

Expression of truncated latent TGF- β -binding protein modulates TGF- β signaling

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Summary

Transforming growth factor- β is released from most cells as an inactive complex consisting of transforming growth factor- β , the transforming growth factor- β propeptide and the latent transforming growth factor- β -binding protein. We studied the role of latent transforming growth factor- β -binding protein in modulating transforming growth factor- β availability by generating transgenic mice that express a truncated form of latent transforming growth factor- β -binding protein-1 that binds to transforming growth factor- β but is missing the known N- and C-terminal matrix-binding sequences. As transforming growth factor- β is an inhibitor of keratinocyte proliferation and is involved in the control of hair cycling, we over-expressed the mutated form of latent transforming growth factor- β -binding protein under the control of the keratin 14-promoter. Transgenic animals displayed a hair phenotype due to a reduction in keratinocyte proliferation, an abbreviated growth phase and an early initiation of the involution (catagen) phase of the hair cycle. This phenotype appears to result from excess active transforming growth factor- β , as enhanced numbers of pSmad2/3-positive nuclei

are observed in transgenic animal skin. These data suggest that the truncated form of latent transforming growth factor- β -binding protein-1 competes with wild-type latent transforming growth factor- β -binding protein for binding to latent transforming growth factor- β , resulting in latent transforming growth factor- β complexes that fail to be targeted correctly in the extracellular matrix. The mis-localization of the transforming growth factor- β results in inappropriate activation and premature initiation of catagen, thereby illustrating the significance of latent transforming growth factor- β -binding protein interaction with transforming growth factor- β in the targeting and activation of latent transforming growth factor- β in addition to previously reported effects on small latent complex secretion.

Supplementary material available online at
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Introduction

Members of the TGF- β superfamily play significant roles in development, cell differentiation and tissue morphogenesis in embryos and adult organisms (Massague, 1990; Taipale et al., 1998). The prototypic members of the family, the TGF- β s, are secreted from cells as biologically latent complexes. The dimeric TGF- β molecule associates noncovalently with its processed dimeric N-terminal propeptide, also called latency-associated protein (LAP) (Fig. 1A). For TGF- β to bind to its signaling receptors and exert its biological functions, the cytokine must be released from the small latent complex (SLC) by a process referred to as activation (Annes et al., 2003). LAP also binds covalently to the latent TGF- β binding protein (LTBP-1, -3 or -4) (Miyazono et al., 1988). The binding of the SLC to LTBP produces the large latent complex (LLC) (Fig. 1A), and it is in this form that TGF- β is secreted from most cell types.

LTBPs are 120-220 kDa glycoproteins composed of multiple epidermal growth factor (EGF)-like repeats and four domains containing eight cysteines – the 8-Cys or cysteine rich (CR) domains (Fig. 1A). EGF-like repeats provide stability to protein structures via calcium binding and participate in noncovalent protein-protein interactions (Davis, 1990). The CR domains are unique to the fibrillin/LTBP superfamily and their function(s) are not well understood. The third CR domains of LTBP-1, -3 and -4 bind LAP (Gleizes et al., 1996; Lack et al., 2003; Saharinen et al., 1996b; Saharinen et al., 1998; Yin et al., 1995), whereas the other CR and hybrid domains may be involved in matrix binding (Unsold et al., 2001). LTBPs are important for the folding, secretion and targeting of TGF- β to the ECM (Miyazono et al., 1991; Taipale et al., 1994). Indeed, null and hypomorphic mutations of the *Ltbp-3* and *Ltbp-4* genes have yielded phenotypes associated

with decreased SLC secretion (Dabovic et al., 2002a; Dabovic et al., 2002b; Sterner-Kock et al., 2002). At least three distinct regions of LTBP-1 associate with the ECM. The first (hybrid), the second and the region around fourth CR repeats of LTBP-1 interact with the ECM (Nunes et al., 1997; Olofsson et al., 1995; Saharinen et al., 1996a; Taipale et al., 1994; Unsold et al., 2001). The model proposed by Unsold et al. (Unsold et al., 2001) suggests that the initial interactions of LTBP-1 with the ECM involves the N-terminal regions of LTBP-1 followed by interaction of the C-terminal region. The N- and C-terminal regions can bind independently to the ECM, and each has the ability to inhibit the association of exogenous LTBP-1 with fibroblast ECM (Unsold et al., 2001). The strong N-terminal binding sites may anchor the protein, and the weaker C-terminal site may subsequently stabilize the binding. LTBP-1 colocalizes with fibronectin (Taipale et al., 1996), elastin (Karonen et al., 1997) and fibrillin-1 (Dallas et al., 2000; Isogai et al., 2003; Raghunath et al., 1998; Sakai et al., 1986).

A significant fraction of the latent cytokine is covalently associated with the ECM (Miyazono et al., 1991; Miyazono et al., 1992; Taipale et al., 1992; Taipale et al., 1995). Liberation of active TGF- β from its ECM-bound form requires several steps (Annes et al., 2003; Koli et al., 2001). In the case of protease-mediated activation, the first step may involve the release of the latent complex from the ECM by proteolytic cleavage of LTBP-1 at protease-sensitive sites (Koli et al., 2001; Taipale et al., 1992; Taipale et al., 1995). The second step, activation of latent TGF- β , liberates TGF- β from its noncovalent complex with LAP. This process is mediated by interaction with proteases, integrins or thrombospondin in cell-type-specific ways (Annes et al., 2003; Koli et al., 2001). As TGF- β regulates the expression of ECM components and the extracellular proteolytic balance, TGF- β activation is a key element in a complex and finely controlled feedback system of ECM remodeling (Koli et al., 2001).

The action of TGF- β has been extensively examined in the skin. Several transgenic mouse models have been generated in which TGF- β and TGF- β receptor function have been perturbed in the epidermis using keratin- or keratinocyte-specific gene promoters to express different forms of the cytokine or its receptor. These models have produced significantly different epidermal phenotypes depending on the specific mode, place and time of expression of different TGF- β isoforms and their receptor, as well as different activation mechanisms (Blessing et al., 1995; Blessing et al., 1996; Cui et al., 1995; Fowles et al., 1996; Ito et al., 2001; Sellheyer et al., 1993; Wang et al., 1997; Wang et al., 1999). However, they all support the conclusion that TGF- β influences proliferation and apoptosis in the epidermis and regulates ECM synthesis in the dermis (Frank et al., 1996; Shah et al., 1999; Streuli et al., 1993). Moreover, TGF- β has been proposed to regulate the apoptosis-driven onset of catagen during hair cycling (Kim et al., 1998; Lindner et al., 1997; Maurer et al., 1997; Weedon and Stratton, 1981). The hair follicle proceeds cyclically through three phases of growth: hair shaft formation (anagen), hair shaft involution (catagen) and quiescence (telogen). The first phase of hair follicle development is called postnatal hair follicle morphogenesis. Studies with TGF- β 1 null mice further support the hypothesis that TGF- β 1 plays an important role in catagen induction (Foitzik et al., 2000; Philpott et al., 1994; Soma et al., 1998). Consistent with this possibility, TGF- β and

TGF- β receptor type II expression is maximal at the anagen-catagen transition (Paus et al., 1997; Welker et al., 1997).

To examine the role of LTBP-1 in latent TGF- β 1 activation, we generated transgenic mice that ectopically express the TGF- β -binding EGF-CR3-EGF region of LTBP-1 (ECR3E, the third CR repeat flanked by two EGF-like repeats) under the control of the keratin 14 (K14) promoter (Wang et al., 1997) (Fig. 1D). We reasoned that ECR3E would exert a dominant negative effect by competing with endogenous LTBP for covalent association with latent TGF- β 1, and there would be no inhibition of SLC secretion, as in the *Ltbp* knockout and hypomorphic mutants (Dabovic et al., 2002a; Dabovic et al., 2002b; Sterner-Kock et al., 2002). Our approach would permit us to distinguish between LTBP effects on latent TGF- β after secretion versus the effect of the earlier reported mutations that impair secretion. As ECR3E lacks the LTBP-1 sequences required for ECM incorporation, latent TGF- β bound to ECR3E will fail to bind to the matrix (Nunes et al., 1997; Taipale et al., 1994; Unsold et al., 2001). Thus, the majority of the latent TGF- β will remain as a soluble latent complex and its activation may be altered (Annes et al., 2004).

The K14-ECR3E transgenic mice displayed decreased keratinocyte proliferation, an abbreviated anagen, early induction of catagen and shorter hair. These phenotypes correlate with the presence of higher levels of active TGF- β 1 in the transgenic skin as measured by increased amounts of nuclear pSmad2/3 in keratinocytes. These data suggest that LTBP-1-facilitated targeting of latent TGF- β 1 into the ECM plays an important role in modulating latent TGF- β 1 activation. In addition, constructs of the type described offer the potential to modulate TGF- β availability in a novel manner in other tissues.

Materials and Methods

Reagents

Agarose, protein-A agarose, Lipofectamine Plus, optiMEM and DMEM were purchased from Gibco Laboratories (Grand Island, NY). Bouin's solution, eosin, hematoxylin and permount were purchased from Sigma Chemical Co. (Springfield, NJ). Proteinase K and salmon sperm were purchased from Roche Applied Science (Indianapolis, IN). Mouse anti-HA IgG was purchased from BabCo (Richmond, CA). ECL and HRP-conjugated goat anti-mouse IgG were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Endonucleases were purchased from Boehringer Mannheim Corp. (Indianapolis, IN) and New England Biolabs (Beverly, MA). [α - 32 P] dCTP was purchased from Perkin Elmer NEN Life Science Products, Inc. (Boston, MA). G418 sulfate was purchased from Mediatech (Herndon, VA). Rabbit polyclonal anti-Myc IgG (A14) and anti-pSMAD2/3 (SC-11769) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). VB3A9, a mouse anti-LAP IgG was produced in our laboratory (Nunes et al., 1998). Normal mouse IgG was purchased from Sigma Chemical Co. Ab39 was a gift from K. Miyazono (Tokyo University), LAP was prepared in our laboratory and anti-TGF- β neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). BrdU was purchased from Sigma. BrdU was revealed using the Boehringer biotinylated anti-BrdU antibody.

Cell culture

B9-293 (a gift from B. Arrick, Dartmouth Medical School, Hanover, NH) is a stable cell line that expresses high levels of SLC (Arrick et al., 1992). These cells were cultured in DMEM (Gibco Laboratories)

supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin-glutamine (PSQ) at 37°C with 5% CO₂. HT1080 cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM supplemented with 10% FBS and PSQ at 37°C with 5% CO₂. Primary murine keratinocytes were isolated from newborn (1-3 day old) ECR3E-Myc transgenic and wild-type littermates as described previously (Glick et al., 1994). Cells were cultured in low calcium (50 μ M CaCl₂) 5-MEM (Life Technologies) containing 8% FBS treated with chelex resin, 100 μ g/ml penicillin G and 100 μ g/ml streptomycin sulfate.

Mammalian cell expression of ECR3E-Myc and ECR3E1-2-Myc

pcDNA3-ECR3E-Myc or -ECR3E-1-2-Myc were transiently transfected into B9-293 cells using Lipofectamine Plus according to the manufacturer's instructions. Cells were grown for 48 hours and incubated in serum-free media (Optimem) for 24 hours. The proteins in the conditioned media were separated by 7% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and western blotted using rabbit anti-Myc IgG and HRP-conjugated anti-rabbit antibody. To detect the formation of the complex between either ECR3E-Myc or ECR3E-1-2-Myc and TGF- β , the samples were separated by 7% PAGE under nonreducing conditions and blotted with either VB3A9 or mouse anti-Myc IgG (A14). The signal was detected by ECL and exposed to Kodak X-OMAT film.

To show that ECR3E-Myc protein competes with LTBP-1 and binds latent TGF- β , HT1080 cells were transfected with either pcDNA3-ECR3E-Myc or pcDNA3-ECR3E-1-2-Myc constructs using Lipofectamine Plus and selected with G418 (500 μ g/ml) for 1 week. Several cell clones were isolated and maintained in DMEM supplemented with G418 (250 μ g/ml), 10% FBS and PSQ. The cells were incubated with Optimem for 2 days, and the conditioned media collected for western blot analysis. The conditioned media were resolved by 7% SDS-PAGE under nonreducing conditions, transferred to a nitrocellulose membrane and immunoblotted with specific antibodies. The immunoblots were performed with anti-Myc antibody to detect the ECR3E-Myc protein, Ab 39 to detect both the free and complexed (to LAP) LTBP-1, and VB3A9 to detect LAP, ECR3E-Myc-LAP complex and LTBP-1-LAP complex. The signal was detected by ECL with Kodak X-OMAT film.

K14-ECR3C-Myc and K14-ECR3E-1-2-Myc transgenic constructs

Myc-tagged ECR3E (a region spanning EGF-like domain 13, CR3 and EGF domain 14 of LTBP-1 (Fig. 1C) was amplified from pcDNA3-LTBP-1S by PCR using the primers 5'-CACTAGTGGATGTGAATGAATGTGAATGCTC-3' and 5'-GCTCTAGAGTCACGTGAGATCCTCCTCAGAAATCAGTTTTGCTCGGTTAACTCCAGGTCAGTCTTTTCTC-3' with restriction sites for directional cloning. The (*SpeI-XbaI*) ECR3E-Myc fragment was subcloned into pRcCMV plasmid containing the BM 40 signal peptide (a gift from R. Timpl, Martinsried, Germany) and sequenced. Subsequently a *HindIII-XbaI* ECR3E-Myc fragment was transferred into pBluescript for mutation/deletion procedures. To convert the LTBP-1 ECR3E into LTBP-2 ECR3E (ECR3E-1-2), the pBlueECR3E-Myc plasmid was subjected to mutation/deletion procedures using Quick-Change Site directed Mutagenesis Kit (Stratagene) and the following primers: 5'-GATAACTGCgacctcTGCCCGGTCTTGGGAAGTGC-3' and 5'-GCAGTTCCCAAGACCGGGCagaggtcGCAGTTATC-3'. The lower case letters represent the sequences where the mutations were introduced. In this construct, the codons for the amino acids E (glutamic acid, residue 48) and I (isoleucine, residue 49) of LTBP-1 CR3 were mutated to resemble the LTBP-2 codons for the amino acids D (aspartic acid) and L (leucine) (Fig. 1B). The glutamic acid and isoleucine residues correspond to amino acids 1058 and 1059 in

LTBP-1S sequence (Lack et al., 2003). At the same time, the FP (phenylalanyl proline) amino acid sequence of LTBP-1 CR3 was deleted with the same primers (Fig. 1B). For expression analyses in mammalian cells, the *HindIII-XbaI* fragment was cloned into pcDNA3 plasmid.

The ECR3E-Myc and ECR3E-1-2-Myc cDNAs were excised from pcDNA3-ECR3E/ECR3E-1-2-Myc by *HindIII* and *BamHI* digestion, blunted by Klenow, and subcloned between the K14 promoter and a 3' noncoding region of the human growth hormone (hGH) gene, which contains an intron and a polyadenylation signal in the K14-hGH cassette (a gift from Elaine Fuchs, Rockefeller University, NY) (Wang et al., 1997). Plasmid DNA was prepared using a Qiagen (Valencia, CA) purification kit. The K14-ECR3E-Myc-hGH and K14-ECR3E-1-2-Myc-hGH transgenes were excised from the pGEM 3Z backbone with *KpnI*, and *HindIII*. The 5.5 kB transgenes were gel purified by electroelution, and re-suspended in the injection buffer (5 mM Tris-HCl pH 8.0, 0.1 mM EDTA). The DNA was forwarded to the Transgenic Mouse and Microinjection Facility at the Skirball Institute (N.Y.U. School of Medicine) for injection into the pronuclei of fertilized FVB/N oocytes of female mice.

Genotyping ECR3E-Myc and ECR3E1-2-Myc transgenic mice

Genomic DNA was prepared by digesting 1 cm of mouse tail in 1 mg/ml of proteinase K in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 50 mM EDTA, 1% SDS for 6-16 hours at 55°C with shaking, followed by isopropanol and 70% ethanol precipitations. Genotyping of the ECR3E-Myc and ECR3E-1-2-Myc transgenic mice was performed with PCR, using primers 5'-CGTTTTTCACAGAAGGCTTACC-3' (anneals to the EGF-like domain 13) and 5'-CTGTGGGTGATGAAAGCCAAG-3' (anneals to the end of the K14 promoter sequence).

For Southern blot analysis, genomic DNA samples (10-20 μ g) were digested with 80 units of *BamHI* (ECR3E-Myc mice) or *SacI* (ECR3E-1-2-Myc mice) and separated by electrophoresis in 1X TAE - 0.8% Agarose gel at 3 V/cm. The treatment of the gel, transfer to Hybond-XL nylon membrane and hybridization was performed according to the manufacturer's protocol (Amersham Pharmacia Biotech, UK) using [α -³²P]-labeled probe, specific activity 3000 Ci/mmol. As a probe, we used the *BamHI-SacI* fragment (500 bp) corresponding to the upstream region of the hGH in the K14-pGEM 32 constructs. Blots were washed twice in 2X SSC, 0.1% SDS, for 15 and 30 minutes at 65°C, once in 0.5% SSC, 0.1% SDS for 10 minutes at 65°C, and exposed to Kodak X-OMAT film at 80°C for 1-3 days.

Co-immunoprecipitation and TGF- β activity assays

For immunoprecipitation of the LLC and/or Myc-tagged transgenes, either anti-LTBP-1 (Ab39) or anti-Myc (A14), were used. Wild-type and transgenic primary keratinocytes were isolated (Glick et al., 1994) and plated at a density of 7×10^6 cells/well on 6-well plates in low Ca⁺⁺ medium. After 3 days, conditioned media were collected and pre-cleared by adding 200 μ l/ml of Protein A Agarose (50% in PBS) and incubating for 45 minutes at 4°C. The cleared supernatants were immunoprecipitated in the presence of an anti-Myc antibody (5 μ g/ml), nonspecific rabbit IgG (5 μ g/ml), and anti-LTBP-1 IgG (2 μ l/ml) or pre-immune rabbit serum (2 μ l/ml). After 2 hours at 4°C, 50 μ l samples of Protein A Agarose (50% on PBS) were added and the incubation continued for an additional 45 minutes at 4°C. Immunoprecipitates were washed three times with 1 ml of washing buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X100) and once with 1 ml of PBS. Each sample was re-suspended in DMEM containing 0.1% BSA and heat activated at 80°C for 10 minutes. The TGF- β activity in immunoprecipitates was determined by the mink lung epithelial cell luciferase assay after heat activation of latent complexes (Abe et al., 1994; Mazziari et al., 2000). Briefly, 2.5×10^4 cells were plated per well in 96-well plates and incubated 3-4 hours at 37°C. At that time, media containing the samples from

immunoprecipitation were added to the cultures. Specificity of the signal was determined by incubation of the samples with either LAP, which inhibits TGF- β activity, or specific anti-TGF- β neutralizing antibodies. After 16–20 hours, the cells were washed with PBS, lysed, and the luciferase measured. The results are presented as % of samples incubated in the absence of LAP or anti-TGF- β .

Measurement of hair length in ECR3E-Myc and ECR3E-1-2-Myc transgenic mice

For measurement of hair length, the pelage hairs were plucked from the back of the neck of ECR3E-Myc, ECR3E-1-2-Myc transgenic and wild-type littermates at various time intervals during the first 21 days of life. The samples were obtained at various stages of the hair cycle, including the neofollicular growth stage, the catagen or regression stage, and the telogen or resting stage (Wilson et al., 1994). Hairs were evaluated using Alpha Imager 2000 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA), which projects a magnified image of the plucked hairs onto a computer screen. Plucked hairs were viewed on the screen and separated by tweezers to identify the various subtypes of hair (guard/monotrich, awl and zigzag). Hair length was measured on the computer screen by dragging the cursor across the image of the hair. For each transgenic or wild-type mouse examined, a range of 10 to 15 of the guard/monotrich hairs and 20 to 30 each of the awl and zigzag hairs were measured.

Histology of ECR3E-Myc and ECR3E-1-2-Myc transgenic mouse skin

Full thickness mouse skin biopsies were taken from the middle of the back from ECR3E-Myc, ECR3E-1-2-Myc transgenic and wild-type littermates at various time intervals during the first 21 days of life. Biopsy specimens were fixed in 4% paraformaldehyde in PBS for 16 hours at 4°C, processed in gradients of alcohol and embedded in paraffin. Sections, 4–5 μ m, were cut and attached to poly-Lysine coated SuperPlus slides (Fisher Scientific, Springfield, NJ). The sections were deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin.

BrdU incorporation

Transgenic and wild-type newborn littermates were injected intraperitoneally with 0.2 mg of BrdU per g body weight 1 hour before killing. Skin was dissected from middorsum, fixed in 70% ethanol for 16 hours at 4°C, and paraffin embedded. 4 μ m skin sections were incubated 20 min at room temperature in 0.3% H₂O₂, washed three times 3 minutes each in dH₂O, denatured for 15 minutes at 37°C in 1.5 N HCl, washed in dH₂O as before, and blocked for 20 minutes at room temperature in PBS and 10% FCS. Cells incorporating BrdU into the DNA were localized by incubating sections for 1 hour at room temperature with a biotinylated anti-BrdU antibody diluted 1:10 in PBS/10% FCS, followed by three washes with PBS for 5 minutes each, and a final incubation with horseradish peroxidase-conjugated streptavidin. Sections were counterstained with hematoxylin. The labeling index was determined by counting BrdU-positive cells per total interfollicular basal cells in ten randomly selected fields per animal.

Detection of Myc and pSmadD2/3 in sections of skin from transgenic mice

Frozen or paraffin sections of skin samples were subjected to immunohistological staining for detection of the Myc antigen and pSmad2/3 using either rabbit anti-Myc or anti-pSmad2/3 antibodies. For detection of Myc, skin biopsies were fixed in 4% paraformaldehyde, frozen in OCT compound and the sections immediately fixed in Bouin's solution for 5 minutes at RT and washed

once in 70% ethanol and twice in PBS (10 minutes each). The slides were blocked in 2% goat serum and incubated overnight with the anti-Myc antibody at 1:100 dilution. After washing, the signal was revealed using the Fast-red system (BioGenex, CA), counterstained, and pictures were taken using a Zeiss light microscope. The detection of pSmad2/3 was performed as follows. Briefly, deparaffinized sections were treated with DAKO® Target Retrieval Solution in a Black & Decker steamer according to the manufacturer's protocol (DAKO Corporation, CA). After blocking with PBS/2% goat serum for 1 hour at room temperature, the samples were incubated with 1:200 dilutions of the pSmad2/3 antibody and nonimmune IgG (as background control) overnight at 4°C. After washing in PBS, the samples were incubated for 1 hour at room temperature with biotin labeled secondary anti-rabbit antibody. The detection of the signal and counterstaining of the nuclei was performed by incubation of the samples with streptavidin Alexa Fluor® conjugate (1 μ g/ml) (Molecular Probes, OR) and DAPI (50 ng/ml) (Roche Applied Science, IN) in PBS for 30 minutes at room temperature. After a brief wash, the slides were mounted in Fluoromount-G (Southern Biotechnology Associates, AL) and viewed using a Zeiss fluorescence microscope. Pictures were taken at random using a Hammatsu digital camera and Openlab 2.2.5 software. The blue DAPI stained nuclei were artificially colored in green using Openlab software so that the overlapping signals with the red stain for pSmad gave a better contrast (yellow-orange). At each time point, four random fields from each of two slides for each genotype were counted. The results are represented as percentage pSmad-positive cells (yellow-orange signal) of the total cell (green plus yellow-orange nuclei) number.

Results

LTBP-1 has been shown to target latent TGF- β 1 to the ECM (Miyazono et al., 1991; Nunes et al., 1997; Taipale et al., 1994; Unsold et al., 2001). To elucidate the significance of the interaction of LLC with ECM on TGF- β activation and signaling, we generated two constructs that coded for truncated forms of LTBP-1: ECR3E-Myc and ECR3E-1-2-Myc (Fig. 1C). The first LTBP-1 construct (ECR3E-Myc) contains the LAP-binding third CR domain flanked by EGF-like domains, but lacks the regions that mediate LTBP-1 association with the ECM (Gleizes et al., 1996; Saharinen et al., 1996b; Unsold et al., 2001). Thus, ECR3E-Myc should compete with the endogenous LTBP-1 for binding to SLC and prevent targeting of SLC to the ECM. The second LTBP-1 construct (ECR3E-1-2-Myc) encompasses the same LTBP region but with two amino acids mutated and two others deleted, thereby altering the sequence to resemble the LTBP-2 CR3 domain (Fig. 1B), which cannot covalently associate with LAP (Saharinen et al., 1998). Thus, the protein encoded by ECR3E-1-2-Myc cannot compete with the native LTBP-1 for binding to SLC and serves as a negative control for the binding of SLC to ECR3E-Myc.

To verify that both constructs functioned as proposed, HT1080 cells were stably transfected with either pcDNA-ECR3E-Myc or pcDNA-ECR3E1-2-Myc and the conditioned media from these cultures analyzed using western blotting with anti-Myc, anti-LAP or anti-LTBP antibodies. As shown in Fig. 2A, both ECR3E-Myc and ECR3E-1-2-Myc were well expressed, but only ECR3E-Myc blocked the association of LAP with the endogenous LTBP-1 (Fig. 2B). Furthermore, the expression of ECR3E-Myc but not that of ECR3E-1-2-Myc resulted in the formation of a slowly migrating complex that contains LAP (Fig. 2C). Similar results were obtained using B9-293 cells (data not shown). These results indicate that ECR3E-Myc and ECR3E-1-2-Myc are expressed, secreted and

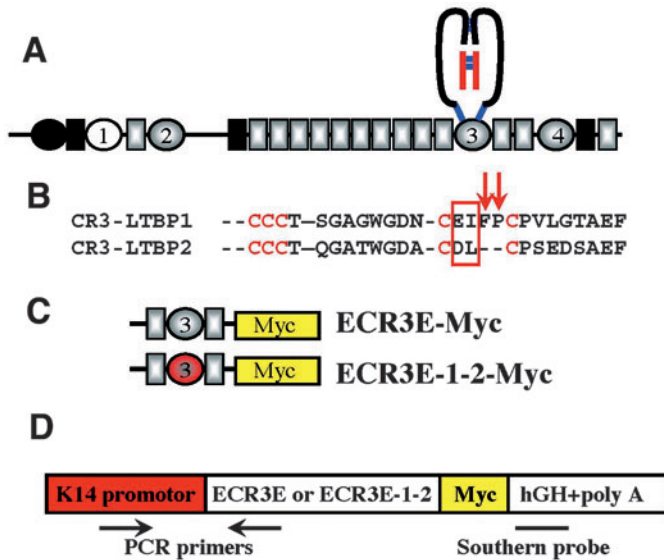


Fig. 1. LTBP-1, ECR3E-Myc, ECR3E-1-2-Myc and transgene constructs. (A) LTBP-1 structure and its binding to LAP and TGF-β through the CR3 region. Ovals represent CR domains, rectangles EGF-like domains; LAP is in black, and mature TGF-β in orange. (B) The amino acid sequence of the CR3 region of the LTBP-1 (ECR3E-Myc) and the mutations introduced to convert ECR3E-Myc into CR3 that resembles that of LTBP-2 (ECR3E-1-2), which does not bind TGF-β. The amino acid changes are boxed and the deleted amino acids are labeled with arrows. (C) Transgene products. The two CR3 regions (oval) surrounded by the EGF-like repeats (rectangles) tagged with Myc tag (long rectangle) that were used in making the transgene constructs represented in D. (D) K14 transgene constructs. The keratin 14 (K14) promoter was used to drive the expression of ECR3E-Myc constructs into the basal layer of the epidermis. The human growth hormone 3' untranslated region and poly A (hGH poly A) were added for stabilization of the transcript. Primers used for PCR are represented by arrows and the fragment of the hGH used for Southern blotting indicated by the bold lines.

behave as predicted with regard to their ability to associate and compete with native LTBP for binding to LAP.

Transgenic mice

To determine the effects of *in vivo* overexpression of ECR3E-Myc and ECR3E-1-2-Myc on TGF-β activation, transgenic mice were produced in which the two deletion mutants of LTBP-1 were expressed under the control of the K14-promoter (Fig. 1C) specific for expression in basal keratinocytes and the outer root sheath (ORS) of the hair follicle (Wang et al., 1997). Basal keratinocytes are known targets of TGF-β and their proliferation is inhibited by TGF-β (Frank et al., 1996; Shah et al., 1999; Streuli et al., 1993). In addition, previous immunohistochemical analyses suggested that the cells primarily affected by TGF-β in the hair follicle during the hair cycle are localized in the ORS during late anagen (follicle morphogenesis) and catagen (Isogai et al., 2003; Paus et al., 1997; Seiberg et al., 1995; Soma et al., 2003; Welker et al., 1997).

ECR3E-Myc mice

Six mice were positive for the K14-ECR3E-Myc transgene both by Southern analysis of tail DNA. (Southern analysis of tail DNA from ECR3E-Myc and ECR3E-1-2-Myc mice as well as PCR analysis for genotyping is illustrated in Fig. S1 in supplementary material.) Five founders showed transmission of the transgene to the F1 generation. Two of these founders were used to establish two independent lines because of the strong skin phenotype shown by their transgenic progeny. There was no loss of ECR3E-Myc embryos based on the Mendelian pattern of transgene transmission in adult animals.

ECR3E-1-2-Myc mice

Six founder mice were positive for the K14-ECR3E-1-2-Myc transgene by both Southern and PCR analysis of tail DNA. However, only four founders transmitted the transgene to their progeny. Two founders were used to establish two lines. As in the case of ECR3E-Myc mice, there was no embryonic lethality of ECR3E-1-2-Myc mice.

Association of ECR3E-Myc with SLC

To verify the ability of ECR3E to associate with the SLC produced by murine keratinocytes, co-immunoprecipitation of conditioned media from wild-type and transgenic cells was combined with a TGF-β activity assay (Fig. 3). Basal keratinocytes were isolated from ECR3E-Myc transgenic and wild-type newborn littermates. When grown in low-Ca²⁺ medium, cultures of primary keratinocytes show a basal cell phenotype (Glick et al., 1994; Li et al., 1995). An anti-Myc antibody was used to immunoprecipitate ECR3E-Myc from transgenic and wild-type keratinocyte-conditioned medium, and an anti-LTBP antibody was used to immunoprecipitate LLC from wild-type keratinocyte-conditioned medium. The co-

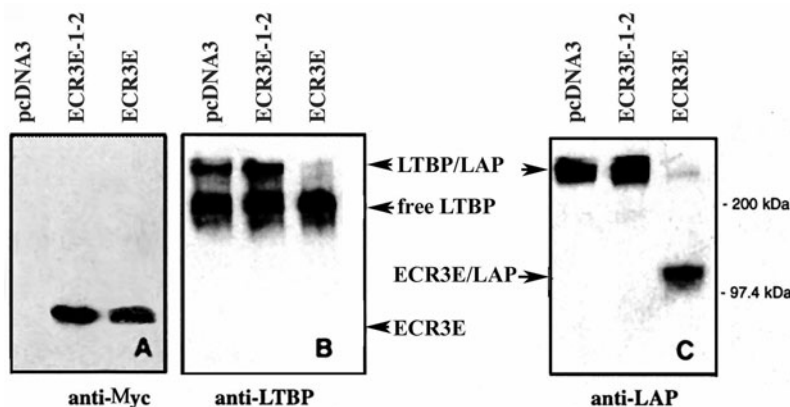
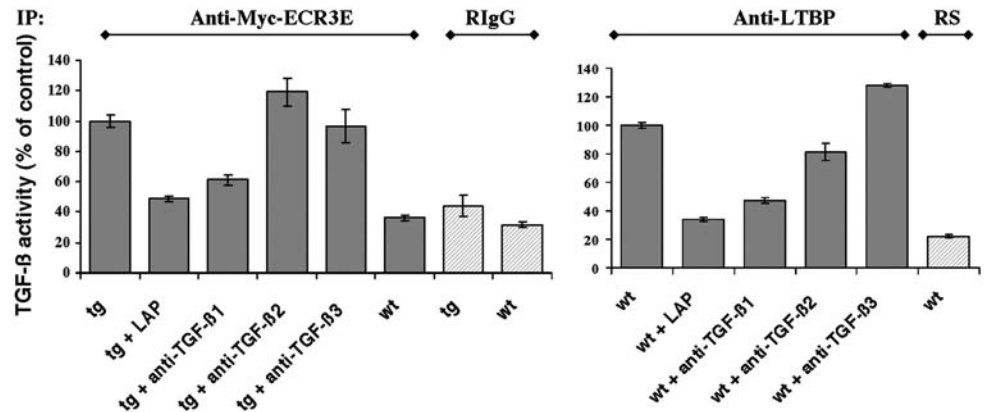


Fig. 2. Expression of the ECR3E-Myc and ECR3E-1-2-Myc proteins and competition with endogenous LTBP for binding to the small latent complex (SLC) in transfected cells. Western blot analyses of conditioned media of ECR3E-Myc, ECR3E-1-2-Myc or mock transfected HT1080 cells. (A) Reducing conditions and anti-Myc antibody. (B) Nonreducing conditions and anti-LTBP antibody. (C) Nonreducing conditions and anti-LAP antibody.

Fig. 3. Co-immunoprecipitation of ECR3E and TGF- β activity from primary keratinocytes. Wild-type and transgenic (ECR3E) primary keratinocytes were isolated and grown in low calcium medium. Conditioned medium was collected and used in immunoprecipitation (IP) experiments with either anti-Myc (A14) or anti-LTBP (Ab39) antibodies. Nonimmune rabbit-IgG (RiGg) or rabbit serum (RS) were used as negative controls. Immunoprecipitates were analyzed for TGF- β activity using the PAI-1-luciferase assay as described in Materials and Methods. PAI-1-luciferase transfected mink lung cells were grown for 16 hours in the presence of heat-activated wild-type (wt) or transgenic (tg) immunoprecipitates in the absence or in the presence of LAP or neutralizing isoform-specific anti-TGF- β antibodies (anti-TGF- β 1, anti-TGF- β 2 and anti-TGF- β 3).



immunoprecipitation of TGF- β was verified using the PAI-1-luciferase TGF- β assay (Abe et al., 1994; Mazziari et al., 2000).

The results of this experiment (Fig. 3) indicate that anti-LTBP antibody co-immunoprecipitates TGF- β from wild-type cell conditioned medium, showing that cultured epidermal keratinocytes produce and secrete the LLC. The fact that the response of the assay is TGF- β -specific is shown by the inhibition of the response by LAP. TGF- β activity is blocked by anti-TGF- β 1 neutralizing antibodies, not by anti-TGF- β 3 antibodies, and only slightly by anti-TGF- β 2 antibodies identifying TGF- β 1 as the major LTBP-associated TGF- β isoform produced by cultured basal keratinocytes. The TGF- β isoform profile of cells in vivo may be different than what is observed in vitro. The anti-Myc antibody co-immunoprecipitates TGF- β from transgenic ECR3E-Myc cell conditioned medium indicating that ECR3E-Myc associates with SLC produced by transgenic basal keratinocytes. Again, TGF- β activity was almost completely blocked by anti-TGF- β 1 neutralizing antibodies or LAP, whereas nonimmune rabbit serum or IgG immunoprecipitated essentially no TGF- β activity. Taken together, these data show that overexpressed ECR3E-Myc associates with latent TGF- β 1 and therefore competes with native LTBP for the binding to LAP in basal keratinocytes derived from ECR3E-Myc transgenic mice.

Phenotype

The in vivo presence of the K14-ECR3E-Myc and K14-ECR3E-1-2-Myc transgenes was examined by immunohistochemical staining of back skin from 9-day-old wild-type and transgenic littermates using an anti-Myc antibody as described in Materials

and Methods. The Myc tag was detected primarily in basal keratinocytes and ORS sheath cells of hair follicles in both ECR3E-Myc and ECR3E-1-2-Myc transgenic mice but not in wild-type littermates (Fig. 4A). RT-PCR experiments revealed

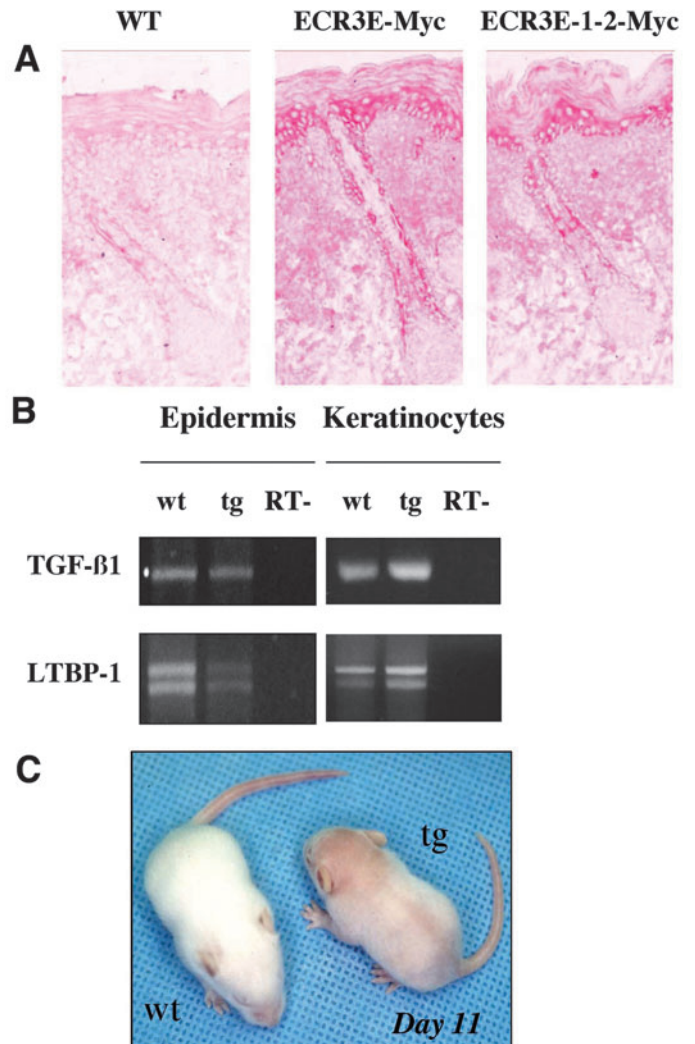


Fig. 4. Expression of the transgenes in vivo. (A) The Myc-tagged transgenes were detected using anti-Myc antibody in sections of back skin from 9-day-old mice. Both ECR3E-Myc and ECR3E-1-2-Myc transgenic animals showed labeling in the basal layer of the epidermis and the outer root sheath (ORS) of the hair follicle. Control (WT) littermates did not show significant labeling of these regions. (B) Expression of the TGF- β 1 and LTBP-1 visualized using RNA extracted from isolated epidermis or cultured keratinocytes followed by RT-PCR. (C) Wild-type and transgenic (ECR3E-Myc) mice at day 11.

expression of TGF- β 1 and LTBP-1 in both ECR3E transgenic and wild-type epidermis and cultured basal keratinocytes (Fig. 4B).

Short hair

Mice from all transgenic ECR3E-Myc lines, but not normal littermates (Fig. 4C) or ECR3E-1-2-Myc mice (not shown), displayed a slight change in color due to the visibility of the skin through the hair. Examination of these animals revealed that their hair was shorter than that of normal littermates. The difference in hair growth was grossly visible in animals by day 12 after birth, became more evident between days 15 and 40, and diminished in older mice presumably because hair growth is synchronized during the first two cycles but not thereafter. To further analyze the hair phenotype, back hairs were plucked from ECR3E-Myc and ECR3E-1-2-Myc transgenic and normal littermates at different ages and examined for morphology and length. No difference in the percentage of each of three major types of hair (guard, awl and zigzag) was observed between transgenic and normal littermates. However, all hair types from the ECR3E-Myc transgenic mice displayed a progressive reduction in length compared with wild-type hair during the first hair cycle. A maximum difference of 30% was reached 15 days after birth (see Fig. S2A in supplementary material). The control ECR3E-1-2-Myc mice showed no reduction in hair length when compared with wild-type littermates (see Fig. S2B in supplementary material).

Early induction of catagen

The hair follicle proceeds cyclically through three phases of growth: hair shaft formation (anagen), hair shaft involution (catagen) and quiescence (telogen). The length of the hair shaft depends primarily on the number of days that a follicle remains in anagen (Stenn and Paus, 2001). This fact and the proposed involvement of TGF- β in the onset of catagen (Foitzik et al., 2000; Paus et al., 1997; Philpott et al., 1994; Seiberg et al., 1995; Soma et al., 2003; Soma et al., 1998; Welker et al., 1997) suggested that the duration of anagen might be modified in the ECR3E-Myc mice due to altered levels of active TGF- β . This hypothesis was evaluated by histological examination of the follicular morphogenesis in transgenic and wild-type mice during the first hair cycle. No apparent histological differences were observed between the transgenic and normal skin of newborn mice (Fig. 5). However, a striking difference was observed in the hair follicles of ECR3E-Myc mice compared with wild-type skin starting from day 6 and especially around day 15 (Fig. 5). Normal murine hair follicles finish postnatal follicle morphogenesis and enter catagen at day 18 post partum (p.p.) (Stenn and Paus, 2001). However, at day 15 p.p., when the wild-type follicles were still in the late follicle morphogenesis phase, ECR3E-Myc transgenic follicles had already entered the catagen phase (Fig. 5), as shown by the fact that the hair bulbs no longer surrounded the

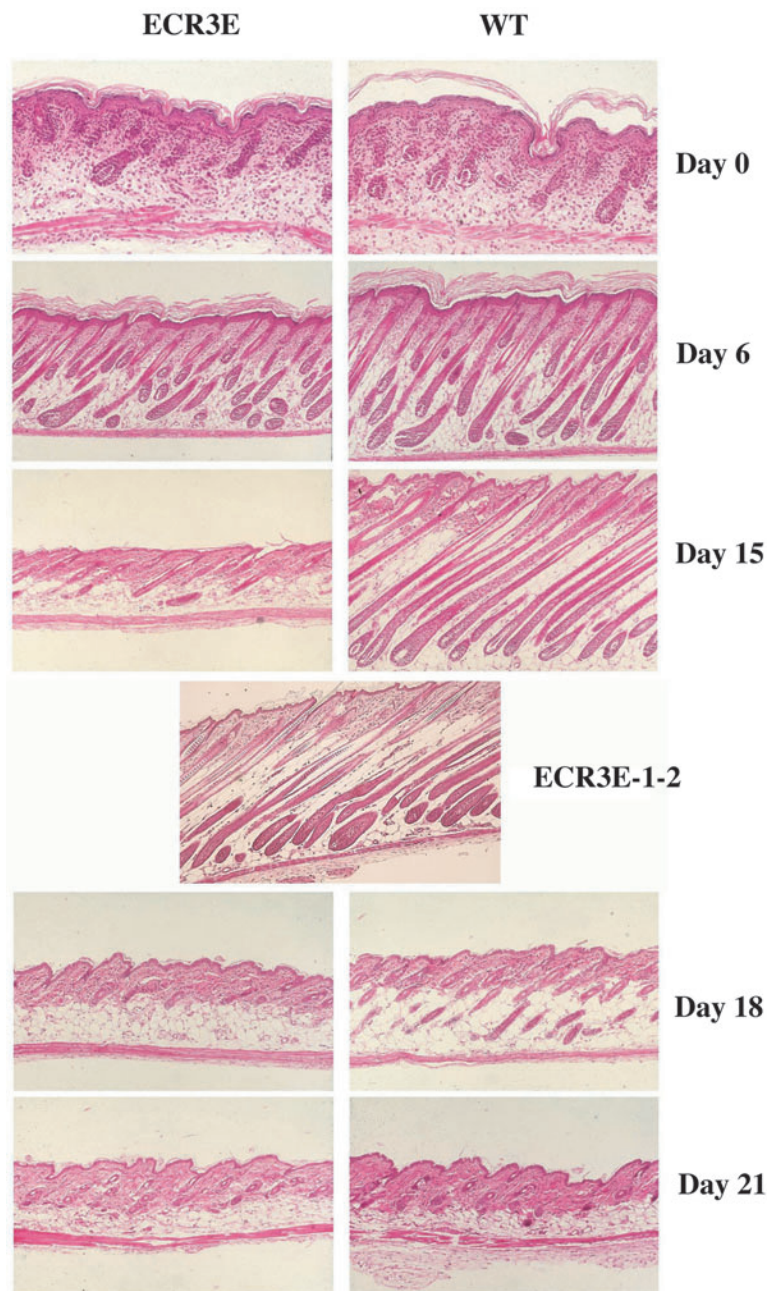


Fig. 5. Histological analyses of the hair cycle. Skin sections from mice stained with hematoxylin and eosin were analyzed at days 0-21. (A) Wild-type (WT) tissues at day 15 displayed hair morphology typical of the late postnatal hair follicle morphogenesis phase, with well-differentiated hair follicles characterized by large oval hair bulbs surrounding the dermal papillae (arrow). At the same day after birth, the ECR3E-Myc transgenic follicles had already entered the catagen phase as evidenced by the regressing follicles with the papilla outside of the hair bulb (arrow). (B) The control ECR3E-1-2-Myc skin had a hair cycle similar to that of the wild-type skin as illustrated by the fact that the hair was in late postnatal hair follicle morphogenesis phase at day 15 and was not distinguishable from wild-type follicles of the same age.

dermal papillae, which were left behind by the regressing follicles (Fig. 5, day 15). At day 18 p.p. transgenic follicles had begun the resting telogen phase, whereas wild-type hair

follicles did not begin telogen until day 21 p.p. (Fig. 5). These changes in hair cycle were also apparent in the first hair cycle (data not shown) and appeared to persist throughout the life of the animals – transgenic ECR3E-Myc mice always had shorter hair even when the hair cycles were no longer synchronized. In addition, skin thickness increased during the anagen phase as the developing follicles elongated into the fat layer. However, skin from ECR3E-Myc mice was thinner than wild-type skin. The difference in skin thickness between wild-type and ECR3E-Myc mice was not due to a difference in the thickness of the epidermis or dermis, but to the adipose layer, which remains thin in the transgenic skin. The control ECR3E-1-2-Myc mice displayed the same skin thickness, follicle morphology and catagen onset as the wild-type mice (Fig. 5), indicating that the phenotypes observed in the ECR3E-Myc mice were due to the association of ECR3E with the endogenous SLC and not to the expression of the transgene product by itself.

Because the main factor in determining hair length is the duration of the growth phase, we conclude that the short hair phenotype observed in ECR3E-Myc mice is mostly due to the reduction of the postnatal follicle morphogenesis phase, resulting from the early onset of catagen. As TGF- β is believed to be involved in the induction of catagen (Foitzik et al., 2000), the early onset of catagen in the ECR3E-Myc transgenic mice suggests that latent TGF- β 1 activation and consequently TGF- β 1 activity are enhanced in the presence of ECR3E-Myc LTBP-1 mutants.

Inhibition of keratinocyte proliferation

Because TGF- β 1 is a potent inhibitor of epithelial cell proliferation both in vitro (Coffey et al., 1988; Shipley et al., 1986; Wilke et al., 1988) and in vivo (Foitzik et al., 2000; Sellheyer et al., 1993; Wang et al., 1997), we determined the rate of proliferation in the epidermis of transgenic and normal newborn littermates from two ECR3E-Myc transgenic lines (44 and 60). Mice were injected with BrdU 1 hour before they were killed, and back skin sections were analyzed for BrdU incorporation as described in Materials and Methods. Both ECR3E-Myc transgenic lines showed a reduction in the proliferation of interfollicular basal keratinocytes of 54.4% and 43.2%, respectively. These results are in agreement with an enhanced TGF- β 1 activity in the presence of ECR3E-Myc LTBP-1 mutants.

Detection of phosphorylated Smad2/3

Our attempts to directly measure active TGF- β in whole tissue using antibody or fluorescence approaches were unsuccessful. Therefore, we indirectly measured TGF- β signaling by immunohistochemical staining for phosphorylated Smad2 and Smad3 (pSmad2/3) on mouse skin from wild-type and transgenic animals at days 0, 2, 6, 9, 12, 15, 18, 21, 23 and 26 throughout the first hair cycle as described in Materials and Methods. Phosphorylation of Smad2/3 is a consequence of TGF- β signaling and the pSmads are translocated to the nucleus where they regulate transcription (Derynck and Zhang, 2003; Kawabata et al., 1998; Shi and Massague, 2003; Stroschein et al., 1999). Thus, nuclear localization of pSmad2/3 is a reflection of TGF- β signaling. When skin

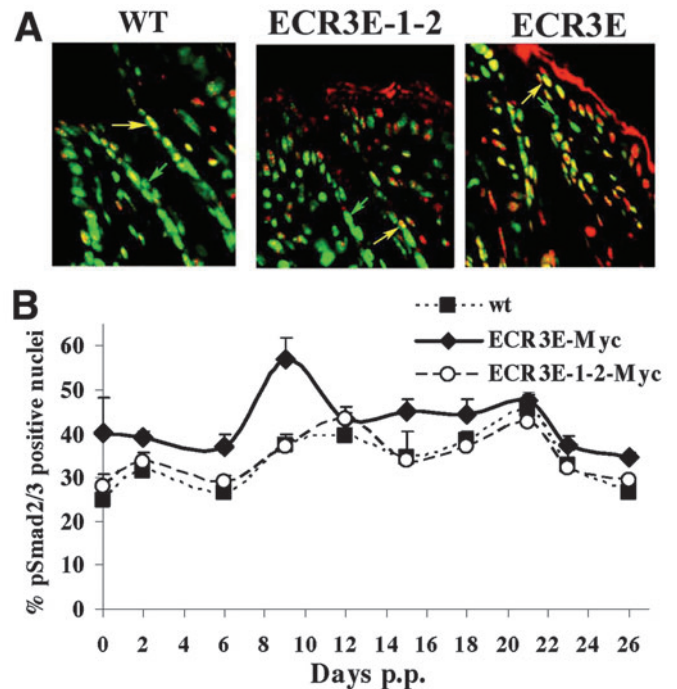


Fig. 6. Detection of phosphorylated Smad2/3 (pSmad2/3) in the skin of wild-type and transgenic animals during the first hair cycle. (A) Immunocytochemistry using pSmad2/3 antibody and Alexafluor visualization of activated Smad2/3 on sections from 9-day-old mice. The positive pSmad2/3 signal stained red. The DAPI-stained nuclei, which were blue, were artificially colored green. Therefore, cells with pSmad2/3 in the nucleus appeared yellow-orange because of the red green overlap. Green arrows indicate pSmad-negative nuclear staining, yellow arrows indicate pSmad-positive nuclear staining. (B) The number of pSmad-positive cells in hair follicles is represented as a percentage of the total number of cells in at least eight fields from each sample of skin from animals 0 to 26 days after birth. The follicles in the skin of ECR3E-Myc animals have higher levels of positive cells compared with the follicles of the wild-type and ECR3E-1-2-Myc.

samples were stained for pSmad2/3 (red), as well as DAPI (green) for nuclei, cells in which pSmad2/3 was present in the nucleus appeared yellow-orange (Fig. 6A). Positive nuclei were counted only in the hair follicles not in the interfollicular regions and the percentage of pSmad positive nuclei for the three mouse types were plotted as a function of day after birth (Fig. 6B). (Although the illustration shows only the upper dermis, the sections used for counting included all regions of the epidermis and dermis.)

The percent of pSmad2/3-positive nuclei in the wild-type follicles was 25% at birth and went through three peaks during the first hair cycle (Fig. 6B). There was a slight rise in the percentage of positive nuclei at day 2, a second larger increase between days 9 and 12, and a third increase between days 18 and 21. Following the third peak, there was a significant decrease in the number of pSmad-positive nuclei back to the level observed at day 0. Skin from animals expressing the control ECR3E-1-2-Myc displayed a pattern of pSmad-positive nuclei in the follicles equivalent to that observed with the wild-type skin, indicating that the overexpression of the non-TGF- β -binding protein had no effect on TGF- β signaling. However, at the initial sampling time, day 0, ECR3E-Myc follicles

contained considerably more pSmad2/3-positive nuclei than controls (40% vs. 25%). The level of TGF- β signaling remained higher than the control values at all times except at days 12 and 21, at which time the wild-type and ECR3E-1-2 follicles showed peak values of pSmad2/3-positive nuclei. The ECR3E-Myc follicles showed peaks of enhanced numbers of positive nuclei in the follicles at day 9. This peak may correspond to the peak observed in the control skin at day 12. A slight increase in the number of pSmad-positive nuclei was also observed in the follicles of ECR3E-Myc skin at day 21, and this was followed by a decline of positive nuclei back to the level observed at day 0. Higher numbers of pSmad-positive cells at day 0 correlate with the BrdU incorporation data and the reduction in keratinocyte proliferation observed in ECR3E-Myc mice. Moreover, the premature high level of TGF- β signaling, as indicated by increased numbers of pSmad-positive cells, at day 9 instead of day 12, correlates with the inhibition of hair growth and the early induction of catagen in the ECR3E-Myc mice.

Discussion

The purpose of the present study was to elucidate the possible function of LTBP-1 in targeting TGF- β to the ECM and the effect of modifying TGF- β ECM interactions on the regulation of latent TGF- β activation. The ECR3E region of LTBP-1, where covalent binding to TGF- β LAP occurs, was expressed under the control of the K14 promoter in the basal cells of the epidermis and ORS of the hair follicle. Basal keratinocytes were chosen as target cells because of the known effects of TGF- β 1 on keratinocyte biology. However, the transgene product should bind all three TGF- β isoforms. Both epidermal and follicular keratinocytes express TGF- β 1, TGF- β receptors and LTBP-1 in a very site- and time-specific manner during the hair cycle (Paus et al., 1997; Tumber et al., 2004; Welker et al., 1997). Mouse skin, therefore, provides a particularly informative *in vivo* animal model system to study TGF- β biology.

Several reports have shown that TGF- β inhibits keratinocyte proliferation and induces keratinocyte apoptosis – events important for both epidermal homeostasis and hair follicle regression during the anagen-catagen transition (reviewed by Massague, 1990; Sakai et al., 1986; Sporn and Roberts, 1990). Overexpression of constitutively active TGF- β in mouse skin results in mice with taut, stretched skin with suppressed proliferation of epidermal and follicular keratinocytes (Sellheyer et al., 1993). By contrast, overexpression of dominant-negative TGF- β receptor type II in mouse skin causes a 2.5-fold increase in the BrdU labeling index in the epidermis and results in mice with wrinkled skin (Wang et al., 1997). In addition, TGF- β 1-deficient mice have a significantly prolonged anagen phase compared with their wild-type or heterozygous littermates, and the local injection of active TGF- β 1 reverts the phenotype (Foitzik et al., 2000).

We concentrated our attention on the postnatal hair follicle development because it is *de novo* and because morphogenesis is synchronized. During this period, the TGF- β RII, which binds active TGF- β and forms heterodimers with the TGF- β R1,

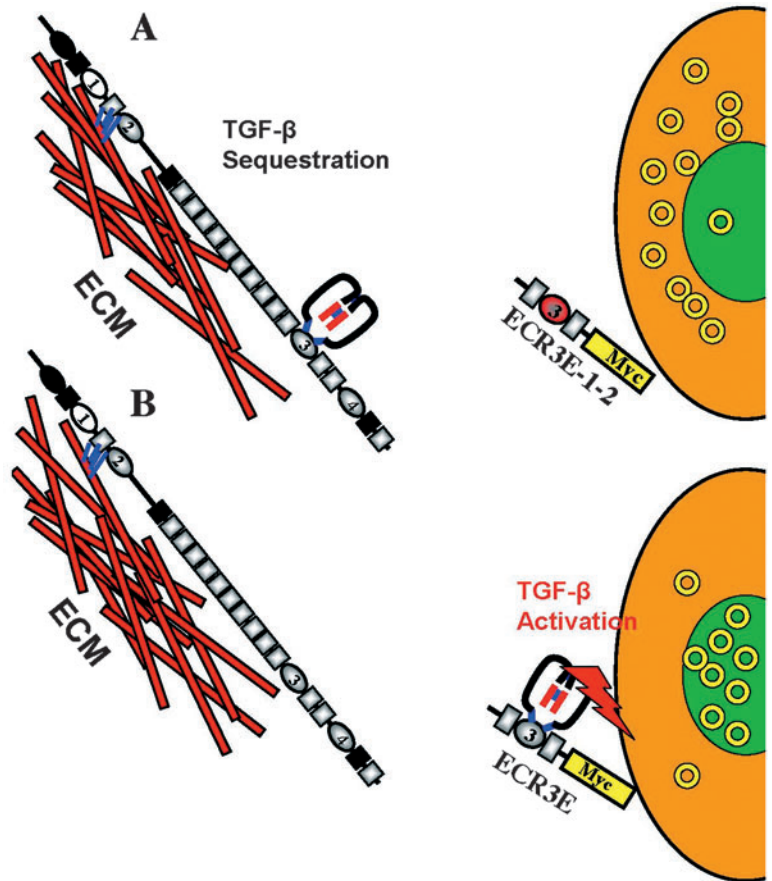


Fig. 7. Model for latent TGF- β activation in ECR3E-Myc and ECR3E-1-2-Myc skin. (A) In the presence of wild-type LTBP-1 and/or ECR3E-1-2-Myc, the latent TGF- β SLC is bound to LTBP-1 and sequestered into the matrix. This prevents latent TGF- β from interacting with cell-associated activators until the appropriate time. (B) In the situation in which ECR3E-Myc is overproduced, it out-competes LTBP-1 for binding to SLC. The complex of ECR3E-Myc and SLC is not incorporated into the ECM as it is missing the N- and C-terminal matrix binding sites of LTBP-1. The soluble latent TGF- β complex, therefore, interacts with cell-associated activators generating active TGF- β , which causes increased signaling through its receptor and accumulation of nuclear pSmad2/3.

is specifically expressed in the epidermal keratinocytes that will form the hair placode and in all keratinocytes of the developing outer root sheath (ORS) in the invaginating and differentiating hair follicles (Paus et al., 1997).

As a consequence of ECR3E expression, we obtained mice with reduced epidermal keratinocyte proliferation, shorter hair, shortened morphogenesis and early onset of growth arrest. All phenotypes correlated with increased numbers of Smad2/3 positive nuclei, a marker of active TGF- β , in the follicles of ECR3E-Myc skin compared with wild-type skin. Transgenic mice overexpressing a mutant form of ECR3E-Myc that is unable to bind latent TGF- β did not display short hair, shortened postnatal hair follicle morphogenesis or increased nuclear pSmad2/3. Therefore, the observed phenotypes are the result of the overexpression of a mutated form of LTBP-1 capable of binding latent TGF- β . Quantitative differences in the skin of wild-type and transgenic animals were observed in the length of the hair and the degree of pSmad2/3 signaling.

Unfortunately, because the ECR3E protein can bind TGF- β 1, TGF- β 2 or TGF- β 3, we cannot define which TGF- β isoform is altered in our system. We propose that ECR3E binding to LAP prevents LTBP-1-dependent latent TGF- β deposition into the ECM, which, in turn generates unbound 'soluble' latent TGF- β (Fig. 7). In this model, an increased soluble pool of latent TGF- β bound to ECR3E causes the inappropriate presentation of the latent TGF- β to activators localized on the cell surface (Annes et al., 2003) and release of active TGF- β . Support for this supposition derives from the work of Neptune and colleagues (Neptune et al., 2003), who showed that in the absence of fibrillin-1, an ECM binding partner for LTBP-1, an excess of active TGF- β 1 is produced in the lungs. Thus, latent TGF- β that is not sequestered in the ECM may be inappropriately activated. The mechanism for this activation is unknown but could involve any one of the known pathways for latent TGF- β activation (Annes et al., 2003).

Previous reports describing the phenotypes of Ltbp null or hypomorphic mutations concluded that the observed phenotypes were the result of a TGF- β deficit, as in the absence of Ltbp the SLC secretion is inhibited (Dabovic et al., 2002a; Sterner-Kock et al., 2002). The results of the experiments presented in this paper are not related to decreased secretion of TGF- β , as latent TGF- β levels were normal. The inappropriate TGF- β signaling we observed strongly implies a function for LTBP modulation of latent TGF- β action after secretion in addition to the previously observed deficits on SLC release.

ECM-associated latent TGF- β may represent a storage form, which allows for a rapid, highly localized TGF- β response, potentially bypassing the need for new protein synthesis (Annes et al., 2003). LTBP-mediated matrix incorporation regulates the soluble pool of latent TGF- β and prevents inappropriate activation of latent TGF- β . The inability to direct latent TGF- β into the matrix facilitates the inappropriate activation of the latent cytokine, resulting in heightened signaling. Thus, the deposition of TGF- β in the ECM, an event facilitated by LTBPs, is important in the regulation and fine-tuning of signaling events that are dependent on TGF- β . In addition, perturbation of LLC interaction with the ECM utilizing mutated LTBPs may yield insights into TGF- β functions.

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