Conditional epidermal expression of TGFβ1 blocks neonatal lethality but causes a reversible hyperplasia and alopecia

Xin Liu, Valarie Alexander, Kinnimulki Vijayachandra, Ervind Bhogte, Ilysa Diamond, and Adam Glick*

Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, MD, 20892

Edited by Francis H. Ruddle, Yale University, New Haven, CT, and approved June 7, 2001 (received for review January 10, 2001)

To study the role of transforming growth factor type β1 (TGFβ1) in epidermal growth control and disease, we have generated a conditional expression system by using the bovine keratin 5 promoter to drive expression of the tetracycline-regulated transactivators tTA and rTA, and a constitutively active mutant of TGFβ1 linked to the tet*O* target sequence for the transactivator. This model allows for induction or suppression of exogenous TGFβ1 with oral doxycycline. Maximal expression of TGFβ1 during gestation caused embryonic lethality, whereas partial suppression allowed full-term development with neonatal lethality characterized by runting, epidermal hypoproliferation, and blocked hair follicle growth. With complete suppression, phenotypically normal double transgenic (DT) mice were born. Acute induction of TGFβ1 in the epidermis of adult mice inhibited basal and follicular keratinocyte proliferation and reentry of telogen hair follicles into anagen. However, chronic expression of TGFβ1 in adult DTs caused severe alopecia characterized by epidermal and follicular hyperproliferation, apoptosis, as well as dermal fibrosis and inflammation. Readministration of doxycycline to tTA DT mice caused hair regrowth within 14 days. The mRNA and protein for Smad7, an inhibitor of TGFβ signaling, were up-regulated in the epidermis and hair follicles of alopecic skin and rapidly induced in rTA mice in parallel with the TGFβ1 transgene, suggesting that the hyperproliferative phenotype may result in part from development of a sustained negative feedback loop. Thus, this conditional expression system provides an important model for understanding the role of TGFβ1 during development, in normal skin biology, and in disease.

Transforming growth factor type β1 (TGFβ1) is a member of a large family of multifunctional secreted polypeptides that are potent regulators of cell growth, differentiation, and matrix production (1). In the mouse skin, members of the TGFβ superfamily are involved in complex biological processes such as the development and normal cycling of the hair follicle, wound healing, and response to tumor promoters (2–4). In the mouse multistage skin carcinogenesis model, inhibition of TGFβ signaling either through reduced expression of the ligand or inactivation of receptor signaling leads to rapid malignant progression (5–7). However, TGFβ1 also may act to promote cancer progression (8), possibly by acting both as an angiogenesis factor (6) as well as an inducer of an epithelial to mesenchymal conversion in malignant tumor cells (8). In human skin, elevated expression of TGFβs are associated with fibrotic skin diseases such as scheroderma, keloids, and hypertrophic scar formation (9–11). Thus the level of TGFβ expression, and the differentiation and neoplastic state of the responding cell, may play a key role in determining the biological response.

Several transgenic mouse models have been generated in which TGFβ1 or TGFβ family members have been targeted to the skin with different keratin or structural gene promoters. These models have produced distinct phenotypes ranging from inhibition of tumor promoter-induced proliferative stimulation (12–14), neonatal lethality, epidermal and follicular hypoproliferation (15, 16), and epidermal hyperproliferation, inflammation, and hair loss (16). Taken together these results suggest that spatial and quantitative differences in expression of TGFβ1 or related molecules can produce significantly different phenotypes in the epidermis. Bitransgenic mice capable of conditional expression of target genes are useful models to overcome neonatal lethality and address the questions involving timing and levels of expression. Such a system has been generated for TGFβ1 by using a loricrin promoter-driven modified progesterone receptor as the transactivator. In this case, induction of TGFβ1 in the skin of adult mice with an antiprogestin blocks phorbol 12-myristate 13-acetate-induced hyperplasia (17). To temporally and quantitatively regulate expression of active TGFβ1 in the epidermis, we have generated transgenic lines in which expression of the tetracycline-regulated transactivators tTA or rTA is targeted to the basal layer of the epidermis and hair follicles with the keratin 5 promoter (18). These mice have been crossed with a second transgenic line expressing a tet*O*-linked active mutant of TGFβ1 (19). In K5/tTA double transgenic (DT) mice, doxycycline suppresses expression of the TGFβ1 transgene, whereas doxycycline induces expression in the K5/rTA (DT) mice. Here we show that induction of active TGFβ1 during development causes epidermal growth inhibition and lethality in the neonate, but hyperplasia and alopecia in the adult.

Materials and Methods

Generation of Transgenic Mice. Mice transgenic for K5/tTA and K5/rTA transgenes were generated as described (18). The tetOTGFβ1 construct was made by subcloning a *Bgl*II fragment containing the constitutively active Cys-Ser$^{223–225}$ double mutant of the porcine TGFβ1 cDNA (19) into a modified plasmid UHD10–3 (20) containing a heptameric tetO sequence, cytomegalovirus minimal promoter, and a β-globin intron. Transgenic mice containing the tet*O*TGFβ1 construct were generated by using standard techniques (21), and founders were identified by Southern blot analysis of tail DNA (18) with the porcine TGFβ1 cDNA as a hybridization probe. To identify inducible founder lines, keratinocytes were isolated from newborn transgenic mice (22), transfected with the K5/tTA plasmid by using Lipofectamine Plus (GIBCO), and serum-free conditioned media made in the presence or absence of 2 ng/ml doxycycline was assayed for the presence of active TGFβ1 with CCL64 PAIL cells as indicator cells (23). Offspring of founders and crosses with the transactivator lines were genotyped by using the following

DEVELOPMENTAL BIOLOGY

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: TGFβ1, transforming growth factor type β1; NT, nontransgenic; ST, single transgenic; DT, double transgenic; RT-PCR, reverse transcriptase–PCR; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

*To whom reprint requests should be addressed at: Building 37 3B26, National Cancer Institute, Bethesda, MD 20892. E-mail: glicka@dc3a.nci.nih.gov.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PCR primers for the tet*O*TGFβ1 transgene: 5′-GTGCTAATGCTGGAAAGCGGC and 5′-CTCCGGTGAGTCACTGGGCGC, and for the tTA or rTA transgene: 5′-CTCGCCCAGAAGCTAGGTGT and CCATCGCGATGACTTAGT.

Induction of TGFβ1 Expression. To suppress TGFβ1 expression, pregnant females from crosses of BK5/tTA × tetOTGFβ1 mice and adult animals were maintained on 10 μg/ml doxycycline in drinking water containing 5% sucrose or on 200 mg/kg doxycycline chow (Bioserv, Frenchtown, NJ). Pregnant females were placed on 1–5 μg/ml doxycycline to achieve partial suppression during gestation. To induce TGFβ1 expression, mice were removed from doxycycline water or chow. Adult BK5/rTA DT were fed 1 g/kg doxycycline chow to induce TGFβ1 expression. To induce the hair cycle in telogen skin, adult K5/rTA DT and single transgenic (ST) mice were shaved, and hair regrowth was monitored for 48 h. Animals without hair regrowth were considered to be in telogen. Liquid Zip wax was applied to the shaved dorsal skin and when hardened peeled off to remove hair follicles (24). To block proliferation, mice were fed doxycyline chow 24 h before depilation. To induce TGFβ1 expression *in vitro* K5/rTA DT keratinocytes were treated with 2 μg/ml doxycycline, whereas K5/tTA DT keratinocytes initially cultured in media containing 2 ng/ml doxycycline were changed to doxycycline-free media.

Quantitation of TGFβ1 Expression. A TGFβ1-specific ELISA (R & D Systems) was used to quantitate levels of total and active TGFβ1 secreted by DT and ST keratinocytes. TGFβ1 was induced by removal of doxycycline for 48 h with the final 24 h in serum-free media.

Total RNA was isolated from keratinocytes and skin by using Trizol Reagent (GIBCO/Life Technologies, Rockville, MD). TGFβ1 mRNA expression was detected by Northern analysis with a ^{32}P-labeled porcine TGFβ1 cDNA or with a semiquantitative reverse transcriptase–PCR (RT-PCR) assay with primers that distinguish porcine TGFβ1 transcript from the endogenous mouse TGFβ1 transcript. Total RNA was treated with RQ1 DNase (Promega) and then 2 μg was reverse-transcribed by using oligo(dT) and superscript RT (GIBCO/Life Technologies). PCR primers specific for porcine TGFβ1 were: 5′-GAAAGCGGCAACCAAATC and 5′-TGACATCAAAGGACAGCCAC.

Additional primers used for RT-PCR were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCCTTCATTGACCTCAACTAC and 5′-CCACCTTCTTGATGTCATCAT-3′, and Tenascin, 5′-CCATCGCCACCAAGTTTACC 3′ and 5′-AACAATCCATCCACCTCCATC-3′.

All RT-PCRs were done for 35 cycles.

Measurement of Cell Proliferation and Apoptosis. Cell proliferation was measured by using an anti-BrdUrd mAb (Becton Dickinson) or with anti-Ki67 staining (Neomarkers, Fremont, CA) after antigen retrieval in a 10 mM sodium citrate buffer (BioGenex Laboratories, San Ramon, CA).

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) was performed with a TACS 2 TdT-Blue label kit (Trevigen, Gaithersburg, MD) according to the manufacturer's specifications, with Mn^{+2} as the divalent cation in the labeling reaction mix. Labeled sections were counterstained with Nuclear Fast red. Labeling was not observed in the absence of terminal deoxynucleotidyltransferase. Cell death was detected with the CellTiter 96 kit (Promega). Apoptosis was quantified with annexin V-biotin (Amersham Pharmacia) followed by strepavidin-APC (Amersham Pharmacia), with propidium iodide (5 μg/ml) according to the manufacturer's specifications. Stained cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson). To assay nuclear condensation cells were stained with 1.5 μg/ml 4′,6-diamidino-2-phenylindole and visualized with a Leitz DMRB fluorescent microscope.

Immunolocalization and Western Blot Analysis. Immunohistochemical detection of keratins was done with monospecific polyclonal antibodies (Babco, Richmond, CA) on 70% ethanol fixed sections. For double indirect immunofluorescence, antibodies specific for tenascin-c or type IV collagen (Chemicon) were detected with a Texas red-labeled anti-rabbit antibody followed by guinea pig anti-keratin 14 and FITC-labeled secondary antibody. Protein extracts were isolated from whole skin by using a 1% Triton X-100 lysis buffer and protease inhibitors. Smad7 protein was detected in immunoblots and ethanol-fixed sections by using anti-Smad7 antisera (C-20 and N-19) (Santa Cruz Biotechnology).

Results

Regulation of TGFβ1 Expression in DT Keratinocytes by Doxycycline. To create a conditional expression system targeting TGFβ1 to the basal layer of the mouse epidermis, we produced transgenic mice in which the Cys-Ser$^{223-225}$ constitutively active mutant of porcine TGFβ1 (19) was linked to the tet*O* recognition site for the tetracycline-regulated tTA and rTA (20, 25). An inducible TGFβ1 founder line was crossed with transgenic lines expressing the tTA or rTA linked to the keratin 5 promoter (26). Transactivation by these tTA and rTA lines is confined to keratin 5-expressing epithelia, predominantly in the basal layer of the epidermis and outer root sheath of the hair follicle (18). Under conditions of maximal suppression (2 ng/ml doxycline for K5/tTA, without doxycycline for K5/rTA), primary newborn DT keratinocyte cultures did not express detectable levels of porcine TGFβ1 mRNA. When doxycycline was removed or added to the K5/tTA DT or the K5/rTA DT keratinocytes, respectively, there was a significant induction of porcine TGFβ1 mRNA without effect on control genotypes (Fig. 1*A*). Secretion of both total and active TGFβ1 in the DT keratinocytes after removal of doxycycline from 39 ± 1.2 pg/ml per 1 × 10^6 cells and 3.1 ± 1 pg/ml per 1 × 10^6 cells, respectively, to 4 ng/ml per 1 × 10^6 cells and 865 pg/ml per 1 × 10^6 cells (Fig. 1*B*). The percent of active TGFβ1 increased from 7.6% to 21%. The uninduced levels of TGFβ1 in the DT were similar to that of controls, and in these genotypes removal of doxycycline had no significant effect on secreted TGFβ1 levels. In rTA DT mice treated with doxycycline chow, porcine TGFβ1 mRNA was detected within 24 h and reached a maximum within 48 h (Fig. 1*C*). In the K5/tTA DTs, a significant increase in TGFβ1 mRNA expression was not detected until 3 weeks after removal of doxycycline chow, most likely due to the time required for clearing of doxycyline from the animal (Fig. 1*D*). These results show that both *in vitro* and *in vivo* the TGFβ1 transgene can be tightly and rapidly regulated by doxycycline in DT animals.

Modulation of TGFβ1 Effects During Development with Doxycycline Levels. To maximally induce TGFβ1 expression during development, mating pairs of ST K5/tTA and tet*O* TGFβ1 mice were maintained without doxycycline. Under these conditions no DT mice were obtained from 30 mice born. This embryonic lethal phenotype could be variably modified by using different doxycycline concentrations. Doxycycline at 1–5 μg/ml, previously shown to cause 25–50% suppression of target gene expression (18), allowed full-term development of DT mice, but these animals were born dead or died shortly after birth and were runted with a shiny taught erythemic skin (Fig. 2*A*). The skin of affected newborns had a thinner epidermis than that of ST or nontransgenic (NT) littermates, with a reduced number of cornified layers, and hair follicle density, whereas the dermis was hypervascular (Fig. 5 *A* and *B*, which is published as supplemental material on the PNAS web site, www.pnas.org). Proliferation

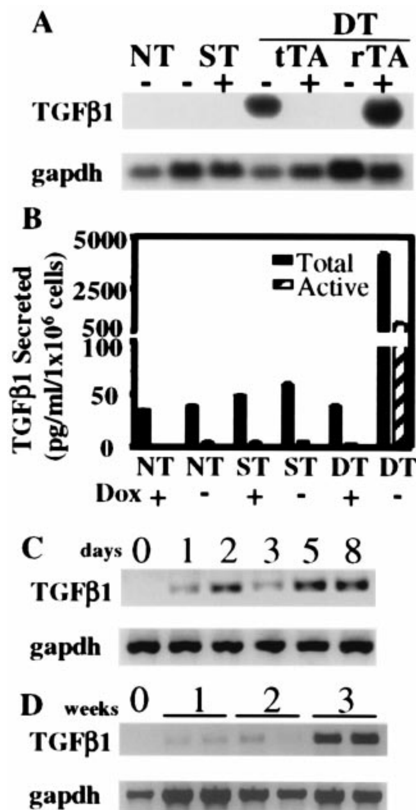

Fig. 1. Conditional regulation of TGFβ1 expression *in vitro* and *in vivo* by doxycycline. (*A*) Northern blot detection of TGFβ1 mRNA induction *in vitro* after removal or addition of doxycycline. (*B*) Detection of total and active TGFβ1 secreted from primary K5/tTA DT, ST and NT keratinocytes by ELISA. (*C*) RT-PCR detection of TGFβ1 mRNA induction by doxycycline in RNA isolated from skin of adult K5/rTA DT mice. (*D*) RT-PCR detection of TGFβ1 mRNA induction by removal of doxycycline in RNA isolated from skin of adult K5/tTA DT mice.

was markedly reduced in the DT epidermis (Fig. 5 *C* and *D*), with no significant alteration of keratins 1, 5, or 10 or loricrin. As seen previously in HK.1-TGFβ1 newborn trangenics (15), keratin 6 was specifically increased in the spinous layers of the DT epidermis (Fig. 5 *E* and *F*), possibly reflecting an alternative pathway of keratinocyte differentiation induced by TGFβ1. When tTA mating pairs were maintained on 10 μg/ml or higher doxycycline, or rTA kept in the absence of doxycycline, phenotypically normal DT mice were born with the expected Mendelian ratio of 25%.

Acute Expression of TGFβ1 in the Adult Epidermis Blocks Cell Proliferation During Induction of Hair Cycle. To test whether induction of active TGFβ1 could inhibit cell proliferation in the adult epidermis, the hair cycle was synchronously initiated by depilation of telogen follicles of rTA bigenic and ST mice in the presence and absence of doxycyline, and cell proliferation was measured by using BrdUrd injection and anti-BrdUrd immunohistochemistry. Consistent with previous studies (27), 24 h after depilation, the percentage of BrdUrd-positive cells in the basal layer of the epidermis increased from 2.5% to 30% in both ST and DT mice kept in the absence of doxycycline (Table 1). However, in DT animals in which TGFβ1 had been induced by doxycycline 24 h before depilation, the percentage of BrdUrd-positive cells was only 5.5, significantly reduced compared with the untreated DT animals ($P = 0.048$). Cell proliferation was completely blocked in hair follicles of DT animals dosed with doxycycline for as long as 72 h after initiation of the hair cycle. Thus, addition of doxycycline to rTA DT mice causes rapid induction of TGFβ1 expression with an immediate inhibition of cell proliferation.

Chronic Expression of TGFβ1 in the Adult Epidermis Causes Alopecia and Fibrosis That Is Reversible by Doxycycline. To examine the long-term consequences of TGFβ1 expression in the adult epidermis, phenotypically normal DT weanlings under conditions of maximal suppression were removed from doxycycline (K5/tTA) or dosed with doxycycline (K5/rTA). Within 3–4 weeks profound phenotypic changes occurred in the epidermis consisting of progressive hair loss, hyperkeratosis, and a dermatitis that initially appeared on the scalp and shoulders as well as the ventral surface, and extended caudally with time (Fig. 2 *B* and *C*). This phenotype took several weeks longer in the K5/rTA mice, possibly reflecting lower induction of target genes compared with the K5/tTA line (18), but was never observed in tet*O*TGFβ1 ST animals in the presence or absence of doxycycline, or in suppressed DT animals.

The epidermis of affected mice was acanthotic and hyperplastic with a variable neutrophilic inflammatory infiltrate characterized by microabscesses within and above the cornified layers. Although there was a significant reduction in the hair follicle density, many follicles appeared hyperplastic with abnormal morphologies (Fig. 2 *E* and *F*). In addition there was a significant thickening and increased cellularity in the dermis associated with increased extracellular matrix gene expression and protein deposition around the hair follicles (Fig. 6 *G–J*, which is published as supplemental material on the PNAS web site). Expression of the basal cell markers keratins 5 and 14 was not altered, but there was focal loss of expression of the suprabasal keratins K1 and K10 in the DT animals, and keratin 6, which is normally expressed only in hair follicles was expressed throughout all layers of the DT epidermis (Fig. 6 *C* and *D*). To test whether the alopecia phenotype depended on continued expression of TGFβ1, affected K5/tTA DT mice were retreated with doxycycline. At doses above 5 μg/ml doxycycline for 1 week, the hyperplastic, hyperkeratotic phenotype was reversed (Fig. 2 *G* and *H*), correlating closely with suppression of TGFβ1 mRNA levels (Fig. 2*I*), and within 14 days, nearly complete regrowth of hair occurred (Fig. 2*D*).

Overexpression of TGFβ1 Causes Increased Proliferation and Apoptosis Accompanied by Sustained Induction of Smad7. Cell proliferation in the hyperplastic epidermis of the DT mice was significantly elevated (Fig. 3*A*), with the percent BrdUrd-positive cells in the basal layer 14.6 ± 5.2 ($n = 9$) compared with 2.1 ± 0.29 ($n = 8$) in the ST mice ($P = 0.016$). Additionally, virtually all hair follicles had proliferating cells in the outer root sheath of the hair follicle particularly in the infundibular region, whereas this was rarely seen in ST mice even in hair follicles in the anagen, or active growth phase.

In addition, significant numbers of TUNEL-positive cells were present in the suprabasal layers of the interfollicular epidermis and in two distinct regions of the hair follicle: the central inner root sheath in the distal follicle, and the inner and outer root sheath in the bulge/isthmus region near the sebacious gland (Fig. 2). TUNEL-positive cells were detected only infrequently in the interfollicular epidermis of control skins. In telogen follicles of nonlesional back skin of the DT animals, there was significant increase in the number of follicles that had five or more apoptotic nuclei compared with ST animals (Fig. 3*A*). Doxycyline treatment of primary rTA DT keratinocytes caused a growth arrest and induced markers of apoptosis (Fig. 3 *B–E*), indicating that induction of apoptosis was a direct effect of TGFβ on the keratinocytes.

To understand the mechanism underlying this hyperproliferative phenotype we examined expression of inhibitors of the

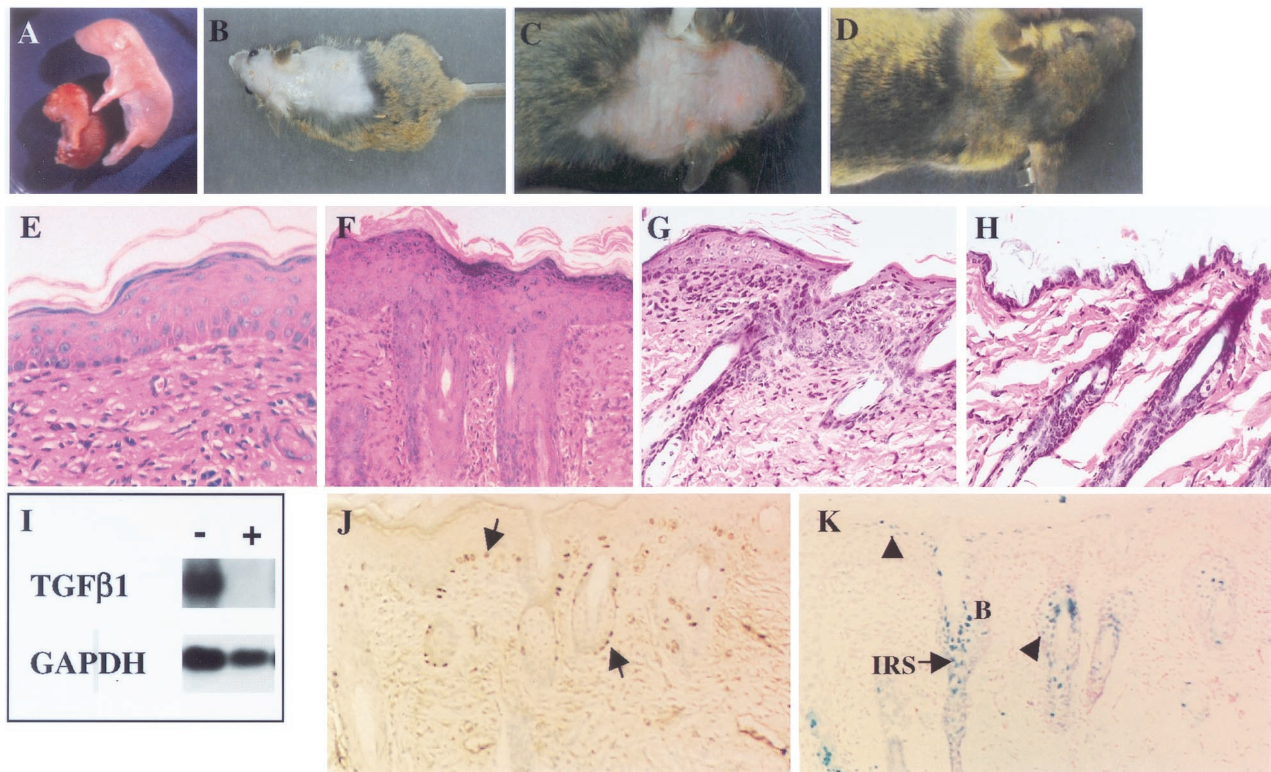

Fig. 2. Neonatal hypoplasia and adult hyperplasia caused by conditional expression of TGFβ1. (*A*) Neonatal runting and erythemic skin phenotype of DT animals produced by partial suppression of TGFβ1 expression by oral dosing of pregnant females with 1 μg/ml doxycycline. (*B*) Alopecia in K5/rTA DT mice 1.5 months after dosing with 1 g/kg doxycycline chow. (*C*) Alopecia in a K5/tTA DT mouse 3 weeks after removal from doxycycline. (*D*) Reversal of alopecia of mouse in *B* after dosing with doxycycline for 14 days. (*E* and *F*) Hematoxylin/eosin-stained sections of dorsal epidermis from K5/rTA DT mice exhibiting alopecia. (*G*) Early lesion in epidermis of hematoxylin/eosin-stained sections of K5/tTA DT mouse. (*H*) Reversal of hyperplastic and hyperkeratotic phenotype in K5/tTA DT mouse after 1 week of treatment with 20 μg/ml doxycycline. (*I*) Northern blot analysis of adult skin RNA showing suppression of porcine TGFβ1 expression after treatment of alopecic mice with 20 μg/ml doxycycline for 1 week. (*J*) Elevated cell proliferation in the basal layer and hair follicles of DT skin detected by anti-BrdUrd immunohistochemistry. (*K*) Elevated apoptosis in the dorsal skin of DT mice detected by using *in situ* TUNEL assay. Arrows, BrdUrd-positive nuclei; arrowheads, TUNEL-positive nuclei. IRS, inner root sheath; B, bulge/isthmus

TGFβ signaling pathway. Smad7 is a TGFβ-inducible gene that specifically blocks phosphorylation of Smad2 and Smad3 by the activated type 1 receptor and inhibits TGFβ signaling (28). Fig. 4 shows that Smad7 mRNA and protein was highly expressed in the DT alopecic skin compared with control, and this increased expression was localized to the epidermis and hair follicles. In the rTA mice induction of Smad7 occurred within 2–3 days after treatment with doxycycline, closely after TGFβ1 induction, indicating that *in vivo* this gene is also a primary target of TGFβ1.

Discussion

Here we describe a conditional expression system in which the onset and expression level of TGFβ1 in the epidermis can be controlled by doxycycline. For tet*O*TGFβ1 ST animals or K5/tTA or rTA DT mice kept in the presence or absence of doxycycline, respectively, there was negligible background expression of porcine TGFβ1 mRNA and no discernible phenotype. Modulation of the level and timing of TGFβ1 expression with doxycycline produced phenotypes that ranged from embryonic and neonatal lethality, coupled with epidermal and follicular hypoplasia, to alopecia, associated with fibrosis and epidermal and follicular hyperplasia. The finding of fibrotic changes in the DT skin is not surprising because TGFβ1 directly regulates extracellular matrix gene expression (1) and overexpression in other transgenic models as well as human diseases is associated with fibrosis (9, 29). Alopecia has not been observed in previous skin targeted TGFβ1 transgenic mice possibly because of low expression levels required for normal development or due to use of suprabasal keratin promoters (12, 13). By controlling expression levels we have produced a phenotype with considerable similarity to K10-BMP6 transgenic mice, which display a progressive alopecia and dermatitis (16) and K14-activin βA transgenic mice, which exhibit epidermal hyperplasia, altered differentiation, and changes in dermal matrix protein expression (30). Thus, this single model encompasses the diverse epidermal phenotypes previously generated by overexpression of TGFβ superfamily members under the monotonic control of different keratin promoters (15, 16, 30). Although these growth factor families interact with distinct surface receptors (31), our results indicate that the diverse phenotypes observed in the transgenic models result from specific levels and timing of transgene

Table 1. Cell proliferation blocked by TGFβ1 after initiation of hair cycle

Genotype	Plucking	Dox	% BrdUrd labeled
ST	−	−	2.5 ± 0.07 (n = 2)
ST	+	−	30 ± 4.7* (n = 3)
DT	+	−	29 ± 8.8 (n = 2)
DT	+	+	5.5 ± 2.4† (n = 3)

Telogen phase K5/rTA DT mice were treated with 1 g/kg doxycycline chow 24 h before initiation of hair cycle with wax depilation, and cell proliferation was measured 24 h later by 1-h injection of BrdUrd and immunohistochemistry on skin sections. n = number of animals analyzed.

*Significantly different from ST not plucked, P = 0.02.

†Significantly different from DT without doxycycline, P = 0.048.

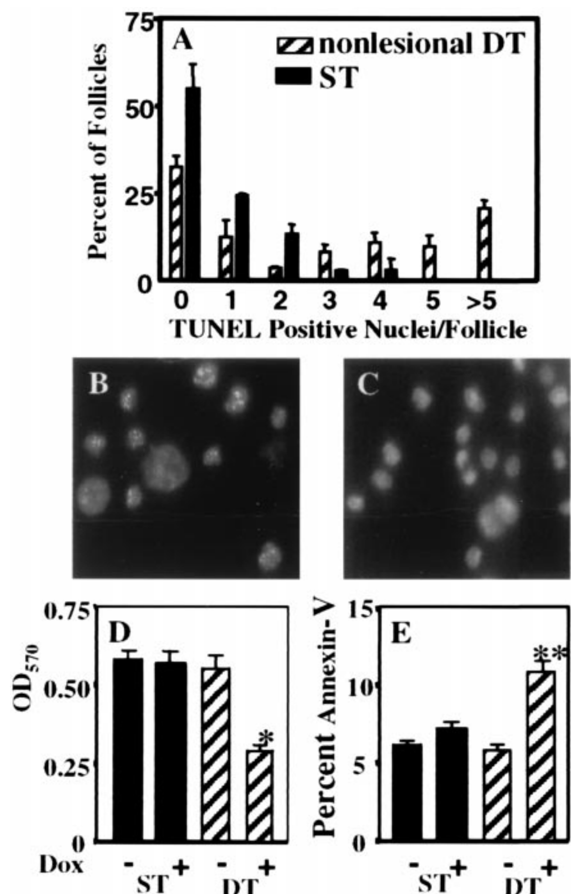

Fig. 3. Apoptosis precedes alopecia and is induced by TGFβ *in vitro*. (*A*) Quantitation of the frequency of hair follicles with the indicated number of TUNEL-positive nuclei in telogen hair follicles from ST and DT mice before onset of alopecia. $n = 3$ for DT, 2 for ST at least 50 follicles counted per mouse. (*B–D*) TGFβ1 was induced in K5/rTA DT and ST primary keratinocytes by addition of 1 μg/ml doxycycline for 48 h. (*B*) 4′,6-Diamidino-2-phenylindole staining showing nuclear condensation in DT with doxycycline (*B*) and without doxycycline (*C*). Quantitation of increased cell death in K5/rTA DT keratinocytes with the MTT assay at 48 after addition of doxycycline. Results are average of two assays done in quadruplicate. (*D*) Increased annexin-V biotin binding of K5/rTA DT but not control keratinocytes 48 h after addition of doxycycline. Results are average of two experiments. *, Significantly different from DT-doxycycline and ST, $P < 0.0001$. **, Significantly different from DT-doxycycline, $P = 0.001$ using unpaired *t* test.

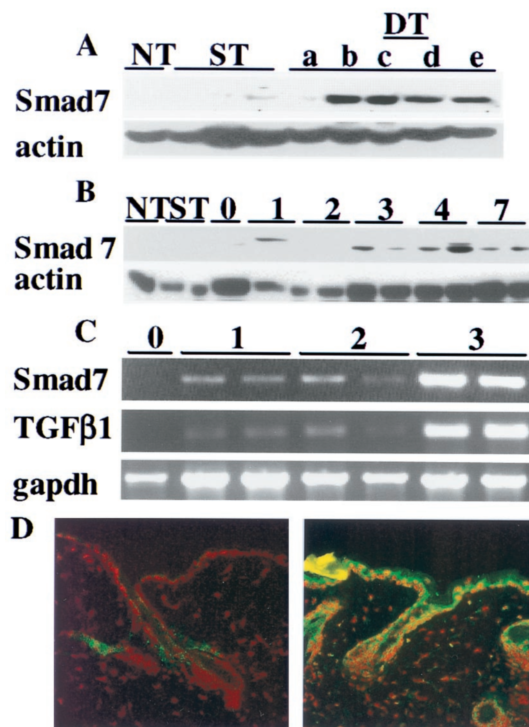

Fig. 4. Rapid and sustained induction of Smad7 by TGFβ *in vivo*. (*A*) Western blot analysis of Smad7 expression in skin extracts from individual mice of the following genotypes: NT, ST, K5/tTA DT with doxycycline (lane a), and alopecic skin of K5/tTA DT without doxycycline (lanes b–e). (*B*) Western blot of skin extracts from NT, ST, or rTA DT at the indicated days after dosing with 200 μg/ml doxycycline. (*C*) RT-PCR analysis of Smad7 and TGFβ1 mRNA levels in RNA isolated from skin of tTA DT mice at the indicated weeks after removal of doxycycline. (*D*) Indirect immunofluorescent localization of Smad7 expression to the epidermis and hair follicles of ST (*Left*) and K5/tTA DT skin (*Right*). No staining was observed in the absence of primary antibody.

expression but reflect activation of an overlapping set of genes and biological responses in keratinocytes and fibroblasts.

Our results highlight the importance of the hair follicle as a target for TGFβ1 action in the epidermis and demonstrate within a normal tissue the ability of TGFβ1 signaling to produce opposing outcomes. Addition of doxycycline to K5/rTA DT mice induced TGFβ1 in the epidermis within 24 h, which was sufficient to inhibit the depilation-induced increase in cell proliferation in both the interfollicular epidermis and hair follicle, and completely block entry of telogen follicles into a new hair cycle. These results demonstrate the utility of the rTA system for rapid induction of target gene expression and, as observed in other transgenic models (12–14, 17), show that overexpression of TGFβ1 acts to inhibit an acute proliferative stimulus. Overexpression of TGFβ1 in the newborn mouse also blocked epidermal and hair follicle cell proliferation and inhibited normal hair follicle morphogenesis consistent with the importance of this signaling pathway in hair follicle development and the early expression of the type II receptor (32), and activation of the K5 promoter between days 11.5 and 13 of gestation (33).

Hair loss in the adult mouse proceeded in an anterior to posterior direction, paralleling the direction of the hair cycle. However despite inhibitory effects on hair development and hair cycling there was elevated proliferation in the basal layer of the epidermis and outer root sheath of hair follicles in the hairless epidermis. There is also elevated basal cell proliferation in K14-activin βA (30), K10-BMP6 transgenic mice (16), and a subset of K6-TGFβ1 and K10-TGFβ1 transgenic lines, which do not have an overt skin phenotype (12, 13), suggesting that this is a consistent and relevant tissue response to overexpression of TGFβ superfamily members in the epidermis. The finding of increased apoptosis in hair follicles of DT animals in the posterior dorsal epidermis before any overt phenotypic changes or hair loss and of apoptosis in primary cultures of DT keratinocytes indicates that this is a primary effect of TGFβ1 that is upstream of the alopecia phenotype, and not simply a secondary result of a inflammation or other pathological changes in the skin. In anagen of a normal hair cycle, apoptotic cells are located in the central inner root sheath and the bulge/isthmus region (34), strikingly similar locations to that observed in follicles of DT animals. These results not only support the concept that one of the major functions of TGFβ1 is the regulation through apoptosis of the anagen-catagen transition during the hair cycle (2), but also suggest that dysregulation of this process can lead to a hyperproliferative phenotype. The reversibility of hair loss in this model suggests that essential cellular components for hair

follicle formation such as the stem cells are resistant to the apoptotic effects of TGFβ1 and can regenerate a functional hair follicle once TGFβ1 expression is suppressed. Given the strong growth inhibitory effects of TGFβ1 on keratinocytes, it seems unlikely that TGFβ1 becomes a direct mitogen in this setting, but rather that growth inhibition by TGFβ1 is blocked. In support of this concept we find that Smad7, an inhibitory Smad that is a direct target of TGFβ1 *in vitro* (28), is rapidly induced *in vivo* in the epidermis and hair follicles after activation of transgene expression with doxycycline. Although more direct studies are necessary, these results suggest that a Smad7-mediated negative feedback loop is generated in the epidermis by continuous expression of TGFβ1, which blocks some of the autocrine functions of TGFβ1 on keratinocytes. Interestingly, in some epithelial cells Smad7 potentiates apoptosis mediated by TGFβ and tumor necrosis factor α (35, 36), although an inhibitory effect also has been observed. Smad7 also is induced by inflammatory cytokines (37, 38) therefore, it is possible that the influx of inflammatory cells in the epidermis of the DT animals perpetuates this inhibitory loop. In contrast, paracrine effects of keratinocyte produced TGFβ1, such as those regulating matrix production in the dermal fibroblasts are not affected. Because Smad7 is elevated in chemically induced mouse skin tumors (42), this type of compartmentalized negative feedback loop may have relevance for differential effects of TGFβ on mesenchymal and epithelial cells in tumor progression. The inducible and reversible nature of this model should allow detailed analysis of the sequential molecular and cellular changes associated with overexpression of TGFβ1 during development, and in normal and pathological conditions of the adult epidermis.

We thank Ms. Katy Bernhard and Katherine Lucas for excellent care and maintenance of transgenic animals, Ms. Susan Garfield for assistance with confocal microscopy, Ms. Jessica Lee for help with genotyping, Dr. Paturu Kondaiah for the gift of pPK9 containing the active TGFβ1 mutant, Dr. Jose Jorcano for the keratin 5 promoter, and Dr. Stuart Yuspa for critical reading of the manuscript.

1. Roberts, A. B. & Sporn, M. B. (1990) in *Handbook of Experimental Pharmacology, Vol. 95/I Peptide Growth Factors and Their Receptors I*, eds. Sporn, M. B. & Roberts, A. B. (Springer, New York), pp. 419–472.
2. Foitzik, K., Lindner, G., Mueller-Roever, S., Maurer, M., Botchkareva, N., Botchkarev, V., Handjiski, B., Metz, M., Hibino, T., Soma, T., *et al.* (2000) *FASEB J.* **14,** 752–760.
3. Ashcroft, G. S., Yang, X., Glick, A. B., Weinstein, M., Letterio, J. L., Mizel, D. E., Anzano, M., Greenwell-Wild, T., Wahl, S. M., Deng, C., *et al.* (1999) *Nat. Cell Biol.* **1,** 260–266.
4. Akhurst, R. J., Fee, F. & Balmain, A. (1988) *Nature (London)* **331,** 363–365.
5. Glick, A. B., Lee, M. M., Darwiche, N., Kulkarni, A. B., Karlsson, S. & Yuspa, S. H. (1994) *Genes Dev.* **8,** 2429–2440.
6. Go, C., Li, P. & Wang, X. J. (1999) *Cancer Res.* **59,** 2861–2868.
7. Amendt, C., Schirmacher, P., Weber, H. & Blessing, M. (1998) *Oncogene* **17,** 25–34.
8. Cui, W., Fowlis, D. J., Bryson, S., Duffie, E., Ireland, H., Balmain, A. & Akhurst, R. J. (1996) *Cell* **86,** 531–542.
9. Querfeld, C., Eckes, B., Huerkamp, C., Krieg, T. & Sollberg, S. (1999) *J. Dermatol. Sci.* **21,** 13–22.
10. Lee, T. Y., Chin, G. S., Kim, W. J., Chau, D., Gittes, G. K. & Longaker, M. T. (1999) *Ann. Plast. Surg.* **43,** 179–184.
11. Wang, R., Ghahary, A., Shen, Q., Scott, P. G., Roy, K. & Tredget, E. E. (2000) *Wound Repair Regen.* **8,** 128–137.
12. Cui, W., Fowlis, D. J., Cousins, F. M., Duffie, E., Bryson, S., Balmain, A. & Akhurst, R. J. (1995) *Genes Dev.* **9,** 945–955.
13. Fowlis, D. J., Cui, W., Johnson, S. A., Balmain, A. & Akhurst, R. J. (1996) *Cell Growth Differ.* **7,** 679–687.
14. Blessing, M., Nanney, L. B., King, L. E. & Hogan, B. L. (1995) *Teratog. Carcinog. Mutagen.* **15,** 11–21.
15. Sellheyer, K., Bickenbach, J. R., Rothnagel, J. A., Bundman, D., Longley, M. A., Krieg, T., Roche, N. S., Roberts, A. B. & Roop, D. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5237–5241.
16. Blessing, M., Schirmacher, P. & Kaiser, S. (1996) *J. Cell Biol.* **135,** 227–239.
17. Wang, X. J., Liefer, K. M., Tsai, S., O'Malley, B. W. & Roop, D. R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 8483–8488.
18. Diamond, I., Owolabi, T., Marco, M., Lam, C. & Glick, A. (2000) *J. Invest Dermatol.* **115,** 788–794.
19. Samuel, S. K., Hurta, R. A., Kondaiah, P., Khalil, N., Turley, E. A., Wright, J. A. & Greenberg, A. H. (1992) *EMBO J.* **11,** 1599–1605.
20. Gossen, M. & Bujard, H. (1996) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
21. Hogan, B., Beddington, R., Costantini, F. & Lacy, E. (1994) *Manipulating The Mouse Embryo* (Cold Spring Harbor Lab. Press, Plainview, NY).
22. Dlugosz, A. A., Glick, A. B., Tennenbaum, T., Weinberg, W. C. & Yuspa, S. H. (1995) *Methods Enzymol*. **290,** 3–20.
23. Abe, M., Harpel, J. G., Metz, C. N., Nunes, I., Loskutoff, D. J. & Rifkin, D. B. (1994) *Anal. Biochem.* **216,** 276–284.
24. Stenn, K. S., Paus, R., Dutton, T. & Sarba, B. (1993) *Skin Pharmacol.* **6,** 125–134.
25. Kistner, A., Gossen, M., Zimmermann, F., Jerecic, J., Ullmer, C., Lubbert, H. & Bujard, H. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10933–10938.
26. Casatorres, J., Navarro, J. M., Blessing, M. & Jorcano, J. L. (1994) *J. Biol. Chem.* **269,** 20489–20496.
27. Wilson, C., Cotsarelis, G., Wei, Z. G., Fryer, E., Margolis-Fryer, J., Ostead, M., Tokarek, R., Sun, T. T. & Lavker, R. M. (1994) *Differentiation* **55,** 127–136.
28. Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H., *et al.* (1997) *Nature (London)* **389,** 631–635.
29. Sanderson, N., Factor, V., Nagy, P., Kopp, J., Kondaiah, P., Wakefield, L., Roberts, A. B., Sporn, M. B. & Thorgeirsson, S. S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2572–2576.
30. Munz, B., Smola, H., Engelhardt, F., Bleuel, K., Brauchle, M., Lein, I., Evans, L. W., Huylebroeck, D., Balling, R. & Werner, S. (1999) *EMBO J.* **18,** 5205–5215.
31. Massague, J. (1998) *Annu. Rev. Biochem*. **67,** 753–791.
32. Paus, R., Foitzik, K., Welker, P., Bulfone-Paus, S. & Eichmuller, S. (1997) *J. Invest Dermatol.* **109,** 518–526.
33. Ramirez, A., Bravo, A., Jorcano, J. L. & Vidal, M. (1994) *Differentiation* **58,** 53–64.
34. Lindner, G., Botchkarev, V. A., Botchkareva, N. V., Ling, G., van der Veen, C. & Paus, R. (1997) *Am. J. Pathol.* **151,** 1601–1617.
35. Lallemand, F., Mazars, A., Prunier, C., Bertrand, F., Kornprost, M., Gallea, S., Roman-Roman, S., Cherqui, G. & Atfi, A. (2001) *Oncogene* **20,** 879–884.
36. Landstrom, M., Heldin, N. E., Bu, S., Hermansson, A., Itoh, S., ten Dijke, P. & Heldin, C. H. (2000) *Curr. Biol.* **10,** 535–538.
37. Bitzer, M., von Gersdorff, G., Liang, D., Dominguez-Rosales, A., Beg, A. A., Rojkind, M. & Bottinger, E. P. (2000) *Genes Dev.* **14,** 187–197.
38. Ulloa, L., Doody, J. & Massague, J. (1999) *Nature (London)* **397,** 710–713.
39. He, W., Cao, T., Smith, D. A., Myers, T. E. & Wang, X. J. (2001) *Oncogene* **20,** 471–483.