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Permalink

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Journal

Journal of Investigative Dermatology, 139(4)

ISSN

0022-202X

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Publication Date

2019-04-01

DOI

10.1016/j.jid.2018.10.031

Peer reviewed



Published in final edited form as:

J Invest Dermatol. 2019 April ; 139(4): 940–950. doi:10.1016/j.jid.2018.10.031.

Smad7 ameliorates TGF β -mediated skin inflammation and associated wound healing defects but not the susceptibility to experimental skin carcinogenesis

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Abstract

We assessed roles of Smad7 in skin inflammation and wound healing using genetic and pharmacological approaches. In K5.TGF β 1/K5.Smad7 bigenic (double transgenic) mice, Smad7 transgene expression reversed TGF β 1 transgene-induced inflammation, fibrosis and subsequent epidermal hyperplasia, and molecularly abolished TGF β and NF- κ B activation. Next, we produced recombinant human Smad7 protein with a Tat-tag (Tat-Smad7) that rapidly enters cells. Subcutaneous injection of Tat-Smad7 attenuated infiltration of F4/80⁺ and CD11b⁺ leukocytes and α SMA⁺ fibroblasts prior to attenuating epidermal hyperplasia in K5.TGF β 1 skin. Further,

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Conflict of Interest

XJW is an inventor of the patent filed by the University of Colorado for Tat-Smad7 biologic and a scientific founder of Allander Biotechnologies, LLC, which has commercial interests in developing Smad7-based therapies. LB, DDW and CDY currently hold part-time employment with Allander Biotechnologies.

topically applied Tat-Smad7 on K5.TGF β 1 skin wounds accelerated wound closure with improved re-epithelialization and reductions in inflammation and fibrotic response. A short treatment with Tat-Smad7 was also sufficient to reduce TGF β and NF- κ B signaling in K5.TGF β 1 skin and wounds. Relevant to clinic, we found that human diabetic wounds had elevated TGF β and NF- κ B signaling compared to normal skin. To assess the oncogenic risk of a potential Smad7-based therapy, we exposed K5.Smad7 skin to chemical carcinogenesis and found reduced myeloid leukocyte infiltration in tumors but not accelerated carcinogenesis compared to wildtype littermates. Our study suggests the feasibility of using exogenous Smad7 below an oncogenic level to alleviate skin inflammation and wound healing defects associated with excessive activation of TGF β and NF- κ B.

Introduction

Transforming growth factor β 1 (TGF β 1) is a growth inhibitor for keratinocytes and a growth factor for fibroblasts; it has complex functions regulating the immune response (Morikawa et al., 2016). Increased TGF β 1 correlates with disease severity in psoriasis patients (Flisiak, 2002). In wound healing, except for the pro-fibrotic effect of TGF β 1, other roles of TGF β 1 are controversial (Amendt et al., 2002, Ashcroft et al., 1999, Gilbert et al., 2016, Jude et al., 2002, Ramirez et al., 2014, Reynolds et al., 2008). To assess pathological effects of constitutive TGF β 1 expression in the epidermis, we developed K5.TGF β 1 mice expressing human TGF β 1 directed by the keratin 5 promoter (Li et al., 2004). These mice developed severe skin inflammation with molecular signatures found in human psoriasis (Swindell et al., 2011). Severe inflammation in K5.TGF β 1 mice compromises wound healing (Wang et al., 2006). Once TGF β 1 initiates inflammation, NF- κ B, another potent inflammation activator, is subsequently activated (Li et al., 2004). It is particularly challenging to treat wounds when TGF β - and NF- κ B-induced inflammation becomes chronic. During wound healing, a transient inflammation stage cleanses the wound bed, followed by cascading stages of re-epithelialization, tissue regeneration and wound remodeling (Eming et al., 2014, Singer and Clark, 1999). When inflammation lingers, TGF β -mediated fibrotic response hinders wound remodeling.

A potential strategy to decrease both TGF β and NF- κ B utilizes the nuclear protein Smad7 that attenuates both TGF β and NF- κ B activation (Han et al., 2013, Hong et al., 2007). In contrast, another study reports that Smad7 is overexpressed in human psoriasis and contributes to hyperproliferation of psoriatic epidermis (Di Fusco et al., 2017). This study raises the question of whether Smad7 functions differently in a chronically inflamed milieu. With respect to cutaneous wound healing, elevated endogenous Smad7 is implied to contribute to delayed re-epithelialization in integrin α 3 β 1 knockout mouse wounds (Reynolds et al., 2008). In contrast, we have shown that Smad7 transgene expression in the epidermis promotes wound healing (Han et al., 2011). These studies raise questions about how Smad7 functions differently in inflammatory conditions vs. non-inflammatory conditions, or in conditions with excessive vs. reduced TGF β signaling. Our study used genetic models and a pharmacological test to address these questions. Further, because Smad7-mediated keratinocyte proliferation and migration promoted wound healing in our model, we evaluated potential oncogenic effects of Smad7 in the skin.

Results

K5.Smad7 transgene reversed phenotypes of K5.TGF β 1 mice

To assess the role of Smad7 in TGF β 1-mediated skin inflammation, we bred K5.Smad7 transgenic mice that express Smad7 at levels ~ the double amount of endogenous Smad7 (Han et al., 2006), with K5.TGF β 1 mice that have full penetrance skin inflammation (Li et al., 2004). Both K5.TGF β 1 and K5.Smad7 mouse lines were generated in either C57BL/6 or ICR strains without strain phenotypic differences (Han et al., 2006, He et al., 2002, Li et al., 2004). K5.TGF β 1 mice must be bred at 8-12 weeks of age before skin inflammation becomes too severe. In total, we generated nine K5.TGF β 1/K5.Smad7 bigenic mice from multiple litters. Macroscopically, K5.TGF β 1/K5.Smad7 mice had sparse hair, similar to K5.Smad7 mice but they strikingly lacked skin inflammation seen in their K5.TGF β 1 littermates (Fig. 1a), the latter progressively worsen and die of inflammation-associated wasting syndrome around six months of age. Microscopically, K5.TGF β 1/K5.Smad7 skin showed mild epidermal hyperplasia, hypotrophic hair follicles and hypertrophic sebaceous glands similar to K5.Smad7 skin (Fig. 1b). However, marked leukocyte infiltration in the dermis and subsequent epidermal hyperplasia seen in K5.TGF β 1 skin was absent in K5.TGF β 1/K5.Smad7 skin (Fig. 1b). *In situ* hybridization using a probe for human TGF β 1 transgene detected this transcript in K5.TGF β 1/K5.Smad7 epidermis and hair follicles comparable to that in K5.TGF β 1 skin (Fig. 1c), indicating that reversal of the inflammatory skin phenotype in K5.TGF β 1/K5.Smad7 skin is not due to reduced TGF β 1 expression. *In situ* hybridization with mouse Smad7 probe (for transgene and endogenous Smad7) shows that the Smad7 transcript intensity was higher in K5.TGF β 1 skin compared to wildtype (WT) skin (Fig. 1c), consistent with endogenous Smad7 being a transcriptional target of TGF β 1 (Nakao et al., 1997). Immunostaining showed that K5.TGF β 1/K5.Smad7 skin had CD45⁺ leukocyte numbers comparable to those in WT littermate skin (Fig. 1d, 1e). Nuclear phosphorylated Smad2 (pSmad2), a TGF β activation marker, and nuclear NF- κ Bp50, a NF- κ B activation marker were also reduced in K5.TGF β 1/K5.Smad7 epidermis and dermis (Fig. 1d, 1e). *In situ* hybridization for type-IA1 collagen (COL1A1), a fibroblast activation marker (Han et al., 2011), showed extensive staining in K5.TGF β 1 skin but was reversed in K5.TGF β 1/K5.Smad7 skin (Fig. 1d).

Subcutaneous Tat-Smad7 injections to K5.TGF β 1 skin reduced inflammation and subsequently alleviated other pathological characteristics

To determine if Smad7 can be utilized therapeutically, we produced a Tat-Smad7 fusion protein that rapidly enters into keratinocytes (Han et al., 2013). We s.c. injected Tat-Smad7, 1 μ g in 30 μ L phosphate buffered saline (PBS)/mouse, 3 times/wk, into the skin of K5.TGF β 1 mice. This regimen was based on the long half-life of Tat-Smad7 and the effective dose used in oral mucositis studies (Han et al., 2013). Because untreated K5.TGF β 1 skin progressively deteriorates, after initial confirmation that vehicle (PBS) did not alleviate the phenotype (not shown), we compared skin biopsies before and after treatment. Among K5.TGF β 1 mice treated with Tat-Smad7, thickened and inflamed skin gradually improved macroscopically beginning 2-3 wks after treatment and was grossly obvious by 4wks (Fig. 2a). Microscopically, Tat-Smad7-treated skin showed reductions in leukocyte infiltration and epidermal hyperplasia (Fig. 2b). Immunofluorescence staining with a V5 antibody

(recognizing the c-terminal V5 epitope of Tat-Smad7) showed Tat-Smad7 protein accumulated in the epidermis and dermis (Fig. 2c). Consistent with decreased leukocyte infiltration seen in H&E sections of Tat-Smad7 treated skin, CD45 staining also showed reduced leukocyte numbers (Fig. 2c). Additionally, Tat-Smad7 treatment reduced α -smooth muscle actin (α SMA), a marker of activated fibroblasts (Fig. 2c). To identify early Tat-Smad7 effects, we examined K5.TGF β 1 skin 6 days after Tat-Smad7 treatment when gross skin thickness and epidermal hyperplasia had not obviously subsided. Tat-Smad7 treatment reduced nuclear pSmad2⁺ and NF- κ Bp50⁺ keratinocytes (Fig. 2d). Among the major leukocyte subtypes found in K5.TGF β 1 skin (Li et al., 2004), the most abundant leukocytes in K5.TGF β 1 dermis were CD11b⁺ (non-overlapping with Ly6G⁺) myeloid cells and F4/80⁺ macrophages; Ly6G⁺ cells were mainly in microabscesses indicating they were neutrophils (Fig. 2d). CD3⁺ T lymphocytes, primarily Th1 CD4 cells and CD8 cells (Li et al., 2004), were much fewer than CD11b⁺ and F4/80⁺ cells but were the main infiltrated leukocytes in the epidermis and hair follicles or surrounding hair follicles (Fig. 2d). The most obvious reductions by Tat-Smad7 treatment were Ly6G⁺ and CD11b⁺ leukocytes (Fig. 2d). Tat-Smad7 treated skin also had moderate reductions in F4/80⁺ macrophages (Fig. 2d). In contrast, Tat-Smad7 treatment did not reduce T cells at this time point (Fig. 2d). Reductions of all these cells occurred at later time points after Tat-Smad7 treatment (not shown) when epidermal hyperplasia was also alleviated (Fig. 2b, 2c). Further, Tat-Smad7 treatment reduced α SMA fibroblasts (Fig. 2d). Therefore, targeting TGF β /NF- κ B-mediated inflammation and fibrotic response appeared to be an early effect of Tat-Smad7.

Topical Tat-Smad7 application alleviated defects of K5.TGF β 1 wound closure and re-epithelialization

Severe inflammation in K5.TGF β 1 skin often induces self-inflicted recalcitrant skin wounds, thus we explored if Tat-Smad7 could treat K5.TGF β 1 wounds. We first tested Tat-Smad7 transduction on open wounds by topically applying 0.5 μ g of Tat-Smad7 in 10 μ L PBS to 6mm diameter excisional wounds; wounds were excised 48h after treatment for analysis. We detected Tat-Smad7 in the wounded epidermis that had reduced pSmad2 and nuclear NF- κ Bp50 compared to PBS treated control wounds (Fig. 3a). Next, we introduced four excisional wounds/mouse by 6mm punch biopsies in the skin of 8-10 wks old K5.TGF β 1 mice, and treated these wounds with Tat-Smad7 or PBS (9 mice/group). Wound closure in WT mice with identical wounds treated with PBS (n=7) was used for comparison. Although K5.TGF β 1 skin had numerous α SMA⁺ fibroblasts (Fig. 2c), wounds did not show obvious contraction as seen in WT mouse skin (Fig. 3b). This is possibly due to severe inflammation and subsequent epidermal hyperplasia and fibrosis. We topically applied 0.5 μ g of Tat-Smad7 in 10 μ L PBS to each wound every other day. Macroscopically, K5.TGF β 1 wounds treated with PBS were not healed by day 15 (Fig. 3b, 3c). In contrast, wound closure in K5.TGF β 1 wounds treated with Tat-Smad7 was completed by day 15 post wounding (Fig. 3b, 3c). Histological analysis showed that K5.TGF β 1 wounds had markedly delayed re-epithelialization compared to WT wounds (Fig. 3e). Tat-Smad7 treatment increased re-epithelialization of K5.TGF β 1 wounds, noticeable on day 5 after wounding through day 11, before re-epithelialization completed (Fig. 3d, 3e). Differences in epidermal migration between PBS and Tat-Smad7 treated wounds were more obvious by double fluorescent immunostaining of α SMA and keratin K14 on day 8 wounds (Fig. 3f). Cells positive for

α SMA (vessel walls or myofibroblasts) were primarily in the granulation tissue of WT and Tat-Smad7 treated K5.TGF β 1 wounds, but extended to dermis adjacent to granulation tissue in K5.TGF β 1 wounds (Fig. 3f). Much longer migrating epithelial tongues (K14⁺) were seen in Tat-Smad7 treated K5.TGF β 1 wounds compared to PBS treated K5.TGF β 1 wounds (Fig. 3f).

Topical Tat-Smad7 application to K5.TGF β 1 wounds alleviated defects in proliferation of wounded epidermis and ameliorated excessive inflammation associated with activation of TGF β and NF- κ B.

To determine if the epidermal proliferation status in K5.TGF β 1 wounds affected wound closure, we performed BrdU labeling and staining for proliferative cells in wounded epidermis. Although K5.TGF β 1 epidermis is hyperplastic, there were few BrdU⁺ cells in the wound area epidermis (Fig. 4a). Tat-Smad7 treatment increased epidermal BrdU⁺ cells (Fig. 4a). Consistent with reduced inflammatory cell numbers in H&E sections of Tat-Smad7 treated wounds, immunostaining showed significant reduction of CD45⁺ leukocytes (Fig. 4b). We further examined CD45⁺ subtypes affected by short-term Tat-Smad7 treatment shown in Fig. 2d. Reductions in F4/80⁺ macrophages and CD11b⁺ myeloid cells were most obvious in Tat-Smad7 treated wounds (Fig. 4c). Ly6G⁺ neutrophils, present in wound stroma albeit much fewer than in wound abscesses, were also reduced by Tat-Smad7 in wound stroma (Fig. 4c).

To determine if Tat-Smad7 can attenuate excessive TGF β and NF- κ B signaling in K5.TGF β 1 skin wounds, we examined nuclear pSmad2⁺ and NF- κ B p50⁺ cells in K5.TGF β 1 wounds treated with PBS or Tat-Smad7. Tat-Smad7 treatment reduced these two markers in wounded epidermis and dermis (Fig. 5a- 5c). To explore if our model is applicable to humans, we examined diabetic wound skin samples for TGF β and NF- κ B activation. A previous study did not find elevated TGF β 1 in diabetic wounds (Jude et al., 2002), possibly because inflammatory cells can both produce and degrade TGF β 1 and activated TGF β 1 protein has a very short half-life (Wakefield et al., 1990). We therefore examined skin at the edge of diabetic wounds that has low-grade inflammation typically seen in diabetic wounds (Maione et al., 2016) without acute infection (n=5). Normal skin samples (n=5) were used for comparison. Among diabetic wound samples, CD45⁺ cell numbers and intensity of TGF β 1 immunostaining varied, but overall were increased compared to normal skin (Fig. 5d, 5e). Nuclear staining of pSmad2 and NF- κ B was consistently increased in diabetic wound skin compared to normal skin (Fig. 5d, 5f, 5g).

Chemical carcinogenesis-induced skin tumors in K5.Smad7 mice were more differentiated and less inflammatory than tumors in WT littermates

To assess if long-term Smad7 overexpression poses an oncogenic risk, we exposed K5.Smad7 and their WT littermates (both genders) to skin chemical carcinogenesis. K5.Smad7 mice developed tumors with kinetics similar to WT littermates (Fig. 6a). Among tumors collected at the completion of the study (week 22), most were benign papillomas with a few SCCs (Fig. 6b, 6c); no significant differences in tumor cell proliferation and apoptosis were found between K5.Smad7 and WT tumors (not shown). K5.Smad7 papillomas appeared to be more differentiated than papillomas in WT mice (Fig. 6b, 6c).

Further, K5.Smad7 papillomas retained more membrane-associated E-cadherin than WT papillomas (Fig. 6c). To determine if Smad7 transgene expression is sufficient to affect elevated TGF β and NF- κ B signaling during skin carcinogenesis, we examined pSmad2 and NF- κ Bp50 staining patterns in these tumors and found that K5.Smad7 papillomas had less nuclear pSmad2 and NF- κ Bp50 than in WT papillomas (Fig. 6c). Consistent with reduced TGF β and NF- κ B activation, major leukocyte subtypes infiltrated into papillomas, i.e., F4/80⁺, CD11b⁺ and Ly6G⁺ cells were markedly fewer in K5.Smad7 papillomas than WT papillomas (Fig. 6d).

Discussion

Smad7 attenuates TGF β 1-initiated inflammation, fibrotic response, and subsequent epidermal hyperplasia

The anti-inflammatory effect of Smad7 is context-specific. Smad7 overproduction and blockage of TGF β signaling in gut mucosa causes inflammatory bowel disease (Monteleone et al., 2015). Conversely, TGF β 1-induced inflammation is unique to stratified epithelial tissues (Han et al., 2013, Li et al., 2004, Lu et al., 2004). Because both TGF β 1 and Smad7 transgenes are expressed during embryonic development before the immune system is developed, a lack of inflammation in K5.TGF β 1/K5.Smad7 skin represents a preventive effect of Smad7. In contrast, Tat-Smad7 treatment in K5.TGF β 1 skin revealed its therapeutic effects. Because Tat-Smad7 penetrated all skin cell types, the cell population targeted was broader than just keratinocytes in K5.TGF β 1/K5.Smad7 skin. The anti-inflammatory effect of Tat-Smad7 occurred sooner (6 days) than the obvious gross improvement (two weeks), coinciding with reduced nuclear pSmad2 and NF- κ B p50. This could explain why short-term Tat-Smad7 treatment reduced the number of infiltrated myeloid cells but not T cells, as TGF β is a potent chemotactic factor for F4/80⁺ and CD11b⁺ leukocytes (Morikawa et al., 2016), and NF- κ B is the most effective pathway to induce inflammation. In this context, infiltrated T cells are likely to be the consequence of myeloid cell infiltration; hence reductions in T cell infiltration required a longer Tat-Smad7 treatment. Additionally, Tat-Smad7 rapidly reduced α SMA (Fig. 2d, Fig. 3f), a marker for fibroblast activation and a direct TGF β transcriptional target (Hu et al., 2003), suggesting that reduced fibroblast activation is both a direct anti-TGF β effect of Tat-Smad7 and the of reduced inflammation at later time points.

Our most unexpected result is attenuation of epidermal hyperplasia by Smad7 in K5.TGF β 1 skin. Because Smad7 is a direct TGF β transcriptional target, it is unsurprising that Smad7 is overexpressed in psoriasis (Di Fusco et al., 2017) when TGF β is activated (Flisiak, 2002). The critical question is whether elevated Smad7 mediates negative feedback or pathogenic processes during psoriasis. Because the reversal of epidermal hyperplasia by Tat-Smad7 occurred after its reduction of inflammation and fibroblast activation, inflammation and fibroblast activation appear to be the major cause of epidermal hyperproliferation attenuated by Smad7 in K5.TGF β 1 skin.

Therapeutic effects of Tat-Smad7 on inflammation-associated chronic wounds

We have shown that K5.TGF β 1 skin expresses TGF β 1 at levels comparable to the peak level of endogenous TGF β 1 during wound healing (Wang et al., 2006). However, unlike transiently surged endogenous TGF β 1, constitutively high levels of TGF β 1 cause unresolved chronic inflammation and refractory wounds. This is supported by our finding that human diabetic wounds with low-grade inflammation also had elevated TGF β 1, nuclear pSmad2 and NF- κ Bp50. Although K5.TGF β 1 epidermis is hyperplastic, K5.TGF β 1 keratinocytes proliferated poorly in wounded epidermis. Additionally, although TGF β 1 has been reported to promote keratinocyte migration (Reynolds et al., 2008, Zambruno et al., 1995), TGF β 1 overexpression in an inflammatory microenvironment inhibits keratinocyte migration (Han et al., 2013). Therefore, anti-TGF β effects of Smad7 on keratinocyte proliferation and migration contributed to more rapid wound closure. Continued Tat-Smad7 treatment was necessary for complete healing through reducing excessive inflammation in K5.TGF β 1 wounds.

Potential for developing Smad7-based biologic that avoids oncogenic effects

To develop a Smad7-based therapy for inflammatory skin conditions and inflammation-associated wound healing defects, we would need to consider its potential oncogenic effects. K5.Smad7 mice expressed Smad7 about double amount of endogenous Smad7 in WT mice (Han et al., 2006), but this modest increase in Smad7 is sufficient to abolish TGF β 1-mediated skin inflammation without inducing massive epidermal proliferation [Fig. 1, (Han et al., 2006)]. With this Smad7 expression level, we did not observe increased susceptibility to chemically induced skin tumors. Our findings are contrary to a recent report showing increased tumor formation in K5.Smad7 mice (Ha Thi et al., 2018). The contradictory results could be due to differences in Smad7 transgene expression levels, strains, and mouse housing environments. Several findings from this study could explain the lack of oncogenic effects of Smad7. In tumor epithelia, the Smad7 expression level was insufficient to increase tumor cell proliferation or decrease apoptosis, but did attenuate the partial loss of E-cadherin that is normally repressed by several TGF β transcriptional targets (Hoot et al., 2008). In tumor stroma, K5.Smad7 tumors exhibited reductions in inflammation. Because the Smad7 transgene is only expressed in keratinocytes, Smad7-targeted chemokines to recruit leukocytes must be secreted from tumor epithelial cells.

In summary, our current study identified Smad7 effects that alleviated inflammatory skin conditions and promoted skin wound healing in a chronic inflammatory milieu. Our data suggest that a Smad7-based biologic may be designed with effective therapeutic doses without triggering potential oncogenic effects. Future studies will identify Smad7 molecular targets in the epidermis and stroma.

Materials and Methods

Reagents and antibodies

Antibodies used in this study: guinea pig anti-K14 (RDI-Fitzgerald, Acton, MA), rat anti-CD45, mouse anti-BrdU, (BD Bioscience, San Jose, CA), mouse anti-V5 (Invitrogen, Grand Island, NY), rabbit anti-pSmad2 and rabbit anti-mouse F4/80 (Cell Signaling Technology,

Danvers, MA), mouse anti- α SMA (Sigma-Aldrich, St. Louis, MO) and rabbit anti-NF- κ Bp50 (Santa Cruz Biotechnology, Dallas TX), rat anti-mouse Ly6G (Biolegend, San Diego, CA), mouse anti-E-cadherin (Biosciences, San Jose, CA), rabbit anti-mouse CD3, (Dako, Carpinteria, CA), rat anti-mouse CD11b (Novus, Littleton, CO). Immunofluorescence used Alexa Fluor 594 (red) or 488 (green) conjugated secondary antibodies (Invitrogen, Carlsbad, CA).

Human specimens

Human skin and wound samples were from patients with written and informed consent under the protocol approved by Yueyang Hospital ethics committee. Diabetic foot ulcer samples from non-healing edges were from either surgical debridement procedures (otherwise discarded) or diagnosis for ruling out malignancy. We obtained normal skin samples from excess skin during cosmetic surgeries. We fixed samples in 10% formalin immediately after excision and processed for paraffin embedding.

Animals

The Institutional Animal Care and Use Committee approved our animal experiments. We bred previously generated K5.TGF β 1 and K5.Smad7 mice (He et al., 2002, Li et al., 2004) to generate K5.TGF β 1/K5.Smad7 bigenic mice and housed them in a specific-pathogen-free facility.

Generation and application of Tat-Smad7 protein

We produced Tat-Smad7 protein in *E. coli* as previously reported (Han et al., 2013, Luo et al., 2018). To treat K5.TGF β 1 skin inflammation, we made a 6 mm punch biopsy from lesional skin before Tat-Smad7 injection. We s.c. injected Tat-Smad7 (1 μ g/30 μ L PBS) close to biopsy three times per week for up to 4 weeks followed by a second biopsy near the first biopsy area. In wound healing experiments, under sterile conditions with anesthesia, four full-thickness excisional wounds were generated on dorsal skin of 8-10 week old mice (both genders) using a 6mm-diameter dermal punch. We topically applied Tat-Smad7, 0.5 μ g/10 μ L PBS to each wound every other day until day 8; allowed treatment solution to dry before mice returned to their cages. After day 10 when wounds were completely covered by scabs, Tat-Smad7 was topically applied to the gap between the scab and wound periphery to avoid the barrier of the hard scab. We used PBS (10 μ L/wound) as a control. We evaluated wound healing by calculating both wound area and histological wound width from wound midline under microscopy.

In situ hybridization

In situ hybridizations on paraffin-embedded tissue sections were performed using digoxigenin-11-deoxyuridine Triphosphate (dUTP) labeled antisense probes for human TGF-beta1 (corresponding to first 500bp human TGF-beta1 mRNA, Li et al., 2005), mouse endogenous or transgenic Smad7 (Han et al., 2006) and mouse COL1A1 (Lakos G, et al., 2004) as previously described. We used sense probes in the same region as negative controls.

Histology analysis and immunofluorescence/immunohistochemical staining

We used an 8mm diameter dermal punch to collect the entire wound including an equal length of wound edge, fixed the wound in formalin and embedded it in paraffin. We cut serial sections from wound midline and stained them with H&E. We used the largest cross section of each wound to measure the wound width. For *in vivo* BrdU labeling, we injected BrdU (125mg/kg, i.p. in 0.9% NaCl) to animals one hour before euthanasia. We performed and quantified immunofluorescent and immunohistochemical staining on frozen or paraffin-fixed samples as previously described (Han et al., 2013, Li et al., 2004, Luo et al., 2018). We counted positive cells in four to five images and averaged them from each wound. We quantified CD45⁺ cells as positive cells/mm² stromal area directly under migrating epithelium including the area closest to the edge of the wound, and pSmad2 and NF- κ Bp50 as percentage of positive cells in the epidermis.

Chemical skin carcinogenesis in K5.Smad7 mice

We applied 50 μ g DMBA (7,12-Dimethylbenz(a)anthracene) in acetone to the shaved back skin of 8-week-old K5.Smad7 mice and their wildtype littermates. One week after, we topically treated mice with 5 μ g TPA (12-O-Tetradecanoylphorbol-13-acetate) in 100 μ l acetone weekly for 20 weeks. We monitored skin tumor development and growth weekly, and collected tumors 22 weeks after DMBA initiation.

Statistical Analysis

Statistical differences were analyzed using two-tailed Student's T-test, except data in Fig. 3 c, in which p-value was determined by repeated measures one way ANOVA with Tukey's multiple comparisons test from day 3 to day 15, and data in Fig. 6b, which were analyzed by Fisher's exact test.

ACKNOWLEDGEMENTS

This work was supported by NIH grants R01AR61792 to XJW and R44 DE028718 to CDY and XJW. FL was sponsored by grant (No. 81673866) from the National Science Foundation of China (NSFC) and the "Dawn" Program of Shanghai Education Commission (17SG41), and is currently supported by Shanghai Science and Technology Committee (No. 16411955000). ZJ was a visiting scholar supported by the NSFC (No. 81402599). GH is currently supported by the NSFC grant (No. 81472903). We thank Miss Min-Feng Wu for assistance and Ms. Pamela Garl for proof reading of this manuscript.

Abbreviation used:

αSMA:	α -smooth muscle actin
BrdU:	bromodeoxyuridine
DMBA:	7,12-Dimethylbenz(a)anthracene
H&E:	Hematoxylin and eosin stain
PBS:	phosphate buffered saline
Smad7:	Mothers against decapentaplegic homolog 7
TGFβ:	transforming growth factor β

TPA: 12-O-Tetradecanoylphorbol-13-acetate

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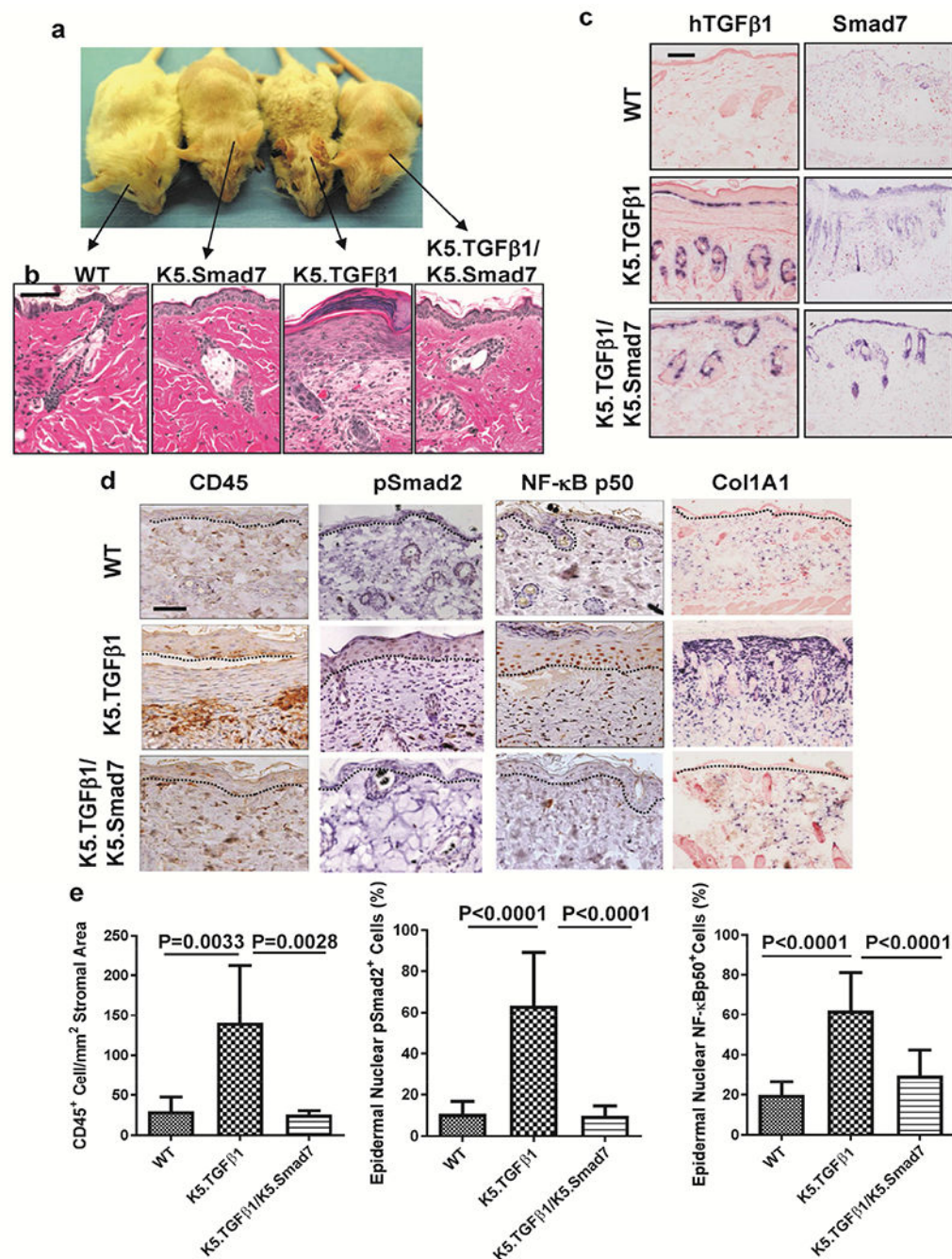


Figure 1. K5.Smad7 transgene expression reversed skin phenotypes in K5.TGFβ1 mice. (a) Gross appearance of transgenic littermates. (b) H&E skin sections from mice in (a). Arrows connect each mouse to its corresponding H&E section. Scale bar = 50μm for all sections. (c) *In situ* hybridization of human TGFβ1 (hTGFβ1) and mouse endogenous and transgenic Smad7 (purple). Fast red: counterstain. Scale bar = 100μm for all panels. (d) Immunostaining for CD45⁺, nuclear pSmad2 and NF-κB p50 cells, and *in situ* hybridization for Col1A1 (fast red: counterstain). Scale bar = 50μm for CD45, pSmad2 and NF-κB

staining, and 100µm for ColA1. Dotted lines: epidermal-dermal boundary. (e) Quantification of CD45, pSmad2 and NF-κB p50 cells (Mean±SD). Each group contains 3 to 6 samples.

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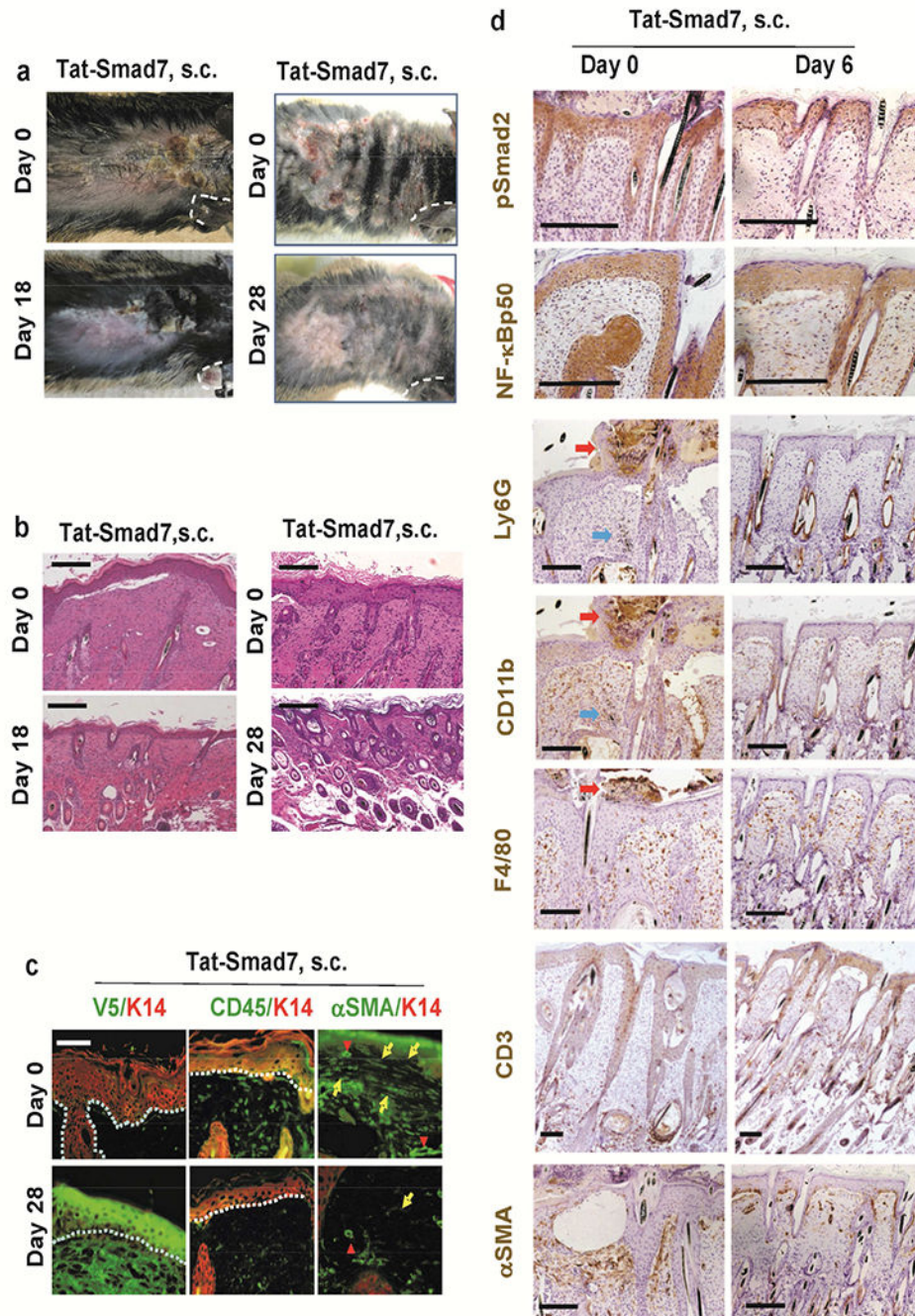


Figure 2. Tat-Smad7 alleviated K5.TGFβ1 skin abnormalities.

(a) Photos of K5.TGFβ1 skin before/after treatment. Dotted lines: the lower ear. (b) H&E skin sections. Scale bar = 100μm. (c) Immunostaining of V5 (for Tat-Smad7 delivery in the dermis and most of the epidermal cells), CD45⁺ cells and αSMA staining in myofibroblasts (yellow arrows) and vessel walls (red arrows). K14 (red): counterstain. Dotted lines: epidermal-stromal boundary. Scale bar = 50μm for all sections. (d) Immunostaining on days 0 and 6 Tat-Smad7 treated K5.TGFβ1 skin. Red arrows in CD11b, Ly6G, and F4/80 staining

point to a microabscess. Blue arrows point to leukocytes positive for both CD11b and Ly6G in the dermis. Hair shafts have non-specific leukocyte staining panels. Scale bar =100µm.

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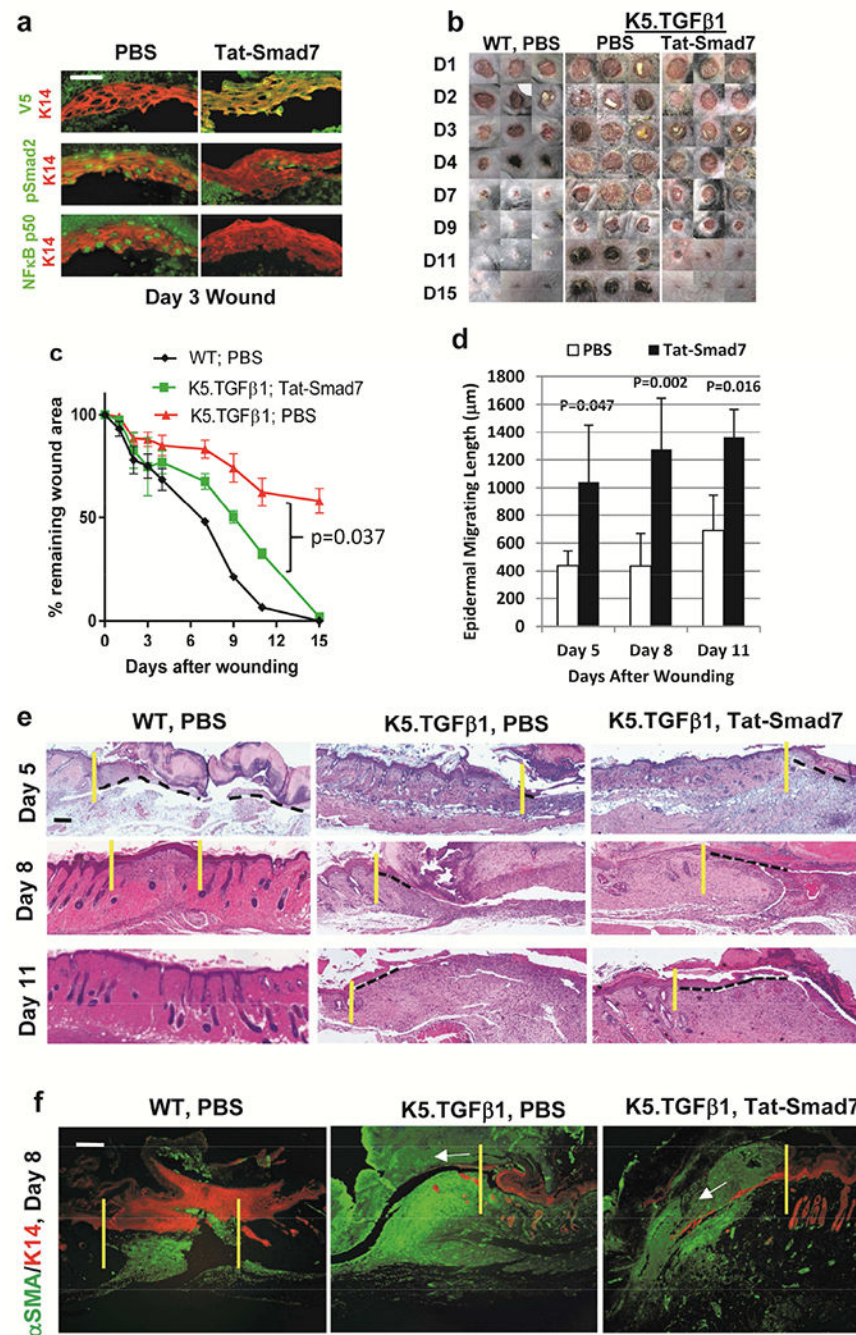


Figure 3. Tat-Smad7 accelerated re-epithelialization in K5.TGFβ1 wounds.

(a) Tat-Smad7 (V5 staining) was biologically active with reduced pSmad2⁺ and NF-κBp50⁺ cells in wounded epidermis. K14 (red): counterstain. Scale bar = 25μm. (b) Gross photos of wound closure. (c) Quantification of unhealed wound areas (Mean±SEM), 5-8 samples/time point. ANOVA for comparison between PBS and Tat-Smad7 treated K5.TGFβ1 wounds. (d) Quantification of epidermal migrating length on H&E images (Mean±SD), 4-8 samples/group/time point. (e) Wound H&E sections. Dotted black lines: migrating tongues. Yellow vertical lines: wound edges (except healed Day11 WT wound). Scale bar = 200μm for all

sections. (f) α SMA staining in granulation tissue (demarked by yellow lines). K14 (red) highlights migrating tongues into granulation tissue. White arrows: migration direction. Scale bar = 200 μ m for all sections.

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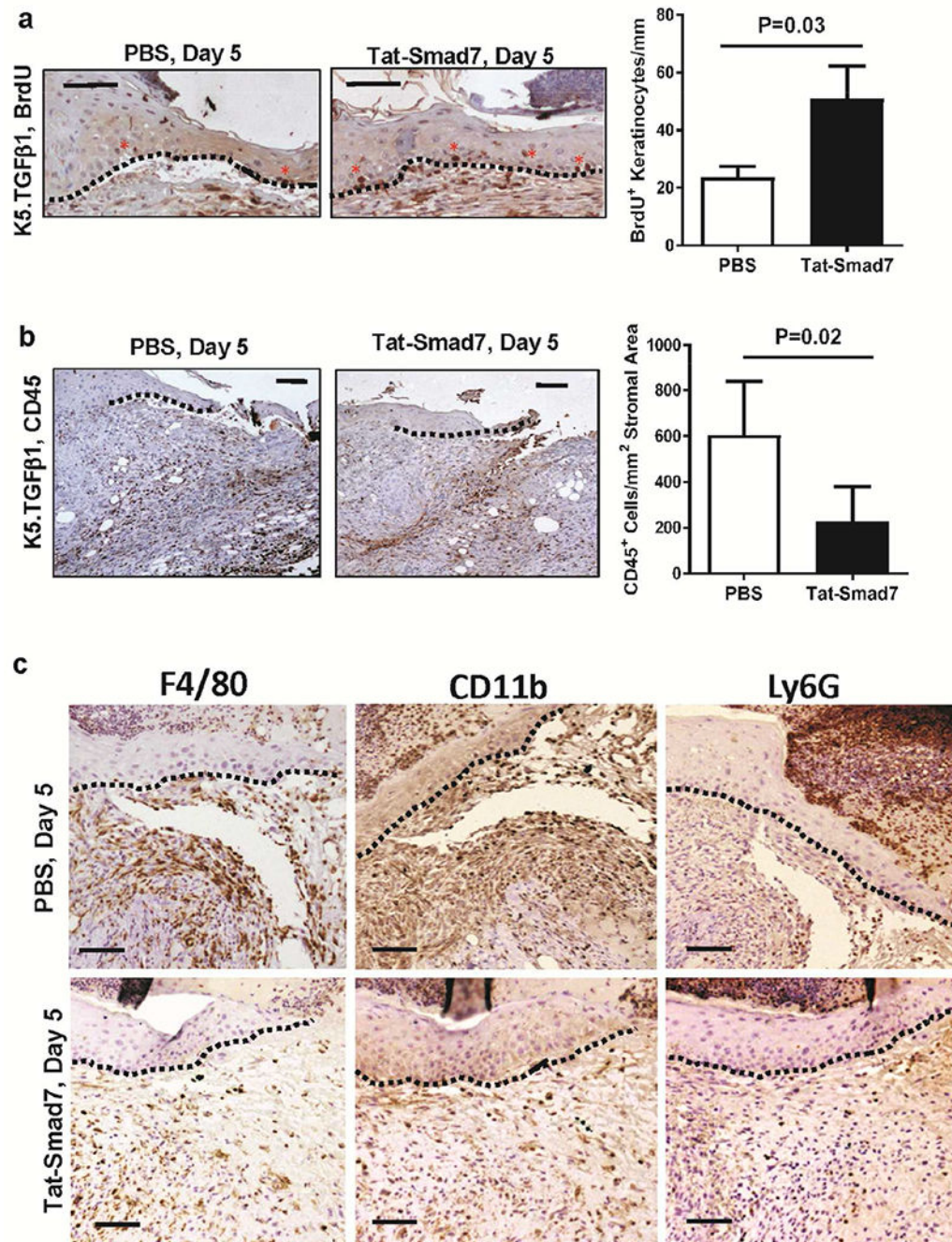


Figure 4. Topical Tat-Smad7 application attenuated proliferation defect and alleviated excessive inflammation in K5.TGFβ1 skin wounds.

(a) BrdU staining images and quantification. *: single or clustered BrdU⁺ basal/suprabasal keratinocytes. Scale bar = 50µm. (b) CD45 staining and quantification. Quantification of cells positive for BrdU in length of migrating tongue and CD45 positive cells in wound stroma area on day 5 wounds (Mean±SD). Each group contains 3 to 6 samples. Scale bar = 50µm (c) Subtypes of inflammatory cells reduced by Tat-Smad7 treatment. Dotted lines in a-c delineate the epidermal-stromal boundary. Scale bar = 50µm.

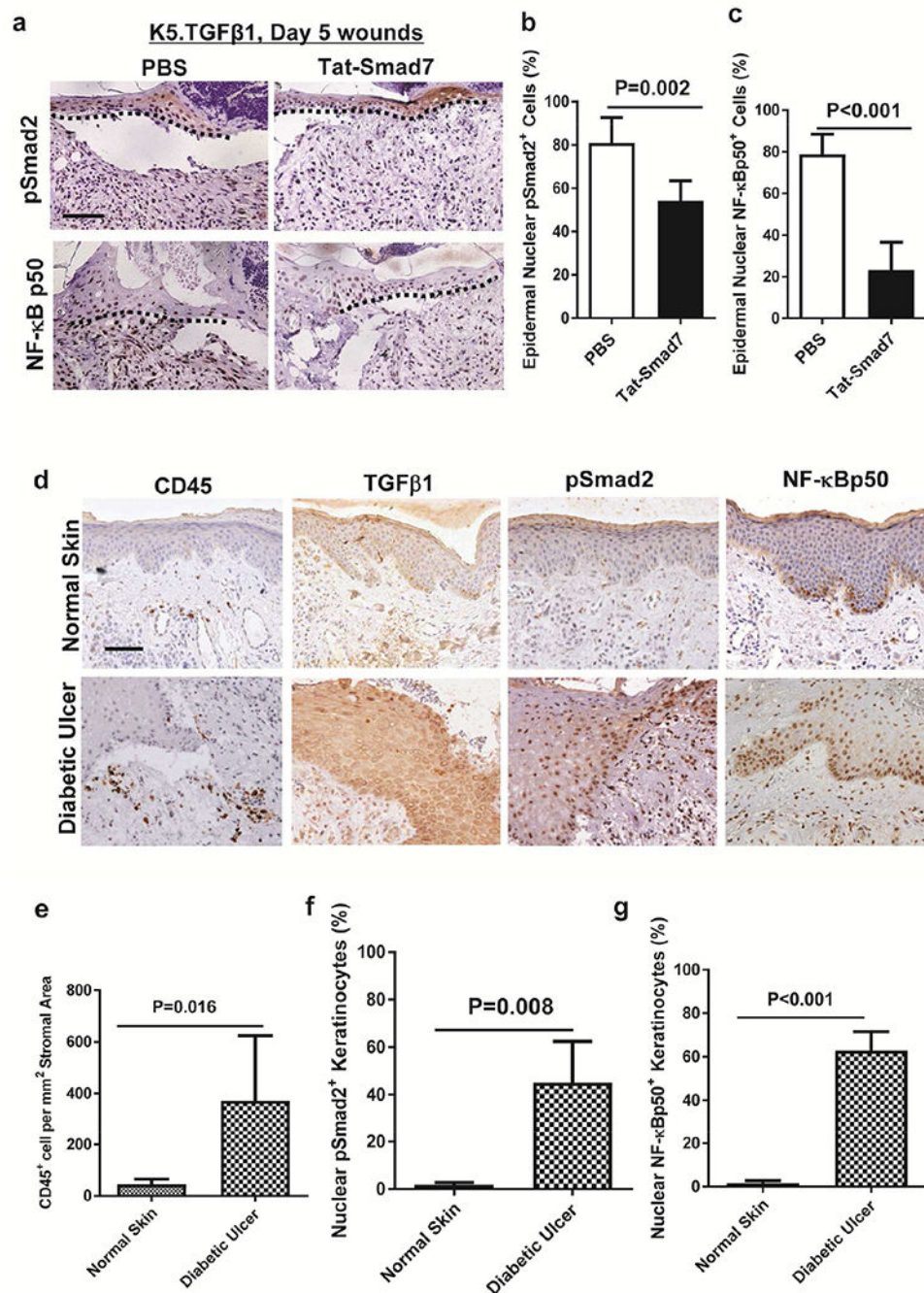


Figure 5. Activation of TGFβ and NF-κB signaling in K5.TGFβ1 mouse skin wounds and human diabetic wounds.

(a) Representative immunohistochemistry staining for pSmad2 and NF-κBp50 on day-5 mouse wounds. Dotted lines delineate the epidermis in the wound area. Scale bar = 50μm for all sections. (b, c) Quantification of percentage of nuclear pSmad2 and NF-κBp50 positive cells in the epidermis (Mean±SD). Each group contains 3 to 6 samples. (d) Representative images of immunohistochemistry staining for CD45⁺ leukocytes, TGFβ1, pSmad2 and NF-κBp50 in normal human skin and diabetic wound skin. Scale bar = 50μm for all sections. (e,

f, g) Quantification of CD45⁺ cells, nuclear pSmad2 and NF-κBp50 positive cells in human normal skins and diabetic wound skins (Mean±SD). 4-5 samples/group.

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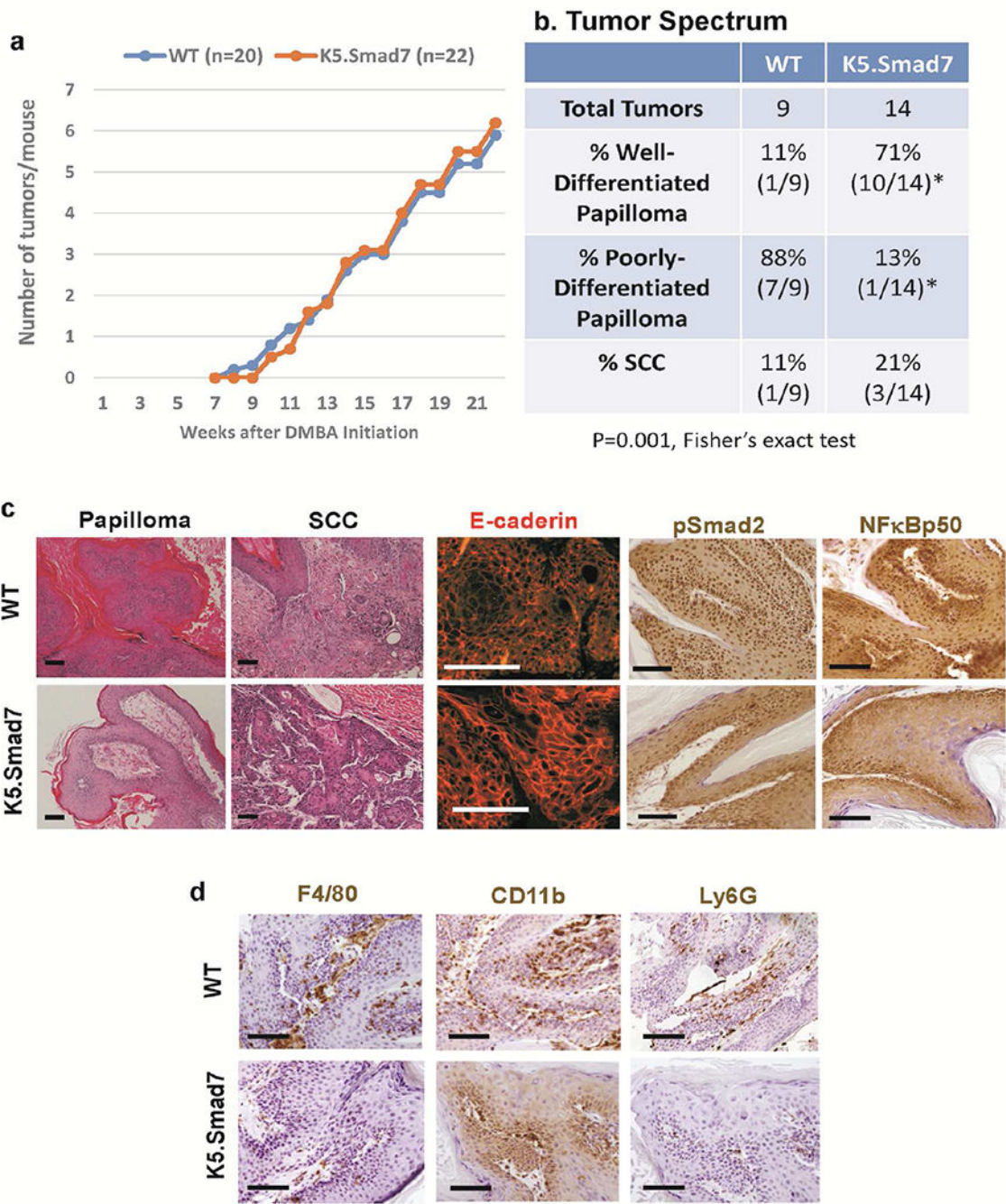


Figure 6. Smad7 overexpression in the epidermis did not affect susceptibility to chemical skin carcinogenesis
(a) Tumor formation kinetics. (b) Summary of histologic changes in K5.Smad7 and WT tumors. (c) Morphological and epithelial differences between WT and K5.Smad7 tumors. WT papilloma and SCC appeared to be less differentiated (lack of spinous layers and stratum corneum) than K5.Smad7 tumors. Membrane E-cadherin staining showed partial loss in WT papilloma. Scale bar = 50µm. (d) Reduced inflammation in K5.Smad7

papillomas. CD11b⁺ cells are identified by intensity (darker than non-specific staining in keratinocytes) and morphology. Scale bar = 50µm.

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