DETERMINING THE FUNCTIONS OF SMAD4 DURING DEVELOPMENT, WOUND HEALING AND CANCER

by

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CERTIFICATE OF APPROVAL

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Abbreviations

ALK Activin Like Kinase
BM Basement Membrane
bFGF Basic Fibroblast Growth Factor (FGF2)
BMP Bone Morphogenetic Protein
CAF Carcinoma Associated Fibroblast
CSF-1 Colony Stimulating Factor
CTGF Connective Tissue Growth Factor
C_t Cycle Threshold
CDK Cyclin Dependent Kinase
DPC4 Deleted in Pancreatic Carcinoma 4
Dsg4 Desmoglein-4
DP Dermal Papillae
ECM Extracellular Matrix
FSP-1 Fibroblast Specific Protein-1
HF Hair Follicle
H&E Hematoxylin-Eosin
HGF Hepatocyte Growth Factor
HHT Hereditary Hemorrhagic Telangiectasia
IF Immunofluorescence
IHC Immunohistochemistry
IFNγ Interferon-γ
IL-1 Interleukin-1
IRS Inner Root Sheath
IGF Insulin Growth Factor
JPS Juvenile Polyposis Syndrome
MHC Major Histocompatibility Complex
MAPK Mitogen Activated Protein Kinase
MCP-1 Monocyte Chemoattractant Protein-1
MMC Mitomycin-C
MMP Matrix Metallo-Protease
OCT Optimum Cutting Temperature
ORS Outer Root Sheath
PBS Phosphate Buffered Saline
PBT Phosphate Buffered Saline + .1% Tween
PCR Polymerase Chain Reaction
PDGF Platelet-Derived Growth Factor
PFA Paraformaldehyde
QPCR Quantitative Polymerase Chain Reaction
QRT-PCR Quantitative Real-Time Polymerase Chain Reaction
RT Room Temperature
RT-PCR Real-Time Polymerase Chain Reaction
SCID Severely Compromised Immunodeficient
SSCP Single Strand Conformation Polymorphism
TIFF Tagged Image File Format

TIMP Tissue Inhibitors of Metalloproteinase
TEA Triethanolamine
TEM Transmission Elecetron Microscopy
TGFβ Transforming Growth Factor β
TNFα Tumor Necrosis Factor α
VEGF Vascular Endothelial Growth Factor
WHN Winged Helix Nude (FoxN1)

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Abstract

Smad4 is at the nexus of many important cell signaling paradigms. The function of Smad4 has been to relay phosphorylated signals from the plasma membrane and shuttle them towards specific regions of DNA to elicit distinct transcriptional programs. These programs can mediate cellular fate, survival, migration, apoptosis and homeostasis. Smad4 was originally identified as a tumor suppressor in the pancreas over ten years ago, but since that time it has been found to be important in every cell type investigated as well as a critical mediator for normal differentiation and development.

This thesis explores how Smad4 functions in skin. Conditionally removing this gene in skin helps determine its role during normal skin development, cutaneous wound healing and cancer. I have found that Smad4 is required for the proper differentiation of the hair follicle by its specific production of normal adhesions through the novel desmosomal cadherin Desmoglein-4 (Dsg4).

Furthermore, I have found that deletion of Smad4 in the epidermis during cutaneous wound healing produces more profound cell non-autonomous defects than defects in Smad4-deficient keratinocytes. Additionally, the defects found in cutaneous wound healing mirror the tumorigenic processes that occur in spontaneous tumors formed in Smad4-deficient skin.

Chapter 1: Introduction

1.1 Smads in skin development

Smads are intracellular proteins that shuttle from the cytoplasm to the nucleus. There are three classes of Smad proteins termed Receptor Smads (R-Smad-1, 2, 3, 5, 8), Inhibitory Smads (I-Smad-6, 7) and the common partner or mediator Smad (Smad4) for a detailed review of Smads see Massague et al., 2005; ten Dijke and Hill, 2004. R-Smads are phosphorylated by the serine/threonine kinase of the TGFβ family receptors once these receptors are activated by their ligands (Figure 1). Phosphorylated R-Smads then bind with Smad4 and enter the nucleus, where the Smad complex transcriptionally regulates gene expression by binding sequence specific elements within the promoters of target genes, regions termed the Smad binding element (SBE). The SBE requires co-factors to elicit a myriad of transcriptional activation, repression and/or attenuation. Among R-Smads, Smad2 and Smad3 (Smad 2/3) are regulated by TGFβ and activin, whereas Smads1, 5 and 8 (Smad 1/5/8) are thought to be primarily activated by BMPs. The I-Smads block the ability of the cognate receptors to phosphorylate R-Smads and can bind to R-Smads thereby inhibiting signaling. Smad6 preferentially antagonizes the signaling of Smad-1, 5, 8, whereas Smad7 preferentially antagonizes the signaling of Smad2 and Smad3 (Figure 1).

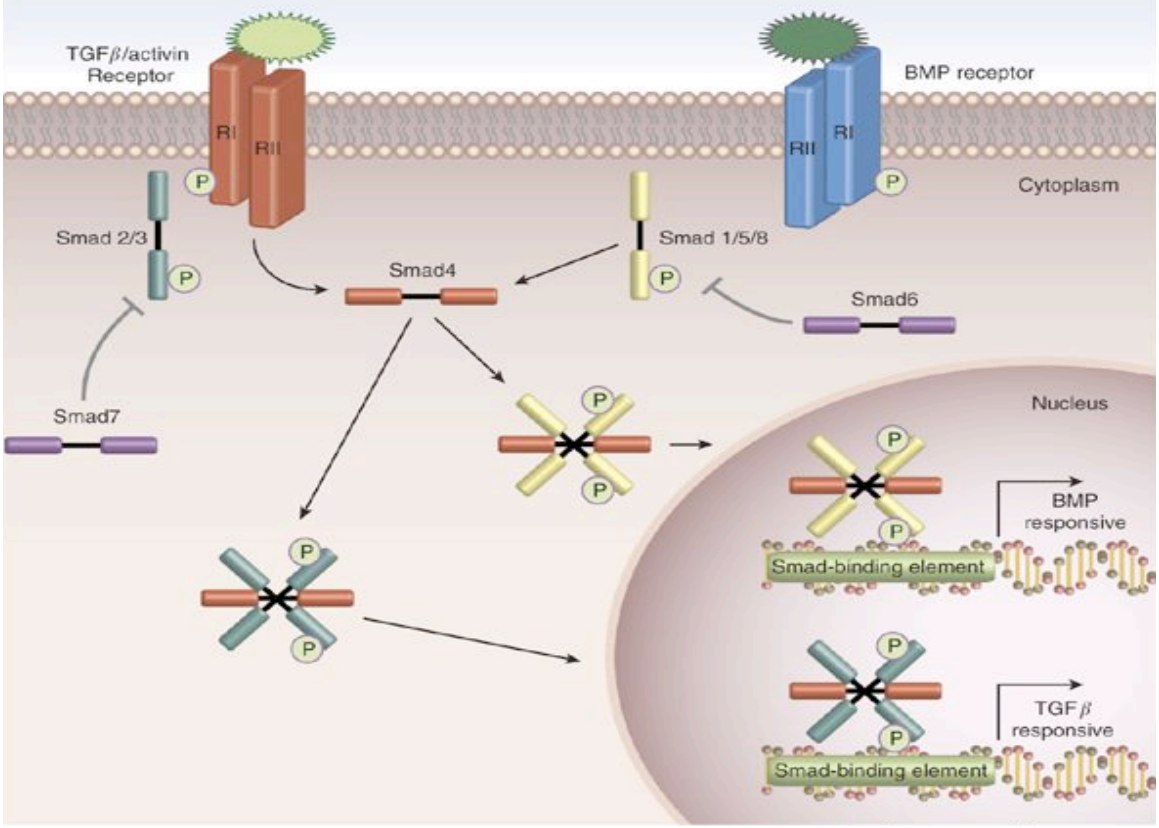


Figure 1. **Smad Signaling.** Smads are intracellular signaling molecules that are phosphorylated by the serine/threonine kinase receptors of transforming growth factor β (TGFβ)/Activin or bone morphogenetic protein (BMP). These receptors are activated (phosphorylated) upon ligand binding to their extracellular domains. Once phosphorylated, receptor-Smads (R-Smads) then partner with Smad4 where they form a hetero-trimeric complex and shuttle into the nucleus. Upon entry to the nucleus, Smads form diverse associations with transcription co-factors and bind site-specific Smad binding elements (SBE). Smad2 and Smad3 (Smad 2/3) are signaled from TGFβ/Activin ligands while Smad-1, 5 and 8 (Smad 1/5/8) are activated from BMP ligands. For each R-Smad group there lies an inhibitory Smad (I-Smad): Smad6 inhibits Smad 1/5/8 and Smad7 inhibits Smad 2/3. Figure from Owens et al., 2008b.

The mammalian epidermis and its appendages develop from a simple ectoderm to a stratified epithelium during embryogenesis. In mice, epidermal development begins at embryonic day 9.5 (E9.5). Upon birth, the epidermis has become fully differentiated, expressing differentiation markers for the terminal differentiation of the epidermis (Figure 2) for a review, see Fuchs and Raghavan, 2002.

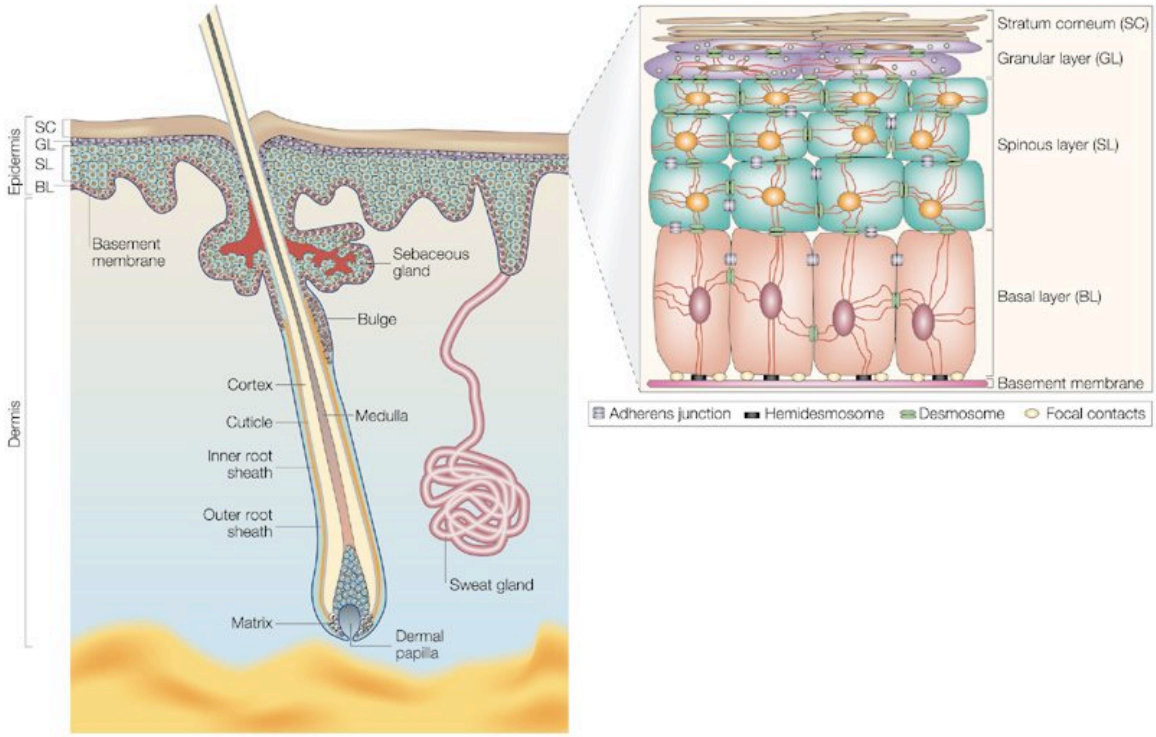


Figure 2**. The skin and its appendages.** Mammalian skin consists of the epidermis and dermis, separated by a basement membrane (BM). The epidermis is a stratified squamous epithelia. On the basement membrane is the basal layer consisting of the proliferating, transit amplifying cells. The differentiated cell layers above are the spinous layer, granular layer and lastly the stratum corneum. Cross-section of a hair follicle, which consists of an outer root sheath (ORS) that is contiguous with the basal epidermal layer. At the bottom of the follicle is the hair bulb, made from proliferating matrix cells. The transit-amplifying matrix cells terminally differentiate to generate the different cell types of the follicle. Also shown is the bulge, which is part of the outer root sheath and is where epidermal stem cells reside. The dermal component of the hair follicle is the dermal papilla. Figure from Fuchs and Raghavan, 2002.

Epidermal appendages (extruding protrusions) consist of hair follicles, sebaceous/sweat glands, mammary glands, teeth, nails/claws as well as external genitalia structures. Of these appendages, the best studied and understood is the hair follicle. The primary murine hair follicle, which gives rise to the guard hair (thicker and sparse), begins to develop at E14.5. Secondary hair follicles (awl and zigzag) begin to develop approximately two days later and make up the majority of hair follicles in mouse skin. When hair follicles develop, epidermal keratinocytes receive instructive signals from the

underlying mesenchyme (dermis), and then aggregate and form a condensation (placode). The placodes then become associated with underlying dermal aggregates and give rise to the hair germ. As hair follicles move downwards, the underlying dermal cells gradually concentrate and are engulfed by the broadened bottom of down-growing hair follicles (hair peg), forming dermal papillae (DP). The instructive signals are from, but not limited to, the pathways of WNT/β-catenin, hedgehog, fibroblast growth factor (FGF) and BMP. Once the hair follicles have been specified, they grow until approximately two weeks postnatally at which point they begin cycling. Hair cycling begins with the catagen or regressing phase. Once they are physically separated from the DP, the hair follicles enter into the telogen or resting phase. The transition from telogen to the anagen or regrowth phase utilizes the molecular mechanisms involved in hair follicle induction during embryonic hair development.

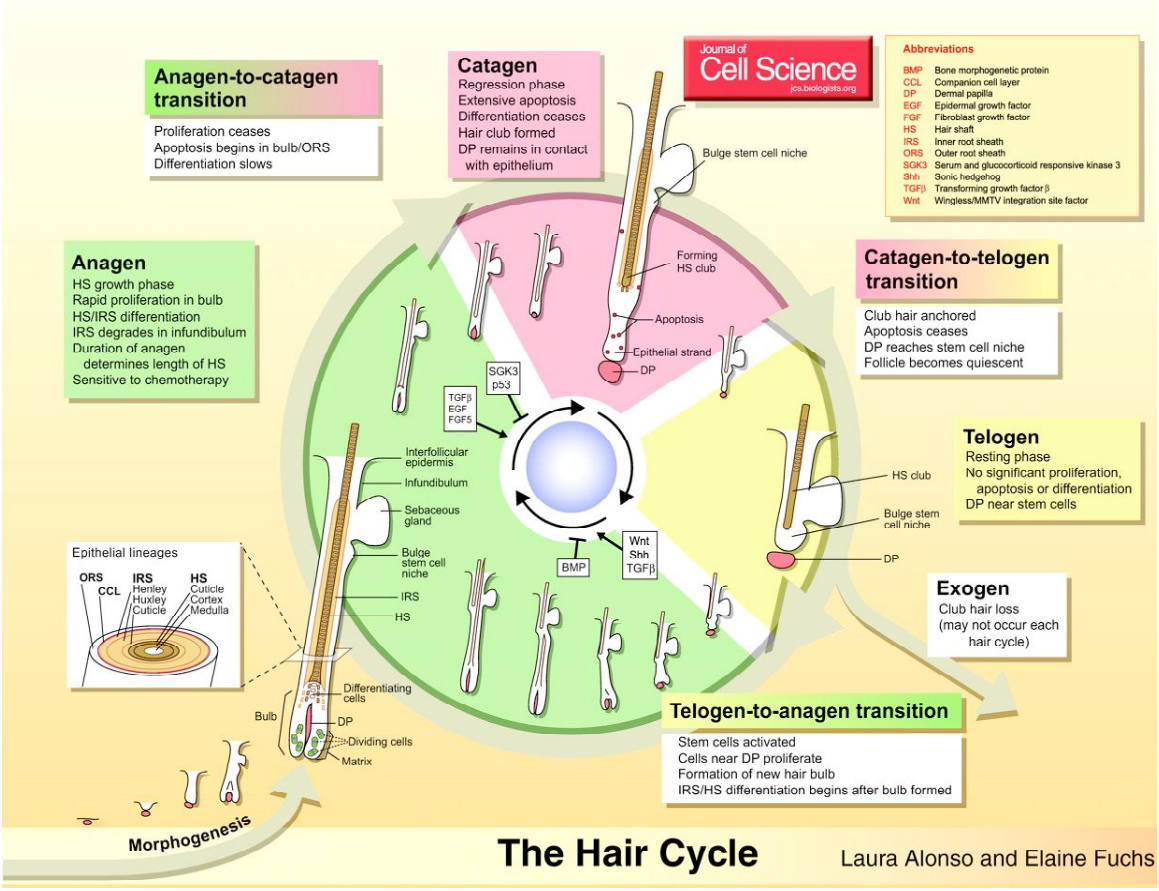


Figure 3. **The Hair Cycle.** Hair follicles alternate between phases of growth (anagen), destruction (catagen) and rest (telogen). Figure from Alonso and Fuchs, 2006.

Sebaceous glands develop in the first week after birth and alter their size proportionally with the associated hair follicles during hair cycling. Underneath the sebaceous glands, the size of the 'bulge' region remains persistent throughout the hair cycle, and has been widely characterized as a region of multipotent stem cells (for reviews, see (Blanpain and Fuchs, 2006; Moore and Lemischka, 2006). Delicate machinery that regulates stem cell fate and progenitor differentiation is thus required for proper skin development during embryonic stages and for the maintenance of tissue homeostasis. Recent studies have shown that expression of Smads and their target genes are enriched in the epidermal stem cell population (Morris, 2004; Tumbar et al., 2004). It

remains to be determined if all Smads play a role in skin development and stem cell homeostasis.

The contribution of the TGFβ superfamily to epidermal development

TGFβ and activin ligands activate Smad 2/3 via the type I receptors Alk-1, 4, 5 and 7. Among three TGFβ isoforms, TGFβ2 is both required and sufficient to induce hair follicles in mice (Foitzik et al., 1999), via a mechanism of RAS/MAPK activation and subsequent Snail transcription (Jamora et al., 2005). Although neither TGFβ1 nor TGFβ3 is required for hair follicle development, TGFβ1 is a potent inducer of catagen (Foitzik et al., 2000), and TGFβ3 has been shown to regulate epidermal migration during wound healing (Bandyopadhyay et al., 2006). In addition to TGFβ ligands, activin plays a role in skin development. Mice lacking activin βA lack vibrissae (whisker) follicles (Matzuk et al., 1995a). Germline deletion of follistatin, an activin-binding protein and antagonist, results in hyperkeratotic epidermis and abnormal development of vibrissae that appear thin and inappropriately orientated (Matzuk et al., 1995b). Coordinately, follistatin knockout mice and activin βA over-expressing transgenic mice show a delay of hair follicle morphogenesis. Treatment of wild-type embryonic skin explants with follistatin protein has been shown to stimulate hair follicle development. This effect was inhibited by addition of recombinant Activin-A protein. Activin βA transgenic mice demonstrate a failure of catagen entry and cannot cycle into the smaller resting hair follicles (telogen) (Nakamura et al., 2003). These findings highlight the important signaling and cross talk from mesenchymal cells of the dermal papillae to differentiated keratinocytes of the hair

follicle. Presumably, these ligands and antagonists coordinate their function via Smad activation.

BMPs consist of soluble proteins that activate Smads 1/5/8 via the type I receptors of ALK-2, 3 and 6. It has been difficult to study the requirement for BMP signaling in the epidermal development because different BMP ligands and receptors are expressed in different epithelial and stromal compartments or in cells of different lineages within the same compartment. Advances have mainly come from keratinocyte specific BMP transgenic mouse models (Botchkarev and Sharov, 2004). A recent study shows that BMP-2 induces transcription of canonical WNT/β-catenin family ligands and receptors to regulate cell fate in human keratinocytes (Yang et al., 2006). Many BMP ligands and their antagonists do not determine specific cell fate but help to regulate its proper timing and behavior of a specific cell lineage. For instance, administration of Noggin protein (subcutaneous bead implantation) into telogen mouse skin revealed that Noggin is sufficient to induce a new localized anagen (Botchkarev et al., 2001). Deletion of Noggin in mouse skin results in a lack of guard or secondary hair follicles (Botchkarev et al., 2001). More recent evidence demonstrates that mice overexpressing Noggin using a keratin 5 promoter were able to expand cell proliferation via affecting cell cycle related genes, thus controlling increased hair follicle size and cell number (Sharov et al., 2006). Furthermore, this study showed that when primary keratinocyte explants were treated with BMPs, they became quiescent and underwent growth arrest (Sharov et al., 2006). Additional evidence for the involvement of BMPs in skin development is presented in a study by Elaine Fuchs' laboratory showing that BMP6 is restricted in the DP and the stem cell niche (Fuchs et al., 2004). This finding suggests that BMP6 may be a potent

inducer of hair follicles from the matrix cells in the developing hair follicle, since transgenic mice overexpressing BMP-6 have enlarged hair follicles and increased hair fiber production (Blessing et al., 1996). Several studies have provided evidence for the requirement of ALK-3 (also known as Bmpr1A) in post-natal hair follicle maintenance. In these studies, ALK-3 was conditionally deleted in keratinocytes, which resulted in the collapse of the hair follicles postnatally (Andl et al., 2004; Kobielak et al., 2003; Ming Kwan et al., 2004; Yuhki et al., 2004). These reports all demonstrated that there was an ALK-3 requirement to make the differentiated matrix cells of the hair follicle. More recently, a study has shown that deletion of ALK-3 in the hair follicle leads to an expansion of hair follicle stem cells, which fail to utilize β-catenin to specify hair follicle lineages. In contrast, activation of the oncogene AKT and loss of tumor suppressor PTEN are found in these stem cells (Zhang et al., 2006). This report suggests that ALK-3 functions to define the progeny from the hair follicle stem cell niche and to regulate a balance of committed and undifferentiated cells (Zhang et al., 2006) It remains to be determined if the effects of these BMP ligands and receptors require spatio-temporal Smad activation.

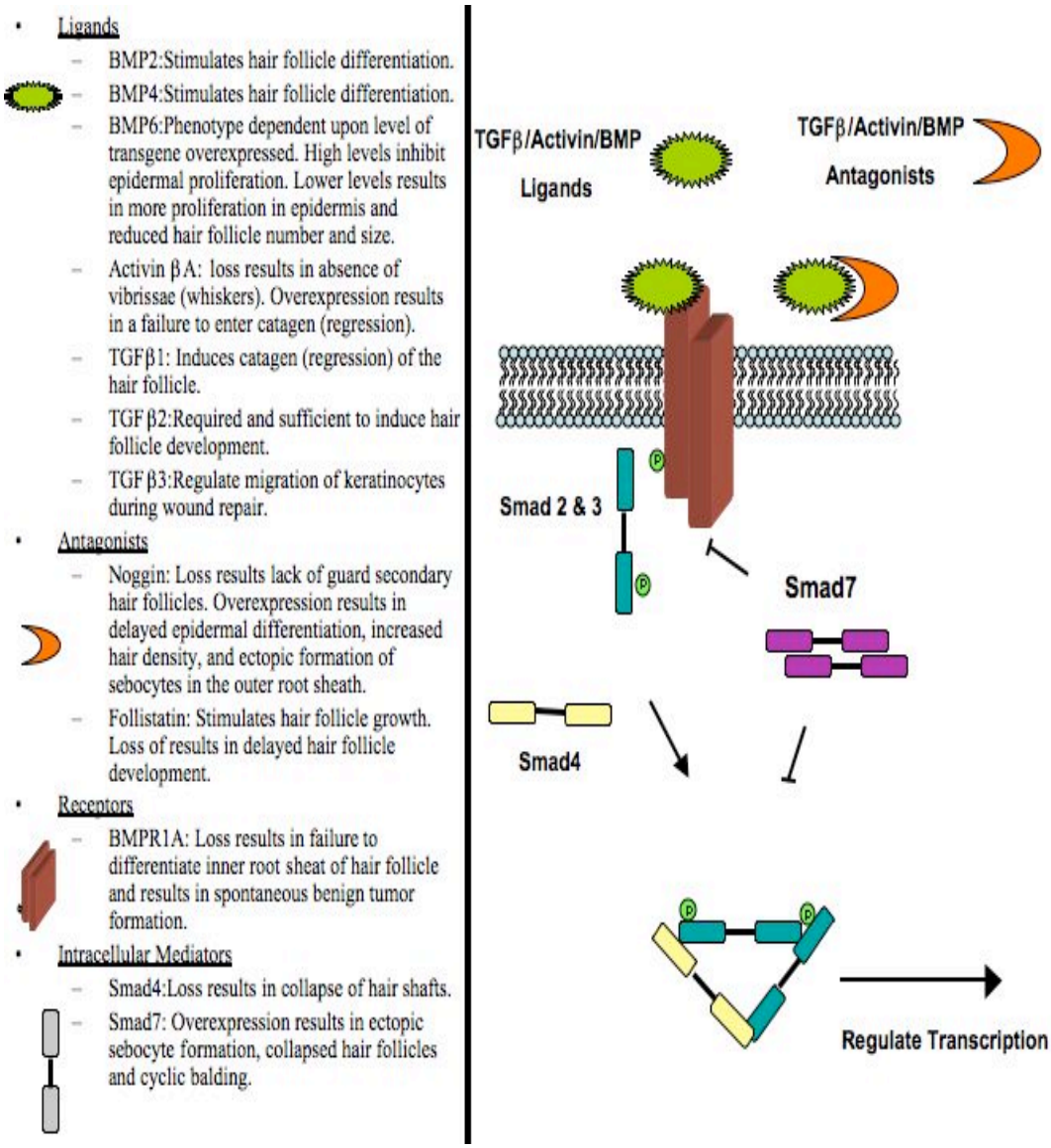


Figure 4. Skin phenotypes of the TGFβ superfamily. A description of the phenotypic consequences when TGFβ is disrupted at each level of signaling; ligand to receptor to transcription factor. This is only a summary of known phenotypes in genetically engineered mice, further development of mice with alterations in TGFβ/Activin/BMP signaling is currently under way. Extracellular ligands and their soluble antagonists regulate the formation of differentiated hair follicles and epidermal differentiation. All three ligand sub family members are sufficient to contribute to proper differentiation of the hair follicle. Antagonists such as Noggin are complicated and function in dose dependent and spatially restricted compartments. Misexpression of Noggin typically results in producing more hair follicles and/or delaying the catagen (regression) phase. Controversy still remains on the exact location and magnitude required for antagonists to specify normal hair follicle growth and cycling. On the receptor level only the type I

BMP receptor A has demonstrated a requirement for lineage of the inner root sheath in the hair follicle. Intracellular mediators Smads 4 and 7 show similar yet different phenotypes when lost or overexpressed respectively. Loss of Smad4 results in failure to maintain normal adhesion in the hair shaft while Smad7 when overexpressed degrades the hair follicle inducing protein βcatenin.

R-Smads: Little effect on skin development

Regardless of the profound effects of TGFβ superfamily members and receptors on skin development and abundant expression levels of Smads 1-5 in the epidermis and hair follicles (He et al., 2001), studies have revealed little effect of individual R-Smads on skin development and differentiation. Germline Smad2 knockout mice die at the embryonic stage prior to hair follicle development due to the failure of the germ layer specification and primitive streak formation (Waldrip et al., 1998). Transgenic mice that overexpress Smad2 by the keratin 14 promoter exhibit delayed hair growth, underdeveloped ears and shortening of their tails. Further analysis suggests that these phenotypes correlated with enhanced signaling from TGFβ and activin (Ito et al., 2001). It remains to be determined if there is any pathological conditions in which Smad2 is overexpressed in the skin. At the physiological level, Smad2 alone appears to have little effect on skin development, as our unpublished data reveal that keratinocyte-specific Smad2 deletion does not result in any abnormalities in skin development and differentiation. Similarly, Smad3 knockout mice also have no apparent phenotype in epidermal development. These Smads are broadly expressed in all cell types of the skin and there expression is not restricted temporally or spatially. With respect to Smad-1/5/8, very little is known about the individual roles of these Smads in the epidermis. Antibody

staining, which recognizes all three phosphorylated forms, thereby serving as a ‘readout’ for BMP activity, revealed abundant pSmad-1/5/8 staining in both interfollicular epidermis and hair follicles (Han et al., 2006). Some evidence for the involvement of these smads in skin development and differentiation has been shown by their known co-factors and binding partners. For instance, immunoprecipitation experiments of Smad1 and Smad4 identify that they form a transcriptional complex on the promoter of the Dlx3 transcription factor (Park and Morasso, 2002). Dlx3 is a homeodomain transcription factor that helps pattern and specify cell fate in keratinocytes, and its expression and is induced by BMP-2 (Park and Morasso, 2002). Another potential link to Smad-1/5/8 may be RUNX3, a transcription factor often demonstrated to partner with Smad-1/5/8 during osteoblast formation. Runx3 was found to be a critical determinant of hair shape as well as nail and gland formation (Raveh et al., 2005).This observation is interesting because of the extensive work demonstrating that Runx family members are important transcriptional co-activators with BMP induced Smad signaling for osteoclast/bone formation . However, direct evidence for a requirement for BMP-specific Smads in skin development is lacking. Our unpublished data reveal that keratinocyte-specific deletion of either Smad1 or Smad5 does not affect skin development and differentiation. To date, there is no report for Smad8 function in the epidermis. Due to the structural similarity and the lack of antibodies specific for individual BMP-specific Smads, it is difficult to determine if they compensate for each other’s loss. Further analysis of Smads 1/5/8 is warranted, and a reexamination of distinct differences in the skin may be revealed with improved experiments.

Smad4: the bridge between R-Smads

The lack of skin phenotypes in R-Smad knockout keratinocytes suggests that individual Smads may be able to compensate for each other's loss during skin development and differentiation. Indeed, when Smad4 is deleted in keratinocytes, which results in abrogation of most Smad signaling, hair follicles collapse and tumors arise from the skin (Qiao et al., 2006; Yang et al., 2005). These mice lose post-natal hair follicles prior to the first catagen entry. They also develop spontaneous squamous cell carcinomas. Interestingly, the hair follicle phenotypes are very similar to those in epidermal specific ALK-3 knockout mice (Andl et al., 2004; Kobielak et al., 2003; Ming Kwan et al., 2004; Yuhki et al., 2004), which illustrates a possible role for Smad4 in skin development downstream of BMP signaling, but not for Activin and TGFβ signaling (Figure 1). Smad4 has been identified to partner with Lef1 at the Msx2 promoter (Hussein et al., 2003); these two proteins are in the WNT/β-catenin signaling cascade, which is important for hair development and differentiation. Therefore, Smad4 may cooperate with WNT/β-catenin signaling at the transcriptional level. Another transcriptional partner of Smads as shown in T-cells is GATA-3. Smad3 was shown to partner with GATA3 and regulate cell fate in helper T-cells (Blokzijl et al., 2002). In the skin, GATA3 is expressed specifically in the inner root sheath (IRS) (Kaufman et al., 2003). Mice with a GATA3 gene deletion exhibited a collapsed hair follicle phenotype (Kaufman et al., 2003) similar to the keratinocyte-specific Smad4 knockout phenotype. It remains to be determined if GATA-3 and Smads 3/4 form a functional transcription complex that specifies the differentiated IRS of the hair follicle. Another interesting Smad partner may be FOXO factors which,

much like Smads, are proteins that shuttle between the nucleus and cytoplasm and are found to be expressed in human keratinocytes (Gomis et al., 2006). FOXO transcription factors belong to a winged helix domain of transcription factors, which is a large family that also includes FoxN1, a transcription factor responsible for the nude mouse phenotype (Mecklenburg et al., 2001). It remains to be determined if FoxN1 has a direct interaction with Smads, as has been demonstrated with other FOXO family members.

Smad4 has been considered an intriguing link among Smad signaling because it is widely required for many Smad-associated processes. This notion has been challenged by the observation that in HaCaT keratinocytes with an engineered and inducible system for Smad4 reduction, Smad transcriptional activity still occurred (Levy and Hill, 2005). Further evidence from the hematopoeitic system has shown that Smad4 loss results in new stoichiometric accumulations of R-Smads with the TIF1γ transcription factor (He et al., 2006). TIF1γ is exclusively nuclear and does not aid in the shuttling of Smad2/3 into the nucleus. This study shows that even when Smad4 is removed, phosphorylated R-Smad complexes may still bind their target genes. For the above reasons, it is not surprising that epidermal and hair follicle development is largely intact when Smad4 is deleted in keratinocytes. However, regardless of the initial normal embryonic skin development, homeostasis in Smad4-deleted keratinocytes apparently is lost, which eventually results in skin tumor formation. Evidence for Smad4 independent signaling has been observed by localizing phosphorylated R-Smads in the nucleus of cells devoid of Smad4 (ten Dijke and Hill, 2004). It is interesting to note that of all phenotypes observed the closest phenocopies are from Smad4 and ALK-3 conditional epidermal

knockouts (Figure 4). This may imply that the role of Smad4 in the epidermis is to specify the hair follicle via BMP signaling.

I-Smads: Restricting and Refining R-Smads

Of the two I-Smads: Smad6&7, abundant Smad6 mRNA was detected in the developing mouse skin at E15 (Flanders et al., 2001). This report also showed that the vibrissae follicles lack Smad6 expression. There have been reports of transient expression or loss of expression of Smad6 from early mouse epidermal development to pathological skin conditions. For instance, Smad6 and Smad7 are lost in keloids, which are benign tumors in the skin that result from over-accumulation of extracellular matrix (ECM) proteins (Yu et al., 2005). As a result, the failure to restrict TGFβ/BMP signaling results in overproduction of pathological levels of ECM. Further evidence of Smad6 functioning in skin pathology remains to be identified. Little or no expression of Smad6 is detected in adult mouse skin (Chen et al., 2002) and the role of Smad6 in skin development is unknown. A thorough examination of Smad6 expression patterns during different developmental stages and physiological/pathological states of the skin will serve as an initial step toward determining which, if any, role Smad6 plays in the skin. It is possible that Smad6 has different roles in mouse skin compared to human skin diseases and development.

To date, Smad7 has shown very dramatic effects on skin development. Smad7 is expressed at a very low level in normal keratinocytes (He et al., 2001), but is often overexpressed under pathological conditions, e.g., in intrinsically aged and photo-aged

human skin (Chen et al., 2002; Quan et al., 2002) and during skin carcinogenesis (He et al., 2001). To assess the overall role of Smad signaling in skin development, our lab previously generated Smad7 transgenic mice, which overexpress Smad7 under the control of a keratin 5 promoter (K5.Smad7) to levels that are sufficient to block Smad signaling from TGFβ/activin and BMP (Chen et al., 2002). These transgenic mice exhibited multiple developmental defects in the stratified epithelia, including decreased hair follicle size. These mice die postnatally due to epithelial hyperkeratosis in the upper digestive tract and severe thymic atrophy (Chen et al., 2002). Since the phenotype severity correlates with the degree of Smad signaling inhibition, these data suggest the importance of Smad signaling in skin development. This information would not necessarily be revealed in individual Smad knockout skin if the Smads have functional redundancy, as previously indicated. However, one K5.Smad7 transgenic line, which expressed a low level of Smad7 transgene, and therefore did not block all Smad2 signaling, still exhibited a hair loss phenotype (Han et al., 2006). The phenotypes observed did not parallel the opposite signaling targets of TGFβ and also did not show a direct phenotype association with BMP mutants. To date, only this component (Smad7) has a specific cycling phenotype of balding.

To further understand the mechanism, our lab had previously created Smad7 transgenic mice, in which the Smad7 transgene expression can be induced in keratinocytes at different developmental stages and with different levels in keratinocytes, including epidermal stem cells (Chen et al., 2002). This inducible transgenic system consists of a transactivator line (GLp65) (Cao et al., 2002; Lu et al., 2004) and a target line (tata.Smad7). The K5 vector was used to target the GLp65 transactivator to the basal

layer of the epidermis and the Outer Root Sheath (ORS) of the hair follicle, including epidermal stem cells (Arin et al., 2001). The target transgene (tata.Smad7) consists of a GAL4 UAS enhancer upstream of a tata minimal promoter (Cao et al., 2002; Lu et al., 2004) and cDNA encoding mouse Smad7 with a Flag tag (Chen et al., 2002). RU486 is used to regulate transgene expression in bigenic mice (GLp65/tata.Smad7). This gave us the ability to control Smad7 expression at a pathologically relevant level and in an acute or a sustained manner, which furthered our analysis of the underlying molecular mechanisms. By using a bimodal system we have been able to successfully identify novel phenotypes from varying the level of the Smad7 transgene. This model has also helped our lab to determine the role of Smad7 overexpression at stages critical for stem cell fate decision, differentiation and in postnatal hair cycling.

Our lab found that Smad7 transgene induction resulted in a significant delay in embryonic hair follicle development and a complete blockade of hair follicle differentiation (Han et al., 2006). When transgenic Smad7 expression was induced in a sustained manner beginning either on E10.5 or on E14.5, the effect on bigenic mouse skin from E16.5 throughout P1 (post-natal day 1) was essentially identical. In both cases, histological examination showed that hair follicle morphogenesis was delayed in Smad7 transgenic skin in comparison with normal skin. On E16.5, hair follicles in normal embryos are in developmental Stage 2 or Stage 3, in which follicles appear to be in the form of a hair germ or peg, respectively. In contrast, only scattered hair follicle placodes were observed in Smad7 transgenic embryos. On E18.5, certain follicles in control skin have entered Stage 4, characterized by the beginning of IRS formation and engulfment of dermal papilla within the hair bulb. In contrast, Smad7 E18.5 hair follicles were in Stages

1 and 2. In P1 normal skin, more than 50% of the hair follicles have passed Stage 4, and some have entered Stage 5, evidenced by the presence of melanin in the precortex and an elongated IRS (Han et al., 2006). However, the majority of the hair follicles in P1 Smad7 transgenic skin were in Stages 2 and 3, equivalent in appearance to the E16.5 normal embryonic hair follicles. After birth, if Smad7 transgene induction was continuously maintained, hair follicle differentiation was abrogated. On P10, normal skin hair follicles have each formed a well-differentiated hair shaft (Han et al., 2006). However, Smad7 transgenic follicles were much shorter and disoriented with prominent sebaceous glands and clustered melanin. AE15 and AE13, hair follicle differentiation markers, normally stain IRS cells and medulla cells of the hair shaft (AE15), and upper cortical and cuticle cells (AE13). This staining is absent in Smad7 transgenic hair follicles, which appear disorganized and lacked any obvious IRS or hair shaft. In contrast, sebaceous gland development was significantly accelerated in Smad7 transgenic skin (Han et al., 2006). Sebocytes are visible on P1 in Smad7 transgenic skin. Again, when the Smad7 transgene is expressed at levels high enough to inhibit Smad signaling, these skin phenotypes are exacerbated and epidermal differentiation is perturbed. However, even when Smad7 is expressed at a lower level, which does not affect Smad signaling, these skin abnormalities still occur (Han et al., 2006).

Further analysis revealed that independent of its role in anti-Smad signaling, Smad7 bound β-catenin and induced β-catenin degradation by recruiting an E3 ligase, Smurf2, to the Smad7/β-catenin complex. Consequently, WNT/β-catenin signaling was suppressed in Smad7 transgenic hair follicles. Co-expression of Smurf2 and Smad7 transgenes exacerbated Smad7-induced abnormalities in hair follicles and sebaceous

glands. Conversely, when endogenous Smad7 was knocked down, keratinocytes exhibited increased β-catenin protein and enhanced WNT signaling. These data suggest that even at a low, physiological level, endogenous Smad7 participates in β-catenin turnover (Han et al., 2006). This mechanism also explains the phenotype difference between Smad4-/- skin and Smad7 transgenic skin, which cannot be explained by the difference in the degree of abrogating Smad signaling. Although Smad4 also potentially affects WNT/β-catenin signaling at the transcriptional level, the direct effect of Smad7 on β-catenin degradation appears to be more potent for affecting WNT/β-catenin signaling. This is likely why Smad7 transgenic, but not Smad4-/- skin, exhibits delayed hair follicle development (Figure 5).

To date, the most significant advances in our understanding of Smad functions in the skin have come from mouse models with spatio-temporal expression/ablation of individual Smad genes. In the future, thorough examination of Smad expression patterns in developmental stages and pathological conditions of the skin (e.g., wound healing, cancer, various skin diseases) will provide the most valuable information about Smad function in the epidermis. More complex genetic approaches such as knocking out more than one Smad gene in keratinocytes and combination of these knockouts with trangenics overexpressing TGFβ family ligands will further elucidate the overlapping and compensatory functions of individual Smads in the skin. The recent finding that Smad7 functions via the WNT/β-catenin pathway demonstrates that Smad proteins are not simply the mediators/antagonists of TGFβ signaling. Smad4 and Smad7 have been the most informative for function in the development of the skin. These similarities and differences are summarized in figure 5. It remains to be determined how other pathways

are integrated by Smads and under what physiological/pathological conditions these interactions occur.

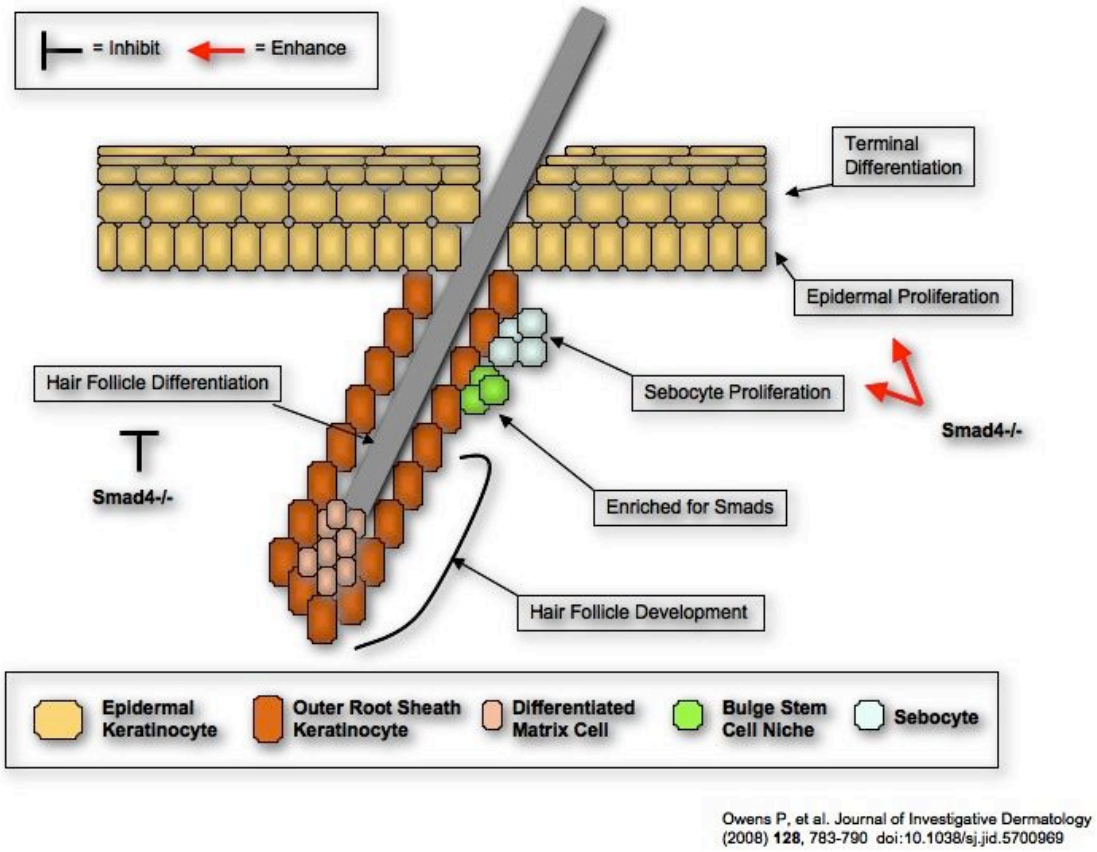


Figure 5. **Smad signaling in skin development.** Smads are highly enriched in the hair follicle bulge stem cell niche. Sebocytes hyperproliferation occur when Smad-4 is absent or Smad-7 is overexpressed. Smad-4 loss or Smad-7 increase also results in epidermal hyperproliferation. Terminal differentiation of the epidermis is blocked by Smad-7 overexpression. Figure from Owens et al., 2008b.

1.2 TGFβ/Smad signaling in cutaneous wound healing

The ability to repair a wound is essential to the success of any living organism. In humans the ability to heal a wound well and without scar are critical determinants to health and conviviality. In addition, scars have profound psycho/social effects that limit quality of life. The skin is on the 'frontlines' to protect from an unending number of pathological insults. In the U.S. over a million people will experience burns annually (Brigham and McLoughlin, 1996) and chronic non-healing wounds are a significant complication to almost all surgical procedures. Progress in studying the mechanisms involved in wound repair and tissue engineering have resulted in improvements in wound care strategies (Clark et al., 2007). Cutaneous wound healing is a very complex, multi-cellular, sequential process that requires extreme cellular organization and extracellular coordination (Figure 5). Wound healing occurs in three phases: inflammation, tissue formation, and tissue remodeling (Figure 5). These distinct processes overlap in time and location. Every process in wound healing is dynamic and for every increase at some point there must be a corresponding decrease to achieve homeostasis. This reason has made therapy extremely difficult because amplifying a single aspect of wound healing does not allow a natural refractory period to coordinate in a native microenvironment. While a breakdown of the individual players helps to understand the forces in a wound, it cannot be underscored enough that all of these events are inter-coordinated and can influence the mutual outcomes.

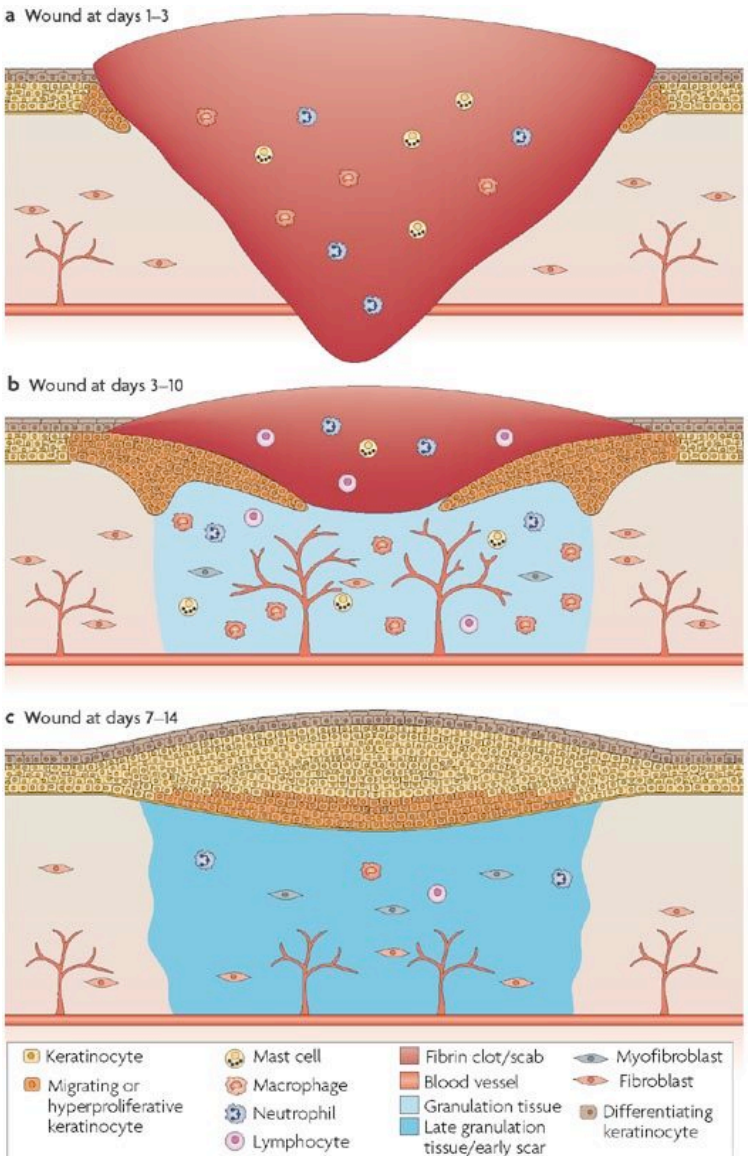


Figure 6. **The phases of skin wound repair.** A) The inflammatory phase. 1-3 days after injury the wound consists of a clot and inflammatory cells (neutrophils, mast cells, macrophages and lymphocytes). B) New tissue formation. 3-10 days after injury, macrophages are abundant in the wound tissue and new blood vessels appear. Fibroblasts migrate, proliferate and deposit ECM. Some of them differentiate into myofibroblasts. The new tissue that fills the wound is known as granulation tissue. At the wound edge, keratinocytes from the injured epidermis and hair follicles migrate along the injured dermis and above the provisional matrix, and their rate of proliferation is increased. C) Tissue remodeling. 1-2 weeks after injury, wound reepithelialization is finished, the cellular density decreases and the ECM is remodeled. This results in the formation of a scar with reduced tensile strength. Figure from Schafer and Werner, 2008.

Inflammation

When a wound breaks the skin through the epidermis or further it causes blood vessels to be broken and release blood. Within the blood are platelets, which begin to immediately form a clot to control further loss of blood (Figure 7). The next major cell type to respond are neutrophils, whose major role is to propagate the inflammation process by secreting cytokines/chemokines/growth factors into the wound. Neutrophils also play a key role in removing debris and contaminants (bacteria, etc.) from the wound. The third major cell type is the monocyte/macrophage which also has an important role in 'cleaning up' the debris and contaminants via phagocytosis. Fibroblasts that have also been recruited to the wound and are actively proliferating, secrete Extra-Cellular Matrix (ECM). This ECM is critical to all these cell types as it will stimulate the ligation of integrins to the matrix and continue the functions of healing the wound (Brown, 1995). Different cell types secrete many important factors, but TGFβ appears to enhance this early inflammatory phase of wound healing (Rappolee et al., 1988). All of these inflammatory cells have competence to respond to TGFβ and monocyte/macrophages are greatly stimulated by its presence. Additionally, all of these cells are sufficient to produce TGFβ, making this a complex and difficult to control system (Figure 7).

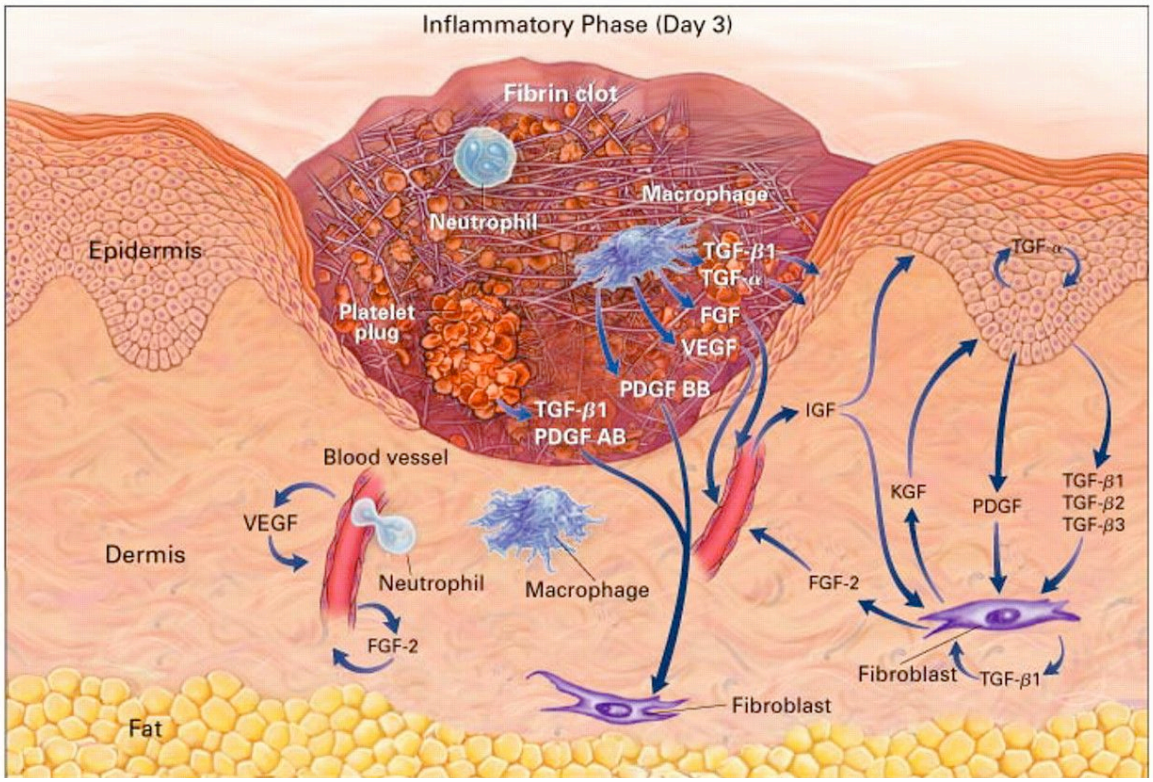


Figure 7. **The Inflammatory phase of wound healing.** Wound healing begins with the appearance of inflammatory cells. First neutrophils and platelets and then macrophages invade the wound. All cells from the epithelium and stroma are capable of secreting a vast array of growth factors and cytokines necessary to perpetuate the inflammatory response. Figure from Singer and Clark, 1999.

Reepithelialization

Keratinocytes once disrupted begin to migrate in search of other keratinocytes across the wound that will close the wound and allow homeostasis (Figure 8). The keratinocytes affected at the 'leading edge' of the wound (also known as a migrating tongue) relax their intracellular adhesion components and form actin based protrusions (fillopodia) in search of ECM to guide them to close the wound (Goliger and Paul, 1995; Paladini et al., 1996). Once the platelets have created a dense provisional 'plug' which is rich in the ECM fibrin, the keratinocytes will begin to migrate underneath and thereby

choose where the new ‘barrier’ will be. Keratinocytes express Matrix-Metallo Proteases (MMPs), which allow for degradation of ECM and obstacles to migrating into the wound bed (Pilcher et al., 1997). As the keratinocytes move farther and farther into the wound the original margins of the wound begin to restore their normal adhesions even prior to full closure (Clark et al., 1982). Normal terminal differentiation of keratinocytes ‘push’ the wound away form the body and through the normal sloughing of squamous cells, the scab will fall away (Figure 8).

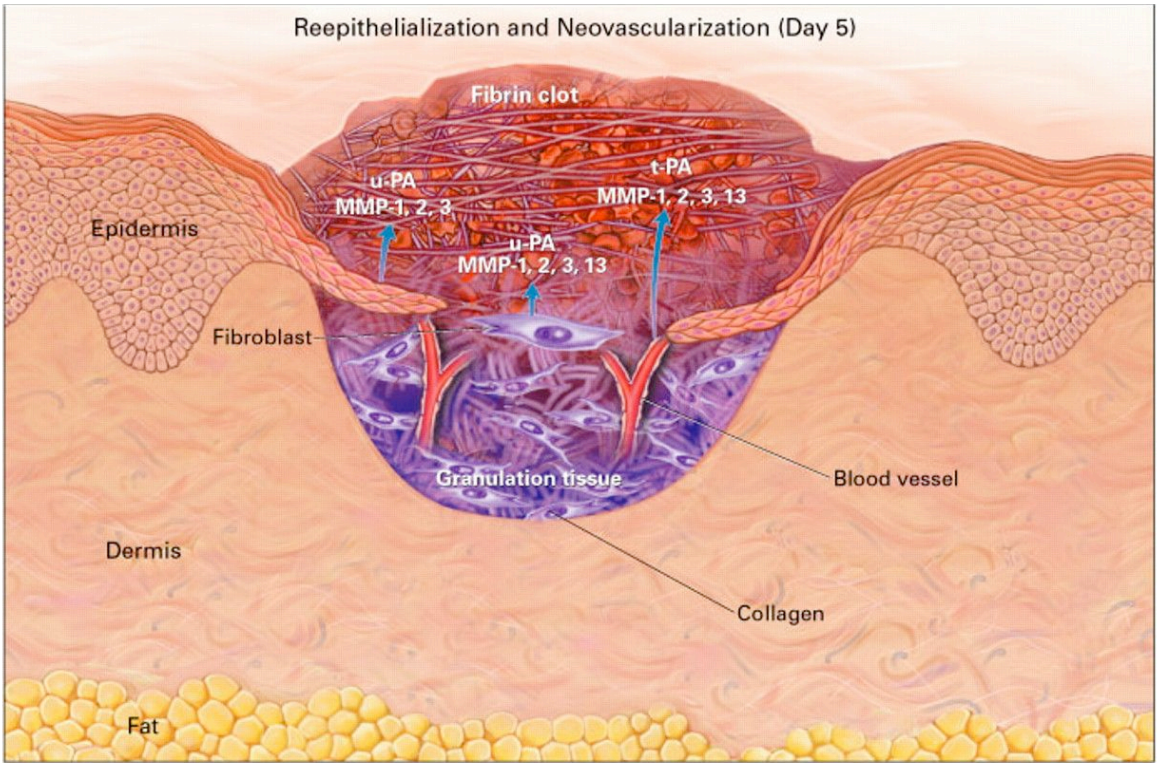


Figure 8. **Reepitheliazation of wounds.** Once a stable blood clot has been formed and inflammation has begun to subside, keratinocytes from the wound edge will begin to migrate into wound bed. Keratinocytes express matrix metallo-proteases (MMPs) used to digest the provisional matrix of the granulation tissue just underneath the fibrin rich clot. Figure from Singer and Clark, 1999.

Matrix Remodeling

The wound has experienced a mechanical disruption of the stroma, inflammatory cells coming and going, and keratinocytes 'chewing' their way through the wound bed. Now the wound must repopulate the dermis with new ECM and resting fibroblasts. New granulation tissue (fibrous connective tissue) begins to grow upwards into the wound space three days after the initial wound (Figure 6). Proliferating fibroblasts respond to TGFβ by secreting ECM and differentiating to myofibroblasts (Gray et al., 1993; Singer and Clark, 1999; Xu and Clark, 1996). This new complement of ECM being secreted is termed the 'provisional' matrix. This new matrix is a necessary scaffold for cell migration, which is directed by integrin specific receptors (McClain et al., 1996; Xu and Clark, 1996). Once the provisional matrix has been replaced by new collagen, the invading cells can no longer make their integrin specific adhesions and begin to die and the dermis reverts to a collagen rich acellular environment (Desmouliere et al., 1995).

Angiogenesis in the healing wound

The disruption of the blood vessels has initiated the wound repair process and during healing must deliver cells and molecules to the wound, but it must also be repaired and form a new homeostatic network throughout the wound. New blood vessels also use the provisional matrix to migrate appropriately into the wound (Madri et al., 1996). Angiogenesis can be stimulated by many signaling molecules; including vascular endothelial growth factor (VEGF), TGFβ, Angiopoietin1&2, and Thrombospondin

(Folkman and D'Amore, 1996; Iruela-Arispe and Dvorak, 1997; Risau, 1997). Many of the angiogenic factors are secreted by the migrating keratinocytes as they 'chew' their way through the provisional matrix (Figure 7)(Brown et al., 1992). Protease and MMP cleavage in the wound from migrating cells actually creates substrates for endothelial cells of blood vessels to stimulate growth and migration into the wound (Figure 8). As soon as new connective tissue has filled the wound, then angiogenesis will cease and without new tissue, many vessels will regress and undergo programmed cell death (Ilan et al., 1998). Angiogenesis can be a 'double-edged sword' whereby too much early while delivering important helpful molecules can also deliver too many factors that prevent useful clotting, alternatively not enough could result in failure to form clotting. Towards the end phases of wound healing too little may cause poorly vascularized tissue leading to necrosis of the skin and too much may lead to inflammatory and painful swelling skin conditions. Angiogenesis must be coordinated within a precise tolerance in defined space and time.

Paradoxical roles of TGFβ during cutaneous wound repair

After injury, TGFβ is rapidly upregulated and secreted by keratinocytes, platelets, and macrophages (Singer and Clark, 1999). TGFβ1 is essential for initiating inflammation and granulation tissue formation (McCartney-Francis and Wahl, 1994). Additionally, TGFβ1 plays an important role in cell migration during wound repair. Some of the proteases that are involved in cell migration, such as MMP1, MMP2, MMP3, and MMP9, are transcriptionally regulated by TGFβ1 (Ellenrieder et al., 2001;

Madlener et al., 1998; Santibanez et al., 2000; Verrecchia et al., 2001). Cell migration-associated integrins, such as β1, α5, αv, β5, and β6, are also regulated by TGFβ1 (Gailit et al., 1994; Zambruno et al., 1995). These integrins affect keratinocyte and fibroblast migration (Gailit et al., 1994; Zambruno et al., 1995). TGFβ1 has also been shown to stimulate wound contraction (Montesano and Orci, 1988) through its direct induction of αSMA expression in fibroblasts (Desmouliere et al., 1993). Furthermore, TGFβ1 stimulates the production of ECM molecules, including collagens and fibronectin, which strengthen the healing wound. Reduction of TGFβl has been observed in humans with impaired wound healing, particularly in diabetic foot ulcers and chronic venous leg ulcers (Cowin et al., 2001; Jude et al., 2002; Schmid et al., 1993). In addition, many animal models of impaired wound healing exhibit reduced TGFβ1 expression and the rate of healing is improved by the application of exogenous TGFβ1 (Beck et al., 1990; Pierce et al., 1989; Salomon et al., 1990). Based on these studies, it is not surprising that TGFβ1 has long been considered as a promising potential therapeutic agent for impaired wound healing. Despite early studies showing that injection of TGFβ1 to wounds accelerated healing in experimental animals (Mustoe et al., 1987; Sporn and Roberts, 1993), clinical trials that exogenously administered TGFβ1 to human chronic ulcers have achieved very limited efficacy (Mulder, 2004).

Recent studies have begun to challenge whether TGFβ1 can be used as a therapeutic agent for impaired wound healing. The first evidence for this was from wound healing using Smad3 null mice, in which TGFβ signaling is partially abolished. Instead of the predicted delay in healing, Smad3 null mice showed accelerated wound healing, as seen by increased keratinocyte proliferation and migration, and reduced

monocyte infiltration (Ashcroft et al., 1999). Consistently, deletion of the secretory leukocyte protease inhibitor, a molecule that is required for normal wound healing, results in delayed wound healing that is characterized by increased TGFβ1 activation and can be attenuated by TGFβ1 antibody (Ashcroft and Roberts, 2000). More direct evidence comes from recent studies, in which transgenic mice expressing the TGFβ1 transgene in keratinocytes exhibited delayed healing after burn injury (Tredget et al., 2005; Yang et al., 2001). Additionally, TGFβ1 knockout mice showed accelerated reepithelialization during incisional wound repair, in comparison with wildtype mice (Koch et al., 2000; O'Kane and Ferguson, 1997). A better wound-healing outcome may be achieved by selectively blocking the negative effects of TGFβ1. In supporting this notion, transgenic mice overexpressing a dominant-negative TGFβ receptor restricted to keratinocytes exhibit increased reepithelialization in full thickness skin wounds. This is associated with an increase in proliferation and reduced apoptosis in keratinocytes at the wound margins, owing to the resistance of keratinocytes to TGFβ-induced growth arrest and apoptosis (Amendt et al., 2002). Similarly, administration of exogenous Smad7, an antagonist of TGFβ signaling, to mouse eyes accelerates corneal wound healing via promoting epithelial cell migration and inhibiting monocyte/macrophage invasion to the wounds (Saika, 2004; Saika et al., 2005). Taken together, selective suppression of TGFβ signaling in certain cell types in cutaneous wounds may potentially benefit wound healing. Both of these systems function by preventing phosphorylation of R-Smads 2&3, which prevents activation of transcriptional target genes.

Constitutive overexpression of TGFβ in keratinocytes delays wound healing owing to its pro-inflammatory functions

Previously reported studies prompted our lab to hypothesize that endogenous TGFβ1 may have a negative impact on wound healing, at least partially owing to its role in the induction of inflammation. TGFβ1 has long been known for its dual effects on inflammatory response and immune modulation. The pro-inflammatory effect of TGFβ1 has been overlooked ever since it was shown that TGFβ1 knockout mice exhibit inflammation in multiple organs and autoimmune conditions, which demonstrate an anti-inflammatory role for TGFβ1 (Kulkarni et al., 1993; Shull et al., 1992). However, the *in vivo* role of TGFβ1 in regulating inflammatory/ immune response may be organ specific. For instance, TGFβ1 knockout mice do not develop any inflammatory phenotypes in the skin and are devoid of Langerhans cells in the epidermis (Kulkarni et al., 1993; Shull et al., 1992). Because TGFβ1 is a potent chemotactic cytokine for virtually all leukocytes as well as endothelial cells and fibroblasts (Wahl, 1999; Wahl and Chen, 2005; Wahl et al., 1993), all of which are involved in the development of inflammation, the proinflammatory effect of TGFβ1 is likely to predominate in the skin. In supporting this notion, overexpression of TGFβ1 in basal keratinocytes and hair follicles initiates chronic skin inflammation, marked by epidermal hyperplasia, leukocyte infiltration, and angiogenesis (Li AG, 2004). In addition, our lab had previously observed that during cutaneous wound healing, endogenous TGFβ1 increased rapidly upon injury and reached a peak level 3 days post wounding, which coincides with the peak of the inflammation

phase during early stages of wound healing. Our lab had further assessed the wound-healing process in K5.TGFβ1 transgenic mice, which spontaneously develop an inflammatory skin disorder which resemble psoriasis (Li AG, 2004). Notably, almost all of the K5.TGFβ1 mice developed spontaneous skin ulcers in the friction-prone areas by 4 to 5 months of age. Histology on the skin ulcers revealed an absence of epidermis, massive inflammatory cells in the dermis, and prominent angiogenesis, resembling granulation tissues during wound healing. To further understand the consequence of TGFβ overexpression, wound healing studies on the dorsal skin of 8-week-old transgenic K5.TGFβ1 mice were undertaken. At this age, the hair follicles are synchronized at the telogen phase (Alonso and Fuchs, 2006), and the inflammation and epidermal hyperplasia are not yet as severe in transgenic skin. Five mice in each group were evaluated at each post-wounding time point. Both male and female mice were used in the study, and there was no noticeable difference in the wound healing kinetics between the sexes. Wound closure in non-transgenic mice occurred visibly during the first week, whereas the wound areas were barely changed in K5.TGFβ1 mice. The scabs on non-transgenic wounds detached at about day 10 post-wounding, but the scabs remained on transgenic wounds, even at day 21 post-wounding. Consistent with delayed wound closure, immunohistochemistry revealed an approximately 3-fold decrease in keratinocyte proliferation in the migrating tongue of K5.TGFβ1wt wounds compared to that in non-transgenic wounds. This observation is in contrary to that observed in unwounded skin, in which TGFβ1 induces expression of growth factors from fibroblasts and leukocytes, resulting in epidermal hyperplasia (Li AG, 2004). Therefore, it appears that these growth factors are not sufficient to overcome TGFβ1-induced growth inhibition at the wound

edge where keratinocytes demand a higher proliferation rate. Grossly, K5.TGFβ1wt wounds displayed delayed reepithelialization and prolonged granulation tissue accompanied by a persistent inflammatory cell infiltration throughout in comparison with non-transgenic wounds. Our lab has shown that the delayed cutaneous wound healing in K5.TGFβ1wt mice can be partially attributed to excessive inflammation throughout all stages of wound healing as well as growth arrest. For instance, inflammatory cells, especially macrophages at sites of injury, produce a large amount of MMPs, which may over-digest the basement membrane between the newly formed epidermis and the underlying granulation tissue, thereby suppressing re-epithelialization to cover the wound. This is consistent with an upregulation of MMP2, MMP3, and MMP9, and a rapid degradation of the basement membrane in K5.TGFβ1wt transgenic skin (Li AG, 2004). More importantly, elevated levels of MMPs, including MMP2 and MMP9, have been reported in wounds of human chronic ulcers, and MMP inhibitors have been considered for a new therapy (Mandal et al., 2003). In addition, excessive TGFβ1 and other inflammatory cytokines expressed by inflammatory cells may directly inhibit expression of genes that promote keratinocyte migration.

Activins in wound healing

Activins are members of the TGFβ superfamily, which like TGFβs are secreted molecules that regulate cell growth and differentiation in various tissues and organs. The first hints for a role of Activin in wound healing came from studies performing full-thickness excisional wounds on mouse skin, which were analyzed for the expression of

Activins at different time points after injury (Hubner et al., 1996b). Expression of the Activin A and to a lesser extent the Activin B subunit was strongly upregulated within 24 hours after wounding and remained elevated until the healing was completed. Follistatin (soluble Activin antagonist) as well as the Activin receptors were also expressed in normal and wounded skin, but their levels were not changed during skin injury (Hubner et al., 1996b; Wankell et al., 2001). *In situ* hybridization staining revealed that Activin A mRNA was mainly expressed in the granulation tissue adjacent to the hyperproliferative keratinocytes and below the scab, whereas the highest levels of Activin B mRNA were restricted to the suprabasal keratinocytes of the hyperproliferative epithelium at the leading wound edge and in the migrating epithelial tongue (Hubner et al., 1996a). The upregulation of Activin expression is likely to be important for normal wound repair, since severe delay in wound healing observed after cyclosporin A treatment of rats was strongly correlated with downregulation of Activin A expression in the granulation tissue fibroblasts (Petri et al., 1998).

To delve further into the function of Activin during wound healing, transgenic mice that overexpress the Activin A subunit specifically in the epidermis were created (Munz et al., 1999). The skin of these animals showed epidermal hyperproliferation and a fibrotic dermis. The latter effect is most likely due to diffusion of Activin from the epidermis to the mesenchyme and suggests a role of the protein in fibrotic stimulation. The keratinocyte hyperproliferation in these transgenic mice was very similar to the phenotype observed in hyperproliferative human skin diseases. The effect of Activin on keratinocyte proliferation *in vitro* may be indirect, since activin was shown to inhibit proliferation of human keratinocytes (Seishima et al., 1999; Shimizu et al., 1998). Thus

Activin may induce the expression of growth factors in dermal fibroblasts, which stimulate keratinocyte proliferation in a paracrine fashion. Also, the differentiation pattern of the epidermal keratinocytes was altered. Full-thickness excisional wounds in these mice revealed a large increase in granulation tissue, with a higher cell number and an increased deposition of ECM, compared with wild-type control mice. The latter effect appears to be at least partially due to an earlier induction of fibronectin and Tenascin-C expression in the wounds of Activin overexpressing mice. In contrast, collagen type I expression was similar in normal and transgenic mice, indicating that the effects of Activin on the synthesis of ECM proteins are variable, whereas TGFβ always seems to increase the synthesis of ECM molecules in a broad manner (Roberts et al., 1986).

The results obtained with the Activin-overexpressing mice demonstrated novel activities of Activin in the regulation of the healing process. However, they do not allow adequate conclusions regarding the roles of endogenous Activin in wound healing. To begin to address this question (Wankell et al., 2001) overexpressed the soluble Activin antagonist Follistatin in the epidermis of transgenic mice (Wankell et al., 2001). The skin of these animals showed a mild dermal and epidermal atrophy. After injury, a severe delay in wound healing was observed. In particular, granulation tissue formation was strongly reduced, leading to a major reduction in wound tensile strength. The wounds, while delayed, did ultimately heal and the resulting scar area was actually smaller when compared with normal controls (Wankell et al., 2001). These results are in parallel to the observation that with Activin overexpressing mice wounds have larger scars and thus provide suggestive evidence for the function of endogenous Activin in the control of wound repair and scar formation.

Bone Morphogenetic Proteins in cutaneous wound healing

In addition to TGFβs and Activins, BMPs have also been suspected to be involved in wound repair. At least fifteen BMPs are known and are shown to exert their functions similarly to TGFβ and Activin. BMP-2, BMP-4, and BMP-7 are all normally secreted in wounded mouse skin (Wankell et al., 2001). The expression pattern of these BMPs in skin wounds and their roles in wound repair have yet to be fully appreciated, but exogenous BMP-2 treatment induces a large dermal and epidermal growth in fetal wounds (Stelnicki et al., 1998). In contrast to those BMPs, much has been learned about the expression of BMP-6 in the healing skin. It is highly upregulated in the neo-epidermis at the wound margins as well as in the fibroblasts of the granulation tissue. When completion of wound closure has occurred, BMP-6 accumulates in the suprabasal layers of the newly formed keratinocytes (Kaiser et al., 1998). This specific localization led to the hypothesis that BMP-6 inhibits keratinocyte proliferation and terminal differentiation, a hypothesis that is supported by the observation that BMP-6 induces keratinocyte terminal differentiation *in vitro* (D'Souza et al., 2001; McDonnell et al., 2001).

To study the functions of BMP-6 in skin, Blessing et al. made transgenic mice overexpressing this protein in the suprabasal epidermis (Blessing et al., 1996). Strong expression of the BMP-6 transgene inhibited cell proliferation but did not impede terminal differentiation. However weak expression of the transgene resulted in an opposite phenotype: keratinocyte hyperproliferation and a psoriatic-like lesion. For wound repair, reepithelialization was delayed in mice that had low expression levels of

BMP-6 in the epidermis (Kaiser et al., 1998), which suggested that this protein inhibits keratinocyte proliferation in wounded skin and is necessary for the reestablishment of a fully differentiated epidermis in a dose dependent manner. Further characterization of wound healing in BMP-6 null mice could shed more light on these results (Solloway et al., 1998). Certainly the roles of other BMPs in wound repair have yet to be discovered and remain an exciting avenue of research.

1.3 Smad4 in Cancer

Smad4 is also known as DPC4 (deleted in pancreatic carcinoma 4) and is located on human chromosome 18q21.1. About 90% of human pancreatic carcinomas show allelic loss at 18q. In this groundbreaking study it was found that the Smad4 gene was homozygously deleted in 25 of 84 tumors and mutations were identified as somatic mutations occurring in 6 of 27 carcinomas that lacked deletions. To directly test the hypothesis that the Smad4 gene is a tumor suppressor that is critical for transmitting signals from the TGFβ superfamily of ligands, Smad4 was deleted through homologous recombination in human colorectal cancer cells (Zhou et al., 1998). This deletion abrogated signaling from TGFβ, as well as from the other TGFβ family members such as Activin/BMP/GDF. These results provided evidence that inactivation of Smad4 causes TGFβ unresponsiveness, and yielded the primary basis for understanding the physiologic role of this gene in tumorigenesis. Smad3 is a direct mediator of transcriptional activation by the TGFβ receptor. Its target genes in epithelial cells include cyclin-dependent kinase (CDK) inhibitors that generate a cytostatic (growth arrest) response. Smad3 was shown to

mediate transcriptional repression of the growth-promoting gene c-MYC. A complex containing Smad3, the transcription factors E2F4, E2F5, and DP1, and the corepressor p107 preexists in the cytoplasm (Chen et al., 2002). In response to TGFβ, this complex moves into the nucleus and associates with Smad4, recognizing a composite Smad-E2F site on MYC for repression. Recipients of CDK regulatory signals, E2F4/E2F5 and p107 act as transducers of TGFβ receptor signals upstream of CDK. Smad proteins therefore mediate transcriptional activation or repression depending on their associated partners and cellular context to enact growth arrest. Three important inhibitors that are controlled via direct Smad4 transcriptional control such as p21, p15 and p16ARF regulate CDK's.

Molecular Genetics of Smad4

Other key early work to demonstrate Smad4 as a tumor suppressor was the observation that it is frequently lost in human sporadic colon cancer tumors (Thiagalingam et al., 1996). A defined minimally lost region on chromosome 18q21, which extended between markers D18S535 and D18S858 was used to show loss. Smad4 was deleted in up to one-third of cases. It was next concluded that Smad4 is altered only infrequently in head and neck squamous cell carcinomas (HNSCCs), but may play some role in the tumorigenesis of a small set of HNSCC, because a nonsense gln526-to-ter mutation (Q526X) was found in the primary tumor and a lymph node metastasis from 1 of 11 patients (Kim et al., 1996).

To further understand the clinical genetics of Smad4 loss, Schutte et al. analyzed 338 tumors, originating from 12 distinct anatomic sites, for alterations in the Smad4 gene

(Schutte et al., 1996). Smad4 sequence alterations were found in 64 specimens selected for the presence of allelic loss of 18q. An alteration of the Smad4 gene sequence was identified in 1 of 8 breast carcinomas and 1 of 8 ovarian carcinomas. These results indicated to them that whereas Smad4 inactivation is prevalent in pancreatic carcinomas (48%), it is distinctly uncommon (less than 10%) in other tumor types.

Kinzler and Vogelstein referred to colorectal cancers developing on the basis of JPS as ”landscaper defects”. This is following the designation “gatekeeper defects” for the mutations in tumor suppressor genes that are known to prevent cancer through direct control of cell growth, including p53, RB, VHL, and APC (Kinzler and Vogelstein, 1998). Inactivation of these genes contributes directly to the neoplastic growth of the tumor; thus, they normally function as 'gatekeepers’. (Kinzler and Vogelstein, 1998) used the designation “caretaker defects” for the susceptibility genes that indirectly suppress neoplasia (for example, XPB, ATM, MSH2, and MLH1). A second class of indirectly acting cancer susceptibility genes was suggested by findings in JPS, which carries an increased risk of colorectal cancer. The polyps in this situation are markedly different from the epithelium-rich adenomatous polyps that give rise to most cases of colorectal cancer. Polyps from JPS patients have a low potential to become malignant and are composed largely of stromal cells, comprising a mixture of mesenchymal and inflammatory elements in which epithelium is entrapped, often forming dilated cysts. The epithelial cells within and surrounding the polyp are initially devoid of neoplastic features but nonetheless are at increased risk of becoming malignant. (Kinzler and Vogelstein, 1998) proposed that the increased cancer susceptibility due to inherited mutations in juvenile polyposis is the product of an abnormal stromal environment. That an abnormal

stroma can affect the development of adjacent epithelial cells is suggested by the experience with ulcerative colitis, which also leads to inflammation and cystic epithelium in the mucosa of the colon. Initially, the embedded epithelium shows no neoplastic changes, but foci of epithelial neoplasia and progression to cancer eventually develops in many cases. The regeneration that occurs to replace damaged epithelium may increase the probability of somatic mutations in this abnormal microenvironment. The increased risk of cancer in JPS and ulcerative colitis patients seems, therefore, primarily the result of an altered terrain for epithelial cell growth and thus can be thought of as a "landscaper" defect. It was found intriguing that the stromal cells, but not the epithelial cells, of most hamartomas from JPS patients contain a clonal genetic alteration. Similarly, clonal genetic changes have been demonstrated in the stroma, but not the epithelial cells, of endometrial polyps. In contrast, clonal genetic alterations have been demonstrated in epithelial cells, but not stromal cells, of polyps arising in patients with familial adenomatous polyposis (due to mutations in the APC gene) or Peutz-Jeghers syndrome, which are morphologically distinct from those of JPS patients. These results add to the emerging realization that solid tumors are not simply composed of neoplastic epithelial cells. Historically, the search for drugs that can modulate neoplasia has focused on such epithelial cells. Targeting specific stromal cells (such as those found in blood vessels) may be more valuable for therapeutic purposes. (Kinzler and Vogelstein, 1998) raised the question: "Could drug targeting of the paracrine factors and other features of the stromal-epithelial interaction be similarly useful?"

Friedl et al. examined 29 patients with the clinical diagnosis of JPS for germline mutations in the Smad4 or BMPR1A genes and identified Smad4 mutations in 7 (24%)

and BMPR1A mutations in 5 patients (17%) (Friedl et al., 2002). A remarkable prevalence of massive gastric polyposis was observed in patients with Smad4 mutations when compared with patients with BMPR1A mutations or without identified mutations. This was claimed to be the first genotype-phenotype correlation observed in JPS.

Animal Models Studying Smad4 Loss

The first mouse model to test *in vivo* Smad4 function, inactivated the mouse Smad4 (DPC4) homolog and found that the homozygous mutants were embryonic lethal, whereas the heterozygotes showed no abnormality (Takaku et al., 1998). The investigators then introduced the Smad4 mutation into the knockout mice for the mouse homolog of the human APC gene, Apc-Δ716, a model for human familial adenomatous polyposis. Because both Apc and Smad4 are located on mouse chromosome 18 and previously shown to be lost in colon cancers, they constructed compound heterozygotes carrying both mutations on the same chromosome by meiotic recombination. In such mice, intestinal polyps developed into more malignant tumors than those in the simple Apc-delta716 heterozygotes, showing an extensive stromal cell proliferation, submucosal invasion, cell type heterogeneity, and in vivo transplantability. Takaku et al. (Takaku et al., 1998) suggested that mutations in Smad4 play a significant role in the malignant progression of colorectal tumors.

It was also found that when Smad4 was deleted specifically in the mouse pancreatic epithelium, that it had no impact on pancreatic development or physiology (Bardeesy et al., 2006). However, when the investigators combined an activated

oncogenic Kras allele, Smad4 deficiency enabled rapid progression to activated Kras-initiated neoplasm. Smad4 deficiency also exacerbated the tumor phenotypes of mice with combined Kras activation and deletion of the tumor suppressor Ink4a/Arf.

A new report has discovered that a new type of immature myeloid cell can be recruited from the bone marrow towards the invasive front of malignant tumors. These immature myeloid cells overexpress MMP9 and MMP2 which are known proteases involved in metastasis (Kitamura et al., 2007). In the subsequent adenocarcinomas that formed, expression of CCL9 (inflammatory secreted chemokine) is produced from the tumor epithelium. These results indicated that loss of Smad4 signaling in the tumor epithelium causes accumulation of immature myeloid cells that promote tumor invasion.

(Kim et al., 2006) showed that selective loss of Smad4-dependent signaling in T cells leads to spontaneous epithelial cancers throughout the gastrointestinal tract in mice, whereas epithelial-specific deletion of the Smad4 gene does not. Tumors arising within the colon, rectum, duodenum, stomach, and oral cavity are stroma-rich with dense plasma cell infiltrates. Smad4-null T cells produce abundant TH2-type cytokines including IL5, IL6, and IL13, known mediators of plasma cell and stromal expression. (Kim et al., 2006) concluded that their results support the concept that cancer, as an outcome, reflects the loss of normal communication between the cellular constituents of a given organ, and indicate that Smad4-deficient T cells ultimately cannot undergo homeostasis with their stromal and epithelial neighbors.

Deletion of *Smad4* in multiple murine tissues results in spontaneous cancers (Qiao W, 2006; Yang L, 2005). Our own studies and others have shown that epidermal-specific *Smad4* deletion blocks the growth inhibitory effect of TGFβ, resulting in

hyperproliferation, with down-regulation of p21 and p27, and upregulation of c-Myc and cyclin D1 (Qiao W, 2006; Yang L, 2005). Further, all *Smad4-/-* mice developed spontaneous skin tumors including primarily SCCs, as well as sebaceous adenomas, basal cell carcinomas, and tricoepitheliomas (Qiao W, 2006; Yang L, 2005). *Smad4-/-* SCCs demonstrated inactivated PTEN and activated AKT, representing activation of a key cell survival pathway (Bardeesy et al., 2006; Izeradjene et al., 2007; Yang L, 2005), in the forestomach (Teng et al., 2006), and liver (Xu et al., 2006) However, *Smad4* deletion in mice also resulted in spontaneous cancer formation of the stomach (Xu et al., 2000), skin and mammary gland (Li et al., 2003b; Qiao et al., 2006; Yang et al., 2005).

Chapter 2: Materials and Methods

2.1 Animals

Smad4 homozygous floxed (f/f) mice (Yang et al., 2002) were mated with K5.Cre.PR1 mice (Zhou et al., 2002). Smad4 deletion in keratinocytes was achieved by daily i.p. injection of 100μg/kg RU486 with 0.5mg progesterone in pregnant mice bred from the above two lines at the time points specified in the Results section, or through topical application of 20ug/mL RU486 to neonatal or adult mouse skin (specified in the Results section), once a day for 5 Days. Genotypes of these mice were identified by PCR as previously described to detect the wildtype, floxed allele, the CrePR1 transgene, and Cre-mediated Smad4 deletion Smad4 fl/fl mice (Yang et al., 2002) were also mated with MMTV-Cre (Wagner et al., 1997) mice. WT mice were lacking Cre and KO mice were homozygous for the Smad4 floxed allele and carried one allele of MMTV-Cre transgene. Mice were maintained and bred under pathogen free conditions. Genotypes of mice and detection of Cre mediated deletion of Smad4 were determined as described (Li et al., 2003b). The protocol for these animal studies was approved by the Animal Care and Use Committee, IUCAC and the department of comparative medicine (DCM) at OHSU. Dsg4 mutant mice were characterized previously(Bazzi et al., 2005).

2.2 Cell Culture

Primary mouse epidermal keratinocytes were isolated following suggested media protocol (CellNTec/Chemicon). Primary mouse hair follicle keratinocytes were isolated from neonatal K5.Smad4-/- mice (in C57BL/6 background) as previously described (Han et al., 2006) in CnT-07 media (Chemicon). Briefly, following dermal-epidermal separation of neonatal skins, dermal pieces were finely minced and digested with collagenase for 1 hour at 37 degrees. These were then centrifuged in a 4% Ficoll gradient before plating. Prior to transfection, cells were placed in CnT-02. Induction of endogenous Dsg4 mRNA was performed by culturing primary keratinocytes isolated from neonatal C57BL/6 (Jax) mice in CnT-02 media in high calcium (1.2mM) for 48 hours with or without treatment of 10ng/mL of TGFβ2, Activin A, and BMP-2 (R&D Systems). Cells were then collected for RNA extraction as previously described (Han et al., 2006). Dsg4 mRNA levels were examined by qRT-PCR as described above. CnT-02 media is used for cells that have been passaged enough times (more than 10-20 times) and considered 'immortal'. CnT-07 media is specifically for primary cells.

2.3 Histology

Embryos, skin, wounds and tumor samples were fixed in 10% neutral-buffered formalin overnight and then placed in 70% ethanol prior to embedding in paraffin, sectioned at 5μM, and stained with Hematoxylin and Eosin (H&E). Sections were stained

with Masson trichrome staining kit (Sigma) according to manufacturer instructions. Frozen sections were cut at 8μM and fixed in 75% Isopropanol for five minutes, washed in PBS and placed in blocking buffer. Samples were imaged on a Nikon E600 wide field light microscope affixed with a Qimaging 10bit RGB camera, and software from Metamorph or Nikon was used to acquire images in Raw TIFF (Tagged Image File Format).

2.4 Immunostaining and Antibodies

Immunofluorescence (IF) or immunohistochemistry (IHC) was performed on OCT-embedded frozen sections or paraffin embedded sections as previously described (Wang et al., 1997). Paraffin slides were dewaxed in xylene twice for 5min and place in an ethanol series to rehydrate for 3min washes culminating in PBS. Slides were then microwaved in pH 6.0citrate buffer and blocked in 5% serum of the secondary antibody host. Sections were incubated in primary antibody at the dilutions listed above overnight at 4 degrees. IHC was performed using the MOM kit (Vector Labs). Secondary AlexaFluor antibodies (IF) were purchased from Invitrogen and used a 1:100 for 10min. Slides were washed four times in PBS and mounted in SlowFade containing DAPI mounting medium (Invitrogen).Antibodies for K5, K6, K14, K10, and BrdU were purchased from Covance Inc.; and β-catenin was from BD Pharmingen. Antibodies to AE13 (hair keratin complex) and AE15 (trycohyalin) were kindly provided by Dr. T.T. Sun at NYU. Antibody to pPten and pAKT were from Cell Signaling, Inc. The following antibodies and their working dilutions were used for Immunohistochemistry (IHC). K14

from RDI-Fitzgerald (1:500). F4/80 (1:200), αSMA from Sigma (1:500), CD31 from BD Pharmingen (1:200). Primary antibodies used for immunostaining included: Adipophilin (1:500) and K14 (1:500) from RDI-Fitzgerald; K1 (1:250), K6 (1:250), Loricrin (1:500), and Filaggrin (1:500) from Covance; AE13 (1:100) from Abcam; Gata3 (1:50) from Santa Cruz; Smad4 (1:200) from Upstate; and E-cadherin (1:200) from BD. The AE15 antibody was a gift from Dr. T.T. Sun. Dsg4 staining was performed using mouse monoclonal anti-Dsg4 (18G8, 1:10) as previously described (Bazzi et al., 2006). Quantification of CD31 positive vessel area was performed with NIH ImageJ following provided instructions (http://rsb.info.nih.gov/ij/docs/examples/stained-sections/index.html).

2.5 ChIP

Fresh skin was removed at postnatal day 6 (anagen), chopped and disrupted using a Dounce homogenizer, and cross-linked with 4% formaldehyde for 20 minutes. The cross-linked chromatin was then sheared using the ChIP-IT Express Enzymatic kit (Active Motif) for 10 minutes. Fifteen μg of sheared chromatin was immunoprecipitated with 1μg of antibody at 4^{o}C for overnight, and precipitated DNA was eluted in 50 μl H2O. Antibodies used for immunoprecipitation included: RNA Pol II, rabbit anti-Smad3, and rabbit anti-Smad4 from Upstate; mouse monoclonal antibodies for Smad1 and Gata-3 were from Santa Cruz; rabbit antibodies for Smad2 and Smad5 from Zymed; and rabbit anti-phospho-Smad1/5 from Cell Signaling. PCR primers encompassing the SBE of the

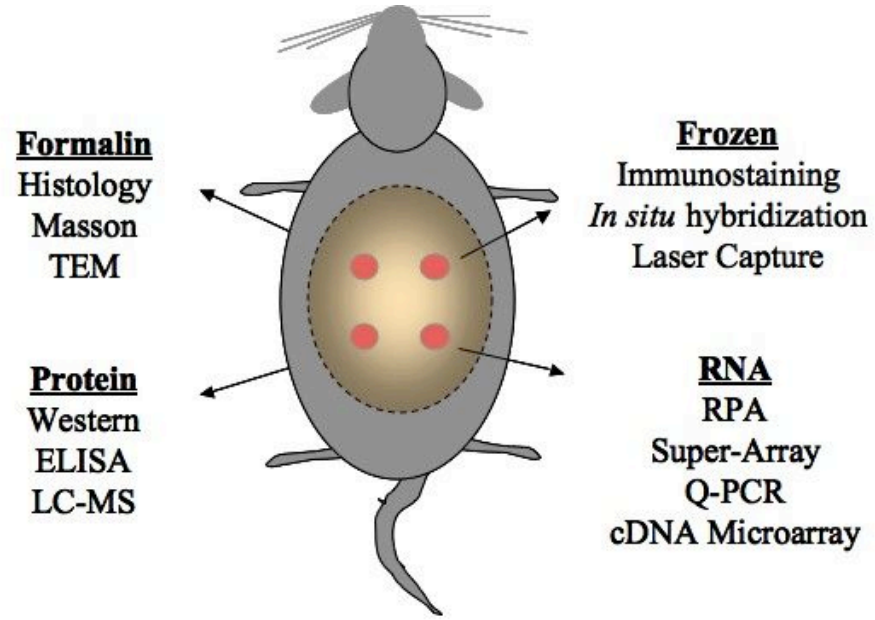
mouse Dsg4 primers are as follows: FWD-5' ACCCCCTGAAATAAACTGGAGC and REV-5' GGTAGGTGCTATGGTGACTAAACCC. PCR Primers encompassing a region of -5kb of the promoter, which does not contain SBE, were used as a negative control: FWD-5' GCTATCGCTGAAACAAAGGTCACAG and REV-5' TGATGAGGGACTCTGGCTAATGC. DNAs precipitated with individual antibodies were used for PCR. Primers for the BMP responsive element in the Msx2 promoter (Brugger et al., 2004), and for the TGFβ responsive Snail1 promoter, were used as controls for binding of Smad1/5 or Smad2/3, respectively.

2.6 Luciferase, Plasmids, Cloning and Mutagenesis

To avoid interference of high levels of endogenous Smad4 with Luc assay, K5.Smad4-/- keratinocytes were used for Luc assay. Keratinocytes were transfected with full length individual Smad expression constructs at 50ng/well each (gifts from XH Feng), 500ng of a firefly luciferase reporter construct containing 3kb upstream mouse Dsg4 promoter in PGL4.26 (Promega), and 10ng of the Renilla luc plasmid PGL4.74 (Promega). For site-directed mutagenesis, the SBE sequence CTGT was mutated to TAGA. Twenty-four hours after transfection, cells underwent a calcium switch (1.2 mM calcium in CnT-02 media) for 48 hours to induce Dsg4 promoter activity. Luciferase activity was then normalized to Renilla luciferase activity. Lysates were collected in passive lysis buffer and analyzed on a Glomax™ luminometer using the dual luciferase assay (Promega).

2.7 Wounding, Morphometry and Sample Collection

12-week-old healthy animals were wounded with a full thickness 6mm punch biopsy at four positions in the flanks of dorsal skin. Animals were anesthetized with isoflurane under appropriate animal care guidelines. No mice were used that displayed any obvious health problems including skin lesions or signs of neoplasm. Animal welfare guidelines were followed in accordance with IUCAC and institutional protocols. Images of wounds were taken with four surrounding reference objects of known size in order to calibrate the area of the wounds. Images were analyzed with ImageJ (National Institutes of Health). Over 40 wounds were analyzed for each time point and respective genotype. Once pixels were converted into area, these areas were subtracted from their starting wound areas to yield a percent of change. Percent of wound closure was analyzed using a box whisker plot to display the distribution of data. Each animal contained four wounds and each location (anterior left, anterior right, posterior left, posterior right) was used for each type of tissue preparation. The first two wounds from freshly euthanized animals would be immediately frozen in liquid nitrogen and used in RNA or Protein extraction. The third wound would be place in OCT and immediately frozen. The fourth and final wound would be placed in 10% neutral buffered formalin overnight. Then placed in 70% ethanol and embedded into paraffin and sectioned into 5μM sections for staining. Frozen or Paraffin embedded wounds were bisected equilaterally prior to sectioning/embedding.



Schematic of the wound biopsy procedure. The bronze area represents the shaved dorsal skin that will be wounded. The four circles represent 6 mm full thickness punch biopsies. The red circles represent the area that will be excised to obtain wound samples. 12 week old mice were used that contained no prior lesions or neoplasms and were healthy in appearance and behavior.

Figure 9 **Wound Strategy.** Depiction of how samples were to be used and organized.

2.8 *in vitro* 'Scratch' Assay

Primary keratinocytes that had not been passaged were treated with RU486 prior to isolation were plated at high density ($1x10^6$ cm^2**).** Cells were allowed to reach full confluence until a uniform 'cobblestone appearance' was attained. A 200ul pipette tip was used to make 1 horizontal and two vertical 'scratches' in a 6-well dish. Two hours prior to scratch fresh media containing 10µg/Ml of Mitomycin C (MMC) was added to some wells to control for proliferation induced closure of the artificial wound. Cells were photographed using phase contrast brightfield on an inverted zeiss axiovision microscope. Images were captured at 0, 24 and 48 hours.

2.9 Quantitative Real Time Polymerase Chain Reaction (QPCR)

RNA from the dorsal skin was extracted in Trizol and further purified using an RNeasy column with on-column DNase treatment (Qiagen). 50ng of RNA per reaction was analyzed and then amplified with Brilliant II 1-Step QPCR reagent (Stratagene). Analysis was carried out using MxPro Software V4.0 (Stratagene). Samples were analyzed in triplicate and normalized to an internal VIC-labeled Taqman probe for GAPDH. Total cellular RNA was isolated from freshly frozen wounds stored at -80 degrees in Trizol. Wounds were placed into Trizol and minced using scissors and then homogenized. RNA was isolated from subsequent Chloroform-Isopropanol-Ethanol purification. Aliquots of RNA were then further purified using RNeasy columns (Qiagen) coupled with on-column DNase treatment. 100ng of total RNA was used per well in triplicate and Brilliant II 1-step QPCR reagent (Stratagene) was used to convert RNA into cDNA and amplify in an Mx3000P QPCR machine (Stratagene). Other probes were FAM-labeled Taqman Assays as follows: Dsg1a: Mm00809994_s1, Dsg2: Mm00514608_m1, Dsg3: Mm00659652_m1, Dsg4: Mm00812608_m1, Msx2: 00442992_m1, Gata-3: 00484683_m1, Krt31: Mm00657991_gH HoxC13: Mm00802798_m1, FoxN1: Mm00433946_m1, Dlx3: Mm00438428_m1, Gli-1: Mm00494645_m1. TGFβ1-Mm03024053_m1, MMP3-Mm00440295_m1, MMP12-Mm00500554_m1, MMP13-Mm00439491_m1, MMP14-Mm00485054_m1. A VIC labeled GAPDH (Applied Biosystems) was used in every well as a normalizing housekeeper gene. Reference dye was ROX for background fluorescence. Relative

quantity or log fold change was calculated using MxPro software V.4 (Stratagene) using all three algorithm enhancements (adaptive baseline, moving average and amplification-based thresholds).

2.10 Electron Microscopy

Fresh skin was isolated from animals and immediately fixed in 4% PFA/1% glutaraldehyde/.1M PBS for 2 hours and then immersed in .2M sucrose overnight. Samples were postfixed in 1% Osmium tetroxide in PBS for 1 hour. Samples were then dehydrated in negative vaccum and received three washes of 50-70-80-90-100% Ethanol under vaccum of at least -20kPa. Samples were next washed in propylene oxide twice for fifteen minutes. Next, samples were placed in araldite/propylene oxide mixtures until 100% araldite was attained with no air bubbles and baked for 48 hours at 60 degrees. Ultrathin sections were cut and stained with urayl acetate and then lead acetate for fifteen minutes on medium grids and imaged. All reagents are available from Energy Beam Sciences. The Transmission Electron Microscope used was the Philips CM100.

2.11 Protein Isolation and Determination of Concentration

Protein isolation was performed using complete lysis M protein extraction buffer (Roche). For 12-well culture plates 300ul of lysis buffer were used per-well. For tissue, 1mL of lysis buffer was placed in a 14mL tube and frozen or fresh tissue was immediately placed in lysis buffer. Tissue was next minced using curved surgical scissors

followed by homogenization. Samples were always maintained on ice and following lysis, samples were centrifuged at maximum speed at 4 degrees and soluble protein supernatant was either frozen or directly used. Concentration of soluble protein in suspension was determined by using BioRad Dc (detergent compatible) protein assay. Standard curves had at least six points and were run in triplicates. Absorbance was measured on a standard plate reader at 560nm. Sample absorbance values were calculated simple fit with acceptable R2 values of linearity for standard curve. Confidence intervals were validated for each sample using software provided with plate reader. Samples were diluted to a lowest common denominator for uniform use in further assays.

2.12 Statistical Analysis

Significant differences between the values obtained in each assay on samples from various genotypes were determined using the Student's t-test and standard deviation expressed as mean ±. Measurements were subjected to paired 2-tailed students t-tests. Significance was determined by p-values lower than 0.05 and when not displayed are assumed to not be significant. Significant data is marked with a symbol.

Chapter 3: Smad4 Dependent Desmoglein4 Expression Contributes to Hair Follicle Integrity

3.1 Introduction

Epidermal development in mice begins in embryos around E9 with a single layer of the epithelium, and continues with stratification and epidermal barrier formation before birth (Fuchs, 2007). Thereafter, the epidermis undergoes constant self-renewal throughout the life of the animal. Epidermal appendages include hair follicles and sebaceous glands. Hair follicle development begins around E14.5 in mice and continues with hair follicle differentiation and production of the hair shaft 1 week after birth (Millar, 2002). Hair follicle cells differentiate into the layers of the outer root sheath (ORS), the inner root sheath (IRS), and the hair shaft. Along with hair follicle morphogenesis, sebaceous glands develop from cells residing in the “bulge” area at the upper portion of the hair follicle. Postnatal hair follicles undergo regenerative cycles of growth (anagen), regression (catagen) and rest (telogen). Following telogen, a new hair shaft is generated adjacent to the previous one through the re-initiation of anagen for review see (Alonso and Fuchs, 2006). Key transcription factors shown to be involved in hair follicle formation and differentiation include Gata-3, Msx-2, FoxN1 among others. Loss of these molecules results in failure of hair shaft formation (Johns et al., 2005; Kaufman et al., 2003; Satokata et al., 2000). Another important molecule in making a normal differentiated hair shaft is Desmoglein-4 (Dsg4). Among the different Dsg isoforms, Dsg4 is the only isoform which is highly expressed in the hair cortex (Green

and Simpson, 2007). Loss of function mutations in Dsg4 in humans, rats and mice result in balding due to aberrant hair shaft production (hypotrichosis) (Bazzi et al., 2005).

Multiple signal transduction pathways tightly control the process of skin morphogenesis, differentiation and renewal. Among them, signaling from TGFβ/Activin/BMP, which requires Smad transcription factors as mediators, plays an important role (Li et al., 2003a). In the TGFβ family, TGFβ2 has been shown to be required for hair follicle development (Foitzik et al., 1999), whereas TGFβ1 is required for the hair follicle to enter into the catagen phase (Foitzik et al., 2000). Activins have also been shown to play crucial roles in hair follicle development, as knocking out the activin ligand, expressing the activin antagonist, Follistatin, or expressing a dominant negative Activin receptor IB, all give rise to abnormal hair follicle development or cycling (Bamberger et al., 2005; Matzuk et al., 1995a; Matzuk et al., 1995b). In the BMP family, a recent study has revealed that dermal derived BMP2 and BMP4 are cyclically expressed, which regulate stem cell activation during hair regeneration (Plikus et al., 2008). Studies have shown that overexpression of BMP ligands promotes differentiation and that BMP antagonists, such as Noggin, are required to maintain the undifferentiated state of epidermal progenitors for review see (Botchkarev and Sharov, 2004). Consistently, keratinocyte-specific deletion of the type 1A BMP receptor (also known as Activin Like Kinase 3; ALK-3) results in alopecia due to failure of IRS differentiation and hair shaft formation (Andl et al., 2004; Kobielak et al., 2003; Ming Kwan et al., 2004; Yuhki et al., 2004).

It has been shown that TGFβ/activin signals mainly through Smad2 and Smad3, whereas BMP signals mainly through Smad1 and Smad5 (Li et al., 2003a). We have

previously deleted Smad4, a common Smad that interacts with both TGFβ/activin-specific Smads and BMP-specific Smads, by crossing Smad4 floxed mice with MMTV-Cre mice (Qiao et al., 2006). In addition to directing Cre expression in mammary epithelia, the MMTV promoter targets Cre expression in keratinocytes, around E13.5 (Wagner et al., 2001). These conditional Smad4 knockout mice develop epidermal hyperplasia, progressive hair loss beginning at the first catagen phase on P16, and spontaneous skin tumor formation later in life (Qiao et al., 2006). Similar phenotypes have also been reported in keratinocyte-specific Smad4 knockout mice when using a truncated keratin 5 (K5) targeting vector, which targets Cre expression in keratinocytes around E13.5 (Yang et al., 2005). It remains to be determined whether the loss of hair follicle differentiation markers is the cause or the consequence of hair follicle degeneration in Smad4 null keratinocytes. Further, it remains to be determined whether Smad4 loss affects epidermal differentiation in addition to epidermal hyperproliferation, particularly when it is lost at stages critical for epidermal development. In the present study, we used a K5 promoter to target the CrePR1 transgene that allows inducible Cre expression in keratinocytes including epidermal stem cells as early as E10.5, when a single epithelial layer begins transitioning into a stratified epidermis (Han et al., 2006; Zhou et al., 2002). When we deleted Smad4 in K5.CrePR1/Smad4flox bigenic mice, we found that Smad4 deletion in keratinocytes either in embryos or after birth did not affect epidermal differentiation. However, Smad4 deletion resulted in degeneration of the hair follicles. We then focused on the identification of direct transcriptional targets of Smad4 that are imperative for hair follicle/hair shaft integrity. We found that Dsg4 is a Smad4 target gene and that loss of Dsg4 expression in Smad4 knockout keratinocytes

contributed at least in part to hair follicle degeneration. The results of this study were published October 1st 2008 in Developmental Biology 322(1):156-166, PMID: 18692037

3.2 Smad4 deletion in keratinocytes resulted in hair follicle degeneration and hypertrophic sebaceous glands

We cross-bred K5-CrePR1 mice (Zhou et al., 2002) with Smad4 floxed mice (Qiao et al., 2006). Smad4 deletion in keratinocytes was induced by either injection of RU486 to pregnant mice from the above mating, or by topical application of RU486 to bigenic mouse skin after birth (designated as K5.Smad4-/-, Figure 10A). The earliest RU486 application began on E9.5, and the embryos were taken on E10.5, E12.5 and E15.5 to examine Smad4 expression patterns. Smad4 gene deletion in embryonic skin was detected as early as E10.5 (Figure 10B). Depletion of existing Smad4 protein, as detected by immunostaining, took a few more days. By E12.5, Smad4 protein in wildtype skin was detected uniformly in the nucleus of epidermal cells and also in some of the stromal cells (Figure 10C). In contrast, Smad4 nuclear positive cells were significantly reduced in K5.Smad4-/- epidermis (Figure 10D). By E15.5, Smad4 nuclear staining in wildtype skin was prominent in the basal layer of the epidermis and newly formed hair follicle placodes (Figure 10E). At this stage, uniform depletion of Smad4 protein in K5.Smad4-/- epidermis became obvious, and only stromal cells had persistent Smad4 nuclear staining (Figure 10F). To verify Smad4 deletion indeed occurs in epidermal stem cells, we applied RU486 to neonatal bigenic skin daily for 5 days, and examined Smad4 expression pattern on P35 skin, when the epidermis had gone through at least two cycles

of self renewal and hair follicles had entered into the second postnatal anagen phase. Nuclear Smad4 staining was detected in the epidermis and throughout all layers of the hair follicle (Figure 10G). At this stage, hair follicles in K5.Smad4-/- skin were degenerated into cysts. The epidermis, hair follicle cysts and sebaceous glands of K5.Smad4-/- skin were negative for Smad4 nuclear staining, whereas Smad4 positive stromal cells were still detectable (Figure 10H, I). This result indicates that epidermal stem cells harboring Smad4 deletion can repopulate the entire epidermis and hair follicles.

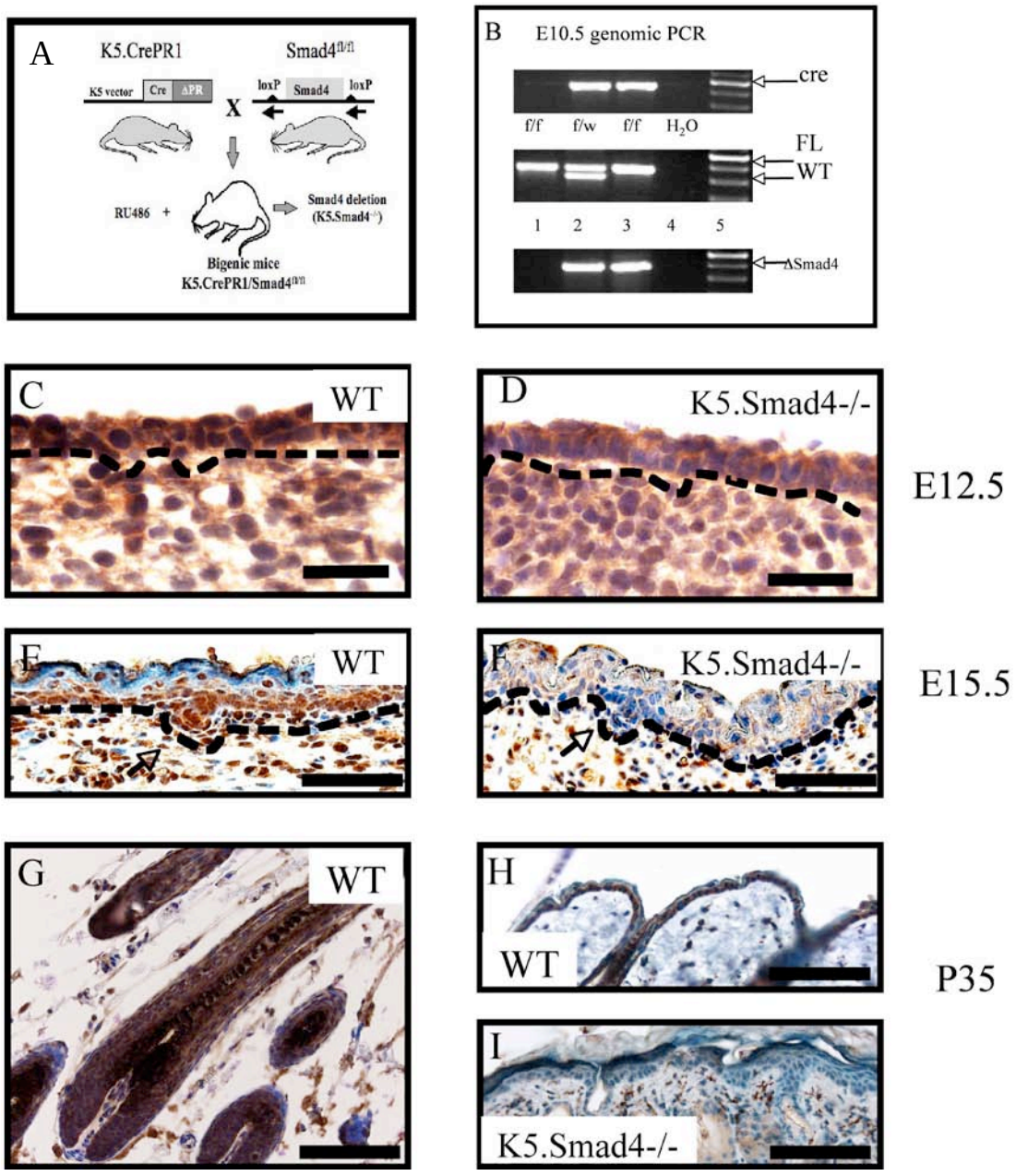


Figure 10. **Keratinocyte-specific Smad4 deletion.** A: Schematic demonstration of generation of K5.Smad4-/- mice by mating K5.CrePR1 mice with Smad4 floxed mice (Smad4f/f), and applying RU486 to bigenic mouse skin. B: Genomic PCR results from E10.5 mouse tail DNA indicate genotype of Smad4 alleles. Lanes 2 and 3 = K5.CrePR1/Smad4f/wt and K5.CrePR1/Smad4f/f, which were heterozygous and homozygous for the floxed allele (FL), respectively and both were positive for the CrePR1 transgene (cre). Smad4 deletion (ΔSmad4) was induced by RU486 application to day 9.5 pregnant bigenic mice. C to I: Immunostaining for Smad4 (brown) of skins of E12.5 (C and D), E15.5 (E and F), and P35 (G to I). Note that Smad4 nuclear staining was uniform in WT E12.5 epidermis, but was patchy and reduced in K5.Smad4-/- E12.5 epidermis. Smad4 nuclear positive cells were predominantly located in the basal layer of

the epidermis and the hair follicle placode (arrow), and stromal cells in WT E15.5 skin. Smad4 protein was ablated in the epidermis and placode (arrow) in K5.Smad4 -/- skin, but was still detected stromal cells. In P35 WT skin, Smad4 staining was positive in all layers of the anagen follicle (G), the epidermis (H), and some stromal cells (G and H). In contrast, in P35 K5.Smad4-/- skin, Smad4 staining was negative in the epidermis, degenerated hair follicle cysts and sebaceous glands (I). The stroma remained Smad4 positive cells. Light brown staining in K5.Smad4-/- epidermis, hair follicle cysts and sebaceous glands represents background staining. Scale bars = C-D: 25μm E-F: 50μm G-I: 100μm.)

Regardless of Smad4 deletion in embryos, K5.Smad4-/- mice were born normally and showed no visible phenotype in neonates. Neonatal K5.Smad4-/- skin displayed normal thickness including dermal and epidermal compartments. Early epidermal differentiation markers, such as keratin 1 (K1), and terminal differentiation markers, such as loricrin and filaggrin, were not altered in K5.Smad4-/- epidermis at all ages even when adult mice developed epidermal hyperplasia. However, K5.Smad4-/- mice developed gross hair follicle abnormalities around P6. At this time point, K5.Smad4-/- pups were slightly smaller than their wildtype siblings (Figure 11A), possibly due to esophageal hyperplasia as previously reported (Yang et al., 2005). Histologically, hair follicles in P6 K5.Smad4-/- skin were shorter and less differentiated (Figure 11C) than wildtype follicles (Figure 11B). Some K5.Smad4-/- hair follicles underwent partial degeneration (Figure 11C, 11D). A similar phenotype was also observed in Dsg4 mutant hair follicles at the same stage (Figure 11E). By P10, K5.Smad4-/- mice exhibited retarded growth in comparison with their littermates (Figure 11F). Hair follicles remained shorter in K5.Smad4-/- skin (Figure 11H) than in wildtype skin (Figure 11G). Degenerated hair follicles and abnormal hair shafts were broadly observed in P10 K5.Smad4-/- skin (Figure 11H).

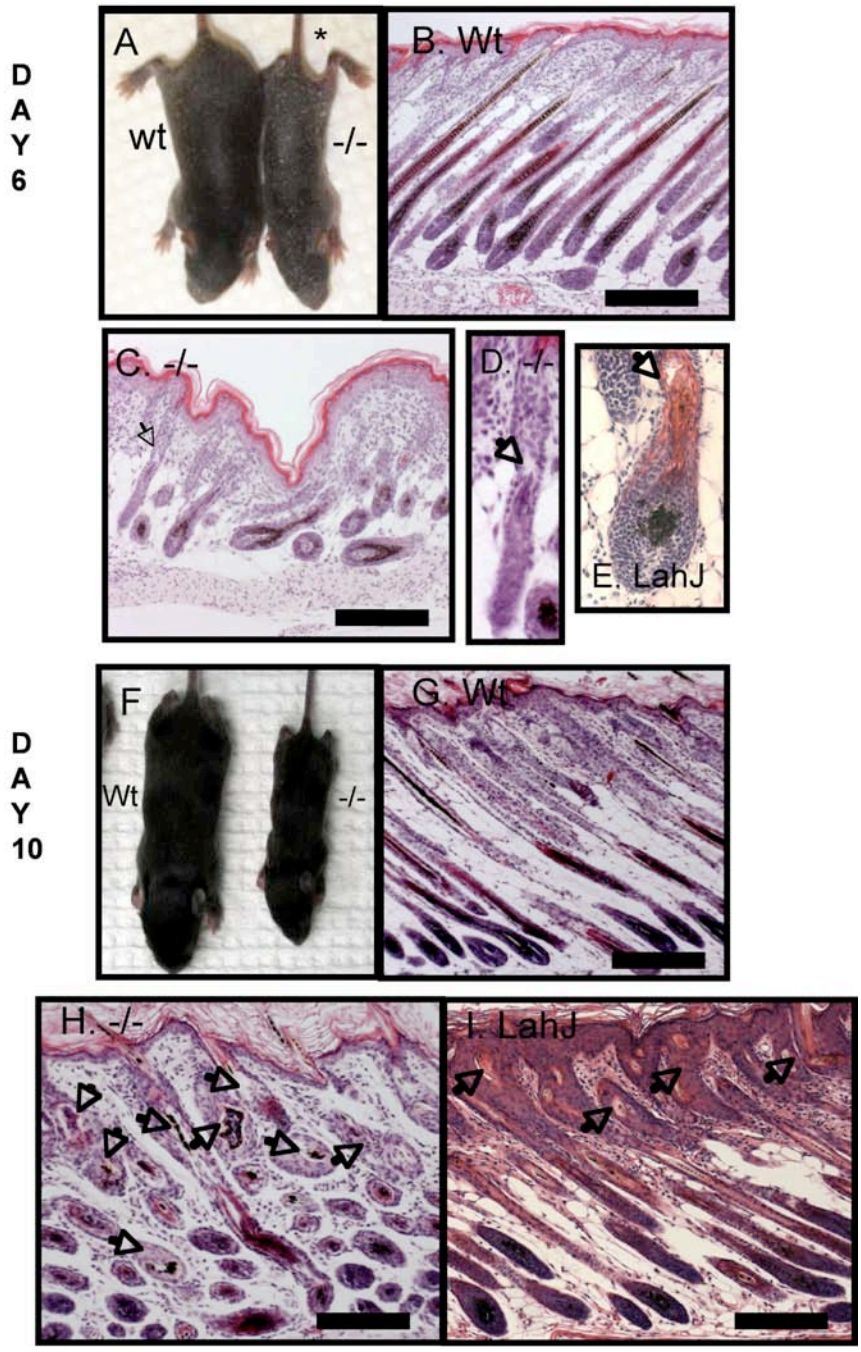


Figure 11. Early onset of hair follicle degeneration in K5.Smad4-/- skin. A: A P6 K5.Smad4-/- mouse (-/-) was slightly smaller than its wildtype (WT) littermate. The arrow in C points to a degenerated hair follicle, which is enlarged in D. E: an example of a degenerated hair follicle from a P8 Dsg4 mutant (LahJ) skin for comparison. The arrow symbols in D and E highlight the degenerated region of the hair follicle. F: A P10 K5.Smad4 -/- mouse (-/-) showed a smaller size than its wildtype (WT) littermate. G-I: Histology of P10 skins from WT (G), K5.Smad4-/- (H, -/-) and Dsg4 mutant (I, LahJ) mice. Arrows in H and I point to examples of degenerated hair follicles. Scale bars: B, C, G: 100μm; H, I: 150μm. Dsg4 mutants (LahJ) were analyzed by Hisham Bazzi.

Similarly, Dsg4 mutant hair follicles also underwent broad degeneration at this stage (Figure 11I). By P14, when hair follicles on the head region transitioned from the late anagen to the catagen phase (Figure 12B), K5.Smad4-/- mice exhibited hair loss in this anterior region (Figure 12A), which progressed as anterior-to-posterior total alopecia correlated with the anterior-to-posterior order of the catagen phase. At this stage, K5.Smad4-/- hair follicles were degenerated to form canals (Figure 12C), which were also seen in Dsg4 mutant hair follicles (Figure 12D). However, hair follicle degeneration in K5.Smad4-/- skin appeared more severe than Dsg4 mutant skin, with shorter hair follicles but prominent sebaceous glands (Figure 12C). K5.Smad4-/- hairs did not grow back after the first catagen phase. After weaning at P21, K5.Smad4-/- mice were provided with soft food. However, significant retarded growth continued in K5.Smad4-/- mice, which rendered a necessity of euthanizing them around P35. At this stage, K5.Smad4-/- mice were completely hairless (Figure 12E). Histology shows that while wildtype hair follicles re-entered into the anagen phase (Figure 12F), K5.Smad4-/- hair follicles further degenerated into cysts, which harbored inward hair shaft growth or degenerated hair shafts (Figure 12G). Similar phenotypes were also observed in Dsg4 mutant skin with the exception of a lack of enlarged sebaceous glands (Figure 12H). Topical application of RU486 to P1 bigenic skin gave rise to the same skin phenotypes except without the severe retarded growth phenotype observed when RU486 was administered *in utero*.

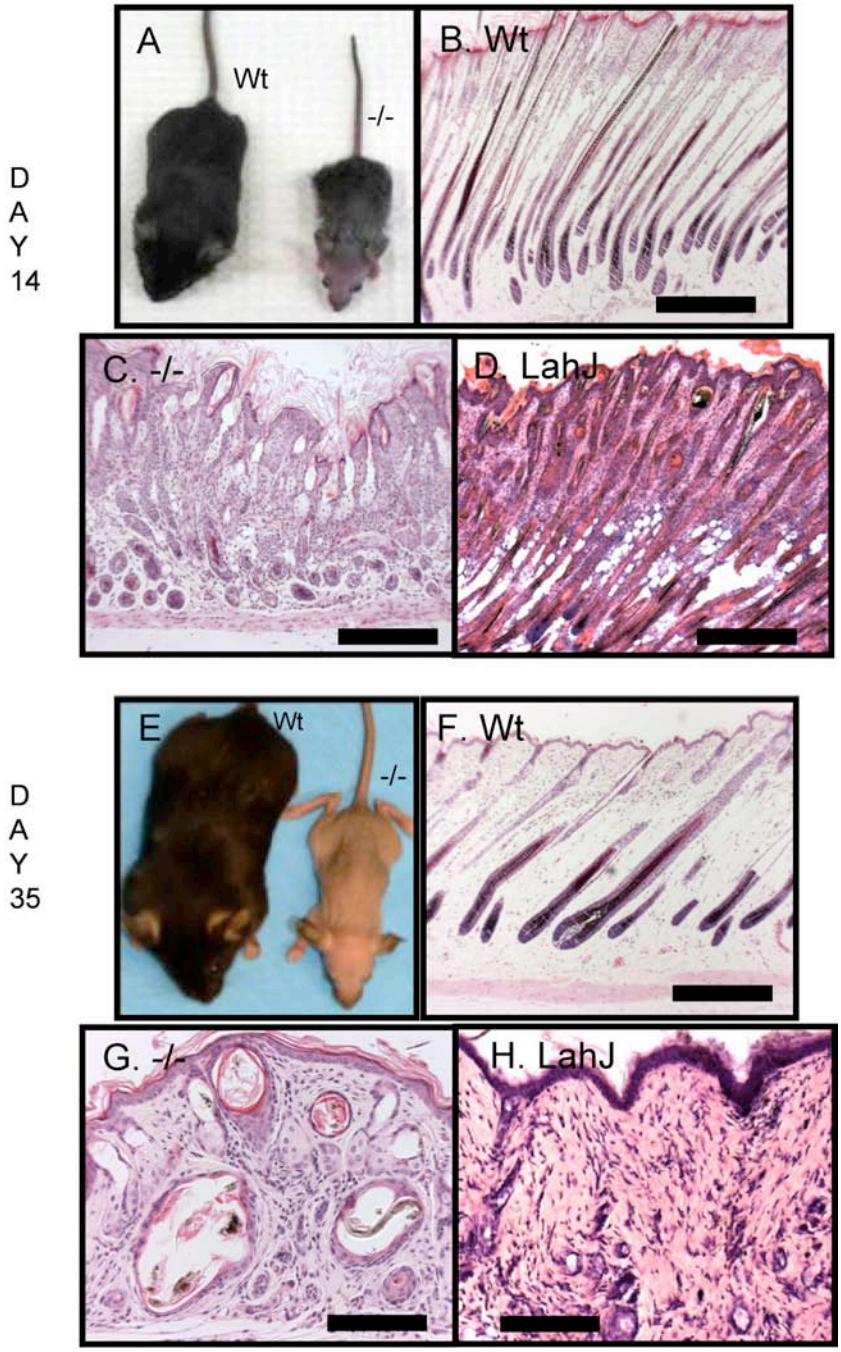


Figure 12 **Loss of Smad4 results in progressive alopecia**. A: Anterior to posterior alopecia in P14 K5.Smad4-/- skin (-/-). When wildtype (WT) hair follicles were preparing to enter catagen (B), K5.Smad4 -/- (-/-) hair follicles (C) were shorter than WT follicles in B. K5.Smad4 -/- (-/-) hair follicles formed canals and lacked hair shafts, which was a more severe phenotype than Dsg4 mutant (LahJ) hair follicle degeneration shown in D. K5.Smad4-/- skin also exhibited prominent sebaceous glands, which was not shown in P14 Dsg4 mutant (LahJ) skin (D). E: Complete hairless of P35 K5.Smad4-/- mouse (-/-). F: Wildtype (WT) hair follicles entered into the second anagen growth phase while the Smad4 -/- hair follicles were degenerated (G). H: Adult Dsg4 mutant (LahJ)

skin showing that all hair follicles were degenerated into cysts. Scale bars: B-D and F: 300μm. G and H:150 um. Dsg4 mutants (LahJ) were analyzed by Hisham Bazzi.

To determine if the enlarged sebaceous glands found in K5.Smad4-/- skin are a consequence of hair follicle degeneration or represent a direct effect of Smad4 on sebaceous gland development, we performed immunofluorescence staining for Adipophilin, a marker for sebocytes (Heid et al., 1998), at stages before and after sebaceous gland development. The number of sebocytes was comparable between wildtype and K5.Smad4-/- skin up until P6, suggesting that Smad4 loss did not accelerate sebaceous gland formation. However, sebaceous glands in adult K5.Smad4-/- skin became hypertrophic and also contained larger numbers of sebocytes in each gland, when compared with those in wildtype skins (Figure 13B and D compared to A and C). PCNA staining showed increased proliferative sebocytes in K5.Smad4-/- skin (Figure 13F) when compared with wildtype skin (Figure 13E), suggesting that increased sizes and numbers of sebaceous glands were the consequence of increased sebocyte proliferation.

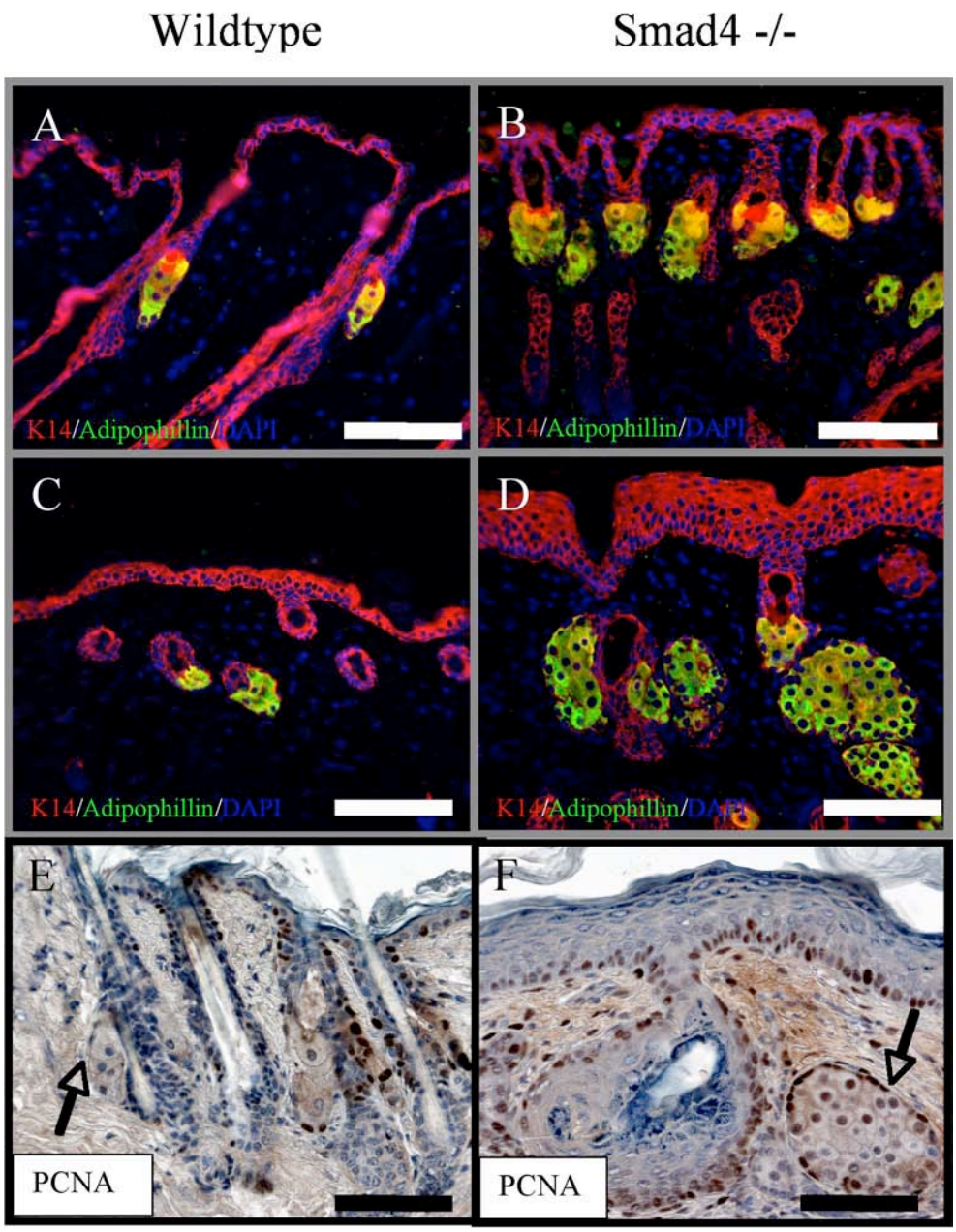


Figure 13. **Sebaceous gland hyperplasia and hyperproliferation in K5.Smad4-/- skin**. A-D.) Immunofluorescence staining for the sebocyte marker Adipophilin (green) counterstaining with K14 (red) shows no accelerated sebaceous gland development prior to P6 but progressively increased sebaceous hyperplasia in K5.Smad4-/- skin. E, F.) IHC for PCNA indicates proliferative cells with nuclei stained dark brown. Scale bars: 100μm.

3.3 Smad4 loss resulted in failure in maintaining hair shaft integrity

To determine if hair follicle degeneration in K5.Smad4-/- skin is due to defects in hair follicle differentiation, we examined several known hair follicle differentiation markers. Keratin K6, which stained in the companion layer of ORS of wildtype hair follicles (Figure 14), also stained in ORS of K5.Smad4-/- hair follicles at all stages, including the layer surrounding the hair follicle cyst. In wildtype hair follicles, the AE13 antibody stained for cortical acidic keratins, and the AE15 (trichohyalin) antibody stained the IRS and medulla cells (Figure 14). These markers were also stained in the same compartments of K5.Smad4-/- hair follicles (e.g., P6) prior to hair follicle collapse (Figure 14), but could not be detected after hair follicles were degenerated into canals and cysts at P35 (Figure 14).

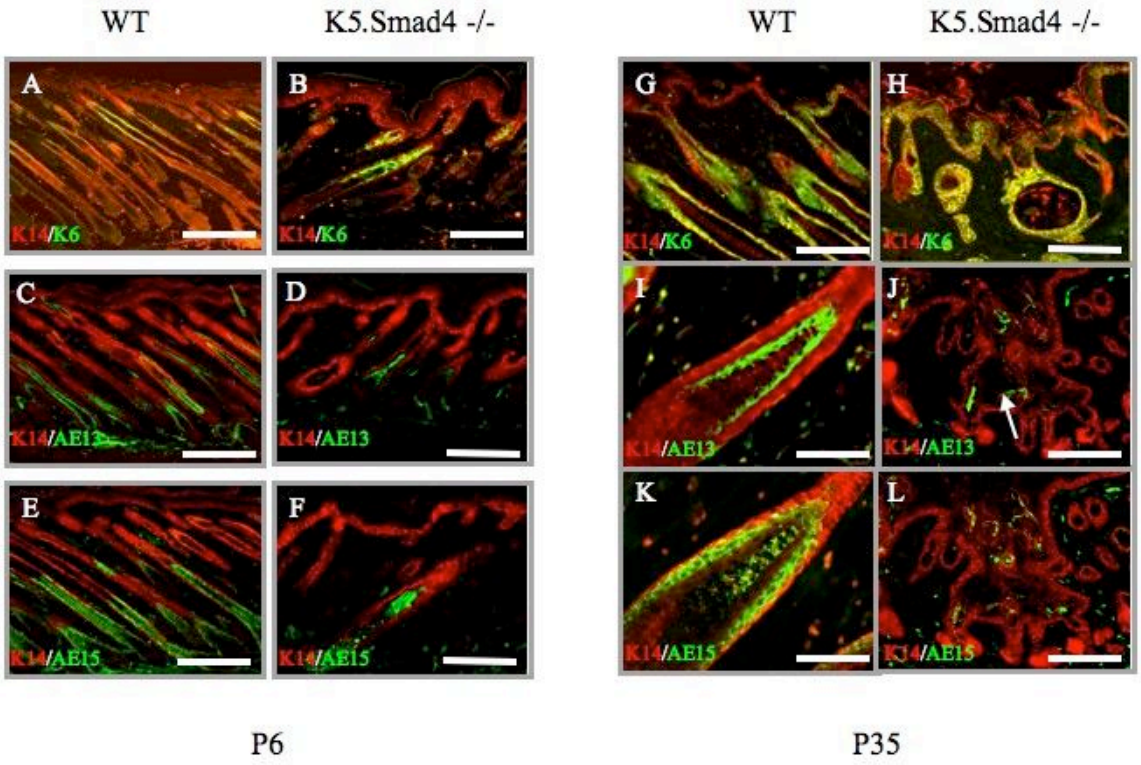


Figure 14 **Gradual loss of hair follicle differentiation markers in K5.Smad4-/- skin.** A & G) Immunofluorescence staining of P6 and P35 wildtype (WT) hair follicles shows normal Keratin6 (K6) (green) expression in upper ORS. B & H) K6 staining (green) in P6 & P35 K5.Smad4-/- skin indicates typical early expression but following hair follicle collapse, K6 positive keratinocytes expand into the epidermis (H). C-D & I-J) Staining for AE13 which indicates IRS was stained normally in wildtype (WT) hair follicles, was still present in early K5.Smad4-/- hair follicles, and could be detected in collapsed cysts at P35 (white arrow). E-F & K-L) Staining for AE15 (trichohyalin) (green) was detected in the cortical and medulla cells of the hair shaft in P6 and P35 wildtype follicles, with similar patterns in P6 K5.Smad4-/- follicles, but was absent in P35 K5.Smad4-/- hair follicles. Scale bars: I, K=50μm, B, D, F, G= 100μm, A, C, E, H, J, L=200μm.

Immunofluorescence staining for Dsg4 revealed that Dsg4 was predominantly expressed in the precortex, cortex and cuticle of the hair follicle in wildtype skin (Figure 15A, C, E), but not in the differentiated layers of the epidermis. When the new hair shaft was formed on P6, Dsg4 protein exhibited patchy staining in K5.Smad4-/- hair follicles (Figure 15B). By P14, it was obvious that hair shafts in K5.Smad4-/- skin have begun to collapse and Dsg4 was significantly reduced. Additionally, K5.Smad4-/- hair follicles,

even without obvious degeneration by histology, still exhibited reduction in Dsg4 staining when compared to wild type hair follicles. (Figure 15D). When the hair follicles eventually collapsed, a complete absence of Dsg4 and an inability to produce hair shafts were observed in K5.Smad4-/- skin (Figure 15F).

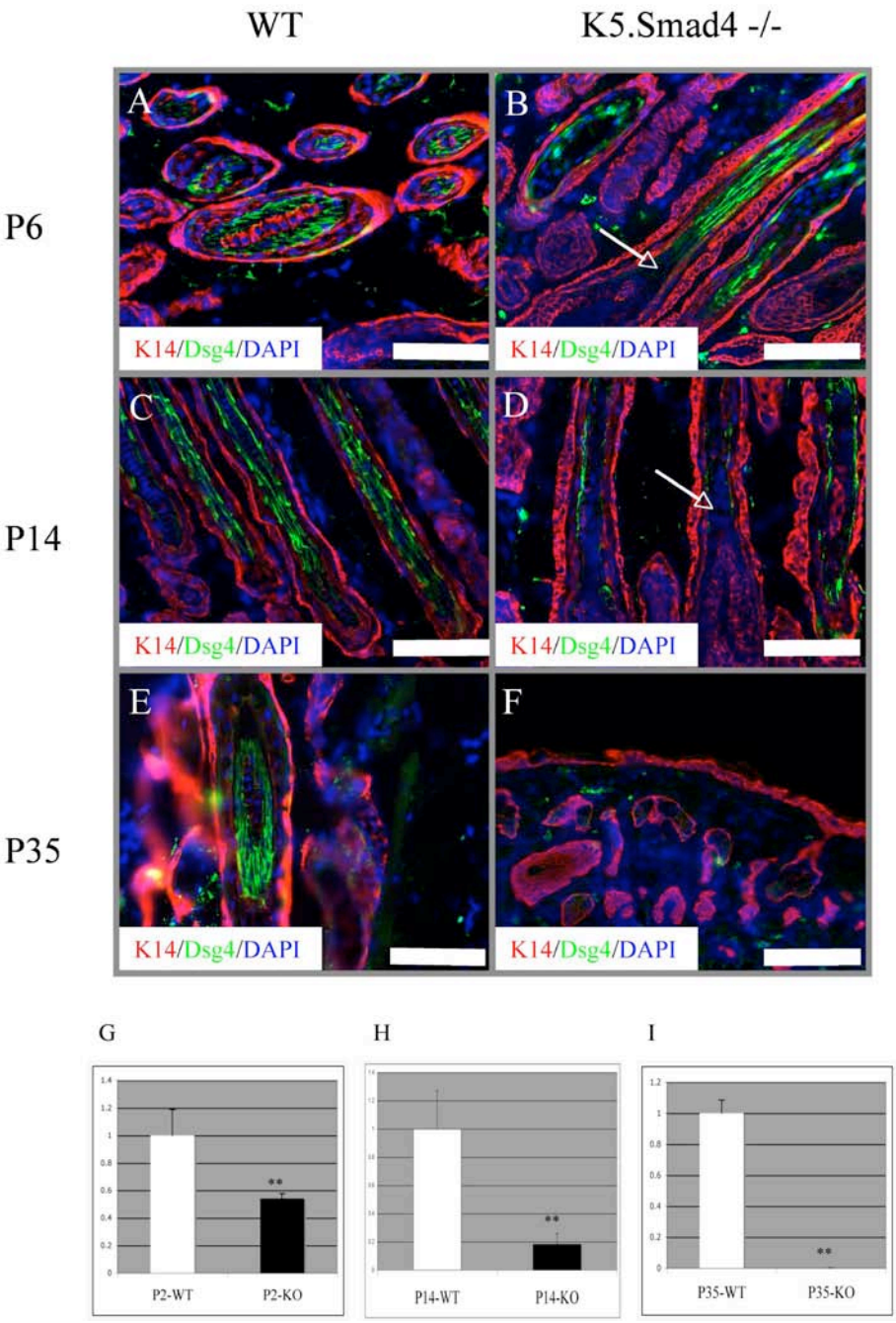


Figure 15 **Loss of Dsg4 in K5.Smad4-/-hair follicles.** A-F: Immunofluorescence of Dsg4 (green) counterstained with K5 (red). Arrows in B and D point to the loss of Dsg4 protein in the precortex region of K5.Smad4-/- hair follicles. Scale bars: A, E=50μm B, C & D = 100μm F = 200μm. G-I: qRT-PCR of *dsg4* transcripts from K5.Smad4-/- dorsal skins (KO) compared to their wildtype (WT) littermate skins (3 skins/group). **: $p<0.01$. Immunostaining for Dsg4 was performed by Hisham Bazzi.

3.4 Smad4 loss resulted in transcriptional downregulation of Dsg4 prior to hair follicle degeneration

To determine if reduced Dsg4 protein in K5.Smad4-/- skin represents a direct effect of Smad4 on Dsg4 expression, we examined Dsg4 mRNA expression at different stages of K5.Smad4-/- skin. In comparison with wildtype skin at the same time points, the Dsg4 mRNA level in K5.Smad4-/- skin was reduced by 50% on P2, further reduced by 80% on P14, and became undetectable by P35 (Figure 15G-I). To determine if loss of Smad4 specifically affects Dsg4 or affects Dsg family members in general, we examined other Dsg family members. To exclude molecular changes that are secondary to degenerated hair follicles, we compared P2 wildtype and Smad4-/- skin. At this stage, no delayed hair follicle development or hair follicle degeneration was observed in Smad4-/- skin. We found that mouse Dsg-1α, -2 and -3 were not reduced in K5.Smad4-/- skin (Figure 16). We also examined expression of several other possible Smad target genes that have been shown to regulate hair follicle formation and differentiation and are potential TGFβ/activin/BMP target genes, such as FoxN1, HoxC13, Msx2, GATA3, and Gli1 (Blokzijl et al., 2002; Dennler et al., 2007; Hussein et al., 2003; Park and Morasso, 2002). We did not find reduction of these genes in Smad4-/- skin prior to hair follicle degeneration (Figure 16). In addition, Krt31 (Ha1), a marker for hair shafts, was expressed in Smad4-/- skin at a level similar to wildtype skin at P6 (Figure 15), even when Smad4-/- skin began to show degenerated hair follicles.

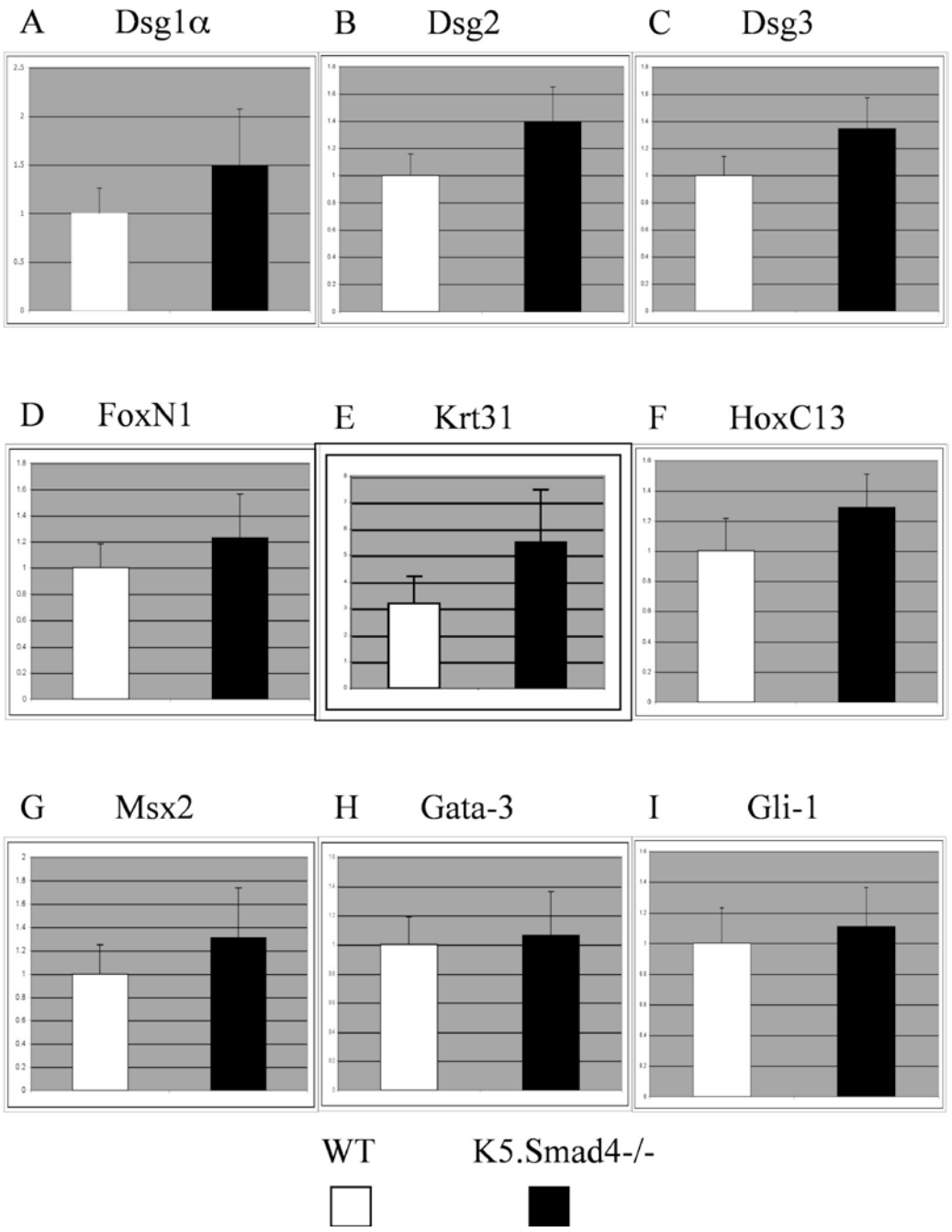


Figure 16. **No change in levels of hair follicle differentiation-associated genes in K5.Smad4-/- neonatal skin prior to onset hair follicle abnormalities.** QPCR was performed on three skin samples from each genotype. Error bars indicate standard deviation. No statistical differences were found between wildtype and K5.Smad4-/- skins. Expression levels of all markers shown here were from P2 skin except for keratin31 (Ha1), which was undetectable in P2 skin (not shown) but showed no change in Smad4-/- P6 skin (E).

Furthermore, we examined expression patterns of the E-cadherin protein, another adhesion molecule critical for epidermal and hair follicle integrity (Tinkle, et al.,2004). We found that E-cadherin staining persisted in the cell membrane of K5.Smad4-/- epidermis and hair follicles (Figure 17). To further assess any adhesion defects in the K5.Smad4-/- epidermis we performed Transmission Electron Microscopy (TEM) to closely inspect the basement membrane (BM) as well as cell-cell junctions. We found that while the epidermis was abnormal, hemidesmosomal plaques could still be seen (Figure 17). Additionally we noticed that adherens junctions between the cells were present yet occasionally would display disruptions along the cell-cell interface (Figure 17). Finally, we noticed the accumulation of mesenchymal cells along the BM that clearly had a unique role in mediating compartment homeostasis in the skin (Figure 17).

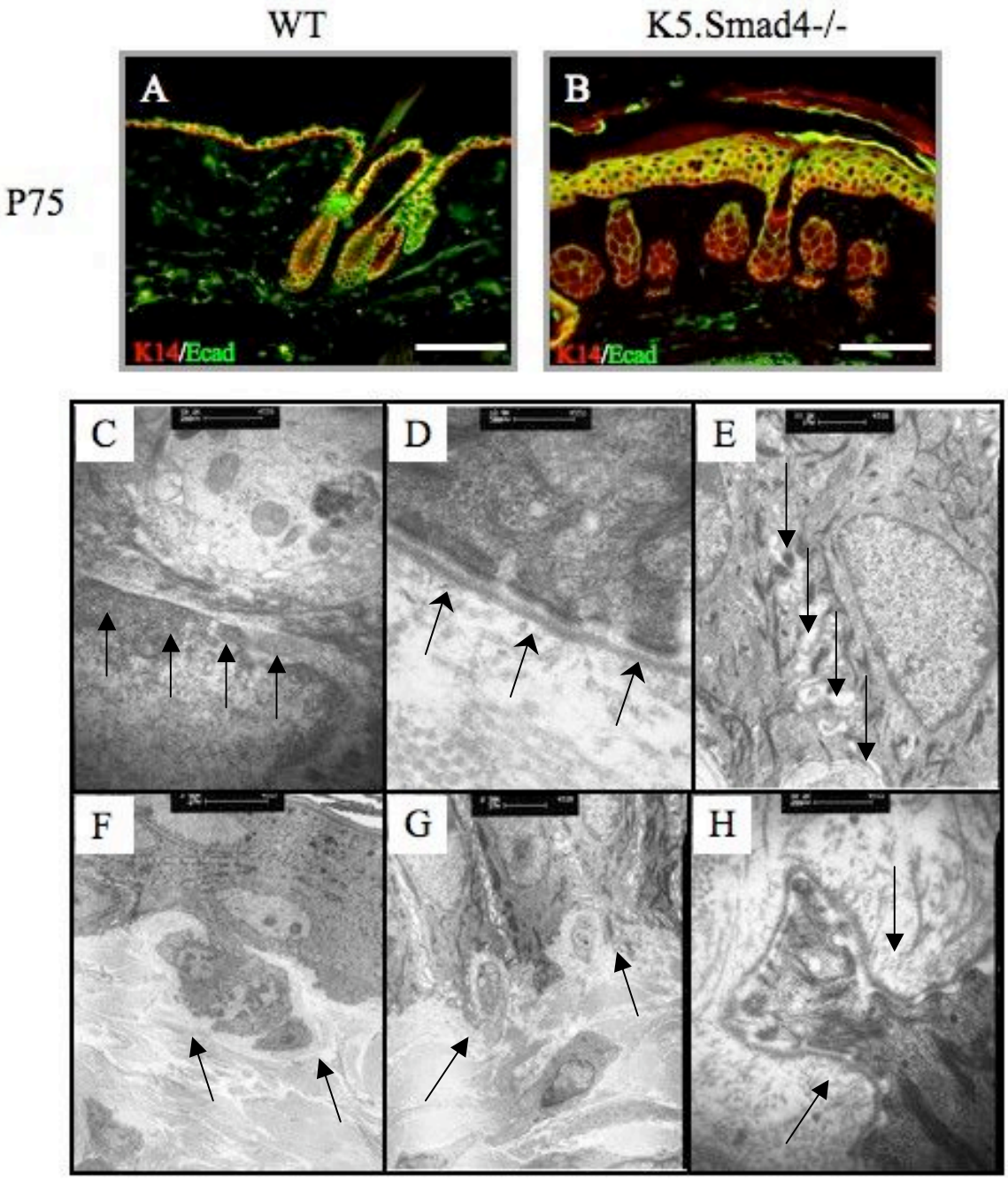
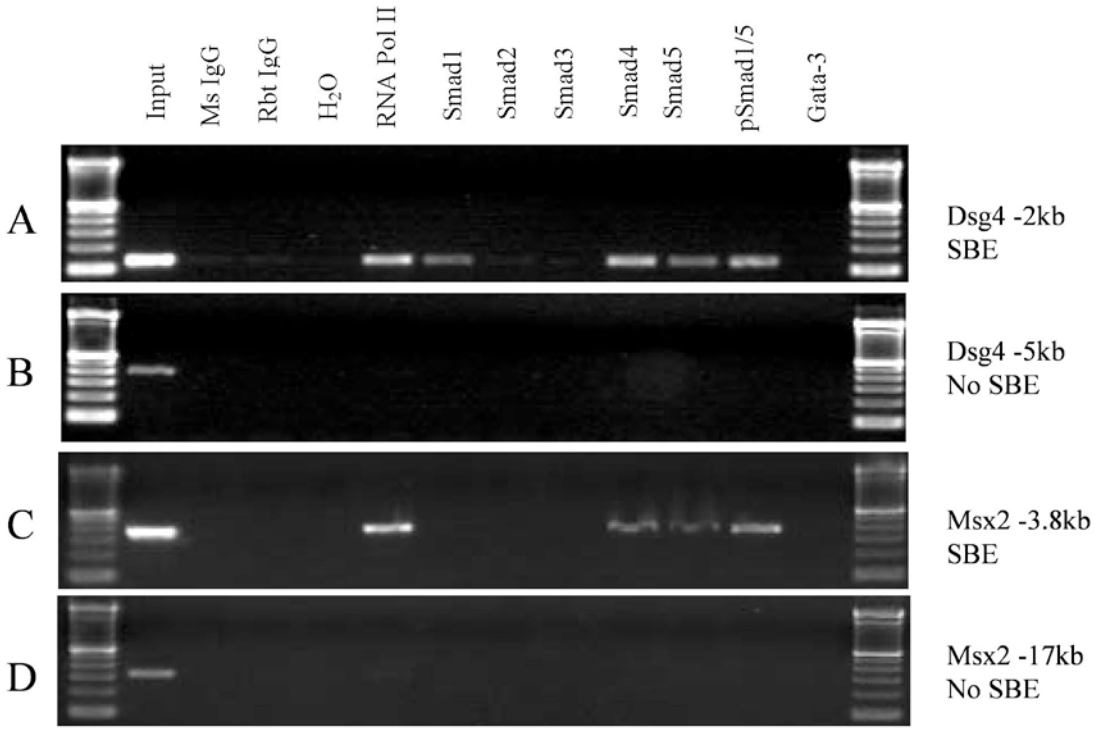
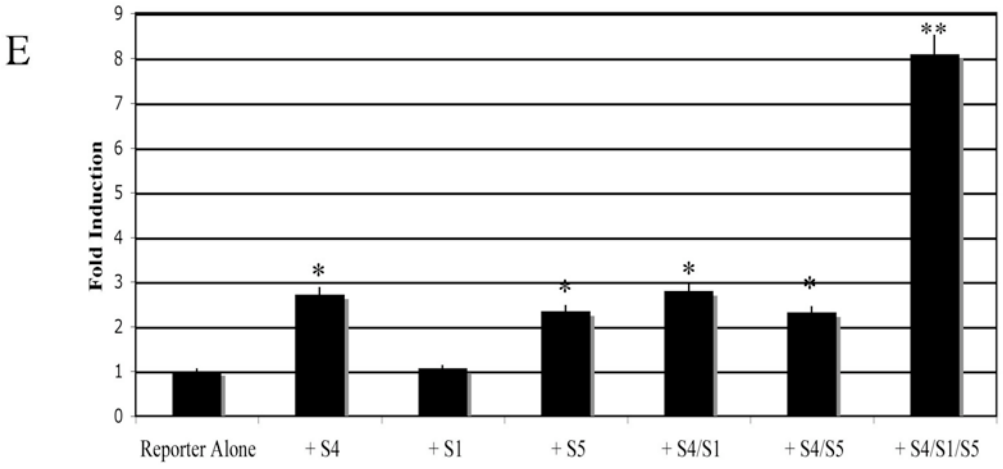


Figure 17. **Adhesion in wildtype (WT) and K5.Smad4-/- skin (P75).** Note that E-cadherin staining (green) was located at the cell membrane of the epidermis and hair follicles of both WT and K5.Smad4-/- skin, the intensity of which was stronger in differentiated cells than in basal proliferative cells. Counterstain in red is K14. C-H) TEM demonstrates that desmosomal plaques are maintained in mutant skin as well as cell-cell contacts. C) WT normal cell-junctions (black arrows) between to basal keratinocytes. D) WT normal BM desmosomal adhesive plaques are shown as dark lateral condensates (black arrows. E) KO cell-cell junctions are still maintained. F) KO BM has uneven BM (black arrows) and keratinocytes that appear to be 'detaching' from epithelium. G) Abnormal stromal cells (black arrows) interacting with Smad4 KO

epidermis. H) Increased magnification of G shows that BM desmosomal plaques (black arrows) while stressed are still present in K5.Smad-/- skin. Assistance with processing tissues and images was provided by Jackie DeGagne.

To further determine if Dsg4 was a transcriptional target of Smad4, we analyzed the mouse Dsg4 promoter sequence and found that a consensus (GTCT) Smad Binding Element (SBE) exists -2173bp upstream of Dsg4 translational start site (TSS). We next sought to identify if endogenous Smads bind to the SBE of the Dsg4 promoter *in vivo.* We performed *in vivo* chromatin immunoprecipitation (ChIP) from P6 dorsal skin of wildtype C57BL/6 mice. Smad1, 4 and 5 bound to the SBE of both the Dsg4 promoter and a known SBE in the Msx2 promoter (Figure 18A and 18C). In contrast, Smad2 and Smad3 bound to the SBE of the Snail promoter but not the Dsg4 promoter (Hoot et al., 2008). To test if Smad1/4/5 binding results in transactivation of Dsg4 expression, we performed a Dsg4-luc reporter assay, using a 3kb mouse Dsg4 promoter sequence upstream of the Dsg4 TSS. In primary epidermal keratinocytes derived from K5.Smad4-/- newborn skins, we switched the culture medium to high calcium (1.2mM) to induce promoter activity of Dsg4, and transfected Dsg4-luc together with full-length Smad1, 4, or/and 5 expression vectors. Among individual Smads transfected, Smad4 and Smad5 individually resulted in a 3-fold increase in Dsg4-luc activity, whereas Smad1 had no effect on Dsg4-luc activity (Figure 18E). However, combination of Smad1, 4, and 5 resulted in a 8-fold increase in Dsg4 promoter activity (Figure 18E). This result suggests that Dsg4 transcription is predominantly BMP-dependent and requires Smad4 for full transactivation.


Input
Ms IgG
Rbt IgG
H2O
RNA Pol II
Smad1
Smad2
Smad3
Smad4
Smad5
pSmad1/5
Gata-3
A
Dsg4 -2kb
SBE
B
Dsg4 -5kb
No SBE
C
Msx2 -3.8kb
SBE
D
Msx2 -17kb
No SBE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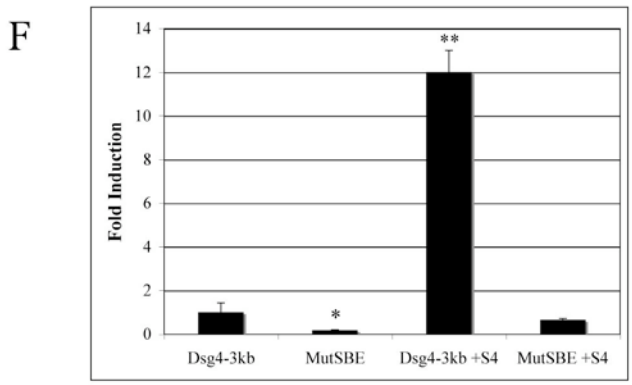
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Dsg4-3kb
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Dsg4-3kb +S4
MutSBE +S4

Figure 18. **Smad4 transcriptionally activates Dsg4 expression**. A: ChIP PCR shows that Smad1, 4 and 5, but not Smad2 and 3 bound to the promoter of Dsg4. B: PCR encompassing the Dsg4 promoter region without the SBE shows no Smad binding. Mouse (Ms) IgG and rabbit (Rbt) IgG were used as negative controls for Smad antibody. Antibody to RNA polymerase (pol) II was used as a positive control for ChIP assay. A Gata-3 antibody was used as a control for specificities of individual Smad antibodies. C: Positive control for BMP-specific Smad binding to the Msx2 promoter. D: PCR encompassing the Msx2 promoter region without the SBE shows no Smad binding. E: Luciferase reporter assay in K5.Smad4-/- keratinocytes for Dsg4 transcription with individual Smad (S) expression vectors. *: $p<0.05$, **: $p<.001$, in comparison with reporter alone transfection. F: Dsg4 luciferase reporter assay in K5.Smad4-/- hair follicle cells showed that transactivation of the Dsg4 promoter by Smad4 (S4) was dependent on wildtype SBE. MutSBE: mutated SBE in the 3kb Dsg4 promoter. *: $p<0.05$, **: $p<.001$, in comparison with transfection with wildtype Dsg4 3kb promoter reporter alone. Original cloning of the mouse Dsg4 promoter was performed by Hisham Bazzi. Mutation of the SBE element was performed by Genscript.

Next, we examined if Smad4 induction of Dsg4 occurs in hair follicle cells as well, as these cells predominantly express Dsg4 in mice. Primary mouse hair follicle keratinocytes from neonatal K5.Smad4-/- mice (in C57BL/6 background) were isolated and cultured as previously described (Han et al., 2006). After switching the culture medium to high calcium (1.2 mM) to induce promoter activity of Dsg4, we transfected a Smad4 expression vector. Smad4 induced Dsg4 luc activity to a level (Figure 18F) similar to that shown in cultured epidermal keratinocytes (Figure 18E). In contrast, mutating the SBE site within the Dsg4 reporter abolished both basal and Smad4-induced luc activity (Figure 18F), suggesting that Smad4 binding to the SBE is necessary for regulating Dsg4 expression. To further test if this is the case for endogenous Dsg4, we treated primary keratinocytes with different TGFβ family member ligands, and examined endogenous Dsg4 mRNA levels. TGFβ2, which is essential for hair follicle development (Foitzik et al., 1999), resulted in a reduction of Dsg4 mRNA level (Figure 19). Activin A had no effect on Dsg4 expression (Figure 18). However, BMP2 increased Dsg4 mRNA

expression in comparison with controls. These inductive experiments support the observation that Dsg4 is a target of BMP signaling. The effect of TGFβ2 on reducing the levels may be attributed to the impairment of terminal differentiation and lack of Dsg4 differentiated cells.

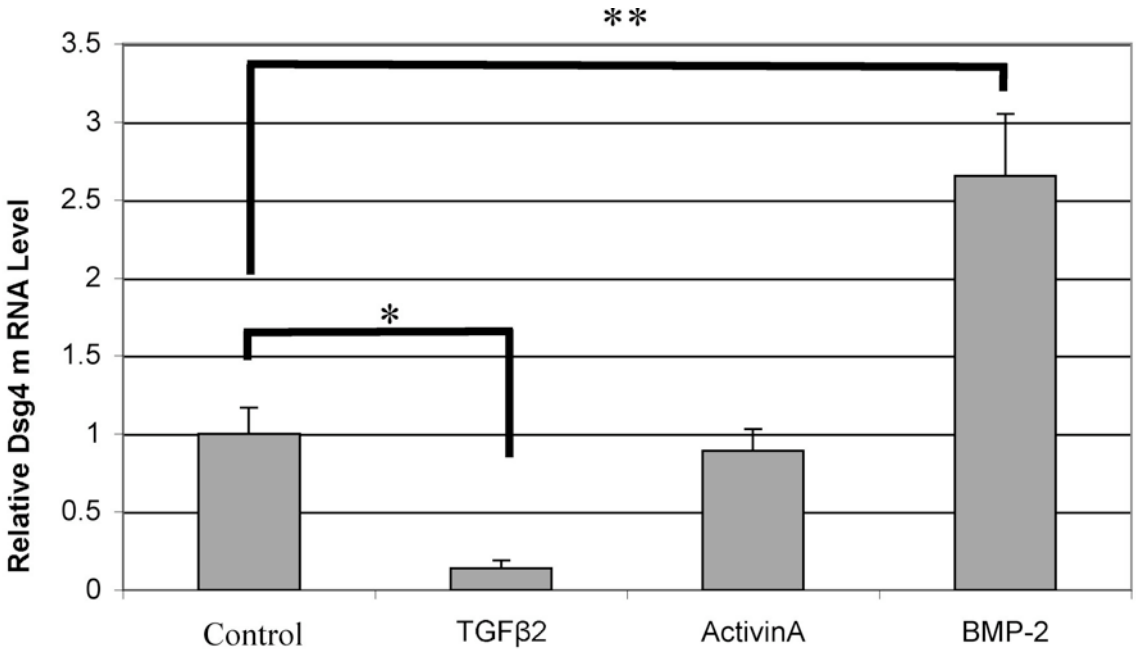

Figure 19 **BMP induction of Dsg4.** Primary wildtype mouse keratinocytes were grown in 1.2mM calcium for 48 hours with or without TGFβ2, activin A or BMP-2 at a concentration of 10ng/ml. All samples were analyzed in quadruplicate, and normalized to GAPDH internal multiplexed control. *: $p<0.05$, **: $p<0.01$ in comparison with no ligand control. Initial culture experiments were performed on primary cells isolated at CellNTec by Peter Girling and associates.

3.5 Discussion

In the present study, we deleted Smad4 in keratinocytes at different stages during skin development. Smad4 deletion did not affect epidermal differentiation or initial events of hair follicle morphogenesis. However, hair follicles were degenerated in

K5.Smad4-/- skin after birth. In comparison with previous studies (Qiao et al., 2006; Yang et al., 2005), the onset of hair follicle defects appeared earlier and more severe, possibly due to Cre-mediated Smad4 excision being more complete by the current K5 promoter (Zhou et al., 2002). The uniform Smad4 deletion in keratinocytes allowed us to further analyze the molecular targets of Smad4 loss-associated hair follicle defects.

Smad4 is required for maximal transactivation of Dsg4 via interaction with BMP-specific Smads

Smad4 is a common Smad that potentially mediates signaling from TGFβ, activin and BMP. We have previously shown that Smad4 loss in keratinocytes results in abrogation of TGFβ-mediated expression of cell cycle inhibitors (Qiao et al., 2006), which explains the hyper-proliferative phenotype in K5.Smad4-/- epidermis, hair follicles and sebaceous glands. However, as ablation of neither TGFβ receptors (Honjo et al., 2007; Wang et al., 1997), Smad2 (our unpublished data), nor Smad3 (Li et al., 2004) resulted in hair follicle abnormalities, Smad4-loss associated hair follicle defects are likely to be a result of blockade of either the BMP pathway alone, or a combination of TGFβ/Activin and BMP. We found that Smad4 loss led to the reduction of Dsg4 mRNA, a protein critical for hair shaft differentiation and adhesion (Kljuic et al., 2003; Qiao et al., 2006), at a stage prior to hair shaft formation in normal mice and to hair follicle degeneration in K5.Smad4-/- mice. Further, Smad1, 4 and 5, but not Smad2 and 3, directly bound to the SBE of the Dsg4 promoter, and the combination of Smad1, 4 and 5 achieved the highest transactivation of the Dsg4 promoter. Lastly, mutating the SBE of

the Dsg4 promoter abolished the effect of Smad4 on Dsg4 promoter activity. These data suggest that Dsg4 is a direct transcription target of Smad which is mainly activated by BMP signaling. In supporting of this notion, the ligand of BMP (BMP2), but not TGFβ or Activin, induced endogenous Dsg4 expression. Consistent with our current finding, keratinocyte deletion of the BMP type I receptor, ALK-3, exhibited a similar hair follicle phenotype, i.e., does not affect initial hair follicle development, but results in degeneration of the hair follicle and hair shaft (Andl et al., 2004; Kobielak et al., 2003; Ming Kwan et al., 2004; Yuhki et al., 2004). However, since the combination of Smad-1, -4, and -5 induced the highest transcriptional activity of Dsg4, and knocking out both Smad1 and Smad5 in keratinocytes did not show abnormalities in hair follicle differentiation (our unpublished data), our findings suggest that Smad4 is indispensable for maximal transactivation of Dsg4.

Downregulation of Dsg4 contributes at least in part to the defects in hair shaft differentiation and adhesion in K5.Smad4-/- skin

Dsg4 has been shown to be critical for the proper differentiation and specification of trichocytes of the hair shaft (Kljuic et al., 2003). Mutations in Dsg4 result in balding due to the lack of proper hair shaft formation and adhesion. Therefore, if Dsg4 is a major Smad4 target gene contributing to the role of Smad4 in hair differentiation, it is not surprising that K5.Smad4-/- skin still undergoes normal hair follicle development during embryogenesis, given the fact that Dsg4 is mainly expressed in differentiated pelage hair shaft after birth. Although it remains to be determined what signal plays a major role in

switching on Dsg4 expression and how it interacts with Smad4, it appears that the initiating signals for Dsg4 expression in trichocytes are sufficient to support the first wave of hair shaft formation even in the absence of Smad4. Alternatively, Smad4 only regulates Dsg4 expression level modestly or to a limited extent, such that the remaining Smad1 and Smad5 together with other unknown cofactors may transcribe the amount of Dsg4 sufficient for the first wave of hair shaft formation. However, sustained Dsg4 expression during hair follicle regeneration was hindered by the loss of Smad4. It is possible that the defects in hair follicle regeneration in Smad4-/- skin largely prevented the renewal of Dsg4 positive cell types. It is also possible that regeneration of Dsg4 positive cells require maximal transcriptional upregulation of Dsg4.

Unlike Dsg3 knockout mice, which show cyclical balding due to adhesion defects of the club hair anchorage (Koch et al., 1997; Pulkkinen et al., 2002), K5.Smad4-/- hair shafts, which are similar to Dsg4 mutant hair shafts, have the combination of defects in both Dsg-mediated adhesion and associated hair shaft differentiation. At early stages, K5.Smad4-/- hair follicles showed phenotypes similar to Dsg4 mutant mouse and human follicles, i.e., degeneration of hair follicles, inward hair shaft growth, fragile hairs which disappear after the first catagen phase, and hair shaft collapsing in epidermoid cysts highlighting adhesion defects. However, we did not observe changes in E-cadherin expression (Figure 17), suggesting that the adhesion defect is restricted to the Dsg4-mediated adhesion but not to the adhesion complex. This result is consistent with the observation that Smad4 is required for suppression of E-cadherin expression (Deckers et al., 2006). In addition to hair follicle abnormalities, Dsg4 mutant mouse epidermis also showed epidermal hyperplasia, possibly as a secondary effect to hair shaft abnormalities.

Similarly, K5.Smad4-/- epidermis also exhibited more obvious epidermal hyperplasia after hair follicle abnormalities became apparent. Therefore, although K5.Smad4-/- epidermis is hyperplastic mainly due to abrogation of TGFβ-mediated growth arrest (Yang et al., 2005), down regulation of Dsg4 in K5.Smad4-/- epidermis could also contribute to epidermal hyperplasia. However, consistent with Dsg4 mutant mice, epidermal differentiation was not altered in K5.Smad4-/- epidermis. Since Dsg4 in differentiated epidermal keratinocytes is expressed at much lower level in mice than in humans (Bazzi et al., 2005), the predominant effect of Dsg4 downregulation was restricted to hair shafts. It is also possible that the epidermis has other Dsg family members that will compensate for Dsg4 loss, whereas Dsg4 is the only predominant Dsg in differentiated hair shafts.

The phenotypes of K5.Smad4-/- skin suggest multiple functions of Smad4 in skin homeostasis

Although K5.Smad4-/- mice had hair follicle defects similar to Dsg4 mutant mice, K5.Samd4-/- mice also showed significant sebaceous hyperplasia. Additionally, there are differences between keratinocyte-specific ALK-3-/- mice and Smad4-/- mice: the former eventually developed hair follicle tumors but did not show expanded sebaceous glands (Andl et al., 2004; Kobielak et al., 2003; Ming Kwan et al., 2004; Yuhki et al., 2004), whereas the latter mainly develop SCC (Qiao et al., 2006; Yang et al., 2005). These observations highlight the multi-functionality of Smad4 and its diversified transcriptional targets that are cell-context specific. For instance, Smad4 loss-associated

hyperproliferation and tumorigenesis are largely attributed to its abrogation of Smad2 and Smad3-mediated transcriptional responses to cell cycle arrest (Qiao et al., 2006), as these processes have been shown to require Smad4 (Levy and Hill, 2005). In contrast, Smad4 loss-associated hair follicle defects are the result of abrogation of BMP signaling. In addition, we have previously shown that Smad4 loss leads to down regulation of Lef1 (Qiao et al., 2006), a key transcription factor for the Wnt signaling. In contrast, Smad4 loss did not alter expression of hedgehog signaling components. Perturbing the balance of these two pathways has been shown to switch the epidermal stem cell decision from the hair follicle fate to the sebocyte fate (Niemann et al., 2003). Thus, reduced Wnt signaling and a relative increase in hedgehog signaling in K5.Smad4-/- skin could contribute to sebaceous hyperplasia. However, different from Smad7 transgenic mice in which sebaceous gland development was accelerated (Han et al., 2006), K5.Smad4-/- skin did not show this phenotype. Unlike Smad7, which induces b-catenin degradation via protein-protein interaction thus has a more potent inhibitory effect on Wnt signaling (Han et al., 2006), Smad4 is only known to affect Wnt signaling via transcriptional regulation (Hussein et al., 2003; Lim and Hoffmann, 2006). Therefore, sebaceous gland hyperplasia in K5.Smad4-/- skin could be the result of a combination of mildly reduced Wnt signaling and direct proliferative effect of Smad4 loss due to abrogation of TGFβ-mediated growth arrest. Notably, although K5.Smad4-/- skin has multiple defects, skin development and epidermal differentiation are not altered. Some of the gene products, which are involved in these processes, e.g., such as Msx2, HoxC13, and Gli1, are known or potential Smad target genes (Blokzijl et al., 2002; Dennler et al., 2007; Hussein et al., 2003; Park and Morasso, 2002). However, expression levels of these genes were not

changed in K5.Smad4-/- skin prior to hair follicle degeneration while other Smad target genes related to cell cycle control were down regulated (Qiao et al., 2006; Yang et al., 2005), suggesting that transcriptional regulation of these genes could be either Smad4 independent, or dispensable for Smad4 effects due to *in vivo* compensatory mechanisms. Indeed, Smad4 independent transcriptional regulation of Smad-dependent genes has been observed (Levy and Hill, 2005). Smad4 has also been shown to be in competition with other transcription factors such as TIF1γ for binding to activate Smads 2/3 (Deckers et al., 2006).

In summary, we identified Dsg4 as a direct transcriptional target of Smad4. Such a Smad4 target gene would not necessarily be identified using routine screening for transcriptional targets, given the fact that Dsg4 expression is restricted to certain developmental stages and in specific differentiated cell compartments *in vivo*. Our data further suggest that loss of Dsg4 expression in K5.Smad4-/- keratinocytes contributes to hair follicle degeneration. However, we should also point out that not all of the phenotypes of K5.Smad4-/- skin can be explained by Dsg4 downregulation and that K5.Smad4-/- and Dsg4 mutant mice have distinguishable phenotypes. These differences highlight the multifunctional nature of Smad4 in the skin and our current study shows the importance of identification of functional Smad transcription targets in a context-specific manner during different developmental stages and physiological/pathological conditions. Future study of expressing a Dsg4 transgene in K5.Smad4-/- skin would ultimately determine to what extent Dsg4 loss mediates hair follicle degeneration associated with Smad4 loss.

Chapter 4: Loss of Smad4 in Skin Results in Aberrant Wound Healing

4.1 Introduction

Cutaneous wound healing is a complex yet highly coordinated process that encompasses distinct yet overlapping processes for review see (Singer and Clark, 1999). Successful wound healing is dependent upon well-regulated inflammation, reepitheliazation and stromal remodeling. Inflammation occurs immediately upon wounding and begins with platelets degranulating and releasing many pro-inflammatory molecules. This triggers the recruitment of neutrophils followed by macrophages, which will phagocytose debris in the wound and subsequently undergo programmed cell death. Concurrently, keratinocytes from the epidermis must begin to reepithelialize into the wound and form what is known as a "migrating tongue" in order to close the wound. Once the wound has been closed and inflammation has subsided, repair of the dermis can begin by the remodeling of the granulation tissue and reorganization of the extracellular matrix (ECM). New blood vessels that helped deliver the inflammatory leukocytes and nutrients regress and become quiescent. Myofibroblasts that were activated to secrete the new ECM and growth factors must undergo programmed cell death after synthesizing the newly arrayed collagen. Finally a scar will remain at the wound site. The scar tissue that remains will never quite attain the original integrity of the non-wounded skin.

Smad4 is at the nexus of the TGFβ/Activin/BMP signal transduction pathways. Smad proteins are cytoplasmic proteins which transmit the signals propagated from

TGFβ/Activin/BMP ligands binding to their cognate receptors (for a detailed review of Smads see Massague, 2005(Massague et al., 2005)). Briefly, receptor Smads (Smad2 and Smad3) are phosphorylated by TGFβ family receptors and associate with the common partner Smad: Smad4 (the co-smad). TGFβ ligands have been shown to be a dominant force in regulating all phases of cutaneous wound healing in every cell type(Wang et al., 2006). TGFβ1 has been shown to exacerbate inflammation during wound healing when overexpressed(Li AG, 2004). Activin and BMP signaling molecules have been observed to also play roles in wound repair and have been shown to be expressed during wounding(Hubner et al., 1996b). Mice that overexpress Activin A in keratinocytes during wound healing show epidermal hyperplasia and fibrosis in the dermis (Munz et al., 1999). There is no current data indicating whether BMP related receptor Smads 1, 5 and 8 or inhibitory Smad 6 play a role in cutaneous wound healing, however, BMP signaling molecules have been shown to be important in this process. BMP-2, when exogenously administered to wounds, causes stimulation of the keratinocytes and the dermal fibroblasts(Stelnicki et al., 1998). BMP-6 is strongly upregulated at the wound edge in keratinocytes and granulation tissue, and when overexpressed in keratinocytes, reepitheliazation and proliferation are impaired(Kaiser et al., 1998). These findings, when taken into consideration together, indicate that selective suppression of TGFβ signaling in certain cell types in cutaneous wounds may benefit wound healing.

Downstream of TGFβ superfamily ligands and receptors, Smads have proved important in many aspects of the wound healing process. Smad2, a TGFβ/Activin related receptor Smad, has been shown to result in keratinocyte restriction of reepithelialization and

wound closure when it is overexpressed(Ito et al., 2001). Smad3 has been extensively studied as it has profound effects on mediating wound healing. For a detailed review of Smad3 see (Roberts et al., 2006). Smad3 knockout mice are viable and their deletion occurs in all cell types including leukocytes, keratinocytes and fibroblasts. Smad3 null mice exhibit accelerated wound healing, indicated by increased keratinocyte proliferation and migration, and reduced monocyte infiltration (Ashcroft et al., 1999). Smad7, an antagonist of TGFβ signaling, which blocks Smads 2 & 3, accelerates corneal wound healing via promoting epithelial cell migration and inhibiting monocyte/macrophage invasion to the wounds as demonstrated when it is exogenously added to mouse eyes (Saika, 2004; Saika et al., 2005). Smad4, however, has only been studied *in vitro* by scratch assays in 2D cell culture in which it has been shown that epithelial cells lacking Smad4 have defective cell migration, especially in response to the TGFβ ligand(Levy and Hill, 2005). Smad4 mediates the signaling of all of the growth factor/cytokines of the TGFβ superfamily including TGFβs, Activins and BMPs, therefore loss of this molecule during cutaneous wound healing could have vast implications. TGFβ/Activin/BMP molecules all demonstrate an ability to induce tissue expansion during wound repair. Defective wound healing can result from too much or not enough of these signaling molecules from imprecise temporal/spatial expression. Smad4 has been shown to have dependent and independent signaling to mediate the diverse effects of TGFβ/Activin/BMP(Levy and Hill, 2005). The role of Smad4 as a tumor suppressor controlling cell autonomous as well as extracellular microenvironments during tumorigenesis therefore warrants a closer look as it functions in the processes of wound healing(Kinzler and Vogelstein, 1998; Kitamura et al., 2007).

Here we show that disruption of Smad4 in the skin results in abnormal cutaneous wound healing. Competing forces in wound healing do not reconcile themselves and belay further pathological consequences of Smad4 loss *in vivo*. Migration of Smad4 deficient epidermal tongues is not impaired, and the compromise made involving an increased inflammatory response promotes excessive scarring and matrix remodeling failures. Smad4 deficient mice have previously been demonstrated to generate spontaneous tumors that mirror the changes in the Smad4 absent epidermis(Qiao et al., 2006). Here we find support of the hypothesis that 'tumors are wounds that do not heal' in that the Smad4 tumor suppressor function relies on cell non-autonomous effects in the stromal microenvironment for proper wound healing.

4.2 Wounds in Smad4 deficient skin heal poorly

Previously we mated Smad4 floxed mice with MMTV-Cre mice and showed that Smad4 was deleted in hair follicles and the epidermis (Xu et al., 2006). Prior reports had demonstrated that MMTV-Cre activity could be extended out of the epithelium and abnormally expressed in various cell types such as B and T cells (Wagner et al., 2001; Wagner et al., 1997). To further assess this possibility we crossed MMTV-cre/Smad4f/f mice with a ROSA26R reporter to make tri-genic mice. We performed wounds on these mice and stained for LacZ. We show that in the day 3 wound margin only the epithelium and no stromal cells were stained indicating recombination of the Smad4 allele was restricted to the epidermis (Figure 20B).

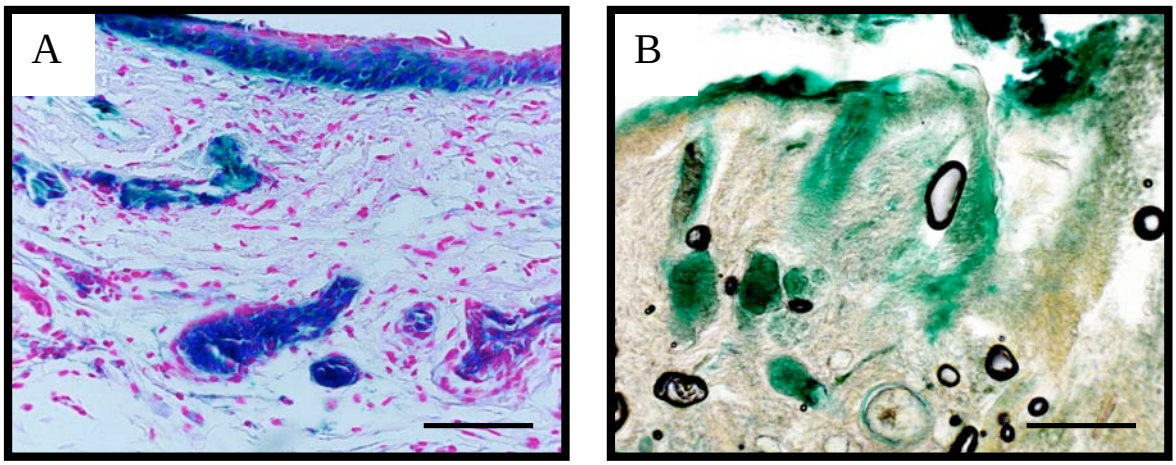

Figure 20. **LacZ staining of MMTV-Cre/Smad4/Rosa26R skin and wounds.** ROSA26 Cre reporter mice were crossed with MMTV-Cre/Smad4 mice to demonstrate where the Smad allele was undergoing recombination. A) In normal skin and hair follicles but not in the stroma MMTV-Cre is directing recombination of the Smad4 allele, B) during the 3rd day of wounding only the hair follicles and epidermal tongue demonstrate Cre activity. Staining was assisted by Tim Cleaver and Stephen Malkowski. Green color was resulted in B from fixation of wounds, which required gradual and careful washes of fixative.

Previously we had isolated primary keratinocytes that were null for Smad4. We first used these cells for comparison with normal Smad4 expressing cells to perform an in vitro 'scratch assay'. We found that these cells had defective migration and did not heal their artificial wound as well as the normal controls (Figure 21). Knockout (KO) cells, if able to use their previously described proliferation advantage, can heal the scratch most likely due to increased cell number. However, when MMC treatment blocks proliferation, and only contributions to cell migration can be considered, KO cells do not populate the 'wound' area as well as WT. Additionally, KO cells appear more fibroblastic in response to MMC treatment and do not show coordinated 'sheet movement' as compared to MMC treated WT controls (Figure 21). One of the great disadvantages to this assay is that these cells, while capable of being cultured on specific substrates (e.g.; collagen IV, Laminin, etc.), do not have blood vessels and leukocytes delivering directed factors for wound

stimulation. Another caveat to this assay is that these cells are migrating as a single layer and do not reflect the stratified epithelium in skin or the hyperproliferation that occurs with migrating epidermal tongues.

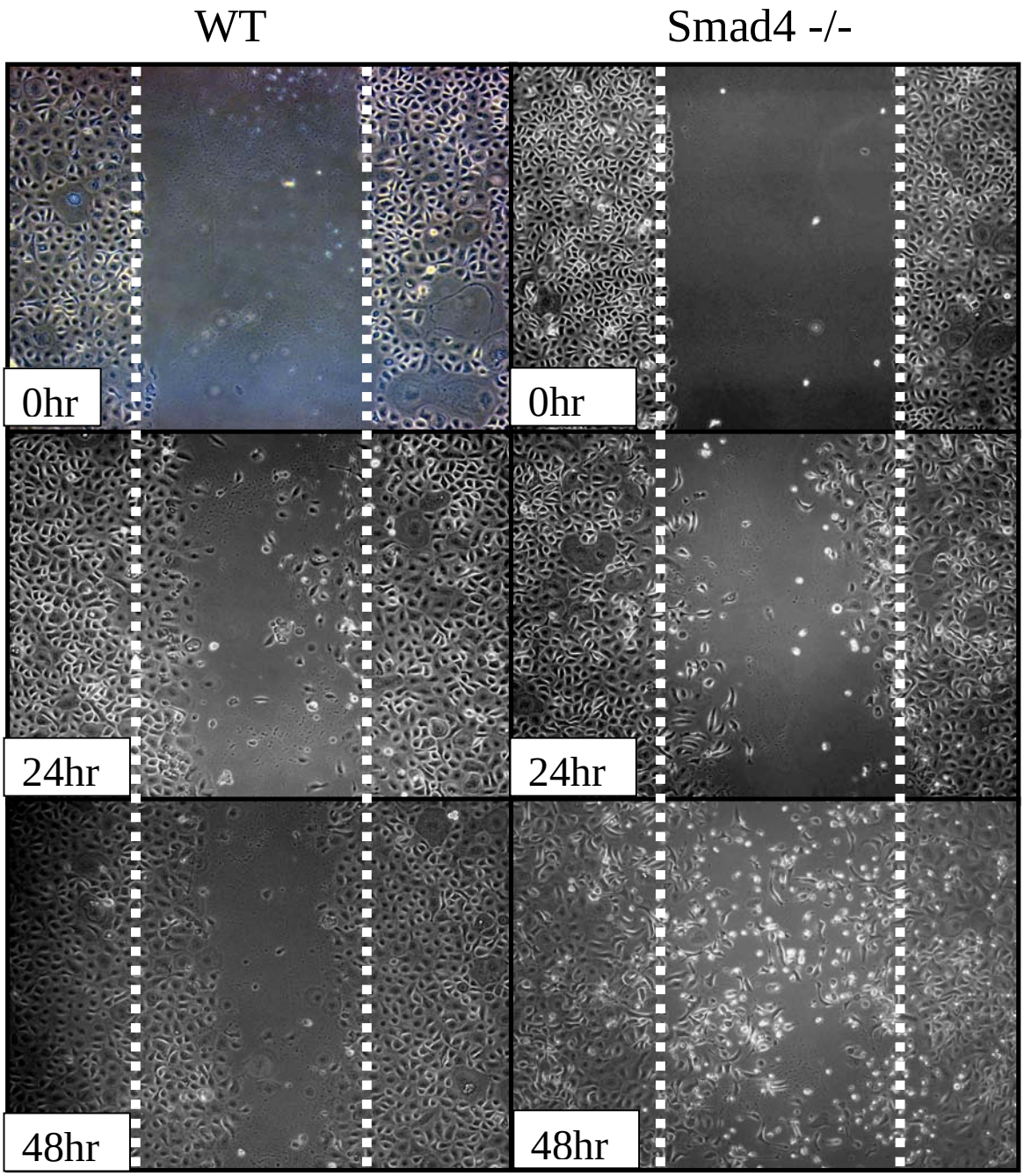

Figure 21. In Vitro Scratch Assay. Primary keratinocytes from wild-type skin or K5.Smad4-/- mice were grown to confluence and scratched with a pipette tip. Another group was treated with MMC to inhibit proliferation. Experiments were done in triplicate

and repeated more than three times. WT cells are primary keratinocytes from C57/BL6 neonatal pups. KO is Smad4 null keratinocytes as previously described above. MMC was treated for two hours prior to 'wounding'. If Smad4 KO primary cells have a proliferative advantage (no MMC treatment) then they can fill the wound, however in response to MMC the migration is perturbed and mostly dead or detached cells appear in the wound margin area.

To further assess the functional consequence of Smad4 loss in the epidermis, we performed cutaneous wound healing *in vivo* by making four 6mm full thickness wounds in compound MMTV-Cre and Smad4 floxed mice (referred to as KO) and mice lacking MMTV-Cre (referred to as WT). We captured images of these wounds and show that in KO animals, wounds are poorly healed (Figure 22A). Wounds at day 3 and day 5 show a larger area of initial wound than the WT controls. Wound images were analyzed for change in the initial area of the wound and it was shown that when compared with WT controls a statistically significant change in wound area was observed (Figure 22B&C). Wounds in WT mice had 47% of their average wound area remaining of 68 wounds analyzed in 17 mice. In comparison, KO mice had only 71% of their average wound area remaining in 56 wounds analyzed in 14 mice. At five days after wounding this discrepancy in healing was even more pronounced. WT wounds after five days had an average of 30% of their initial wound area remaining while KO wounds had 48% of their initial respective wound area remaining (Figure 22C). When these data are displayed as box-whisker plots to adequately display the distribution and inherent variability associated with cutaneous wound healing, it can be seen that the inner quartiles did not overlap by comparison.

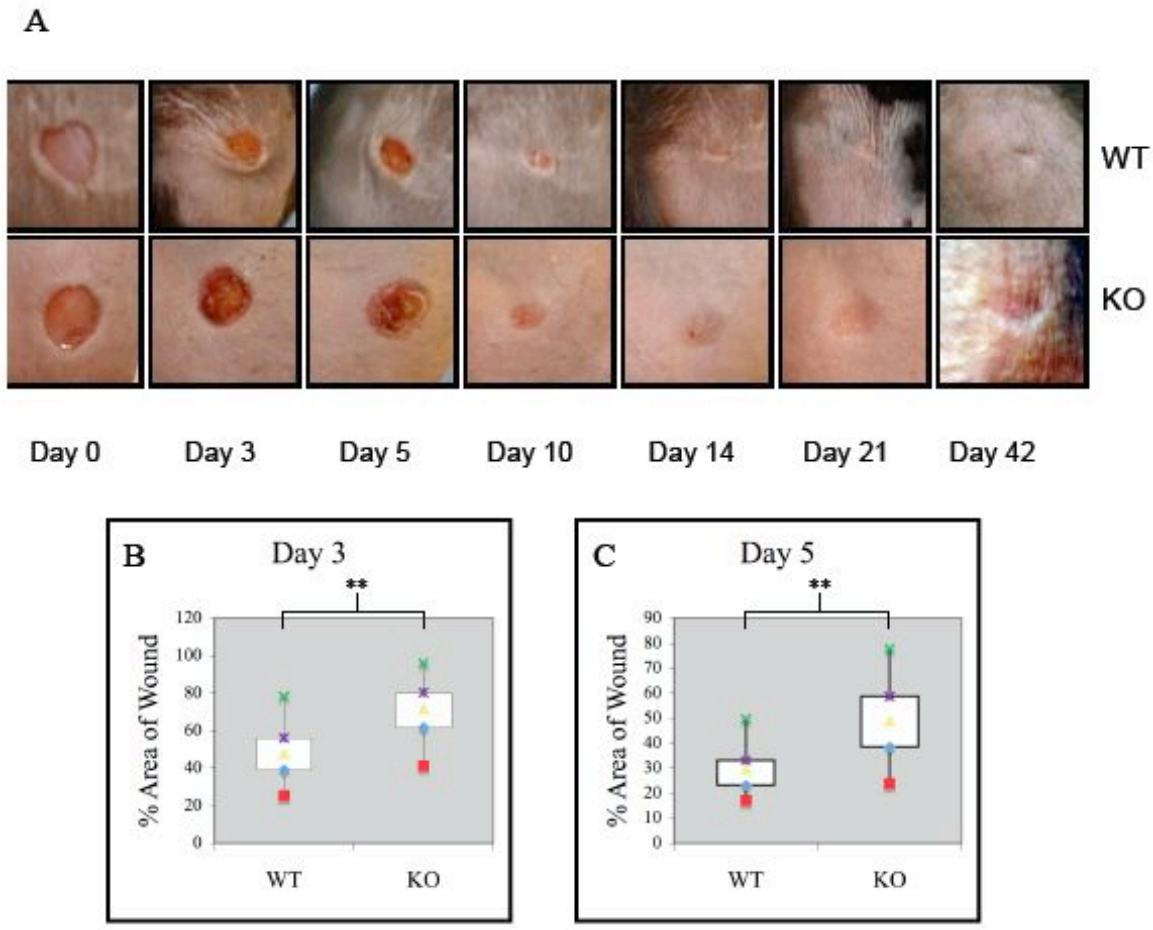

Figure 22. Smad4 deficient skin has poorly healed wounds. A) 6mm full thickness excisional wounds were made on the dorsal skin of WT and Smad4 KO mice and photographed at Day 0, 3, 5, 10, 14, and 42. B&C) The remaining unhealed wound areas were expressed as % area of wound. The percent of wound remaining was calculated for each individual wound and displayed with a box-whisker plot highlighting the distribution of inner and outer quartiles. **: $p<0.01$. Technical assistance was provided by Gangwen Han for wounding surgery advice. Statistical analysis was assisted by the OHSU bioinformatics core facility.

4.3 Smad4 deficient wounds do not have defects in reepitheliazation

To further assess why KO wounds fail to close as quickly WT wounds we stained wounds with H&E and investigated the lengths of the epidermal tongue (Figure 23). Because the wound areas were larger in KO wounds we suspected that Smad4 deficient keratinocytes may be defective in performing reepitheliazation. It has been observed that

when Smad4 is knocked down using siRNA that cancer cells or keratinocytes to do not migrate efficiently (Jazag et al., 2005). However when we examined the leading edge of KO wounds, the keratinocyte tongue was not shortened compared with WT controls (Figure 23A-D). We measured the lengths of these tongues and we found no significant statistical difference. In fact, several of the KO tongues were longer than WT tongues (Figure 23E).

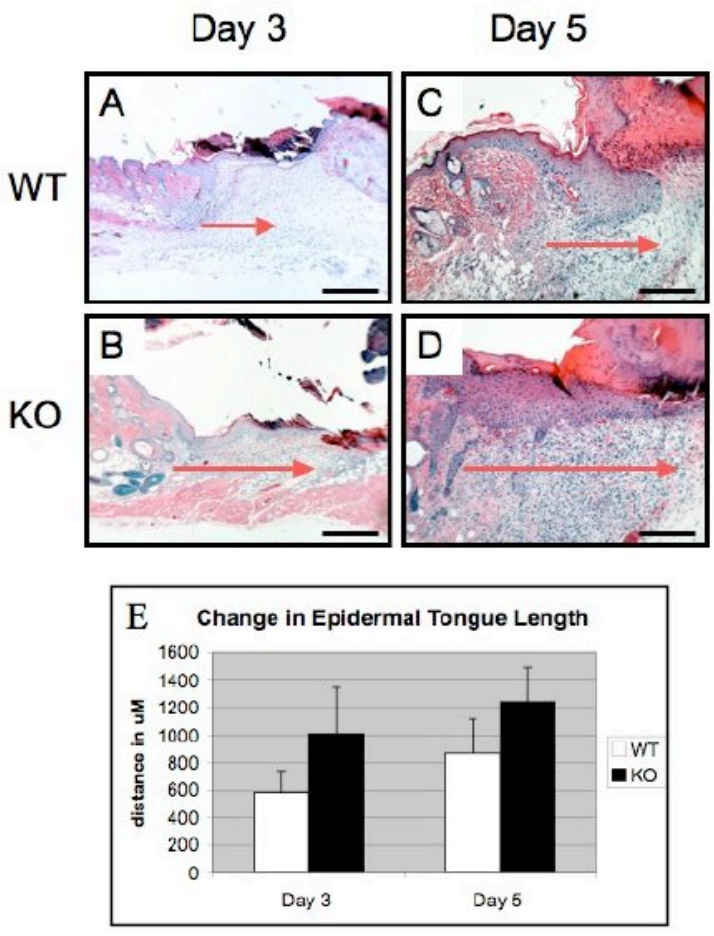

Figure 23 Keratinocytes in KO wounds are not retarded. A-D) Epidermal migrating tongues (denoted in length by red arrows) were not impaired and many times longer in KO(B&D) wounds than WT(A&C). E) Quantitation of epidermal tongue length revealed no statistical significance, yet KO tongues were longer than WT on average. All scale bars are = 200μM.

4.4 Smad4 deficient wounds have increased neo-angiogenesis

Upon histological examination of wounds we first noticed a striking phenotype in KO wounds compared with WT. Abnormally large blood vessels were apparent in the wound bed and continued to be present throughout wound healing up to six weeks post wounding (Figure 24A-H black arrows). In order to visualize the blood vessels we stained for CD31 (PECAM) to mark endothelial cells. We observed that while more vessels appeared in Smad4 KO skin prior to wounding, that this was in large part due to collapsed hair follicle epidermoid cysts (Figure 24J). Hair follicles are typically well vascularized and in our wound healing protocol the WT skin has resting or telogen hair follicles which are small and relatively quiescent in their angiogenic properties compared to the large cysts that exist in the skin of Smad4 KOs (Figure 24J). However, after the wounding of both WT and KO skin, both will require neovascularization into the wound bed. While at later timepoints we observed a similar network of new blood vessels at post-wound days 5, 10 and 14. However, There was a profound early migration of new blood vessels into the wound margins at day 3 in KO wounds in the granulation tissue (Figure 24L). This is in stark contrast to WT wounds whose angiogenesis is restricted behind the epidermal tongue (Figure 24K). We could quantify this by analyzing the signal generated by IF staining of CD31 (Figure 24O). We found that there was almost no neo-angiogenesis into the wound margins at day 3 in WT. Most strikingly, the KO wounds at day 3 contained new blood vessels. To further quantify this angiogenic phenotype we performed real-time PCR on wounds and found that not only at day 3 but

day 5 demonstrated increased VEGFA mRNA in KO wounds when compared to WT control wounds (Figure 24O&P). VEGFA has been shown to be a potent stimulator of angiogenesis (Folkman, 1997). Wounds that have exogenous VEGFA stimulation have increased healing (Nissen et al., 1998). So while Smad4 null wounds have increased angiogenesis their wounds have impaired healing, which leads us to ask what the significance of early blood vessels may be. This suggests that Smad4 deficient wounds are overhealing and the resultant expanse of wound area is from an increased healing response. Angiogenesis is required to drive the early events in wound healing by delivering important cells and molecules that will perform or assist the repairs. Increased vessels early should correlate with more opportunity to heal.

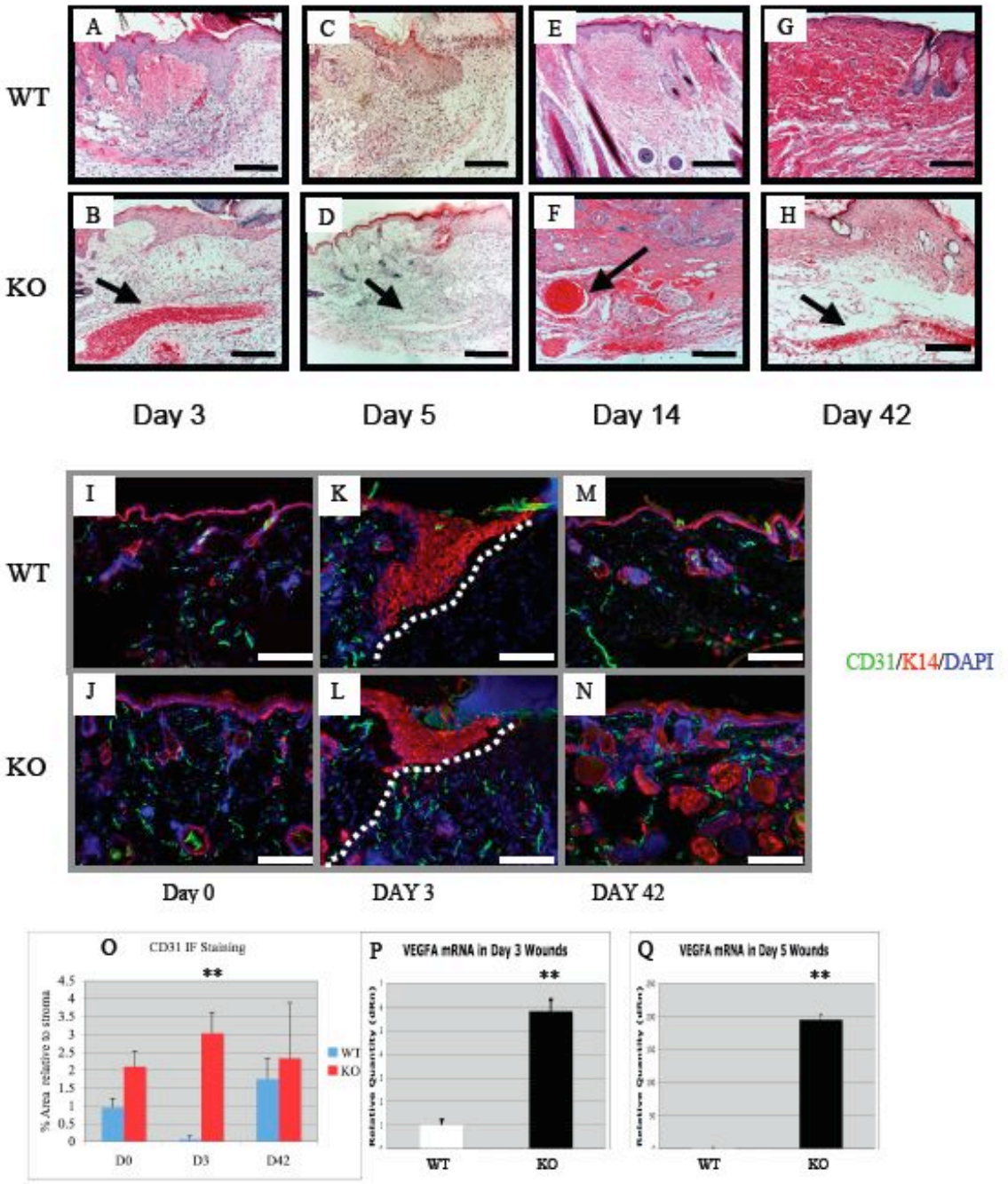

Figure 24 Angiogenesis is increased in Smad4 deficient wounds. A-H) H&E staining was informative to demonstrate abnormally large blood vessels (black arrows) in KO wounds compared to WT counterparts. I-N) IF staining for CD31 (green) demonstrated how well vascularized the dermis was in KO skin. Dotted white lines indicate new provisional matrix and granulation tissue below this line O). Quantitation of CD31 staining using ImageJ revealed increased vessels per area, yet these were not statistically significant except when measure adjacent to the migrating tongue at day3 which showed enhanced angiogenesis in KO wounds. P-Q) QPCR demonstrated the upregulation of

VEGFA mRNA in day 3 and 5 KO wounds respectively. Each group had at least four samples. Scale bars = 200uM.

4.5 Smad4 deficient wounds have increased inflammation

To determine if inflammation was abnormal in KO wounds we first investigated markers for inflammatory cells in biopsies of KO skin and compared them to WT biopsies. No significant changes were noted in most inflammatory cells as assessed by immunohistochemistry. However, we found a dramatic increase in the presence of F4/80 positive monocyte/macrophages in KO skin prior to wounding (Figure 25B) and throughout the wound healing process (Figure 25C-H). We next sought to asses the chemokines that may play a role in the increased number of monocyte/macrophages and discovered that the chemokines CCL5 and CCL20 were significantly upregulated during the inflammatory phase of wound healing (day 3).

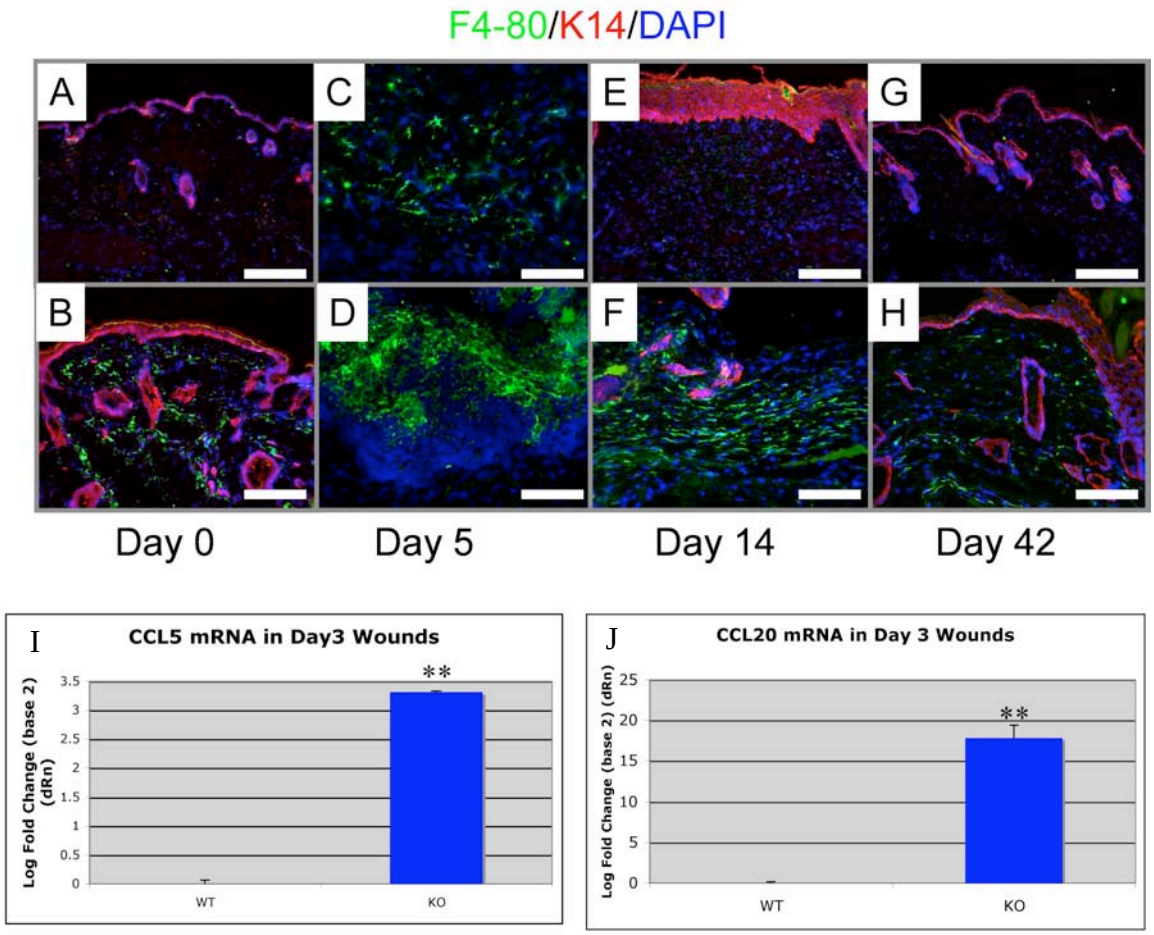

Figure 25 Inflammatory changes in Smad4 deficient skin and wounds. A-H) F4/80 staining (green) shows that Smad4 deficient skin prior to wounding has a large accumulation of macrophages (B). In the wound bed of KO animal are much densely packed accumulation of macrophages (D) compared with WT wound beds (C). At day 14 macrophages have regressed in WT (E) whereas in KO (F) wounds they are still present in and remain six weeks post wounding in the KO (H) but not in the WT (G). Scale bars = 200uM. I&J) QPCR for relative mRNA upregulation of CCL5 & CCL20 at the inflammatory phase (day 3) wound healing in Smad4 deficient wounds. Samples were performed in quadruplicate.

4.6 Smad4 deficient wounds have stromal alterations

We first noticed that beyond the hair follicle phenotype in Smad4 deficient mice, there are other alterations in the stroma (Owens et al., 2008a). Specifically we observed that in the stroma of adjacent wound margins there exists a densely packed array of cells not seen in controls (Figure 26B&D). We identified these cells via staining for αSMA as myofibroblasts (Figure 26). We followed this observation with αSMA staining during wound healing and observed that Smad4 deficient wounds had an earlier and greater increase in the number of αSMA positive cells in the underlying stroma. Specifically we noticed that at day 3 post wounding KO wounds had rapidly accumulated myofibroblasts while WT controls did not (Figure 26A&B). Myofibroblast are differentiated fibroblast that produce ECM and are highly secretory and help propagate the wound healing response. A potent activator of fibroblasts to myofibroblasts is TGFβ. We observed that KO skin had a profound increase in TGFβ1 mRNA prior to and during wound healing, culminating during scar formation (Figure 29). While keratinocytes are incapable of normal response to TGFβ, fibroblasts are fully competent to 'read' TGFβ as they still possess Smad4.

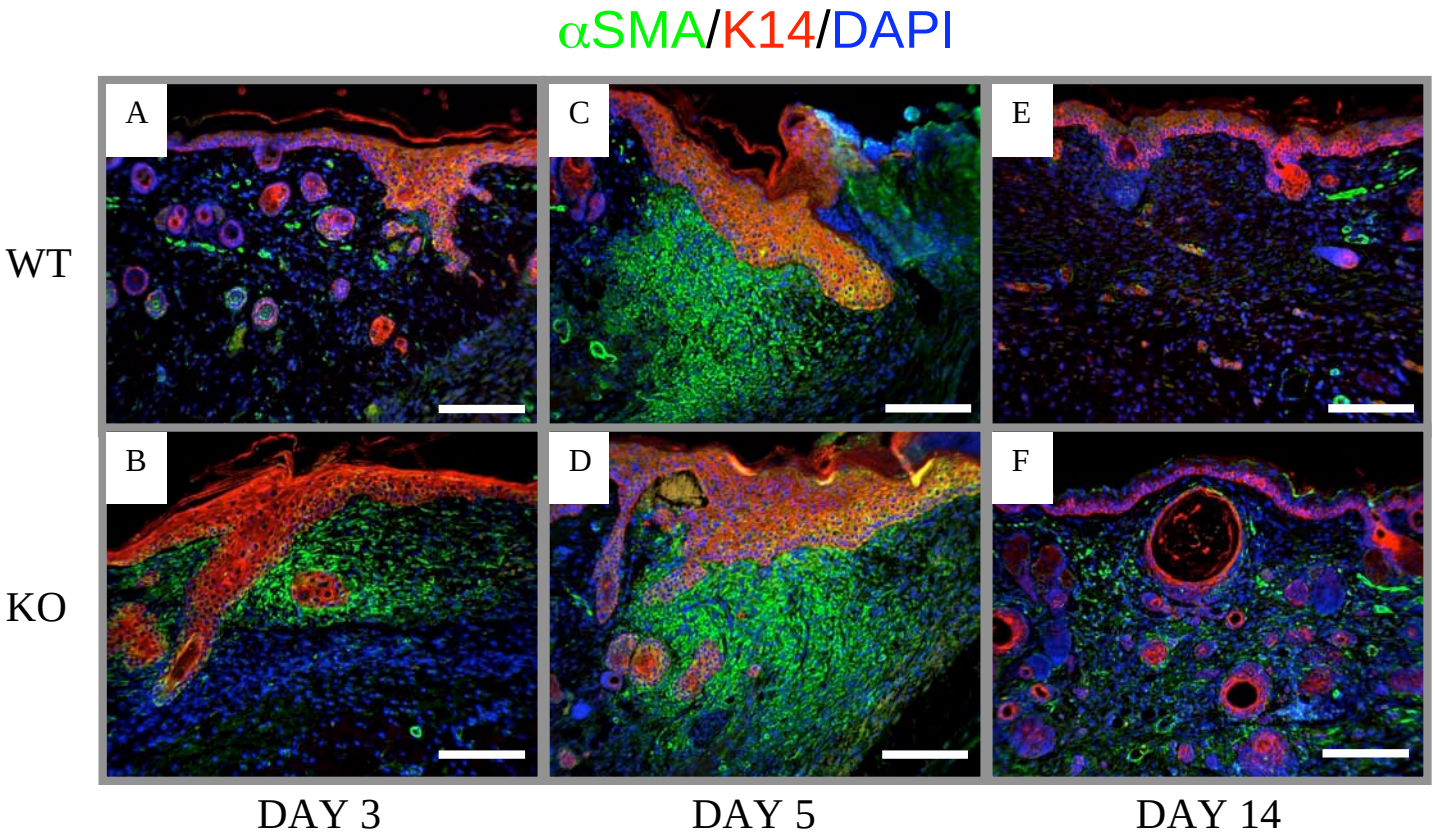

Figure 26. Myofibroblast accumulation in cutaneous wounds. At the early inflammatory phase (day 3) KO wounds have many more myofibroblasts (A-B). During reepitheliazation αSMA demonstrates equal myofibroblast presence (C-D), however at day 14 when most of the WT myofibroblasts have undergone programmed cell death (E), the KO wound at day 14 still has a large number of myofibroblasts compared to control (F). Scale bars = 200uM.

Matrix changes and increased MMP levels in skin and wounds lacking Smad4

We next observed by Masson's trichrome staining that the ECM appeared less organized and that granulation tissue appeared expanded (Figure 27A-D). Excessive scars in KO skin after six weeks of healing showed a more provisional styled matrix with less dense accumulation of collagen fibrils (Figure 27D). Using real-time PCR we assayed the

comparative levels of MMPs. MMP3, 12,13 and 14 were found to be significantly upregulated in skin prior to wounding and throughout the healing in KO skin and wounds (Figure 28).

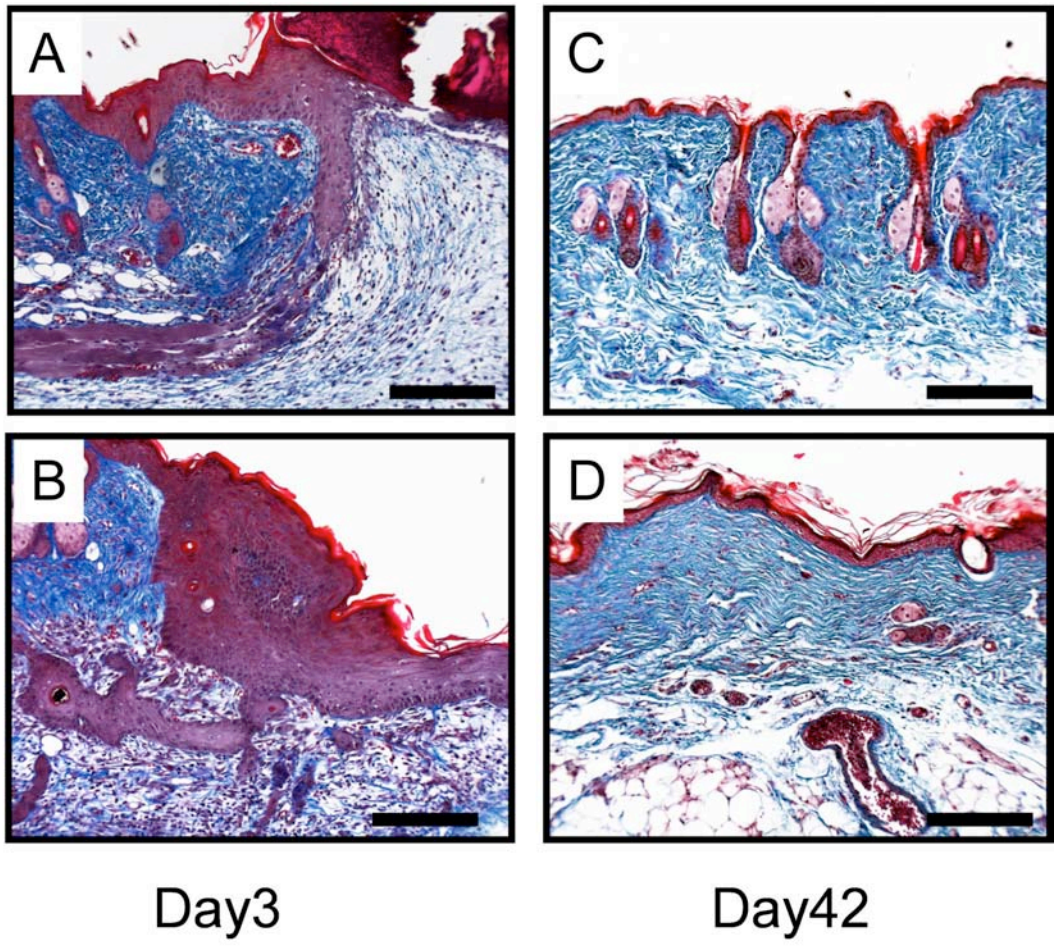

Figure 27. Changes in stroma of Smad4 deficient wounds. Masson trichrome staining illustrates how well organized the ECM in the stroma is. A) WT wounds at day 3 have a mostly acellular and ECM free wound bed, in KO (B) there are many more cell infiltrates and increased degradation adjacent to the migrating epidermal tongue. After six weeks of healing, the WT dermis (C) has well arrayed and dense collagen ECM while the KO dermis (D) is loosely packed and less densely organized. Scale bars = 200uM.

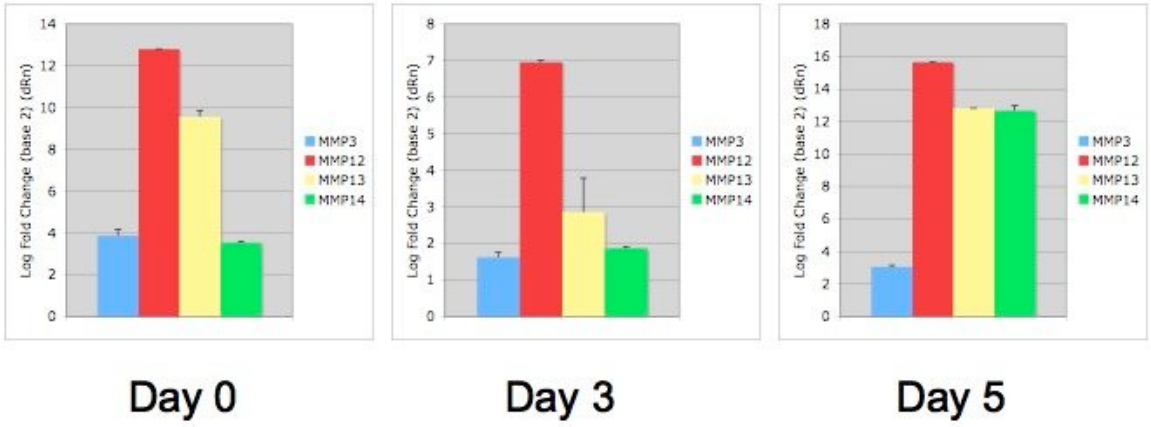

Figure 28. MMP levels in Smad4 deficient skin and wounds. QPCR of no less than four samples for each genotype and timepoint were assessed. Prior to wounding, Smad4 deficient skin had significant increased MMP mRNA at day 0. At day 3 and day this trend progressed irrespective of inflammatory phase (day 3) migratory cells and reepitheliazation phase (day 5) of keratinocytes. Graphs are normalized to WT expression and reflect the increase from their counterpart controls.

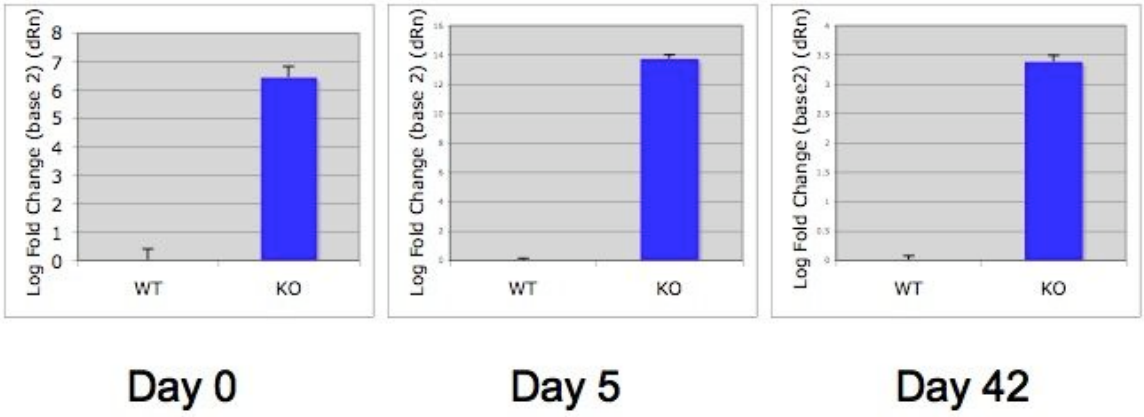

Figure 29 Relative TGFβ mRNA in Smad4 deficient skin and wounds. QPCR of no less than four samples for each genotype and timepoint were assessed.Prior to wounding TGFβ mRNA was upregulated in Smad4 deficient skin. Additionally, at day five during the peak of TGFβ expression KO wounds were still upregulated. Six weeks after healing levels of TGFβ are still upregulated and more than prior to wounding. Graphs demonstrate increase scaled to a log score from a normalized WT control.

4.7 Smad4 deficient wounds recapitulate molecular and cellular hallmarks of spontaneous skin SCC formation in Smad4 deficient tumors.

We next hoped to determine whether changes in Smad4 null wounds parallel with the spontaneous tumors that form in the mice skin. First we noticed that just like the skin and wounds Smad4-/- tumors were highly vascularized (Figure 30A) as shown by staining of CD31 for endothelial vessels. Smad4-/- tumors also had abundant monocyte/macrophage infiltrates as shown by staining for F4/80 (Figure 30C). αSMA staining also demonstrated increased myofibroblast presence in the tumor stroma (Figure 30B). When Smad4-/- tumors were stained with Masson trichrome it was apparent that ECM (blue) was significantly degraded in the stroma (Figure 30D).

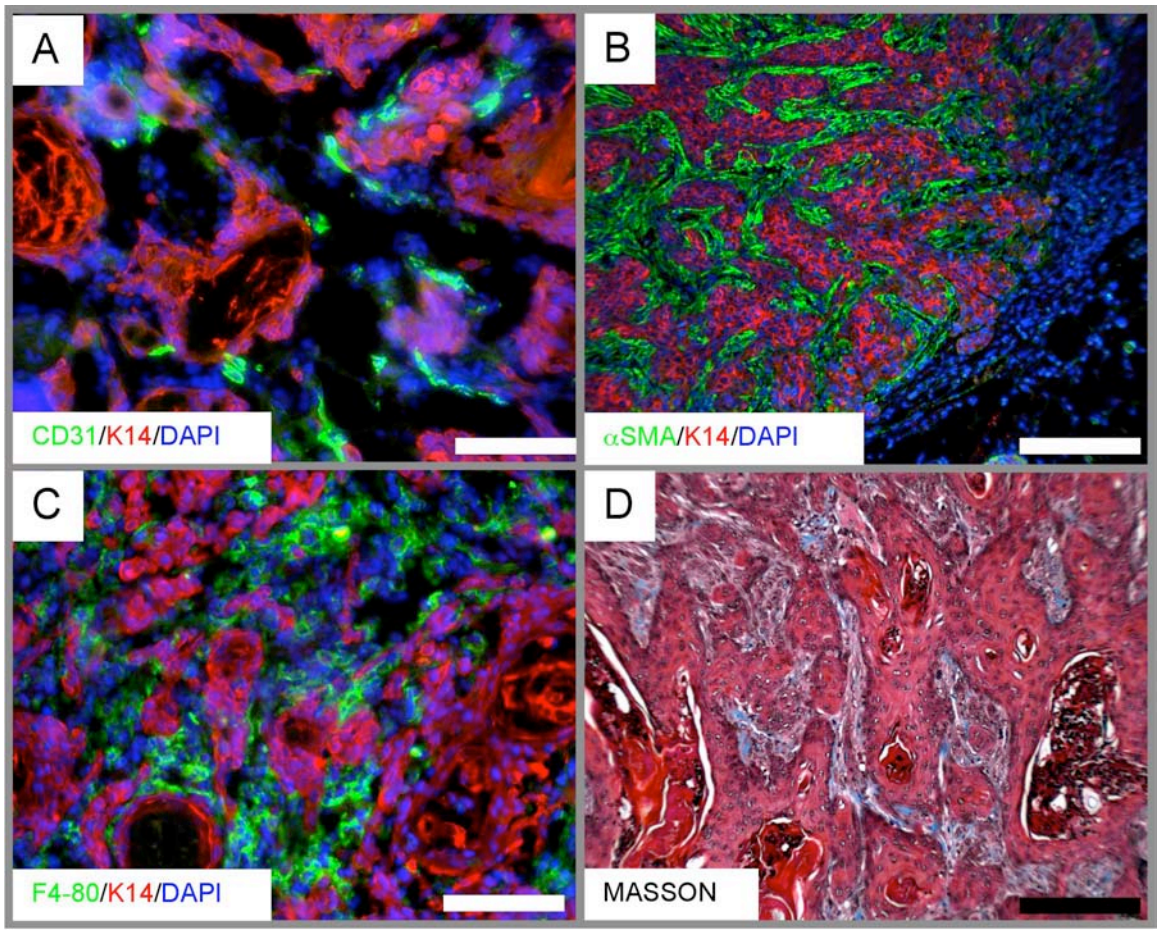

Figure 30. Comparative tumors histopathology. A) IF staining for CD31 (green) reveals the well vascularized tumor stroma. B) IF staining for αSMA (green) demonstrates the dramatic infiltrating myofibroblasts. C) IF staining for monocyte/macrophages illustrates the presence of tumor associated macrophages. D) Masson trichrome staining shows how loss of aniline blue (blue) results from a degraded ECM in the surrounding stroma. B&D scale bars = 200uM. A&C scale bars = 100uM.

To assess the molecular changes between KO skin and tumors we performed real-time PCR comparing KO skin with SCC's that had spontaneously developed on separate mice.

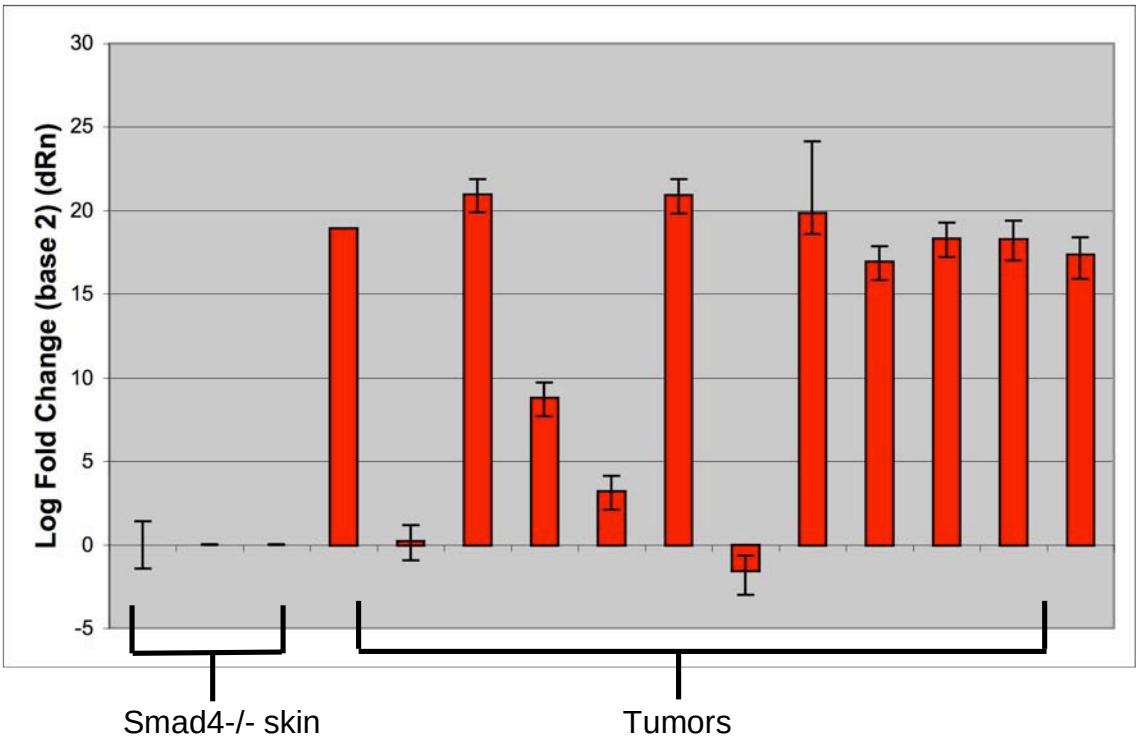

Figure 31. Change in VEGFA mRNA expression. QPCR of KO skin vs. Smad4 spontaneous tumors. Three skins were designated as relative 1 and compared to RNA from 12 tumors. Error bars represent deviation amongst technical replicates of three.

We had previously noticed that KO skin showed an elevation in angiogenic VEGFA mRNA (Figure 31). This increase was furthered in the majority (10/12) of tumors (Figure 31). Interestingly, there were two SCC's who had comparable levels of VEGFA mRNA, and one of those two sample even demonstrated a slight reduction in relative VEGFA expression. While both stroma in tumors and skin are abnormal, KO

skin with the accumulation of epidermoid cysts does not invade below the subcutis, whereas the tumors do.

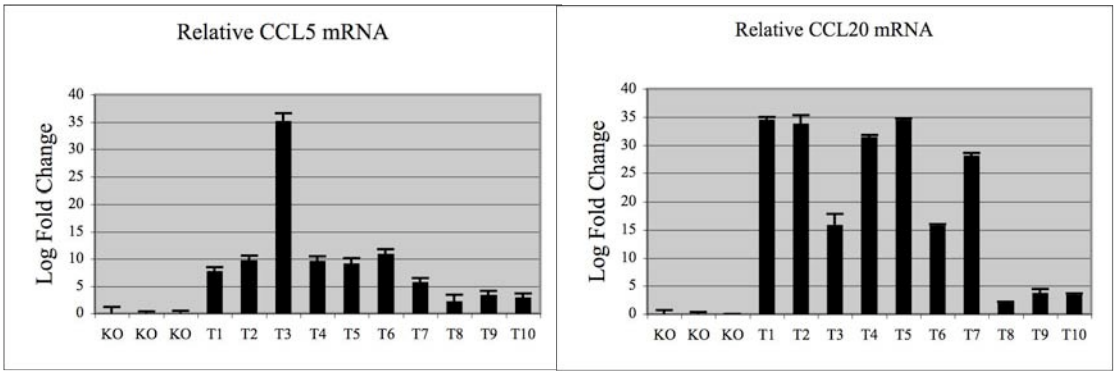

Figure 32. Change in CCL5 & CCL20 mRNA expression. QPCR of KO skin vs. Smad4 spontaneous tumors. Three skin were designated as relative 1 and compared to RNA from 10 tumors. Error bars represent deviation amongst technical replicates of three.

In excess of KO skin we also found that inflammatory chemokines CCL5 and CCL20 were upregulated in spontaneous Smad4-/- tumors (Figure 32). We also found that MMP14 levels were dramatically increased in all of the tumor samples (Figure 33).

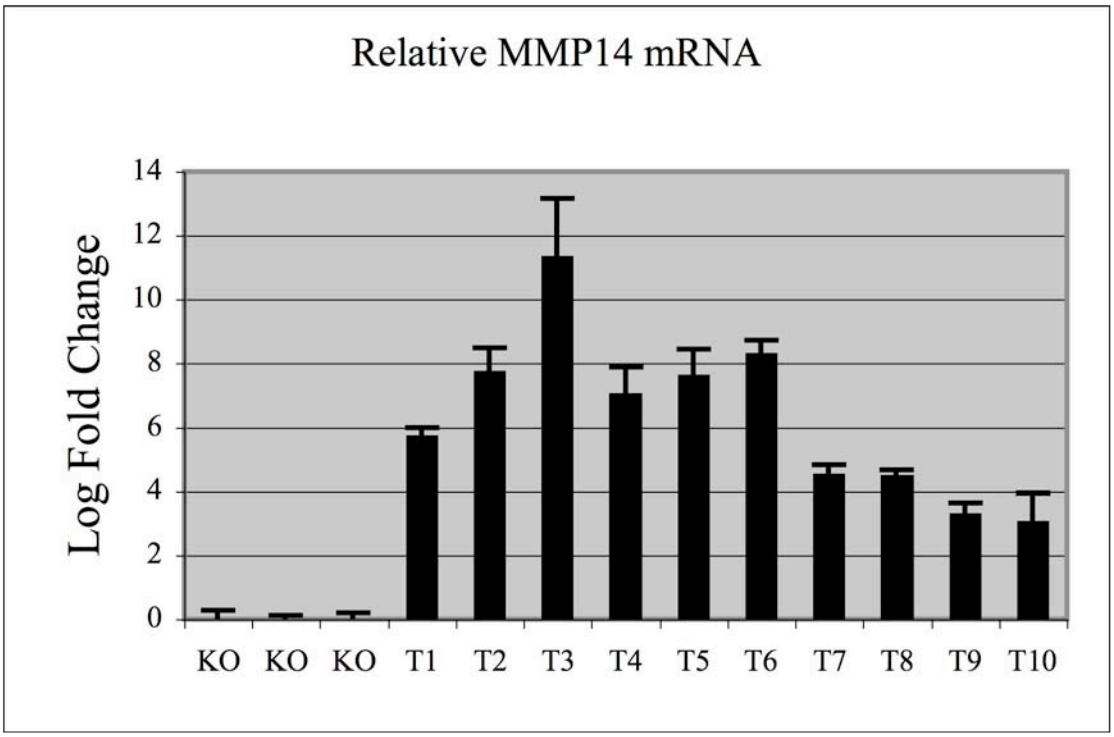

Figure 33. Change in MMP14 mRNA expression. QPCR of KO skin vs. Smad4 spontaneous tumors. Three skins were designated as relative 1 and compared to RNA from 10 tumors. Error bars represent deviation amongst technical replicates of three.

Finally, we were curious to see if TGFβ1 levels were changed and found that in accordance with its role as a late stage tumor promoter (Li et al., 2005), it was greatly increased in the tumors as compared to the KO skin (Figure 34). TGFβ only has tumor suppressor functions on epithelial cells that have intact Smad4, without Smad4 in the keratinocytes secreted TGFβ is free to stimulate tumorigenesis throughout the microenvironment.

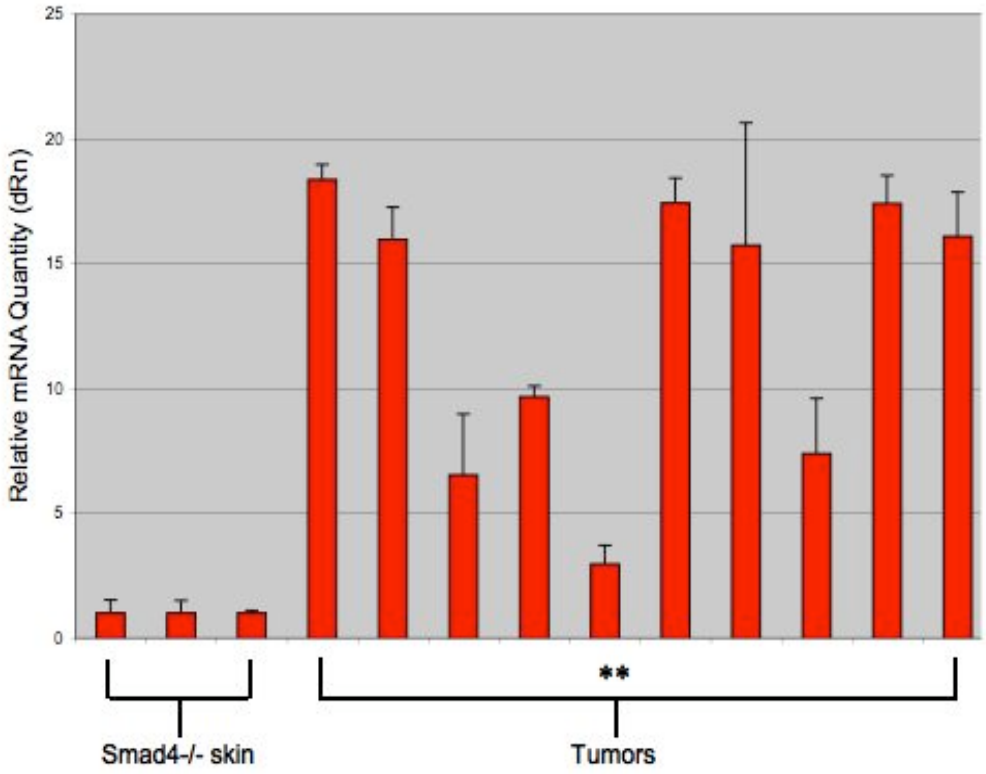

Figure 34. Change in TGFβ mRNA expression. QPCR of KO skin vs. Smad4 spontaneous tumors. Three skins were designated as relative 1 and compared to RNA from 10 tumors. Error bars represent deviation amongst technical replicates of three.

4.8 Discussion

Mechanism of the effects of epithelial Smad4 loss on the stromal compartment in vivo

Here we report for the first time the *in vivo* consequences of Smad4 loss during cutaneous wound healing. We find that wound closure is defective, yet the migrating epidermal tongues that lead the reepithelialization into the wound are not defective and show no restriction at coordinated cell movement. The epidermal tongues of Smad4 deficient wounds, while hyperproliferative, do not show impairment in migration. The

defect in the wound healing process therefore must result from excessive angiogenesis/inflammation and not from the epithelium where Smad4 is disrupted.

Smad4 has been widely described as an important tumor suppressor (Bardeesy et al., 2006; Kim et al., 2006; Kitamura et al., 2007; Qiao et al., 2006; Xiao et al., 2006; Yang et al., 2005). Recently, experiments in cultured cells have shown that a knockdown of Smad4 results in defective migration ability (Jazag et al., 2005; Levy and Hill, 2005). We have also observed that primary keratinocytes exhibit this defect in tissue culture as well as on a variety of ECM substrata (Fig. 21 and data not shown). These *in vitro* observations are in conflict with the fact that Smad4 deletion results in more invasive tumors, which exhibit accelerated cell migration. Our data show that *in vivo*, the hyperproliferative effects of Smad4 loss and perhaps also the stromal changes caused by this deficiency, can overcome the intrinsic migration defect of Smad4 KO cells shown in tissue culture.

Previously our laboratory has found that increased expression of TGFβ is commonly found in human skin cancers (Han et al., 2005). We also find in this study that even prior to spontaneous tumor formation in Smad4 deficient skin, there exists an increase in TGFβ. While this elevation of TGFβ probably allows for endogenous tumor suppressive roles, the lack of Smad4 makes these benefits arbitrary, as the cells are no longer capable of the signaling required to induce growth arrest. We show here that epithelial defects such as hyperproliferation, are not the sole causes of tumor formation. Elevation in key chemokines and cytokines that have pro-tumorigenic function such as CCL-5 (Borczuk et al., 2008; Karnoub et al., 2007; Soria and Ben-Baruch, 2008) and

CCL-20 (Abiko et al., 2003; Campbell et al., 2005; Giuliani et al., 2008; Kimsey et al., 2004; Qiao et al., 2006) contributes to paracrine alterations resulting from an epithelial loss of Smad4. Further evidence from deletion of the type II TGFβ receptor, as in invasive breast carcinomas, also demonstrates the increase in these pro-inflammatory and tumor promoting secreted molecules (Bierie et al., 2008). Further studies have demonstrated that a loss of Smad4 combined with a loss of WNT/β-catenin signaling is accompanied by an elevation of inflammatory processes that dramatically increases metastatic invasion (Kitamura et al., 2007). Over ten years ago, Kinzler and Vogelstein first described the loss of Smad4 to elicit 'landscape' changes in the tumor microenvironment (Kinzler and Vogelstein, 1998). This description was indicated by the changes that appeared outside the tumor and resulted from an abnormal microenvironment. We demonstrate that the real significance of the loss of Smad4 in epithelia is the inability of these cells to maintain normal cellular crosstalk between compartments to maintain homeostasis. Smad4 spontaneous tumors do not occur immediately, in fact, they tend to develop between 3 months to 13 months after birth (Qiao et al., 2006). While this observation suggests that additional genetic alterations are required for carcinoma formation, our data suggests that there may be an additional threshold of paracrine effectors that promote malignant transformation. Further experiments testing the function of these molecules during wound healing and tumorigenesis will open up new avenues for targeted therapy.

Increased angiogenesis in Smad4 KO skin contributes to delayed wound healing and remodeling

Angiogenesis, the process of blood vessel formation, is necessary in normal wound healing in order to reestablish blood flow to newly formed epithelia. However, it is important that the 'right' amount of angiogenesis occur for proper wound healing. Previously we have observed that hair follicles are collapsed and the epidermis is hyperproliferative in Smad4 deficient skin (Owens et al., 2008a; Qiao et al., 2006). It is not surprising that keratinocytes which are incapable of normal TGFβ signaling and therefore unable to elicit cytostasis or growth arrest become not only hyperproliferative, but also demand an elevated supply of nutrients via increased blood vessel formation. The collapsed hair follicles form epidermoid cyst-like structures, which increase in size with the age of the animal. This increase in cyst expansion would ultimately require fresh blood supply to fuel their aberrant growth. The fact that these abnormal hair follicles also began to develop normally but subsequently collapsed hints that they came with the normal pre-existing vasculature, and that once hair shafts were destroyed and cysts were formed that these abnormal adnexae were already 'wired' for angiogenic control(Owens et al., 2008a). Furthermore, it is not surprising that angiogenesis is increased in Smad4 deficient skin with the elevated levels of TGFβ1, a potent stimulator of new blood vessel formation. This highlights the requirement for Smad4 in the epithelium to regulate levels of TGFβ1 to maintain vascular homeostasis. Angiogenesis is required for proper wound healing however, excessive angiogenesis may contribute to a delay in wound healing. The delay we observe here is not in the closure of the wound but in gross appearance and scar formation. Wound healing is a bimodal process and must be finely regulated 'up' and 'down', here we show that excessive 'up' processes can lead to poor wound healing.

Excessive inflammation in Smad4 KO skin contributes to delayed wound remodeling

Inflammation is a process necessary in normal wound healing in order to recruit the molecules necessary for wound healing processes to occur. For example, inflammation brings macrophages to the wound, which is necessary for phagocytosis of debris in the wound area. However, if these inflammatory molecules remain in the wound for an extended period, they can actually do more bad than good. The excessive presence of macrophages in Smad4 deficient epithelia, does not affect early stages of wound healing such as reepitheliazation, but has a greater impact on wound healing during the later stages. Their prolonged presence and activity do not allow for normal remodeling of the stroma. This exacerbation of macrophage response impairs wound healing when it competes with the repopulation of the dermis by ECM molecules. Here we find that their failure to leave the wound area can result in an excessive scar and inhibit proper matrix remodeling.

Tumor Associated Macrophages (TAMs) are known to promote carcinoma formation. Furthermore, it has been recently shown that an elevation of TGFβ in skin tumors results in the recruitment and progression of TAMs(Byrne et al., 2008). Previous work from this same group has also shown that TGFβ can suppress dendritic cell activation which can also facilitate tumor promotion(Weber et al., 2005). The simultaneous restriction of immune surveillance and TAM activation due to elevated levels of TGFβ is observed here in Smad4 KO wounds and tumors via elevated macrophages as well as increased TGFβ1.

Persistent myofibroblast accumulation in Smad4 KO skin contributes to delayed wound remodeling

Myofibroblasts are cells that are between the fibroblast and smooth muscle cell in their development. They are required in normal wound healing in order to contract the edges of the wound. The persistence of myofibroblasts beyond their needed time frame in wound healing, however, can cause fibrosis and delay wound healing. Carcinoma Associated Fibroblasts (CAFs) are common within tumors that display malignancy. TGFβ has shown a role in promoting the activation of fibroblast differentiation to the myofibroblast state(Narine et al., 2004) and these myofibroblast cells are sufficient to become CAFs. Fibrosis from the stroma has been shown to be causative in facilitating tumor progression via the activation of myofibroblasts (De Wever et al., 2008). Again, the failure of normal TGFβ signaling due to a deficiency of Smad4 in the epidermis results in an abundance of TGFβ1 mRNA in skin, wounds and tumors. These facts taken together with the observations in this study indicate that the Smad4 deficiency in the epithelium is sufficient to result in stromal accumulation of myofibroblasts, ultimately to pathological levels, which results in abnormal wound healing via excessive inflammation and angiogenic processes and cancer progression. In Smad4 deficient epithelia, remodeling myofibroblasts do not go through their normal programmed cell death and macrophages compete with ECM molecule repopulation in the wound, both due to the excessive presence of TGFβ. These processes combine to prevent appropriate closure of the wound and the stroma from being acellular and composed mainly of ECM. It is therefore not surprising that excessive scarring is the result in Smad4 deficient epithelia.

Tumors as 'wounds that do not heal' or tumors as wounds that 'overheal'

It has been postulated that tumors are 'wounds that do not heal' (Dvorak, 1986). This hypothesis was driven by observations in the surrounding stroma and the observation of inflammatory infiltrates common to both wounds and tumors. Recently, this hypothesis has been given a new look with the postulation that a wound may, in fact, be 'overhealing' (Schafer and Werner, 2008). We extend this hypothesis in our findings, which indicate that synchronous, step-wise cutaneous wound healing requires discrete timing and localization of the healing processes. While all of the initial phases of wound healing can be considered 'healing' they must occur within a reasonable timeframe for the wound to be considered 'healed.' So perhaps it is presumptive to suggest that these wounds 'overheal' or that the tumors 'overheal' and it seems more appropriate to say that tumors are 'wounds that do not heal.'

In Smad4 deficient skin, there is no reduction in the initial wound healing processes. In fact, there is excessive inflammation, macrophage and myofibroblast accumulation and angiogenesis. However, Smad4 deficient wounds have delayed healing and excessive scarring. Therefore, a further characterization of matrix remodeling and the later stages of wound healing is necessary, particularly angiogenesis and apoptosis of inflammatory cells, in order to reconcile how the abnormal healing and tumorigenic processes result in inadequate healing and promotion of malignancy in Smad4 deficient wounds and tumors. The study of these later processes that reduce angiogenesis and

apoptose inflammatory cells and fibroblasts therefore must be more thoroughly studied to further our understanding of how we can effectively heal wounds and treat tumors.

Chapter 5: Discussion

5.1 Smad4 requirement during skin development

Our system of deleting Smad4 earlier than previous studies uncovered new roles for Smad4 in epidermal development. Smad4 deletion did not affect epidermal terminal differentiation (Descargues et al., 2008) or initial events of hair follicle specification (Owens et al., 2008a). The hair follicles in K5.Smad4-/- skin were abnormal shortly after birth of in mutant mice. Previous results using alternate methods showed, a more patchy and gradual loss of hair follicles, most likely due to incomplete Cre-mediated recombination in epidermal stem cells (Qiao et al., 2006; Yang et al., 2005). Our study utilized a system that has more robust Cre by the K5 promoter (Zhou et al., 2002). This early deletion also allowed us to uncover the transcriptional target Dsg4 here in this study.

Smad4 is required for sebocytes homeostasis

We found that after the hair follicle collapsed and formed cysts (Figure 12G), the accompanying sebaceous glands remained. But beyond just persisting they began to hyperproliferate (Figure 13). This finding should be followed up more closely especially with the observation that sebaceous adenomas can spontaneously form in the skin of mice lacking Smad4 (Qiao et al., 2006). While these sebocytes are abnormal, their architecture is well intact, and they appear to be still connected to the epidermis. We do not know if

these mice have excess sebum or are ‘oily’. A measurement of lipids on the skin of these mice may be useful to determine whether Smad4 is required to process the normal excretion of these cells.

BMP specific functions of Smad4

We have also uncovered the primary phenotype of Smad4 loss in skin, which is not TGFβ/Activin, but closely phenocopies the loss of the ALK-3 (BMP receptor 1A). To further demonstrate BMP specific functions of Smad4 we showed by ChIP and qPCR of endogenous mRNA that Dsg4 was responsive to the BMP specific stimulus. Interestingly when TGFβ2 ligand was added to cells expressing Dsg4 there was a net repression of Dsg4 mRNA expression. Smad4 loss did not affect terminal differentiation, yet it is known to participate with Smads 2&3, which have been implicated independent of Smad4 to be required for terminal differentiation (Descargues et al., 2008). A careful examination of how BMPs regulate terminal differentiation may be warranted from these results. Could different ligand/antagonists and receptors possibly specify each differentiated layer in a unique fashion? Markers for these lineages are available and morphological and ex vivo systems are available to perform this analysis.

Non-cell autonomous roles of Smad4 in skin development

We have shown in our wound healing studies that non-keratinocyte mechanisms mediate the phenotypes of impaired wound closure. We have not identified in the early

development of the hair follicle a role for inflammatory cell infiltrates in the collapse of the hair follicle. We do not observe cyclic balding such as in Smad7 transgenic overexpressing mice. Smad4 collapsed hair follicle cysts never ‘rebuild’ and do have a desmoplastic stroma. It will be useful to see in the future whether intervention in the immune system could alter this phenotype. Mating Smad4 mutant mice with mice that are deficient in macrophage or even T-cell signaling may uncover even more novel phenotypes in the paracrine control of the hair follicle. It is also noteworthy to point out that the hair follicle is composed of non-epithelial cells. The dermal papillae (DP) is of mesenchymal origin and has been shown to be a potent source of BMP signaling (Raveh et al., 2005). If the hair follicle keratinocytes can no longer read the BMP induction than what is the fate of these cells? They could perhaps be attempting to induce other epithelial cells or they could apoptose. The ability to track and trace these cells could be vital to determining the effect on this novel mesenchymally derived cell population.

5.2 Smad4 mediates cell non-autonomous defects in wound healing

Cell non-autonomous control of migration

Smad4 has been identified as a critical tumor suppressor which ultimately protects organisms from metastatic and highly invasive tumors (Massague, 2008). Recently, experiments in cultured cells have shown that knockdown of Smad4 results in defective migration (Giehl et al., 2007; Jazag et al., 2005; Levy and Hill, 2005; Xiao et al., 2006). We have also observed that primary keratinocytes exhibit this defect in tissue culture as well as a variety of ECM substrates. It was puzzling to think that loss of Smad4 in cells resulted in cells that could not migrate well. Smad4 as a tumor suppressor should lead one to hypothesize that it restricts invasive migration. It is curious to think that Smad4 deletion results in more invasive tumors usually more typical of accelerated cell migration. We would hypothesize that Smad4 loss would result in accelerated keratinocyte migration based on clinical observations of Smad4 loss in tumors. However, we have shown *in vitro* (as others have as well) that smad4 results in defective coordinated sheet migration. Smad4-/- keratinocytes cannot effectively migrate and appear to have defects in coordinated sheet migration when compared to wild type cells. Here we report for the first time the *in vivo* consequences of Smad4 loss during cutaneous wound healing. We find that wound closure is defective, yet the migrating epidermal tongues are not defective and show no restriction at coordinated cell movement. These epidermal tongues while hyperproliferative, demonstrate an ability to close the wounds.

Paracrine changes when Smad4-/- is lost

Loss of Smad4 resulted in increased levels of TGFβ in skin, wounds and tumors. TGFβ is a tumor suppressor mainly by its exerted functions on epithelial cells to induce cell cycle growth arrest, apoptosis and/or differentiation. Smad4 has previously been shown to be a critical mediator of these functions in epithelia (Levy and Hill, 2005). However, TGFβ can function as a tumor promoter in epithelia cells indirectly by its concerted stimulus of monocyte/macrophages and carcinoma-associated-fibroblasts (CAFs) (Figure 35). The functional implication of Smad4 loss-TGFβ increase is that it implies a control system between epithelial and stromal compartments. Cells/tissues can only find homeostasis if the epithelium can satisfy a Smad4 dependent response in keratinocytes. Deletion of Smad4 in the stromal fibroblasts would be insightful to see if this would also affect this feedback mechanism.

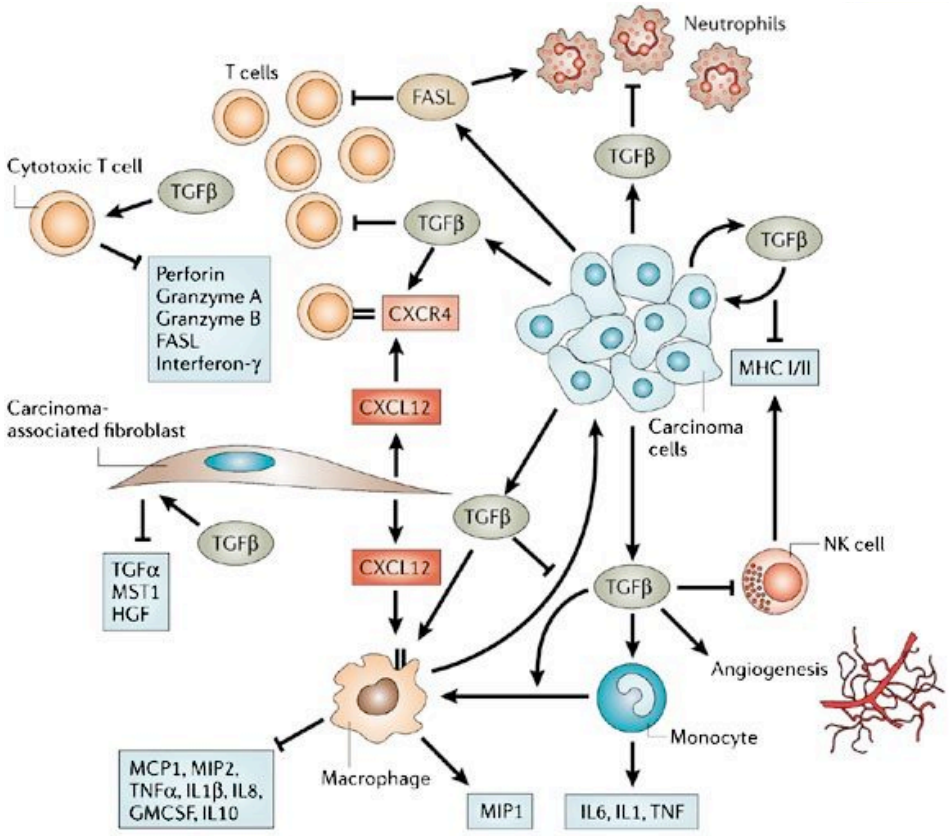

Figure 35. TGFβ in the tumor microenvironment. TGFβ is a potent regulator of T-cell, neutrophil, monocyte, macrophage, natural-killer (NK)-cell, carcinoma associated fibroblast (CAF) and carcinoma-cell-autonomous signaling in the tumor microenvironment. In addition, TGFβ contributes to the regulation of angiogenesis. TGFβ stimulates the migration of fibroblasts, T cells, neutrophils and monocytes. TGFβ inhibits cytotoxic T cells and stimulates monocytes to differentiate into macrophages. Figure from Bierie and Moses, 2006.

Smad4 mediated TGFβ/activin/bmp functions

Previously we demonstrated that loss of Smad4 in skin and tumors abrogated not only signaling from TGFβ but BMPs as well (Xu et al., 2006). We have yet to identify whether Activin and other TGFβ superfamily member signaling is disrupted. Certainly Smad4 can mediate signaling downstream of these molecules, yet there appears to be cell type specific contexts for functional phenotypes. While other Smads participate and receptors mediate tumor suppressor function, only Smad4 shows spontaneous tumor formation when lost. However benign tumors are formed in mice lacking ALK-3 (Andl et al., 2004; Kobielak et al., 2003; Ming Kwan et al., 2004; Yuhki et al., 2004). The diverse effects of the TGFβ superfamily can be altered and potentiated by the microenvironment and differentiation of the cells themselves. Certainly, Smad4 has been shown here in these studies to result in quantitative phenotypes that can be exaggerated and become progressive most likely due to a failure to send and receive proper signals of the TGFβ superfamily.

Smads cell types specific function in cutaneous wound healing

In this study we show that Smad4 loss in keratinocytes mediates a myriad of cellular phenotypes independent of the keratinocytes themselves. This is in stark contrast to Smad3 studies, which lack Smad3 in every cell. Smad3 and Smad4, while partners in transcription of TGFβ target genes, have demonstrated opposing functions. When Smad4 is lost in skin, spontaneous tumors form (Xu et al., 2006). However, Smad3 null mice are

resistant to skin carcinogenesis (Li et al., 2004). It is tempting to speculate that Smad3 loss in keratinocytes could have the opposite effects as Smad4 phenotypes, yet tissue specific loss of Smad3 remains to be demonstrated. Because Smad4 and Smad3 bind and elicit shared transcriptional targets, it is interesting that they have such different effects. Because Smad3 is restricted to TGFβ signaling and independent of BMP/Activin/GDF signaling this might indicate that Smad4 may exist in competition with stoichiometric quantities of R-Smads such as Smad3. Because Smad4 loss phenocopies BMP signaling more than TGFβ or Activin phenotypes there may be more skin dependent functions for BMPs than previously appreciated, which are masked by the redundancy of Smads 1/5/8. Further targeting of both combined with a functional phenotypic screen such as wound healing or carcinogenesis should unlock novel roles for these proteins.

Fibroblasts and myofibroblasts in healing and cancer

The fibroblast is responsible for the creation of most of the components of the ECM, including collagens, structural molecules, as well as the enzymes and growth factors stored in the stroma. Each tissue has very specific requirements, and therefore the fibroblasts at these sites must respond to their organ-specific requirements. In response to different physiologic signaling molecules, whether from normal or pathological conditions, fibroblasts will alter their phenotype and function. Pathologists were the first to notice that fibroblasts inside tumors were unique and undergoing dynamic changes accompanying tumor progression. In tumors, fibroblasts have been referred to as carcinoma-associated fibroblasts (CAFs). They typically exhibit a higher proliferative

index as compared with fibroblasts in normal tissues, often express α-SMA, and are commonly surrounded by dense accumulations of fibrillar collagens. This phenotype, common to several types of human cancer, e.g., breast, prostate, pancreatic, colon, and lung is termed desmoplasia and is associated with the recruitment of inflammatory cells and activation of angiogenic programs. CAFs isolated from malignant tissues exhibit altered phenotypes including disorganized patterns of growth, and enhanced proliferation. These phenotypes have been shown to aid in tumor progression and are similarly observed in Smad4 deficient wounds and tumors. Tissue mixing experiments using cultured fibroblasts and epithelial cells have demonstrated the role of stromal fibroblasts in carcinogenesis. Tumorigenic epithelial cells within recombinant grafts respond to fibroblast-derived signals and the response depends on the types of genes mutated in the adjacent epithelial cells. CAFs when perturbed can send signals that either initiate abnormal epithelial growth or enhance the progression of non-tumorigenic cells to tumorigenic states. The combination of normal human prostatic epithelial cells with CAFs demonstrated an interaction that limited growth potential of the epithelial cells while reinstating their ability to form ductal structures resembling prostatic intraepithelial neoplasia. It remains to be seen whether TGFβ and the other molecules we have identified are sufficient to recapitulate these phenotype in the epithelium.

Fibroblasts also play a very important role in scarring and the production of benign skin tumors known as keloids. These usually result from an over accumulation or uncontrolled proliferation of the fibroblasts, resulting in fibroplasia. Fibrosis in the skin has been demonstrated to be driven by increased levels of TGFβ, which coincidentally we observe with our results in this study. Interestingly, a lack of Smad3 results in

resistance to carcinogenesis and a lack of Smad4 results in spontaneous tumorigenesis, which we find duplicated in wound healing. Smad3 null wounds have much less fibroplasia and scarring or ECM production. However in our study we demonstrate the opposite is true in Smad4 null skin. It will be exciting to test what the consequence of combined Smad4 and Smad3 loss is during both wound healing and tumorigenesis. The roles of these two molecules as competing factors for opposite stromal effects remain to be determined.

Macrophages in tumorigenesis and wound repair

Macrophages are an important part of the innate immune system and play many roles in all aspects of immunity. They can be a very heterogeneous population of cells. Just like CD4 T cells, macrophages have anti-tumor function, and alternatively facilitate the growth and metastasis of tumors. Monocytes that are activated to become macrophages by IFNγ and LPS will destroy tumor cells through their synthesis of nitric oxide and cytokines and chemokines. These macrophages can also function as antigen-presenting cells, which will stimulate cytotoxic CD8+ T. Macrophages activated with TGFβ promote tumor progression by enhancing angiogenesis and producing different cytokines and chemokines. Macrophages are a continuum of phenotypes with M1 and M2 serving as the polarized extremes of differential activation. Most progressively growing tumors are infiltrated by large numbers of macrophages. These tumor-associated macrophages (TAM) are a key component of the tumor stroma and are essential for the angiogenesis and matrix remodeling that support progressively growing neoplasms.

Using a spontaneous mouse mammary tumor model, the transition from premalignant to malignant phenotype was associated with increased blood vessel formation, and the elimination of TAMs blocked neo-angiogenesis, while early infiltration of TAM-enhanced angiogenesis (Bierie et al., 2008). Metastasis is also enhanced by TAMs when they promote the intravasation of tumor cells into local blood vessels, as graphically shown by intravital multiphoton imaging of live mammary tumors *in situ* (Sidani et al., 2006). TAMs are often called M2 macrophages because they promote tumor progression. Gene expression profiling of TAMs have confirmed that TAMs and M2 macrophages express many of the same molecules. It has recently been shown that epithelial deletion of TGFβ-RII in mammary carcinoma also instructs F4/80 positive macrophages to malignant tumors (Bierie et al., 2008). We find similar results in Smad4 deficient skin, however only our results of smad4 loss resulting in spontaneous tumor formation are contrasted to these other models, which require an additional oncogene to be present. In our results, macrophages only required the lack of Smad4 to be activated, which could suggest the difference may lie in additional functions of Smad4 yet to be discovered independent of the TGFβ ligands and receptors.

Many pathological conditions including cancer are associated with chronic inflammation, which often involves macrophages. The excessive appearance of TAMs correlates with poor prognosis in well over 80% of published studies (Bingle et al., 2002). Macrophages appear to be directly involved in tumor progression and metastasis. Removal of macrophages in mice through a homozygous null mutation of the gene that encodes the macrophage growth factor, colony-stimulating factor-1 (CSF-1) can reduce the rate of tumor progression and almost eliminate metastasis of the tumor in a mouse

model of breast cancer. In contrast, overexpression of CSF-1 accelerated tumor progression and metastasis in this mouse model (Lin et al., 2001). Blocking expression of mouse CSF-1 in a xenograft model (mice engrafted with human tumor cells) reduced the growth and metastatic capacity of the tumor cells, and this was associated with reduced invasion of host-tumor associated macrophages. These data suggest that a causal relationship exists between poor prognosis in a variety of reproductive tumors and overexpression of CSF-1, which recruits macrophages. Indeed, in human breast cancers, there is a positive correlation between poor prognosis and the density of tumor-associated macrophages (Lin et al., 2002). During wound healing, macrophages are sentinel cells that organize immune defenses and coordinate the tissue repair process, which involves epithelial migration, matrix remodeling, and angiogenesis (Coussens and Werb, 2002). We show here that tumors recruit macrophages and create a microenvironment that causes macrophages to suppress immune functions and instead adopt roles found during development and repair. However, in contrast to normal tissue, tumor cells have lost their "off switches" due to intrinsic mutations and no longer respond to positional information. Therefore, they continue to grow, invade the surrounding tissue, and escape to distant sites.

Macrophages also play important roles in many inflammatory processes, and at different sites of the body macrophages have very unique and heterogeneous functions. In cutaneous wound repair the macrophage is responsible for efficient phagocytosis of all the undesirable foreign and damaged cells. Lack of efficient macrophage activity has been shown to allow wounds to heal normally and without scar formation (Martin et al., 2003). However these animals were in pathogen free environments and most animal

models of wound healing do not realistically subject the wound to 'real world' exposure to infection and foreign invaders. While it is tempting to speculate that depletion of macrophages could be an effective treatment, macrophages provide a very important role in clearing the wound of debris and assisting in remodeling.

Tumors as wounds that 'do not heal' or tumors are wounds that 'overheal'

Over twenty years ago the idea that tumors were wounds that did not heal was established (Dvorak, 1986). Recently, this hypothesis has been extended to 'that a tumor may be an overhealing wound' (Figure 36) (Schafer and Werner, 2008). The early hypothesis that "tumors are wounds that do not heal" has parallels observed in our study here as well as other studies. We have revealed that Smad4 deficient tumors activate the wound-healing program, but in an exaggerated and increased fashion. Most of the genes that regulate the wound-healing process are found as stimulators of cancer growth and progression. However, there are fundamental differences between Smad4 null wounds and tumors. Spontaneous tumors never arose from these wounds and did not activate malignant conversion. This demonstrates that an additional threshold is still yet required for the process of cancer formation. Another important difference in tumor progression to wounds is the fact that most tumors harbor mutations and epigenetic changes in the tumor cells themselves, possibly also in the stroma, yet in the wound no mutation occurs. Mutations and epigenetic changes in tumors change their metabolism, cause them to lose their differentiation capacity, and migrate into adjacent non-cancerous tissue and metastasize to foreign sites. Inflammation in tumors, unlike wounds, is not resolved

within a couple of weeks, as continuous activation of the wound-healing-esque inflammatory program occurs. Exacerbated inflammation in chronic skin wounds and other diseases increases mutagenesis risks. Uncontrolled cell proliferation in chronic lesions might further lead to the multiplication of cells with a potentially dangerous cancer mutation, thus increasing the risk that an already mutated cell accumulates additional deleterious mutations. Therefore, it will be a major challenge for the future to reduce chronic inflammation, to improve the healing capacity of chronic wounds and to identify the factors that control the termination of the wound-healing process. Identification of theses key molecular and cellular programs will and have begun to open up new therapies that may diminish inflammatory contributions to cancer and other diseases.

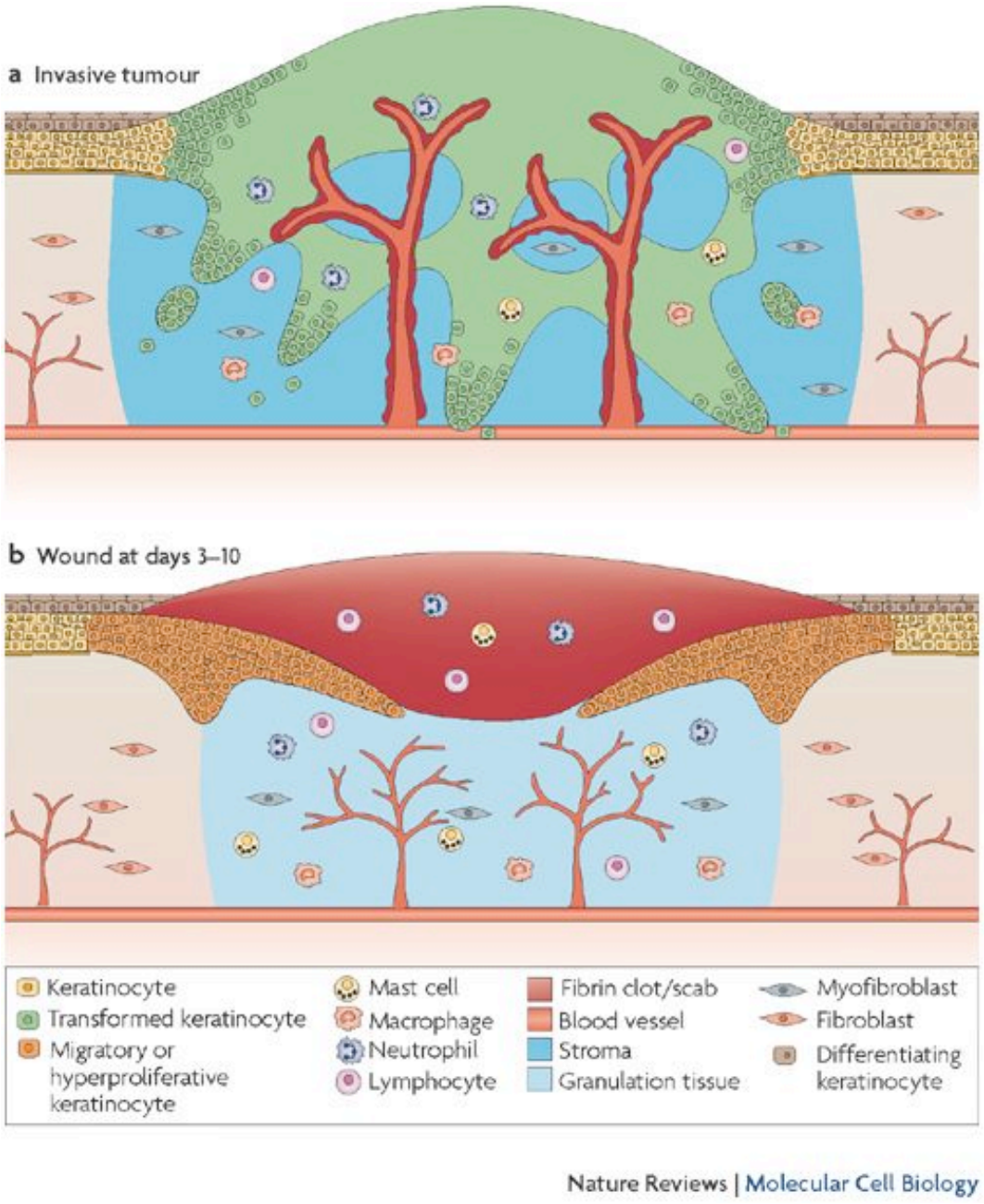

Figure 36. Cellular parallels between a tumor and a skin wound. Schematic representation of an epithelial cancer (**a**) and a skin wound in the phase of new tissue formation after wounding (**b**). Note that both types of tissue are characterized by the presence of a fibrin clot, inflammatory cells (neutrophils, macrophages, mast cells and lymphocytes), newly formed blood vessels, and a large number of fibroblasts and myofibroblasts. These are components of the wound granulation tissue, which strongly resembles the tumor stroma. In addition, migrating and proliferating keratinocytes are present in the wound and in the cancer tissue. The main difference between tumors and wounds is the invasive growth of the transformed cells, which have somatic mutations keratinocytes (which fill the tumor). Figure from Schafer and Werner, 2008.

Chapter 6: Summary, Conclusions and Future Directions

Smad4 regulates hair follicle integrity via transcription of desmoglein4

We have found in this study that Smad4 is required in skin to maintain homeostasis of the epidermis, sebaceous glands and hair follicles. Specifically we find that Smad4 is a transcription factor, which regulates the synthesis of dsg4 RNA. Furthermore we find that this occurs in concert with BMP specific R-Smads 1&5 and is induced by BMP ligands. Future studies will focus on the mechanism of sebaceous gland proliferation and epidermal hyperplasia. Additional questions remain on which transcriptional co-factors mediate Smad4 functions specifically on the dsg4 promoter as well as possible other target genes. Because Smads are enriched in the putative stem cell niche at the bulge, it will be interesting to determine with the collapse of the hair follicle in Smad4 null skin, what is the ultimate fate of stem cells in Smad4-/- skin. New approaches could determine what parallels aged skin has with Smad4 loss.

Smad4 loss in keratinocytes during wound healing results in multiple cell non-autonomous defects

We have also found in this study that the function of Smad4 in keratinocytes encompasses cells that have normal Smad4 function. Keratinocytes are cells that can ‘wear many hats’ by secreting cytokines, presenting antigens and simultaneously mediating barrier function. We have found that loss of Smad4 in keratinocytes results in

poorly healing wounds that do not have defects in reepitheliazation yet have excessive angiogenesis, inflammation and myofibroblast accumulation during wound repair. For all of our molecular analysis we have yet to determine which cells produce the signaling molecules responsible for amplifying or potentiating the resulting phenotype. Careful dissection of the tissue using navigated laser capture microdissection could determine which cells contribute which factors. Furthermore, functional blocking of angiogenesis with pharmacological agents as well as against inflammation would be worthwhile to determine the efficacy of the therapy.

Developing translational treatments and therapies of an abnormal stroma

If the stroma is altered as a result of genetic change in the neighboring epithelia, then perhaps removal or direct intervention such as gene replacement therapy will be required to treat these pathologies. Identification beyond malignant phenotypes for somatic changes in epithelia could allow for more thorough targeting of diseased tissue. Extracellular proteases in early cancer progression, malignant conversion, and metastatic processes have demonstrated and prompted the first clinical testing of non-cytotoxic therapeutics. Unfortunately, results with MMP inhibitors thus far have been less than effective and may reflect their broad usage by many cell types. Fibrotic breast disease can also predispose one to breast cancer, and environmentally induced fibrotic disorders can increase the incidence of lung and skin cancer, underscoring the importance of developing therapeutic modalities that target tumor microenvironments. One exciting potential agent is Halofuginone, which can inhibit collagen synthesis. Halofuginone

inhibits type I collagen synthesis by blocking TGFβ mediated activation of Smad3, and has demonstrated efficacy in reducing liver disease and inhibiting myofibroblast proliferation in various cancers (Genin et al., 2008; Pines et al., 2003; Sheffer et al., 2007; van Kempen et al., 2008). This inhibitor was recently approved as a therapeutic agent for scleroderma and is currently in clinical trials for treatment of various types of solid tumors (Pines et al., 2003). Because remodeling of collagen fibrils is also involved in the alignment and formation of endothelial tubular structures, inhibition of collagen synthesis may limit tumor-associated angiogenesis. Collagen is an important scaffold for a large array of mitogens and morphogens; therefore, disrupting the balance of regulated collagen metabolism may alter the bioavailability of these factors and subsequently inhibit neoplastic programming. Preliminary phase 1 trials are very promising with this agent (de Jonge et al., 2006).

Targeting fibroblasts for therapy

Fibroblasts appear to be strong mediators of the inflammation and excessive hypertrophic scarring seen in many diseases and cancers. These alterations have been observed in this study as well. Further experiments that can ‘rescue’ the phenotype are needed. Blocking the elevated levels of TGFβ may be an important start, however targeting the CAF’s more directly through identification of yet unknown activated myofibroblast receptors and secreted molecule could make excellent druggable targets. In addition, fibroblast alterations may provide a gene expression signature that helps to predict the clinical course of disease. Since few reports of mutation or genetic lesions

have been identified in stromal cell populations, dramatic and complicated therapies such as gene transduction or ‘rescue’ may not be necessary. Instead there are mostly likely secreted molecules that are binding to specific receptors and either of these could be blocked with functional antibodies. Additionally, if any of these targets have enzymatic properties such as phosphorylation then specific chemical inhibitors could be rapidly identified. Further identification is clearly needed of the signals that can stimulate the disappearance of fibroblasts, such as the apoptosis that they normally undergo with the cessation of the normal wound healing process.

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APPENDIX I: Reagent Information

Table 1. IHC and IF antibody information.

Antibody	Host	Vendor	Catalog #	Primary Concentration
αSMA	Mouse	Sigma	A2547	1:500
ECadherin	Mouse	BD Bioscience	610182	1:100
F4/80	Rat	Invitrogen (Caltag)	MF48000	1:100
Keratin 13	Mouse	Chemicon	CBL176	1:100
Keratin 14	Giunea Pig	RDI-Fitzgerald	20R-CP002	1:400
Keratin 8/18	Giunea Pig	RDI-Fitzgerald	20R-CP004	1:100
Keratin 6	Rabbit	Covance	PRB-169P	1:500
Keratin 1	Rabbit	Covance	PRB-165P	1:500
Smad4	Mouse	Santa Cruz	sc-7966	1:200
AE13	Mouse	T.T. Sun	Columbia U.	1:50
AE15	Mouse	T.T. Sun	Columbia U.	1:10
Adipophillin	Giunea Pig	RDI-Fitzgerald	20R-AP002	1:500
PCNA	Mouse	Santa Cruz	sc-7907	1:200
CD31	Rat	BD Bioscience	553708	1:200

Table 2. ChIP Antibody Information.

Antibody	Host	Vendor	Catalog #
IgG	Rabbit	Santa Cruz Biotechnologies	sc-2027
IgG	Mouse	Santa Cruz Biotechnologies	sc-2025
RNA Pol II	Mouse	Upstate	05-623B
Smad1	Mouse	Santa Cruz	sc-7965
Smad2	Rabbit	Zymed	51-1300
Smad3	Rabbit	Upstate	06-920
Smad4	Rabbit	Upstate	06-693
Smad5	Rabbit	Zymed	51-3700
Gata-3	Mouse	Santa Cruz	sc-268
pSmad1/5	Rabbit	Cell Signaling	9516

Table 3. qRT-PCR Probe Information.

Gene ID	Dye	Catalog # or Probe ID
GAPDH	VIC	4352339E
Dsg1α	FAM	Mm00809994_s1
Dsg2	FAM	Mm00514608_m1
Dsg3	FAM	Mm00659652_m1
Dsg4	FAM	Mm00812608_m1
Msx2	FAM	Mm00442992_m1
Gli-1	FAM	Mm00494645_m1
Krt31	FAM	Mm00657991_gH
HoxC13	FAM	Mm00802798_m1
FoxN1	FAM	Mm00433946_m1
Gata-3	FAM	Mm00484683_m1
MMP3	FAM	Mm00440295_m1
MMP12	FAM	Mm00500554_m1
MMP13	FAM	Mm00439491_m1
MMP14	FAM	Mm00485054_m1
CCL5	FAM	Mm01302428_m1
CCL20	FAM	Mm00444228_m1
TGFβ1	FAM	Mm03024053_m1
VEGFA	FAM	Mm00437306_m1

Table 4. Genotyping primer sequence information.

Gene	Primer Sequences
K5CrePR1	CGGTCGATGCAACGAGTGAT
	CCACCGTCAGTACGTGAGAT
Smad4 floxed	GGGCAGCGTAGCATATAAGA
	GACCCAAACGTCACCTTCA
Smad4 Deletion	AAGAGCCACAGGTCAAGCAG
	GACCCAAACGTCACCTTCA

APPENDIX II: List of Contributors

Hisham Bazzi & Angela Christiano-histopathology of dsg4 mutant mice (LahJ), antibody to dsg4 and luciferase reporter to Dsg4.

Peter Girling, CellNTEC Corporation-primary keratinocyte cell isolation establishment.

Tim Cleaver & Steve Malkowski- β-gal staining of tissue biospsies

Jackie DeGagne & Dennis Trune- transmission electron microscope assistance.

Chuxia Deng-Smad4 floxed mice and MMTV skin samples.

Department of Comparative Medicine (DCM)-care of aniumals-wounds and runts.

W. Harv Flemming Laboratory-Use of inverted microscope.

Carolyn Gendron-Histology.

Genscript Corporation-SBE mutation in Dsg4 reporter.

Gangwen Han-Scratch assay.

Maranke Koster & Dennis Roop- In vivo ChIP assay development.

Allen Guanqun Li – Wound experiments, design and implementation.

Oregon Cancer Institute (OCI) Biostatistics Core- Statistical analysis consultation.