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RESEARCH PAPER

Transient stimulation of TRPV4-expressing keratinocytes promotes hair follicle regeneration in mice

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Background and Purpose: Hair follicle telogen to anagen transition results in a break in cellular quiescence of the hair follicle stem cells, which subsequently promotes hair follicle regeneration. Many critical molecules and signalling pathways are involved in hair follicle cycle progression. Transient receptor potential vanilloid 4 (TRPV4) is a polymodal sensory transducer that regulates various cutaneous functions under both normal and disease conditions. However, the role of TRPV4 in hair follicle regeneration in vivo remains incompletely understood.

Experimental Approach: Using adult C57BL/6J mice, keratinocyte (*K14*^{Cre}; *Trpv4*$^{f/f}$) and macrophage (*Cx3cr1*^{Cre}; *Trpv4*$^{f/f}$) *Trpv4* conditional knockout (*cKO*) mice, *Trpv4*$^{-/-}$ mice, we investigated the effect of a single intradermal injection of GSK1016790A, a potent and selective small molecule TRPV4 activator, on hair follicle regeneration. Chemical cues and signal molecules involved in hair follicle cycle progression were measured by immunofluorescence staining, quantitative RT-PCR and western blotting.

Key Results: Here, we show that a single intradermal injection of GSK1016790A is sufficient to induce telogen to anagen transition and hair follicle regeneration in mice by increasing the expression of the anagen-promoting growth factors and down-regulating the expression of growth factors that inhibit anagen. The action of GSK1016790A relies largely on the function of TRPV4 in skin and involves activation of downstream ERK signalling.

Conclusion and Implications: Our results suggest that transient chemical activation of TRPV4 in the skin induces hair follicle regeneration in mice, which might provide an effective therapeutic strategy for the treatment of hair loss and alopecia.

1 | INTRODUCTION

The hair follicle is the most prominent mini-organ found in the skin. It is composed of three main components: the outer root sheath, the inner root sheath and the hair shaft. The hair follicle cyclically transforms from rapid growth (anagen), to apoptosis-driven regression (catagen) and last to relative quiescence (telogen) phases (Muller-Rover et al., 2001; Oshima, Rochat, Kedzia, Kobayashi, & Barrandon, 2001). Many critical molecules and signalling pathways are involved in these distinct phases of hair follicle cycle progression. The quiescence of hair follicle stem cells during telogen is largely maintained by bone morphogenetic proteins (BMPs) derived

Abbreviations: BMP, bone morphogenetic protein; GSK101, GSK1016790A; GSK219, GSK2193874; TRP, transient receptor potential; wt, wild type.

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from both bulge cells and many other types of dermal cells. For instance, the subcutaneous adipocytes and dermal fibroblasts produce **BMP-2A** and **BMP-2B** that inhibit anagen (Plikus et al., 2008) and the inner bulge layer secrets high levels of **VG-1-related protein** (BMP-6) and **FGF18** that suppress the proliferation of hair follicle keratinocytes (Hsu, Pasolli, & Fuchs, 2011). On the other hand, dermal papilla-derived anagen-promoting **FGF7** and **Noggin** are elevated when the hair follicle progresses from early telogen to late telogen. Moreover, Noggin expression is rapidly induced following the activation of bone morphogenetic protein receptors and therefore acts as an effective feedback antagonist against bone morphogenetic protein signalling (Gazzerro, Gangji, & Canalis, 1998; Ito, Akiyama, Shigeno, & Nakamura, 1999).

Transient receptor potential (TRP) channels are versatile players in the skin and regulate various cutaneous functions under both normal and disease conditions besides serving as molecular sensors for detecting many distinct sensory modalities (Nilius & Szallasi, 2014). Many TRP channels, especially TRP vanilloid 3 (**TRPV3**) and TRP vanilloid 4 (**TRPV4**) are extensively expressed in the skin-resident cells and play critical roles in skin physiology and diseases (Caterina & Pang, 2016; Luo & Hu, 2014; Toth, Olah, Szollosi, & Biro, 2014). TRPV3 plays important roles in hair morphogenesis in mice (Cheng et al., 2010) and gain-of-function TRPV3 mutations in humans cause Olmsted syndrome which is associated with severe chronic itching and skin disorders (Lin et al., 2012). Moreover, TRPV3 was reported to play different roles in hair follicle cycling (Borbiro et al., 2011; Imura et al., 2007). TRPV4 is a polymodal sensory transducer that integrates a variety of thermal, mechanical and chemical stimuli including warmth (27–35°C), hypo-osmotic stimulation and many inflammatory metabolites (Nilius & Szallasi, 2014; Voets, 2014), contributing to pain sensation, epidermal barrier homeostasis and the formation of tight junctions (Denda, Sokabe, Fukumi-Tominaga, & Tominaga, 2007; Kida et al., 2012; Mamenko, Zaika, Boukelmoune, O'Neil, & Pochynyuk, 2015; Sokabe, Fukumi-Tominaga, Yonemura, Mizuno, & Tominaga, 2010; Sokabe & Tominaga, 2010). Our previous study further demonstrated that TRPV4 is highly expressed by both skin keratinocytes and dermal macrophages and contributes to the pathogenesis of both allergic and non-allergic chronic itch in mice (Luo et al., 2018). Although a recent study showed that activation of TRPV4 induces apoptosis and drives early catagen transition in human hair follicles, which inhibits human hair growth ex vivo (Szabo et al., 2019), the role of TRPV4 in hair follicle regeneration in vivo remains unclear.

In the present study, we demonstrate that transient chemical activation of TRPV4 is sufficient to trigger hair follicle regeneration through regulating hair follicle telogen to anagen transition in *wild-type* (*wt*) but not TRPV4 KO mice. Mechanistically, TRPV4 activation disrupts the balance of anagen-promoting and inhibiting signalling pathways that control hair follicle regeneration. Moreover, TRPV4-mediated response requires ERK signalling pathway in keratinocyte. These findings identify the keratinocyte-expressed TRPV4 as an effective promoter of hair follicle regeneration in mice.

What is already known

- Bone morphogenetic proteins signaling suppresses while overexpression of FGF7 and Noggin promotes hair follicle induction.
- These molecular cues originate from both hair follicle bulge and other types of dermal cells.

What this study adds

- Activation of TRPV4 is sufficient to regulate hair follicle telogen to anagen transition in mice.
- TRPV4 activation disrupts the balance of anagen-promoting and inhibiting pathways that control hair follicle regeneration.

What is the clinical significance

- This study suggests that keratinocyte-expressed TRPV4 is a potential target to promote hair follicle regeneration
- TRPV4 is a novel target for the treatment of clinically relevant hair regeneration disorders.

2 | METHODS

2.1 | Animals

Adult (7–10 weeks, around 20-g body weight) male and female C57BL/6J mice (Jackson Laboratories, Ellsworth, ME, USA), $K14^{Cre}$ (Jackson Laboratories), $Cx3cr1^{Cre}$ (Jackson Laboratories) and $Trpv4^{-/-}$ (provided by Masashi Imai, Jichi Medical School, Tochigi, Japan) (Suzuki, Mizuno, Kodaira, & Imai, 2003) were used in this study. To generate the $Trpv4^{f/f}$ mice, three of the properly targeted ES cell clones were obtained from the KOMP Repository and used for blastocyst injections and one clone led to high contribution chimaeras that produced germline transmitted offspring as assayed by black coat colour. This chimera line was then mated to FLPo mice (Jackson Laboratories) to remove the neomycin cassette and generate heterozygous $Trpv4^{f/+}$ mice, which were subsequently crossed with $K14^{Cre}$ and $Cx3cr1^{Cre}$ to generate both Cre^{+} and Cre^{-} $Cx3cr1^{Cre}$; $Trpv4^{f/f}$ and $K14^{Cre}$; $Trpv4^{f/f}$ mice, respectively. All mice were housed under a 12:12-h light/dark cycle with food and water provided ad libitum. All animal care and experimental procedures were performed using protocols approved by the Animal Studies Committee at Washington University School of Medicine and were in accordance with the guidelines provided by the National Institutes of Health. All mice were randomly allocated to different experimental groups by the lab members who were blinded to the experimental design. Data analysis was also

performed in a manner completely blind to experimental groups. At the end of experiments, mice were killed by CO_2 asphyxia. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010; McGrath & Lilley, 2015) and with the recommendations made by the *British Journal of Pharmacology*.

2.2 | Animal treatment

The second telogen of C57BL/6J mice starts at day 49 after birth and it will last around 5 weeks (Muller-Rover et al., 2001). On day 48 after birth, the mice were shaved. On day 49 after birth, *wt* and *Trpv4*$^{-/-}$ mice were intradermally injected with 30-μM **GSK1016790A** (GSK101, a selective TRPV4 agonist) (50 μl), which is diluted in saline from the 100 mM stock solution. **GSK2193874** (GSK219, a selective TRPV4 antagonist) (25 mg·kg^{-1}, 200 μl dilution in saline from 50 mg·ml^{-1} stock solution) (Thorneloe et al., 2012) or **PD98059** (10 mg·kg^{-1}, 200 μl dilution in saline from 37.5 mg·ml^{-1} stock solution) was applied through intraperitoneal injection on day 48 after birth or 1 hour prior to GSK1016790A injection. Insulin syringes with 30-G needle (UltiCare) were used for intradermal injection and 1-ml Sub-Q syringes with 26-G needle (BD) were used for intraperitoneal injection. Seventeen days after GSK1016790A injection, the mice were killed and the skin samples of the injection area were collected for analysis. In mice with newly generated hair, generally, there was a small patch of skin with dark colour and longer hair shaft at the injection sites compared with that in the surrounding area.

2.3 | Histology, immunofluorescence staining

Dorsal skin samples were fixed overnight at 4°C in Zamboni's fixative (2% paraformaldehyde, 15% [v/v] saturated picric acid, 0.1-M PB, pH 7.3), dehydrated in ethanol, embedded in paraffin and sectioned at 10 μm. Haematoxylin and eosin (*H&E*) staining was performed according to standard protocols to analyse the hair follicle cycle stages as described, including the length of the hair follicle, the size and location of dermal papilla and the position of the hair shaft and the inner root sheath (Muller-Rover et al., 2001). For immunofluorescence detection, sections were deparaffinized, rehydrated and subjected to antigen retrieval in 10-mM citrate buffer (pH 6.0). After blocking, sections were stained with primary antibody against Ki67 (1:1,000; Thermo Fisher Scientific, Cat# PA1-29503, RRID:AB_1955602), phosphop-p44/42 MAPK (1:200; Cell Signaling Technology, Cat# 4370S, RRID:AB_2315112), p44/42 MAPK (1:1,000; Cell Signaling Technology, Cat# 4695, RRID:AB_390779), keratin 14 (1:1,000; BioLegend, Cat#, RRID:AB_2616962) and keratin 15 (1:1,000; BioLegend, Cat# 833901, RRID:AB_2564970) at 4°C overnight in a humid chamber, followed by incubation for 1 h at room temperature (RT) with Cy3-conjugated secondary antibody (1:500; Jackson ImmunoResearch, Cat# 111-005-003, RRID:AB_2337913) and Alexa Fluor 488-conjugated secondary antibody (1:500; Jackson ImmunoResearch, Cat# 703-005-155, RRID:AB_2340346). Nuclei were stained with DAPI (SouthernBiotech). The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* Alexander et al., 2018.

2.4 | Quantitative RT-PCR

For real-time PCR, skin sample was collected 24 h after GSK1016790A injection. Total RNA was extracted from mouse skin tissue using RNeasy kit (Qiagen, Germantown, MD, Germany) according to the manufacturer's instructions. RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and the cDNA was synthesized in vitro using ThermoScript® RT-PCR System kit (Invitrogen). Primers used for PCR were listed in Table S1. Reactions were carried out in a volume of 20 μl per reaction containing 10-μl SYBR Green master mix (2×) (Roche, Indianapolis, IN, USA), 0.5-μl cDNA, 1.2-μl 5-μM primer mix and 8.3-μl water using StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative expression of genes was normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ methods.

2.5 | Western blotting

The skin was lysed in RIPA lysis and extraction buffer (Thermo Fisher Scientific, USA). Following cell protein quantitation, 10 μg of protein per sample were subjected to 12% SDS-PAGE. The proteins separated on the SDS-PAGE gel were transferred to a PVDF membrane (Millipore Corp., Billerica, MA, USA), which was blocked for 1 h at RT. The membrane was then incubated overnight at 4°C with monoclonal antibodies against β-actin (1:5,000; Sigma-Aldrich, Cat# A5441, RRID:AB_476744) or phosphop-p44/42 MAPK (1:200; Cell Signaling Technology, Cat# 4370S, RRID:AB_2315112) and p44/42 MAPK (1:1,000; Cell Signaling Technology, Cat# 4695, RRID:AB_390779) followed by incubation with a secondary antibody conjugated with HRP. Immunoreactive bands were visualized by an enhanced chemiluminescence (ECL) system.

2.6 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Data are expressed as the mean ± SEM. No statistical power calculation was conducted before the study. Sample sizes subjected to statistical analysis at least 5 animal per group (n=5), where n= number of independent values. The differences of hair follicle cycling were compared by Fisher's exact test. The significance was determined by one-way ANOVA for comparisons among three or more groups and Student's unpaired *t*-test for comparisons between two groups with GraphPad Prism 7 (GraphPad Software Ltd., San Diego, CA, USA, RRID:SCR_002798). A value of

$P < 0.05$ was considered significantly different. The post hoc tests were conducted only if F in ANOVA achieved $P<0.05$ and there was no significant variance inhomogeneity.

2.7 | Materials

PD98059 was from Cell Signaling Technology (Danvers, USA). GSK1016790A was from Sigma-Aldrich Inc. (St. Louis, MO, USA). GSK2193874 was purchased from Tocris (Minneapolis, USA). Stock solutions of drugs were made in DMSO and diluted to working concentrations in saline immediately before use.

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | TRPV4 activation by a small molecule activator induces hair follicle regeneration

To check whether activation of TRPV4 could affect the normal hair follicle cycling in mice, we first examined whether genetic ablation of TRPV4 function affects general hair follicle cycling. We found that there was no difference in the hair follicle cycling between the TRPV4 KO mice and their wild type (*wt)* littermates (Figure S1). Moreover, pharmacological inhibition of TRPV4 function with the TRPV4 antagonist GSK2193874 had no effect on the normal transition of first telogen to second anagen (Figure S1). These results suggest that TRPV4 is not involved in general hair follicle cycling under normal condition.

To further investigate the function of TRPV4 in hair follicle, we performed a single intradermal injection of GSK1016790A, a potent and selective small molecule TRPV4 activator (Thorneloe et al., 2008) into the back skin of 7-week-old *wt* mice, where the hair follicles were in telogen, that is the entire hair follicle resides in the upper dermis and is associated with ball-shaped dermal papilla (Muller-Rover et al., 2001; Paus & Cotsarelis, 1999). Surprisingly, 17 days after the GSK1016790A injection, newly generated hair coat could be clearly observed at the injection site in a dose-dependent manner but not in mice with vehicle injection (Figures 1a and S2). After GSK1016790A injection, the hair follicles were elongated by more than fourfold with the bulbs resided in the deep subcutis, reminiscent of anagen phase (Figure 1b,c). Furthermore, most of the hair follicles had entered anagen III–VI phases after GSK1016790A injection, whereas most of hair follicles were in the telogen or anagen I phase after vehicle treatment (Figure 1d). Importantly, the GSK1016790A-induced hair regeneration was completely abolished by pretreatment with TRPV4 antagonist GSK2193874. Moreover, the GSK1016790A-induced hair regeneration was completely absent from the global TRPV4 KO mice (Figure 1a–c). Taken together, these results suggest that a single intradermal injection of GSK1016790A is sufficient to promote hair follicle regeneration. Given that GSK1016790A selectively activates TRPV4, it is conceivable that this receptor is the sole mediator of GSK1016790A-induced hair follicle transition from telogen to anagen.

3.2 | TRPV4 activation by GSK1016790A increases proliferation of hair follicle keratinocytes

Since the early stage of hair follicle development is characterized by intensive proliferative activity (Schmidt-Ullrich & Paus, 2005), we examined if a single intradermal injection of GSK1016790A affects the proliferative activity of hair follicle keratinocytes at the site of injection using Ki67 staining. Indeed, the number of Ki67-positive K14-expressing hair follicle keratinocytes was significantly increased in comparison with that in mice injected with vehicle only (Figure 2a). Furthermore, we also observed increased number of Ki67-positive K15-expressing hair follicle stem cells in the bulge of the mice subjected to GSK1016790A injection, suggesting that hair follicle stem cell proliferation might be involved in GKS101-induced hair follicle regeneration (Figure 2b). Therefore, transient chemical activation of TRPV4 in the skin can promote proliferation of keratinocytes and stem cells in telogen.

3.3 | TRPV4 activation shifts the balance of chemical cues that inhibit or promote activity of hair follicle stem cells

Multiple signalling pathways including these for Wnt/β-catenin, bone morphogenetic protein and FGFs are critically involved in hair follicle development and cycling (Hsu et al., 2011; Plikus et al., 2008). From early to late telogen, the expression of several hair germ activation cues is increased, including FGF7 and bone morphogenetic protein inhibitor Noggin, while the BMPs are down-regulated, which facilitates activation of the hair follicle stem cells (Greco et al., 2009; Plikus et al., 2008). Moreover, Wnt signalling in dermal papilla also plays an important role in hair germ activation (Enshell-Seijffers, Lindon, Kashiwagi, & Morgan, 2010; Greco et al., 2009; Kandyba et al., 2013). Therefore, both an increase in expression of anagen-promoting chemical cues and a decrease in expression of anagen-inhibiting growth factors induce hair follicle telogen to anagen transition.

We thus tested if TRPV4-mediated hair follicle regeneration involves changes in the expression of signalling molecules that either promote or inhibit hair follicle telogen to anagen transition by using real-time RT-PCR 24 h after GSK1016790A injection (Figure 3a). Indeed, GSK1016790A treatment resulted in a significant decrease in

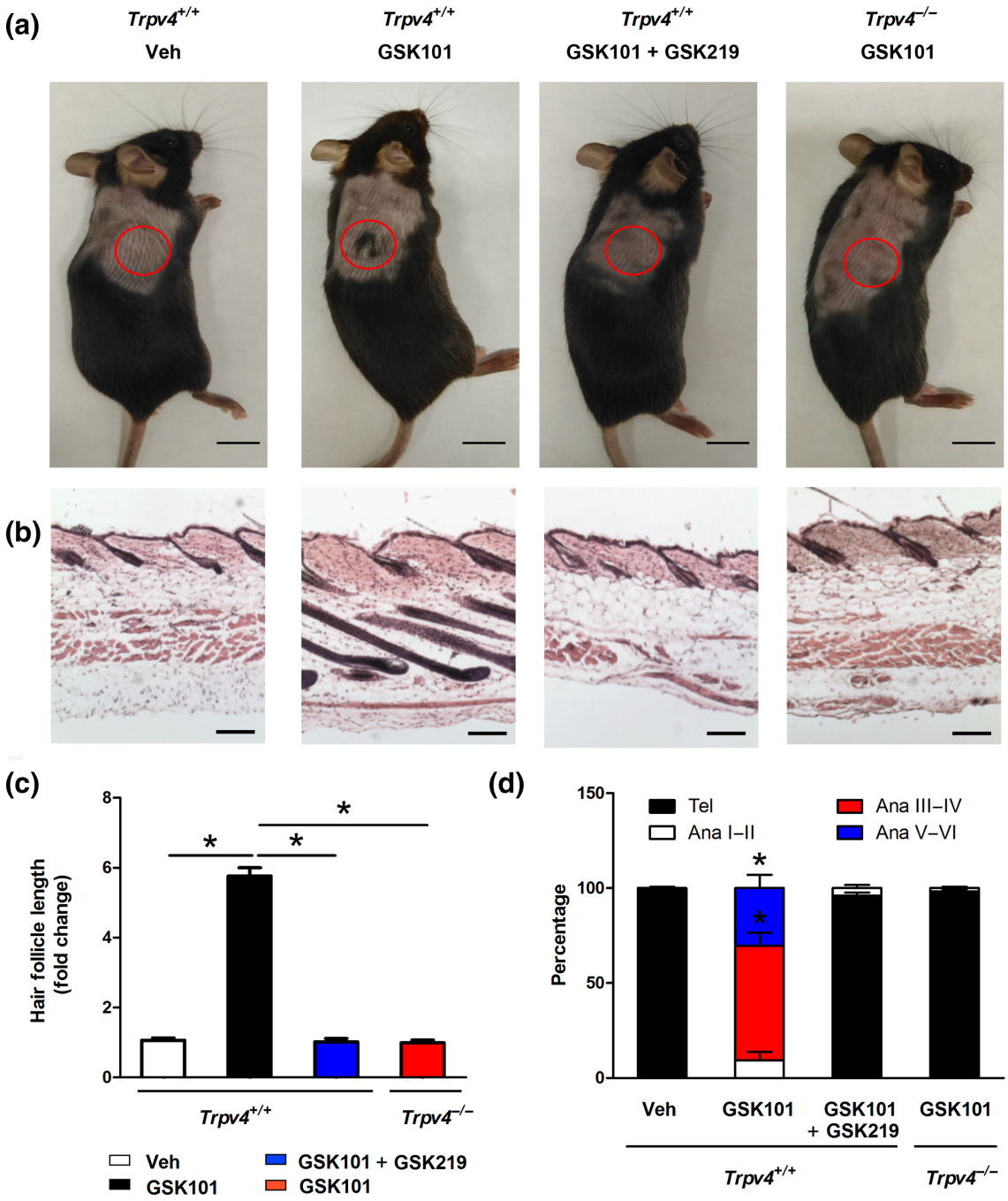
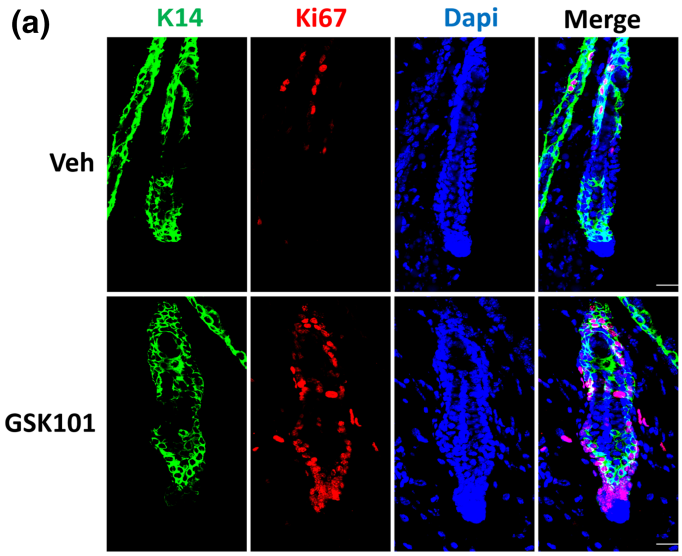


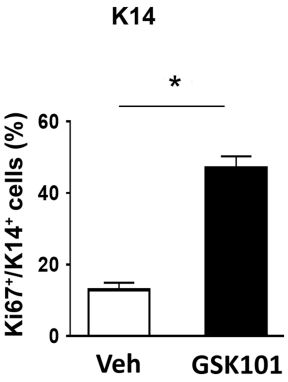
FIGURE 1 TRPV4-mediated hair genesis. (a) Representative images showing hair growth in *Trpv4*$^{+/+}$ mice (n = 10) intradermally injected with vehicle(Veh), 30 μM GSK1016790A (GSK101), GSK1016790A with 25 mg·kg^{-1} GSK2193874 (GSK219) pretreated and in *Trpv4*$^{-/-}$ mice (n = 10) injected with GSK1016790A. Scale bar = 1 cm. (b) Representative images of H&E staining showing back skin sections from *Trpv4*$^{+/+}$ mice treated with vehicle, GSK101, GSK101 + GSK219and *Trpv4*$^{-/-}$ mice with GSK1016790A injection (n = 6). Scale bar = 100 μm. (c) Quantification of hair folliclelength in each group. (d) Stacked bars showing the percentage of hair follicless at the different stages of the hair cycle in each group (n = 6). Data are expressed as mean ± SEM. *P < 0.05

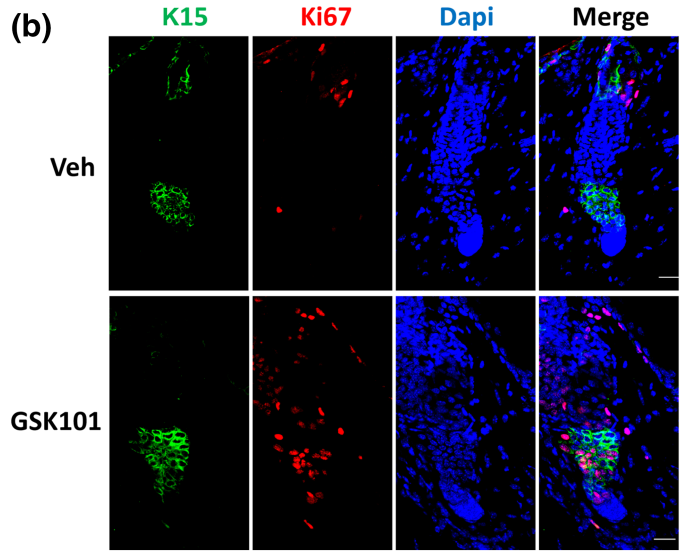
the mRNA transcripts of both *Bmp6* and *Fgf18*, while markedly increased the mRNA transcripts of *Fgf7*, *Wnt16* and *Nog* (Figure 3b–f). Both pharmacological inhibition by GSK2193874 and genetic ablation of the TRPV4 function significantly attenuated the effect of GSK1016790A (Figure 3b–g), suggesting that TRPV4 is responsible for GSK1016790A-induced changes in expression of these signalling molecules.

3.4 | ERK signalling is critically involved in TRPV4-mediated hair follicle regeneration

Previous studies showed that TRPV4-mediated Ca^{2+} influx activates downstream ERK signalling in mouse skin resulting in an acute scratching behaviour to exogenously applied histamine (Chen et al., 2016). To investigate if ERK signalling is also involved in







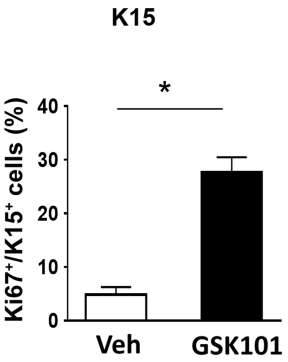


FIGURE 2 TRPV4 activation induces hair follicle keratinocytes proliferation. Representative immunofluorescent images of back skin sections from *Trpv4*$^{+/+}$ mice (n = 6) at 48 h after injection with vehicle (Veh) or GSK1016790A (GSK101) reveal an increase of Ki67-positive cells in K14-positive (a) and K15-positive (b) cells. Bar charts on the right show the percentage of Ki67-positive cells. Data are mean ± SEM. Scale bar = 100 μm. *P < 0.05

TRPV4-mediated hair follicle regeneration, we used PD98059, a selective inhibitor of MEK1 and the MAPK cascade, in mice subjected to a single intradermal injection of GSK1016790A. Strikingly, hair growth was completely absent from the PD98059-pretreated mice 17 days after GSK1016790A injection, while GSK1016790A persistently induced hair growth in vehicle-treated mice (Figure 4a). In line with this finding, we also found that ERK1/2 phosphorylation was significantly increased 15 min after a single intradermal injection of GSK1016790A, which was severely attenuated by pretreatment with either GSK2193874 or PD98059 (Figures 4b,c and S3), further confirming that the activation of ERK signalling is required for TRPV4-mediated hair follicle regeneration.

To further determine whether ERK signalling is involved in TRPV4-mediated disruption of the balance of the inhibitory and activation factors that regulate hair follicle stem cell activity, we measured GSK1016790A-induced changes in expression of *Bmp6*, *Fgf18*, *Fgf7*, *Wnt16* and *Nog* in the skin preparations of mice treated with PD98059 and vehicle control using real-time RT-PCR. Remarkably, PD98059 completely reversed the GSK1016790A-induced decrease in the expression of *Bmp6* and *Fgf18* and increase in the expression of *Fgf7*, *Wnt16* and *Nog* (Figure 4d), suggesting that ERK signalling downstream of TRPV4 activation is responsible for hair follicle regeneration by differentially regulating the expression of both inhibitory and activation factors that influence hair follicle stem cell telogen to anagen transition.

3.5 | TRPV4-expressing keratinocytes are critically involved in GSK1016790A-induced hair follicle regeneration

Recent studies have demonstrated that skin-resident macrophages also contribute to hair follicle stem cell activation and hair follicle regeneration, especially when intensive macrophage infiltration occurs under inflammatory states induced by wound injury or topical application of imiquimod, an immune response modifier (Amberg, Holcmann, Stulnig, & Sibilia, 2016; Chen et al., 2015; Wang et al., 2017). Moreover, Ali et al. (2017) showed that the skin regulatory T (Treg) cells

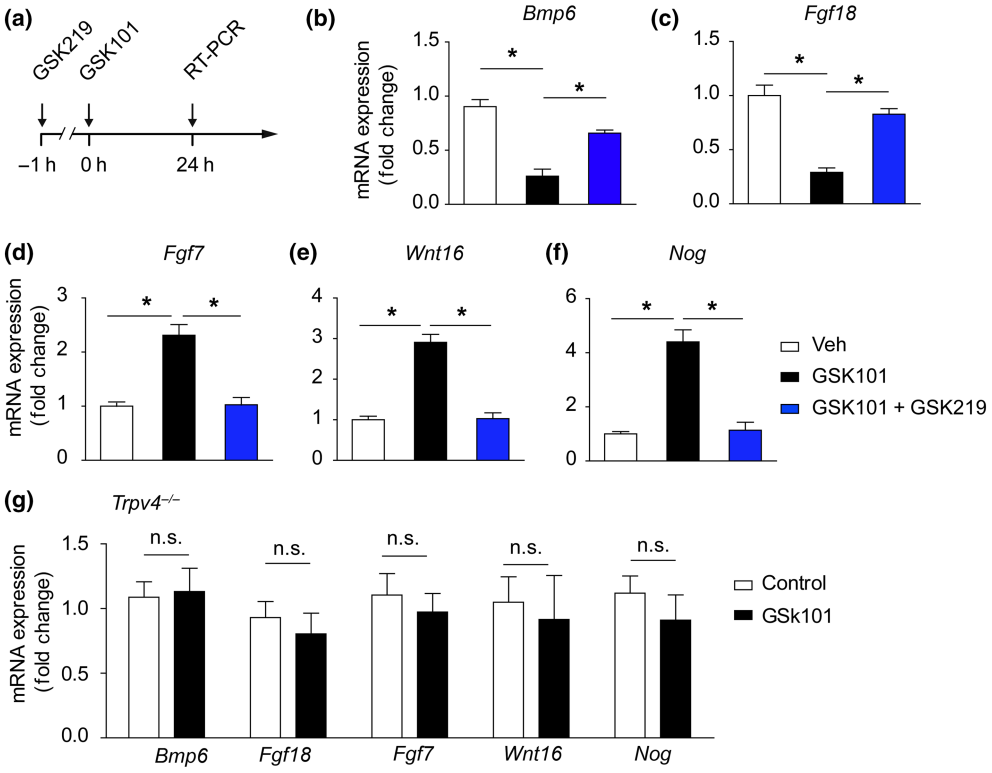


FIGURE 3 TRPV4 activation disrupts the balance of hair follicle stem cell inhibiting and activating factors. (a) Schematic diagram illustrates experimental protocol. Quantitative RT-PCR data obtained from whole skin lysates of *Trpv4*$^{+/+}$ mice (n = 5) 24 h after indicated treatments showing gene expression for *Bmp6* (b), *Fgf18* (c), *Fgf7* (d), *Wnt16* (e) and *Nog* (f). (g) *Trpv4*$^{-/-}$ mice (n = 5) were intradermally injected with vehicle (Veh) or 30-μM GSK1016790A (GSK101). As for the *Trpv4*$^{+/+}$ mice, the expression of target mRNAs was examined 24 h after GSK1016790A injection. Data are expressed as mean ± SEM. *P < 0.05; n.s., non-significance

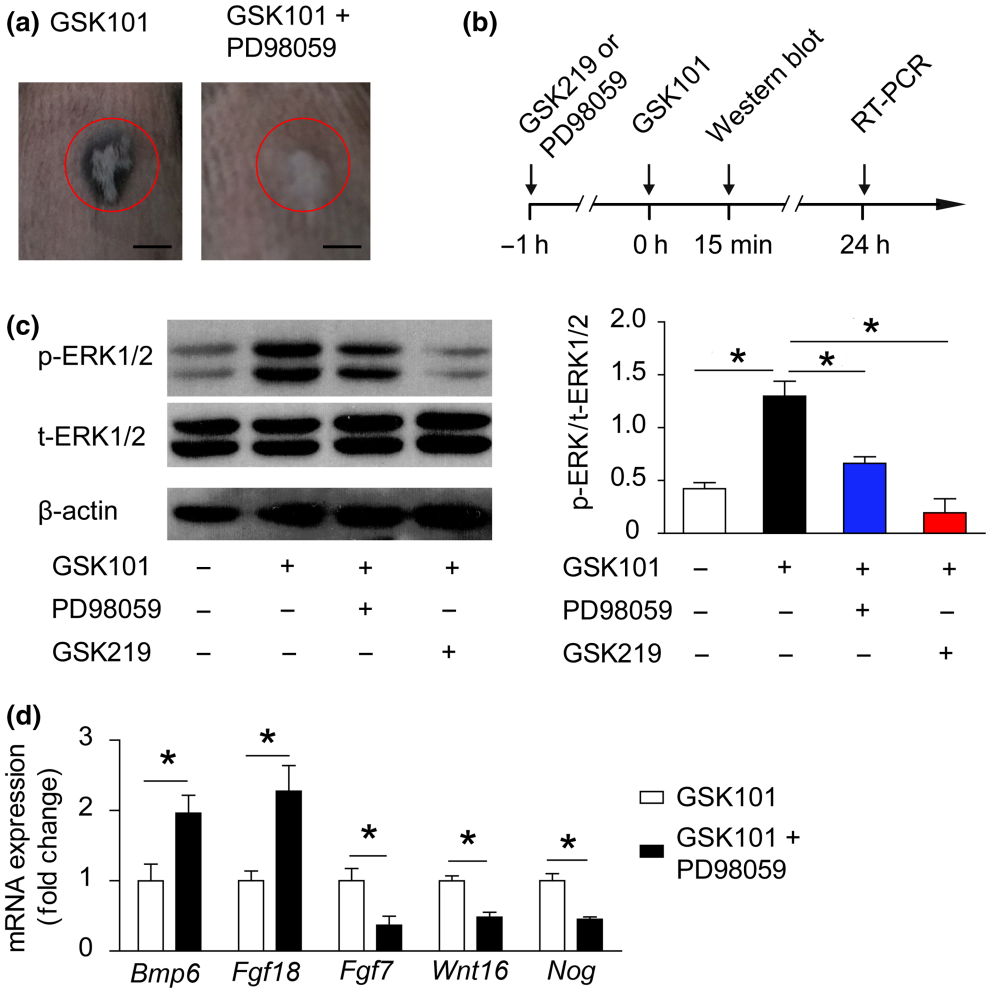


FIGURE 4 TRPV4-mediated hair regeneration requires ERK signalling. (a) Representative images showing new hair growth in *Trpv4*$^{+/+}$ mice injected with 30-μM , GSK1016790A (GSK101) in the absence (n = 5) or presence (n = 5) of PD98059. Scale bar = 0.5 cm. (b) Schematic diagram illustrates the experimental protocol. (c) Western blot data obtained from whole skin lysates subjected to different treatments using antibodies against phosphorylated ERK (p-ERK), total ERK (t-ERK), or β-actin (n = 5, respectively). Bar charts in the right illustrate the quantification of p-ERK expression. Data are expressed as mean ± SEM. (d) Quantitative RT-PCR data obtained from whole skin lysates showed that PD98059 pretreatment reversed the GSK1016790A-induced disruption of hair follicle stem cell inhibiting and activating factors. Data are mean ± SEM and normalized to the PD98059 presence group. Five independent experiments were performed. *P < 0.05

could enhance the activation and differentiation of epithelial stem cells, thereby promoting hair follicle cycling. Together, these findings highlight the importance of a crosstalk between the epithelial stem cell niche and micro-environment in hair follicle regeneration (Amberg et al., 2016; Wang et al., 2017). Since TRPV4 is also functionally expressed in dermal macrophages in addition to keratinocytes, we thus engineered both macrophage-specific and keratinocyte-specific TRPV4 conditional KO mice by crossing *Cx3cr1*^{Cre} and *K14*^{Cre} mice with *Trpv4*$^{f/f}$ mice, respectively, to determine whether TRPV4-expressing dermal macrophages and/or keratinocytes contribute to GSK1016790A-induced hair regeneration (Figure 5a).

Interestingly, although TRPV4 is expressed by dermal macrophages, a single intradermal injection of GSK1016790A induced comparable hair regeneration in both *Cx3cr1*^{Cre}; *Trpv4*$^{f/f}$ mice and their *Cre*$^{-}$ *wt* littermates (Figure S4). On the other hand, GSK1016790A-induced hair regeneration was completely absent from the *K14*^{Cre}; *Trpv4*$^{f/f}$ mice (Figure 5b), recapitulating the hair growth phenotype seen in the global TRPV4 KO mice, while their *Cre*$^{-}$ *wt* littermates persistently had hair regeneration. Moreover, GSK1016790A treatment did not affect the mRNA expression levels of *Bmp6* and *Fgf18* in the *Cre*$^{+}$ *K14*^{Cre}; *Trpv4*$^{f/f}$ mice (Figure 5c), further confirming keratinocytes origin of *Bmp6* and *Fgf18*. Although the expression of *Fgf7*, *Wnt16* and *Nog* was still increased after the GSK1016790A injection in the *Cre*$^{+}$ *K14*^{Cre}; *Trpv4*$^{f/f}$ mice (Figure 5c), it was significantly decreased compared with that in *wt* mice injected with GSK1016790A (Figure 5d). Taken together, these results suggest that acute chemical activation of TRPV4-expressing keratinocytes but not dermal macrophages is critical for breaking the quiescence of hair follicle stem cell to initiate hair follicle telogen to anagen transition and induce hair regeneration.

4 | DISCUSSION

In this study, we demonstrated that transient activation of TRPV4 by a single intradermal injection of a small molecule agonist successfully induced telogen to anagen transition and hair follicle regeneration and growth of new hair in mice. This hair follicle regeneration occurred through TRPV4-mediated activation of ERK signalling that broke the hair follicle stem cell quiescence through both increasing the expression of chemical cues that promoted hair follicle stem cell activity and reducing the expression of chemical cues that suppressed hair follicle stem cell activity. Although recent studies showed that skin-resident macrophages can modulate keratinocyte proliferation and contribute

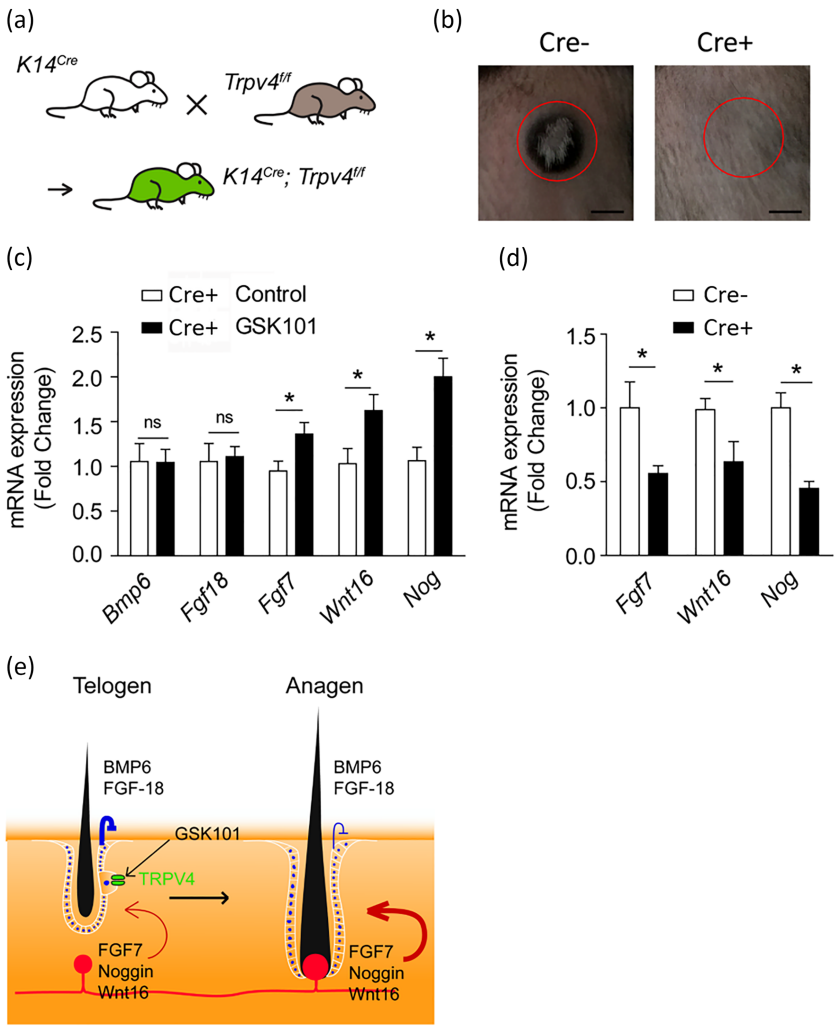


FIGURE 5 Keratinocyte-specific ablation of TRPV4 function abolishes GSK1016790A (GSK101)-induced new hair growth as well as changes in *Bmp6* and *Fgf18* expression. (a) Schematic diagram showing the generation of *K14*^{Cre}; *Trpv4*$^{f/f}$ mice. (b) Representative images showing new hair growth in the *Cre*$^{-}$ but not the *Cre*$^{+}$ *K14*^{Cre}; *Trpv4*$^{f/f}$ mice in response to intradermal GSK1016790A injections. Scale bar = 0.5 cm. (c) Quantitative RT-PCR data obtained from whole skin lysates from the *Cre*$^{+}$ *K14*^{Cre}; *Trpv4*$^{f/f}$ mice show that GSK1016790A did not change the expression of *Bmp6* and *Fgf18* but increase the expression of *Fgf7*, *Wnt16* and *Nog*. (d) Quantitative RT-PCR data showed that the increase of *Fgf7*, *Wnt16* and *Nog* was significantly less in *Cre*$^{+}$ *K14*^{Cre}; *Trpv4*$^{f/f}$ than that in the wild-type mice. (e) Schematic diagram showing the telogen to anagen transition induced by GSK1016790A activation of TRPV4. Five independent experiments were performed. Data are mean ± SEM. *P < 0.05; n.s., non-significance

to hair follicle regeneration induced by wound injury or imiquimod (Amberg et al., 2016; Wang et al., 2017), our results showed that the activation of TRPV4-expressing keratinocytes was required to induce hair follicle stem cell proliferation and regeneration. However, other types of TRPV4-expressing cells might also contribute to sustain anagen entry by regulating the expression of *Fgf7*, *Wnt16* and *Nog*. These findings significantly advanced our understanding of the regulation of hair follicle stem cells by TRP channels and might help to develop effective hair follicle regenerative therapies.

Mouse skin hair follicle morphogenesis follows a precise timescale and there are two hair cycles in C57BL/6 mice during the first 14 weeks after birth (Muller-Rover et al., 2001; Paus & Cotsarelis, 1999). Many types of skin cells including stem cells in the bulge and dermal papilla, dermal fibroblasts, adipocytes and dermal macrophages can produce a variety of chemical cues to regulate hair follicle stem cell activity (Greco et al., 2009; Hsu et al., 2011; Plikus et al., 2008). Our results showed that TRPV4 activation by GSK1016790A caused a down-regulation of the expression of *Fgf18* and *Bmp6*, both of which are anagen-inhibiting factors. On the other hand, the expression of promoting factors *Fgf7*, *Wnt16* and *Nog* was significantly up-regulated following a single intradermal injection of GSK1016790A. Moreover, the decrease of *Fgf18* may also initiate an up-regulation of *Nog* expression, whose gene product will further inhibit the expression of *Bmp6* (Reinhold, Abe, Kapadia, Liao, & Naski, 2004; Song et al., 2010). These results suggested that transient chemical activation of TRPV4 shifted the balance of hair follicle stem cell inhibiting and promoting factors in early telogen, which initiated hair follicle regeneration. Additionally, we showed that GSK1016790A had no effect on the expression of *Bmp6* and *Fgf18* in mice with epithelial cell-specific ablation of the TRPV4 function, which is consistent with a previous report that both *Bmp6* and *Fgf18* derive from skin keratinocytes (Hsu et al., 2011). Although the expression of three promoting factors was still increased after GSK1016790A injection in the keratinocyte-specific conditional TRPV4 KO mice, the increase was significantly reduced when compared with that in the Cre^{-} *wt* littermates in response to GSK1016790A treatment. The increase of these genes in $K14^{Cre}$; $Trpv4^{f/f}$ mice indicated that other cell types expressing TRPV4 might also contribute to the increased expression of these genes, such as fibroblasts and adipocytes (Sharma et al., 2017; Ye et al., 2012). Therefore, although the absence of hair follicle regeneration in $K14^{Cre}$; $Trpv4^{f/f}$ mice suggesting that K14-expressing keratinocytes play an essential role in GSK1016790A-induced hair follicle regeneration, we could not exclude the possibility that neighbouring fibroblasts, adipocytes and dermal macrophages might also play some direct or indirect roles. Together, these results suggest that down-regulation of *Bmp6* and *Fgf18* likely is a prerequisite for TRPV4-mediated hair follicle regeneration and a combination of reducing the expression of the inhibiting factor and up-regulation of promoting factors of hair follicle function leads to hair follicle anagen initiation.

TRPV4 is a Ca^{2+}-permeable cation channel, activation of which leads to increased intracellular Ca^{2+} levels (Voets et al., 2002). Interestingly, Ca^{2+}-dependent ERK phosphorylation is required for VEGF-induced proliferation of dermal papilla cells (Li et al., 2012). Recent studies have also shown that ERK signalling pathway contributed to TRPV4-mediated acute chemical itch sensation and UVB-induced sunburn pain (Chen et al., 2016; Moore et al., 2013). Consistent with these findings, we showed that GSK1016790A application induced a rapid ERK phosphorylation around the site of injection, which was blocked by the ERK inhibitor PD98059. More importantly, PD98059 pretreatment reversed both the changes in expression of hair follicle stem cell inhibiting and promoting factors as well as hair follicle regeneration induced by GSK1016790A, further supporting that ERK signalling was critical to TRPV4-mediated hair follicle regeneration.

Among TRP channels, TRPV3 and TRPV4 share many functional similarities in the skin:- (1) both channels are expressed by skin keratinocytes and are activated by warm temperatures, (2) both channels are important for skin barrier function and (3) like TRPV4, genetic ablation of TRPV3 function has no effect on general hair follicle cycling in mice (Cheng et al., 2010). TRPV3 was reported to play a differential role in hair follicle cycling. For instance, chemical activation of TRPV3 in cultured human hair follicle inhibited the hair shaft elongation and promoted the early catagen transformation (Borbiro et al., 2011). However, the gain-of-function mutation of TRPV3 inhibited the anagen to catagen transition, resulting in prolonged anagen phase (Imura et al., 2007). One explanation for this discrepancy might be that the cellular toxicity produced by the constitutive channel activity of the gain-of-function TRPV3 mutation causes death of hair follicle keratinocytes as gain-of-function TRPV3 mutations induced marked cell death in vitro (Lin et al., 2012; Xiao, Tian, Tang, & Zhu, 2008). In marked contrast to a recent study showing that TRPV4 activation by GSK1016790A decreased the ratio of proliferating cells and increased the number of apoptotic cells in cultured human hair follicles ex vivo and presumably resulted in an inhibition of hair growth (Szabo et al., 2019), our results showed that transient stimulation of the TRPV4-expressing hair follicle keratinocytes by a small molecule activator promoted hair follicle regeneration in vivo in mice. The discrepancy in these two studies might be caused by species differences in TRPV4 function in hair follicles between mice and humans as well as differences in experimental settings in which our studies examined hair follicle regeneration in vivo, while Szabó et al. evaluated anagen maintenance ex vivo using cultured human hair follicles. Due to the species difference of mice and human skin where the anagen and telogen phases are interspersed, the dosage and timing of a small molecule activator as a potential use in human skin should be carefully evaluated. It would be also interesting to analyse the effect of TRPV4 activation in humans in vivo during a complete hair cycle, to determine whether anagen to catagen transition also occurs in humans.

In summary, we demonstrate that transient chemical activation of TRPV4-expressing hair follicle keratinocytes shifts the balance of hair follicle stem cell inhibiting and promoting factors in telogen through ERK signalling, thereby promoting the telogen to anagen transition and hair follicle regeneration. Our study advanced our understanding of the importance of TRPV4-expressing hair follicle keratinocytes in hair follicle regeneration, which might provide mechanistic insights

into the development of effective therapeutic strategies for the treatment of hair loss and alopecia.

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AUTHOR CONTRIBUTIONS

Hongzhen Hu designed the research. Pu Yang , Ping Lu and Jialie Luo performed the majority of the experiments. Lixia Du, Jing Feng, Tao Cai, Yi Yuan and Hunter Cheng performed some in vitro experiments. Pu Yang , Ping Lu and Jialie Luo analyzed the data. Pu Yang and Jialie Luo wrote the manuscript. Hongzhen Hu, Pu Yang, and Ping Lu revised the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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