TAK1 Is Required for Dermal Wound Healing and Homeostasis

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Dermal connective tissue is a supportive structure required for skin's barrier function; dysregulated dermal homeostasis results in chronic wounds and fibrotic diseases. The multifunctional cytokine transforming growth factor (TGF) β promotes connective tissue deposition, repair, and fibrosis. TGF-β acts through well-defined canonical pathways; however, the non-canonical pathways through which TGF-β selectively promotes connective tissue deposition are unclear. In dermal fibroblasts, we show that inhibition of the non-canonical TGF-β-activated kinase 1 (TAK1) selectively reduced the ability of TGF-β to induce expression of a cohort of wound healing genes, such as collagens, CCN2, TGF-β1, and IL-6. Fibroblast-specific TAK1-knockout mice showed impaired cutaneous tissue repair and decreased collagen deposition, α-smooth muscle actin and CCN2 expression, proliferating cell nuclear antigen staining, and c-Jun N-terminal kinase and p38, but not Smad3, phosphorylation. TAK1-deficient fibroblasts showed reduced cell proliferation, migration, cell attachment/spreading, and contraction of a floating collagen gel matrix. TAK1-deficient mice also showed progressively reduced skin thickness and collagen deposition. Thus, TAK1 is essential for connective tissue deposition in the dermis.

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INTRODUCTION

Stromal–epithelial interactions, involving the autocrine and paracrine effects of a variety of growth factors, are essential for adult wound healing and connective tissue homeostasis (Barrientos *et al.*, 2008). Of these growth factors, the transforming growth factor-β (TGF-β) family, consisting of TGF-β1, β2, and β3 isoforms, is especially important (Gordon and Blobe, 2008). In the ''canonical'' TGF-β signaling pathway, active TGF-β binds to the TGF-β receptors type I (called activin linked kinase (ALK) 5 in fibroblasts) and II; ALK5 phosphorylates Smad2/3 that dimerizes with Smad4. The resultant complex migrates into the nucleus to activate transcription (Feng and Derynck, 2005; Leask, 2008). TGF-β is pleitropic; TGF-β suppresses proliferation in epithelial cells but stimulates proliferation and extracellular matrix (ECM) expression in mesenchymal cells (Rahimi and Leof, 2007). 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 ECM deposition and remodeling, including the differentiation of resident fibroblasts to myofibroblasts, the critical effecter cell type of wound repair and fibrosis (Leask, 2008). Failure to properly initiate tissue repair causes in chronic wounds, which often result in limb amputations, whereas excessive tissue repair causes fibrotic disease, which collectively account for 45% of the deaths in the western world (Wynn, 2008; Elliott and Hamilton, 2011; Rafehi *et al.*, 2011).

Subcutaneous injection of TGF-β1 has been shown to increase connective tissue deposition and to accelerate wound healing (Roberts *et al.*, 1986; Cromack *et al.*, 1987). However, the specific mechanism of TGF-β action on any individual cell type within tissues is still not understood. These differences are likely to occur through the activation of ''non-canonical'' signaling pathways, which are activated in parallel to the Smad pathway (Leask, 2008; Rahimi and Leof, 2007). Owing to the pleitropic nature of TGF-β, broad targeting of canonical TGF-β signaling *in vivo* to combat connective tissue disease is likely to have unintended deleterious consequences, necessitating the targeting of TGF-β in the right cell type and at the right time (Leask, 2008; Dooley and ten Dijke, 2012). In this regard, targeting non-canonical TGF-β signaling pathways is likely to be useful, as these might be expected to mediate gene- or pathway-specific effects. Thus, targeting these pathways is likely to be useful to control the ability of TGF-β to modify specific effects on cells (e.g., in the control of wound healing or fibrosis).

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Abbreviations: ALK, activin linked kinase; ECM, extracellular matrix; dcSSc, diffuse cutaneous systemic sclerosis; JNKs, c-Jun N-terminal kinases; PBS, phosphate-buffered saline; RT-PCR, real-time PCR; TAK, TGF-β-activated kinase; TGF, transforming growth factor

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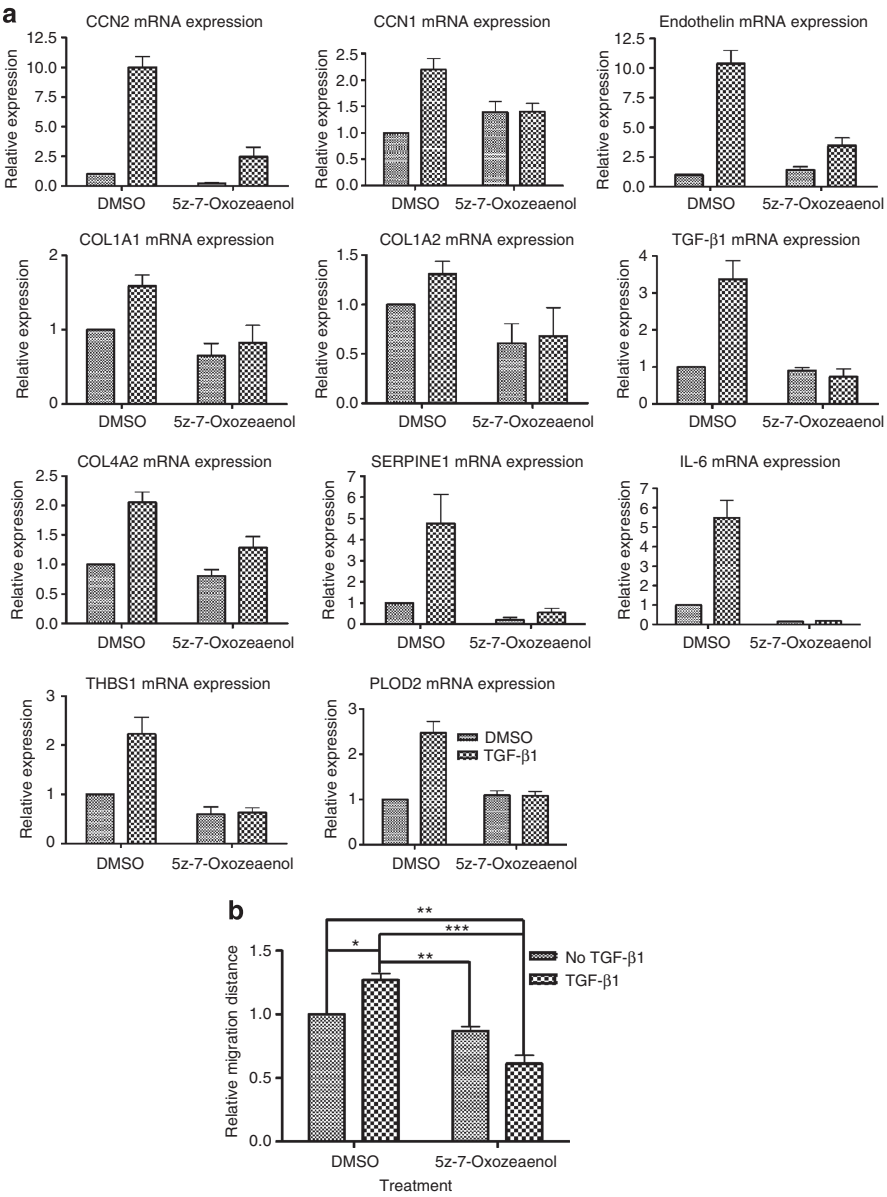

Figure 1. Transforming growth factorβ (TGF-β)–activated kinase 1 (TAK1) inhibition impairs the ability of TGF-β1to induce an *in vitro* "wound healing" phenotype. (**a**) Human dermal fibroblasts were treated with or without TGF-β1 (4 ng ml^{-1} (160 pM)) in the presence of DMSO or TAK1 inhibitor (5*Z*)-7-oxozeaenol (400 nM). Six hours post TGF-β1 addition, RNAs were harvested and subjected to real-time PCR analysis using the indicated primers. 18S RNA was used as the internal control. ($N=3$, *$P<0.05$, Student's *t*-test). (**b**) Cells were subjected to a scratch wounding assay before the addition of TGF-β1 and inhibitor. Data represent averages and SD ($N=9$, *$P<0.05$, two way analysis of variance). **$P<0.01$; ***$P<0.001$.

The non-canonical TGF-β signaling pathways essential for connective tissue function *in vivo* are largely unknown. The MAPKKK TGF-β-activated kinase (TAK)1 has been suggested to participate in the signal transduction of TGF-β receptors, and activates stress-activated kinases: p38 through MKK6 or MKK3 and c-Jun N-terminal kinases (JNKs) via MKK4 (Wang *et al.*, 2001; Yamashita *et al.*, 2008). TAK1 also activates NF-κB via Toll-like receptors and the receptors for IL-1, tumor necrosis factor-α, and TGF-β (Shuto *et al.*, 2001; Takaesu *et al.*, 2003). TAK1 downstream molecules NF-κB and JNK can have opposite effects on cell death and carcinogenesis; therefore, the role of TAK1 in the skin is unpredictable. Indeed, loss of TAK1 in the liver results in inflammation, carcinogenesis, and fibrosis (Inokuchi *et al.*, 2010), whereas loss of TAK1 in kidney results in resistance to the unilateral ureteral obstruction model of kidney fibrosis (Ma *et al.*, 2011).

TAK1-knockout mice die *in utero* (Shim *et al.*, 2005; Jadrich *et al.*, 2006). Immortalized embryonic fibroblasts (E 9.75) isolated from these mice show impaired pro-fibrotic signaling in response to TGF-β (Shi-wen *et al.*, 2009). TAK1 is constitutively phosphorylated in fibroblasts isolated from fibrotic lesional skin of patients with the autoimmune connective tissue disease diffuse cutaneous systemic sclerosis (Shi-wen *et al.*, 2009). However, whether TAK1 is

essential for the function of cutaneous connective tissue is unknown. In this report, we use a chemical TAK1 inhibitor *in vitro* and a previously unreported fibroblast-specific TAK1-knockout mice model to investigate the role that TAK1 has in dermal fibroblasts.

RESULTS

The ability of TGF-β1 to induce expression of wound healing genes is selectively impaired in dermal fibroblasts treated with TAK inhibitor

To begin to assess whether, in principle, TAK1 could contribute to TGF-β gene expression in dermal fibroblasts, we first evaluated whether a selective TAK1 inhibitor could impair the ability of TGF-β to induce gene expression in cultured human dermal fibroblasts. Cells were cultured until 80% confluence, serum-starved for 24 hours, and treated in the presence or absence of TGF-β1 (4 ng ml^{-1} (160 mM)) for an additional 6 hours in the presence or absence to TAK1 inhibitor (400 nM). Total RNA was prepared, reverse transcribed, and subjected to Affymetrix genome-wide expression profiling. Experiments were performed twice, and average induction values were obtained. TGF-β1 induced 1,049 transcripts (775 of which had Gene Symbols associated with them) >1.7 fold in DMSO. Of these, 741 transcripts (513 of which had Gene Symbols associated with them) were not induced in the presence of inhibitor. Cluster analysis using DAVID revealed that TGF-β-induced expression of wound healing and ECM genes were selectively sensitive to treatment with TAK1 inhibitor (Supplementary Table SI online). These transcripts included: TGF-β1, endothelin-1, CCN2 (cyr61), PLOD2, IL-6, and collagen type IV (COL4A2) (Supplementary Table SI online). Results were verified using real-time PCR (RT-PCR) analysis of RNA (Figure 1a). To further confirm that TGF-β-induced expression of wound healing and ECM genes were sensitive to TAK1 inhibition, RT-PCR analysis was conducted to show that TGFβ induced CCN2 (connective tissue growth factor), type I collagen (COL1A1 and COL1A2), thrombospondin (THBS), 1 and serpine 1 mRNAs (Figure 1a). TAK inhibition reduced the ability of TGF-β to induce endothelin-1, CCN2, and collagen protein (Figure 2), and the ability of TGF-β1 to induce cell migration in a scratch wound assay (Figure 1b). Collectively, these results suggest that TAK1 is required for a subset of TGF-β responses in fibroblasts, namely those involved with wound repair. These data suggest TAK1 may mediate wound repair responses *in vivo*.

Deletion of TAK1 causes delayed cutaneous wound repair

On the basis of these data, we then examined whether TAK1 was required for wound healing *in vivo*. To do this, we generated mice that were (a) hemizygous for an allele in which a tamoxifen-inducible cre recombinase is expressed under the control of a fibroblast-specific collagen type I promoter and (b) homozygous for a TAK1 allele flanked by lox P sites. Four-week-old mice were injected with corn oil or tamoxifen to generate control mice (C/C) or mice deleted for TAK1 in fibroblasts (K/K). Genotyping was confirmed by PCR (not shown). Ten days later, mice were subjected to the dermal punch model of cutaneous tissue repair. Deletion of

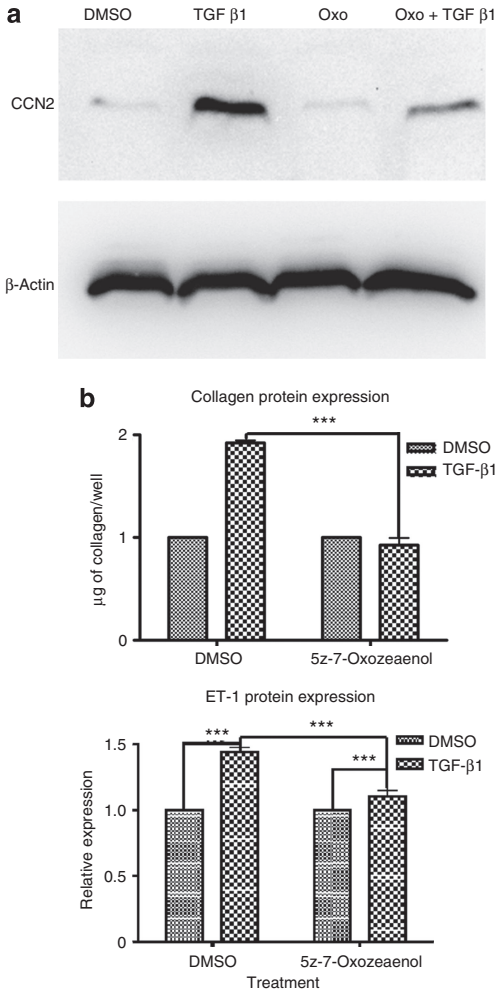

Figure 2. Transforming growth factorβ (TGF-β)–activated kinase 1 (TAK1) inhibition impairs the ability of TGF-β1 to induce CCN2, collagen, and endothelin-1 (ET-1) protein. Human dermal fibroblasts were treated with or without TGF-β1 in the presence of DMSO or TAK1 inhibitor (5*Z*)-7-oxozeaenol (Oxo). (**a**, **b**) Twenty-four hours post TGF-β1-addition, proteins were harvested and (**a**) CCN2 was detected by western blot analysis using anti-CCN2 antibody while anti-GAPDH antibody was used as a control or (**b**) collagen and ET-1 were detected by ELISA. ($N=3$, ***$P<0.001$, two way analysis of variance).

TAK1 in fibroblasts was confirmed with indirect immunofluorescence analysis of skin and isolated fibroblasts using an anti-TAK1 antibody, as well as RT-PCR and western blot analyses of isolated fibroblasts (Figure 3a) Compared with wounded control animals (C/C), TAK1-deficient (K/K) animals possessed a reduced rate of wound closure (Figure 3b). TAK1-deficient animals were examined 7 days post wounding and displayed reduced collagen production and granulation tissue (Figure 4a and b), and CCN2, α-SMA- and proliferating cell nuclear antigen (PCNA)–expressing myofibroblasts (Figure 4c; Supplementary Table SII online). Loss of TAK1 did not appreciably impair Smad3 phosphorylation, but displayed a reduction in p38 and JNK phosphorylation (Figure 4c; Supplementary Table SII online). The ability of TGF-β1 to induce Smad3 phosphorylation was not appreciably affected in fibroblasts derived from control (C/C) or knockout (K/K) mice (Supplementary Figure S1 online).

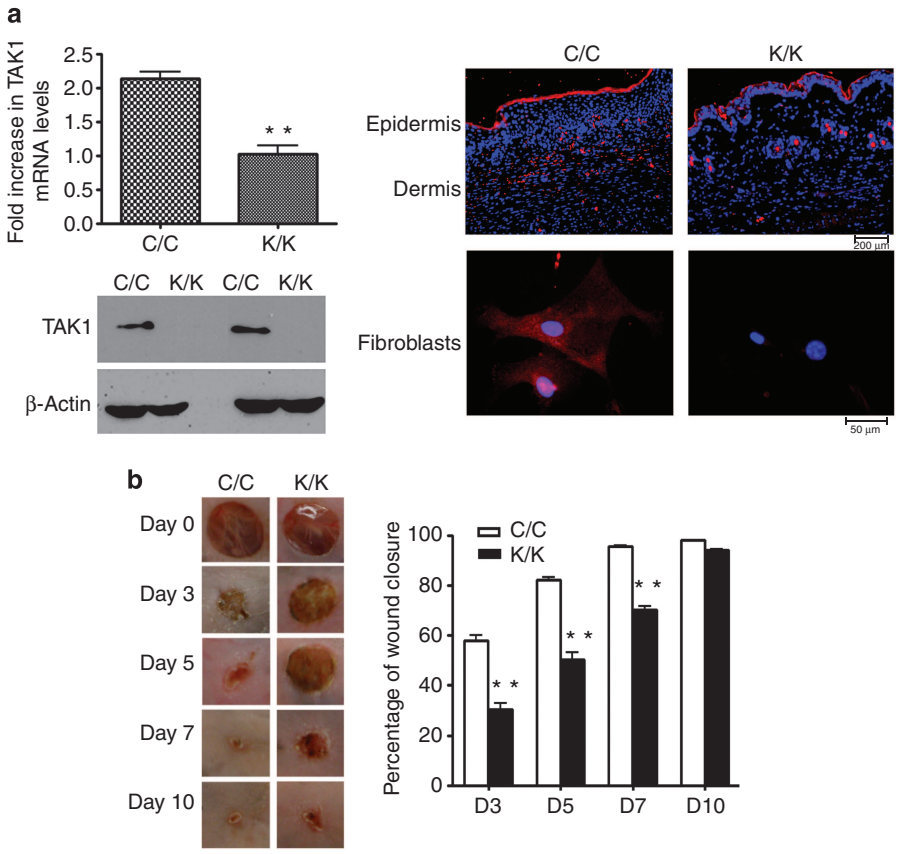

Figure 3. Conditional deletion of transforming growth factorβ (TGF-β)–activated kinase 1 (TAK1) in fibroblasts results in impaired cutaneous wound healing. Fibroblasts or mice containing the TAK1 gene or not are designated (C/C) or (K/K). (**a**) Loss of TAK1 in fibroblasts was confirmed using real-time PCR using primers detecting TAK1, and western blot and indirect immunofluorescence analyses of isolated fibroblasts with anti-TAK1 antibodies ($N=3$; $^{**}P<0.01$, Student's *t*-test) or by immunohistochemistry of skin with an anti-TAK1 antibody ($N=7$). (**b**) Mice were wounded and wound closure was measured as described in Materials and Methods. For all assays, six mice per group were analyzed. Data represent averages and SD from all these mice ($^{**}P<0.01$, Student's *t*-test).

Deletion of TAK1 causes impaired migration, adhesion, proliferation, spreading, and ECM contraction

Consistent with our *in vivo* data showing reduced PCNA staining in 7-day wounds of TAK1-deficient animals, isolated TAK1-deficient dermal fibroblasts showed reduced proliferation compared with wild-type fibroblasts (Figure 5a). Moreover, TAK1-deficient dermal fibroblasts showed a reduced ability to adhere to fibronectin (Figure 5b). Cell spreading was also monitored post-adhesion microscopically, using rhodamine phalloidin and anti-vinculin antibodies. Loss of TAK1 also resulted in impaired spreading on fibronectin, as revealed with anti-vinculin antibody (green) and rhodamine-phallodin (red) staining to detect actin (Figure 5c). Moreover, TAK1-deficient cells showed reduced vinculin, integrin β1, and αSMA protein expression (Figure 5d). TAK1-deficient fibroblasts were less able to contract floating collagen gel matrices in the presence or absence of added TGF-β(Figure 5e). Finally, TAK1-deficient fibroblasts showed less migratory ability either in the presence or absence of added TGF-β (Figure 6a and b). In our studies, we noted a significant decrease in the skin thickness of TAK1-deficient mice even 10 days post-cessation of tamoxifen injection (i.e., when we initiated our wounding experiments). To assess whether TAK1 was required for dermal homeostasis, we monitored mice until 40 days post-cessation of tamoxifen injection; the dermis became progressively thinner, concomitant with a progressive loss of collagen (Supplementary Figure S2 online). Thus, TAK1 is required for dermal homeostasis.

DISCUSSION

The pleitropic cytokine TGF-β executes essentially all its functions via the canonical Smad/ALK5 pathway; however, the importance of non-canonical TGF-β signaling pathways in mediating cell-, gene-, and pathway-selective effects is being increasingly appreciated (Leask, 2008; Rahimi and Leof, 2007; Trojanowska, 2009). TAK1 mediates the ability of TGF-β to induce JNK and p38 (Yamashita *et al.*, 2008; Shi-wen *et al.*, 2009). In this study, we show that TAK1 mediates wound healing responses to TGF-β. *In vivo,* fibroblast-specific TAK1-knockout mice show reduced rates of wound closure and decreased skin thickness.

The contribution of TAK1 to connective tissue function is controversial. Although loss of TAK1 expression resulted in resistance to the unilateral ureteral obstruction model of kidney fibrosis (Ma *et al.*, 2011), hepatocyte-specific deletion of TAK1 in mice resulted in spontaneous hepatocyte death, inflammation, fibrosis, and carcinogenesis

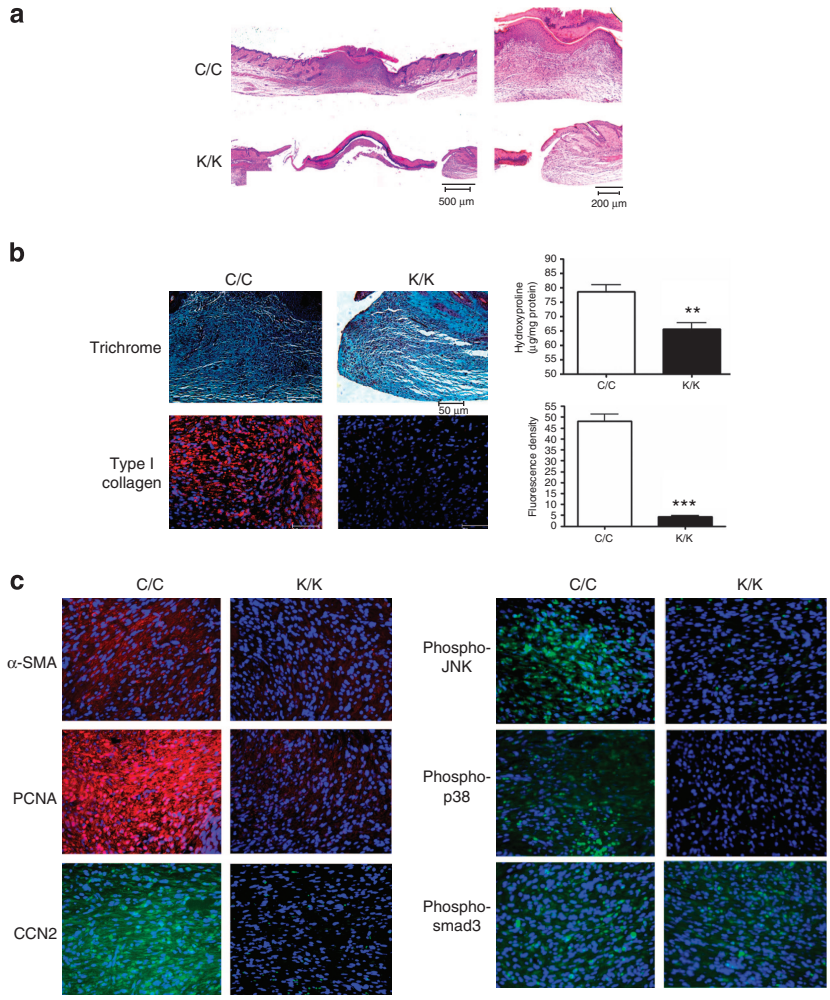

Figure 4. Conditional deletion of transforming growth factorβ (TGF-β)–activated kinase 1 (TAK1) in fibroblasts results in impaired cutaneous wound healing: histological examination. Seven days post wounding mice were examined and measured as described in Materials and Methods using (**a**) hematoxylin and eosin (H and E) analysis, (**b**) trichrome stain, anti-type I collagen antibody, and hydroxyproline levels to detect collagen. For all assays, six mice per group were analyzed. (**$P<0.01$, ***$P<0.001$, Student's *t*-test). Note that TAK1-deficient wounds had defects in wound closure as revealed by reduced granulation tissue in the wound. (**c**) Seven days post wounding mice were examined and measured as described in Materials and Methods using proliferating cell nuclear antigen (PCNA) to detect proliferation, anti-CCN2, α-SMA antibody to detect myofibroblasts, and anti-phospho-JNK, anti-phospho-p38, and anti-phospho-Smad3 antibodies. For all assays, at least four mice per group were analyzed. A representative photograph is shown. For **b** and **c** wound edges are shown.

that was partially mediated by tumor necrosis factor receptor signaling (Inokuchi *et al.*, 2010). In heart, TAK1 is activated in response to stress, and an activating mutation of TAK1 expressed in myocardium of transgenic mice was sufficient to produce p38 mitogen–activated protein kinase phosphorylation *in vivo*, cardiac hypertrophy, interstitial fibrosis, severe myocardial dysfunction, ''fetal'' gene induction, apoptosis, and early lethality (Zhang *et al.*, 2000). Our data showing that TAK1-deficient mice showed reduced dermal thickness, delayed wound closure, and impaired JNK and p38, but not Smad3, phosphorylation are consistent with this report.

TAK1-deficient fibroblasts showed reduced adhesion and spreading on collagen concomitant with reduced integrin β1 and vinculin protein expression. Integrin β1-deficient mice show reduced rates of tissue repair, yet they retain their TGF-β responsiveness. Instead activation of latent TGF-β is impaired in these cells (Liu *et al.*, 2010). Moreover, although integrin β1 mice show skin thinning and a reduction in basal collagen production (Liu and Leask, 2012), this process is much more pronounced in TAK1-deficient mice in that a skin thinning phenotype is observed earlier post-deletion of the gene. Vinculin is a cytoplasmic actin-binding protein enriched in focal adhesions that has a critical role in regulating integrin clustering and force generation, and controls cell proliferation, adhesion strength, and acts as a scaffold of cell proliferation (Carisey and Ballestrem, 2011). The effect of loss of vinculin expression in fibroblasts *in vivo* has not yet been examined. Thus, the overall phenotype of TAK1-deficient mice is likely to arise through at least two independent yet complementary mechanisms, namely an impaired TGF-β response and a reduction in integrin β1 and vinculin expression.

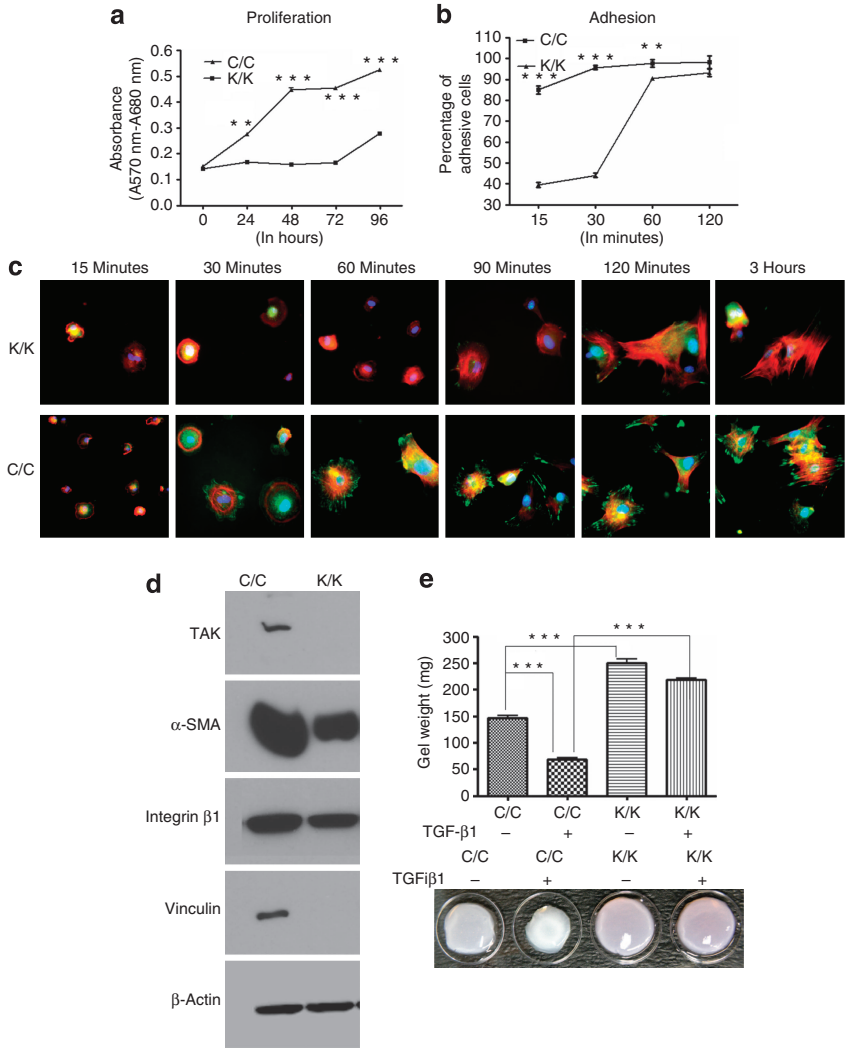

Figure 5. Loss of transforming growth factor-β (TGF-β)–activated kinase 1 (TAK1) results in a reduced ability of fibroblasts to proliferate, to adhere on extracellular matrix (ECM), and to contract a collagen gel matrix. Fibroblasts were isolated by explant culture from mice containing the TAK1 gene (C/C) or not (K/K). Cells were subjected to (**a**) a proliferation assay and (**b**) a fibronectin adhesion assay as described in Materials and Methods. (**c**) Cell spreading was monitored by plating cells on fibronectin, and fixing and staining cells with anti-vinculin antibody (to detect focal adhesions) and rhodamine phalloidin to detect actin. For all assays, fibroblasts from six mice per group were analyzed. Data represent averages and SD from all these mice. The number of focal adhesions per cell were counted (30 C/C or K/K cells per mouse line; $^{**}P<0.01$, $^{***}P<0.01$, Student's *t*- test). (**d**) Cells were subjected to western blot analysis with an anti-α-SMA, anti-integrin β1, and anti-vinculin antibodies. (**e**) The effect of loss of TAK1 expression on ECM contraction generated by fibroblasts embedded in a floating collagen gel matrix was assessed over a 24-hour period. Contraction was assessed photographically and by measuring diameter of contracted gels (fibroblasts from three separate animals were used, and experiments were performed in triplicate; average ± SD is indicated). Note that wild-type (C/C) fibroblasts were able to contract a collagen gel matrix (Student's *t*-test; $^{**}P<0.01$, $^{***}P<0.01$), relative to TAK1-deficient cells (K/K).

Collectively our data suggest that TAK1 is essential for proper cutaneous tissue repair and dermal homeostasis.

MATERIALS AND METHODS

Expression profiling

Expression profiling was performed as described in prior publications (Guo *et al.*, 2011a,b) at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). Briefly, 5.5 μg of single-stranded cDNA was synthesized from 200 ng of total RNA, end-labeled, and hybridized, for 16 hours at 45 °C, to Human Gene 1.0 ST arrays. GeneChips were scanned with the GeneChip Scanner 3000 7G and probe level (.CEL file), and the data were generated using Affymetrix Command Console v3.2.4 (Santa Clara, CA). Probes were summarized to gene level data in Partek Genomics Suite v6.6 (Partek, St Louis, MO) using the RMA algorithm. Experiments were performed twice, and fold changes and *P*-values were generated using analysis of variance in Partek. Genes that significantly changed (at least 1.7 fold change, *P*-value <0.05) in the presence or absence of inhibitor were compiled and exported into DAVID (http://david.abcc.ncifcrf.gov/) for further analysis.

Generation of TAK1 conditional knockout mice

Mice possessing a tamoxifen-dependent Cre-recombinase under the control of a fibroblast-specific regulatory sequence from the

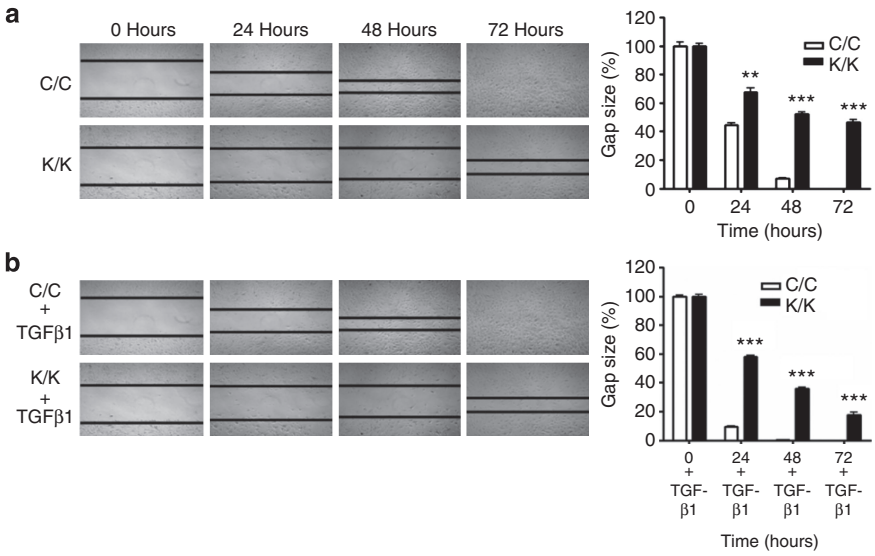

Figure 6. Loss of transforming growth factorβ (TGF-β)–activated kinase 1 (TAK1) results in a reduced ability of fibroblasts to migrate on extracellular matrix (ECM). Fibroblasts were isolated by explant culture from mice containing the TAK gene (C/C) or not (K/K). Cells were subjected to scratch wound assay of cell migration in the absence (**a**) or presence (**b**) of added TGF-β as described in Materials and Methods. Fibroblasts from six mice per group were analyzed. Data represent averages and SD from all these mice (**$P<0.01$, ***$P<0.01$, Student's *t*-test).

proα2(I) collagen gene (Zheng *et al.*, 2002) were crossed with mice homozygous for a conditional TAK1 allele (B6;129S7-*Map3k7*^{tm1Mds}/J; Jackson Laboratories; Xie *et al.*, 2006) to generate Cre/TAK1 heterozygote mice, which were mated to generate mice hemizygous for Cre and homozygous for TAK1. Animals used for experiments were genotyped by PCR (Zheng *et al.*, 2002; Xie *et al.*, 2006). To delete TAK1 in fibroblasts (K/K), 3-week-old mice were given intraperitoneal injections of tamoxifen suspension (0.1 ml of 10 mg ml^{-1} 4-hydroxitamoxifen, Sigma, St Louis, MO) over 5 days. Littermate mice of identical genotype were injected with corn oil and were used as controls (C/C). All animal protocols were approved by the appropriate regulatory authority.

Wound surgery

Littermate mice homozygous for the loxP-TAK1 allele and hemizygous for type I collagen-cre were treated with tamoxifen (''knockout TAK1'', K/K) or corn oil (''conditional TAK1'', C/C). Two weeks post-cessation of tamoxifen injection, wounding experiments were conducted essentially as previously described (Liu *et al.*, 2009). Wounds were separated by a minimum of 6 mm of uninjured skin, and photographed at 0, 2, 4, 7, and 10 days post wounding using a Sony D-9 digital camera. The wound area was determined using Northern Eclipse (Empix, Mississauga, Canada) software, and wound closure was expressed as percentage of initial wound size.

Immunohistochemistry and assessment

Sections were cut and processed as described above. Immunolabeling of α-SMA, CCN2, p-Smad3, p-p38, p-JNK, and PCNA was conducted and average fluorescence intensity percentage was calculated using image analysis software (Northern Eclipse, Empix) (Liu *et al.*, 2010). To assess the effects of TAK1 deletion on wound collagen synthesis, trichrome collagen stain was also used. The amount of collagen was calculated using a kit, as described by the manufacturer (Quickzyme, Leiden, The Netherlands).

Cell culture, immunofluorescence, and western analysis

Mouse dermal fibroblasts were isolated from explants (4- to 6-week-old animals) as described (Liu *et al.*, 2009, 2010), and cultured in DMEM and 10% fetal bovine serum (Life Technologies, Burlington, Canada). Cells were subjected to indirect immunofluorescence analysis as described (Liu *et al.*, 2009, 2010) using anti-α-SMA, rhodamine phalloidin (Sigma), and anti-vinculin (Sigma) antibodies, followed by an appropriate secondary antibody (Jackson Immunoresearch, West Grove, PA). Cells were photographed (Zeiss Axiphot B-100, Empix). Alternatively, cells were lysed in 2% SDS, proteins quantified (Fisher, Nepean, Canada), and subjected to western blot analysis as described (Liu *et al.*, 2009, 2010). Endothelin-1 and collagen levels were measured by ELISA, as described by the manufacturers (Medicorp Montreal, QC and Quickzyme, respectively). Human dermal fibroblasts were purchased (ATCC, Manassas, VA). When appropriate, cells (70% confluence) were serum-starved (DMEM, 0.5% fetal bovine serum) for 18 hours. Cells were then incubated for 45 minutes in the presence or absence of DMSO or 400 μM TAK inhibitor ((5*Z*)-7-oxozeaenol; Tocris, Bristol, UK) before incubation for 6 hours (for RNA extraction) or 24 hours (for protein analysis), indicated in the presence or absence of 4 ng ml^{-1} TGF-β1 (R and D Systems, Minneapolis, MN).

Real-time PCR

RT-PCR was performed essentially as described (Guo *et al.*, 2011a,b). Three independent experiments were conducted. Cells were cultured until 80% confluence, serum-starved for 24 hours, and total RNA was isolated (Trizol). Total RNA (25 ng) was then reverse transcribed and amplified (TaqMan Assays on Demand; Life Technologies) as described (One-step Mastermix; Life Technologies) using the ABI Prism 7900 HT sequence detector (Perkin-Elmer-Cetus, Vaudreuil, Quebec, Canada). Triplicates of each samples were run, and expression values were standardized to values obtained with control 18S RNA primers using the delta delta ct method.

Adhesion assay

Fibroblasts were isolated and cultured as described above. Adhesion assays were performed essentially as previously described (Liu *et al.*, 2009, 2010; Guo *et al.*, 2011a). Wells of 96-well plates were incubated overnight, 4 °C, with 10 μg ml^{-1} fibronectin (Sigma) in 0.5% BSA, 1X phosphate-buffered saline (PBS). Subsequently, cells were blocked for 1 hour in 10% BSA in PBS at room temperature. Fibroblasts were harvested with 2 mM EDTA in PBS (20 minutes, room temperature), washed twice with DMEM serum-free medium containing 1% BSA (Sigma), resuspended in the same medium at 2.5×10^5 cells ml^{-1}, and 100 μl of suspension was incubated in each well for the times indicated. Non-adherent cells were removed by washing with PBS. Adherent cells were quantified by incubation with 10 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution for 4 h at 37 °C, after which formazan reaction products in each well were dissolved in 100 μl of dimethyl sulfoxide and A_{570} was measured. Comparison of adhesive abilities was performed by using Student's unpaired *t*-test. A *P*-value < 0.05 was considered as statistically significant.

Collagen gel contraction

Experiments were performed essentially as described (Liu *et al.*, 2010). For a floating gel assay, 24-well tissue culture plates were pre-coated with BSA. Cells were used at passage 3. Trypsinized fibroblasts were suspended in MCDB medium (Sigma) and mixed with collagen solution (one part of 0.2 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH8.0; four parts collagen (Nutragen, Advanced Biomatrix, San Diego, CA, 3 mg ml^{-1}) and five parts of MCDB X 2) for a final concentration of 80,000 cells per ml in 1.2 mg ml^{-1} collagen. Collagen/cell suspension (1 ml) was then added to each well to polymerize. Gels were then detached from wells by adding 1 ml of MCDB medium. Gel contraction was quantified by measuring changes in diameter using image analysis software (Empix).

Migration and proliferation assays

For *in vitro* wounding (migration) experiments, fibroblasts obtained from TAK1 conditional (C/C) and knockout (K/K) mice were cultured in 12-well plates. Medium was removed, and cells rinsed with serum-free medium + 0.1% BSA and cultured for an additional 24 hours in serum-free medium + 0.1% BSA. The monolayer was artificially injured by scratching across the plate with a blue pipette tip (~1.3 mm width). Cells were washed two times to remove detached cells or cell debris, and cultured in serum-free medium in the presence of mitomycin C (10 μg ml^{-1}, Sigma) to prevent cell proliferation. After 24 and 48 h, images of the scratched areas under each condition were photographed. For the cell proliferation assay, cells were seeded in 96-well plates at 2,000 cells per well. Cell number was determined after 24, 48, and 72 h incubation using a kit (Roche, Laval, Canada).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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