Biochemical and Biophysical Research Communications 456 (2015) 721–726

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Trps1 deficiency inhibits the morphogenesis of secondary hair follicles via decreased Noggin expression

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ARTICLE INFO

Article history:
Received 1 December 2014
Available online 13 December 2014

Keywords:
Trps1
Noggin
Hair follicle
Development
BMP

ABSTRACT

A representative phenotype of patients with tricho-rhino-phalangeal syndrome (TRPS) is sparse hair. To understand the developmental defects of these patient's hair follicles, we analyzed the development of hair follicles histologically and biochemically using Trps1 deficient (KO) mice. First, we compared the numbers of primary hair follicles in wild-type (WT) and KO embryos at different developmental stages. No differences were observed in the E14.5 skins of WT and KO mice. However, at later time points, KO fetal skin failed to properly develop secondary hair follicles, and the number of secondary hair follicles present in E18.5 KO skin was approximately half compared to that of WT skin. Sonic hedgehog expression was significantly decreased in E17.5 KO skin, whereas no changes were observed in Eda/Edar expression in E14.5 or E17.5 skins. In addition, Noggin expression was significantly decreased in E14.5 and E17.5 KO skin compared to WT skin. In parallel with the suppression of Noggin expression, BMP signaling was promoted in the epidermal cells of KO skins compared to WT skins as determined by immunohistochemistry for phosphorylated Smad1/5/8. The reduced number of secondary hair follicles was restored in skin graft cultures treated with a Noggin and BMP inhibitor. Furthermore, decreased cell proliferation, and increased apoptosis in KO skin was rescued by Noggin treatment. Taken together, we conclude that hair follicle development in Trps1 KO embryos is impaired directly or indirectly by decreased Noggin expression.

1. Introduction

The Trps1 gene encodes an atypical member of the GATA-type family of transcription factors. Mutations in the human TRPS1 gene lead to Tricho-rhino-phalangeal syndrome (TRPS), an autosomal dominant malformation characterized by sparse and slow-growing scalp hair as well as craniofacial and skeletal abnormalities [1]. Trps1 deficient (KO) mice die shortly after birth from respiratory failure due to tracheal cartilage malformation and present craniofacial and skeletal anomalies that mimic those in TRPS patients. Hair follicle formation in Trps1 KO mice seems to be negatively affected, as demonstrated by the complete loss of vibrissae and the reduction of approximately 50% of pelage hair follicles [2,3].

Morphogenesis of hair follicles (HFs) is a result of reciprocal signaling and crosstalk between epidermal and dermal cells. HFs form as a result of a competition between promoters and repressors of placodes [4–6]. Wnt signaling functions as a key stimulator of HF development, whereas TGF-β superfamily members are the most prominent inhibitors as is reviewed elsewhere [6,7]. In mouse fur, HF morphogenesis is induced at embryonic day 14.5 (E14.5) and results in the formation of four hair types, including guard hair, also known as primary or tylotrich hair, which makes up approximately 5–10% of all HFs in the mouse dorsal skin and is characterized by a large hair bulb, a long straight hair, and two sebaceous glands [8–10]. Induction of the secondary or nontylotrich (awl and zig-zag) HFs that produce thinner and shorter hair shafts with one sebaceous gland occurs from E16.5 to postnatal day 0.5 (P0.5) [8–10]. Several studies have suggested that the induction of primary and secondary HFs requires different signaling pathways as exemplified by guard and zig-zag hairs, whose development specifically depends on the signaling of the new TNF receptor, ligand family members Eda/Edar and the downstream transcription factor NF-κB [11–13]. Mice with a Noggin mutation or that overexpress Dkk4 were reported to have malformed secondary HF formation [14,15].

Trps1 is expressed throughout the morphogenesis of HFs. During the hair germ stage at E14.5, Trps1 expression was observed in

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http://dx.doi.org/10.1016/j.bbrc.2014.12.039
0006-291X/

the nuclei of dermal condensate cells. By E16.5 and E17.5, Trps1 expression had become restricted to the nuclei of mesenchyme-derived dermal papilla cells and the mesenchymal cells surrounding hair pegs and underlying the epidermis [16]. Trps1 has been reported to function by activating a network of secreted Wnt inhibitors and transcription factors or by suppressing Sox9 to guide the proper initiation and differentiation of vibrissa follicle morphogenesis [17,18]. However, until now, there have been no reports on the mechanisms that lead to decreased pelage HFs.

In this study, we examined the development of pelage HFs at different time points throughout embryonic development in Trps1 KO and wild-type (WT) mice to identify the role of Trps1 in pelage HF development.

2. Materials and methods

2.1. Trps1 KO mice and tissue preparation

Trps1 KO mice were generated as previously described [2]. Heterozygous Trps1-deficient (Trps1 HT) mice were mated, and the appearance of the vaginal plug was defined as embryonic day 0.5 (E0.5). The Committee on Animal Care and Use at Wakayama Medical University approved all animal experiments conducted in this study.

2.2. Organ culture of the developing mouse skin

Developing mouse skin was cultured as previously described [19] with minor modifications. The entire layer of back skin was carefully peeled off from E16.5 embryos under a dissecting microscope and spread with the epidermal side up onto Nuclepore filters (pore size 8 μm; Whatman) coated with type I collagen (BD biosciences). The skin grafts were covered with a drop of culture medium and were incubated while floating on 2 ml of medium in 35-mm plastic culture dishes or 6-well plates (Thermo Scientific) in a humidified atmosphere with 5% CO_2 at 37 °C for 2 days. Culture medium was DMEM/F12 (Gibco) supplemented with 1% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma). For rescue experiments, recombinant mouse Noggin (0.3 μg/ml or 0.6 μg/ml) (R&D) and DMH 1 (2 μM or 5 μM) (BMP inhibitor II, Calbiochem) were added to the culture medium.

2.3. Preparation of histological sections and immunohistochemical staining

Cultured skin grafts or E13.5–18.5 embryos were washed with PBS, fixed in 4% paraformaldehyde overnight, and embedded in paraffin blocks in the same direction. The skin grafts and embryos were sectioned longitudinally into 3 μm thick sections that were used for the subsequent HE staining or immunostaining. For immunofluorescence of Noggin, 8 μm cryosections were used. Immunostaining was performed using an autoclave-based antigen retrieval technique. Primary antibodies used in this study include anti-Noggin (goat polyclonal, 1:50, R&D), anti-phosphoSmad1/Smad5/Smad8 (rabbit polyclonal, 1:100, Millipore), and anti-PCNA (mouse polyclonal, 1:1000, Chemicon) antibodies. After incubation with primary antibodies, sections were treated with a Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. For immunofluorescence, FITC- or Cy3-conjugated secondary antibodies were used (Invitrogen).

2.4. Western blot analysis

Dorsal skin pieces that were peeled from age matched WT and Trps1 KO embryos were homogenized in cold tissue lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 2.5 mM Sodium orthovanadate, 10 mM NaF, and 10 mM β-glycerol phosphate) containing a Complete Protease Inhibitor Mixture (Roche). Twenty micrograms of tissue lysate was separated by SDS–PAGE and blotted on PVDF membranes (Millipore). Blotted membranes were incubated with a primary goat anti-mouse Noggin antibody (R&D) and a secondary anti-goat antibody (Sigma Aldrich); signals were developed using the ECL Plus detection system (Amersham Bioscience).

2.5. Quantitative real-time polymerase chain reaction (PCR)

SYBR Green Supermix (Bio-Rad) was used for quantitative real-time PCR. Gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Sequences of the primers used were as follows: Noggin forward 5′-AGCACCCAG-CGACAACCT-3′ and reverse 5′-GTCTCGTTCAGATCCTTCTCC-3′; Eda forward 5′-CACTGACTTTGCCAGCTAC-3′ and reverse 5′-TCTTCCCTG-TCTCAATGCT-3′; Edar forward 5′-AGCTGTGAGTGATGCTATC-3′ and reverse 5′-TTGGTTCATTTGAGTGCTC-3′; Shh forward 5′-GTGAAAG-CAGAGAACTCCGT-3′ and reverse 5′-ACGTAAGTCCTTCACCAGCT-3′.

2.6. Hair follicle number counting

The number of hair follicles was counted from E14.5 to E18.5 embryos or from cultured skin grafts ($n = 3$). For each sample, the total number of HFs from 6 nonconsecutive sections (every 30 μm per section) was counted at a low power field under a microscope. To calculate the total length of the 6 sections, each section was consecutively photographed at a low power field under a microscope. The summed length of the basal cell layers was measured using Image J software.

2.7. TUNEL staining

TUNEL staining was performed on paraffin sections of embryonic skin or cultured skin grafts using the ApopTag peroxidase in situ apoptosis detection kit (Millipore) or the TMR red in situ cell death detection kit (Roche) according to the manufacturer's instructions. For counter-staining of nuclei, 0.5% methyl green or DAPI was used.

2.8. Statistical analysis

The data were analyzed in Excel with an unpaired 2-tail Student's t-test and expressed as the mean ± SD. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Initiation of secondary hair follicles was inhibited in Trps1 KO embryos

E13.5–E18.5 embryonic skin samples from WT and Trps1 KO mice were examined in HE stained sections. The number of HFs was counted on each gestation day. In WT fetal skin, the first wave of hair bud formation was initiated at E14.5 (primary hair follicles), followed by the second wave of hair bud formation at E16.5 (secondary hair follicles), represented by a dramatic increase in the number of hair buds. In Trps1 KO mice, primary hair follicles were initiated normally at E14.5. No difference was observed in the number of hair buds between WT and Trps1 KO fetal skins until E15.5. However, a sharp increase in the number of HFs was observed at E16.5 in WT fetal skin. By E18.5, the number of HFs present in KO fetal skin was approximately half the number of

HFs in WT skin (Fig. 1A and B). Sonic hedgehog (Shh) is one of the first signals sent by epithelial cells to direct the condensation of dermal cells [20,21]. At E14.5 Shh expression levels remained similar between WT and Trps1 KO fetal skins. However, Shh expression was greatly decreased in Trps1 KO fetal skin compared to WT fetal skin at E17.5 when robust secondary HF formation has taken place in WT fetal skin (Fig. 1E and F). The expression of Eda and Edar, which are largely regulated in primary HF development, was not changed at E14.5 and E17.5 in KO fetal skin compare to WT fetal skin (Fig. 1C and D).

3.2. Noggin expression is impaired in Trps1 KO fetal skin

Because Noggin KO mice are characterized by a lack of secondary HFs induction [15], we examined Noggin expression in Trps1 KO mice. In WT fetal skin, Noggin expression was observed in dermal papillae and dermal cells underlying the epidermis (Fig. 2A). Noggin expression at the protein and mRNA level was greatly reduced in Trps1 KO mice compared to WT mice at E14.5 and E17.5 (Fig. 2B–D). Immunostaining for Noggin at E14.5 was not detected in either WT or KO fetal skin.

Because Noggin is a well-known BMP antagonist [22], we next examined Smad1/5/8 phosphorylation to investigate the activation of BMP signaling. At E14.5, immunostaining for phosphorylated Smad1/5/8 (pSmad1/5/8) was even across the epidermis with no significant differences between WT and KO fetal skins. At E16.5, as secondary HFs initiated, simultaneously overall signals decreased and negative patches of pSmad1/5/8 staining were observed in the interfollicular epidermis (arrows in Fig. 2E) of WT skin, whereas in KO skin these negative patches were absent. At E17.5, pSmad1/5/8 immunostaining was significantly decreased in both WT and KO skins. However, the overall signals in KO skin were still more intense than in WT skin (Fig. 2E).

3.3. Exogenous Noggin treatment rescued the number of HFs

Based on the results described above, we hypothesized that decreased Noggin expression was a critical reason for the reduction of HF numbers in Trps1 KO dorsal fetal skin. To confirm our hypothesis, in vitro skin graft cultures were performed in the presence or absence of recombinant mouse Noggin or the BMP inhibitor Dmh1. When skin grafts from E15.5 fetuses were cultured in control medium (without the addition of Noggin or Dmh1) for 2 days, Trps1 KO skin grafts produced 50% less HFs than their WT counterparts. Noggin or DMH1 rescued the number of HFs to levels similar to WT skin grafts (Fig. 3A and B). Noggin treatment increased Shh mRNA expression (Fig. 3C) and effectively inhibited Smad1/5/8 phosphorylation (Fig. 3D).

3.4. Proliferation and apoptosis were changed by Trps1 deficiency and reversed by Noggin treatment

To investigate cell proliferation and apoptosis, TUNEL staining and immunostaining for PCNA were performed. In E18.5 KO fetal skin, epidermal cell proliferation was greatly inhibited and TUNEL-positive cells were greatly increased compared to their WT counterparts (Fig. 4A). No difference was detected at E14.5 between WT and Trps1 KO skins (data not shown). Noggin treatment in culture medium increased cell proliferation, reducing apoptosis of epidermal cells in Trps1 KO skin (Fig. 4B). The cell proliferation was restricted to keratinocytes in the basal layer, and TUNEL positive cells were mainly located in the epithelial cells of hair follicles and the surrounding mesenchymal cells.

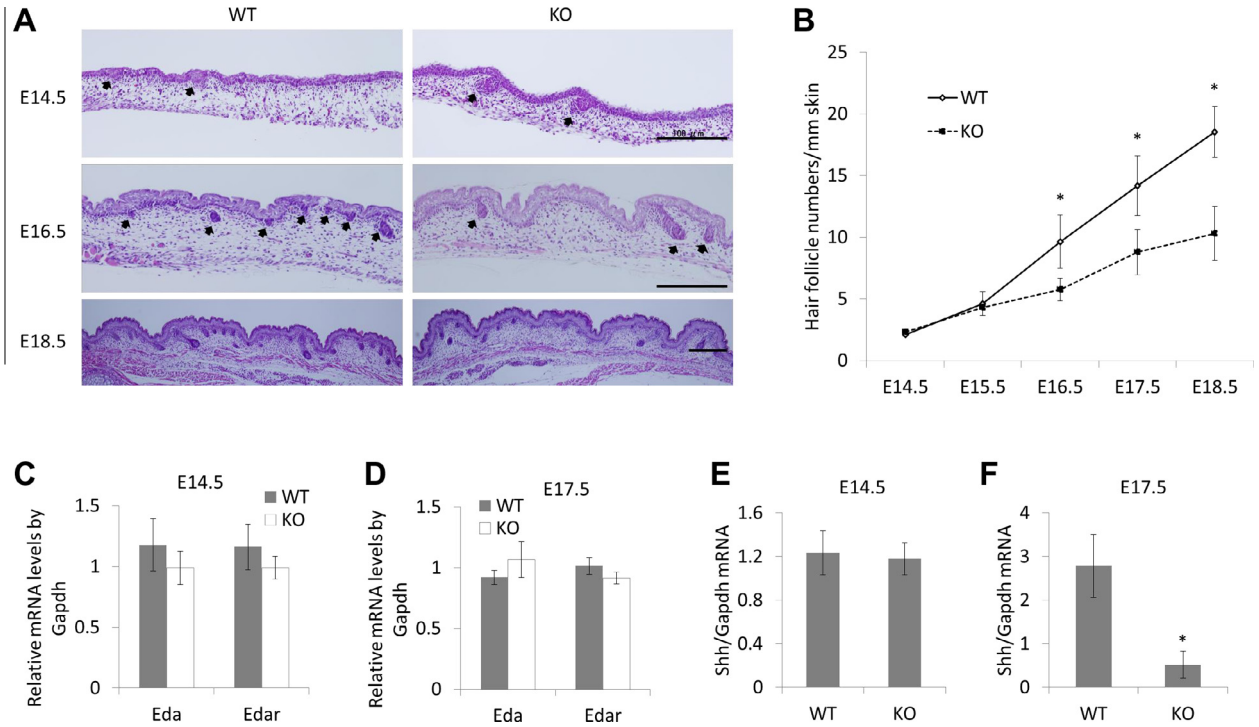

Fig. 1. The initiation of secondary hair follicles is inhibited in Trps1 KO embryos. (A) Representative H.E. stained sections of back skins from E14.5 to E18.5 wild-type (WT) and Trps1 KO (KO) embryos. Initiation of primary HFs in KO skin is the same as in WT skin at E14.5. Secondary HF formation was held back at E16.5. The total HF numbers were decreased by half in KO examined at E18.5. Scale bar: 100 μm. (B) Quantification of HF numbers as described in Section 2. (C)–(F) Real-time PCR examination of Eda, Edar, and Shh at E14.5 and E17.5. mRNA expression levels of Eda or Edar were not changed either E14.5 (C) or E17.5 (D). Shh expression was not changed at E14.5 (E) but was greatly suppressed at E17.5 (F). Data are presented as means ± S.D. $^{*}P < 0.05$.

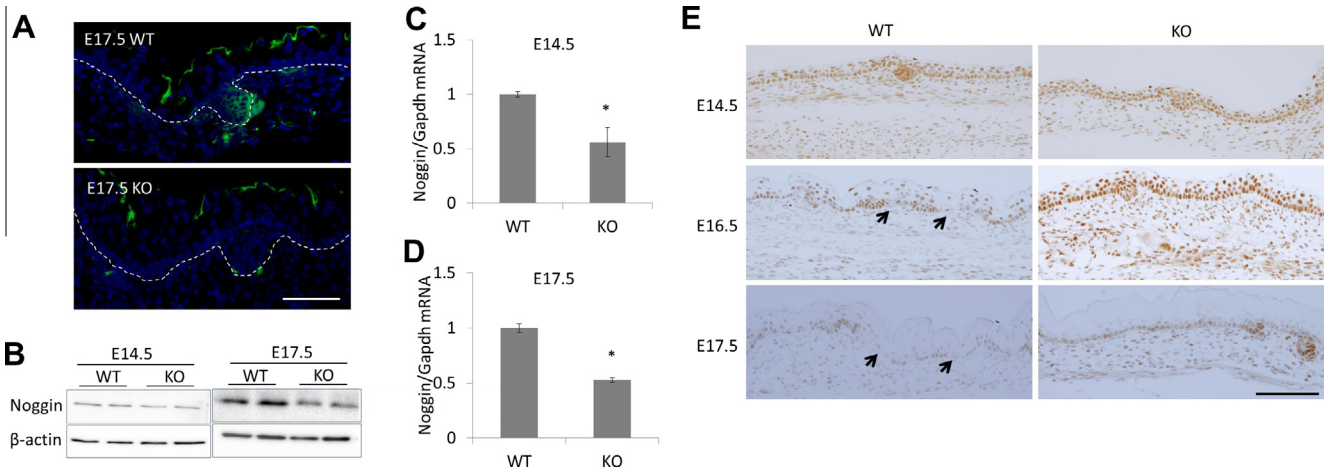

Fig. 2. Noggin expression is impaired in Trps1 KO fetal skin. (A) Detection of Noggin protein using E17.5 back skin sections. Dotted lines indicate the border between the epidermis and dermis. Scale bar: 20 μm. (B) Western blotting for Noggin at E14.5 and E17.5. (C) and (D) Noggin expression levels at E14.5 (C) and E17.5 (D) detected by real-time PCR. Data are presented as means ± S.D. $^{*}P < 0.05$. (E) Immunohistochemistry staining for pSamd1/5/8. Arrows indicated patched decreasing signals in the interfollicular epidermis. Scale bar: 50 μm.

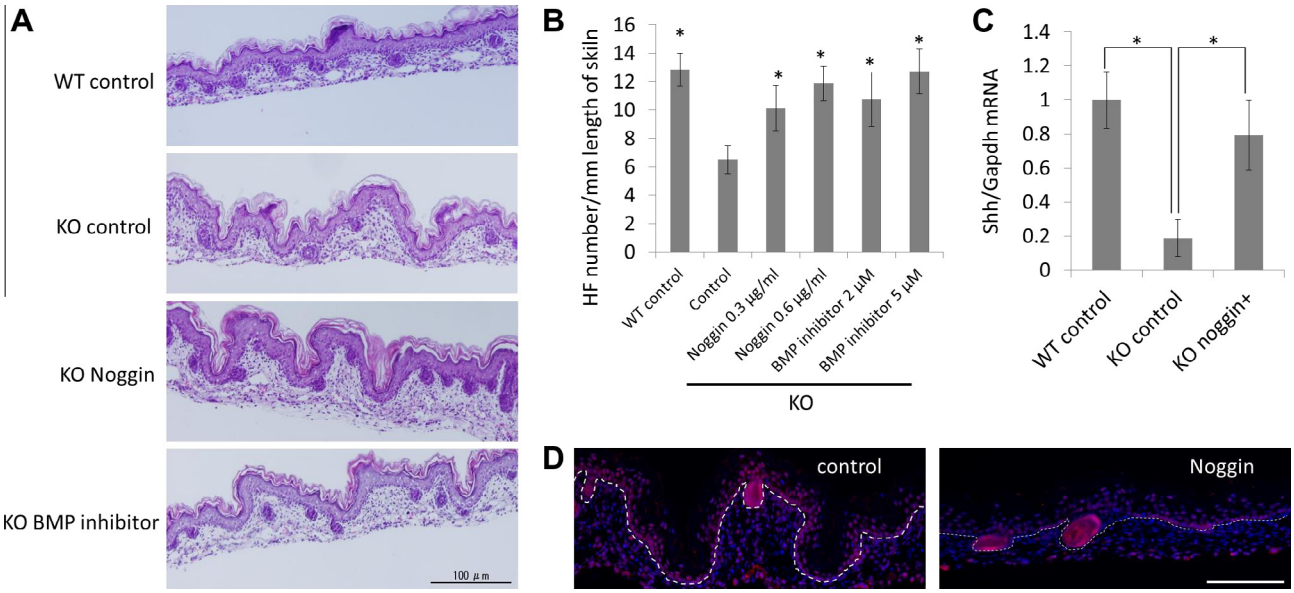

Fig. 3. Noggin treatment rescues HF numbers. (A) H.E. stained sections of E15.5 embryonic skin grafts underwent 2 days in vitro culture in conditioned culture medium. Scale bar: 100 μm. (B) Quantification of HF numbers of skin grafts after 2 days culture using a method described in Section 2. (C) Real-time PCR for Shh after culture in conditioned medium. Data are presented as means ± S.D. $^{*}P < 0.05$. (D) Immunofluorescent staining for pSmad1/5/8 in Trps1 KO skin grafts with or without Noggin treatment. Dotted lines indicate the border between the epidermis and dermis. Scale bar: 50 μm.

4. Discussion

In this study, we reported that Trps1 deficiency led to impaired production of secondary (nontylotrich) HFs without affecting the initiation of primary HFs. In addition, we demonstrated that Trps1 deficiency reduced Noggin expression, which may be the underlying cause of decreased HF formation through overactivation of BMP signaling, which results in a reduction of cell proliferation and the promotion of apoptosis.

Trps1 KO embryonic skins have normal HF initiation at E14.5 and E15.5; however, the sharp increase in the number of HFs observed in WT embryonic skins occurs for unknown reasons. It has been suggested that the induction of primary and secondary HFs may require different signaling pathways. Shh is one of the signature genes that induces epidermal placodes, and its expression reflects HF generation [21]. Shh expression had similar levels in WT and Trps1 KO embryonic skins during primary HF generation (E14.5); subsequently, Shh dramatically decreased in Trps1 KO skin compared to WT skin. From these data, we hypothesized that the secondary but not primary HFs were affected by the loss of Trps1 because the suppression was not derived from Eda/Edar expression, which induces primary HFs [13].

In Trps1 KO embryonic skin, Noggin expression was greatly suppressed compared to WT skin at both E14.5 and E17.5. Noggin is a mesenchymal cell-derived stimulator that is indispensable for secondary HF induction because of its inhibition of BMP signaling [15,23]. In Noggin deficient mice, HFs arrest at stage 2 and secondary HFs are unable to initiate [15]. Dermal Noggin secretion is essential to rescue BMP mediated inhibition. Recent evidence has shown that sustained Shh expression relies on BMP inhibition mediated by dermal Noggin [24,25]. During HF induction, Noggin localizes to the mesenchymal condensation, dermal papilla, and

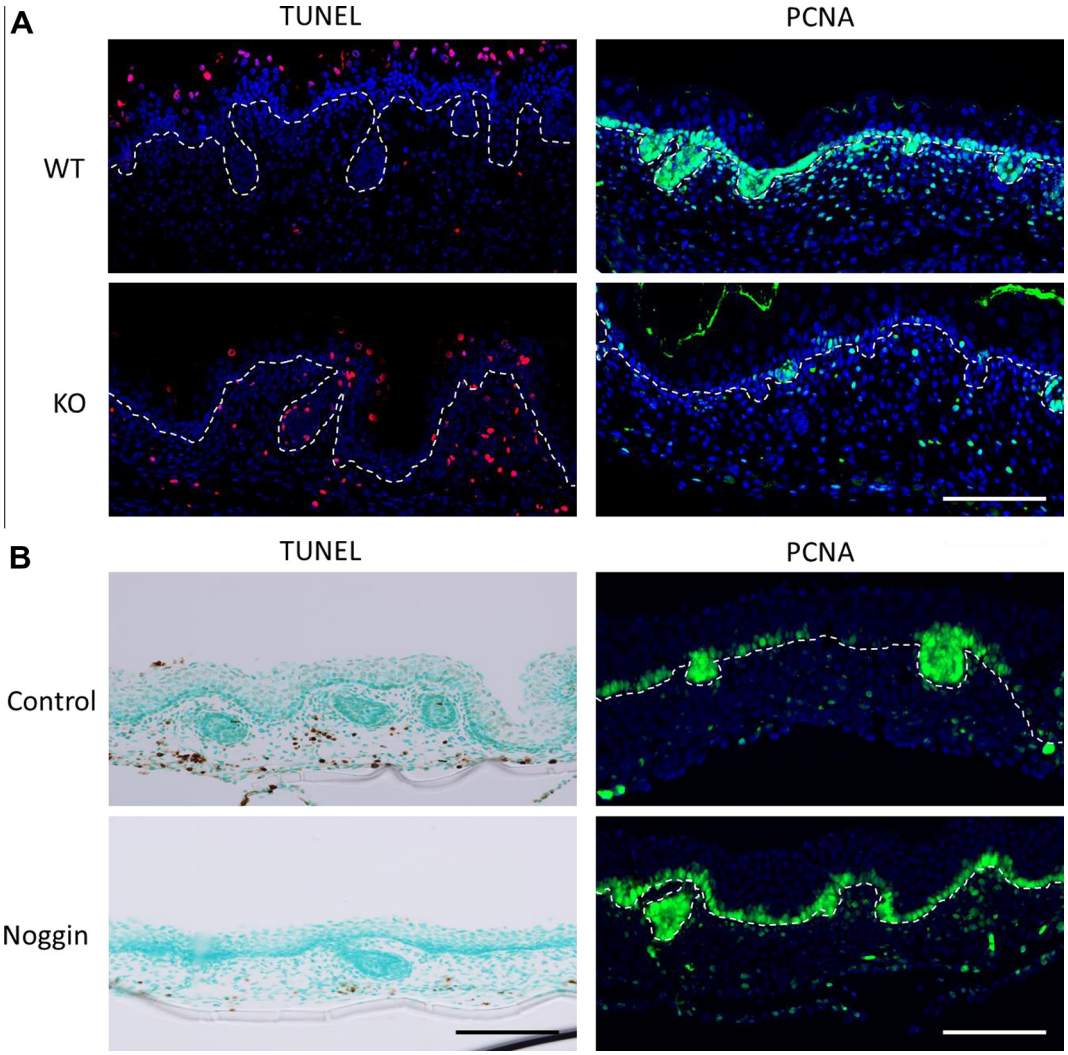

Fig. 4. Decreased cell proliferation and increased apoptosis at E18.5. Trps1 KO embryonic skin and post-cultured skin grafts were rescued by Noggin treatment. (A) TUNEL assay and Immunofluorescent staining for PCNA of E18.5 WT and Trps1 KO (KO) embryonic skins. (B) TUNEL assay and Immunofluorescent staining for PCNA of Trps1 KO post-cultured skin grafts with or without Noggin treatment. Dotted lines indicate the border between the epidermis and dermis. Scale bar: 50 μm.

the proximal connective tissue sheath of hair follicles [23], which correspond to locations where Trps1 is expressed [16]. We hypothesized that for some unknown reasons (direct or indirect), loss of Trps1 leads to decreased Noggin expression, which in turn causes impaired secondary HF generation. We confirmed our hypothesis using an in vitro skin graft culture system. Exogenous recombinant Noggin treatment rescued HF generation in KO skin grafts, simultaneously increased Shh mRNA expression levels, and inhibited Smad1/5/8 phosphorylation.

Noggin is thought to exert its role in HF induction via inhibition of BMP signals [23]. In our study, we demonstrated that Trps1 KO embryonic skin had decreased Noggin expression and increased pSmad1/5/8 signals. Moreover, in vitro studies showed that treatment with either Noggin protein or the BMP inhibitor Dmh1 rescued the number of HFs in KO skin grafts, indicating that Noggin functions as a BMP inhibitor. This is in agreement with previous studies: that retardation of HF development in E17.5 Noggin knockout mice is associated with alterations in Lef1 expression in hair placodes and of p75NTR in mesenchyme [23] and that long term deletion of Noggin leads to strong upregulation of BMP2 and BMP4 in the epidermis and dermis of Noggin null skin transplants [15].

Overexpression or loss of Noggin is reportedly related to cell proliferation and apoptosis during HF development or tumorigenesis in the skin [23,26,27]. At E18.5, in Trps1 KO skin, cell proliferation was inhibited, whereas apoptosis was increased compared to WT counterparts. We hypothesized that this was a result of a Noggin deficiency. Noggin treatment in the culture system reversed the relationship between proliferation and apoptosis, which corroborates our hypothesis.

In this study, we failed to demonstrate a direct interaction between Trps1 and Noggin. However, because Trps1 is a well-known transcriptional repressor, the decreased expression of Noggin through Trps1 deficiency may be an indirect reaction induced by other factors, such as Wnt [18] or Sox9 [17].

In conclusion, we report, for the first time, that Trps1 plays an important role in the morphogenesis of secondary but not primary HFs via an interaction with the BMP inhibitor Noggin. We hope that our study will provide deeper insights into the developmental regulation of hair follicles and contribute to a clinical application for treatment of patients with TRPS.

Conflict of interest

The authors declare no conflicts of interests, financial or otherwise.

Acknowledgments

We thank Ms. Mayumi Akira for her technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (24590488) from the Ministry of Education, Science, Sports, and Culture of Japan (to Y.M.).

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