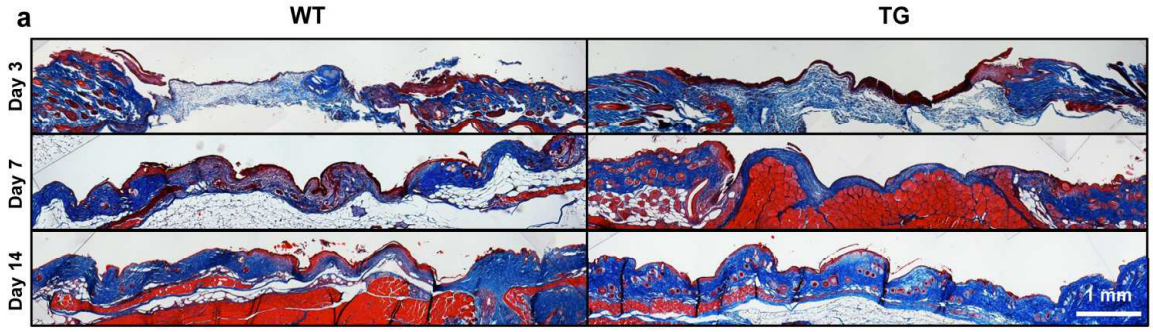

Figure 4: Masson Trichrome staining of wild type and CD109 transgenic mouse skin at 3, 7, and 14 days after wounding. At day 7, a significant decrease in dermal thickness is observed in transgenic (TG) mice overexpressing CD109 in the epidermis when compared to wild-type (WT) mice.

Adapted from Vorstenbosch *et al*., 2013 [74]

1.8 DERMAL-EPIDERMAL INTERACTIONS

Dermal–epidermal interactions have long been known to be important in skin development and homeostasis. It was first discovered in the early 70s that dermal skin fibroblasts were crucial in regulating epidermal growth and differentiation[76, 77]. Essentially, epidermal cells were able to reconstruct the intact epidermis *in vitro* when incubated in fibroblast conditioned media[76]. Soon after, the pioneering work of Rheinwald and Green showed that keratinocyte growth can only be maintained in culture when co-cultured with irradiated fibroblast cells[78]. The co-culture setup is based on culturing irradiated fibroblast feeder cells in the postmitotic state with keratinocytes. It is now widely accepted that keratinocyte growth is regulated by fibroblasts through what is known as double paracrine signaling. This essentially involves keratinocyte-fibroblast cross talk through releasable growth factors, whereby cytokines released by keratinocytes stimulate the release of cytokines in fibroblasts, which in turn act back on keratinocytes and modulate their function (Figure 5) [79]. Maas-Szabowski *et al*. provided the first line of evidence for the double paracrine mechanism in regulating keratinocyte growth using the feeder co-culture method. Keratinocytes were found to release interleukin-1α (IL-1α), which acts on fibroblasts to release keratinocyte growth factor (KGF). KGF then acts on keratinocytes and stimulates keratinocyte proliferation[79] (Figure 5). Interestingly, scleroderma and keloid fibroblasts have been found to express constitutive levels of KGF[80]. This finding suggests that constitutive KGF production (possibly through unregulated IL-1α stimulation) may be partially responsible for thickening the epidermis – an observation often seen in keloid patients.

With respect to wound healing, epidermal cells release an array of cytokines, including IL-1, IL-8, and IL-6, while at the same time, dermal cells rapidly induce KGF upon injury [79]. It is therefore believed that the aforementioned paracrine signaling pathway is responsible for the rapid induction of both cytokines during wound healing, which contribute to tissue repair through epidermal growth and re-epithelialization.

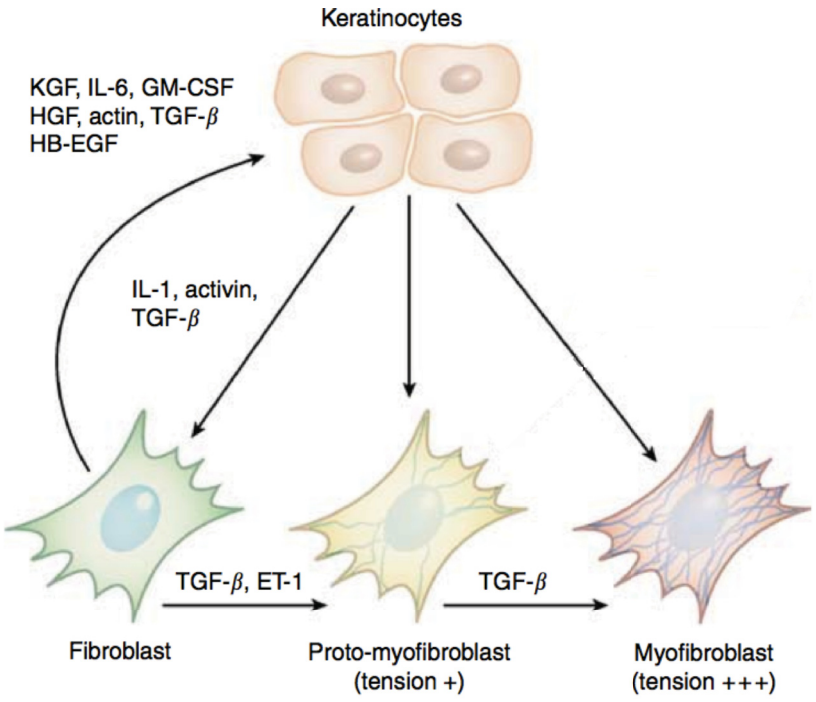

Figure 5: Schematic representation of keratinocyte-fibroblast crosstalk through double paracrine signaling. The double paracrine mechanism in the skin is based on the premise that growth factors released from keratinocytes stimulate fibroblasts to differentiate into myofibroblasts and/or release other growth factors that can, in turn, influence keratinocyte function – and vice versa.

Adapted from Werner *et al*., 2007 [81]

CHAPTER 2: PURPOSE OF THE CURRENT STUDY

Research conducted in our laboratory has demonstrated the significance of CD109 action on TGF-β signaling[59-64, 74, 75, 82, 83]. Specifically, *in vivo* studies carried out by Vorstenbosch *et al*. (2013) have highlighted the therapeutic potential of CD109 on the wound healing and fibrotic response. Of particular importance to my thesis was the observation that a reduction in dermal thickness was observed at day 7 post-wounding[74, 75]. This led us to hypothesize that overexpression of CD109 in the epidermis can translate its effects to the dermis and can lead to an alteration in dermal function. We therefore set out to understand and model the interaction between CD109 overexpressing keratinocytes and fibroblasts *in vitro*. Thus, the project was initially designed using isolated keratinocyte populations from newborn mice. However, due to difficulties encountered with primary mouse keratinocyte isolation and *in vitro* maintenance, we developed a novel co-culture model using intact epidermal sheets, and analyzed the effect of CD109 overexpressing epidermis on wild-type fibroblast function. The main objectives of this thesis are:

1) To develop a novel co-culture model to study dermal-epidermal interactions
2) To demonstrate the application of our co-culture model by comparing the effect of wild-type and transgenic epidermis on fibroblast function, specifically fibronectin expression
3) To compare our co-culture results from objective 2 with results obtained from conditioned media studies looking at the effect of conditioned media collected from wild-type and transgenic epidermis on fibroblast function

CHAPTER 3: MATERIALS AND METHODS

3.1 Generation of K14-CD109 transgenic Mice

CD109 transgenic mice were generated by Dr. Joshua Vorstenbosch, a former PhD student in our laboratory[74]. The sequenced CD109 cDNA was cloned and inserted into the pGEM-3Z vector (Promega), downstream of a K14 promoter that restricts CD109 overexpression to the basal keratinocytes of the epidermis. The transgene was microinjected into fertilized FVB mouse oocytes by the McGill Transgene Core Facility (McIntyre Building, McGill University). Founder mice were screened for the transgene using southern blot and results were confirmed using the polymerase chain reaction (*see below for conditions*). The mice were bred and skin was harvested from one- or two-day old litters.

3.2 Genotyping

Tails were snipped and genotyped in order to distinguish transgenic from wild-type mice. DNA was extracted by boiling tails in 500 μL of 100 mM NaOH for 30 mins. The solution was neutralized with 50 μL of 100 mM Tris buffer (pH 7) and centrifuged to remove debris. PCR reactions were performed using 2 μL of 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. The following primers corresponding to the transgene were used: Forward Primer 5'-GGCGAATACGATCACAAGTT-3'; Reverse Primer 5'-TCCTGGGTACGTCCGGTTACA-3'.

3.3 Primary mouse fibroblast isolation and culture

Primary mouse fibroblast cultures were established from dermal preparations of 1- or 2-day old newborn mice. Skin was isolated from newborn mice (Figure 6A) as per the protocol described by Lichti *et al*. [84]. Skin was floated on 0.25% tissue trypsin (325-043-CL, Wisent) overnight for complete separation of the dermal and epidermal layers. The dermis was separated from the epidermis using sterilized tweezers, and the dermis was then washed with PBS and minced on tissue culture plastic using a scissor pattern with two sterile blades. Minced dermis was transferred to a 15 ml canonical tube with 10 ml of collagenase (C9891, Sigma) for 3 hours. The suspension was then centrifuged for 10 mins at 500 g and re-suspended in 10 ml of DMEM, Dulbecco's Modified Eagle Medium (11995-073, Invitrogen) with 5% serum. Cells were plated on T-175 tissue culture flasks, and media was changed the next day. Once at 90% confluency, cells were trypsinized and re-suspended in freezing medium.

3.4 Preparation of epidermal explants

Newborn mouse skin was isolated as per the protocol described by Lichti *et al*. [84]. The skin was spread on a tissue culture dish, dermis side facing up, and punch biopsies were taken. A maximum of six 8 mm punch biopsies were obtained per skin explant (Figure 5). The skin explants were trypsinized overnight, and the epidermis was separated from the dermis the following day. A sterilized, curved surgical tweezers was used to grab the dermis of the floating explant and pull it away from the epidermis. The epidermal explants were then washed once with PBS at which point they were ready for the co-culture set up.

3.5 Co-culture study

Mouse fibroblasts were seeded in a 6 well plate at a density of 400,000 cells/well, and grown overnight. The following day, cells were serum starved for 4 hours while epidermal explants were prepared, as previously described. After serum starvation, fibroblasts were incubated in 1 ml of 1:1 serum-free DMEM: serum-free EMEM, Eagle's Minimal Essential Medium (06-174G, Lonza) and co-culture inserts were placed in each well. Millicell-CM (Cat no. PICM03050, Millipore) co-culture inserts with a 30 mm diameter and 0.4 μm pore size were used for this study (Figure 6B). The epidermal explant was then floated on 1 ml of 1:1 serum-free DMEM: serum-free EMEM in the upper chamber of the insert. Cells were incubated for 48 hours, followed by conditioned media collection and cell lysis. Treatment groups were done in duplicates per plate (Figure 6C).

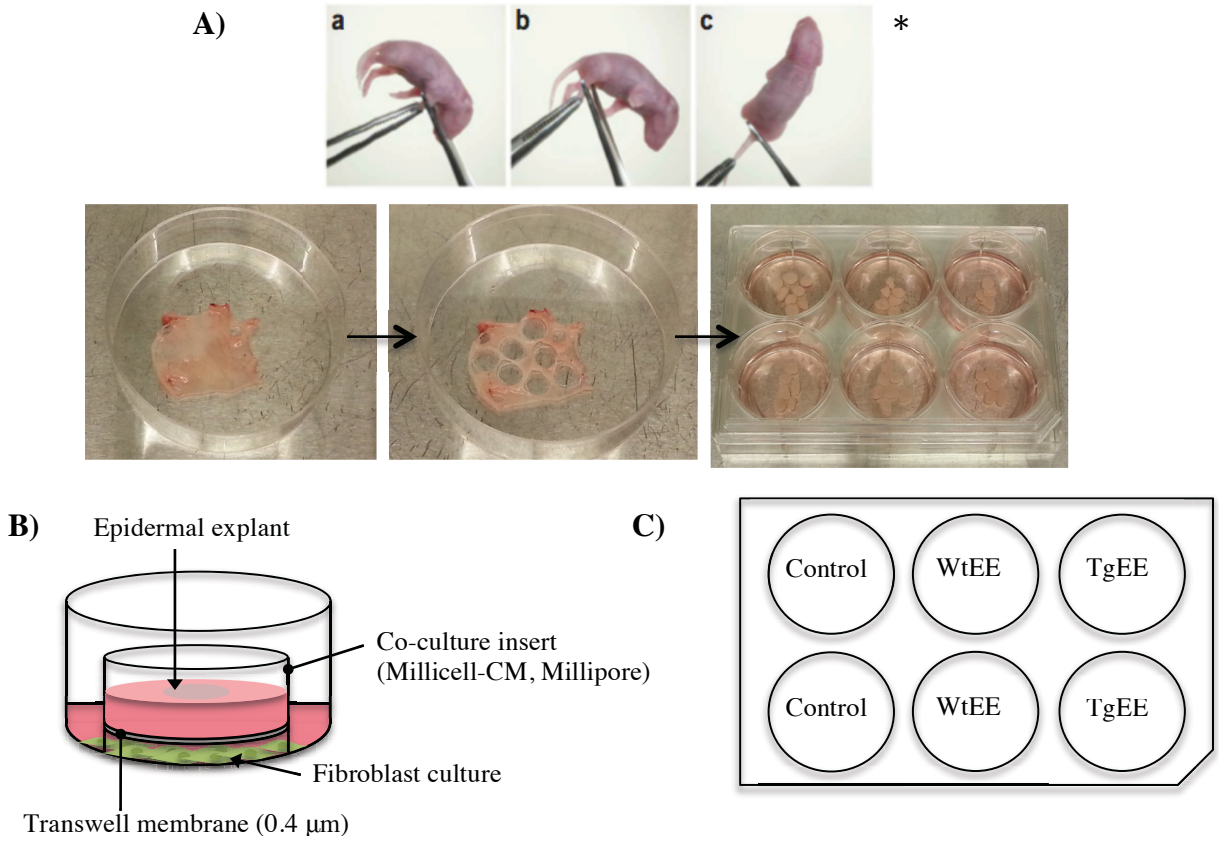

Figure 6: Co-culture study design. A) Harvesting epidermal explants. Skin harvested from newborn mice is spread on tissue culture plastic, dermis side facing up. Explants are excised using an 8mm punch biopsy and floated on 0.25% tissue trypsin overnight in order to separate the dermis from the epidermis. **B) Schematic model of co-culture set up.** Fibroblasts seeded in the lower chamber are co-cultured with epidermal explants in the top chamber for 48 hours. **C) Experimental design of co-culture study.** Treatment groups were done in duplicates and pooled for analysis. Control – fibroblasts seeded alone; WtEE – fibroblasts seeded with an epidermal explant isolated from a wild-type mouse; TgEE – fibroblasts seeded with an epidermal explant isolated from a CD109 transgenic mouse. *Image adapted from Lichti *et al*. (2008) [84].

3.6 Conditioned media study

Epidermal explants were floated on 700 μL of serum-free EMEM per well in a 24 well plate. After 48 hours, conditioned media was collected and frozen at -80°C. Conditioned media of epidermal explants derived from the *same* mouse were pooled such that a minimum of 1.5 ml of medium was collected (3 explants per mouse per group, WT or TG). Fibroblasts were seeded at a density of 400,000 cells / well using a 6-well plate and grown overnight. The following day, cells were serum-starved for 4 hours and treated with 500 μL of 1:1 serum-free DMEM: conditioned media (Table 1). Fibroblast conditioned media and cell lysates were collected after 48 hours (Figure 7). Treatment groups were done in duplicates per 6-well plate and the duplicate samples were pooled upon collection. In a second set of experiments, the effect of TGF-β on fibroblast fibronectin expression was analyzed using the same set-up described above. Cells were treated with 500 μL of epidermal explant media, then 100 pM of TGF-β in DMEM was pipetted into each well. Fibroblasts were incubated for 48 hours, followed by conditioned media and cell lysate preparation.

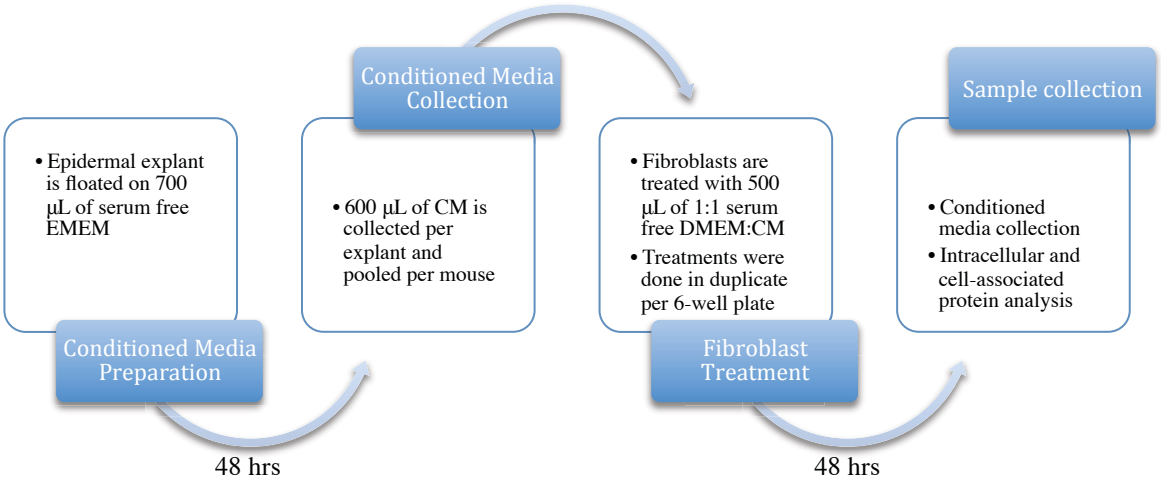

Figure 7: Conditioned media study design and timeline. Epidermal explants are floated on serum-free EMEM and conditioned media is collected after 48 hours. Fibroblast cells are seeded in a 6-well plate at a density of 400,00 cells/well and left to grow overnight. Cells are then serum-starved for 4 hours and treated with conditioned media for 48 hours. Fibroblast conditioned media is then recollected and protein is extracted from cells.

Treatment group	Media preparations (1:1)
Control	Serum-free DMEM: serum-free EMEM
WtCM	Serum-free DMEM: conditioned media from WT epidermal explant
TgCM	Serum-free DMEM: conditioned media from CD109 TG epidermal explant

Table 1: Treatment groups of conditioned media study. Table summarizes the treatment groups and corresponding media preparations of the conditioned media study. Fibroblasts were treated with one of three conditions: Control, Wild-type Conditioned Media (WtCM), or Transgenic Conditioned Media (TgCM).

3.7 Ethanol precipitation of conditioned media

Conditioned media proteins were concentrated by precipitating 200 µL of conditioned media with 1800 µL of 100% EtOH, overnight. The tubes were centrifuged for 20 minutes at 1200 rpm, followed by gentle aspiration of EtOH. The pellet at the bottom of the tube was then dissolved in 40 µL of lysis buffer and 10 µL of loading buffer, and vortexed every 10 mins for 1 hour. The suspension was heated at 95°C for 10 mins and 45 µL were subsequently loaded on the gel.

3.8 Intracellular and cell-associated protein lysis

Cells were washed very gently with ice-cold 1X PBS and lysed on ice in 50 µL of RIPA buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Duplicate samples were pooled per treatment group and placed on ice for 1 hour, vortexing every 20 minutes. The lysates were then centrifuged for 20 minutes at 1200 rpm and the supernatant was collected in new tubes.

3.9 Protein assay and sample preparation

The protein concentration of each sample was measured using the DC™ Protein Assay kit (Bio-Rad), according to the manufacturer's instructions. Samples and BSA standards of known protein concentration (0 µg/µL, 0.3 µg/µL, 0.6 µg/µL, 1.25 µg/µL, 2.5 µg/µL, 5 µg/µL, 10 µg/µL) were assayed in triplicates and incubated at room temperature for 15 minutes. The wavelength of 650 nm was obtained using a spectrophotometer and microplate reader (BioTek). The OD650 reading of each sample was then plotted against a standard curve created from the known BSA protein

concentrations. Samples were normalized for equal protein loading. Samples containing 35 μg of protein were heated at 95°C for 10 mins and allowed to cool before loading onto the gel.

3.10 SDS/Polyacrylamide Gel Electrophoresis (SDS/PAGE) and Immunoblotting

Lysates and precipitated media samples were loaded on a 10% SDS-PAGE gel and run at 100V. After electrophoresis, proteins were transferred onto a 0.45 μm nitrocellulose membrane (Whatman Protran). Effective transfer of proteins was confirmed by staining the membrane with Ponceau Red (Sigma-Aldrich). The membrane was then washed to remove the stain, and non-specific sites were blocked by incubating the membrane in 5% milk in TBST (Tris buffer saline, 1% Tween) for 1 hour at room temperature. After blocking, the membrane was incubated overnight with anti-fibronectin antibody (Cat no. 610078, BD Transduction) at a 1:2500 dilution in incubation buffer (2% milk in TBST) at 4°C. The membrane was then washed three times in TBST for 15 minutes per wash and incubated in secondary antibody, HRP-linked anti-mouse IgG (Cat no. 7076, Cell Signaling) diluted in 5% milk (1:2500 dilution), for 1 hour at room temperature. The membrane was then washed 3 times in TBST buffer for 5 minutes per wash before treatment with detection reagents.

3.11 Detection system and quantification

Enhanced chemiluminescent (ECL) substrate (Thermo Scientific) was used to detect HRP on the membrane, as per the manufacturer's instructions. The membrane was subsequently developed on photographic film and band intensities were quantified using ImageJ software.

3.12 Collagen assay

The amount of soluble collagen in media samples collected from the co-culture and conditioned media study was assayed using the commercial Quickzyme Collagen Assay (QZBcol1-1, Quickzyme Biosciences, Netherlands), following the manufacturer's instructions. The assay is based on binding of the Sirius Red dye to collagen, precipitating the collagen-dye complex, and detecting the precipitate using a microplate reader at a wavelength of 540 nm.

Note: Analysis of type I collagen levels was attempted by Western blotting, however, results were inconsistent likely due to lack of an appropriate primary antibody as well as, difficulties in reproducing the experimental conditions for the primary antibody.

3.13 Statistics

Each immunoblot experiment was performed three times, and data from each sample from each experiment was averaged in the densitometry analysis. Intensity of the bands was quantified using ImageJ software. Statistical significance was determined using Students T-Test and differences were considered statistically significant at $p < 0.05$.

CHAPTER 4: RESULTS

4.1 CD109 transgenic mice

The CD109 transgenic (TG) mice were generated in the Philip laboratory using the K14/ CD109 transgene construct [74]. The CD109 cDNA was inserted downstream of a K14 promoter, which targets expression of the transgene to the basal keratinocytes of the epidermis as well as the keratinocytes of the outer root sheath of the hair follicles. Detection of the transgene was confirmed in three founder mouse lines, and the line which exhibited the highest level of expression of CD109 was selected for this study.

4.2 Genotyping mice

One- to two-day old litters were used for all experiments. In order to distinguish wild type from transgenic mice, the tail of each mouse was cut during the skin harvesting procedure. Genotyping the tails had to be done on the same day so as to properly set up the treatment groups for the co-culture study. DNA was extracted from each tail, and subject to southern blot analysis using primers specific for the transgene that spanned the K14 promoter and the CD109 cDNA sequence. Once genotyped (Figure 8), the co-culture study and conditioned media study could be set up with the appropriate treatment groups on the next day.

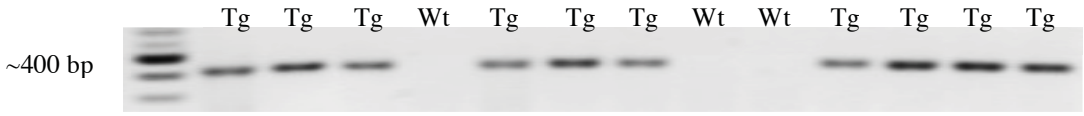

Figure 8: Genotyping mice tails by PCR. One- to two-day old newborn mice were identified as either transgenic or wild-type using primers specific to the CD109 transgene and CD109 cDNA sequence. DNA from mice tails was extracted and subject to southerblot analysis; the CD109 band is detected on an agarose gel at approximately 400 bp (basepairs).

4.3 Co-culture study

4.3.1 Rationale for development of the co-culture study

Epithelial-mesenchymal interactions play an important role in pre- and post-natal skin development, wound healing, and tumor biology. Various *in vitro* models exist to study dermal-epidermal interactions, including the conditioned media technique, the monolayer co-culture model, and organotypic model – with the latter being most representative of the *in vivo* setting. The use of conditioned media to study the effect of soluble factors on another cell type represents the earliest contribution to our understanding of epithelial-mesenchymal interactions. It does however, come with many drawbacks, most limiting of which is its poor representation of the interplay between the many cell types of the skin. On the other hand, Rheinwald and Green (1975) first introduced the simplest co-culture method using irradiated fibroblast feeder cells seeded along with keratinocyte cells. Prior to their work, keratinocyte cultures were difficult to propagate past 20-50 cell generations[78], owing to their terminal differentiation under inappropriate conditions[85]. Their study demonstrated the importance of fibroblasts in maintaining the proliferative capabilities of keratinocyte cultures, and has since been optimized and reproduced by many other laboratories. The two-chamber co-culture model is also often used to study cell-cell interactions and involves culturing keratinocytes and fibroblasts on separate chambers separated by a semi-permeable transwell membrane. Keratinocytes are first cultured on the membrane of a transwell insert, which is then placed on top of fibroblasts cultured on cell-culture plastic. Organotypic cultures are most useful in recapitulating the three-dimensional stratified structure of the skin. It usually involves a collagen scaffold embedded with fibroblast

cells, to which keratinocyte cells are seeded on top. With time, keratinocytes proliferate and are eventually elevated to the air-liquid interface to induce their stratified differentiation[86].

In an attempt to model the interaction between our genetically modified mouse keratinocytes and wild-type fibroblasts, we decided to use the two-chamber co-culture system using Millicell-CM (Millipore) inserts. The use of keratinocyte cultures requires laying down a collagen matrix on the transwell membrane of our insert to allow for better cell adhesion and proliferation. This limited our ability to study the effect of CD109 overexpression on fibroblast collagen expression due to cross-contamination of collagen into the media. In addition, we found that cells tended to clump in the middle of the collagen-coated membrane upon seeding, thus perturbing optimal growth conditions. The aforementioned limitations, as well as difficulties faced with isolating and maintaining keratinocyte populations, led us to develop a novel co-culture method using intact epidermal sheets rather than using single cell populations.

We first attempted to isolate intact epidermal sheets from adult mice; however, the adult epidermis is too fragile and did not separate into an intact sheet. From our experience with keratinocyte isolations from newborn mice, we knew we could easily obtain an intact epidermal sheet using one- to two-day old mice. We controlled for equal size of the epidermises using a 8mm punch biopsy.

After harvesting skin and preparing 8 mm punch biopsies, skin samples are floated onto trypsin overnight to allow for easier separation of the dermal and epidermal layers. When comparing genetically modified mice and wild-type mice from the same litter, genotyping the tails needs to be done the same day to differentiate the mice. With

respect to experimental setup, fibroblasts need to be seeded one day prior to separating the dermis from the epidermis to allow for sufficient time for cell adhesion and to assemble the co-culture model immediately after obtaining the epidermis.

Maintaining the integrity of the epidermis provides us with a better representation of the *in vivo* context. Thus, our co-culture model allows us to 1) look at fibroblast extracellular matrix production, including collagen type I and 2) compare the effects of genetically modified mouse epidermises and wild-type mouse epidermises. Furthermore, as previously mentioned, using epidermal explants as opposed to isolated keratinocytes allows us to determine the overall effect of genetically modifying a protein in the epidermis, while cell contacts with the other residing cells such as Langerhans and Merkel cells remain intact. Though organotypic cultures are more representative of the *in vivo* context, the majority of studies conducted using this model are limited to analysis of keratinocyte proliferation and differentiation. Fibroblast isolation in the embedded matrix of organotypic cultures is more tedious and the embedding could result in an alteration in gene expression. Furthermore, it would be much more difficult to study extracellular matrix production by fibroblasts whilst having fibroblasts embedded in a collagen scaffold. Our co-culture model tackles the aforementioned limitations, making it easier to assess the effect of intact epidermis on fibroblast function.

As with most co-culture models, our model allows us to assess the conditioned media for altered expression of growth factors after treatment. Our co-culture setup does come with its own limitations, especially with respect to the true nature of epithelial-mesenchymal interactions. Our model excludes a basement membrane that separates the dermal and epidermal layers, and lacks keratinocyte-fibroblast communication through the basement

membrane. Thus, future studies may find it more beneficial to manipulate the model in such a way to increase cell-cell contact by either 1) decreasing the volume of media within the cell culture insert or 2) coating the transwell membrane with an extracellular matrix resembling the constituents of the basement membrane *in vivo*. Our model therefore, provides a basis for the development of more sophisticated co-culture models, which can offer better insight into the physiological state of the mouse skin.

Thus far, we have summarized the co-culture models of the literature and described our novel co-culture set up in detail. In the next section, we apply our co-culture model and analyze fibronectin expression in fibroblasts exposed to either a wild-type or transgenic epidermis.

4.3.2 Evaluation of fibronectin expression by fibroblasts co-cultured with wild-type or transgenic epidermal explants

Wound healing studies conducted in our laboratory have shown several differences in the wound healing response between CD109 transgenic mice compared to wild-type mice. Of particular interest, when compared to the control, transgenic mice displayed reduced dermal thickness and reduced extracellular matrix expression, including fibronectin[74, 75]. We therefore set out to investigate the effect of CD109 overexpression in the epidermis on fibroblast function. In the first set of experiments, we used our novel co-culture set up to analyze fibronectin expression. Fibroblasts were seeded in the lower chamber while epidermal explants from either WT or TG mice were floated in the top chamber. Fibroblasts seeded without exposure to epidermal explants were used as a control. After 48 hours, conditioned media was collected and fibroblasts

were lysed in order to analyze soluble fibronectin and cell-associated fibronectin levels, respectively. A statistically significant decrease in both cell-associated fibronectin ($p=0.014$, Figure 9A) and soluble fibronectin ($p=0.001$, Figure 9B) can be seen in fibroblasts co-cultured with a transgenic epidermal explant (TgEE) when compared to fibroblasts co-cultured with a wild-type epidermal explant (WtEE). A statistically significant ($p=0.012$) increase in cell-associated fibronectin expression was observed by fibroblasts co-cultured with a wild-type epidermal explant (WtEE) when compared to the control (Figure 9A). The same trend was observed when analyzing soluble fibronectin levels in the conditioned media (Figure 9B). No significant differences were observed between fibroblasts co-cultured with a TgEE and control (Figure 9A, 9B).

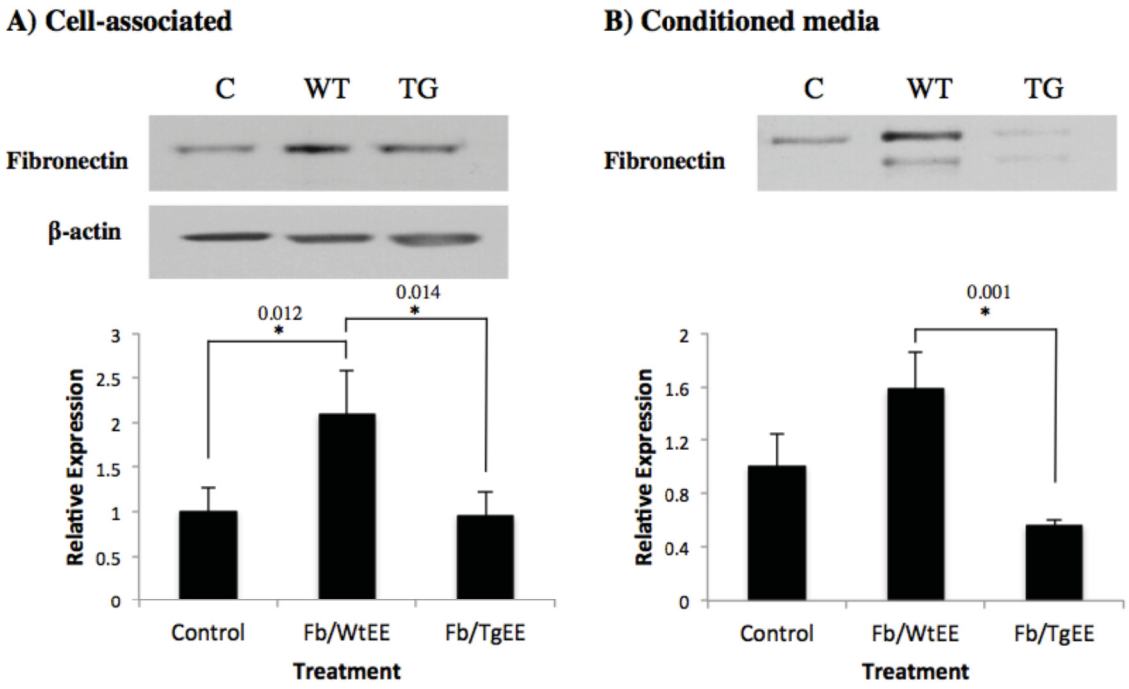

Figure 9: Fibronectin expression by fibroblasts grown in co-culture with epidermal explants isolated from WT and TG mice. A) Intracellular and cell-associated fibronectin levels. Fibroblast fibronectin expression is significantly decreased when fibroblasts are co-cultured with TgEE compared to WtEE. **B) Fibronectin levels in conditioned media.** Soluble fibronectin expression by fibroblasts were also significantly decreased in the Fb/TgEE co-culture, compared to the Fb/WtEE co-culture. (*$p<0.05$)

4.4 Evaluation of fibronectin expression by fibroblasts treated with conditioned media from epidermal explants

In a second set of experiments, wild-type and transgenic epidermal explants were incubated in serum-free media for 48 hours. The conditioned media, WtCM and TgCM respectively, was then collected and used to treat fibroblast cultures. After 24 hours, the resulting conditioned media was recollected and cells were lysed for fibronectin expression analysis. Fibroblasts incubated in serum free DMEM were used as a control. With respect to cell-associated fibronectin expression, a statistically significant ($p=0.041$) decrease was observed by fibroblasts treated with TgCM compared to fibroblasts treated with WtCM (Figure 10A). Unexpectedly, a statistically significant ($p=0.04$) increase in soluble fibronectin was observed by fibroblasts treated with TgCM compared to fibroblast treated with WtCM (Figure 10B). When compared to the control, fibroblasts treated with WtCM show an opposing effect in cell-associated versus soluble fibronectin levels (Figure 10). There was a statistically significant ($p=0.023$) increase in cell-associated fibronectin, but a statistically significant ($p=0.025$) decrease in soluble fibronectin by fibroblasts treated with WtCM compared to control (Figure 10A and 10B, respectively). The decrease in soluble fibronectin observed by fibroblasts treated with WtCM is an observation that is consistent with the literature[87]. No statistical difference was observed in either cell-associated or soluble fibronectin levels of fibroblasts treated with TgCM compared to the control (Figure 10).

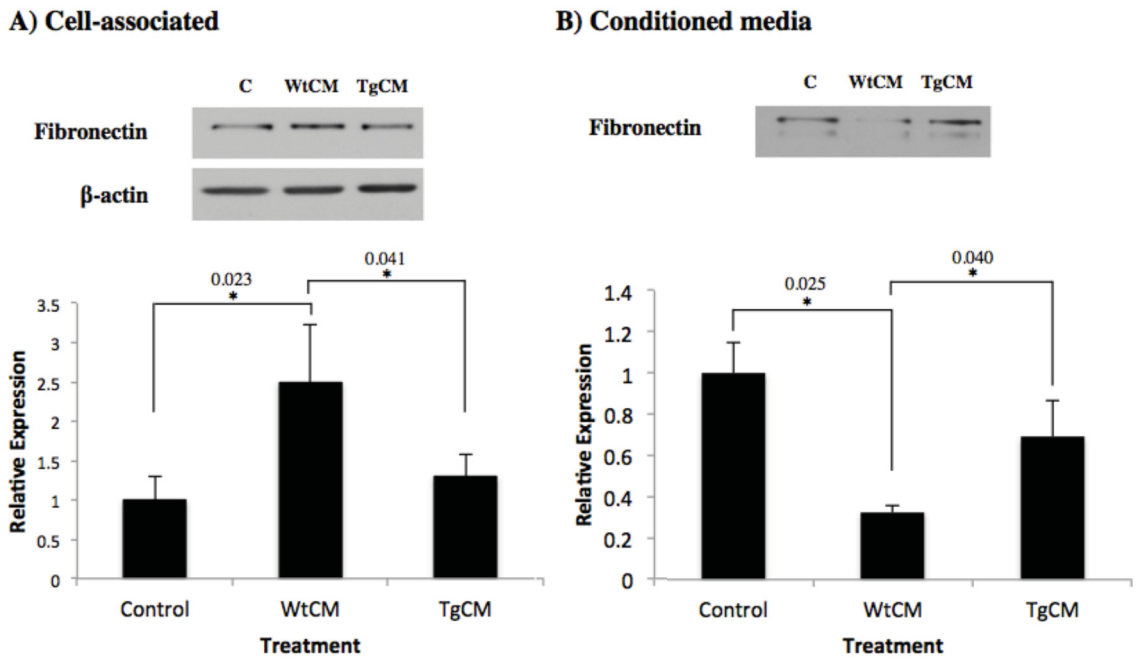

Figure 10. Fibronectin expression by fibroblasts treated with conditioned media collected from wild-type (WtCM) and transgenic (TgCM) epidermal explants. A) Intracellular and cell-associated fibronectin levels. Cell-associated fibronectin levels were significantly decreased in fibroblasts treated with TgCM compared to fibroblasts treated with WtCM1. **B) Soluble fibronectin expression levels.** Unexpectedly, a statistically significant increase in soluble fibronectin was observed by fibroblasts treated with TgCM compared to WtCM. Control: fibroblasts treated with serum free DMEM. (*$p<0.05$)

4.5 Evaluation of fibronectin expression in fibroblasts treated with conditioned media from epidermal explants *in the presence of TGF-β*

As previously mentioned, it is well documented that fibroblasts treated with TGF-β undergo myofibroblast transformation and a corresponding increase in extracellular matrix production, especially fibronectin and collagen type I. We therefore set out to determine whether an increase in fibronectin is observed in fibroblasts treated with epidermal explant conditioned media in the presence of TGF-β. Control fibroblasts treated with serum-free DMEM + 100 pM of TGF-β exhibited a statistically ($p=0.039$) significant increase in soluble fibronectin, with no difference in cell-associated fibronectin levels (Figure 11). No difference was observed in cell-associated fibronectin levels amongst all groups (Figure 11A). With respect to fibronectin release into the media, soluble fibronectin levels did not differ between fibroblasts treated with TGF-β in TgCM and fibroblasts treated with TGF-β in WtCM (Figure 11B). However, both treatments resulted in a significant ($p=0.001$, $p=0.022$) decrease in soluble fibronectin compared to control fibroblasts treated with TGF-β.

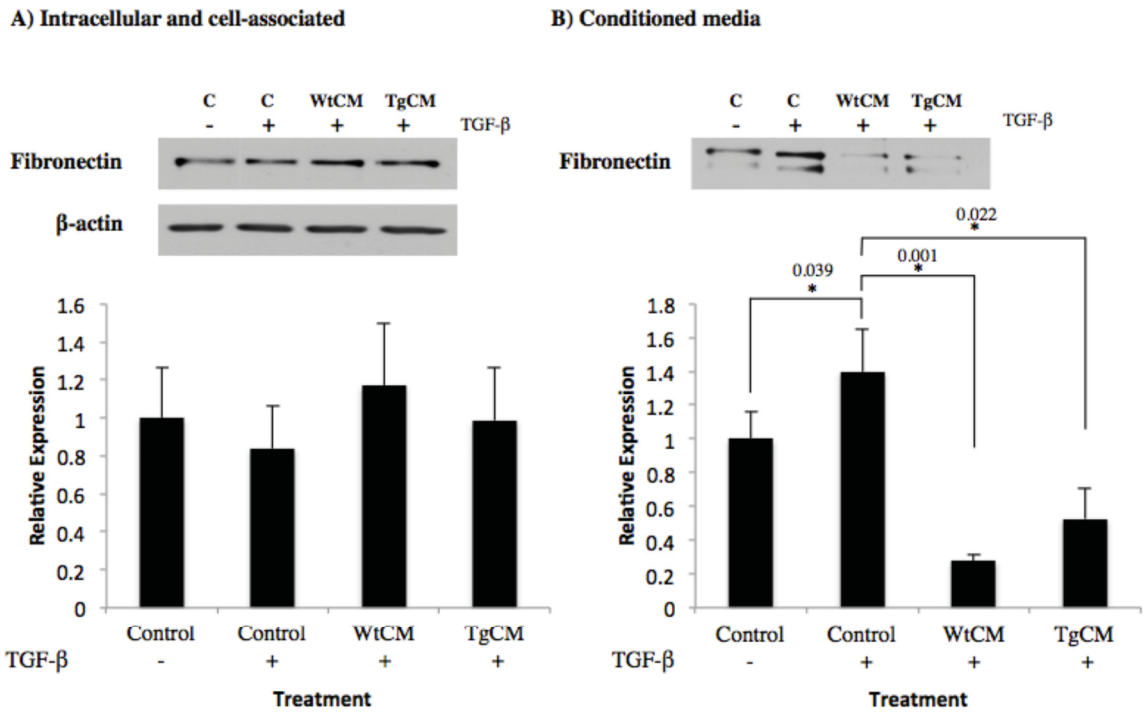

Figure 11: Effect of TGF-β on fibronectin expression in fibroblasts incubated with epidermal explant conditioned media. A) Intracellular and cell-associated fibronectin levels in response to TGF-β. No difference in cell-associated fibronectin levels was observed between fibroblasts treated with TgCM and those treated with WtCM. **B) Soluble fibronectin levels in response to TGF-β.** No difference in soluble fibronectin is seen between fibroblasts incubated with TgCM and WtCM in the presence of TGF-β. We observe a significant increase in soluble fibronectin levels in fibroblasts treated with TGF-β. However, this response is significantly decreased in fibroblasts incubated in epidermal explant conditioned media plus TGF-β. (*$p<0.05$)

4.6 Evaluation of soluble collagen levels in conditioned media from the co-culture and conditioned media study

As a preliminary experiment, we analyzed soluble collagen levels using the Quickzyme Collagen Assay (Quickzyme Biosciences) to determine the effect of CD109 overexpression in the epidermis on collagen levels. A statistically significant ($p=0.049$) decrease in soluble collagen levels is observed by fibroblasts co-cultured with the CD109 transgenic epidermis compared to WtEE (Figure 12A). Furthermore, we observe a statistically significant increase in soluble collagen levels by fibroblasts co-cultured with TgEE ($p=0.018$) and ($p=0.0004$) when compared to the control (Figure 12A). However, this increase is more moderate in fibroblasts co-cultured with TgEE compared to those co-cultured with WtEE (Figure 12A). Our conditioned media study also showed a statistically significant ($p=0.019$) decrease in soluble collagen levels by fibroblasts treated with TgCM compared to those treated with TgCM (Figure 12B). No significant difference was observed between both treatments and the control.

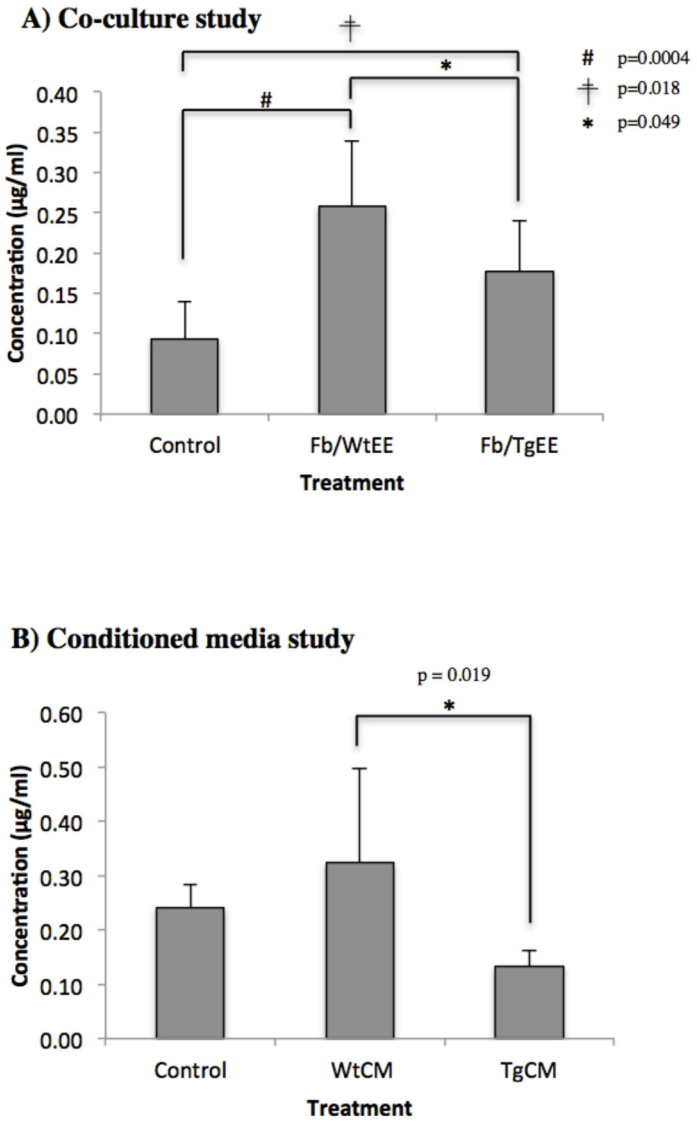

Figure 12: Soluble collagen levels in the conditioned media. A) Co-culture study. A statistically significant (p=0.049) decrease in soluble collagen levels was observed with the Fb/TgEE co-culture compared to the Fb/WtEE co-culture. In addition, a statistically significant increase in soluble collagen levels is observed in both the Fb/TgEE (p=0.018) and Fb/WtEE (p=0.0004) co-culture compared to the control (fibroblasts alone). **B) Conditioned media study.** Fibroblasts treated with TgCM exhibited a statistically significant (p=0.019) decrease in soluble collagen levels compared to fibroblasts treated with WtCM. (*p<0.05)

CHAPTER 5: DISCUSSION

The skin and its response to injury involve the intimate interaction of a number of cell types. When intact, there is constant communication between the dermis and epidermis to maintain skin integrity and homeostasis. Once skin is insulted or wounded, several factors can contribute to scar formation or abnormal healing. Fibroblasts are the primary sources of extracellular matrix deposition and are believed to be the culprit cell type in fibrosis and abnormal wound healing[80]. In pathological skin conditions such as hypertrophic scarring, scleroderma, and keloid formation, a common characteristic is the overproduction of extracellular matrix by fibroblasts. The exact mechanism by which fibroblasts are activated to overproduce ECM is still unclear. However, of all the cytokines involved in wound healing, TGF-β is one of the most potent stimulators of ECM production in fibroblasts, and is therefore believed to be a key player in abnormal wound healing.

CD109, a negative regulator of the TGF-β pathway, holds therapeutic potential in preventing the progression of skin pathologies due to abnormal TGF-β signaling. Our group developed a mouse model overexpressing CD109 in the epidermis, and evaluated the effect of CD109 overexpression on normal wound healing and in a bleomycin-induced model of skin fibrosis[74, 75]. In the wound healing study, transgenic mice exhibited accelerated wound healing and decreased scarring parameters when compared to wild-type mice[74]. In the bleomycin-induced fibrosis study, transgenic mice exhibited decreased ECM deposition and downregulated TGF-β responses [75]. Both studies showed a reduction in dermal thickness in transgenic mice when compared to

wild-type mice, and this led us to hypothesize that CD109 overexpression can translate its effects to the dermis.

Indeed, recent studies show that epithelial-mesenchymal crosstalk is critical to normal healing and, if disturbed, can contribute to fibrotic disorders[80, 88-91]. For instance, when normal keratinocytes and psoriatic fibroblasts were grafted into immunodeficient mice, a thickened epidermis characteristic of psoriasis was observed[92]. This suggests that diseased fibroblasts may be partially responsible for thickening of the epidermis often seen in psoriatic patients. In another study, it was found that keratinocytes isolated from hypertrophic scars induced dermal thickening and significantly increased collagen levels by normal fibroblasts compared to co-cultures of normal keratinocytes and fibroblasts[91]. Thus, based on current research, it is clear that the epidermis plays a significant role in regulating dermal function and that dermal-epidermal cross talk modulates normal wound healing. With this in mind, we explored the effect of CD109 overexpression in the epidermis on fibroblast function *in vitro* using a novel co-culture method.

To assess fibroblast function, we developed a co-culture model using intact epidermal sheets and a two-chamber co-culture system. We then analyzed both cell-associated and soluble fibronectin expression by fibroblasts using our co-culture model. We observe an increase in both cell associated and soluble fibronectin levels by fibroblasts co-cultured with a wild-type epidermal explant (WtEE) when compared to fibroblasts seeded alone; though this increase was only found to be significant in cell-associated fibronectin levels ($p=0.012$). Our finding conflicts with previous co-culture studies that have found 1) decreased levels of soluble fibronectin levels in the

conditioned media of a reconstituted model of the skin, involving fibroblasts and keratinocytes that had been assumed the skin's morphology when incubated with a de-epidermized acellular dermis [87] and 2) decreased mRNA levels of fibronectin in co-cultured fibroblasts [93]. This discrepancy in our findings may be due to several factors such as differences in the composition of treatment media, whereby we used serum-free treatment media while the aforementioned studies used media consisting of 5-10% serum that could potentially alter cell function. Additionally, both aforementioned studies used isolated, early-passage human keratinocyte populations that were subject to manipulations before co-culture, while our study used intact, mouse epidermal sheets that were isolated and floated straight onto our co-culture insert positioned on top of fibroblasts. The epidermal sheet most likely still maintains its diverse cellular composition, consisting of not only epidermal cells but also Langerhans and Merkel cells that may alter keratinocyte function. On the other hand, when comparing fibroblast fibronectin expression in response to epidermal explants, we observe a statistically significant decrease in both cell-associated ($p=0.014$) and soluble ($p=0.001$) fibronectin expression by fibroblasts co-cultured with a TgEE when compared to fibroblasts co-cultured with WtEE. Our findings confirm the *in vivo* observations obtained by Vorstenbosch *et al*. (2013) [74, 75].

In our second set of experiments, we treated fibroblasts with conditioned media from either wild-type (WtCM) or transgenic (TgCM) epidermal explants to look at fibroblast function without direct epidermal contact. With respect to cell-associated fibronectin expression, we see a trend similar to that obtained in our co-culture model. When treated with WtCM, control fibroblasts observed a significant increase in cell-

associated fibronectin ($p=0.023$), however this increase is diminished when fibroblasts are treated with TgCM (Figure 10A). Interestingly, this trend is not observed in soluble fibronectin expression. A significant decrease in soluble fibronectin levels was observed in fibroblasts treated with WtCM compared to control (Figure 10B), suggesting that epidermal explants secrete factors that negatively influence soluble fibronectin expression in fibroblasts. Again, when treated with TgCM, this inhibitory effect was not observed by fibroblasts treated with TgCM, whereby soluble fibronectin levels were comparable to that of the control. Overexpression of CD109 in the epidermis seems to override the effects observed in WtCM-treated fibroblasts, thus reverting their phenotype back to the control – possibly by acting on the soluble factors released by wild-type epidermal explants.

Under the same conditions used in our conditioned media study, we then looked at the effect of TGF-β on fibroblast fibronectin expression while incubated in epidermal explant conditioned media. No difference was observed in cell-associated fibronectin amongst all treatments. When we compare cell-associated fibronectin levels in response to TGF-β treatment with those from our previous experiment (without TGF-β treatment), we observe that TGF-β treatment reverts the decrease seen in fibroblasts treated with TgCM compared to WtCM (Figure 10A and 11A). This is in agreement with our hypothesis; whereas CD109 overexpression in the epidermis downregulates TGF-β responses – as observed in TgCM-treated fibroblasts –, the addition of external TGF-β to our treatment media brings cell-associated fibronectin levels back up. On the other hand, the characteristic increase in soluble fibronectin by control fibroblasts in response to TGF-β was decreased in the presence of epidermal explant conditioned media. This

decrease was found to be significant in WtCM ($p=0.001$) and TgCM ($p=0.022$) when compared to control, plus TGF-β. Our results are interesting considering no study to our knowledge has looked at the effect of conditioned media on TGF-β responses in fibroblasts. Analogous to our earlier experiments using conditioned media in the absence of TGF-β, wild-type epidermal explants may secrete inhibitory factors that act on TGF-β and inhibit TGF-β-induced fibronectin increase. However, it seems that CD109 acts through a different mechanism since our earlier experiments using TgCM alone did not show a corresponding decrease in soluble fibronectin levels, but rather rescued the inhibitory effect of WtCM.

Finally, we assessed soluble collagen levels as a preliminary experiment to analyzing the effect of CD109 overexpression in the epidermis on yet another extracellular matrix protein – particularly one that is upregulated by TGF-β stimulation. Again, as observed in our earlier studies, we do not see the inhibitory influence of keratinocytes on fibroblast collagen expression as cited in the literature[93, 94]. As stated earlier, this is likely due to several factors including differences in treatment conditions. Of particular importance, we used mouse epidermal explants whereas the aforementioned studies used isolated human keratinocytes. Chaturved *et al*. (2004) have highlighted a couple of differences between mouse and human keratinocyte behavior, particularly with reference to their differentiation state[95]; however it is important to note that our study is solely focused on comparing wild-type and CD109 transgenic epidermises. With respect to our treatments, both our co-culture and conditioned media study showed statistically significantly decreased levels of soluble collagen in Fb/TgEE

co-culture and TgCM treatment compared to wild-type. An observation similar to our soluble fibronectin levels in co-culture but not in our conditioned media study.

Our results suggest that overexpression of CD109 in the epidermis affects dermal function through releasable factors, which may act through a double paracrine loop. Keratinocytes secrete a number of growth factors, including TGF-β. In fact, Bellemere *et al*. (2005) have found that in a reconstructed skin model *in vitro*, TGF-β1 is highly expressed by basal layer in the epidermis[91]. Thus, CD109 overexpression in the epidermis, which could also involve the secreted form of the protein, may alter TGF-β responses at the epidermis first prior to reaching the dermis. Alternatively, CD109 can alter fibroblast function through keratinocyte-released vesicles known as exosomes. Exosomes are single-membrane endocytic vesicles that act as reservoirs for a number of different proteins, including TGF-β[47, 51]. They have been found in the conditioned media of several cell types and are capable of secreting stored protein into the extracellular space[96]. Thus, the CD109 protein could be packaged into exosomes, which then reach the dermis and alter dermal function directly. Due to the conflicting increase in soluble fibronectin when fibroblasts are treated with TgCM compared to the decrease we see when in co-culture (Fb/TgEE), we believe a double paracrine mechanism may be involved. CD109 may stimulate or inhibit the secretion of other factors that act on fibroblasts, which in turn are stimulated to release factors that act back on keratinocytes. However, in order to test this hypothesis, further studies need to be conducted that focus on factors secreted by our CD109 transgenic epidermis.

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