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Overexpression of Smad2 Reveals Its Concerted Action with Smad4 in Regulating TGF-β-Mediated Epidermal Homeostasis

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Members of the transforming growth factor-β (TGF-β) superfamily are critical regulators for epithelial growth and can alter the differentiation of keratinocytes. Transduction of TGF-β signaling depends on the phosphorylation and activation of Smad proteins by heteromeric complexes of ligand-specific type I and II receptors. To understand the function of TGF-β and activin-specific Smad, we generated transgenic mice that overexpress Smad2 in epidermis under the control of keratin 14 promoter. Overexpression of Smad2 increases endogenous Smad4 and TGF-β1 expression while heterozygous loss of Smad2 reduces their expression levels, suggesting a concerted action of Smad2 and -4 in regulating TGF-β signaling during skin development. These transgenic mice have delayed hair growth, underdeveloped ears, and shorter tails. In their skin, there is severe thickening of the epidermis with disorganized epidermal architecture, indistinguishable basement membrane, and dermal fibrosis. These abnormal phenotypes are due to increased proliferation of the basal epidermal cells and abnormalities in the program of keratinocyte differentiation. The ectodermally derived enamel structure is also abnormal. Collectively, our study presents the first *in vivo* evidence that, by providing an auto-feedback in TGF-β signaling, Smad2 plays a pivotal role in regulating TGF-β-mediated epidermal homeostasis.

Key Words: **skin; Smad2 transgenic; Smad4; TGF-β signaling; tooth.**

INTRODUCTION

The transforming growth factor-β (TGF-β) family includes a large number of structurally related polypeptide growth factors, each capable of regulating a fascinating array of cellular processes, including cell proliferation, lineage determination, differentiation, and apoptosis. TGF-β signaling occurs through ligand-initiated heteromeric complex formation of type II and type I receptors with serine/threonine-kinase activity. The activated TGF-β receptor then propagates the signal through transient interaction with, and phosphorylation of, TGF-β pathway-restricted Smad2 and Smad3 proteins. Smad4, a common Smad for all members of the TGF-β family, forms hetero-oligomeric complexes with phosphorylated Smad2 or Smad3. Subsequently, the Smad complexes translocate into the cell nucleus, regulate the expression of transcription factors, and affect the transcriptional status of target genes (Massague, 1998).

TGF-β is an important regulator for skin development and maintenance of tissue homeostasis. Like its function in the development of many organs, TGF-β often has opposite effects on different cell types. In the epidermis of skin, cell division is usually restricted to the basal layer, whereas cells in the suprabasal layers are going through the process

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of terminal differentiation as they move toward the surface. TGF-β is a potent epithelial growth inhibitor and can alter differentiative properties of keratinocytes (Tucker *et al.*, 1984; Shipley *et al.*, 1986; Reiss and Sartorelli, 1987; Fuchs, 1990; Fowlis *et al.*, 1996; Wang *et al.*, 1997). In the dermis, however, TGF-β acts as a positive growth factor, inducing the synthesis of extracellular matrix proteins during wound healing (Roberts *et al.*, 1986; Pittelkow *et al.*, 1988; Sporn and Roberts, 1992; Foitzik *et al.*, 1999). In another well-studied organogenesis model, tooth development is initiated at the specified oral ectoderm and depends on continued epithelial–mesenchymal interaction that is mediated by growth and transcription factors. Among them, TGF-β acts as an inhibitor of enamel organ epithelial cell proliferation while promoting extracellular matrix formation and differentiation of mesenchymally derived preodontoblasts (Chai *et al.*, 1994, 1999; Thesleff and Sharp, 1997; D'Souza *et al.*, 1998; Unda *et al.*, 2000). Elucidation of the molecular mechanism of control of epithelial homeostasis by TGF-β is therefore critical to our understanding of the dynamic molecular regulatory mechanism during ectodermally derived organogenesis.

Studies on TGF-β and TGF-β receptor transgenic mice have supported the concept that TGF-β is primarily an ectodermal growth inhibitor both *in vivo* and *in vitro* (Sellheyer *et al.*, 1993; Fowlis *et al.*, 1996; Wang *et al.*, 1997). The discovery of Smad proteins has clearly advanced our understanding on how TGF-β signaling is related from its cognate receptor to the nucleus. In particular, Smad2 and Smad3 are mediators of TGF-β and activin signals. Targeted mutations of Smad gene have revealed important biological functions of Smads in regulating embryogenesis. Smad2-deficient mouse embryos fail to form an organized egg cylinder and lack mesoderm (Nomura and Li, 1998; Waldrip *et al.*, 1998; Weinstein *et al.*, 1998). In contrast, Smad3, thought to act downstream in the TGF-β/activin signaling pathway, is not essential for embryonic development. Instead, adult Smad3-null mutant mice develop immunological defects and colon tumors (Zhu *et al.*, 1998; Yang *et al.*, 1999; Datto *et al.*, 1999). When Smad4 was mutated, mouse embryos died shortly after implantation due to defects in primitive endoderm formation (Yang *et al.*, 1998; Sirard *et al.*, 1998). Moreover, all Smad proteins are highly conserved across species and there are possible regulatory interactions among different Smads in transducing TGF-β signal. But, because of the early embryonic lethality of Smad-null mutation (e.g., Smad2$^{-/-}$), it is desirable to generate tissue-specific alteration of Smad expression in order to investigate the function of Smad during organogenesis.

Here, we investigated the functional role of Smad2 in regulating ectodermally derived organogenesis by using keratin 14 (K14) promoter. Overexpression of Smad2 targeting the basal layer of ectoderm affected the proliferation and differentiation of epidermal keratinocytes. Interestingly, endogenous levels of Smad4 were altered based on the availability of Smad2, hence suggesting a concerted action of these two Smads in regulating TGF-β-mediated ectodermal homeostasis. Finally, these transgenic mice also showed an increase of endogenous TGF-β1 expression in the skin, indicating the positive impact on TGF-β signaling by Smad2 overexpression.

MATERIALS AND METHODS

Plasmid Constructs and Generation of Transgenic Mice

Human keratin 14 (K14) promoter-driven mouse Smad2-encoding transgene was prepared as follows: an *Eco*RI–*Xho*I blunted fragment (1.5 kb) encoding mouse Smad2 was subcloned into the *Bam*HI blunted sites of the pGEM 3Z-K14 vector to produce pG3ZK14-Smad2. This construct thus contained a K14 promoter (2.1 kb), the β-globin intron (736 bp), the coding sequence for Smad2 (1.5 kb), and K14 polyadenylation signal (500 bp). An *Eco*RI–*Hin*dIII (4.7 kb) fragment was isolated free of vector sequence by preparative gel electrophoresis. DNA was further purified by using an Elutip column (Schleicher and Schuell, Dessell, Federal Republic of Germany) and microinjected in the pronuclei of fertilized oocytes (Jackson/B6D2F F1) by standard procedure.

Identification of Smad2 Transgenic and Mutant Mice

Transgenic founder mice were identified by Southern blot analysis of DNA obtained from the tails. Tails were lysed with 100 mM Tris–HCl, pH 8.5, 5 mM EDTA, pH 8.0, 0.2% SDS, 200 mM NaCl/proteinase K, and then treated with phenol/chloroform, 1:1 (by volume), precipitated with ethanol, and dissolved in TE buffer (10 mM Tris–HCl, pH 7.5/1 mM EDTA). After transfer to nylon membranes (Dupont N+), Southern hybridization was performed by using probes labeled with ^{32}P of K14 gene by random priming. For PCR analysis, tail genomic DNAs (0.5–1 μg) were submitted to 35 cycles of amplification (with each cycle consisting of 94°C, 62°C, 72°C, 1 min for each) on a thermal cycler. An aliquot (15 μl) of each reaction was resolved in a 1% agarose gel, and amplified fragments were visualized by ethidium bromide staining. The PCR primers used here were 5′-ACA CCT CCA AAG CAG GAC CAA GTG G-3′ and 5′-ATT TAC GCC TCT GTG ACC CAG GGC TTC-3′. The PCR product size was 487 bp. Genotypes of Smad2 and Smad3 mutant mouse embryos were determined by PCR as previously described (Weinstein *et al.*, 1998; Yang *et al.*, 1999).

RNA Isolation and Northern Blot

Total cellular RNA was prepared by following the RNA isolation method (TRIzol, GIBCO BRL) developed by Chomczynski and Sacchi (1987). The total RNA was further purified by using RNeasy Kits (Qiagen). Aliquots of the total RNA were electrophoresed in 1% agarose gels. Subsequently, the samples were transferred to nylon filters (Hybond-N; Micron Separations Inc.) by electroblotting. Filters were prehybridized for 4 h at room temperature. Each cDNA was labeled by random primer method using the NEBlot kit (New England Biolabs Inc.) and [α-^{32}P]dCTP (NEN Life Science Products). Hybridization was performed at 65°C overnight by using the ExpressHyb hybridization solution (Clontech, Palo Alto, CA). Filters were washed twice in 2× SSC, 0.1% SDS for 15 min at 55°C, then in 1× SSC, 0.1% SDS for 20 min at 55°C. Washed filters were exposed to x-ray film at −80°C for various lengths of time depending upon the intensity of the signal.

Western Blot Analysis

The BMP2/4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), Smad2, Smad4 antibodies (Transduction Laboratories), Smad3 antibody (Zymed, South San Francisco, CA), and TGF-β1 antibody (R & D Systems) were used for Western analysis as previously described (Chai *et al.*, 1999). β-Actin was used as internal control.

Histological Analysis

Mouse tails, incisors, and skin were fixed in 10% buffered formalin overnight at 4°C and embedded in paraffin. Tissue sections (6 μm) were stained with hematoxylin–eosin.

Evaluation of DNA Synthesis Activity in the Skin

Mice were injected (i.p.) with BrdU (5-bromo-2′-deoxy-uridine; Sigma) at 100 mg/kg body weight and sacrificed 3 h after injection. Tail specimen were harvested and fixed in Carnoy's solution for immunostaining (Chai *et al.*, 1998). Ten sections of tail skin were randomly selected from each mouse to evaluate the number of BrdU-labeled cells in the epidermis. Four Smad2 transgenic mice and four wild-type littermates were used for this analysis.

In Situ Hybridization

Both Smad2 and TGF-β RNA probes were labeled with Fluorescein labeling mix (Boehringer Mannheim). Tail-skin sections from wild-type and K14-Smad2$^{Tg/+}$ were mounted on the same slide to ensure equal exposure to the probes. Hybridization was performed as previously described (Zhao *et al.*, 1996). Positive signal was shown as green fluorescence in dark field.

Immunohistochemistry

Mouse tail skin was obtained from control and Smad2 transgenic mice and was processed according to standard procedures (Chai *et al.*, 1999). Serial sections (6 μm) of control and Smad2 transgenic mouse tail were cut and mounted onto the same slide to ensure equal exposure to the same antibody concentration. The following antibodies were used for immunostaining: anti-Smad2 (1:100) (Transduction Laboratories); anti-BrdU (1:200); anti-K14 (1:100); anti-laminin (1:50) (Sigma–Aldrich); anti-loricrin (1:2000); anti-filaggrin (1:1000) (BabCo); anti-K10 (1:100) (Dako); anti-TGF-β1 (1:100) (R & D Systems); and anti-fibronectin (1:100) (Santa Cruz Biotechnology).

Scanning Electron Microscopy

Mouse-tissue specimen were processed and viewed according to standard procedures (Chai *et al.*, 1997).

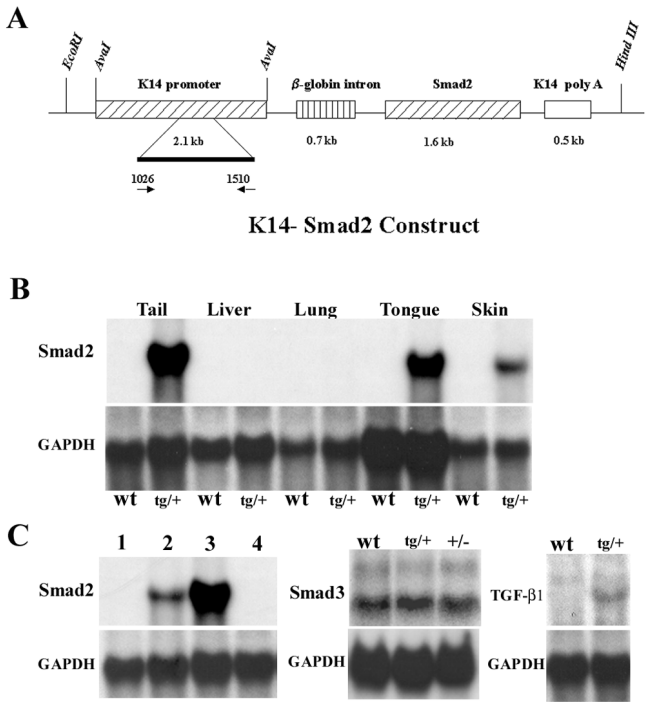


FIG. 1. Transgene construct and expression. (A) Schematic drawing shows the structure of K14-Smad2 transgenic construct. PCR primers were designed to amplify human K14 promoter (1026–1510) for genotyping transgenic mice. (B) Northern analysis of Smad2 mRNA expression in different tissues of wild-type (wt) and transgenic mice (tg/+). The expression of Smad2 transgene was detected in the tail, tongue, and skin but not in liver and lung of 2-wk-old transgenic mice. The transgene expression pattern faithfully correlated with the *in vivo* activity and specificity of human K-14 promoter. The endogenous Smad2 expression was not seen in the wild-type tissue sample because the amount of total RNA loaded in each lane (20 μg) was not sufficient for endogenous Smad2 to be detected. (C) The amount of Smad2 transgene expression was correlated with the severity of phenotype of Smad2 transgenic mice. Fifteen micrograms of total RNA were loaded in each lane. Lane 1, wild-type littermate. Lane 2, lower level Smad2 transgene expression was associated with less severe skin phenotype. Lane 3, higher level of Smad2 transgene expression was correlated with severe skin phenotype (see Figs. 3 and 4). Lane 4, Smad2 heterozygous mutant. Overexpression or underexpression of Smad2 did not affect the endogenous Smad3 expression, as indicated by using tail samples (2 weeks postnatal) from wild-type (wt), K14-Smad2 overexpression transgenic (tg/+), and Smad2 heterozygous mutant (+/−) mice. Overexpression of Smad2 (tg/+) elevated the endogenous TGF-β1 expression while it remained undetectable in the wild-type littermate.

RESULTS

Generation of Transgenic Mice with Targeted Expression of Smad2 to Basal Keratinocytes

A 4.9-kb K14-Smad2 gene construct was generated (Fig. 1A) to target Smad2 expression to the basal keratinocytes of transgenic mice. Human K14 promoter has successfully targeted gene expression to the basal compartment of the epidermis and to the outer root sheath of hair follicles (Vassar *et al.*, 1989; Byrne *et al.*, 1994; Gat *et al.*, 1998). The purified transgene was microinjected into the pronuclei of fertilized eggs harvested from C57BL6/J × CBA/J mice. Southern blot analysis identified three founder mice and

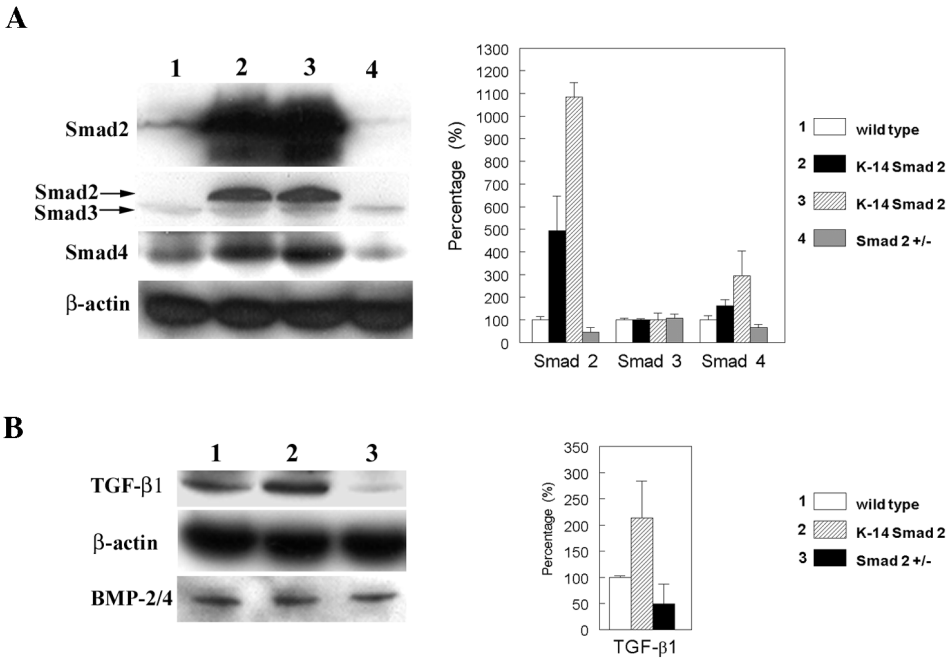


FIG. 2. Western analysis showing overexpression of Smad2 in transgenic mice and changes of Smad4 expression resulted from the alteration of Smad2 expression. (A) Lane 1, wild-type mouse tail. Lane 2, K14-Smad2 transgenic mouse tail (with less severe phenotypic appearance). Lane 3, K14-Smad2 transgenic mouse tail (with severe skin malformation). Lane 4, $Smad2^{+/-}$ heterozygous mutant mouse tail. K-14-Smad2 transgenic mice showed a significant increase in Smad2 expression, while heterozygous loss of Smad2 significantly reduced the endogenous Smad2 expression. Interestingly, either over- ($Smad2^{Tg/+}$) or under- ($Smad2^{+/-}$) expression of Smad2 did not affect the endogenous Smad3 expression. Because of the overwhelming production of Smad2 in the transgenic mice Smad3 antibody cross-reacted with Smad2 by showing an extra band above the Smad3 in transgenic samples. Endogenous Smad4 expression was positively regulated by the overexpression of Smad2 in the transgenic mice while negatively regulated by the underexpression of Smad2 in its heterozygous mutant. β-Actin was used as a loading standard. Densitometric analysis (on the right) indicated the relative amount of protein expression when compared to the wild-type control (as 100%). Each bar represents the mean and SD of at least 14 samples. The two K14-Smad2 transgenics (labeled as 2 and 3) are correlated with lanes 2 and 3 mentioned above. (B) The dosage of Smad2 regulated TGF-β1 expression. Overexpression of Smad2 (tg/+) increased TGF-β1 expression in the skin while underexpression of Smad2 (+/−) reduced TGF-β1 expression. The expression of BMP2/4 was not affected by the dosage of Smad2. Densitometric analysis (right) indicated the relative amount of TGF-β1 expression when compared with the wild-type control (as 100%). Each bar represents the mean and SD of at least 14 samples.

their transgenic lines. Two of the three lines exhibited identical phenotypes, with descendents of one line showing more severe defects than the ones of the other line. In particular, Smad2 transgenic mice had defects in the skin, hair, tail, tongue, ear, and overall size of the body (see Figs. 3 and 4). Because we successfully obtained two lines of Smad2 transgenic mice, we were confident that the observed phenotypes could be attributed to Smad2 transgene expression and not chromosomal integration site.

The expression of human keratin 14 gene is tissue-specific and differentiation-specific in transgenic mice (Vassar *et al.*, 1989). Here, we demonstrated that K14-Smad2 transgene was expressed in the tail, tongue, and skin, but not in liver and lung (Fig. 1B), completely matched with the tissue-specificity of K14-driven transgene expression in mice. When we examined the Smad2 transgene expression level in the two different lines of transgenic mice, it was apparent that a higher level of Smad2 expression was associated with more severe phenotypes (Fig. 1C).

Because of the high homology between Smad2 and Smad3, we examined the endogenous Smad3 expression. There was no alteration of Smad3 expression level in either K14-Smad2 transgenic or Smad2 heterozygous knockout (which were maintained on the same genetic background as the transgenic and tissue samples were collected from newborn $Smad2^{+/-}$ mutant) mice (Fig. 1C).

When we examined the protein expression levels, we found that there was also variation in the overexpression of Smad2 (Fig. 2A), which was correlated with the severeness of macroscopic phenotypes. In particular, there was a 5-fold increase of Smad2 expression in K14-Smad2 mice with less severe phenotypes and more than 10-fold increase on Smad 2 in transgenic mice with severe phenotypes (Fig. 2A). The difference of phenotype severeness was only quantitative (such as the thickness of epidermis), but not qualitative, between the two lines of transgenic mice. When we examined Smad2 expression in its heterozygous mutant mice, it was about one-half of

the endogenous Smad2 expression level when compared to the wild-type littermates (Fig. 2A).

Smad4, but Not Smad3, Is Induced in Response to Smad2 Overexpression while Heterozygous Loss of Smad2 Reduces Smad4 Expression

Both TGF-β and activin engage their receptors that phosphorylate Smad2 and Smad3. Phosphorylated Smad2 and Smad3 associate with Smad4 for the assembly of transcriptional complex that is then transported into the nucleus for TGF-β target gene regulation (Massague *et al.*, 2000). Endogenous Smad3 expression was not affected in either Smad2 transgenic overexpression or Smad2 heterozygous mutant mice (Fig. 2A). Interestingly, endogenous Smad4 expression directly correlated with the level of Smad2. In K14-Smad2 transgenic mice, endogenous Smad4 showed significant ($P < 0.05$) increases, with a correlation to the intensity of Smad2 overexpression. Evaluating the Western analysis results from 14 different K14-Smad2 transgenic mice, densitometric analysis indicated that the average endogenous Smad4 increased by 2- to 3-fold compared with the wild-type control (Fig. 2A). Meanwhile, heterozygous loss of Smad2 ($n = 18$) significantly ($P < 0.05$) reduced endogenous Smad4 expression to about 65% of the wild-type control level (Fig. 2A). Collectively, there is an apparent direct correlation between levels of Smad2 and Smad4 in regulating TGF-β signals in the skin.

Overexpression of Smad2 Positively Regulates Endogenous TGF-β1 Expression

TGF-β1 and its type II receptor are part of the endogenous homeostatic regulatory machinery of the epidermis. Overexpression of TGF-β1 in the epidermis resulted in the elevation of its type II receptor expression, indicating their concerted action during TGF-β signaling (Cui *et al.*, 1995). Here, we examined the possible feedback on endogenous TGF-β1 expression by its signaling molecule Smad2. Overexpression of Smad2 elevated the endogenous TGF-β1 level while heterozygous loss of Smad2 reduced TGF-β1 expression (Figs. 1C and 2B). Densitometric analysis indicated that Smad2 overexpression resulted in a twofold increase of endogenous TGF-β1 expression while haploinsufficiency of Smad2 reduced TGFβ1 by about 50%. To further examine the specificity of TGF-β signaling Smad on other members of the TGF-β superfamily, we evaluated the expression of BMP2 and -4 in both Smad2 transgenic and heterozygous knockout mice and did not see any alteration of BMP expression (Fig. 2B), indicating that the positive feedback on endogenous TGF-β expression by Smad2 was pathway-specific.

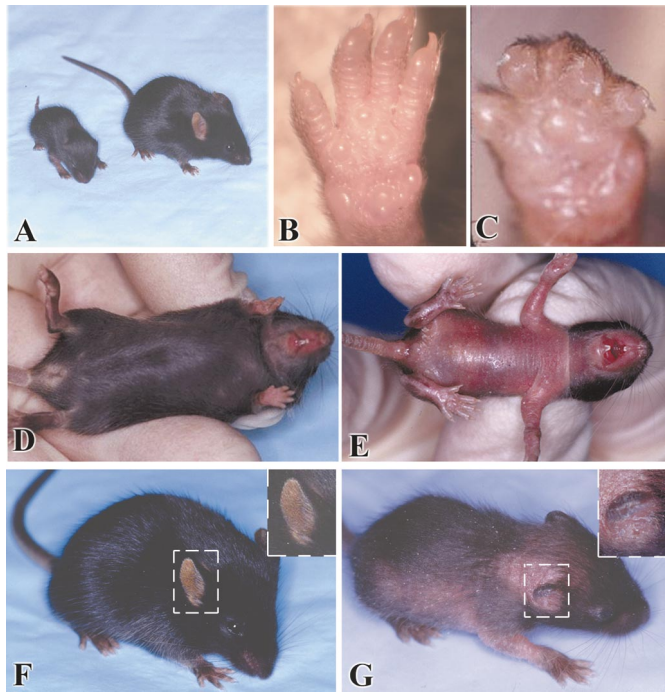


FIG. 3. Macroscopic phenotypes of the Smad2 overexpressing mice. Two-week-old wild-type and transgenic littermates are shown here. (A) Strikingly, the K14-Smad2 transgenic mice (represented by the one on the left) were about one-half the size of their wild-type littermates (shown on the right) and remained to be small until adulthood (3 months). (B, C) Apparent skin defect was present in these transgenic mice. Thick, flaky, and tight skin surface was apparent in the paws of transgenic mice (C), when compared to the control (B). In addition to the skin defect, the hair development was also disturbed in these transgenic mice. (D) Normal hair distribution was shown at the ventral surface of a wild-type mouse. (E) In the Smad2 transgenic animal, the hyperkeratinized (with shiny appearance) ventral skin surface was covered with sparse hair. (F) In the wild-type littermates, ear development was normal with an elongated pinna (inset). (G) In Smad2 transgenic animals, pinna development was severely retarded (inset) and the skin surrounding the pinna was flaky.

The Severity of Phenotypes of Transgenic Mice Is Correlated with the Level of Smad2 Overexpression

At birth, macroscopic phenotypes of transgenic mice were only observed in about 20% of pups ($n = 235$) with short/necrotic tip of tail. Despite strong transgene activity by E14, the remaining transgenic mice did not show overt phenotypes until 10–14 days after birth. They appeared small, with the most severely affected ones only half the size, compared with their wild-type littermates (Fig. 3A). Both front and hind paws showed thickened and flaky skin surface compared with the controls (Figs. 3B and 3C). The skin defects of these transgenic mice varied from macroscopically normal to severely flaky with sparse hair, especially at the ventral surface of transgenic animal (Fig. 3E). In addition, these mice had smaller ears (Fig.

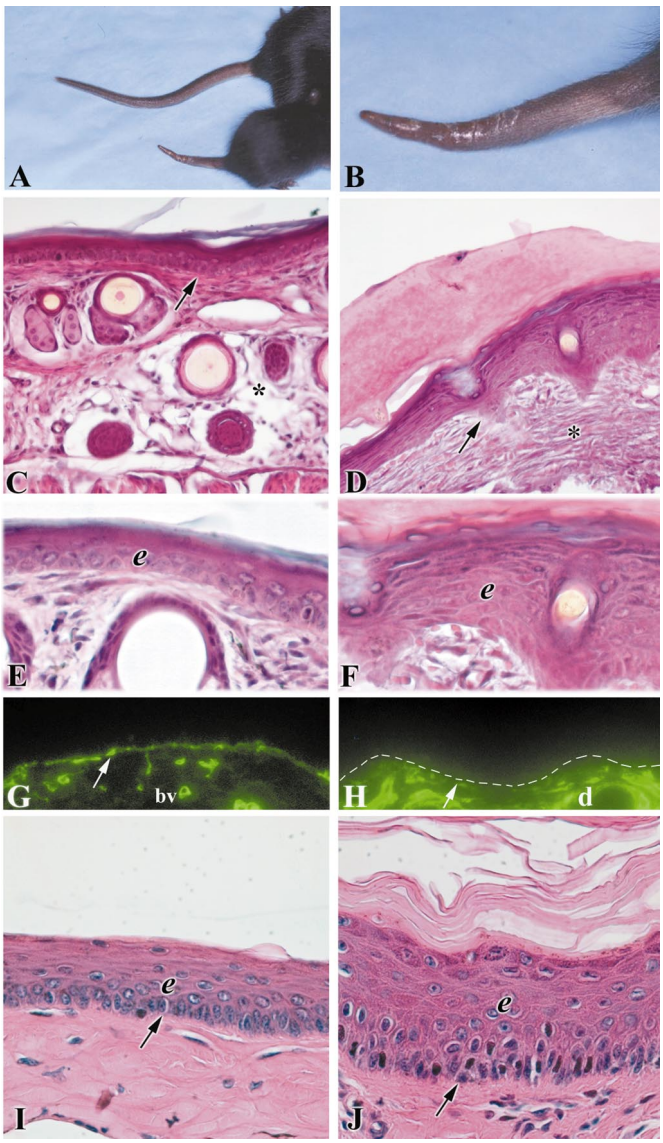


FIG. 4. Histological changes in Smad2 transgenic mouse tail skin. (A) Compared with its wild-type littermates, the tail of the Smad2 transgenic mouse was significantly shorter (lower mouse). (B) When examined closely, there was severe tail malformation in the transgenic animal, with apparent flaky and cracked skin surface. (C, E) Histology of tail skin from a 2-wk-old wild-type littermate mouse. The basement membrane (arrow) was well defined and there were very few suprabasal and granular cell layers in the epidermis (e). (D and F) In Smad2 transgenic mice (littermates of the wild-type control), the architecture of epidermis was severely disturbed. The basement membrane became undefined (arrow). There were more suprabasal and granular cell layers in the epidermis and a severe thickening of the stratum corneum. Interestingly, there was also alteration of the dermis structure. In the control littermates, fatty tissue was localized under the dermis (* in C), while it was replaced by connective tissue in the transgenic animal (* in D). Additionally, Smad2 transgenic mice had fewer hair follicles compared with their wild-type littermates. Anti-laminin staining was used to evaluate the integrity of basement membrane. (G) In the control, laminin expression indicated a well-defined and continuous basement membrane (arrow). Anti-laminin antibody also stained blood vessels (bv) in dermis. (H) In K14-Smad2 transgenic mice, the basement membrane (indicated by the white dotted line) was not well defined and discontinuous (arrow). Anti-laminin antibody also cross-reacted with the increased fibrous tissue in dermis (d). (I, J) The architectural alteration in epidermis persisted in the tail skin of 9-month-old transgenic mice (J) when compared with their wild-type littermate (I). Arrow points to basement membrane. e, epidermis.

3G), compared with their wild-type littermates (Fig. 3F). When examined closely, the rudiment of external ear was present but failed to develop (Fig. 3G, inset). The severity of macroscopic phenotypes correlated with the level of Smad2 transgene expression, with almost no macroscopic alterations in transgenic mice that expressed very low level of Smad2 transgene.

Distinctive features of these transgenic mice were the short tail and flaky skin at 2 weeks after birth (Figs. 4A and 4B). Significant histological changes in the tail skin were observed in both lines of transgenic mice. The epidermis of transgenic mice was much thicker (Fig. 4D) compared with the skin of controls (Fig. 4C). The basement membrane was irregular and not well defined (Fig. 4D, arrow) and the epidermal architecture was highly disorganized. In addition, there were fewer hair follicles ($P < 0.05$) in the transgenic mice. The subcutaneous fatty tissue, which was normally present in the controls (Fig. 4C), was replaced by fibrous connective tissue in K14-Smad2 mice (Fig. 4D). These skin defects were also associated with the body skin and ear. When we examined the different cellular layers of epidermis, we found that there was disorganization of the basal layer and thickening of the suprabasal layer (Fig. 4F). Unlike the controls (Fig. 4E), extra granular cell layers were also present in these transgenic mice (Fig. 4F). At the skin surface, there was significant increase in thickness of stratum corneum (8 to 10 times thicker). To further analyze the integrity of basement membrane, we examined the expression of laminin. In the control, a well-defined and continuous laminin expression was present at the basement membrane (Fig. 4G, arrow). In K14-Smad2 mice, the basement membrane was interrupted as indicated by the pattern of laminin expression (Fig. 4H). As these transgenic animals grew older, their macroscopic phenotypes gradually became less obvious, although both ears and tails remained abnormal. At 9 months, hyperkeratinized epidermis was still observed in the tail skin (Fig. 4J).

Smad2 Transgene Expression in Epidermis, Its Effects on Keratinocyte Proliferation and Differentiation

TGF-β regulates keratinocyte proliferation and differentiation in skin. Endogenous Smad2 expression in the epi-

dermis has been demonstrated and may be altered in epithelial skin tumors (Dick *et al.*, 1998; Lange *et al.*, 1999). Hence, we examined the Smad2 expression and keratinocyte proliferation as well as differentiation in our transgenic mice. K14-Smad2 transgenic mice revealed strong expression of Smad2 throughout the epidermis (Fig. 5B), providing the physical evidence that overexpression of Smad2 might be responsible for both macroscopic and microscopic changes in the skin. In the wild-type control, however, there was very low level of endogenous Smad2 expression in the epidermis while most of the Smad2 was localized to dermis (Fig. 5A). Furthermore, phosphorylated Smad2 was localized within keratinocyte nuclei throughout all layers of epidermis (Fig. 5B inset), indicating that the overexpressed Smad2 was able to be phosphorylated, translocate into the nuclei, and regulate TGF-β target gene expression. We also examined localization of Smad2, Smad3, and Smad4 mRNA in the skin. There was a very low level of Smad2 expression in basal layer of epidermis and diffused expression in dermis (Fig. 5A′) in the control sample while there was a significant increase in Smad2 expression in the epidermis of transgenic mice (Fig. 5B′). Expression of Smad4 mRNA was significantly expanded throughout all layers of epidermis in the transgenic mice (Fig. 5B″), while it was at a very low level in the control sample (Fig. 5A″), indicating that overexpression of Smad2 up-regulated the expression of Smad4 in epidermis. There was no difference in Smad3 expression pattern in epidermis between wild-type control and Smad2 transgenic mice (data not shown).

BrdU incorporation analysis revealed that overexpression of Smad2 significantly increased the proliferation ($P < 0.05$) of basal layer keratinocytes (Fig. 5D) when compared to the control group (Fig. 5C). To examine the terminal differentiation of keratinocytes in transgenic mice, we used several keratinocyte differentiation-specific markers. Loricrin is a major component of the cornified envelope in the granular layer of epidermis (Fuchs, 1990, and references therein). In the control sample, loricrin expression was confined to one or two cell layers in granular layer of epidermis (Fig. 5E), while it was expressed through seven to eight cell layers in the transgenic mice (Fig. 5F). Additionally, filaggrin, another granular layer-specific protein, was also widely expressed through multilayers of epidermis in the Smad2 transgenic mice (Fig. 5F, inset). The normal epidermis usually has four to eight layers of suprabasal spinous cells that are postmitotic, but metabolically active and produce two new keratins (K1 and K10). In the control samples, K10 was localized to the suprabasal spinous cells, but not in the basal layer of epidermis (Fig. 5G). In the transgenic mice, K10 expression was found in all layers of the expanded stratum spinosum, with some of the basal layer cells also showing positive staining (Fig. 5H). The basal layer of epidermis has the capacity of DNA synthesis and mitosis. These cells produce two distinct keratin proteins (K5 and K14). K14 was exclusively expressed in the single basal cell layer of epidermis in the wild-type control (Fig. 5I). In K14-Smad2 mice, K14 was expressed in basal, suprabasal, and granular layers of epidermis (Fig. 5J).

Because our transgenic construct targeted the basal layer of epidermis, we examined the downstream effects of Smad2 overexpression, which might be responsible for the structural changes in dermis (Fig. 4D). TGF-β expression has been shown to be up-regulated in an autoregulatory fashion (van Obberghen-Schilling *et al.*, 1988; Bascom *et al.*, 1989). Hence, we examined the expression of TGF-β in K14-Smad2 mice. There was a twofold increase of endogenous TGF-β1 expression in K14-Smad2 mice (Figs. 1C and 2B). Immunolocalization analysis showed that TGF-β1 was expressed at a very low level in the dermis in the control mice (Fig. 6A), while it was highly expressed in both the epidermis and dermis of transgenic mice (Fig. 6B). This observation was further supported by examining TGF-β1 mRNA expression, which was at a very low level in the basal layer of epidermis (Fig. 6A′) in the control sample. In Smad2 transgenic mice, TGF-β1 mRNA was detected throughout all layers of epidermis (Fig. 6B′). TGF-β1 is known to enhance the production of fibronectins, which are important elements in connective tissue for cross-linking to collagen (Vaughan *et al.*, 2000). In Smad2 transgenic mice, fibronectin expression was elevated in the dermis of transgenic mice (Fig. 6D), which provides potential explanation for dermal fibrosis.

Smad2 Regulates Proper Enamel Formation

Smad2 is expressed during early tooth development and may regulate ameloblast differentiation (Dick *et al.*, 1998). Both newly erupted upper and lower incisors appeared chalky-white and were frequently chipped in Smad2 transgenic mice (Fig. 7B), while the ones from wild-type littermates appeared normal with a shiny and grayish enamel surface (Fig. 7A). Additionally, the tongue was substantially larger in the transgenic animal (Fig. 7B), which possibly interfered with food intake by these transgenic mice. The incisors had blunt tips while the molars had fewer and underdeveloped cusps in transgenic animal (Fig. 7D) when compared with the controls (Fig. 7C). In this study, we focused on the developmental analysis of incisors for the following reasons. First, rodent incisors continue to grow throughout life, which offers a good model to examine the continued differentiation of ameloblasts that are targeted by K14 promoter. Second, only the labial surface of rodent incisors is covered by enamel. Any alteration in the enamel formation can be easily monitored. Under the scanning electron microscope, the enamel surface of control incisors appeared smooth and the tip was pointed (Figs. 7E and 7G), while the incisor surface of transgenic mice was irregular and wavy and the tip was blunt (Figs. 7F and 7H). The difference of enamel surface topography between the wild-type and transgenic mice made it clear that the chalky-white appearance of transgenic incisors was due to light being scattered by the irregular enamel structure.

To evaluate the maturation and differentiation of amelo-

blasts, we first compared the histological appearance of these cells in both control and transgenic mice. In wild-type mice, ameloblasts were well formed and arranged in an orderly fashion (Fig. 8A). In Smad2 transgenic mice, ameloblasts appeared to be arranged in spiral fashion, which resulted in the cross-sectional appearance (Fig. 8B). We then examined the expression of amelogenin as a differentiation marker for these ameloblasts. In a cross-section of lower incisor, amelogenin was localized towards the anterior portion of incisor in both wild-type and transgenic mice (Figs. 8C and 8D). When examined closely, amelogenin was secreted by the ameloblasts and deposited into the enamel matrix at the apical surface of ameloblasts in the wild-type mice (Fig. 8E). In transgenic mice, however, amelogenin was localized between the ameloblasts (Fig. 8F), instead of being in enamel matrix at the apical region of ameloblasts. This inaccurate deposition of amelogenin may have interfered with the enamel crystal structure build-up that ultimately resulted in the irregular enamel surface in Smad2 transgenic mice. Amelogenin expression in the transgenic mice, however, indicated that these ameloblasts were able to terminally differentiate during tooth morphogenesis.

DISCUSSION

Through the generation of our K14-Smad2 transgenic mice, we have learned that the concerted action of Smad2 and Smad4 is important in control of TGF-β-mediated epidermal homeostasis. The level of Smad expression is critical for the regulation of TGF-β signals. The receptor-regulated Smad2 has an auto-feedback effect on TGF-β, which is ultimately responsible for regulating the proliferation and differentiation of keratinocyte as well as ameloblast during epidermally derived organogenesis.

In order to attribute the phenotypes of transgenic mice to the overexpression of Smad2, we first examined the localization of Smad2. Both unphosphorylated and phosphorylated (PS2) Smad2 were localized throughout the epidermis, indicating that the overexpressed Smad2 was activated to initiate TGF-β-mediated genetic response. This PS2 antibody has been used in various studies to demonstrate its specificity in recognizing the phosphorylated Smad2 upon TGF-β stimulation (Nakao *et al.,* 1999; Stopa *et al.,* 2000). Second, we examined some of the possible target genes that are regulated by the increased Smad2 expression. Smad4, but not Smad3, was up-regulated in Smad2 overexpression mice. In tandem, using samples from Smad2 heterozygous mutant mice, we found that Smad4 was down-regulated while the expression of Smad3 remained unaffected. This observation suggests that TGF-β/activin signaling-specific Smad2 positively regulate the expression of Smad4 (the common Smad), which mediates the signaling from diverse members of TGF-β superfamily, and this direct correlation of Smad2 and Smad4 may play an important role in adjusting different Smad pathways. *In vitro* studies have shown that Smad4 acts as a TGF-β-inducible DNA-binding protein and is up-regulated by Smad2 overexpression (Yingling *et al.,* 1997; Li *et al.,* 1998). Smad2 and Smad4 form heterodimers to regulate TGF-β-induced signaling and their mutations may cause carcinogenesis (Jayaraman and Massague, 2000; Tsujiuchi *et al.,* 2000). The expanded expression of Smad4 (as demonstrated by *in situ* hybridization, Fig. 5B″) provides supportive evidence that the elevated Smad4 expression is the result of up-regulated Smad4 gene expression, not of delayed Smad4 degradation due to the excessive amount of Smad2 in our transgenic mice. Collectively, these studies present an important relationship between Smad2 and Smad4, and our study here provides the first *in vivo* evidence of concerted action between Smad2 and Smad4 in regulating TGF-β-mediated epidermal homeostasis.

Smad3 shares more than 95% homology with Smad2 but they may function differently during embryonic development (Baker and Harland, 1996; Wu *et al.,* 1997; Nomura and Li, 1998; Weinstein *et al.,* 1998; Zhu *et al.,* 1998; Yang *et al.,* 1999). In particular, Smad2 may play a more important role in regulating embryonic development while Smad3 is more involved in pathogenesis or the wound-healing process (Ashcroft *et al.,* 1999; Massague *et al.,* 2000). This argument is further supported by the Smad2- and Smad3-null mutation studies, in which $Smad2^{-/-}$ mutant is early embryonic lethal, while $Smad3^{-/-}$ mice develop normally. On the other hand, an alternatively spliced form of Smad2 can apparently substitute for the DNA binding activities of and, therefore, function as Smad3 (Yagi *et al.,* 1999). In general, studies suggest that Smad2 and Smad3 regulate distinct sets of target genes, although on certain target they are possibly interchangeable (see review by Massague *et al.,* 2000).

Additional supportive evidence of overexpressed Smad2 capable of transducing TGF-β-mediated signaling was demonstrated by the elevated expression of endogenous TGF-β1 in Smad2 transgenic mice. TGF-β1 has been shown to increase steady-state levels of its own expression and to affect the expression of its type II receptor (van Obberghen-Schilling *et al.,* 1988; Cui *et al.,* 1995). Overexpression of Smad2 is colocalized with TGF-β1 expression in human pancreatic cancer (Kleeff *et al.,* 1999). Smad3 also appears to be critical for the auto-induction of TGF-β in keratinocytes and macrophages (Ashcroft *et al.,* 1999). Apparently, there is a positive auto-feedback mechanism that regulates endogenous TGF-β expression. Our study has demonstrated that the level of Smad2 expression is responsible for mediating the auto-feedback on TGF-β signaling in epidermis in a cell-autonomous manner. We have also examined the endogenous TGF-β2 and -β3 expression in Smad2 transgenic mice and did not see any alteration (data not shown), indicating the other isoforms of TGF-β may be less sensitive to the level of Smad2 or they are less involved in regulating the fate of epidermal keratinocytes.

A major function of TGF-β is to regulate the expression of genes whose products contribute to building the ECM. TGF-β1 stimulates the production of collagen and fibronec-

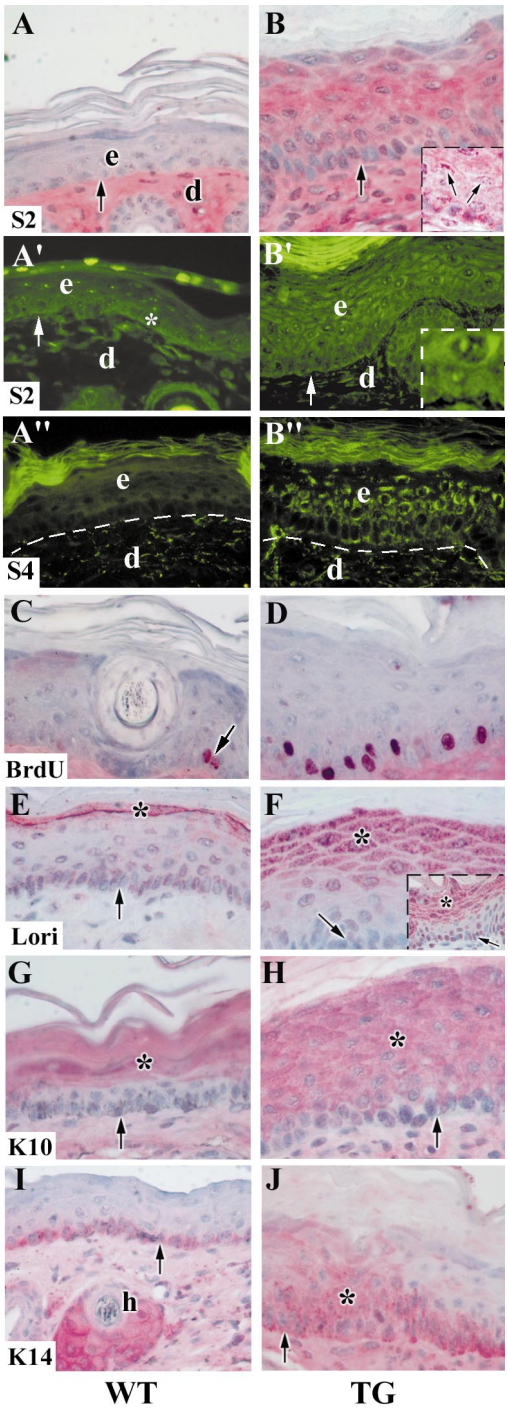
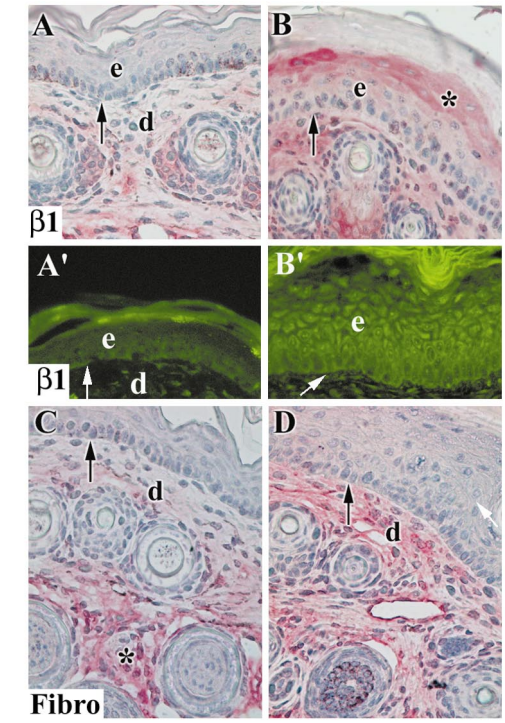
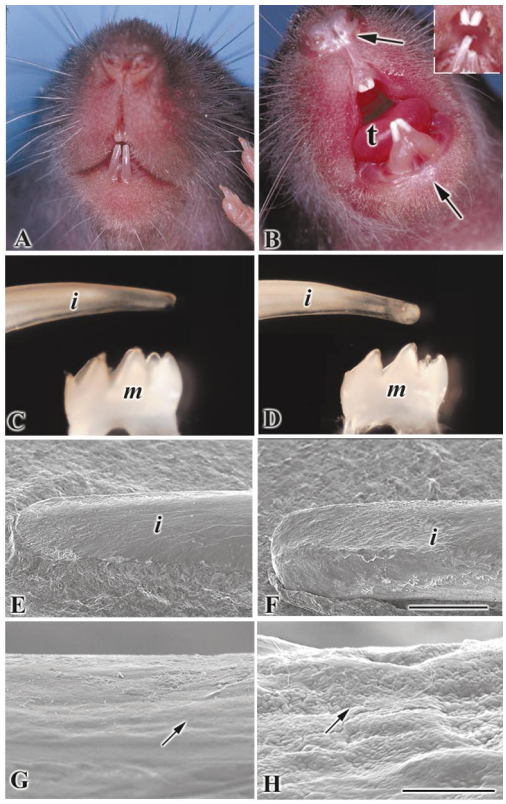


FIG. 5. Overexpression of Smad2 affected proliferation and differentiation of keratinocytes in epidermis of transgenic mice. Positive staining was shown in red. (A) At 2 weeks after birth, endogenous Smad2 (S2) was mainly localized in the dermis (d) and was almost not detectable in the epidermis (e) with the antibody concentration used here. The basement membrane (arrow, and references herein) was well defined in the control tail sample. (B) In the Smad2 transgenic mice, however, Smad2 was highly expressed throughout all layers of epidermis, while its high expression was maintained in the dermis of tail skin. Noticeably, the basement membrane was not well defined in the transgenic sample. Phosphorylated Smad2 was also detected in the nuclei of keratinocyte (arrows) throughout all layers of epidermis (inset). (A′) Smad2 mRNA was present mainly in the basal layer of epidermis (*) and as diffused expression in the dermis of 2-week-old wild-type control sample. (B′) In Smad2 transgenic mice, Smad2 mRNA was detected throughout all layers of epidermis with both cytoplasmic and nuclear localization (see inset). (A″) Very low level of Smad4 mRNA was detected in the suprabasal layer of epidermis (e) of wild-type control sample. Nonspecific signal was observed within stratum corneum. White dotted line marks the basement membrane. (B″) Smad4 mRNA expression expanded throughout all layers of epidermis (especially in suprabasal and granular layers) in transgenic mice. (C) Cell proliferation analysis indicated that basal keratinocytes maintained a low level of DNA synthesis (arrow) in the control sample. (D) In the Smad2 transgenic mice, there was a 3- to 4-fold increase in the proliferation rate of basal keratinocytes. (E) Loricrin (Lori) was localized to one to two layers of granular cells (*) in the control epidermis. (F) In the transgenic sample, loricrin-expressing cells had expanded to five to six layers in the epidermis. When we examined the expression pattern of filaggrin (another granular cell layer specific protein), it showed identical expression pattern (*) in the granular layer comparing to loricrin expression (inset). (G) In the control, cytokeratin 10 (K10) was expressed in the suprabasal layer (*) of epidermis. (H) In Smad2 overexpressing transgenic sample, K10 expression expanded throughout both suprabasal and granular layers of epidermis. (I) Cytokeratin 14 (K14) was only expressed in the basal keratinocytes of epidermis and hair follicles (h) in the control. (J) In the transgenic sample, K14 expression had spread into all layers of epidermis (*).

tin (Ignotz and Massague, 1987; Massague, 1990). *In vitro* study shows that overexpression of Smad2 and Smad3 inhibits adipogenesis (Choy *et al.*, 2000). Within the dermis, long-term stimulation by TGF-β or activin may gradually alter extracellular matrix molecule metabolism and cause the replacement of fatty tissue by connective tissue (Munz *et al.*, 1999). Targeted deletion of one allele of TGF-β prevents the increase of skin thickness and reverses dermal fibrosis in tight skin mice (McGaha *et al.*, 2001). Epidermally synthesized TGF-β1 can certainly reach the dermis and cause such paracrine effects (Fowlis *et al.*, 1992). With the discontinuous basement membrane, as demonstrated by anti-laminin staining (Fig. 4H), it is easier for epidermally overproduced TGF-β1 to reach dermis. Hence, the elevated TGF-β1 expression in the dermis is at least partially responsible for the replacement of fatty tissue by connective tissue (in a cell nonautonomous manner) in K14-Smad2 mice. Further supportive evidence for diffusion of TGF-β1 into the underlying mesenchyme in Smad2 transgenic mice comes from the severely hypertrophic ear cartilage, which is a known effect of TGF-β or activin (Quatela *et al.*, 1993; Munz *et al.*, 1999).

Activin is a critical regulator for keratinocyte proliferation and differentiation as well as in dermal fibrosis and cutaneous wound repair. Overexpression of activin A in the





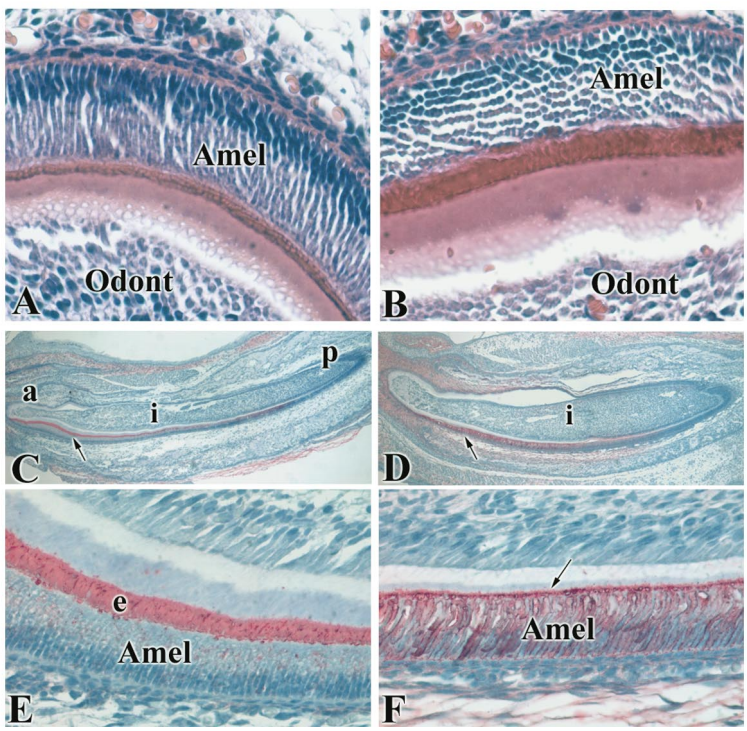


FIG. 6. Overexpression of Smad2 resulted in up-regulation of TGF-β1 and fibronectin expression in dermis of transgenic mice. Positive staining was shown in red. (A) At 2 weeks after birth, TGF-β1 (β1) was mainly localized within the connective tissue under the tail skin. Both epidermis (e) and dermis (d) had very low TGF-β1 expression in wild-type mice. (B) In Smad2 transgenic sample, strong TGF-β1 expression was localized in the epidermis, mainly in the suprabasal layer (*), and dermis. (A′) TGF-β1 mRNA was present (low level) in the

skin using the K14 promoter results in almost identical, although less severe, phenotypes (Munz *et al.*, 1999) when compared with our Smad2 transgenic mice. In this study, we were not able to detect any alteration of endogenous activin expression (mainly due to the nonspecificity of activin antibody). However, given the phenotype similarity between our Smad2 and the activin transgenic mice, we suggest that overexpression of signaling intermediate may, to certain extent, mimic overexpression of ligands which activate Smad2, including both activin and TGF-β. Furthermore, both Smad2 and Smad3 are the cognate Smad proteins downstream of TGF-β as well as activin receptors. Protein structural analysis has revealed, however, that Smad2 and Smad3 behave differently in regulating the transcription of a target gene. Smad3, but not Smad2, alone can stimulate the transcriptional activity of the activin-responsive element derived from *Xenopus* Mix.2 promoter (Nagarajan and Chen, 2000). In human keratinocytes, activin A preferentially activates Smad3 (Shimizu *et al.*, 1998), indicating that Smad3 may more preferentially regulate activin signals. Collectively, this information supports the argument that different Smads may differentially regulate the different members of TGF-β family, as each one of them exerts unique activities in the skin.

In reviewing both the macroscopic and microscopic phenotypes of K14-Smad2 transgenic mice, they were significantly smaller compared to their wild-type littermates at 2 weeks after birth. This might be due to the severely enlarged tongue and epithelial defects in tongue as well as esophagus. The elevated basal keratinocytes proliferation (about threefold increase) activity was associated with the severely flaky skin surface and consistent with the microscopic epidermal hyperkeratosis and acanthosis. While both TGF-β and activin inhibit growth of basal cells, their natural expression in epidermis is predominately in suprabasal differentiating layers, indicating their involvement in maintaining the cessation of growth in the differentiating cells of epidermis (Fuchs, 1990; Hubner *et al.*, 1996). *In vivo* functional studies, however, have revealed that both TGF-β1 and activin A can inhibit or stimulate the proliferation of basal keratinocytes, depending on the level of their expression in the epidermis (Sellheyer *et al.*, 1993; Cui *et al.*, 1995; Fowlis *et al.*, 1996; Munz *et al.*, 1999). The tissue samples presented here were collected from hemizygous Smad2 transgenic mice (by mating transgenic with wild-type mice). When we used transgenic pairs to mate, however, a large number of newborns ($n = 32$, possible homozygous as determined by Southern blot analysis) died at birth with tight skin surface (unpublished data). To date, we have not been able to produce a litter of all transgenic pups by crossing our K14-Smad2 with a wild-type mouse. Microscopic analysis revealed that epidermis was extremely thin with reduced basal cell proliferation in homozygous Smad2 transgenic mice. Clearly, the level of Smad2 expression is critical for mediating the inhibitory or stimulatory function of TGF-β in regulating the proliferation of basal keratinocytes.

Overexpression of Smad2 also altered the program of keratinocytes differentiation. In particular, the boundaries of each epidermal compartment, as defined by using differentiation-specific protein markers, were disturbed and, consequently, there was significant overlap between two adjacent compartments. Keratin 14 expression extended into both suprabasal and granular layers of epidermis, indicating that the keratinocytes enter an accelerated differentiation process as they move toward the epidermal surface while maintaining their basal cell characteristics. Expanded K10 expression was detected in the suprabasal

basal layer of epidermis of control sample. (B′) In Smad2 transgenic sample, TGF-β1 mRNA was present throughout all layers of epidermis, corresponding to the expression pattern of Smad2 transgene. (C) Fibronectin (Fibro) was localized within the connective tissue (*) of wild-type mice. (D) In Smad2 transgenic sample, the expression of fibronectin expanded into the dermis (d). Arrow points to the basement membrane.

FIG. 7. Abnormal upper and lower incisors in Smad2 transgenic mice. (A, C) In the wild-type control mice, incisors (i) and molars (m) were well formed and the enamel surface appeared as shiny and smooth at 2 weeks after birth. (B, D) In Smad2 transgenic mice, incisors (i) appeared to be chalky-white and fragile (chipped easily, see inset in B). The molars of Smad2 transgenic mice had fewer or less developed cusps (m). Smad2 transgenic mice also had severely enlarged tongue (t) that protruded between the upper and lower arch (B). Skin surface of the nose and upper and lower lip appeared shiny (arrow), which resulted from increased thickness of epidermis (especially the stratum corneum). (E, G) When the incisors were examined under scanning electron microscope the labial enamel surface (arrow in G, with higher magnification) of incisor (i) showed a smooth texture in the wild-type mice. (F, H) In Smad2 transgenic mice, the enamel surface of incisor was rough and irregular (arrow). Scale bar = 200 μm in (E) and (F). Scale bar = 10 μm in (G) and (H).

FIG. 8. Alteration of ameloblast organization and enamel matrix formation in Smad2 transgenic animals. (A) At birth, ameloblasts (Amel) were well organized to form enamel matrix, while odontoblasts (Odont) had generated dentine matrix. (B) In the Smad2 transgenic sample, ameloblasts were not organized as well as in the wild-type control. In particular, ameloblasts were not positioned parallel with each other, which had resulted as the multiple cross-section appearance when compared with the control. (C, E) In mandibular cross-section of newborn wild-type mouse, amelogenin was expressed (arrow) in the enamel matrix mainly in the anterior part (a) of the incisor. In the posterior part (p) of the incisor, ameloblasts were going through differentiation and maturation process and were yet to produce amelogenin. At higher magnification (E, 40×), amelogenin was detected in the well-defined enamel matrix (e) at the apical region of the well-organized ameloblasts. (D, F) In Smad2 transgenic mice, amelogenin was not confined within the enamel matrix (arrow) but was in between the less organized ameloblasts (Amel).

and granular layers of epidermis of Smad2 transgenic mice, indicating that keratinocytes entered differentiation process. As we examined the terminal differentiation of keratinocytes by using both loricrin and filaggrin antibodies, we found that the granular layer had expanded to overlap with the suprabasal layer, again indicating an accelerated keratinocyte-differentiation process in Smad2 transgenic mice. These expanded patterns of K14, K10, loricrin, and filaggrin expression are different from the ones in activin-overexpressing transgenic mice, in which K14 and loricrin expression is reduced in epidermis (Munz *et al.*, 1999). Despite the similarity in macroscopic phenotypes between Smad2 and activin A transgenic mice, the alteration of epidermal differentiation was quite different, which might have resulted from the differential regulation of epidermal homeostasis by different members of TGF-β family through the selective activation of Smad2 or Smad3 in transducing TGF-β signaling.

TGF-β controls the proliferation of enamel organ epithelium and regulates ameloblasts differentiation during tooth morphogenesis (Vaahtokari *et al.*, 1991; Coin *et al.*, 1999; Chai *et al.*, 1999). Both Smad1 and Smad2 are present in epithelially derived ameloblast and mesenchymally derived odontoblasts, indicating that both BMP and TGF-β are critical regulators during tooth development (Dick *et al.*, 1998). We have recently shown that both positive and negative TGF-β signaling regulatory Smads are expressed during early tooth development and haploinsufficiency of Smad2, but not Smad3, accelerated TGF-β-mediated tooth development (manuscript under review). Here, overexpression of Smad2 did not block the differentiation of ameloblasts, as demonstrated by the production of amelogenin, but did alter the pattern of ameloblasts assembly, deposition of amelogenin into enamel extracellular matrix, and consequently affected the topography of enamel surface.

We have not observed any skin abnormalities in Smad2 heterozygous mutant mice, which are maintained on the same genetic background as our K14-Smad2 transgenic mice. This is not surprising because about 30% of the $Smad2^{+/-}$ mice died before birth, indicating that the level of Smad2 is critical for the proper TGF-β signaling (Nomura and Li, 1998). Apparently, the $Smad2^{+/-}$ survivors have a sufficient amount of Smad2 to carry out TGF-β-mediated signaling events in the epidermis.

In conclusion, the concerted action of Smad2 and Smad4 may selectively regulate TGF-β-mediated epidermal homeostasis and provide a positive feedback on the endogenous TGF-β1 expression. The biological function of Smad2 in mediating keratinocyte proliferation and differentiation is sensitive to the level of its expression. Our *in vivo* Smad2 functional analysis provides invaluable information toward a better understanding of the complexity of TGF-β-mediated homeostatic equilibrium within the skin.

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