Profile of Transforming Growth Factor-β Responses During the Murine Hair Cycle

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Transforming growth factor-β (TGF-β) appears to promote the regression phase of the mammalian hair cycle, *in vivo* in mice and in organ culture of human hair follicles. To assess the relationship between TGF-β activity and apoptosis of epithelial cells during the murine hair cycle, we identified active TGF-β responses using phospho-Smad2/3-specific antibodies (PS2). Strong, nuclear PS2 staining was observed in the outer root sheath throughout the anagen growth phase. Some bulb matrix cells were positive for PS2 during late anagen. Extensive, but weak, staining was observed in this region at the anagen-catagen transition. We also examined expression of TGF-β-stimulated clone-22 (TSC-22), which is associated with TGF-β-induced apoptosis of some cell lines. Recombinant rat TSC-22 was used to generate a rabbit anti-TSC-22 antibody useful for immunohistochemistry. TSC-22 RNA accumulation and immunoreactivity were observed in follicles throughout the murine hair cycle, including the dermal papilla and lower epithelial strand of late-catagen hair follicles. Neither the expression pattern nor the presence of nuclear TSC-22 correlated with the sites of apoptosis, suggesting that TSC-22 is not an effector of apoptosis in mouse catagen hair follicles. These studies support a complex role for TGF-β in regulating the regression phase of the cycle, with potential for indirect promotion of apoptosis during the anagen–catagen transition. *Key words: Smad/TSC-22/apoptosis/morphogenesis. J Invest Dermatol 121:969–975, 2003*

Transforming growth factor-β (TGF-β) has potent antiproliferative effects on most cultured epithelial cells, including epidermal keratinocytes. This activity appears to be important in the mammalian hair cycle *in vivo*. Mature hair follicles cycle through growth (anagen), regression (catagen), and resting (telogen) phases in a species-specific pattern. In TGF-β1-null mice, initial hair follicle formation is slightly advanced (Foitzik *et al*, 1999); for mature follicles, the regression phase of the hair cycle is delayed (Foitzik *et al*, 2000). TGF-β1 immunoreactivity is present in the outer root sheath (ORS) throughout the murine hair cycle (Foitzik *et al*, 2000). In human hair, TGF-β2 immunoreactivity appears in the boundary area between the bulb matrix and the dermal papilla (DP) during the anagen–catagen transition (Soma *et al*, 2002). In cultured human hair follicles, application of TGF-β2 promotes morphologic changes similar to catagen (Soma *et al*, 1998). Taken together, these data indicate that TGF-β can promote hair follicle regression, a recurring program of tissue remodeling and apoptosis in adult mammals.

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Abbreviations: DP, dermal papilla; EGF, epidermal growth factor; GST, glutathione *S*-transferase; ORS, outer root sheath; PS2, phospho-Smad2; TGF-β, transforming growth factor-β; TSC-22, TGF-β-stimulated clone-22.

During catagen, massive apoptosis occurs in the epithelial components of the hair follicle (Lindner *et al*, 1997; Soma *et al*, 1998). TGF-β2 can induce apoptosis of epithelial cells in cultured human hair follicles (Soma *et al*, 1998). TGF-β2 is expressed during the anagen–catagen transition of the human hair cycle, and sites of TGF-β type II receptor immunoreactivity correlate with the sites of apoptosis in the regressing epithelial strand of catagen hair follicles of humans (Soma *et al*, 2002) and mice (Foitzik *et al*, 2000). These data suggest a simple hypothesis that apoptosis of the follicular epithelial cells in catagen hair follicles is a response to TGF-β. Such a response could be direct, mediated by TGF-β signal transducers in an immediate early response, or it could be indirect, mediated by TGF-β regulation of downstream target genes. Here we examine a direct response to TGF-β, phosphorylation of the Smad signal transducers, and a potential indirect pathway, expression of the transcriptional regulator, TGF-β-stimulated clone *22* (TSC-22; Shibanuma *et al*, 1992).

To understand the roles of TGF-β ligands during the endogenous hair cycle, it is important to know which cells are actively responding to TGF-β signals. A universal TGF-β response is the phosphorylation of Smad proteins, which can then accumulate in the nucleus (Raftery and Sutherland, 1999). Nuclear Smad proteins interact with tissue-specific transcription factors to regulate the expression of TGF-β target genes. Because phosphorylation of Smad proteins is an immediate response to TGF-β receptor activation (reviewed in Massague, 1998), anti-phospho-Smad antibodies allow us to directly visualize the cells that are responding to TGF-β signals. In other developmental systems, this technological advance has provided great insight into *in vivo* roles of TGF-β family signals (Persson *et al*, 1998; Faure *et al*, 2000).

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Recent studies have suggested that TGF-β can promote apoptosis by inducing expression of the TSC-22 transcriptional regulator. TSC-22 was identified as a TGF-β immediately early response gene in mouse osteoblasts (Shibanuma *et al*, 1992). TSC-22 is a transcription factor that has a leucine zipper similar to the bZIP family of transcription factors (Seidel *et al*, 1997) but differs from this family in the DNA-binding domain (Dobens *et al*, 1997). Members of the TSC-22 family share a distinct and conserved sequence in place of the basic DNA-binding domain of bZIP proteins (Ohta *et al*, 1996; Dobens *et al*, 1997; Kester *et al*, 1999; Seidel *et al*, 1997). TSC-22 can act as a sequence-specific DNA-binding protein (Ohta *et al*, 1996) and as a transcriptional repressor (Kester *et al*, 1999). Overexpression of TSC-22 induces apoptotic morphology in a human gastric carcinoma cell line under serum-free conditions (Ohta *et al*, 1997). In a salivary gland cancer cell line, overexpression of TSC-22 increased sensitivity to chemotherapeutic agents that induce cell death (Kawamata *et al*, 1997; Omotehara *et al*, 2000; Uchida *et al*, 2000). The mammalian hair cycle provides an *in vivo* model to examine the role of TSC-22 in apoptotic events that are promoted by TGF-β.

Here, we analyzed the profile of phospho-Smad2 (PS2) antigen localization in the murine hair cycle, to determine the distribution of responses to endogenous TGF-β. To assess whether apoptosis of the follicular epithelial cells is an immediately early response to TGF-β, we compared the accumulation of PS2 with the sites of apoptotic cells during mouse catagen. To reveal whether TSC-22 is involved in apoptosis during the hair cycle, we also examined the distribution of TSC-22 immunoreactivity. These studies support a more complex role for TGF-β in the anagen–catagen transition.

MATERIALS AND METHODS

Hair cycle induction Anagen hair follicles were induced in the dorsal skin of 7-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) by depilation as described (Paus *et al*, 1990). Back skin was harvested on days 0, 2, 6, 10, 12, 17, 18, and 25 after depilation. Skin samples were fixed with phosphate-buffered formalin (pH 7.2) at room temperature for 1 week or 4% paraformaldehyde in phosphate buffer (pH 7.2) at 4°C overnight and then embedded in paraffin wax. Little difference was observed between samples prepared by these fixation protocols. All animal studies had the approval of the Massachusetts General Hospital Animal Care and Use Committee.

Anti-PS2 immunostaining A small aliquot (100 μL) of crude anti-PS2 antiserum was affinity-purified using PS2 peptide (KKK-SSpMSp) coupled to NHS-activated Sepharose gels (Amersham, Piscataway, NJ). Both antiserum and phospho-peptide were gifts from P. ten Dyke (Persson *et al*, 1998). Antibodies were eluted with 0.1 M glycine (pH 2.5) and 0.1 M ethanolamine (pH 12) as described (Harlow and Lane, 1988). High-pH eluates were routinely used for immunohistochemistry at 1:200 dilution in Tris-buffered saline (pH 7.5) containing 0.1% Triton X-100, 10% normal goat serum, and 3% bovine serum albumin. Immunoreactivity was visualized with biotinylated antirabbit IgG and peroxidase-conjugated streptavidin (Laboratory Vision) using paraffin sections. To conserve the purified antibody, the tyramide signal amplification kit (Perkin-Elmer, Boston, MA) was used after the application of peroxidase-conjugated streptavidin according to the vendor's protocol. Sections were developed with diaminobenzidine solution, followed by counterstaining with hematoxylin.

Specificity of PS2 antiserum was tested on primary keratinocytes prepared from newborn Swiss mice and cultured on chambered slides in minimal essential medium with 0.05 M $CaCl_2$, 4% chelex-treated fetal calf serum, and 10 ng per mL epidermal growth factor (EGF; Collaborative Research) as described by (Hennings *et al*, 1980). TGF-β1 (R & D Systems, Minneapolis, MN) was added at 10 ng per mL for 15 or 30 min before processing for immunostaining as described above.

In situ hybridization *In situ* hybridization to sectioned skin samples was performed as previously described (Dohrmann *et al*, 1999), using a 0.9-kb *Pst*I–*Bgl*II fragment of the 3′-untranslated region to generate TSC-22 sense and antisense probes. Sense probes gave little or no staining in control experiments; an example is shown in Dohrmann *et al* (1999).

Anti-TSC-22 antiserum and immunoblotting Rat TSC-22 cDNA (GenBank Accession No. L25785) was subcloned into pGEX-4T2 vector (Amersham) for bacterial production of TSC-22 fusion protein. Recombinant TSC-22 protein was purified according to the vendor's protocol. Antiserum was produced in rabbits at Poconos Farm and Research Laboratory (Canadensis, PA). One anti-TSC-22 antiserum sample was selected for use based on reactivity in immunoblotting using recombinant TSC-22 proteins as briefly described below. Recombinant TSC-22 protein was digested with thrombin, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane (Millipore, Bedford, MA). After being blocked with nonfat dry skim milk, the membrane was reacted with anti-TSC-22 antisera at a 1:5000 dilution. Signal was visualized with anti-rabbit IgG antibody conjugated to alkaline phosphatase (Zymed, South San Francisco, CA) using BM-purple substrate (Hoffmann-La Roche, Basel, Switzerland).

Anti-TSC-22 immunostaining To remove anti-glutathione *S*-transferase (GST) activity, anti-TSC-22 antiserum was passed through a HiTrap column (Amersham) coupled with recombinant GST proteins twice. Affinity purification was against recombinant TSC-22 polypeptide that was cleaved from GST protein and then bound to Affi-Gel 15 (Bio-Rad, Hercules, CA). Immunostaining with anti-TSC-22 antibodies (at 2–4 μg/mL) was performed as described above, without amplification by the tyramide signal amplification system. Sections were counterstained with methyl green.

Double fluorescence labeling with TUNEL and antibodies Paraffin sections were stained with a TUNEL kit (Promega, Madison, WI) by the vendor's protocol, and then sections were incubated with anti-PS2 antibodies (at 1:200 dilution) or anti-TSC-22 antibodies (at 0.1 μg/mL) overnight at 4°C. Signals by the antibodies were amplified with the tyramide signal amplification kit combined with cyanine 3 dye-conjugated streptavidin (Zymed).

RESULTS

PS2 accumulates during the active phases of the mouse hair cycle To determine the sites of TGF-β activity during the hair cycle, we used affinity-purified anti-PS2 antiserum to detect phospho-Smad accumulation in murine hair follicles. This antiserum detects Smad2 and Smad3 after they have been C-terminally phosphorylated by serine-threonine receptor kinases of the TGF-β receptor family (Persson *et al*, 1998). To conserve the purified antibody, we used the tyramide amplification system for PS2 immunostaining. The affinity-purified anti-PS2 antibody detected nuclear antigens in mouse primary keratinocytes that were treated with 10 ng per mL TGF-β1. Incubation with untreated primary keratinocytes gave low levels of diffuse staining (data not shown). Thus, nuclear staining with affinity-purified PS2 antibodies is a specific response to TGF-β family signals.

A synchronized hair cycle was artificially induced by depilation on day 0. Before depilation, telogen hair follicles had only a few cells (**Fig 1*A***, *arrows*) that were positive for PS2 immunostaining. At anagen stage II (day 2 after depilation), epithelial cells in the upper follicle (**Fig 1*B***, *arrowheads*) and around the DP (**Fig 1*B***, *arrows*) were strongly stained by anti-PS2 antibodies. At anagen stage III (day 6), when the epithelia of the follicle become organized, the nuclei of the developing ORS (**Fig 1*C***, *arrows*) and some cells of the dermal sheath (**Fig 1*C***, *arrowheads*) were clearly positive for PS2 staining. In anagen hair at stage V (at day 10), we detected nuclear staining not only in the ORS (**Fig 1*D***, *arrowheads*) but also in the cuticle layer of the IRS (**Fig 1*D***, *arrows*). In anagen VI hair follicles (day 12), some of the bulb matrix cells (**Fig 1*D***, *arrows*) adjacent to the DP were clearly positive for PS2 staining; staining continued to be present in some ORS cells and the cells of the cuticle layer of the IRS (**Fig 1*E***). Interestingly, the intensity of staining varied from cell to cell in the ORS of late-anagen hair follicles (**Fig 1*D,E***). The staining pattern of early-catagen hair follicles (day 17, **Fig 1*F***) was not distinguishable from that of anagen VI hair follicles (see **Fig 1*E***). In late-catagen hair follicles with a long epithelial strand (day 18),

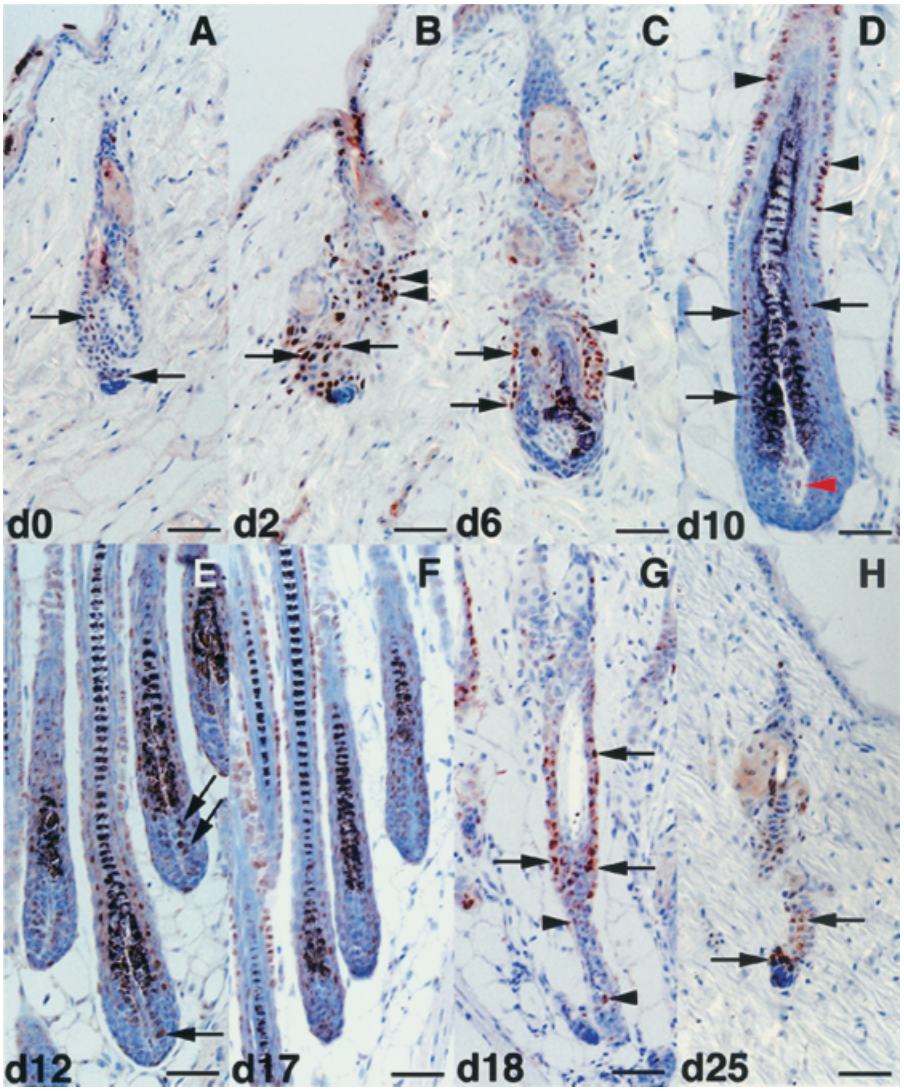

Figure 1. PS2 immunoreactivity during the murine hair cycle. Mouse dorsal skin sections were stained with affinity-purified anti-PS2 antibodies at the indicated time point in a depilation-induced hair cycle. (*A*) Before depilation, only a few PS2-positive cells (*arrows*) were seen in telogen hair follicles. (*B*) Nuclear staining (*brown*) appeared in the epithelial cells (*arrows*) above the DP and the bulge (*arrowheads*) in anagen II hair follicles (at day 2). (*C*) Both developing ORS (*arrowheads*) and dermal sheath (*arrows*) were positive for anti-PS2 antibody staining in anagen III hair follicles (at day 6). (*D*) In anagen V hair follicles (at day 10), nuclear staining was evident in the ORS (*arrowheads*) and the cuticle layer of the IRS (*arrows*). (*E*) Bulb matrix cells (*arrows*) adjacent to the DP were positively stained with anti-PS2 antibody in addition to the ORS and the IRS of anagen VI hair follicles (at day 12). (*F*) The staining pattern of the catagen II hair follicles (at day 17) was almost the same as seen in anagen VI hair follicles. (*G*) Although many nuclei were stained in the ORS (*arrows*), only a few cells (*arrowheads*) were positive in the epithelial strands of catagen VII hair follicle (at day 18 after depilation). (*H*) The residual epithelial components were still nuclear positive for PS2 immunostaining in telogen hair follicles (at day 25). *Bars*, 50 μm.

PS2 immunoreactivity was still detected in the nuclei of the ORS cells (**Fig 1*G***, *arrows*), as well as in a few cells in the epithelial strands (**Fig 1*G***, *arrowheads*). In the telogen stage after depilation (day 25), some nuclei within the regressed epithelial components (**Fig 1*H***, *arrows*) were still positive for PS2 immunostaining. In sum, PS2 staining was present in epithelial components of the follicle both during anagen and catagen phases of the hair cycle. This staining was most pronounced in the ORS. Weaker levels of staining were detected in the bulb matrix as early as anagen stage V.

During the anagen phase, we also detected anti-PS2 staining in occasional nuclei within the DP (**Fig 1*D***, *red arrowhead*). Nevertheless, this staining was quite weak compared to the ORS or the cuticle layer of the IRS. Because the staining was nuclear, we expect this reflects a response to a TGF-β-family signal.

TSC-22 is expressed and present in nuclei throughout the murine hair cycle We were interested to determine whether TSC-22 could be a mediator of TGF-β-induced apoptosis during the hair cycle. First, we examined TSC-22 RNA accumulation during the postdepilation hair cycle, using *in situ* hybridization to a probe from the 3′-untranslated region of the cDNA (Dohrmann *et al*, 1999). TSC-22 RNA accumulation was detected in limited regions of the IRS and ORS, just above the bulb region (**Fig 2**). Staining in this region persisted during anagen stages III (day 5, **Fig 2*A***), V (day 8, **Fig 2*B***), and VI (day 12, **Fig 2*C***). The level of RNA accumulation was very low during catagen (day 19, **Fig 2*D***). The pattern of TSC-22 RNA accumulation was different from the pattern of TGF-β responses, indicating that TSC-22 gene expression is not strongly responsive to TGF-β signaling during the hair cycle.

It has been reported that TSC-22 moves from the cytoplasm to the nucleus following DNA-damage-induced apoptosis (Hino *et al*, 2000, 2002). We wanted to determine whether this change in subcellular localization was associated with programmed cell death during tissue remodeling *in vivo*. In addition, immunohistochemical staining can be a more sensitive method to detect expression. To examine TSC-22 protein localization in murine hair follicles, we generated anti-TSC-22 antiserum. Rat TSC-22 cDNA (Hamil and Hall, 1994) was used to make a bacterially expressed recombinant TSC-22 protein. This was used to generate rabbit anti-TSC-22 antiserum, which recognized TSC-22 fragments by Western blot analysis using the digested recombinant fusion proteins (**Fig 3*A***). The specificity of anti-TSC-22 for immunostaining was confirmed in two ways. First, the crude antiserum was preadsorbed against recombinant TCS-22 protein and used for immunohistochemical staining. Skin sections incubated with this preadsorbed antiserum lacked all immunostaining (data not shown). Second, the antiserum was tested for immunoreactivity on sections of skin from mice with a disrupted TSC-22 gene (C. Dohrmann, T. Soma, J. Brissette, and L. Raftery, unpublished data); the epithelium and

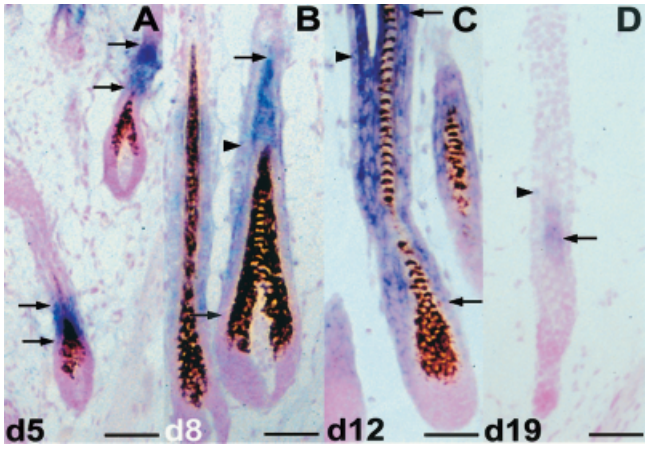

Figure 2. TSC-22 mRNA accumulation during the murine hair cycle. *In situ* hybridization to reveal accumulation of TSC-22 RNA. (*A*) In anagen III hair follicles (at day 5), TSC-22 expression was observed in restricted areas of the ORS and the IRS (*between arrows*). (*B*) The IRS of anagen V hair follicles (*between arrows*) was intensely positive for TSC-22 ISH. Lower levels of TSC-22 mRNA were observed in the ORS (*arrowhead*) compared to the IRS (at day 8). (*C*) In anagen VI hair follicles, the expression pattern of TSC-22 mRNA was not distinguishable from that in anagen V hair follicles. (*D*) The level of TSC-22 mRNA expression was remarkably low in the late-catagen hair follicles. The IRS (*arrow*) and the ORS (*arrowhead*) was slightly positive for TSC-22 ISH (at day 19). *Bars*, 50 μm.

follicles of this tissue were negative for immunostaining. For this study, we used affinity-purified antiserum, because it gave a stronger signal.

TSC-22 immunoreactivity was apparent in multiple cell types in and around mouse hair follicles (**Fig 3*B***). Before induction of the hair cycle by depilation (day 0), occasional nuclei (**Fig 3*B–A***, *arrows*) were positive for anti-TSC-22 staining near the bulge area. In early-anagen hair follicles (day 2 after depilation), anti-TSC-22 staining (**Fig 3*B–B***, *arrows*) was mainly detected in the differentiating follicular keratinocytes above the DP. In anagen IV (day 6), positive signals were observed in the IRS (**Fig 3*B–C***, *arrowheads*) and in the outer most cell layers of the ORS (**Fig 3*B–C***, *arrows*) and also within the DP. In anagen V hair follicles (day 10), when the DP reaches its largest size, a few DP cells (**Fig 3*B–D***, *arrows*) were positive for TSC-22 immunostaining, with continued positive staining in the ORS and the IRS. In anagen VI (day 12; **Fig 3*B–E***), the DP is smaller and all DP nuclei appeared to be positive for anti-TSC-22 staining. At early catagen (day 17; **Fig 3*B–F***), the staining pattern was not distinguishable from that in late-anagen hair follicles (compare to **Fig 3*B–E***). TSC-22 immunoreactivity (**Fig 3*B–G***, *arrows*) was mainly detected in the IRS of early catagen hair follicles and in the DP. In late-catagen hair follicles (at day 18; **Fig 3*B–G***), TSC-22 immunoreactivity was observed in the DP (**Fig 3*B–H***, *arrow*) and the regressing epithelial strand (**Fig 3*B–H***, *arrowheads*). In the telogen stage after depilation (day 25), the staining pattern returned to that observed before depilation. Throughout the hair cycle, TSC-22 was observed to accumulate strongly in the nuclei of some cells (**Fig 3*B–A***, ***B–D***, *arrows*) and to be predominantly cytoplasmic in others (**Fig 3*B–C***, *arrowheads*). At present, the mechanisms that determine nuclear localization of TSC-22 are unclear. Nevertheless, nuclear localization of TSC-22 did not correlate with the onset of catagen, suggesting that it is not linked to programmed cell death during tissue remodeling. As observed for the pattern of RNA accumulation, the pattern of TSC-22 protein localization is distinct from that of the phospho-Smad responses, indicating that TSC-22 function is regulated by other mechanisms during the hair cycle.

Apoptosis in the bulb matrix lags behind the Smad response In anagen VI, some cells of the bulb matrix consistently exhibited a weak phospho-Smad response (**Fig 1*E***), with an increasing proportion of cells showing a response by early catagen (**Fig 1*F***). We directly compared the sites of PS2 accumulation with the sites of apoptosis to further assess whether apoptosis of hair follicle epithelial cells is an immediately early response to TGF-β. If so, the sites and timing of apoptosis should be very similar to the pattern of PS2 accumulation. To compare these events, we used immunofluorescent detection with the TUNEL assay to mark apoptotic cells and amplified immunofluorescent detection of PS2 staining. No TUNEL-positive cells were detected in the lower bulb matrix of late anagen hair follicles (data not shown and Lindner *et al*, 1997), even though PS2 staining was detected at this stage (**Fig 1*E***). In catagen V hair follicles, at day 17 after depilation, numerous bulb matrix cells around the DP were undergoing apoptosis (**Fig 4*A***, *green*). With immunofluorescence, PS2 staining was clearly present in the bulb matrix and was broadly detected in the ORS (**Fig 4*A***, *red*). Nevertheless, PS2 did not costain the apoptotic cells detected by the TUNEL assay. The TUNEL assay detects a late event of apoptosis, so that spatial overlap with nuclear PS2 may not be expected. The spatial and temporal relationship of PS2 staining and TUNEL staining indicate that the direct phospho-Smad response to TGF-β signals is extensive within the bulb matrix, but that TGF-β responses are present several days before the onset of apoptosis. The 5-day lag between the appearance of PS2 staining and onset of apoptosis is inconsistent with the model that apoptosis is a direct response to increased TGF-β signaling.

TSC-22 was not expressed in the bulb matrix during the anagen–catagen transition; however, TSC-22 immunoreactivity was observed in the epithelial strand of catagen VII hair follicles (see **Fig 3*B–G***). We examined the location of TUNEL-positive apoptotic cells relative to TSC-22 staining by immunofluorescence. Even in late-catagen hair follicles (catagen VII, day 18), the regions with TUNEL-positive cells (**Fig 4*B***, *green*) and TSC-22 immunofluorescence (**Fig 4*B***, *red*) were distinct. TSC-22 expression did not precede apoptosis either spatially or temporally, suggesting that the two events are unrelated.

In sum, these double-immunofluorescence experiments indicate that apoptosis is not an immediately early response to TGF-β signaling, because there is a 5-day lag between the time when TGF-β responses can be detected and the time when apoptosis occurs. Nevertheless, most of the epithelial cells in the transient portion of the hair follicle exhibit TGF-β responses during the regression phase of the murine hair cycle. Thus, TGF-β activity may indirectly promote apoptosis in these cells. Although TSC-22 is associated with TGF-β-induced apoptosis in some systems, our data indicate that it is not likely to be an effector of apoptosis during the murine hair cycle.

DISCUSSION

Mammalian hair follicles go through a cyclic process of profound morphologic changes. The closely related TGF-β ligands are thought to be important extracellular signals controlling this process. Each of the three TGF-β genes has been disrupted in mice. Analysis of TGF-β1-null mice showed that TGF-β1 is a negative regulator for hair morphogenesis (Foitzik *et al*, 1999) and anagen progression (Foitzik *et al*, 2000). In contrast, the TGF-β2 gene is required early to induce murine hair follicle morphogenesis, whereas TGF-β3 gene disruption was not associated with any defects in hair follicles morphogenesis (Foitzik *et al*, 1999). Immunoreactivity to each of the three TGF-β ligands was detected in distinct areas of human hair follicles (Soma *et al*, 2002). These observations suggest that each TGF-β isoform may play a distinct role *in vivo*, owing to their nonoverlapping expression patterns. Nevertheless, in organ culture of human hair follicles, TGF-β2

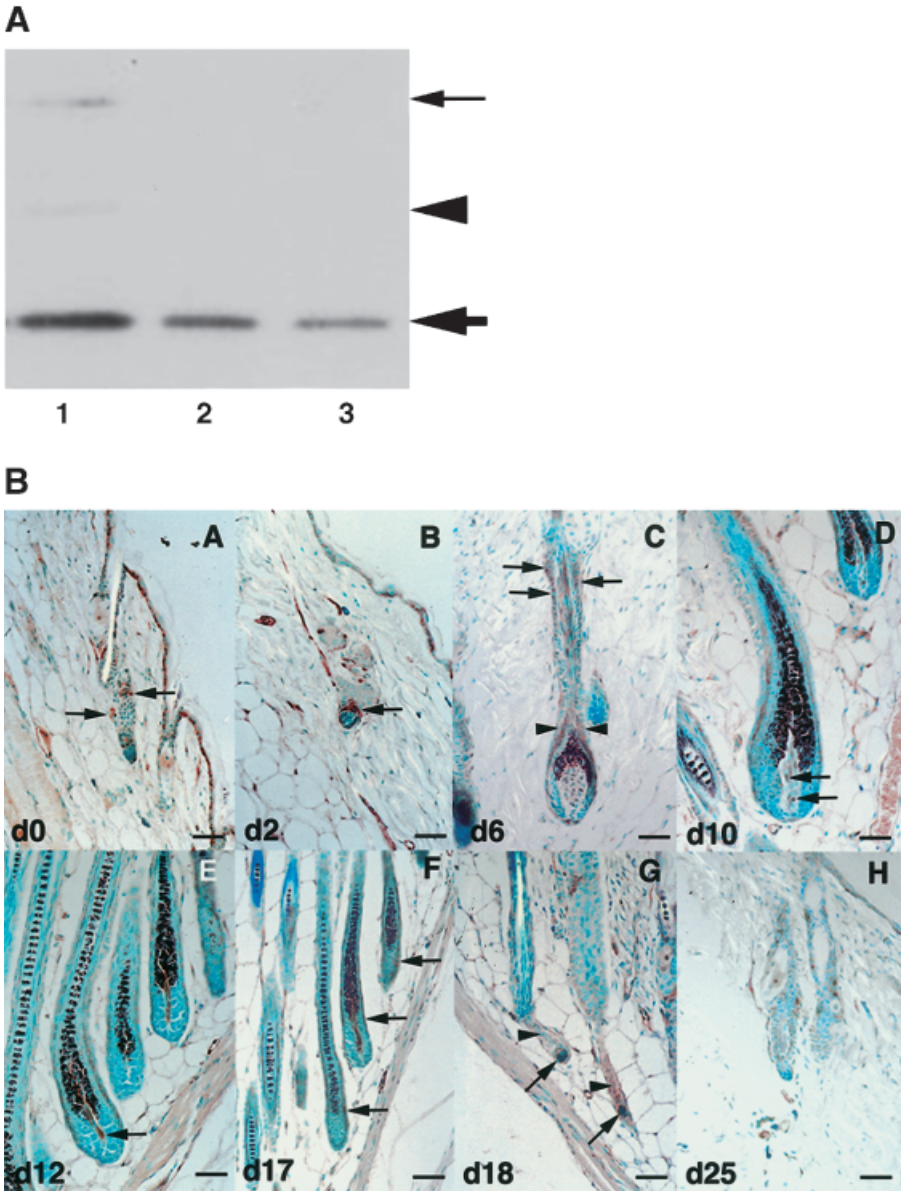

Figure 3. TSC-22 immunoreactivity during the murine hair cycle. (*A*) Western blot analysis of recombinant TSC-22 with anti-TSC-22 antiserum. Recombinant TSC-22 proteins (*lane 1*, 3 ng; *lane 2*, 1 ng; and *lane 3*, 0.3 ng) after digestion with thrombin were used for this analysis. The antiserum detected TSC-22 fragments (*thick arrow*) at a 1:5000 dilution and also recognized fusion proteins (*thin arrow*) and GST fragments (*arrowhead*) weakly. (*B*) Immunohistochemical analysis of mouse tissue sections at distinct stages of depilation-induced hair cycle using affinity-purified anti-TSC-22 antibodies. (*A*) TCS-22 immunoreactivity (*brown*) was detected in nuclei of cells near the bulge area (*arrows*) of telogen hair follicles before depilation. (*B*) The differentiating follicular keratinocytes above the DP (*arrow*) were positive in anagen II hair follicles (at day 2). (*C*) In anagen III hair follicles (at day 6), positive staining was clearly observed in the ORS (*arrows*) and the IRS (*arrowheads*). Staining in the IRS was predominantly cytoplasmic. (*C–F*) The TSC-22 immunoreactivity in the IRS was continuously observed from anagen III phase to catagen I phase. (*D*) Nuclei for some of the DP cells (*arrows*) were clearly positive for TSC-22 immunostaining in anagen V hair follicles, which have the largest DP size during mouse hair cycle (at day 10). (*E*) In anagen VI hair follicles, TSC-22 immunoreactivity covered the entire DP (at day 12). (*F*) Catagen II hair follicles showed the same staining pattern observed in anagen VI hair follicles (at day 17). (*G*) The regressing epithelial strand (*arrowheads*) and the DP (*arrow*) both stained with anti-TSC-22 antibodies (at day 18). (*H*) TCS-22 immunoreactivity was detected near the bulge area of postdepilation telogen hair follicles, similar to before depilation (at day 25). *Bars*, 50 μm.

accelerated the onset of catagen (Soma *et al*, 1998), similar to the *in vivo* activity of TGF-β1 on murine hair follicles. We have taken advantage of an antibody reagent to investigate endogenous responses to TGF-β signals during the murine hair cycle.

The major substrates for TGF-β receptor serine–threonine kinases are Smad2 and Smad3 (reviewed by Massague, 1998). Thus, the presence of phospho-Smad protein is a direct indication of an active response to TGF-β or one of the other ligands that directs phosphorylation of Smads 2 and 3. We affinity-purified the PS2 antiserum provided to us by P. ten Dijke (Persson *et al*, 1998) to detect endogenous responses to TGF-β receptor activation within the cells of murine hair follicles. The affinity-purified antisera strongly stained many nuclei in the ORS layer throughout the murine hair cycle, with the exception of telogen. Although other ligands of the TGF-β superfamily can stimulate PS2 staining, this localization pattern is quite consistent with the immunolocalization pattern for TGF-β1 ligand (Foitzik *et al*, 2000). Thus, we conclude that the ORS is a major target tissue for TGF-β activity during both the growth and the regression phases of the murine hair cycle.

At the end of anagen, TGF-β2 accumulation was detected at the boundary between the DP and the bulb matrix of human hair follicles (Soma *et al*, 2002). The timing of this expression was appropriate for a role in the induction of epithelial cell apoptosis during early catagen. Surprisingly, we detected low levels of PS2 staining in some murine bulb matrix cells at day 12, during anagen stage VI. Thus, TGF-β-like activity is present in the murine bulb matrix throughout late anagen. As previously reported, TUNEL-positive apoptotic cells were not detected before day 17. Many cells of the early catagen bulb matrix have a weak PS2 signal, although the TUNEL-positive cells and the phospho-Smad-positive cells are distinct populations. The TUNEL-positive stage is a very advanced stage of apoptosis, so that the significance of this distinction is not clear. In sum, phospho-Smad responses are detected in the murine bulb matrix 5 days before the onset of apoptosis. This observation leads us to conclude that epithelial cell apoptosis is not coincident with the activation of a direct cellular response to TGF-β signals. Activated Smad proteins transduce TGF-β family signals to the nucleus and thus mediate TGF-β regulation of gene expression. Nevertheless, biochemical studies have suggested that other proteins may be involved in TGF-β-induced apoptosis (Yamaguchi *et al*, 1999; Larisch *et al*, 2000). The septin-like ARTS protein can enhance TGF-β-induced apoptosis, and TGF-β signals induce the

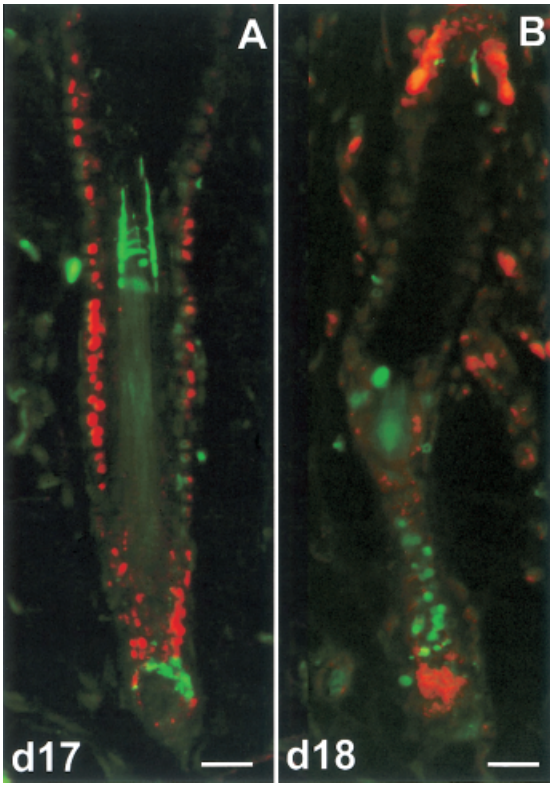

Figure 4. Immunofluorescence detection of PS2- or TSC-22-positive cells compared with apoptotic cells in catagen hair follicles. Mouse dorsal skin sections containing catagen hair follicles from a depilation-induced hair cycle were costained with the TUNEL method and either anti-PS2 or anti-TSC-22 antibodies. The tyramide amplification system was used for immunofluorescence detection with anti-TSC-22 antibodies. (*A*) Cells labeled by the TUNEL assay (*green*) were directly adjacent to those stained with anti-PS2 antibodies (*red*) in the bulb matrix of catagen V hair follicles (at day 17 after depilation), but there were no double-stained cells. (*B*) Many TUNEL-positive cells were observed in the regressing epithelial strand (*green*) of catagen VII hair follicles, however, these did not overlap with TSC-22 immunofluorescence (*red*). *Bars*, 25 μm.

ARTS protein to translocate from the mitochondria to the nucleus (Larisch *et al*, 2000). The relative timing of these events has not been investigated.

In sum, our experiments do not support a model for TGF-β as a direct inducer of epithelial apoptosis during catagen regression *in vivo*. At present, it has not been established that apoptosis is a direct response to TGF-β application in cultured keratinocytes. It may be that TGF-β sensitizes the epithelial cells of hair follicle to undergo apoptosis in response to other signals. The presence of TGF-β-responding cells throughout both the growth and the regression phases of the hair cycle suggests that the cells of hair follicles may be continuously exposed to both catagen-promoting and catagen-inhibiting signals. The rate of growth and the timing of entry into catagen may be determined by the balance between these two types of signal.

A number of reports suggested a link between TSC-22 and TGF-β-induced apoptosis (Ohta *et al*, 1997; Omotehara *et al*, 2000; Uchida *et al*, 2000); however, other studies have suggested a more fundamental role in epithelial morphogenesis in *Drosophila* (Dobens *et al*, 2000) and chick (Dohrmann *et al*, 2002). In mouse hair follicles, nuclear TSC-22 was not exclusively associated with apoptotic cells. For example, nuclear staining was observed in IRS cells and DP cells of anagen hair follicles. Furthermore, there was no overlap of TSC-22 immunofluorescence and TUNEL-positive apoptotic cells in catagen hair follicles. Thus, all our data suggest that TSC-22 does not play a direct role in the induction of apoptosis in catagen phase. Progress on the mouse genome project has revealed additional proteins that are related to TSC-22 (Kester *et al*, 1999). One of these, glucocorticoid-induced leucine zipper shows considerable similarity in its overall structure, as well as in the sequence of the DNA-binding domain. In contrast to TSC-22, glucocorticoid-induced leucine zipper protects T lymphocytes from T cell receptor/CD3-activated cell death (DíAdamio *et al*, 1997). The role of the TSC-22 family in cell survival and cell death may be complex.

Little is known about roles of TSC-22 in mammalian hair. During mouse embryogenesis, TSC-22 RNA accumulates at the sites of epithelial-mesenchymal interactions (Dohrmann *et al*, 1999; Kester *et al*, 2000). The *Drosophila bunched* gene, a member of the TSC-22 family, is required to set a morphologic boundary in response to bone morphogenetic protein and EGF signals during oogenesis (Dobens *et al*, 2000). During feather tract development, TSC-22 was upregulated by a BMP antagonist noggin or receptor tyrosine kinase signals such as EGF, transforming growth factor-α, and fibroblast growth factor, whereas BMP-2/4 repressed TSC-22 expression (Dohrmann *et al*, 2002). The affinity-purified anti-TSC-22 antisera detects an antigen that is highly localized during the mouse hair cycle. Intense TSC-22 immunoreactivity was observed in the IRS, an epithelial cell layer that requires the EGF receptor or TGF-α for proper morphogenesis (Luetteke *et al*, 1993, 1994). Thus, TSC-22 may have a role in organizing the follicular epithelial cell layers in response to the EGF signaling pathway. Future studies of TSC-22-deficient mice, or mice lacking other TSC-22 family members, will enable us to elucidate the function of this family of transcriptional regulators.

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