

Dermal Transforming Growth Factor- β Responsiveness Mediates Wound Contraction and Epithelial Closure

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Stromal-epithelial interactions are important during wound healing. Transforming growth factor- β (TGF- β) signaling at the wound site has been implicated in re-epithelization, inflammatory infiltration, wound contraction, and extracellular matrix deposition and remodeling. Ultimately, TGF- β is central to dermal scarring. Because scarless embryonic wounds are associated with the lack of dermal TGF- β signaling, we studied the role of TGF- β signaling specifically in dermal fibroblasts through the development of a novel, inducible, conditional, and fibroblastic TGF- β type II receptor knockout (Tgfb2^{dermalKO}) mouse model. Full thickness excisional wounds were studied in control and Tgfb2^{dermalKO} back skin. The Tgfb2^{dermalKO} wounds had accelerated re-epithelization and closure compared with controls, resurfacing within 4 days of healing. The loss of TGF- β signaling in the dermis resulted in reduced collagen deposition and remodeling associated with a reduced extent of wound contraction and elevated macrophage infiltration. Tgfb2^{dermalKO} and control skin had similar numbers of myofibroblastic cells, suggesting that myofibroblastic differentiation was not responsible for reduced wound contraction. However, several mediators of cell-matrix interaction were reduced in the Tgfb2^{dermalKO} fibroblasts, including α 1, α 2, and β 1 integrins, and collagen gel contraction was diminished. There were associated deficiencies in actin cytoskeletal organization of vasodilator-stimulated phosphoprotein-containing lamellipo-

dia. This study indicated that paracrine and autocrine TGF- β dermal signaling mechanisms mediate macrophage recruitment, re-epithelization, and wound contraction. (Am J Pathol 2010, 176:98–107; DOI: 10.2353/ajpath.2010.090283)

Skin wound healing in adult humans after full-thickness injury results in scar tissue, which is prone to contracture, loss in elasticity, and altered tensile strength. The characteristics of a healed wound, unlike the original dermal tissue, include an extracellular matrix without the normal basket weave organization of collagen fibers that can be further exaggerated as a hypertrophic scar. In scars, there is a loss of normal tissue architecture and function due, at least in part, to altered cell-matrix interaction and the assembly of the actin cytoskeleton that occurs during wound repair.^{1,2} The actin cytoskeleton is important in processes that are fundamental to wound healing: migration; contraction; adhesion; and proliferation.³ The vasodilator-stimulated phosphoprotein (VASP) is associated with filamentous actin formation and likely plays a role in cell adhesion and motility.⁴ VASP may also be involved in the intracellular signaling pathways that regulate integrin-extracellular matrix interactions.

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The importance of stromal-epithelial interactions in development and wound healing is well established. These interactions likely involve autocrine and paracrine action of multiple growth factors, including members of the transforming growth factor- β (TGF- β) family. TGF- β 1, β 2, and β 3 isoforms signal by binding to the TGF- β receptors type I and type II at the cell surface. The ligand-receptor complex formed stimulates multiple parallel signal pathways in the cytoplasm. TGF- β is a growth inhibitor for many cell types and stimulates extracellular matrix expression in mesenchymal cells. In the injured skin, macrophages, endothelium, fibroblasts, and epithelia are all sources of elevated TGF- β expression. TGF- β signaling at the wound site is thought to be important for extracellular matrix deposition and remodeling. The application of TGF- β 1 to wounds has been shown to accelerate wound healing.⁵ In apparent contradiction, a genetic knockout of the TGF- β effector, Smad3, in mice exhibited accelerated skin and lens wound healing.^{6–8} However, the epithelial and stromal compartments can respond differentially to TGF- β signaling, and the specific mechanism of TGF- β action on any individual cell type within tissues is still not understood. Wounding of embryonic skin, in contrast to that in adults, is not associated with remodeling deficiencies associated with scarring. Interestingly, embryonic dermal fibroblasts lack expression of TGF- β receptor type II.^{9,10}

In the present study, we specifically address the role of TGF- β signaling in dermal fibroblasts by the development of a novel dermal fibroblast-inducible, conditional, TGF- β type II receptor knockout (Tgfb2^{dermalKO}) mouse model. The generation of Tgfb2^{dermalKO} mice provided an opportunity to test the role of TGF- β in stromal cells in wound healing. The full-thickness skin excision wounds in Tgfb2^{dermalKO} mice had accelerated re-epithelization compared with control, impaired collagen organization associated with a lack of wound contraction, and reduced macrophage recruitment. The studies indicate that paracrine and autocrine TGF- β dermal responsiveness mechanisms impact skin wound healing.

Materials and Methods

Generation of Tgfb2^{dermalKO} Mice

To study the role of stromal TGF- β signaling in dermal fibroblasts, we generated a conditional mesenchymal Tgfb2 knockout mouse. We used the Cre expressing transgenic mouse model that is driven by the COL1A2 proximal promoter coupled to a mutated estrogen receptor that only binds tamoxifen. These COL1A2-Cre-ER mice^{11,12} were crossed with Tgfb2^{flloxE2/flloxE2} mice¹³ in the C57BL/6 background (Tgfb2^{ColTKO}). The resulting mouse was further crossed with the Rosa26 line¹⁴ to enable visualization of Cre-mediated recombination by staining for β -galactosidase activity. The mice were genotyped from ear DNA by PCR amplification of the Cre and Tgfb2 genes. The studies described were performed with mice more than or equal to eight generations into the C57BL/6 background. Administration of

4-OH tamoxifen (10 mg/ml in sterile vegetable oil, 200 μ l) activated Cre-mediated recombination at the site of interest (eg, skin). The mice were maintained in 12 hours dark/light cycles and were fed standard chow and water. All animal procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Excisional Wounding Experiments

Adult Tgfb2^{ColTKO} mice (8 to 15 weeks old) were anesthetized with isoflurane, shaved, and cleaned with Beta-dine (Purdue Products L.P., Stamford, CT). Excisional wounds were made 7 days after administration of 4-OH tamoxifen or oil. In experiments for wound closure, two 4-mm full thickness excisional wounds were made with a biopsy punch. After wounding, a metal lock washer of 4-mm inner diameter was secured onto the wound site. Wounds were made on healthy mice in similar locations on either side of the back skin to ensure no site bias. Three to fourteen days after excisional wounding the mice were sacrificed, and the wound sites were removed and processed for histology and immunostaining.

Histology and Immunohistochemistry

Wound specimens, including a margin of nonwounded skin, were collected and fixed in 4% paraformaldehyde buffered with PBS (pH 7.2) and embedded in paraffin. Sections (5 μ m) were stained with H&E for histological analysis. β -galactosidase staining was performed to visualize Cre-mediated recombination in the stroma.¹⁵ For immunohistochemical analysis, after antigen retrieval in 10 mmol/L citrate buffer (pH 6.0), tissues were incubated with antibodies for phosphorylated-Smad 2 (1:1000; Cell Signaling, Boston, MA), keratin 5 (1:600 dilution; Covance Research Products, CA), Ki-67 (1:1000 dilution; Upstate USA Inc., Charlottesville, VA), F4/80 (1:50 dilution; Serotec, Raleigh, NC), von Willebrand factor VIII (1:400 dilution; Dako, Carpinteria, CA), and α -smooth muscle actin (Dako) overnight in a humid chamber at 4°C. All immunohistochemistry was detected by using Envision+ system peroxidase kit (Dako) according to the manufacturer's instructions. Masson's trichrome stain was used to evaluate collagen deposition. Picosirius red staining¹⁶ was used to assess alignment and organization of collagen as well as a means of quantitating mature collagen fibers.

Analysis of Re-Epithelization

To measure the degree of re-epithelization, the wound section was viewed under \times 100 and \times 400 power. The width of the wound as well as the distance that the epithelium had traversed was measured. The percentage of re-epithelization was calculated based on the following formula: % re-epithelization = (distance covered by the epithelium/width of wound bed) \times 100%.^{17,18}

Isolation of Dermal Fibroblasts

Skin tissue from newborn mice (24 to 48 hours) was isolated and rinsed in cold HBSS containing 100 U/ml

Table 1. Primer Sets Used for Real Time RT-PCR Analysis

Primer	Sense	Antisense
Glyceraldehyde-3-phosphate dehydrogenase	5'-CGTGCCGCCCTGGAGAAAC-3'	5'-AGTGGGAGTTGCTGTGTAAGTC-3'
α 1 integrin	5'-TCTCTCGCCAGCTTTGGAAGTCAT-3'	5'-TACTGGAGTTGGGCAGCATGGTAA-3'
α 2 integrin	5'-TGCCGTCGATGGAACAGAAGTAA-3'	5'-TGAAGCCTTGAAAATGATGGCCG-3'
β 1 integrin	5'-TTCAGACTTCCGCATTGGCTTTGG-3'	5'-TGGGCTGGTGCAGTTTTGTTCAC-3'
Collagen I α 1	5'-TTCTCCTGGCAAAGACGGACTCAA-3'	5'-AGGAAGCTGAAGTCATAACCGCCA-3'
Collagen I α 2	5'-AGGCGTGAAGGACACAGTGGTAT-3'	5'-TCCTGCTTGACCTGGAGTTCATT-3'
Collagen III	5'-AGCTTTGTGCAAAGTGAACCTGG-3'	5'-CAAGTGGCTGCATCCCAATTCAT-3'

penicillin, 0.01 mg/ml streptomycin, and 50 μ g/ml gentamicin. To trypsinize the skin, tissue was incubated in 0.2% trypsin in HBSS containing penicillin-streptomycin and gentamicin at 4°C. After overnight incubation, the dermis was isolated and digested by using 0.2% collagenase type I in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C for 1 hour. Digested skin was filtered by using a 70- μ m cell strainer and centrifuged at 1000 g for 5 minutes. The pellet was resuspended in stromal media (DMEM F12, 10% FBS, 50 μ g/ml gentamicin, 10% Nu serum, 100 U/ml penicillin, 0.01 mg/ml streptomycin, 0.25 μ g/ml Fungizone, and 0.1% insulin), and incubated at 37°C. After cells were confluent they were split and used for experiments.

Western Blotting

Dermal fibroblasts were sonicated in 25 mmol/L HEPES buffer, pH 7.5 (containing 300 mmol/L NaCl, 20 mmol/L β -glycerol phosphate, 2 mmol/L sodium pyrophosphate, 1 mmol/L EDTA, 0.2 mmol/L EGTA, 0.1% SDS, 1% Triton X-100, 10% glycerol, 1 mmol/L sodium vanadate, 10 nmol/L microcystin, and 1 mmol/L phenylmethylsulfonyl fluoride), and 40 μ g of protein were subjected to SDS-polyacrylamide gel electrophoresis for subsequent immunodetection by blotting on to polyvinylidene difluoride membranes. Membranes were incubated with antibodies for phosphorylated-Smad 2 (1:1000; Cell Signaling), Smad 2 (1:500; Santa Cruz, Inc., Santa Cruz, CA), β 1 integrin (1:1000; BD Pharmingen, San Jose, CA), and collagen type IV (gift from Dr. Billy Hudson, Vanderbilt University). The immunoblots were visualized by using the enhanced chemiluminescence detection (Amersham, GE Health care, Piscataway, NJ).

RNA Isolation and Real Time Quantitative RT-PCR

RNA was isolated from dermal fibroblasts by using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was quantified and reverse transcribed by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Control reactions in which reverse transcriptase was omitted were included for each sample to confirm the absence of DNA in the RNA sample. Thermal cycling on an iCycler (Bio-Rad) real-time PCR machine

was performed by using SYBR supermix (Bio-Rad) in a total volume of 25 μ l, and running for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Quantitation of the results was performed, as previously described, by using the relative standard curve method with control, glyceraldehyde-3-phosphate dehydrogenase expression.¹⁹ No PCR product was detected in samples in which reverse transcriptase was omitted. Expression of α 1 integrin, α 2 integrin, β 1 integrin, collagen type I α 1, collagen type I α 2, and collagen type III was determine in dermal fibroblasts treated with 4-OH-tamoxifen. The primer sequences are listed in Table 1.

Collagen Gel-Contraction Assay

The gel contraction assay is based on previously described methods.²⁰ Briefly, primary dermal fibroblasts at a density of 10⁵/ml were suspended in DMEM-F12 and bovine type I collagen. After the suspension gelled in 48-well plates, an equal volume of DMEM-F12 supplemented with 0.5% FBS was added, and the gels were released with a spatula. The gels were maintained for 3 days in culture medium containing 200 pM TGF- β or PBS, and the capacity of the dermal fibroblasts to contract was determined by gel diameter measurement. Medium containing 10% FBS was used as a positive control. Experiments were performed three times in triplicate.

Immunofluorescence and Confocal Microscopy

Immunofluorescent staining for actin and VASP was performed on dermal fibroblasts grown on coverslips coated with collagen. Fluorescein isothiocyanate conjugated phalloidin (Sigma-Aldrich, St. Louis, MO) was used to label F-actin in mouse dermal fibroblasts according to the manufacturer's instructions. Briefly, cells were fixed in 3.7% formaldehyde, washed in PBS, dehydrated in cold acetone, permeabilized with 0.1% Triton X-100, stained with a 50- μ g/ml fluorescent phalloidin conjugate solution, washed in PBS, and mounted. VASP was localized by fixing the cells in cold methanol at -20°C for 10 minutes, permeabilized in PBS with 0.3% NP40 for 10 minutes, and incubated in 10% FBS blocking buffer with anti-VASP (1:100; Cell Signaling) overnight at 4°C followed by incubation with Alexa 488 goat anti-rabbit (1:1000; Invitrogen Corporation, Carlsbad, CA). Slides were examined under a laser scanning confocal microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany) equipped with an LSM510

META scan head (Carl Zeiss). Argon 488 and helium-neon 543 lasers were used to generate the excitation lines. Z-stacks were collected and images were acquired by using two-line mean averaging in a Z-series typically containing X-Y overlapping sections of 0.61 μm depth.

Statistical Analysis

Statistical analysis was performed by using the one-way analysis of variance test for the comparison of multiple variables, and Student's *t* test was used for comparing two variables. A *P* value <0.05 was considered statistically significant. Results are shown as mean \pm SD. Graphs were generated by using GraphPad Prism version 4 (Graph Software, San Diego, CA).

Results

TGF- β Dermal Responsiveness Mediates Epithelial Wound Closure

The skin of *Tgfr2^{CoITKO}* mice was characterized following s.c. injection with 4-OH tamoxifen and oil (control). There were no observable differences in the histology of skin from *Tgfr2^{CoITKO}* mice injected with oil (Figure 1A) or 4-OH tamoxifen (Figure 1B) compared with wild-type mice by H&E staining. β -galactosidase staining was per-

formed to visualize Cre-mediated recombination in the stroma. Control *Tgfr2^{CoITKO}* mice showed no positive staining for β -galactosidase (Figure 1C). *Tgfr2^{CoITKO}* mice injected with 4-OH tamoxifen were positive for β -galactosidase in the dermis, indicating that Cre-mediated recombination occurred in the stroma and not in the epithelium (Figure 1D). Immunohistochemical localization of phosphorylated-Smad2 was performed to determine the status of TGF- β signaling activity in the dermis. Control *Tgfr2^{CoITKO}* mice had nuclear localization of phosphorylated-Smad2 (brown nuclei, Figure 1E) in nearly all cells in the skin, whereas *Tgfr2^{CoITKO}* mice injected with 4-OH tamoxifen had few dermal fibroblasts with phosphorylated-Smad2 expression (blue nuclei, Figure 1F). However, hair follicle-lining cells and keratinocytes from *Tgfr2^{CoITKO}* mice injected with 4-OH tamoxifen displayed TGF- β signaling, as indicated by positive phosphorylated-Smad2 staining. It is important to note there are many cell types in the dermal compartment, only those cells expressing collagen 1 α 2 would have the capacity to mediate Cre-mediated *Tgfr2* DNA recombination after 4-OH-tamoxifen injection. The inducible, conditional *Tgfr2^{CoITKO}* mice are referred to as *Tgfr2^{dermalKO}* or control when injected subcutaneously with 4-OH-tamoxifen or oil, respectively.

TGF- β has an important role in the regulation of wound healing and tissue repair.²¹ We tested the role of TGF- β signaling in dermal fibroblasts in full thickness 4-mm excisional wounds. Control and *Tgfr2^{dermalKO}* mice healing were followed up to 14 days after wounding. At 4 days after injury, *Tgfr2^{dermalKO}* skin wounds showed little contraction and a reduced organization of granulation tissue when compared with controls (Figure 2A). However, wounds from *Tgfr2^{dermalKO}* mice showed significantly accelerated re-epithelization when compared with control wounds (Table 2). Because dominant mechanism of wound closure in mice is contraction, a metal washer was fixed to the skin immediately after wounding to limit contraction in further studies of re-epithelization.²² The H&E-stained paraffin sections, representing the longitudinal diameter of the wound, showed minimal re-epithelization in control wounds at 4 days (Figure 2B). In comparison, *Tgfr2^{dermalKO}* wounds had complete re-epithelization in the same period of time but a less complete formation of granulation tissue. The injection of either oil or 4-OH tamoxifen 7 days before wounding of wild-type mice produced no observable differences in the rate or quality of repair (data not shown).

Re-epithelization was visualized by keratin 5 immunohistochemical staining of the basal layer of the epidermis. The loss of TGF- β responsiveness in dermal fibroblasts resulted in increased abundance of keratin 5-positive keratinocytes in a hypertrophic epidermis and evidence of suprabasal expression of keratin 5 (Figure 2C). To examine the role of proliferation in the accelerated re-epithelization of *Tgfr2^{dermalKO}* mice, cell proliferation was assessed by immunolocalization of Ki-67. Control mice showed keratinocyte proliferation at the distal wound edges (Figure 2D). However, wounds on *Tgfr2^{dermalKO}* mice exhibited keratinocyte proliferation extending over the entire wound surface. Accordingly,

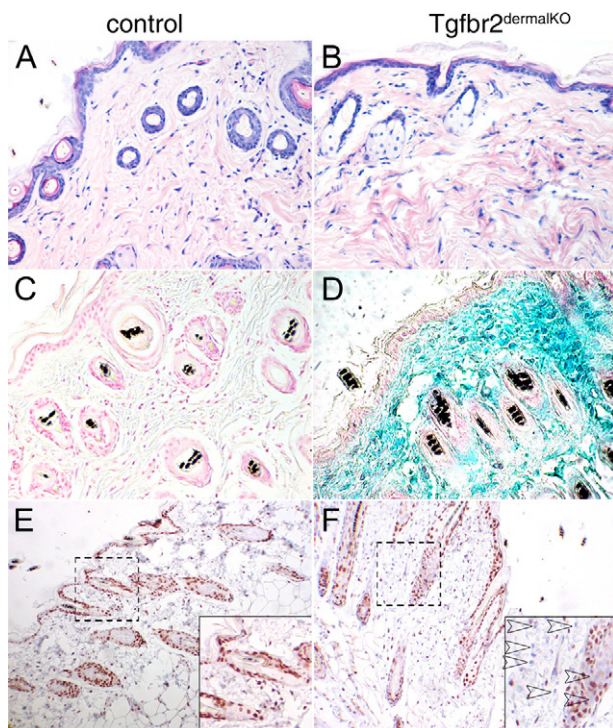


Figure 1. *Tgfr2^{dermalKO}* mice showed Cre-mediated recombination and loss of TGF- β responsiveness in the dermis. Histology of back skin from control and *Tgfr2^{dermalKO}* mice was determined by H&E staining (A and B). β -galactosidase activity, associated with DNA recombination, was localized in back skin of control and *Tgfr2^{dermalKO}* mice (C and D). Immunolocalization for phosphorylated-Smad2, indicating downstream TGF- β signaling, had differential expression in dermal fibroblasts of control and *Tgfr2^{dermalKO}* mice (E and F). Scale bars: 20 μm (A–F); 10 μm (dashed area magnified in inset).

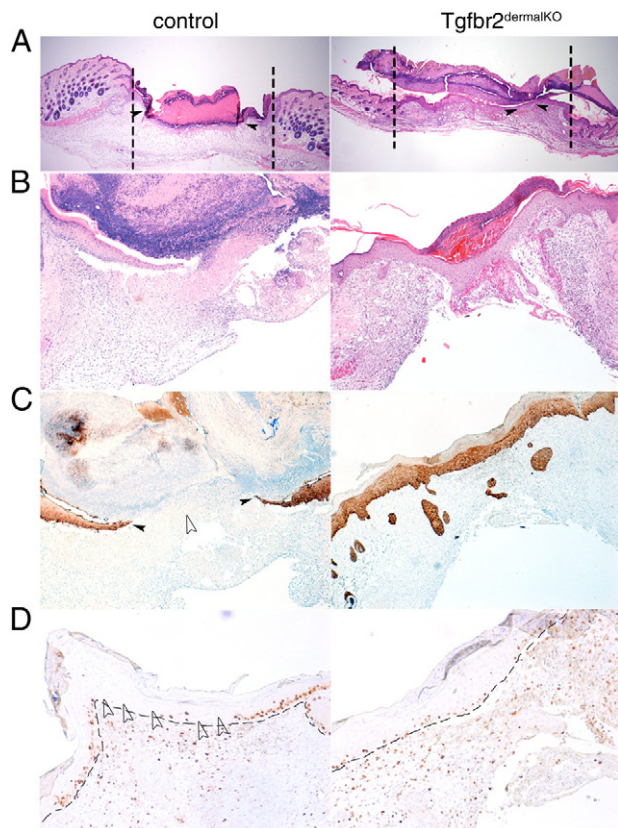


Figure 2. Wounding of *Tgfb2^{dermalKO}* and control skin result in differential contraction and re-epithelization. Full thickness excisional wounds were made on the shaved back of 6- to 8-week-old *Tgfb2^{dermalKO}* and control mice, and healing was examined. Four days after wounding, the control and *Tgfb2^{dermalKO}* mice healed with dermal contraction (vertical dotted lines indicate wound edge) and re-epithelization (A). To limit wound contraction, washers were implanted for the subsequent wounds. H&E staining revealed differential re-epithelization in control and *Tgfb2^{dermalKO}* mice after 4 days of healing (B). Keratin 5 staining indicated abundance of keratinocytes covering the wound with stark differences in control and *Tgfb2^{dermalKO}* mice (C). The epithelial compartment indicated by the dashed line of control and *Tgfb2^{dermalKO}* wounds had similar keratinocyte Ki-67 positive staining at the distal wound edges. *Tgfb2^{dermalKO}* wounds had elevated keratinocyte proliferation at the center of wound compared with control wounds (D). **Filled arrowheads** indicate leading edge of epithelia. **Empty arrowheads** indicate area lacking immunohistochemical staining. Scale bars: 400 μ m (A); 20 μ m (B and C); 10 μ m (D).

the loss of dermal TGF- β responsiveness resulted in accelerated re-epithelization of wounds associated with decreased dermal contraction.

TGF- β Dermal Responsiveness Differentially Affects Granulation Tissue Formation

TGF- β signaling has been implicated in multiple roles in wound healing, including collagen deposition and myo-

Table 2. Effect of the Loss of Dermal TGF- β Signaling on Day 4 of Wound Re-Epithelization

Strain	n	Percent re-epithelization
Control	8	22.5 \pm 2.5
<i>Tgfb2^{dermalKO}</i>	8	58.0 \pm 2.9*

Each value represents mean \pm SE. Original magnification, \times 1000. * P < 0.001 versus control.

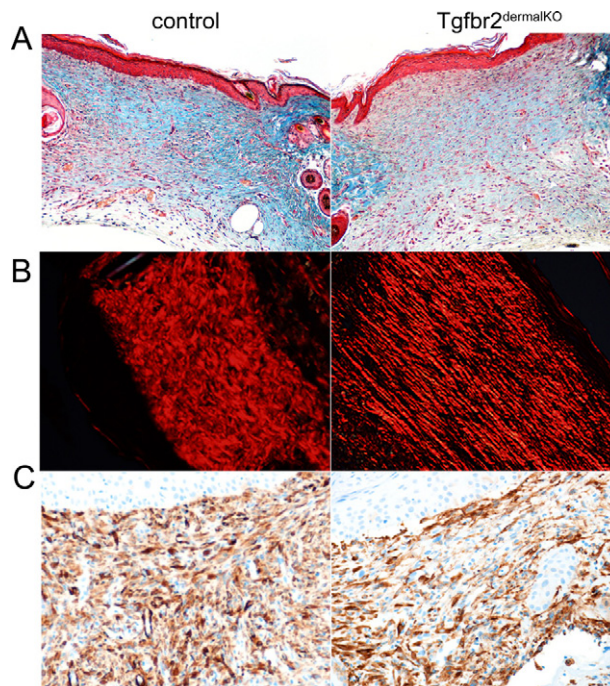


Figure 3. Dermal TGF- β signaling mediates extracellular matrix deposition and organization. *Tgfb2^{dermalKO}* mice had less collagen deposition 14 days after wounding when compared with control mice based on Masson's Trichrome blue staining (A). Collagen fiber organization was visualized by picrosirius red staining and polarized light microscopy. The birefringent collagen fibers in control 14-day wounds had a woven architecture through the span of the wound similar to adjacent nonwounded areas, while *Tgfb2^{dermalKO}* wounds had less refractile collagen fibers in nearly parallel fiber architecture (B). Wounds of control and *Tgfb2^{dermalKO}* mice had similar α -smooth muscle actin immunohistochemical staining (C). Scale bars: 40 μ m (A); 20 μ m (B and C).

fibroblastic differentiation as important components of granulation tissue formation.²³⁻²⁷ In examining collagen deposition in skin wounds, sections stained with Masson's trichrome suggested wounds on *Tgfb2^{dermalKO}* mice had less collagen deposition at day 14 after wounding, compared with control mice (Figure 3A). As a measure of collagen fiber orientation, wounds stained with picrosirius red were observed with polarized light microscopy. Collagen in control mice had abundant, refractile fibers in a woven architecture throughout the 14-day wound, similar to adjacent nonwounded areas (Figure 3B). However, wounds on *Tgfb2^{dermalKO}* mice had fewer refractile collagen fibers compared with control mice with nearly parallel fiber architecture. The adjacent nonwounded areas in the skin of *Tgfb2^{dermalKO}* mice maintained collagen fiber organization similar to control mice. Quantitation of picrosirius red staining indicated a statistically greater collagen expression in control compared with *Tgfb2^{dermalKO}* wounds (18.1 \pm 3.2 and 11.0 \pm 3.1, respectively, P = 0.004; values indicate mean \pm SD). The lack of collagen maturation by 14 days in *Tgfb2^{dermalKO}* reinforced the concept that dermal TGF- β signaling in the stroma potentiates both deposition and maturation of collagen in wound healing. Myofibroblasts are thought to be a transient differentiation state associated with wound healing and are attributed to collagen maturation. The authors of *in vitro* studies suggest TGF- β to be the medi-

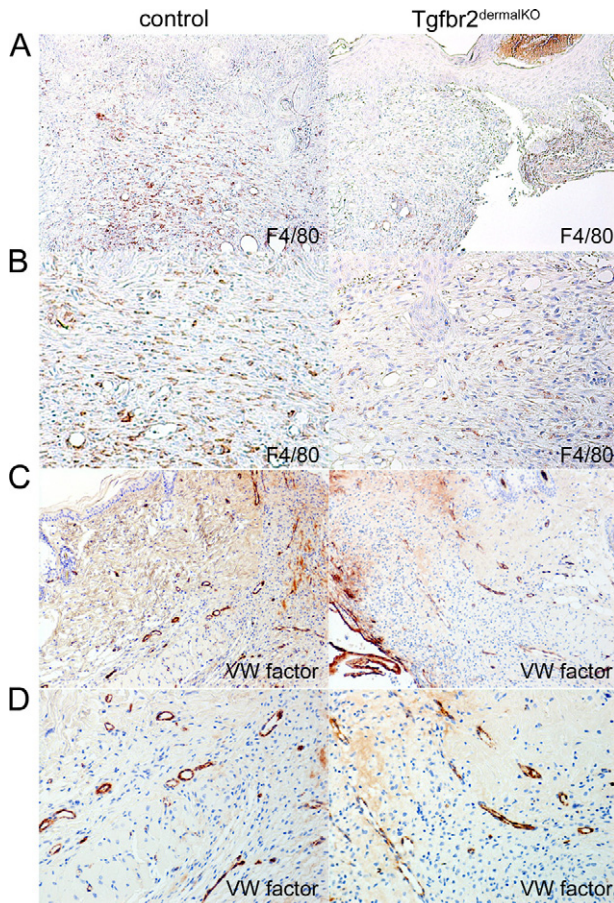


Figure 4. Dermal TGF- β signaling mediates inflammatory infiltration of wounds. Macrophage infiltration was attenuated in the *Tgfb2^{dermalKO}* wounds compared with control localized, as localized by immunohistochemistry for F4/80 expression (**A** and **B**; $P < 0.003$). However, vasculature density did not significantly differ between the control and *Tgfb2^{dermalKO}* wounds as determined by endothelial cell staining by Von Willebrand (VW) factor VIII expression (**C** and **D**). Scale bars: 40 μ m (**A** and **C**); 20 μ m (**B** and **D**).

ator of myofibroblastic differentiation.^{28–30} However, wounds of control and *Tgfb2^{dermalKO}* mice had similar α -smooth muscle actin expression (Figure 3C).

We further analyzed the extent of inflammatory infiltrates and neovascularization in granulation tissue. The control wounds recruited significantly greater numbers of F4/80-positive macrophages than wounds in *Tgfb2^{dermalKO}* skin (21.5 ± 0.5 and 7.3 ± 0.6 , respectively, $P < 0.003$; values indicate mean \pm SE) (Figure 4, A and B). Angiogenesis was assessed by immunohistochemical staining for von Willebrand factor VIII. No significant difference was found in vessel density between the control and *Tgfb2^{dermalKO}* wounds (Figure 4, C and D). These results would indicate that TGF- β signaling by the dermal fibroblasts predominantly mediated collagen deposition and organization while not having a rate-limiting role in stromal differentiation. Reduced TGF- β signaling suppressed mononuclear cell recruitment in granulation tissue, which likely diminished the expression of other paracrine signals.

Extracellular Matrix and Cytoskeletal Organization Mediated by TGF- β Signaling

The remodeling phase of wound healing is characterized by wound contraction due to the action of fibroblasts. Dermal fibroblasts from both control and *Tgfb2^{dermalKO}* wounds had similar myofibroblastic conversion, yet they had vastly different contractile properties. Myofibroblasts have dense arrays of α -smooth muscle actin filaments that crosslink with myosin to produce tension. To measure the contractile capacity in the context of an identical matrix environment, cultured primary dermal fibroblasts from control and *Tgfb2^{dermalKO}* mice were embedded in collagen I gels. The gel diameter was measured after 3 days of incubation in media containing 10% serum. *Tgfb2^{dermalKO}* fibroblasts had markedly reduced gel contraction compared with control fibroblasts (Figure 5A). The *Tgfb2^{dermalKO}* fibroblasts mediated 3.4-fold less gel contraction than that mediated by control fibroblasts ($P < 0.0001$). These results corroborated previous reports that TGF- β mediates contraction by fibroblasts *in vitro*^{31–33}; however, reduced collagen accumulation may have been a secondary factor in the observed reduction in wound contraction in the *Tgfb2^{dermalKO}* wounds.

TGF- β signaling in cultured cells was examined by Western blotting of protein extracts from control and *Tgfb2^{dermalKO}* fibroblasts treated with or without TGF- β 1 for 6 hours. As expected, control fibroblasts had elevated phosphorylated-Smad2 expression when treated with TGF- β 1 (Figure 5B), while *Tgfb2^{dermalKO}* fibroblasts had little phosphorylated-Smad2 activation, based on similar expression levels of total Smad2. However, β 1 integrin expression was up-regulated by TGF- β 1 in the control fibroblasts but not in the *Tgfb2^{dermalKO}* fibroblasts. The expression of basement membrane collagen type IV expression was similar in all cell extracts. The lack of integrin induction by the *Tgfb2^{dermalKO}* fibroblasts could certainly contribute to the reduced dermal contraction observed in the wounds.

To further confirm the involvement of specific integrins and collagen, cultured cells were examined by real time RT-PCR of mRNA from control and *Tgfb2^{dermalKO}* fibroblasts. Integrins are transmembrane proteins that form $\alpha\beta$ heterodimers and act as receptors for extracellular matrix proteins.³⁴ Integrins are also critical in keratinocyte adhesion and migration,³⁴ and they are the principal means of transducing forces and signals between the cytoplasm and the extracellular matrix in mesenchymal cells by linking the extracellular environment to the actin cytoskeleton via connections with adaptor proteins such as talin and filamin.³⁵ The expression of α 1 integrin, α 2 integrin, and β 1 integrin, which constitute the principal collagen receptors, was significantly decreased+ in *Tgfb2^{dermalKO}* fibroblasts compared with control by 3.5-fold ($P < 0.001$), 2.1-fold ($P < 0.001$), and 2.1-fold ($P < 0.001$), respectively (Figure 5C). COL1A1 and COL1A2 expression were reduced by 3.5-fold ($P < 0.001$) and 2.7-fold ($P < 0.001$), respectively (Figure 5D). In addition, COL3A1 mRNA expression was significantly ($P < 0.001$) reduced by 4.4-fold in *Tgfb2^{dermalKO}* fibro-

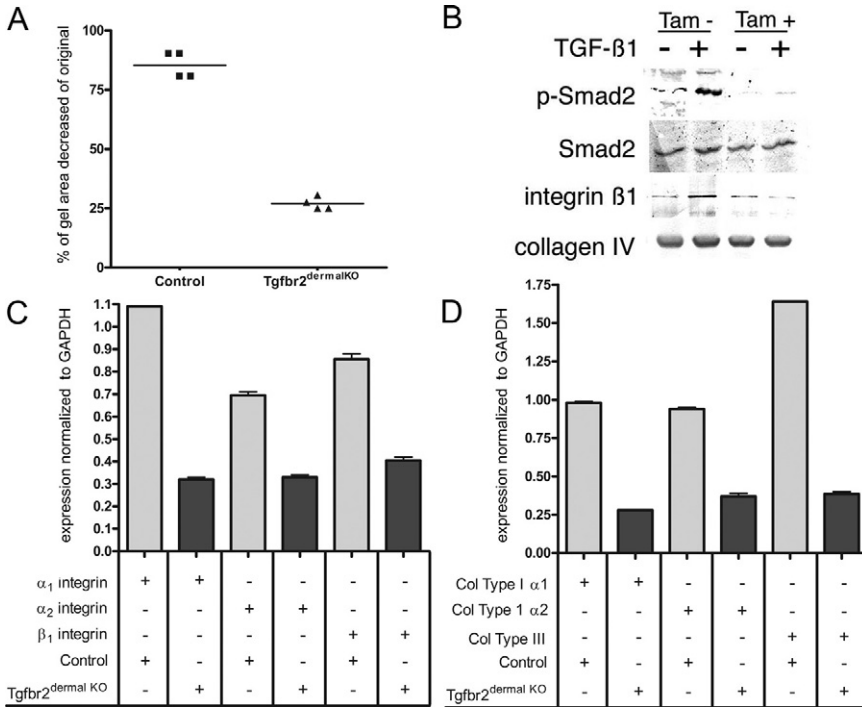


Figure 5. TGF- β signaling mediates dermal contraction. Control and Tgfb2^{dermalKO} dermal fibroblasts had significantly different *in vitro* collagen I gel contractile ability after 3 days of culture (mean indicated by line, $P < 0.0001$) (A). Cells treated with or without TGF- β for 6 hours were examined by Western blotting to determine the activation of Smad 2, expression of β 1 integrin, and collagen type IV (B). Real time RT-PCR indicated mRNA expression of α 1 integrin, α 2 integrin, and β 1 integrin (C); and collagen type I α 1, collagen type I α 2, and collagen type III (D). Control fibroblast expression (light colored bars) had generally greater integrin and collagen isoform expression compared with that of Tgfb2^{dermalKO} fibroblasts (dark bars). Error bars indicate SD ($n = 3$).

blasts compared with control (Figure 5D). Thus, the loss of dermal TGF- β responsiveness inhibited dermal contraction *in vivo* and *in vitro*. The contractile phenotype correlated with dermal collagen organization and fibroblastic expression of interstitial collagens and their integrin receptors.

Reorganization of the actin cytoskeleton is a fundamental process during wound remodeling. To observe F-actin distribution and stress fiber development, control and Tgfb2^{dermalKO} fibroblasts were grown on collagen I coated coverslips and stained with phalloidin. As expected, extensive arrays of F-actin fibers were present in control dermal fibroblasts, while F-actin distribution and development were diminished in Tgfb2^{dermalKO} fibroblasts (Figure 6). VASP is associated with filamentous actin formation, cell adhesion, and motility.⁴ Confocal microscopy revealed markedly diminished lamellipodia expression of VASP in Tgfb2^{dermalKO} fibroblasts. Together, the data emphasize the importance of dermal TGF- β responsiveness in promoting wound remodeling through the interdependent processes of extracellular matrix organization, integrin expression, and intracellular cytoskeletal organization.

Discussion

Paracrine signaling universally mediates stromal-epithelial interactions in cancer initiation, development, and wound healing. In contrast to typical mouse skin wounds, the Tgfb2^{dermalKO} excisional wounds healed by re-epithelization rather than wound contraction and granulation tissue formation. To isolate the first process, we fixed metal stents to the wound periphery. This model limited the contractile response and facilitated the study of re-

epithelization. We found that loss of TGF- β responsiveness in the wounded dermis potentiated the proliferation and migration of the adjacent keratinocytes as the wound re-epithelized. The postnatal knockout of dermal TGF- β signaling did not affect the histological organization of intact skin, but neoderms was markedly altered in syn-

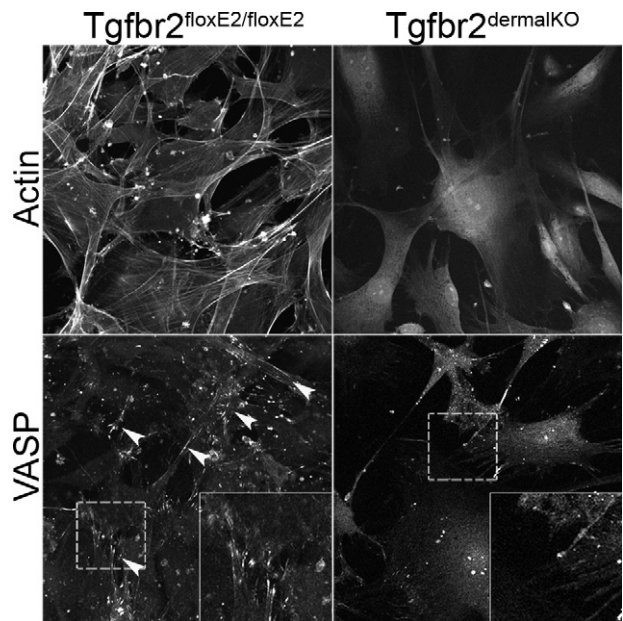


Figure 6. Confocal fluorescent microscopy localized actin and VASP in control and Tgfb2^{dermalKO} dermal fibroblasts grown on collagen-coated coverslips. Actin stress fibers were reduced in Tgfb2^{dermalKO} fibroblasts when compared with control. VASP expression was diminished in Tgfb2^{dermalKO} fibroblasts when compared with control. **Arrowheads** indicate VASP localization at lamellipodia in control fibroblasts. **Insets** reflect a higher magnification of the area in the **dashed boxes**.

thetic and contractile properties. The $Tgfb2^{dermalKO}$ mouse model revealed the paracrine role of TGF- β signaling in re-epithelization of the wound.

Adult skin wound healing involves overlapping phases of acute inflammation, rapid proliferation of epithelial and dermal cells, and the formation of a persistent scar, while embryonic and fetal skin wound healing is rapid, with minimal inflammation, and it is scarless.^{36,37} During development of mouse skin, the transition from scarless to fibrotic healing occurs after embryonic day 16. Intrinsic differences between fetal and adult fibroblasts can influence the quality of wound repair.³⁸ Scarless healing in regenerating fetal skin has been attributed, in part, to the lack of dermal TGF- β signaling.^{39,40} While this article was in review, Denton et al³⁹ reported a similar, conditional knockout of $Tgfb2$ in dermal fibroblasts, where excisional wounding resulted in a phenotype similar to the $Tgfb2^{dermalKO}$ phenotype, characterized by reduced and delayed dermal collagen organization and elevated epidermal proliferation. Changes in macrophage recruitment was not noted, but the authors suggested that myofibroblast abundance could have been due to differentiation of bone marrow-derived fibrocytes.^{39,41} Importantly, human and mouse wound healing differ in that mouse skin produces little scar tissue because dermal contraction is the predominant mechanism of wound closure. Many investigators have proposed that the acquisition of dermal TGF- β signaling later in embryogenesis anticipates scarring in adults. Indeed, induction of TGF- β in fetal dermis produces precocious scarring.⁴² We developed transgenic mice that have an inducible, conditional knockout of the TGF- β type II receptor in dermal fibroblasts in the hope of mimicking the embryonic wound healing phenotype. The $Tgfb2^{dermalKO}$ adult mice exhibited accelerated re-epithelization, reduced inflammatory cell recruitment, and deficient dermal collagen re-organization (Figures 2–4).

Wound healing is classically characterized by the transient development of granulation tissue that supports rapid proliferation, migration, and differentiation of the adjacent epithelium.⁴³ The transient reactive stroma includes vascularization of the wound, infiltration of inflammatory cells, and differentiation of the dermal fibroblasts. Vascularity (determined by von Willebrand factor VIII expression by endothelial cells) was not significantly altered in the $Tgfb2^{dermalKO}$ compared with the control wounds. However, macrophage infiltration (F4/80 expression) was nearly absent at 7 days of wounding in the $Tgfb2^{dermalKO}$ wounds relative to control wounds. The $Smad3$ transgenic knockout wound healing model, in which TGF- β signaling is attenuated, was reported to have a similar decrease in inflammatory infiltration.⁶ Although TGF- β appears to be immunosuppressive in immune cells per se, its chemotactic activity at low concentrations promote inflammatory infiltration during wound healing.^{44–46} The previously reported $SMAD3$ knockout mouse also exhibited a markedly reduced inflammatory response after skin wounding.^{47,48} The $Tgfb2^{dermalKO}$ mouse model suggests the wound-associated inflammatory response to be a result of dermal TGF- β responsiveness.

The granulation tissue is associated with the differentiation of dermal fibroblasts into myofibroblasts. This process, which involves the co-expression of vimentin and α -smooth muscle actin, is often attributed to TGF- β signaling. However, the loss of TGF- β responsiveness in the stromal fibroblasts did not seem to alter myofibroblastic differentiation of the dermis. Denton et al³⁹ recently reported that the myofibroblasts in the conditional knockout wound were also CD34-positive, suggestive of bone marrow origin. Although inflammation can be coupled with myofibroblastic differentiation,^{47–49} this association did not seem to be true in the $Tgfb2^{dermalKO}$ mice. The wounds did not recruit monocytes, yet they had myofibroblastic enrichment. It is possible that an alternative activation of connective tissue growth factor may contribute to this phenomenon.³⁹ Wound contraction is frequently associated with myofibroblastic differentiation, and it is well established that contraction is the main mechanism by which many loose-skinned animals close full-thickness wounds.⁵⁰ Contraction was not observed in $Tgfb2^{dermalKO}$ skin or in isolated $Tgfb2$ -KO dermal fibroblasts. The loss of $\beta 1$ integrin expression and lack of collagen organization are two likely mechanisms by which contraction may be uncoupled in the $Tgfb2^{dermalKO}$ skin, despite the clear myofibroblastic differentiation-expression of α -smooth muscle actin. Dermal contraction requires that (myo)fibroblasts have a means of coupling to the extracellular matrix, and proper matrix interaction is required for concerted cellular movement into and within the wound space. Matrix binding and contractility are interdependent because collagen organization in part requires cellular tension. The observations made in $Tgfb2^{dermalKO}$ skin wound healing suggest that the role for TGF- β dermal responsiveness in terms of vascularization and myofibroblastic differentiation has redundant mechanisms, possibly related to the participation of marrow-derived progenitor populations. The bone marrow contributes significantly to the formation of granulation tissue through the recruitment of fibrocytes and marrow-derived fibroblast progenitors.⁴¹ If $COL1A2$ expression was inactive in these cells at the time of tamoxifen administration, they may have escaped inactivation and thus contributed to the α -smooth muscle actin-positive myofibroblast population. If these circulating cells were TGF- β responsive, one might expect a capacity to compensate for loss of dermal fibroblast collagen production. However, dermal TGF- β responsiveness is necessary for inflammatory cell recruitment and wound contraction in the healing process.

In an attempt to establish the mechanism by which $Tgfb2^{dermalKO}$ fibroblasts may have reduced wound contraction, the expression of integrins and cytoskeletal proteins was studied. Previous studies have shown that mice lacking β_3 integrin had accelerated re-epithelization compared with wild-type mice.⁵¹ Reports from Grenache et al,⁵² Zweers et al,⁵³ and Parks⁵⁴ indicated that skin develops normally in mice deficient in the $\alpha 2$ -integrin subunit. In those mouse models, the authors found changes in other $\beta 1$ integrins that may compensate for the loss of the $\alpha 2\beta_1$ integrin. The loss of TGF- β signaling may well have prevented the activation of compensatory

adhesion receptors. In our model, the loss of Smad activation was associated with decreased expression of integrins, an alteration of F-actin distribution and decreased expression of VASP. Marked morphological alterations of actin stress fibers and VASP expression localization may be associated with reduction in contraction in Tgfr2^{dermalKO} fibroblasts (Figure 6). VASP proteins are involved in numerous processes regulated by actin,⁴ including promotion of actin filament elongation.⁵⁵ Wounding induces the activation of focal adhesion proteins and integrins.³⁵ The loss in the expression of α 1 integrin, α 2 integrin, β 1 integrin, actin stress fibers, and lamellipodia-associated VASP by Tgfr2^{dermalKO} skin fibroblasts can be the rationale for both the lack of mature collagen organization and dermal contraction.

The mechanisms whereby ablation of mesenchymal TGF- β signaling lead to enhanced epithelialization are not well understood. The authors of previous studies of the Smad3 null phenotype in skin and lens wounds had enhanced epithelialization.^{6–8} Possible mechanisms for the elevated epithelialization is increased proliferation or motility. Because TGF- β itself can regulate both mechanisms and the positive feed-back loop of TGF- β expression may be disrupted by the knockout of Tgfr2 in the dermal fibroblasts, we examined the expression of the three TGF- β isoforms as well as hepatocyte growth factor and connective tissue growth factor. Apparently, knocking out TGF- β signaling does not seem to inhibit the expression of the TGF- β 1 and TGF- β 3 ligands or the expression of hepatocyte growth factor and connective tissue growth factor (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). But, we found a significant decrease in TGF- β 2 expressed by Tgfr2 knocked out fibroblasts. The relevance of the reduction of TGF- β 2 in light of similar levels of the other two isoforms of TGF- β is not known. However, it is important to revisit our data in Figure 2D where we found that there was no significant difference in proliferation of the epithelia. So, a likely mechanism for accelerated re-epithelialization is increased motility of the keratinocytes. Together, it seems the specific role of dermal TGF- β responsiveness in wound healing involves the recruitment of macrophage, wound contraction, and epithelial motility. The mechanism for dermal contraction is likely a direct result of TGF- β signaling. However, the paracrine mechanisms re-epithelialization and macrophage recruitment remain unclear in our mouse model.

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