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Tsukushi controls the hair cycle by regulating TGF-β1 signaling

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ABSTRACT

The hair follicle contains stem/progenitor cells that supply progeny for skin development and the hair cycle. Several signaling molecules belonging to the Wnt, BMP, shh, and transforming growth factor β (TGF-β) signaling cascades are involved in the normal hair follicle cycle. However, the systemic mechanism of how these humoral factors are controlled remains largely unknown. Previously, we reported that Tsukushi (TSK), a member of the small leucine-rich repeat proteoglycan family, functions extracellularly as a key coordinator of multiple signaling networks. Here, we show that TSK is expressed at the restricted areas of hair follicle during the morphogenesis and the hair cycle. Targeted disruption of the TSK gene causes the hair cycle to be delayed with low levels of TGF-β1 and phosphorylated Smad2/3 (pSmad2/3) expression. Biochemical analysis indicates that TSK directly binds to TGF-β1. Our data suggest that TSK controls the hair cycle by regulating TGF-β1 signaling.

Introduction

The hair follicle is a complex mini-organ that undergoes repeated sequences of regeneration and degradation known as hair cycles. Hair follicular stem cells (HFSCs) are found in the hair follicle bulge area (Blanpain and Fuchs, 2006; Cotsarelis, 2006). The HFSC population is composed of multipotent keratinocyte stem cells that are responsible for the cyclic regeneration of hair follicles and provide a transient supply of progeny to the interfollicular epidermis (IFE) and to sebaceous glands after wounding (Blanpain and Fuchs, 2006; Cotsarelis, 2006; Oshima et al., 2001). Several signaling molecules belonging to the Wnt, BMP, shh, and TGF-β signaling cascades are involved in the normal hair follicle cycle (Alonso and Fuchs, 2006; Blanpain and Fuchs, 2009). However, the systemic mechanism of how these humoral factors are controlled remains unclear. Knowledge of how HFSCs acquire their function will provide insight into the molecular control mechanism underlying these processes. Moreover, this knowledge is important for skin cell biology, including the study of skin tumors. Here, we focus on the roles of Tsukushi (TSK) in the hair cycle.

Previously, we reported that TSK, a member of the secreted small leucine-rich repeat proteoglycan (SLRP) family, interacts with and regulates essential signaling cascades (Hocking et al., 1998; Schaefer and Iozzo, 2008; Merline et al., 2009). TSK functions as an organizer inducer by inhibiting BMP signaling in cooperation with chordin (Ohta et al., 2004) and interacts with Vg1 to induce the primitive streak and Hensen's node formation in the chick embryo (Ohta et al., 2006). Recently, our group reported that TSK functions as a Wnt signaling inhibitor by competing with Wnt2b for binding to the transmembrane protein Frizzled4 (Ohta et al., 2011). Thus, TSK is a key coordinator of multiple pathways outside the cells through the regulation of an extracellular signaling network (Dellett et al., 2012).

In this study, we demonstrate that TSK controls differentiation during the hair cycle by regulating TGF-β1 activity. We found that TSK is expressed at the restricted areas of hair follicle during the morphogenesis and the hair cycle. A depletion of TSK results in a delay of the hair cycle with low levels of TGF-β1 and pSmad2/3 expression. Our biochemical analysis indicated that TSK can directly bind to TGF-β1. Consequently, TSK is an essential component of the molecular pathways controlling the hair cycle.

Materials and methods

Mice

Tsukushi-null mutant mice were generated by inserting a LacZ/Neo cassette into the TSK coding exon (Ito et al., 2010). The mice used in these studies were backcrossed to the C57BL/6 J strain for at least six generations and can be considered to have a nearly uniform genetic background. All experiments on mice were

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conducted in accordance with the guidelines of the Kumamoto University Center for Animal Resources and Development. The patterns of TSK expression in pelage hair follicles during development and follicle cycling were studied in heterozygous TSK mice by staining for the LacZ gene in the targeted allele. Skin was examined from the developmental stages (E12, E14.5, E16 and E18.5) to the postnatal hair follicle stages (P0, P2, P4, P7, P10, P14, P20, P23, P25, P30, P35 and P45), which allowed for the study of all 8 stages of hair follicle development (Hardy, 1992; Paus et al., 1999) and hair cycling (Müller-Röver et al., 2001). Slides were dried for 30 min at 37 °C and stained for 6–24 h at 37 °C in a solution containing 400 μg/ml X-gal. After washing in H_2O for 1 min at room temperature (RT), the slides were counterstained with eosin or a 1% Neutral Red (Wako) solution for 3 min, washed, dehydrated in graded ethanol and xylene and mounted with a xylene-based medium.

Tissue processing

Deeply anesthetized mice were fixed by transcardial perfusion with 4% paraformaldehyde (PFA) in PBS. Dorsal skin was harvested and preserved with 20% sucrose overnight at 4 °C. These samples were embedded in a mold containing cold Tissue Tek® OCT compound and stored at −80 °C. Frozen samples were sliced into 10-μm-thick sections using a cryostat (Leica).

Immunohistochemistry

The fluorescence antibody technique was performed as follows. Sections were blocked with PBS containing 5% normal goat serum or skim milk for 30 min at RT. The following antibodies diluted in PBS were applied to the sections: mouse anti-AE13 (1:200, Abcam), mouse anti-AE15 (1:100, Abcam), rabbit anti-keratin14 (1:500, Covance), rabbit anti-keratin1 (1:500, Covance), rabbit anti-lorichrin (1:500, Covance), Cy3-conjugated anti-mouse IgG (1:500, Jackson Immuno Research) and Cy3-conjugated anti-rabbit IgG (1:500, GE Healthcare). The sections were incubated with the primary antibody at RT for 16 h in a humidified chamber. After washing, the secondary antibody was applied to the sections for 1–2 h at RT. During washing with PBS, the slides were counterstained with Hoechst 33342 and mounted. When using mouse monoclonal antibody as the primary antibody, blocking and dilution of the antibody were performed using an M.O.M. kit (Vector).

For the enzyme antibody assay, rabbit anti-keratin15 (1:300, Bioworld Technology) and goat anti-rabbit HRP (1:300, Jackson) were used as primary and secondary antibody, respectively, and visualized by DAB (DACO).

In situ hybridization

In situ hybridization was performed as previously described (Ohta et al., 2004) using digoxigenin (DIG)-labeled antisense probes for the human TSK coding region.

Bromodeoxyuridine (BrdU) immunostaining

Deeply anesthetized mice were intraperitoneally injected with 40 mg/kg BrdU (SIGMA) and sacrificed 2 h later. Embryos and skin were harvested, and sections were prepared. Slides were preserved with 4 N HCl for 20 min at RT, washed with 0.1 M sodium tetraborate (3 × 5 min) and PBST (3 × 5 min), and incubated for 2 h at RT with rat anti-BrdU antibody (1:700, Abcam). After washing with PBS, the slides were incubated for 1 h with Cy3-conjugated anti-rat IgG antibody (1:300, Jackson).

Quantitative PCR

Total RNA was prepared from the normal skin of WT and TSK-/- mice. To detect the mRNA expression levels throughout the hair cycle, dorsal skin was harvested from P10, P20, P30, and P40 mice. Total RNA was extracted from each sample using Qiagen RNeasy spin columns (QIAGEN) and transcribed to cDNA using a high-capacity RNA-to-cDNA kit (Applied Biosystems). The cDNA was stored at −20 °C until needed for quantitative PCR. The mRNA expression levels of TSK, TGF-β1, TGF-β2, Wnt5a, BMP2, Gli1 and PTCH1 were examined by quantitative PCR using sequence-specific primers and probes, pre-developed TaqMan assay reagents (40 cycles of denaturing at 92 °C for 15 s and annealing at 60 °C for 60 s), and an ABI StepOne™ system (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the mRNA levels. The relative levels of expression were determined using the ΔΔCt method (Meijerink et al., 2001) to compare target genes and housekeeping gene mRNA expression levels.

Co-precipitation

The M-TSK-Myc-His plasmid was introduced into COS-7 cells with Lipofectamine 2000 (Invitrogen). After 16 h, the cells were washed twice with PBS and then cultured for 4 days with Opti-MEM buffer (Invitrogen). Co-precipitation assays were performed as previously described (Ohta et al., 2004). Briefly, Myc-tagged M-TSK protein and TGF-β1 protein (Cell Signaling Tech.) were incubated overnight at 4 °C and pulled down with protein G-Sepharose (Pharmacia). M-TSK and TGF-β1 proteins bound to the beads were subjected to SDS-PAGE and blotted onto a nylon membrane. Myc-tagged M-TSK protein and TGF-β1 protein were detected by immunoblotting with an anti-Myc antibody [9E10 (Developmental Studies Hybridoma Bank)] and anti-TGF-β1 antibody (1:1000, Santa Cruz).

Statistical analysis

A student's *t*-test was used to determine the levels of significance between the samples. A P value of < 0.05 was considered statistically significant.

Results

Expression of TSK during hair follicle morphogenesis and the hair cycle

We examined the expression of TSK using β-galactosidase (β-gal) staining of TSK heterozygous dorsal skin samples from E12 to P30 (Fig. 1). TSK was expressed from morphogenesis to the hair cycle. TSK was expressed in the undifferentiated epithelium at E12 (Fig. 1A), hair placode cells at E14.5 (Fig. 1B) and germ cells at E16 (Fig. 1C). During the embryonic stage, TSK was expressed in mesenchymal cells of the dermis (Fig. 1A–D). After birth, strong TSK expression was observed in hair bulbs and sebaceous glands (Fig. 1E–I) but disappeared in the dermis. During the morphogenesis of the hair follicle, TSK expression in dermal papilla was weak. In the catagen stage at P16, TSK expression remained in regressive hair follicles (Fig. 1J). In the second anagen stages, TSK was expressed around the bulge area (Fig. 1K and L). Thus, TSK expression was detected in the restricted areas of hair follicle during the morphogenesis and the hair cycle.

We performed in situ hybridizations to verify that β-gal staining indicated actual TSK expression. The expression pattern of TSK transcripts revealed the same pattern as shown by β-gal

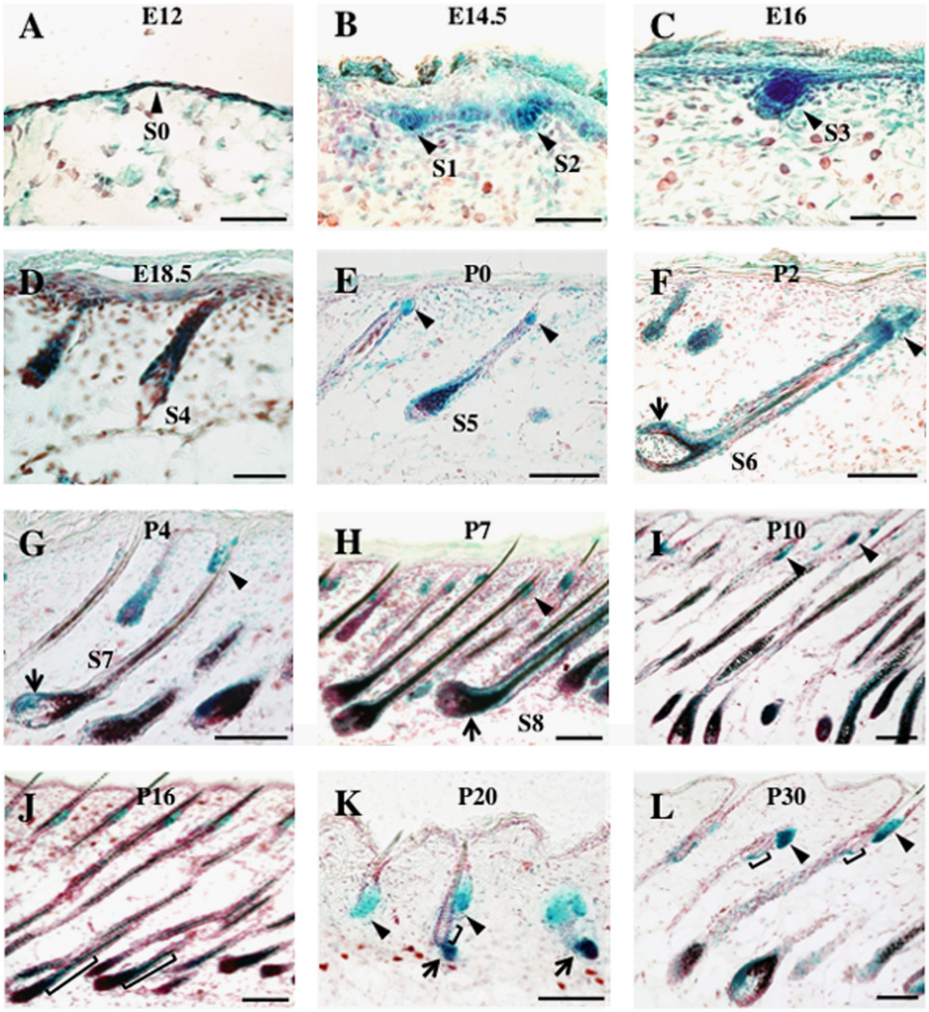

Fig. 1. The TSK expression patterns during hair follicle morphogenesis and hair cycling. Hair follicle development was assessed during eight different stages [referred to as stages 1 (S1) to 8 (S8) in the figures (Hardy, 1992; Paus et al., 1999)]. (A–L) β-Galactosidase staining of dorsal skin cross-sections from TSK heterozygous mice. Counter staining was performed using Neutral Red. Scale bars indicate 100 μm in (A–D) and 50 μm in (F–L). (A) TSK is expressed in the undifferentiated epithelium (arrowhead) at E12. (B) TSK is strongly expressed in hair placode cells (arrowheads) at E14.5. (C) TSK is strongly expressed in germ cells (arrowhead) at E16. (D) TSK is expressed in hair pegs at E18.5. (E) TSK is expressed in the bulbs, inner root sheath and sebocytes (arrowheads) at P0. (F) TSK is expressed in the bulb (arrow), bulge cells, outer root sheath and sebaceous glands (arrowhead) at P2. (G–I) TSK is expressed in bulbs (arrows) and sebaceous glands (arrowheads) at P4 (G), P7 (H) and P10 (I). (J) TSK is expressed in the regressive cells at P16 (brackets). (K, L) TSK is expressed in the bulge cells (brackets) and sebaceous glands (arrowheads) at P20 (K) and P30 (L).

staining (Fig. S1A). Moreover, human TSK mRNA was expressed in the bulge and bulb areas, mirroring the mouse expression pattern (Figs. S1B and C). Further, we examined the mRNA expression levels of TSK throughout the hair cycle. We performed quantitative PCR analyses and determined that the mRNA level of TSK was downregulated at the first telogen stage, although it was upregulated at the secondary anagen (Fig. S1D). This result was consistent with the β-gal expression pattern.

TSK expression is related to hair cell differentiation

We performed immunostaining to determine whether TSK expression cells merge with HFSC. TSK β-gal expression was merged with K15, which is an HFSC marker (Fig. S2A) (Liu et al., 2003). To investigate the proliferative ability of TSK-expressing cells, mice were intraperitoneally injected with BrdU at P20. BrdU was incorporated into hair germ cells but was not incorporated into bulge cells and sebaceous glands, in which TSK is expressed. These results indicated that the proliferative ability of TSK-expressing cells was low (Fig. S2B).

TSK deletion causes a delay in the hair cycle

To perform a phenotypic analysis of skin of the TSK-/- mice, we examined their skin morphology using HE staining (Fig. 2A). At P10 (first anagen), the skin thickness and pilosebaceous unit formation of TSK-/- mice were similar to those of the WT mice. At P14, the hair cycle of WT mice was at the catagen stage, although the hair follicles of TSK-/- mice remained at the anagen stage. At P20, the hair cycle of WT mice was at the telogen stage, although the hair follicles of TSK-/- mice remained at the degraded catagen stage. Subsequently, the hair cycle of TSK-/- mice was gradually delayed relative to WT mice (Fig. 2A and B). Similarly, the skin pigmentation after depilation was different between WT and TSK-/- mice at P30 (Fig. 2C). The skin color of WT mice was gray, which is indicative of the anagen stage, whereas that of TSK-/- mice was pink, which is indicative of the telogen stage (Alonso and Fuchs, 2006).

Subsequently, we verified the difference in hair morphogenesis during development between WT and TSK-/- mice. The density of hair placodes and HFs at P1 was not significantly different between WT and TSK-/- mice (Fig. S3A). High-magnification images of WT

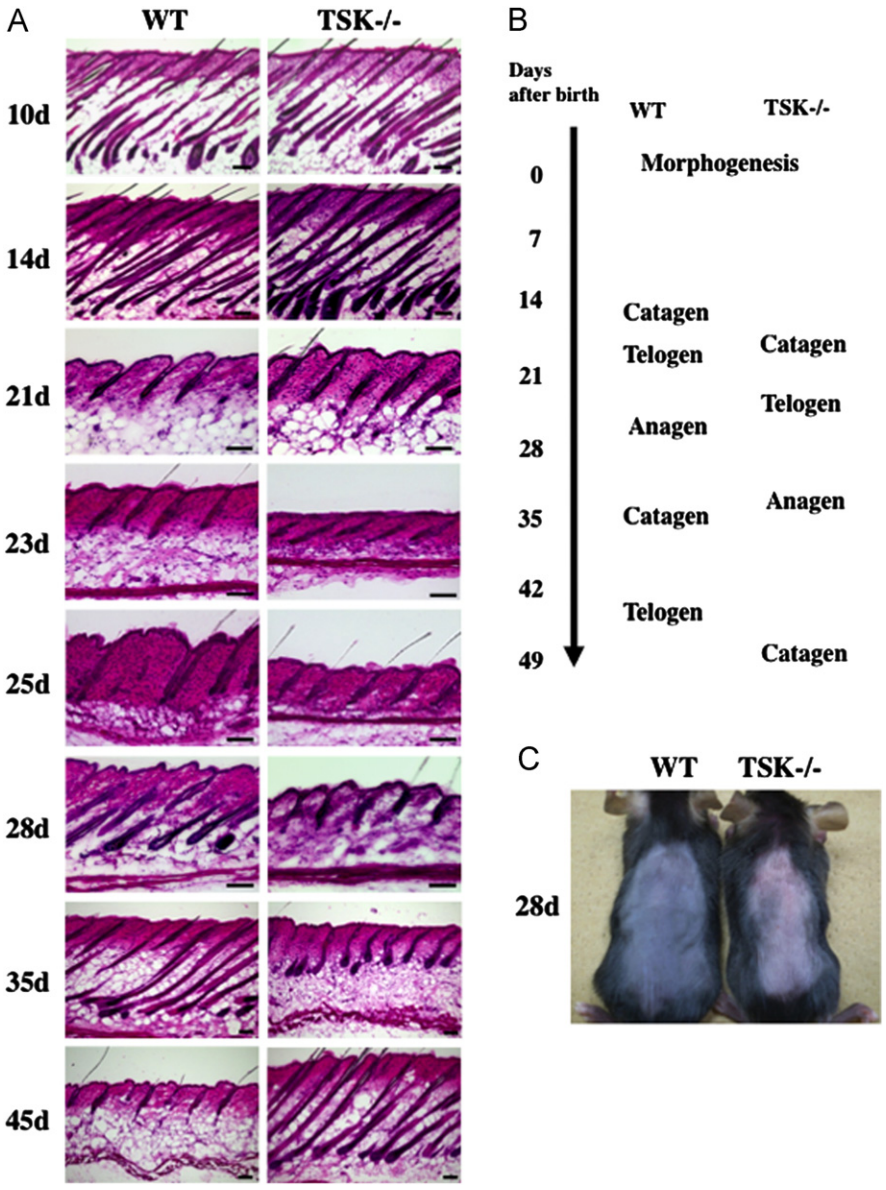

Fig. 2. The hair cycle is delayed in TSK-/- mice. (A) Representative HE images of dorsal skin sections. Scale bars indicate 100 μm. (B) The hair cycle time scale of WT and TSK-/- mice during the first 7 weeks after birth. (C) Macroscopic phenotype of 28-day-old mice. The WT mouse shows a gray color after depilation, which indicates the hair development stage, although the TSK-/- mouse shows a pink color, which indicates that the hair follicles are in a resting state.

and TSK-/- HFs at the P1, P10 and telogen stages revealed no difference in the HF morphology and pigmentation (Fig. S3B). To determine whether there were structural abnormalities in the hair follicles, we performed immunostaining using hair follicle markers. There was no difference in the AE13 (cortex), AE15 (medulla and inner root sheath), and K14 (outer root sheath) between WT and TSK-/- mice (Fig. S4). Further, we studied the development of the epidermis using immunostaining for markers of keratin differentiation (Yuspa et al., 1989). No difference was observed in K1, K14, K17, or lorichrin expression between WT and TSK-/- mice at P10 (data not shown).

The loss of TSK affects hair cycle regulatory molecules at the mRNA expression level

To further assess the cause of the delayed hair cycle in the TSK-/- mice, the mRNA expression levels of hair cycle regulatory molecules (Lin et al., 2004) were examined by quantitative PCR with WT or TSK-/- mice (Fig. 3). TGF-β1 is reported to be a catagen inducer (Foitzik et al., 2000), and TGF-β2 is considered to be transiently activated from the telogen to anagen phases (Oshimori and Fuchs, 2012). The mRNA expression levels of TGF-β1 were significantly downregulated in the TSK-/- mice throughout the hair cycle (Fig. 3A). By contrast, the mRNA expression level of TGF-β2 was low in the TSK-/- mice compared with WT mice at P30 (Fig. 3B).

Wnt and BMP signaling are activated at the anagen stage (Lin et al., 2004; Blanpain and Fuchs, 2009). The mRNA expression level of Wnt5a was not significantly different at P10 and P20 between WT and TSK-/- mice, although the expression level became lower at P30 and higher at P40 in TSK-/- mice compared to WT mice at the anagen phase (Fig. 3C). The mRNA expression level of Bmp2 was lower in TSK-/- mice compared to WT mice from P10 to P30 but higher at P40 (Fig. 3D).

Shh signaling is required for the induction of the early anagen phase (Lin et al., 2004). The mRNA expression levels of the

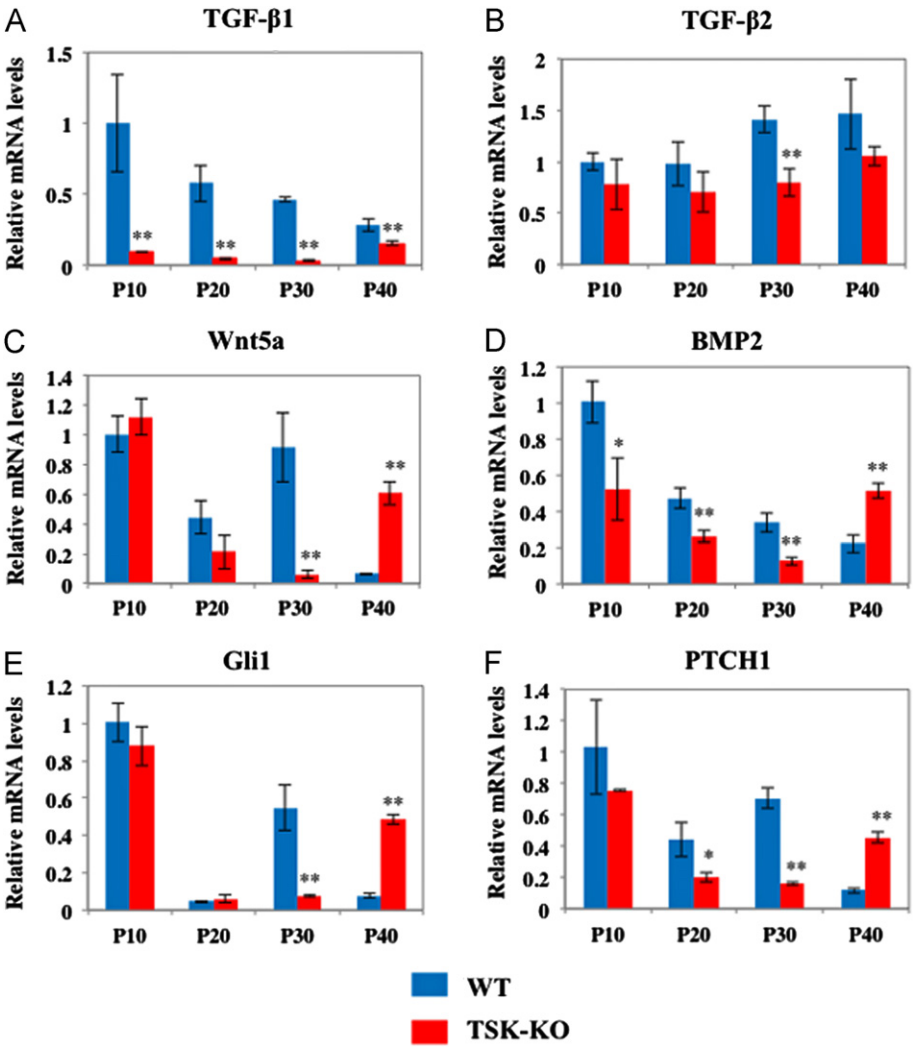

Fig. 3. Comparison of the mRNA expression levels of hair cycle regulatory molecules between WT and TSK-/- mice. The relative mRNA expression levels of TGF-β1 (A), TGF-β2 (B), Wnt5a (C), BMP2 (D), Gli1 (E) and PTCH1 (F) were quantified by quantitative PCR and normalized relative to the expression of endogenous GAPDH. $^{*}p < 0.05$. $^{**}p < 0.005$. Error bars indicate the standard deviation. Six samples were used in each group.

downstream molecules of shh signaling, Gli1, and PTCH1 were downregulated at P30 and upregulated at P40 in TSK-/- mice (Fig. 3E and F). Our data suggest that the observed differences between WT and TSK-/- are most likely due to a shift in the hair cycle and indicate that the loss of TSK affects hair cycle regulatory molecules, thereby delaying the hair cycle.

TSK controls TGF-β1 signaling during the hair cycle

To examine the possibility of a biochemical interaction between TSK and TGF-β1, we performed co-precipitation assays by transfecting tagged constructs into COS-7 cells. When Myc-His-tagged M-TSK was co-precipitated with TGF-β1, bound M-TSK protein and TGF-β1 protein were pulled down (Fig. 4A). These results indicate that TSK binds directly to TGF-β1 and modulates TGF-β1 signaling. Because the TGF-β1 signal causes the phosphorylation of Smad2/3, we analyzed the expression of pSmad2/3 by immunostaining using the anti-pSmad2/3 antibody. The expression of pSmad2/3 was detected in the outer root sheath at the catagen stage and the bulge area at the telogen stage of WT mice, respectively, but was not detected in TSK-/- mice (Fig. 4B). These data suggest that TSK is necessary for TGF-β1 signaling during the hair cycle.

Discussion

We demonstrated that TSK is expressed in the hair bulb, bulge cells, and sebaceous gland throughout the hair cycle. These TSK expression patterns were not merged with BrdU positive cells, and thus, we assumed that TSK is related to cell differentiation and maintenance but not cell proliferation.

The SLRP family, which includes biologically active components of the extracellular matrix, interacts with a host of different cell surface receptors, cytokines, and growth factors, leading to the modulation of cellular functions (Schaefer and Iozzo, 2008; Merline et al., 2009). In our study, the biochemical results indicate that TSK can directly bind to TGF-β1. In addition, other SLRP family molecules, such as decorin, are known to control each cycle by activating or inhibiting TGF-β activity (Merline et al., 2009). Our data indicate that TSK-/- mice show a delayed hair cycle and reduced expression of pSmad2/3, which is downstream of TGF-β signaling. The mRNA levels of TGF-β1 were significantly

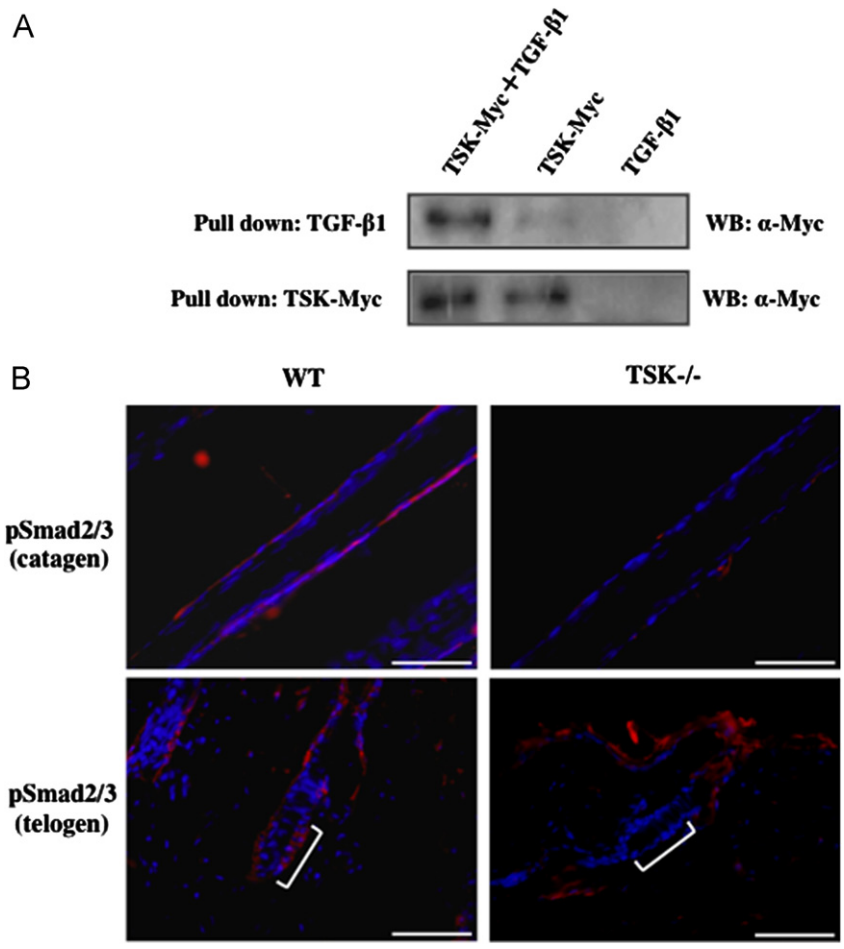

Fig. 4. TSK downregulates TGF-β1 signaling. (A) TSK directly binds to TGF-β1. These figures show the results of the immunoprecipitation assays for TSK-Myc and TGF-β1. (B) pSmad2/3 was downregulated in the bulge area of TSK-/- mice relative to WT mice. Immunostaining of the dorsal skin from WT and TSK-/- mice at the catagen and telogen stages. Brackets indicate the bulge area. Scale bars indicate 50 μm.

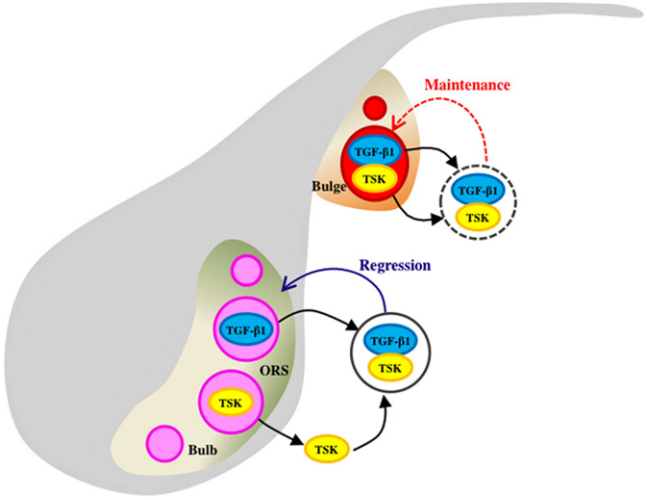

Fig. 5. A schematic model of TSK and TGF-β1 during the hair cycle. TSK is secreted from the lower follicle, binds to TGF-β1 outside the cell and causes cell regression. Furthermore, TSK may maintain HFSCs that are TGF-β dependent. ORS; outer root sheath.

downregulated in the TSK-/- mice throughout the hair cycle. TGF-β1 null mice showed delayed catagen entry similar to that of TSK-/- mice, and the injection of TGF-β1 into the back skin of mice induced premature catagen development (Foitzik et al., 2000). Based on these observations, we demonstrated that TSK secreted from lower follicles binds to TGF-β1 outside the cell and controls cell regression at the catagen stage (Fig. 5). A role for TGF-β signaling in stem cell quiescence has been previously suggested for hematopoietic cells, neural stem cells, and prostate cells (Batard et al., 2000; Salm et al., 2005; Larsson et al., 2003; Karlsson et al., 2007; Falk et al., 2008; Watabe and Miyazono, 2009). Recently, TGF-β signaling has been reported to be important for the homeostasis of the epidermis and hair follicles (Yang et al., 2009; Nishimura et al., 2010; Tanimura et al., 2011). TSK may maintain cells that are TGF-β1 dependent, such as HFSCs, because TSK is a secreted protein and interacts with TGF-β1 outside of cells. Therefore, we suggest that TSK secreted by bulge cells control the hair cycle by extracellularly regulating TGF-β1 activity (Fig. 5)

Furthermore, TSK may control hair cell differentiation in cooperation with TGF-β2 (Fig. 3B). TGF-β2 signaling is required during hair follicle morphogenesis to activate the HFSCs and induce the anagen stage (Foitzik et al., 1999; Oshimori and Fuchs, 2012). TSK-/- mice displayed a prolonged telogen stage, and therefore, TSK may induce the anagen phase by interacting with TGF-β2.

In summary, we determined that TSK functions as a niche molecule by extracellularly interacting with TGF-β1 to control the hair cycle. These findings could help to elucidate the HFSC environment. Moreover, because human TSK is expressed in a pattern similar to that observed in mouse skin, these findings

could provide new evidence regarding the regenerative processes that regulate the hair cycle.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.08.030.

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