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Development of gene-switch transgenic mice that inducibly express transforming growth factor β1 in the epidermis

(inducible/growth inhibition/angiogenesis)

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ABSTRACT **Previous attempts to establish transgenic mouse models to study the functions of transforming growth factor β1 (TGFβ1) in the skin revealed controversial roles for TGFβ1 in epidermal growth (inhibition vs. stimulation) and resulted in neonatal lethality in one instance. To establish a viable transgenic model for studying functions of TGFβ1 in the skin, we have now developed transgenic mice, which allow focal induction of the TGFβ1 transgene in the epidermis at different expression levels and at different developmental stages. This system, termed "gene-switch," consists of two transgenic lines. The mouse loricrin vector targets the GLVPc transactivator (a fusion molecule of the truncated progesterone receptor and the GAL4 DNA binding domain), and a thymidine kinase promoter drives the TGFβ1 target gene with GAL4 binding sites upstream of the promoter. These two transgenic lines were mated to generate bigenic mice, and TGFβ1 transgene expression was controlled by topical application of an antiprogestin. On epidermal-specific induction of the TGFβ1 transgene, the BrdUrd labeling index in the transgenic epidermis decreased 6-fold compared with controls. Induction of the TGFβ1 transgene expression also caused epidermal resistance to phorbol 12-myristate 13-acetate-induced hyperplasia, with a reduction in both epidermal thickness and BrdUrd labeling compared with those in controls. In addition, TGFβ1 transgene expression induced an increase in angiogenesis in the dermis. Given that the TGFβ1 transgene can affect both the epidermis and dermis, this transgenic model will provide a useful tool for studying roles of TGFβ1 in wound-healing and skin carcinogenesis in the future.**

Transgenic mice have proven to be a powerful system to study normal and pathological functions of genes. To study functions of genes in the epidermis, we have generated transgenic mouse models by using epidermal-specific vectors (1, 2). Unfortunately, constitutive overexpression of some genes or more than one oncogenic transgene often results in neonatal lethality because of severe epidermal abnormalities (3–5). To circumvent this problem, it was desirable to develop a new transgenic system that allows focal induction of transgenes in the epidermis after topical application of an inducer. To this end, we used the "gene-switch" system (6–8). This gene-switch was developed after discovering that the human progesterone receptor with a carboxy-terminal deletion failed to bind progesterone but bound progesterone antagonists, which paradoxically transactivated progesterone responsive genes (6). By replacing the DNA binding domain of this truncated progesterone receptor with that of the yeast transcription activator GAL4 (progesterone receptor–GAL4), the possibility of simultaneous activation of progesterone responsive genes is eliminated. To increase the transactivational potential, the transactivation domain of the herpes simplex virus protein VP16 was fused to the C-terminal of the progesterone receptor–GAL4 to form the chimeric regulator GLVPc (7). The unique feature of the GLVPc is the exclusive activation of target genes with promoters that contain a consensus GAL4 binding site (17-mer). Because GAL4-activated genes do not exist in mammalian cells, this regulator specifically activates target genes containing GAL4 binding sites in an antiprogestin dose-dependent manner (7). By placing the regulator gene under the control of an epidermal-specific expression vector, and a target gene under the control of a promoter containing GAL4 binding sites, bigenic mice can be produced that express the target gene in the epidermis in response to topical application of an antiprogestin. Unlike other inducible reagents, antiprogestins, such as RU486, do not have toxic effects, even in long term medical use up to 20 mg/kg in humans (9).

The target transgene presented here is the transforming growth factor β1 (TGFβ1). TGFβ1 is a multifunctional cytokine that regulates cell growth, tissue remodeling, and angiogenesis (for reviews, see refs. 10–14). TGFβ1 is secreted in a biologically latent form in which the mature TGFβ1 is noncovalently bound to a dimer consisting of the "latency-associated peptide" (LAP). Activation of TGFβ1 results from dissociation of the mature TGFβ1 from the LAP dimer. Constitutive activation of TGFβ1 has been achieved by expressing a TGFβ1 cDNA with two site-specific mutations of Cys-223 → Ser and Cys-225 → Ser in the LAP ($TGF\beta1^{S223/225}$) (15). These mutations preclude dimerization of the LAP and result in the secretion of active TGFβ1 (15).

An initial attempt to understand functions of TGFβ1 in the skin *in vivo* used a targeting vector based on the human keratin 1 gene (HK1) to overexpress $TGF\beta1^{S223/225}$ in the epidermis of transgenic mice (5). Essentially all basal cell proliferation ceased in the epidermis, which caused neonatal lethality in these mice. Although the HK1.TGFβ1 mice provided important insights into TGFβ1 function in regulating epidermal proliferation, the neonatal lethality of the HK1.TGFβ1 mice precluded their use in investigating functions of TGFβ1 in adults. When the $TGF\beta1^{S223/225}$ transgene was expressed by using the keratin 10 or keratin 6 promoter (16, 17), TGFβ1 transgenic mice were viable. However, in contrast to both previous *in vitro* and *in vivo* reports, these adult transgenic mice showed an increased epidermal mitotic rate with no histological changes (16). These contradictory data highlight the complex nature of TGFβ1 functions, which may depend on expression levels, expression at varying differentiation stages (proliferative vs. differentiated cells), or physiological condi-

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Abbreviations: TGFβ1, transforming growth factor β1; ML, mouse loricrin; TK, thymidine kinase; ZK, the antiprogestin ZK98.734; RPA, RNase protection assay; LAP, latency-associated peptide; HK1, human keratin 1; RT, reverse transcription; PMA, phorbol 12-myristate 13-acetate.

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tions (neonatal vs. adults) of epidermal cells. Therefore, to further delineate the role of TGFβ1 in growth regulation in the epidermis, the ideal transgenic mouse model would allow TGFβ1 induction at different levels and at different developmental stages to avoid the lethal phenotype resulting from high constitutive levels of TGFβ1 in the epidermis. Here we report a transgenic mouse model that inducibly expresses TGFβ1 in the epidermis by using the gene-switch system (6–8). We show that TGFβ1 transgene expression can be induced in the epidermis by topical application of a progesterone antagonist. Induction of the TGFβ1 transgene exhibits a growth inhibitory effect in the epidermis and resistance to phorbol 12-myristate 13-acetate (PMA)-induced hyperproliferation. Additionally, TGFβ1 transgene expression increased angiogenesis in the dermis.

MATERIALS AND METHODS

Construction of Transgenes. To achieve epidermal specificity, the truncated mouse loricrin promoter (ML), derived from the 6.5-kilobase fragment of the mouse loricrin gene (18), was used to drive GLVPc (Fig. 1*A*). Like the HK1 vector, the ML vector drives transgene expression in both the proliferative and differentiated compartments of the epidermis, but at a higher level (2). The ML.GLVPc transgene was sequenced to confirm that the PCR generated product was correct and then released from the pGem7Z-pSL1180 fusion plasmid by digestion with *Bam*HI (Fig. 1*A*). The target transgene was constructed by using the *Bam*HI-*Cla*I fragment of the porcine TGF$\beta1^{S223/225}$ cut from the HK1.TGFβ1 construct (5) and was ligated to the *Bgl*II-*Cla*I sites of the thymidine kinase (TK) promoter, which contains four copies of the 17-mer GAL4 binding site upstream of the promoter (TK.TGFβ1; Fig. 1*B*). The TK.TGFβ1 transgene was released from the pBLCAT cloning vector by partial restriction digestion with *Hin*dIII and *Kpn*I (Fig. 1*B*).

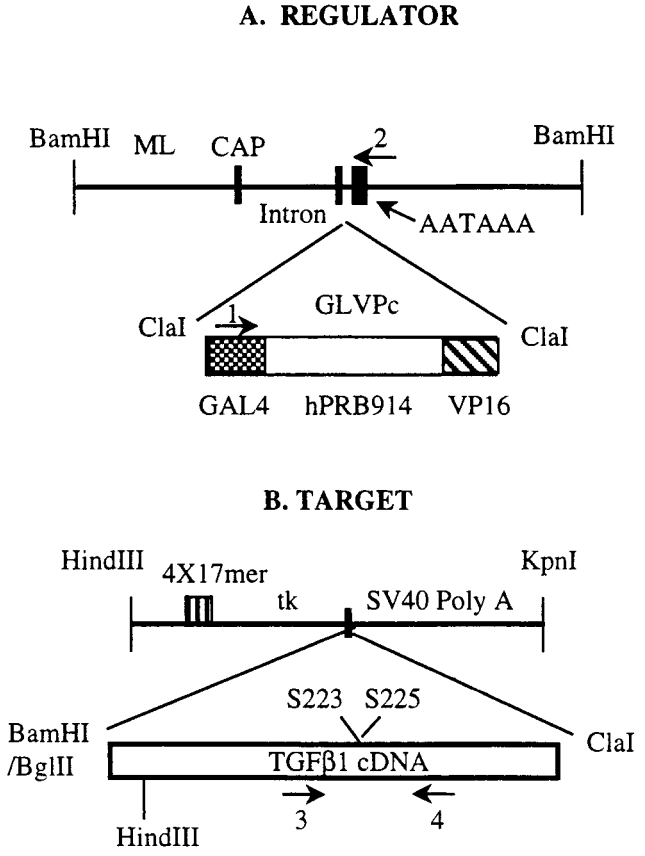

FIG. 1. Constructs of the gene-switch-TGFβ1 transgenes. (*A*) The regulator transgene was inserted into the *Cla*I site of the ML expression vector. The GLVPc regulator comprises the DNA binding domain of the GAL4 activator (residues 1–93), the ligand binding domain of the truncated human progesterone receptor, which lacks 19 amino acids at the C terminus (hPRB914), and the herpes simplex virus VP-16 transactivation domain (residue 411–487). Primers 1 and 2 were used in PCR to identify the GLVPc transgene. (*B*) The target transgene, the porcine TGF$\beta1^{S223/225}$ cDNA, was inserted into a TK promoter with four copies of the 17-mer GAL4 consensus binding sequence situated upstream and the SV40 Poly(A) region at the 3′ terminal of the TK promoter. Primers 3 and 4 were used in PCR to identify the TGFβ1 transgene.

Generation and Identification of the Gene-Switch-TGFβ1 Mice. ML.GLVPc and TK.TGFβ1 transgenes were microinjected into mouse embryos obtained from mating ICR females to strain FVB males. After birth, transgenic mice were confirmed by PCR analysis of tail DNA. The ML.GLVPc transgene was identified by using specific oligos 1 (5′-CCGAAGTGCGCCAAGTGTCT-3′) that recognizes the GAL4 DNA binding domain of GLVPc and 2 (5′-CCAGCTCTGTTGTCTCCGTT-3′) that binds to 3′ end of ML vector (Fig. 1*A*). TK.TGFβ1 transgene was identified by using oligos specific to porcine TGFβ1 (Fig. 1*B*) 3 (5′-GTGGAAAGCGGCAACCAA-3′) and 4 (5′-GGCGAAAACCCTCTATAGCC-3′). The PCR conditions were the same as described (3) except annealing at 60°C for 1 min for TK.TGFβ1 and extension at 72°C for 3 min for ML.GLVPc.

Preparation and Analysis of RNA. The epidermis of newborn pups was separated from the dermis as described (4). The epidermis from the adult ear was separated after incubating the ear biopsy in PBS with 3 mM EDTA at 37°C for 70 min. Total epidermal RNA was isolated with RNAzol B (Tel-Test, Friendswood, TX) as described (4). To determine expression levels of the GLVPc and TGFβ1 transgenes, reverse transcription (RT)–PCR or RNase protection assay (RPA) was performed. In RT-PCR assays, RNA was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (Promega) and pdN6 random primers (Boehringer Mannheim). The resultant cDNA was amplified by PCR by using the primers specific for porcine TGFβ1 (described above). RPA was performed by using an RPA II kit (Ambion, Austin, TX) and a ^{32}P-labeled riboprobe specific for either the VP16 transactivation domain (subcloned into pBS) (Stratagene) or porcine TGFβ1 cDNA (subcloned into pGem3Z) (Promega). To normalize each RNA sample for differences in loading, a ^{32}P- glyceraldehyde-3-phosphate dehydrogenase riboprobe was used. The intensity of protected bands was determined by densitometric scanning of x-ray films.

Tissue Histology, Immunofluorescence, and Immunohistochemistry. Biopsied skins were fixed in 10% neutral-buffered formalin at 4°C overnight, were embedded in paraffin, were sectioned to 6-μm thickness, and were stained with hematoxylin and eosin. Immunofluorescence analysis for keratins 1 (K1), 6 (K6), and 14 (K14), and terminal differentiation markers loricrin and filaggrin were performed on OCT (Miles)-embedded frozen sections as described (4). Immunofluorescence staining and quantitative analysis of vascular density were performed as described (19), using a rat anti-mouse CD31 [platelet/endothelial cell adhesion molecule 1 (PECAM-1)] antibody (PharMingen). Three to five skin samples were analyzed in each group, and the percentage of the dermis covered by vessels is expressed as mean ± SD. As described (19). Immunohistochemistry for TGFβ1 expression was performed on formalin-fixed tissue sections by using the TGFβ1 antibody (named "Karen"; a gift from K. Flanders, National Institutes of Health), which recognizes the LAP of TGFβ1.

BrdUrd Uptake and Staining. Transgenic and control littermates were injected (i.p.) with BrdUrd (Sigma) 125 mg/kg

in 0.9% NaCl and were killed after 1 h. Skin sections were fixed, were processed, and were stained with FITC-conjugated monoclonal antibody to BrdUrd (Becton Dickinson), as described (2). Each tissue section was measured by using a micrometer, and the average number of BrdUrd positive cells/mm epidermis ± SD represents the labeling index.

RESULTS

Generation of Gene-Switch-TGFβ1 Mice. Two separate transgenic lines (c4486 and c4509) carrying ML.GLVPc transgene were confirmed to express the GLVPc regulator by RPA. Both lines exhibited similar transactivational activity: i.e., induction of the TGFβ1 transgene by topical application of the antiprogestin ZK98.734 (ZK) (see below). Three transgenic lines carrying TK.TGFβ1 transgene (c1822, c1849, and c1942) did not express the TGFβ1 transgene, as determined by RT-PCR (data not shown). To generate bigenic TGFβ1 mice that contain both components of the gene-switch, ML.GLVPc mice were mated with TK.TGFβ1 mice (Fig. 1). Bigenic TGFβ1 mice carrying both transgenes were identified by using PCR analyses of tail DNA. None of the bigenic mice, regardless of the parental line, exhibited an abnormal skin phenotype before induction of TGFβ1 expression by ZK application. To determine whether ZK had any adverse effect on the skin before use as the transgene inducer, adult nontransgenic ML.GLVPc and TK.TGFβ1 mice were treated with ZK topically, 10 μg, five times a week for 4 months. No obvious changes in the skin were observed macroscopically or histologically.

Inducible Expression of TGFβ1 Transgene in the Epidermis. To determine expression levels of the TGFβ1 transgene induced by ZK, the ears of bigenic TGFβ1 mice were treated with either ZK (10, 1, 0.1, and 0.01 μg in ethanol) or ethanol alone. Expression of TGFβ1 transgene in bigenic epidermis was detected by RT-PCR as early as 7 h after ZK treatment (data not shown). Expression of TGFβ1 transcripts in the epidermis was detected by RPA 15 h after ZK treatment. All three TK.TGFβ1 lines were mated with ML.GLVPc line c4486. The GLVPc transactivator was expressed at consistent levels in both ethanol and ZK-treated bigenic TGFβ1 epidermis (Fig. 2*A*) whereas the TGFβ1 target gene was only expressed in ZK treated epidermis (Fig. 2*A*). Bigenic mice from c4486 × c1849 showed the highest expression of TGFβ1 on ZK induction (Fig. 2*A*). In line c4486 × c1849, the TGFβ1 transgene was induced 10-fold and 7-fold in response to 10 and 1 μg of ZK, respectively, in comparison to ethanol-treated bigenic control (Fig. 2*A*). Induction of the TGFβ1 transgene was lowest in response to 0.1 μg of ZK and could only be detected by RT-PCR (Fig. 2*B*). TGFβ1 expression was not detectable in response to 0.01 μg of ZK, as determined by RT-PCR (Fig. 2*B*). To detect TGFβ1 transgene induction at the protein level, immunohistochemistry was performed by using an antibody specific for the LAP of the TGFβ1. Fifteen hours after ZK application to bigenic TGFβ1 mouse skin, TGFβ1 transgene expression was detected in the cytoplasm of keratinocytes throughout the epidermis (Fig. 2*C*). Ethanol-treated bigenic TGFβ1 mouse skin did not stain for TGFβ1 (Fig. 2*C*).

Effects of TGFβ1 Induction on Epidermal Proliferation and Differentiation. To determine the effect of TGFβ1 induction on epidermal proliferation, *in vivo* BrdUrd labeling was performed in adult bigenic TGFβ1 mice 15 h after single ZK treatment. In ethanol-treated bigenic TGFβ1 mouse skin, BrdUrd labeling index was 2.77 ± 2.3 cells/mm ($n = 6$) (Fig. 3*A*), which is similar to ZK-treated monogenic ML.GLVPc or TK.TGFβ1 skin (data not shown). However, the labeling index in bigenic TGFβ1 epidermis treated with 10 μg of ZK was only 0.4 ± 0.09 cells/mm ($n = 6$, $P < 0.01$; Fig. 3A), a 6-fold decrease compared with that of ethanol controls. Topical application of 0.1 μg of ZK to bigenic mouse skin did not affect BrdUrd labeling (data not shown).

Previously, HK1.TGFβ1 transgenic epidermis did not show aberrant differentiation before death at birth (5). To determine whether TGFβ1 induction affected epidermal differentiation postnatally, neonatal bigenic TGFβ1 skin was analyzed

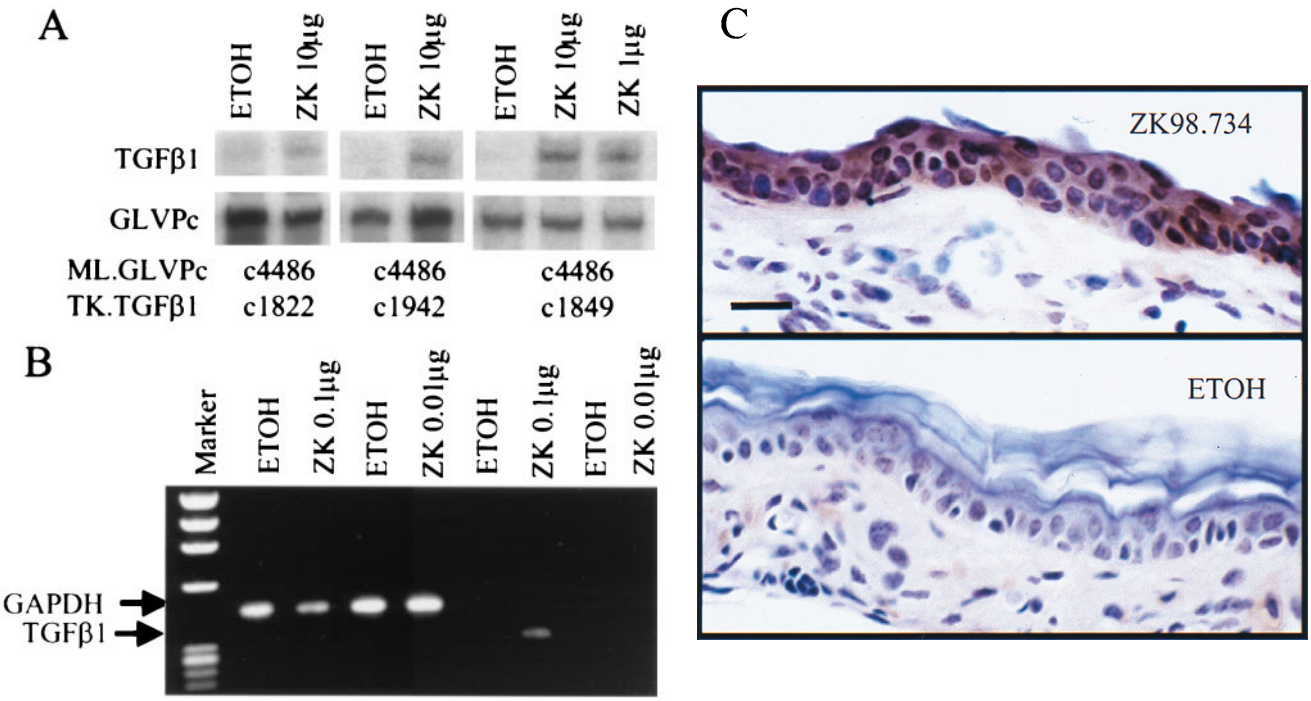

FIG. 2. Inducible expression of the TGFβ1 transgene in bigenic epidermis. Mouse ears were treated with either 10 μg of ZK or 100% ethanol and were excised 15 h after the treatment. Shown is RPA (*A*) and RT-PCR (*B*) on epidermal RNA from treated ears. Note that TGFβ1 expression was only detected in ZK-treated bigenic epidermis. The DNA marker in *B* is the ϕX174/*Hae*III fragments. (*C*) TGFβ1 transgene expression (brown) detected by immunohistochemistry. (Bar = 27 μm.) Tissues in *B* and *C* are from ML.GLVPc line c4486 × TK.TGFβ1 line c1849.

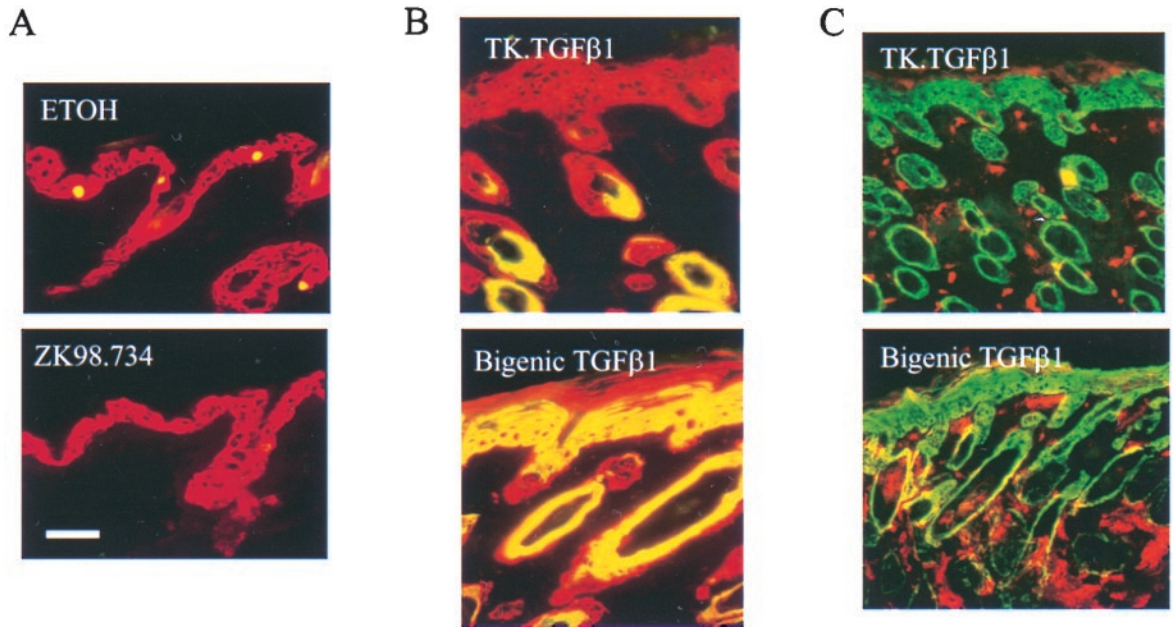

FIG. 3. (*A*) Reduced BrdUrd labeling in bigenic TGFβ1 mouse epidermis. Shown is BrdUrd labeling (yellow) in bigenic adult skin 15 h after either single ZK (10 μg) or ethanol (ETOH) treatment. K14 (red) highlights the epidermis and hair follicles. (*B*) Aberrant K6 expression in bigenic TGFβ1 epidermis after consecutive treatments with ZK. TK.TGFβ1 or bigenic TGFβ1 mice were treated with ZK, 1 μg/day for 7 days. K6 expression (yellow) in TK.TGFβ1 skin was restricted in hair follicles. However, K6 (yellow) was aberrantly expressed in the bigenic TGFβ1 epidermis after ZK treatments. K14 (red) highlights the epidermis and hair follicles. (*C*) Increased CD31 staining in TGFβ1-induced bigenic skin. Newborn pups of TK.TGFβ1 or bigenic TGFβ1 were treated with 1 μg/day ZK for 7 days. CD31 (red) highlights endothelial intercellular junctions. K14 (green) highlights the epidermis and hair follicles. (Bar = 57 μm.)

for epidermal differentiation markers after daily treatment of neonates for 7 days with 1 μg of ZK. Expression patterns of K1, loricrin, and filaggrin in ZK-treated bigenic TGFβ1 mice were normal (data not shown). Expression of K6 remained exclusively in the hair follicles of ML.GLVPc and TK.TGFβ1 pups treated with ZK or bigenic TGFβ1 skin treated with ethanol solvent (Fig. 3*B*). However, consistent with previous observation in HK1.TGFβ1 mice (5), bigenic TGFβ1 epidermis treated with ZK aberrantly expressed K6 (Fig. 3*B*).

Paracrine Effect of TGFβ1 Transgene on the Dermal Stroma. To examine whether TGFβ1 induction in the epidermis exerts a paracrine effect on the dermis, histological analysis was performed on adult bigenic TGFβ1 skin after 10 days of daily 10-μg ZK treatments. No obvious histological changes were observed in the dermis of bigenic TGFβ1 skin (data not shown), suggesting that the TGFβ1 transgene did not have a paracrine effect on fibroblasts.

Because TGFβ1 has been implicated in playing an important role in angiogenesis (11, 20), we examined vascular density of mouse skin after TGFβ1 transgene induction. Bigenic and nonbigenic TGFβ1 neonates were topically treated daily with either 1 μg of ZK or ethanol for 7 days. The treated skin then was subjected to immunofluorescence staining by using an antibody against CD31, a marker of endothelial intercellular junctions as well as platelets and leukocyte subsets (21). Increased neovascularization was shown in bigenic TGFβ1 skin treated with ZK in comparison with either ZK-treated TK.TGFβ1 (Fig. 3*C*) or ML.GLVPc mice. The percentage of the dermis covered by vessels in bigenic TGFβ1 skin treated with ZK increased ≈5-fold to 19.2 ± 8.4% ($P < 0.01$), compared with 4.1 ± 1.4 and 4.6 ± 1.5% in ML.GLVPc and TK.TGFβ1 skin, respectively.

Induction of TGFβ1 Resulted in Resistance to PMA-Induced Epidermal Hyperproliferation. Although TGFβ1 transgene induction resulted in decreased BrdUrd labeling and aberrant K6 expression, the thickness of the epidermis was not significantly changed. It is possible that the proliferation rate of the epidermis is gradually decreased from neonates to adults (22); thus, TGFβ1 may induce obvious morphological changes under conditions of epidermal hyperproliferation. To test this hypothesis, 10 μg of PMA was topically applied to back skin of adult bigenic TGFβ1 mice. ZK (10 μg) or 100% ethanol (50 μl) was applied to PMA-treated skin at time points of 0, 12, 24, and 36 h after PMA application. Forty-eight hours after the PMA treatment, ZK-treated skin from nontransgenic, ML.GLVPc, and TK.TGFβ1 as well as ethanol-treated bigenic TGFβ1 skin exhibited significant epidermal hyperplasia (Fig. 4 *A*, *C*, and *E*). However, the bigenic TGFβ1 skin treated with PMA followed by ZK showed 2-fold thinner epidermis than the controls (Fig. 4 *B*, *D*, and *F*). Bigenic mice also were injected with BrdUrd (125 mg/kg) 48 h after PMA application and were killed 1 h after injection. The BrdUrd labeling index was 48.8 ± 16.2 cells/mm in bigenic TGFβ1 epidermis treated with ethanol ($n = 4$; Fig. 4*A*) but was only 19.5 ± 8.6 cells/mm in bigenic TGFβ1 epidermis treated with ZK ($n = 4$, $P < 0.01$; Fig. 4*B*). Immunohistochemistry using the TGFβ1 antibody detected elevated endogenous TGFβ1 in suprabasal cells of PMA-treated bigenic epidermis (Fig. 4*E*). However, in the PMA-treated bigenic skin simultaneously treated with ZK, stronger staining for the TGFβ1 (including both the endogenous and the transgene) was shown throughout the entire epidermis (Fig. 4*F*).

DISCUSSION

Although TGFβ1 has been well documented as a potent growth inhibitor for epithelial cells *in vitro*, previous studies on the effect of TGFβ1 on epidermal growth using transgenic mice have shown contradictory results. In the HK1.TGFβ1 mice, all basal cell proliferation ceased (5). Similarly, when PMA was topically applied to the skin of K6.TGFβ1 transgenic mice to induce both epidermal hyperplasia and the TGFβ1 transgene, TGFβ1 transgenic epidermis showed a lower proliferation rate than that of control mice (17). Conversely, in K10.TGFβ1 mice, or a K6.TGFβ1 transgenic line that showed a leaky, constitutive TGFβ1 expression, the quiescent adult

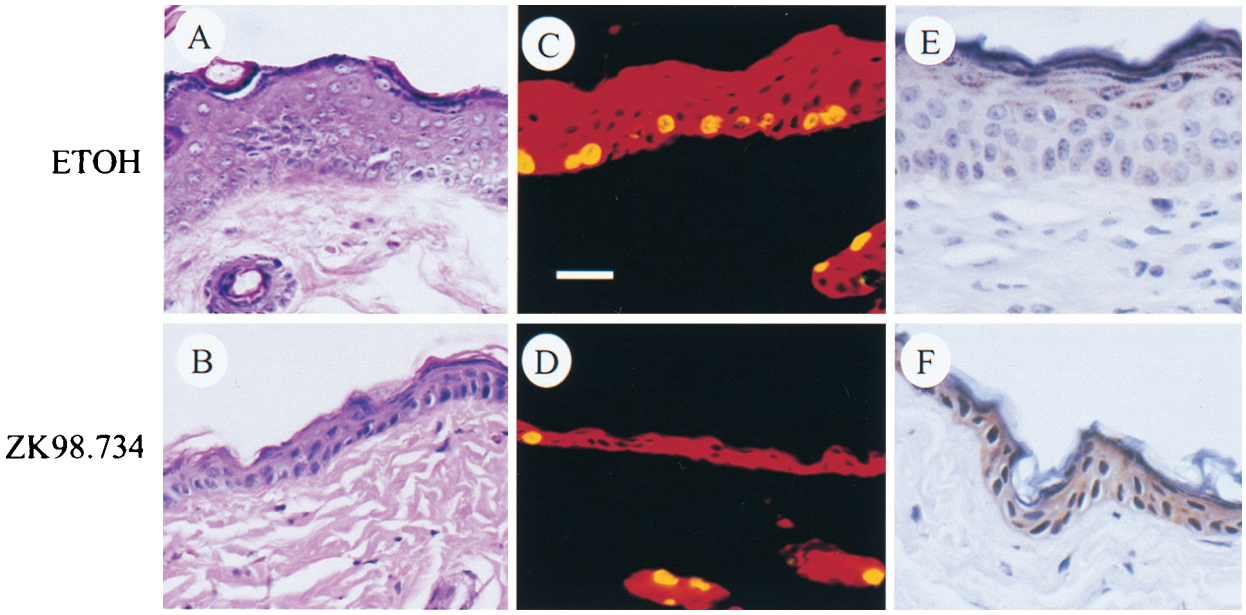

FIG. 4. Comparison of ZK- and ethanol-treated bigenic TGFβ1 mouse epidermis 48 h after PMA application. ZK (10 μg) or 100% ethanol (50 μl) was applied to PMA-treated skin at time points of 0, 12, 24, and 36 h after PMA application. Mice were injected with BrdUrd 48 h after PMA application and were killed 1 h after injection. Histology shows significant epidermal hyperplasia in ethanol control skin 48 h after PMA treatment (*A*) whereas ZK-treated skin shows a 2-fold thinner epidermis (*B*). BrdUrd labeling (yellow) also shows a significant reduction in ZK-treated epidermis (*D*) compared with that of control (*C*). K14 (red) highlights the epidermis and hair follicles (*C* and *D*). The endogenous TGFβ1 (brown cells) was induced by PMA in the suprabasal layers of the epidermis (*E*). However, ZK-treated bigenic TGFβ1 skin shows uniformly expressed TGFβ1 throughout the entire epidermis (*F*), which includes both the endogenous and the TGFβ1 transgene. (Bar = 57 μm.)

epidermis exhibited an increased mitotic rate but failed to produce any notable histological change (16). Because neonatal or PMA-treated epidermis has a higher proliferation rate than adult epidermis (22), it is possible that TGFβ1 acts differently in the proliferative vs. quiescent epidermis. To test this scenario, in the current study we developed gene-switch-TGFβ1 mice, which allow specific induction of the TGFβ1 transgene expression in both of these conditions. On TGFβ1 transgene induction, the adult epidermis exhibited decreased proliferation under both PMA-treated and untreated conditions (Figs. 3 and 4). These data suggest that TGFβ1 not only inhibits cell growth of hyperproliferative cells but also inhibits cell growth in quiescent skin. Another potential explanation for the TGFβ1-induced hyperproliferation previously observed in K10.TGFβ1 or K6.TGFβ1 epidermis is that lower TGFβ1 transgene levels may induce rather than inhibit epidermal proliferation. This is suggested by the fact that TGFβ1 transgenic protein was detected in HK1.TGFβ1 epidermis but could not be detected in either K10.TGFβ1 or K6.TGFβ1 epidermis. However, because these are different transgenic models, it is difficult to make a direct comparison. The advantage of gene-switch-TGFβ1 mice is that TGFβ1 transgene expression levels can be controlled dose-dependently with ZK, thus allowing us to assess whether different levels of TGFβ1 transgene expression have a different effect on epidermal proliferation. Higher TGFβ1 transgene induction (10-μg or 1-μg ZK treatment) exhibited a growth inhibitory effect whereas lower TGFβ1 transgene induction (0.1-μg ZK treatment) did not affect BrdUrd labeling in the epidermis, indicating that low levels of TGFβ1 may not have an effect on keratinocyte growth. Our results are consistent with the skin phenotype observed in both TGFβ1 knockout mice and the transgenic mice expressing a dominant negative TGFβ type II receptor (ML.ΔβRII). These models exhibited epidermal hyperproliferation in response to the lack of the TGFβ1 ligand or TGFβ signaling (2, 23). Therefore, the unexpected effect of TGFβ1 on epidermal proliferation in K10.TGFβ1 or K6.TGFβ1 mice may have resulted from expression exclusively in differentiated cells (16, 17).

In addition to the growth inhibitory effect on keratinocytes, TGFβ1 exhibits a growth stimulatory effect on dermal fibroblasts *in vitro* (24, 25) and stimulates collagen synthesis (12, 20). Therefore, if TGFβ1 produced in the epidermis is able to cross the basement membrane, it should exhibit a paracrine effect on the dermis. However, previous TGFβ1 transgenic mice that overexpressed the constitutively active mutant, $TGF\beta1^{S223/225}$, in the epidermis did not exhibit changes in the dermis (5, 16, 17). There are several possible explanations for this observation. First, a high level of TGFβ1 expression, especially in basal cells, may be essential for secretion into the dermis. Because the K10 and K6 vectors target transgene expression in differentiated cells (16, 17) and the HK1 vector only directs transgene expression in 30% of the basal cells (5), these models may not produce sufficient amounts of TGFβ1 close enough to the basal layer to be secreted to the dermis. Secondly, although HK1.TGFβ1 mice have a higher transgene expression level than that of K10 or K6 TGFβ1 mice, they died soon after birth. If the effect of TGFβ1 on the dermis requires a longer latency, the neonatal lethality of HK1.TGFβ1 mice would have precluded detecting such effects. To further investigate these possibilities, we used gene-switch-TGFβ1 mice. The truncated ML promoter targets the transgene to both basal and suprabasal cells with 5- to 10-fold higher expression levels compared with the HK1 promoter. In addition, to achieve a normal life span of the transgenic mice, TGFβ1 was expressed only when ZK was topically applied; therefore, a chronic effect of TGFβ1 on a small area of the skin could be monitored. Under these conditions, we observed increased angiogenesis but no fibrotic response in the bigenic dermis (Fig. 3). It is possible that the fibrotic response has a higher threshold than angiogenesis in response to the paracrine effect of TGFβ1. Alternatively, expression of the TGFβ1 transgene may induce expression of other angiogenic factors in the epidermis, which are secreted into the dermis. For instance, TGFβ1 has been reported to stimulate expression of vascular endothelia growth factor in keratinocytes (26). If angiogenesis in the bigenic TGFβ1 dermis is a result of an indirect rather than a direct paracrine effect of the TGFβ1 transgene, it is likely that the active form

of TGFβ1$^{S223/225}$ does not have the ability to cross the basement membrane of the epidermis. Because the TGFβ1 antibody (Karen) only recognizes the LAP, we were unable to evaluate whether the mature TGFβ1 was able to migrate into the dermis. It is known that TGFβ1$^{S223/225}$ constitutively releases mature TGFβ1 by failing to associate with the LAP (15). Because mature TGFβ1 has a very short (2–3 min) half-life (27), it is possible that the mature TGFβ1 is quickly bound to the receptors (including nonsignaling betaglycan) of adjacent cells and the rest is rapidly degraded before reaching the basement membrane. Therefore, transport of TGFβ1 across the basement membrane may require association with the LAP, which prolongs the half-life of TGFβ1 to 100 min (28). This hypothesis can be tested by targeting the wild-type TGFβ1 transgene into the transgenic epidermis in the future.

Although TGFβ1 has been suggested to play pivotal roles in wound-healing, because of its complex functions in the epidermis, vascularization, and extracellular matrix remodeling, the exact role of TGFβ1 at different stages of wound-healing have not been defined. TGFβ1 also has complex functions in carcinogenesis. Because TGFβ1 is a potent growth inhibitor for epithelia, it is believed that TGFβ1 acts as a tumor suppressor whose effect must be overcome before progression can occur (29, 30). In contrast, TGFβ1 has been implicated in playing a promotion role in late-stage carcinogenesis (29, 31, 32). However, it is not clear at which stage or under what conditions TGFβ1 switches its role from a tumor suppressor to a tumor promoter. Our current study has shown that TGFβ1 transgene expression and its functions can be focally turned on/off in the gene-switch-TGFβ1 mouse epidermis. Therefore, by using this transgenic model, it should now be feasible to study the stage-specific roles of TGFβ1 in wound-healing and carcinogenesis.

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1. Greenhalgh, D. A., Wang, X. J. & Roop, D. R. (1996) *J. Invest. Dermatol.* **1,** 162–176.
2. Wang, X. J., Greenhalgh, D. A., Bickenbach, J. R., Jiang, A., Bundman, D. S., Krieg, T., Derynck, R. & Roop, D. R. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2386–2391.
3. Greenhalgh, D. A., Quintanilla, M. I., Orengo, C. C., Barber, J. L., Eckhardt, J. N., Rothnagel, J. A. & Roop, D. R. (1993) *Cancer Res.* **53,** 5071–5075.
4. Wang, X. J., Greenhalgh, D. A., Lu, X. R., Bickenbach, J. R. & Roop, D. R. (1995) *Oncogene* **10,** 279–289.
5. 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.
6. Wang, Y., O'Malley, B. W. J., Tsai, S. Y. & O'Malley, B. W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8180–8184.
7. Wang, Y., Xu, J., Pierson, T., O'Malley, B. W. & Tsai, S. Y. (1997) *Gene Ther.* **4,** 432–441.
8. Wang, Y., DeMayo, F. J., Tsai, S. Y. & O'Malley, B. W. (1997) *Nat. Biotechnol.* **15,** 239–243.
9. Handerson, D. (1987) in *Pharmacology and Clinical Uses of Inhibitors of Hormone Secretion and Action*, eds. Furr, F. J. A. & Wakeling, A. E. (Bailliere Tindall, London) 184–210.
10. Bottinger, E. P., Letterio, J. J. & Roberts, A. B. (1997) *Kidney Int.* **51,** 1355–1360.
11. Pepper, M. S. (1997) *Cytokine Growth Factor Rev.* **8,** 21–43.
12. Roberts, A. B., McCune, B. K. & Sporn, M. B. (1992) *Kidney Int.* **41,** 557–559.
13. Roberts, A. B. & Sporn, M. B. (1993) *Growth Factors* **8,** 1–9.
14. Sporn, M. B. & Roberts, A. B. (1992) *J. Cell Biol.* **119,** 1017–1021.
15. Brunner, A. M., Marquardt, H., Malacko, A. R., Lioubin, M. N. & Purchio, A. F. (1989) *J. Biol. Chem.* **264,** 13660–13664.
16. Cui, W., Fowlis, D. J., Cousins, F. M., Duffie, E., Bryson, S., Balmain, A. & Akhurst, R. J. (1995) *Genes Dev.* **9,** 945–955.
17. Fowlis, D. J., Cui, W., Johnson, S. A., Balmain, A. & Akhurst, R. J. (1996) *Cell Growth Differ.* **7,** 679–687.
18. DiSepio, D., Jones, A., Longley, M. A., Bundman, D., Rothnagel, J. A. & Roop, D. R. (1995) *J. Biol. Chem.* **270,** 10792–10799.
19. Go, C., Li, P. & Wang, X. J. (1999) *Cancer Res.* **59**, in press.
20. Roberts, A. B. & Sporn, M. B. (1989) *Am. Rev. Respir. Dis.* **140,** 1126–1128.
21. Charpin, C., Devictor, B., Bergeret, D., Andrac, L., Boulat, J., Horschowski, N., Lavaut, M. N. & Piana, L. (1995) *Am. J. Clin. Pathol.* **103,** 443–448.
22. Bertsch, S. & Marks, F. (1982) *Cell Tissue Kinet.* **15,** 81–87.
23. Glick, A. B., Kulkarni, A. B., Tennenbaum, T., Hennings, H., Flanders, K. C., O'Reilly, M., Sporn, M. B., Karlsson, S. & Yuspa, S. H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6076–6080.
24. Varga, J. & Jimenez, S. A. (1986) *Biochem. Biophys. Res. Commun.* **138,** 974–980.
25. Heckmann, M., Aumailley, M., Chu, M. L., Timpl, R. & Krieg, T. (1992) *FEBS Lett.* **310,** 79–82.
26. Frank, S., Hubner, G., Breier, G., Longaker, M. T., Greenhalgh, D. G. & Werner, S. (1995) *J. Biol. Chem.* **270,** 12607–12613.
27. Coffey, R. J. J., Kost, L. J., Lyons, R. M., Moses, H. L. & LaRusso, N. F. (1987) *J. Clin. Invest.* **80,** 750–757.
28. Wakefield, L. M., Winokur, T. S., Hollands, R. S., Christopherson, K., Levinson, A. D. & Sporn, M. B. (1990) *J. Clin. Invest.* **86,** 1976–1984.
29. Filmus, J. & Kerbel, R. S. (1993) *Curr. Opin. Oncol.* **5,** 123–129.
30. Satterwhite, D. J. & Moses, H. L. (1994) *Invasion Metastasis* **14,** 309–318.
31. Fynan, T. M. & Reiss, M. (1993) *Crit. Rev. Oncog.* **4,** 493–540.
32. Wright, J. A., Turley, E. A. & Greenberg, A. H. (1993) *Crit. Rev. Oncog.* **4,** 473–492.