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**Pharmacological activation of thermoTRPV3 channels inhibits hair growth by inducing cell death of hair follicle outer root sheath**

**Authors:**

Kangjiao Yan<sup>1</sup>, Xiaoying Sun<sup>1</sup>, Gongxin Wang, Yani Liu and KeWei Wang\*

<sup>1</sup>These two authors contributed equally to this paper.

\*Correspondence

**Affiliation:**

Department of Pharmacology, School of Pharmacy, Qingdao University, 38 Dengzhou Road, Qingdao 266021, China

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**Corresponding author:** Professor KeWei Wang

**Address:**

Department of Pharmacology, School of Pharmacy, Qingdao University, 38 Dengzhou Road, Qingdao 266021, China

Tel: 86-532 82991070; E-mail: wangkw@qdu.edu.cn

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**Abbreviations:** DMEM, Dulbecco's modified Eagle's medium; FB, forsythoside B; MSCM, Mesenchymal Stem Cell medium; ORS, out root sheath; OS, Olmsted syndrome; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; TRPC1, transient receptor potential canonica 1; TRPC4, transient receptor potential canonica 4; TRPM8, transient receptor potential melastatin 8; TRPV3, transient receptor potential vanilloid 3; TRPV4, transient receptor potential vanilloid 4; TRPV5, transient receptor potential vanilloid 5

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## ABSTRACT

Hair growth starts from hair follicles that reside in dermis, and abnormal hair growth is an early sign of hair follicle disease or systemic illness such as alopecia or hair loss. Therefore, identifying a target critical for dysfunctional hair follicles is fundamental to alleviating dermatologic or systemic diseases with hair abnormalities. The warm temperature-activated  $\text{Ca}^{2+}$ -permeable TRPV3 channel protein is abundantly expressed in the skin keratinocytes, and dysfunctional TRPV3 causes human congenital Olmsted syndrome characterized by skin diseases and alopecia, indicating an important role of TRPV3 in hair follicle development and hair growth. To validate TRPV3 as a therapeutic target, we investigated the impact of pharmacological modulation of TRPV3 on hair growth using a combination of biochemical and cell biology, immunohistochemical, whole-cell patch clamp, RNA interference and pharmacological approaches. We find that functional TRPV3 channel proteins are highly expressed in hair follicle outer root sheath (ORS) cells as detected by Western blot analysis, immunohistochemical staining and electrophysiological techniques. Pharmacological activation of TRPV3 by agonist natural carvacrol induces cell death of ORS cells and topical application of carvacrol to mouse dorsal skin also inhibits hair growth. Conversely, specific inhibition of TRPV3 by inhibitor natural forsythoside B (FB) and shRNA reverses the cell death induced by carvacrol-mediated TRPV3 activation in human ORS cells. Furthermore, forsythoside B results in a significant reversal of hair growth inhibition induced by agonist carvacrol. Altogether, our findings demonstrate that TRPV3 channel is critical for regulation of hair growth, and inhibition of TRPV3 may represent a promising therapy for hair loss or hair follicle-related skin diseases.

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## Introduction

The hair follicle residing in the dermal layer of the skin is a tunnel-like segment of the epidermis and grows different types of hair. Abnormal hair growth and appearance are early signs of dysfunctional hair follicles including well known alopecia or hair loss or systemic diseases (Spencer and Callen, 1987; Sperling, 2001). Within the hair growth cycle there are three sequential phases consisting of anagen, catagen and telogen (Paus, 1998; Muller-Rover et al., 2001). The anagen, also known as the growth phase, is characterized by the change of skin pigmentation that is considered as a transition from telogen phase to anagen phase (Slominski and Paus, 1993; Slominski et al., 1994; Muller-Rover et al., 2001). Hair follicle as a dynamic organ is featured of outer root sheath (ORS) cells that are essentially a stratified epithelium contiguous with the epidermis. The hair follicle surrounding the hair fiber and inner root sheath also serves as an immunologic sentinel and a sensory organ of the skin (Paus and Cotsarelis, 1999), whereas the ORS cells have been suggested to be fundamental for maintaining the intact structure of hair follicles and regulating hair growth (Paus and Cotsarelis, 1999). Therefore, identifying a target critical for functional and dysfunctional hair follicle is important for diagnosis and treatment of dermatologic or systemic diseases with hair abnormalities.

The warm temperature-activated TRPV3 channel is immunostained in the ORS cells of hair follicles (Borbiro et al., 2011), suggesting a role of the channel in regulation of hair growth. Temperature is a significant factor for regulation of animal hair growth in natural environment (Kunz et al., 2009; Chen and Chuong, 2012). The nonselective cation  $\text{Ca}^{2+}$ -permeable TRPV3 channel is activated by temperature from warm ( $> 33^{\circ}\text{C}$ ) to hot (David E, 2003; Jordt et al., 2003), and is abundantly expressed in the keratinocytes (Peier et al.,

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2002; Chung et al., 2004b) and hair apparatus (Borbiro et al., 2011). TRPV3 participates in numerous physiological processes such as skin barrier formation, cutaneous sensation, and hair growth (Cheng et al., 2010; Aijima et al., 2015; Wang and Wang, 2017). In rodents, the spontaneous gain-of-function mutation (Gly573 substituted by Ser or Gly573 substituted by Cys) of *TRPV3* gene causes hair loss and AD-like dermatitis in both *DS-Nh* mice and WBN/Kob-Ht rats (Asakawa et al., 2006; Steinhoff and Biro, 2009). In humans, we and others identified the gain-of-function mutations (W692G, G573C and G573S) of *TRPV3* gene that cause rare congenital disorder of Olmsted syndrome (OS) characterized by skin inflammatory diseases such as palmoplantar, intense itch and alopecia in most cases (Lai-Cheong et al., 2012; Lin et al., 2012; Choi et al., 2018). Conversely, TRPV3-deficient mice exhibit phenotypes of curly whiskers, a thin cuticle, wavy hair and misaligned hair follicles (Moqrich et al., 2005; Schneider et al., 2008; Cheng et al., 2010), indicating a critical role of TRPV3 in normal morphogenesis of hair and hair follicles.

The polymodal TRPV3 channel is also activated by several natural compounds such as carvacrol, camphor, isoborneol and thymol (Chung et al., 2004a; Xu et al., 2006; Bang et al., 2010; Cao et al., 2012; Cui et al., 2018). Carvacrol (5-Isopropyl-2-methylphenol), a major active component from oregano, activates TRPV3 with an EC<sub>50</sub> value  $0.49 \pm 0.07$  mM (Vogt-Eisele et al., 2007). Carvacrol (250  $\mu$ M) also activates TRPA1 current that is quickly desensitized in the continuous presence of this agonist (Xu et al., 2006; Cui et al., 2018), whereas TRPV1 and TRPV4 are insensitive to carvacrol (1 mM) (Xu et al., 2006). In TRPV3 agonist carvacrol-induced scratching, knocking out TRPV3 suppresses scratching behavior (Cui et al., 2018). Silencing TRPV3 also can reverse the cell death or Ca<sup>2+</sup> signals induced by carvacrol in normal human epidermal keratinocytes and human

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sebocytes (Szollosi et al., 2018; Szanto et al., 2019). These results indicate the specificity of carvacrol on activation of TRPV3 that plays a critical role in itching. However, there is a significant lack of specific TRPV3 blockers. At present, TRPV3 can only be selectively inhibited by natural forsythoside B (FB), an active ingredient of *Lamiophlomis rotata* plant leaves (Zhang et al., 2019).

In this study, we investigated the effect of pharmacological modulation of TRPV3 channel function on hair growth in mice using a combination of biochemical and cell biology, immunohistochemical and pharmacological approaches. Our findings show that TRPV3 is robustly expressed in ORS cells of hair follicle, and pharmacological activation of TRPV3 by agonist carvacrol results in a time- and concentration-dependent delay of hair growth by decreasing hair follicles and inhibiting hair shaft elongation. Conversely, inhibition of TRPV3 by forsythoside B can significantly reverse the hair growth inhibition induced by carvacrol. Our findings demonstrate that topical application of TRPV3 channel modulators may hold a promising therapy for treatment of either hair loss or growth and dysfunctional hair follicle-related diseases.

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## Materials and Methods

### Animals

Male C57BL/6 mice (7-8 weeks) were obtained from Beijing Vital River Laboratory. Mice were housed at least for one week for their adaptation of environment controlled at temperature of  $23 \pm 2^{\circ}\text{C}$  and a 12 h light-dark cycle. For the establishment of carvacrol-induced hairless model, mouse dorsal skin ( $2.5 \text{ cm} \times 3.5 \text{ cm}$ ) was depilated with electric hair clipper and depilatory paste 24 h before starting an experiment. All experiments were approved by the Animal Care and Use Committee of Qingdao University Health Science Center.

### Drugs

Natural monoterpenoid phenol carvacrol (5-Isopropyl-methylphenol) (MW: 150.2) was obtained from Sigma-Aldrich Company and stored at  $4^{\circ}\text{C}$ . Natural compound forsythoside B (FB) (MW: 756.7) was purchased from Shanghai Taoto Biotech Corporation, Ltd. Forsythoside B (100 mM as stock) was dissolved in dimethyl sulfoxide (DMSO). For whole-cell patch clamp recording experiments, compounds were diluted in perfusion solution. For cell death and MTT assays, compounds were prepared using cell culture medium. For *in vivo* experiments, all different concentrations (0.3, 1, 3% equivalent to 19.1, 63.7 and 191.1 mM) of TRPV3 agonist carvacrol were prepared in 50% ethanol and locally applied to the shaved dorsal area in  $150 \mu\text{L}$ . The different concentrations (0.3, 1, 3 mM) of TRPV3 antagonist forsythoside B were prepared in normal saline and intradermally injected into the modeling area in  $50 \mu\text{L}$ . For *in vitro* or *in vivo* experiments, serial dilutions of compounds with buffer were made with final concentration of DMSO less than 0.1% or 3%, respectively.



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## **Cell Culture**

HEK293 cells were cultured in DMEM with 10% fetal bovine serum (FBS) under the condition of 5% CO<sub>2</sub> and 37°C. HEK293 cells were transiently transfected with hTRPV3 cDNA plasmids tagged with GFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The human hair outer root sheath (ORS) cells isolated from scalps were purchased from ScienCell Laboratories (ScienCell, USA). ORS cells were maintained in the culture of Mesenchymal Stem Cell Medium (MSCM) in an incubator under the condition of 5% CO<sub>2</sub> and 37°C. HEK293 cells and ORS cells were seeded on glass coverslips for whole-cell patch clamp recordings.

## **Western Blot**

Lysates of ORS cells were subjected to SDS-PAGE separation before transferred to polyvinylidene fluoride (PVDF) membrane for Western blotting, and first probed with anti-TRPV3 antibody (1:1000, Abcam) and followed by secondary anti-rabbit IgG antibody (1:5000, Abcam). The protein bands in the PVDF membrane were detected using a SuperSignal<sup>TM</sup> West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, USA).

## **Immunohistochemistry**

Skin tissue sections were incubated using anti-TRPV3 antibody (1:100, Abcam) at 4°C overnight, and subsequently incubated with secondary anti-rabbit IgG antibody (1:2000, Abcam) for 30 min at room temperature before further treatment with streptavidin peroxidase complex and stained with chromogen.

## **Whole-cell patch clamp recordings**

Whole-cell patch clamp recordings were carried out using a HEKA EPC10 amplifier with PatchMaster software (HEKA, Instrument Inc, Lambrecht/Pfalz, Germany). Patch pipettes

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were pulled with borosilicate glass using a puller (DMZ-Universal, Zeitz-Instruments GmbH, Martinsried, Germany), and fire polished to a resistance of 3-5 M $\Omega$ . For ramp recordings of HEK293 cells, both pipette solution and bath solution contained 130 mM NaCl, 0.2 mM EDTA, and 3 mM HEPES (pH 7.2). For ramp recordings of ORS cells, the bath solution contained 139 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM MgCl<sub>2</sub>, and 1.8 mM CaCl<sub>2</sub>, pH 7.4 with NaOH. The recording pipette was filled with a solution containing 120 mM CsCl, 5 mM EGTA, 10 mM HEPES, 2 mM CaCl<sub>2</sub>, 0.3 mM Na-GTP, 2 mM Mg-ATP, and 8 mM NaCl, pH 7.3 with CsOH. Membrane potential was held at 0 mV and current was recorded in response to 500 ms voltage ramps from -100 mV to +100 mV. Current amplitude was analyzed at  $\pm$  80 mV. All data were analyzed using Origin 8.6 software (OriginLab, Northampton, MA).

### **Cell death assay**

The cultured ORS cells of human hair follicles were divided into different groups in 6-well plate. All cell nuclei were fluorescently stained with blue dye of Hoechst 33258 (Thermo Fisher Science, USA) and the nuclei of dead cells were labeled with red fluorescent propidium iodide (PI) (Thermo Fisher Science, USA) after treatment of different compounds for 12 h. Images were randomly collected under the microscope (ECLIPSE Ti-S, Nikon, Tokyo) from each group and were counted automatically using a confocal microscopy (A1R MP, Nikon). The average of PI stained cells over cells stained by Hoechst dye from five images represented the cell death ratio.

### **RNA interference**

*TRPV3* shRNAs (Y5919, Y5920 and Y5921) were purchased from Obio Technology Corporation Ltd. (Shanghai, China) for transfection of ORS cells using Lipofectamine

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2000 Transfection Reagent (Invitrogen, Carlsbad, CA). After 24 h of transfection, western blotting was carried for confirmation of gene knockout efficiency and the most effective shRNA was selected for subsequent experiments.

### **MTT assay**

ORS cells were cultured in 96-well plate, and the tetrazolium salt MTT for cell viability stain was added after treatment of carvacrol for 24 h. Formazan dyes from the reduction of tetrazolium salts by mitochondrial dehydrogenases were detected for measurement of cell viability, and the number of viable cells by absorbance at 490 nm.

### **Hair length measurements**

Re-grown hairs were randomly plucked from shaved dorsal areas of mouse skin at time point of day 9, 12, 15, 18 and 21. The average hair length from 10 hairs per mouse was manually measured.

### **Histology**

Dorsal skin was excised after topical applications of TRPV3 pharmacological modulators (carvacrol or forsythoside B), and maintained in 4% paraformaldehyde at 4°C overnight before embedded in paraffin blocks for further transverse and longitudinal sectioning in about 5-10  $\mu\text{m}$  thickness. The tissue sections were stained with hematoxylin and eosin. Images of tissue sections were selected and photographed under a microscope (ECLIPSE Ti-S, Nikon).

### **Hair follicle counting**

Five randomly selected areas (0.09  $\text{mm}^2$  per area) from each tissue section stained with H&E were manually counted for an average number of hair follicles. Images of tissue

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sections in H&E staining were examined and photographed under the microscope (ECLIPSE Ti-S, Nikon).

### **Data analysis**

All data were expressed as the mean  $\pm$  S.D. For two-group comparison statistical significance was assessed by Student's t test (two-tailed, unpaired), and multiple-comparison test was assessed by one-way ANOVA or two-way ANOVA followed by Dunnett's test, Bonferroni's test or Sidak's test using GraphPad Prism 7.0 software. A value of  $p < 0.05$  was considered to be statistically significant.

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## Results

### **Functional expression of TRPV3 channel in the skin keratinocytes and hair follicle outer root sheath cells**

To recapitulate TRPV3 expression in the skin keratinocytes (Paus and Cotsarelis, 1999; Stenn and Paus, 2001) and ORS cells of hair follicles (Borbiro et al., 2011), we started examining TRPV3 protein expressions in the skin keratinocytes from C57BL/6 mice and primary ORS cells of hair follicles using Western blot assay. As shown in Fig. 1A left panel, a sharp band of TRPV3 proteins was detected in both primary culture of ORS cells and mouse dorsal skin, as compared with untransfected or HEK293 cells transiently expressing TRPV3 currents were activated by agonist carvacrol and inhibited by antagonist forsythiaside B (Fig. 1A, right panel). Immunohistochemistry staining further confirmed that TRPV3 proteins were mainly distributed in the skin keratinocytes and hair follicles of mouse dorsal skin (Fig. 1B). Whole-cell patch clamp recordings of ORS cells further confirmed that TRPV3 current was also activated by repeated applications of carvacrol (300  $\mu$ M), and TRPV3 current was inhibited by forsythoside B (100  $\mu$ M) about  $56.5 \pm 6.8$  % and the effect could be washed out (Fig. 1C). These results demonstrated that hair follicle ORS cells indeed could express the functional TRPV3 channel, suggesting its role in hair growth. These results are also consistent with TRPV3 expression in human HF-derived ORS keratinocytes (Borbiro et al., 2011).

### **Activation of TRPV3 by agonist carvacrol induces death of hair follicle outer root sheath (ORS) cells**

TRPV3 agonist carvacrol induces cell death in HEK293 cells expressing TRPV3 channels, HaCaT cells, human epidermal keratinocytes and human sebocytes (Szollosi et al., 2018;

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Zhang et al., 2019). To further confirm the functional expression TRPV3 in ORS cells, we also examined the effect of TRPV3 activation by carvacrol on human hair follicle ORS cells. As shown in Fig. 2A and 2B, ORS cells treated with carvacrol at different concentrations (10-300  $\mu$ M) for 12 h exhibited dose-dependent increases of cell death ratio in cell death assay, as compared with vehicle. In contrast, adding different concentrations of forsythoside B (3-100  $\mu$ M) in the presence of carvacrol (300  $\mu$ M) significantly reversed ORS cell death induced by carvacrol in dose-dependent manner (Fig. 2C). Forsythoside B at 3  $\mu$ M and 100  $\mu$ M reduced ORS cell death ratio to  $23.07 \pm 2.45\%$  ( $p < 0.05$ ) and  $13.78 \pm 2.57\%$  ( $p < 0.0001$ ), respectively, as compared with the cell death ratio at  $31.18 \pm 2.62\%$  for 300  $\mu$ M carvacrol alone (Fig. 2D).

We also determined the cell viability ratio in ORS cells transfected with *TRPV3* shRNA. Transfecting *TRPV3* sh-RNA resulted in significant reduction of TRPV3 protein expression in ORS cells (Fig. 2E), and significantly reversed the carvacrol-induced effect by increasing ORS cell viability to  $78.8 \pm 3.3\%$  from  $40.8 \pm 3.8\%$  (Fig. 2F). These results demonstrated that pharmacological activation of TRPV3 induced ORS cells death and inhibition of overactive TRPV3 by inhibitor forsythoside B or shRNA reversed the cell death, demonstrating a critical role of TRPV3 in regulation of ORS cell viability.

### **Time-dependent delay of hair growth by agonist carvacrol-induced activation of TRPV3**

To examine the impact of TRPV3 modulation on hair growth, we carried out topical applications of different concentrations of carvacrol (19.1, 63.7 and 191.1  $\mu$ M in 150  $\mu$ l) to the shaved dorsal area (2.5 cm  $\times$  3.5 cm) of the skin once a day (Fig. 3A top panel, n=8), and evaluated the time-dependent hair growth by observing the skin black pigmentation

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and hair growth (Fig. 3A). Black pigmentation in the skin is considered as a transition from telogen to anagen phase (Paus and Cotsarelis, 1999; Paus and Foitzik, 2010). We observed the three sequential stages of hair growth: pink skin turning black, black skin growing hair and full hair (Fig. 3A and Table 1). Topical applications of TRPV3 agonist carvacrol (19.1, 63.7 and 191.1 mM) caused a concentration-dependent delay of pink skin turning black from day 9 to day 12 (Fig. 3A and Table 1), as compared with the blank control (50% ethanol) or 0.3 mM forsythoside B group that exhibited a visible black coloration on day 9 (Fig. 3A). Similarly, topical applications of carvacrol (19.1, 63.7 and 191.1 mM) also caused a concentration-dependent delay of black skin starting to grow hairs from day 12 to day 15 (Fig. 3A), as compared with blank control or forsythoside B group that started to grow hairs on day 12 (Fig. 3A and Table 1). For measurement of hair length, applications of carvacrol (19.1, 63.7 and 191.1 mM) resulted in concentration-dependent reduction of hair length, as compared with blank control or 0.3 mM forsythiaside B (FB) group (Fig. 3A and 3B). These results indicate that activation of TRPV3 by carvacrol inhibits hair growth in both time- and concentration-dependent manner.

We also measured the hair growth by comparing the left and right side of dorsal skin in a same mouse after topical applications of 191.1 mM carvacrol on the left, and 50% ethanol on the right as control. As shown in Fig. 3C, the left side of dorsal skin treated with 191.1 mM carvacrol only started to become dark on day 12, and hair began to grow on the day 15. In contrast, the right side of the skin treated with 50% ethanol started to become dark on day 9 and hair already grew on the day 12, indicating at least a 3-day delay of hair growth on the left part of the skin treated with TRPV3 agonist carvacrol. To further confirm hair growth inhibition by carvacrol, we measured the length of hairs plucked from mice on

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day 9, 12, 15, 18 and 21. The hair length on the left dorsal skin was significantly shorter than the right side in time-dependent manner (Fig. 3D,  $n=8$ ,  $p < 0.0001$ ).

### **Inhibition of TRPV3 by natural inhibitor forsythoside B reversed hair growth inhibition induced by carvacrol**

To further confirm the effect of TRPV3 activation on hair growth inhibition, we carried out an intradermal injection of forsythoside B in different concentrations (0.3, 1, 3 mM in 50  $\mu$ l) into mouse dorsal skin 30 min before topical application of carvacrol (63.7 mM) onto the same area. Compared with the group of carvacrol (63.7 mM) alone, intradermal injections of forsythoside B together with carvacrol (63.7 mM) reversed hair growth inhibition by shortening the time of pink skin turning to black (reduced to 7 days from 10 days), and the time from black skin to hair growth (reduced to 3 days from 4 days) and to full growth of hair (reduced to 9 days from 10 days) in both concentration-dependent and time-dependent fashion (Fig. 4A and Table 2). Similarly, the length of hair in carvacrol (63.7 mM) treated group was significantly shorter than the blank control or forsythoside B groups (Fig. 4B). These results demonstrated that pharmacological activation of TRPV3 by carvacrol inhibited hair growth, and inhibition of TRPV3 by forsythoside B reversed hair growth inhibition induced by carvacrol.

### **Reversal of TRPV3 activation-mediated decrease of hair follicles by inhibitor forsythoside B**

Anagen phase is critical for hair follicle development (Ogawa and Hattori, 1983), and the telogen phase is the resting phase of hair follicle (Datta and Singh, 2009). For examination of TRPV3 modulation on hair growth cycle, hematoxylin-eosin staining of skin tissue sections was performed. In the vehicle or forsythoside B treated groups, hair follicles were



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densely distributed in transverse tissue sections and extended in a fixed direction of growth in longitudinal tissue sections (Fig. 5A), as compared with sparse hair follicles and their irregular growth directions in either 63.7 mM or 191.1 mM carvacrol treated groups (Fig. 5A). Similarly, dermal injection of forsythoside B (3 mM in 50  $\mu$ l) significantly reversed the loss of hair follicles caused by topical application of 63.7 mM carvacrol (Fig. 5A,  $n=8$ ,  $p < 0.01$ ). Further analysis confirmed that the number of hair follicles per area (0.09 mm<sup>2</sup>) in carvacrol treated groups was significantly less than vehicle or forsythoside B treated groups (Fig. 5B). These results indicated that inhibition of TRPV3 by forsythoside B reversed the decrease of hair follicles induced by TRPV3 agonist carvacrol.

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## Discussion

Based on ours and others previous investigations that gain-of-function mutations in TRPV3 cause genetic Olmsted syndrome characterized by alopecia in humans and hair loss in rodents, we in this study, therefore, tested the hypothesis that TRPV3 in the keratinocyte of the skin is essential for hair development and growth. Using approaches in a combination of biochemistry, cell biology, immunohistochemistry and *in vivo* models, we examined the effect of TRPV3 channel modulation on hair follicles and hair growth. Our findings show that pharmacological activation of TRPV3 by carvacrol inhibits hair growth in mice by suppressing hair shaft elongation and reducing hair follicles. Pharmacological activation of TRPV3 also leads to death of ORS cells, and specific inhibition of TRPV3 by natural compound forsythiaside B reverses hair growth inhibition and death of ORS induced by carvacrol. Mechanistically, we propose that activation of TRPV3 results in an excessive increase of intracellular calcium in ORS cells that are critical for maintaining the intact structure of hair follicles, thus leading to ORS cell death via activation of an unidentified  $Ca^{2+}$ -dependent kinase, and also inhibition of hair growth (Fig. 6).

Overactive TRPV3 has been shown to be involved in dermatitis and hair loss. In WBN/Kob-Ht rats or DS-*Nh* mice, the spontaneous gain-of-function mutation (G573C or G573S) of *TRPV3* gene causes cutaneous inflammation, itchiness and hair loss (Asakawa et al., 2006). We and others have previously identified the gain-of-function mutations (G573C, G573S and W692G) in the same gating area of *TRPV3* channel gene from humans that lead to genetic disease of Olmsted syndrome characterized by dermatitis, palmoplantar keratosis, severe itching and alopecia (Lai-Cheong et al., 2012; Lin et al., 2012; Choi et al., 2018). Although genetically linked between hair loss and dermatitis, whether and how

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TRPV3 directly affects hair follicles and hair growth still remain largely unknown. This is largely due to the fact that there is a significant lack of specific modulators for TRPV3, which has impeded our understanding of TRPV3 channel function in skin pharmacology and pathology. Our recent investigations demonstrate that pharmacological activation of TRPV3 channel function by carvacrol (5-isopropyl-2-methylphenol), a natural skin sensitizer and relatively specific agonist for TRPV3 (over TRPA1), can evoke dermatitis characterized by pruritus in mice (Cui et al., 2018), suggesting the involvement of TRPV3 in hair growth. To verify this notion, we used the carvacrol as a tool and tested its effect on skin and hair growth in this study. Pharmacological activation of TRPV3 by carvacrol can indeed cause hair loss by suppressing hair shaft elongation and reducing hair follicles. To validate the role of TRPV3 in hair loss, we also took the advantage of selective TRPV3 inhibitor forsythoside B as a tool in this investigation. Forsythoside B is an active ingredient of *Forsythia suspensa* fruits commonly used as a traditional Chinese herbal medicine for alleviation of inflammation and detumescence (Jiang et al., 2010; Muluye et al., 2014; Zhu et al., 2014), and it also attenuates dermatitis induced by either pruritogen or TRPV3 agonist carvacrol (Zhang et al., 2019). In this study, we are able to show that inhibition of TRPV3 by natural forsythoside B reverses hair growth inhibition caused by carvacrol and also reduces ORS cell death. These findings provide an evidence for cutaneous TRPV3 that is critical for hair growth, and pharmacological modulation of TRPV3 channel function in the keratinocytes may be beneficial for hair growth and care involving the hygiene and cosmetology.

Besides TRPV3, a few other TRP channels have been suggested to play a role in regulation of hair growth. The immunoreactivity of capsaicin receptor TRPV1 channel has

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been detected in epidermal hair follicle keratinocytes, sebocytes, mast cells and dendritic cells (Denda et al., 2001; Bodó et al., 2004). Stimulation of TRPV1 induces apoptosis of ORS keratinocytes, and causes the release of proinflammatory cytokines (Bodo E et al., 2005; Biro et al., 2006), and TRPV1 knockout results in a delay in hair follicle cycling (Biro et al., 2006). Another thermosensitive TRPV4 is also expressed in sebaceous gland cells and ORS keratinocytes (Garciaelias et al., 2014; Szabo et al., 2018), and TRPV4 activation can dose-dependently inhibit hair shaft elongation *in vitro* (Szabo et al., 2018). Other TRP channels such as TRPC1, TRPC4, TRPV5, TRPA1 and TRPM8 have also been reported to be expressed in cutaneous keratinocytes, but whether they play some roles in regulation of hair growth remains to be explored (Bezzerrides et al., 2004). Nevertheless, we propose that TRPV3 is essential and critical for regulation of hair growth.

In summary, we show that pharmacological activation of TRPV3 channels inhibits hair growth by inducing death of ORS cells, and inhibition of TRPV3 attenuates hair growth inhibition caused by the overactive channel function. These results demonstrate the critical role of TRPV3 channel function in hair follicles for regulation of hair growth, and topical inhibition of TRPV3 may represent a promising therapeutic strategy for prevention or treatment of hair follicle-related hair loss or skin disease.

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### **Authorship contributions**

Participated in research design: Kangjiao Yan, Xiaoying Sun, Kewei Wang

Conducted experiments: Kangjiao Yan, Xiaoying Sun, Yani Liu

Performed data analysis: Kangjiao Yan, Xiaoying Sun, Gongxin Wang

Wrote or contributed to the writing of the manuscript: Kangjiao Yan, Xiaoying Sun,

KeWei Wang

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## Figure legends

**Figure 1. Functional expression of TRPV3 proteins in mouse skin keratinocytes and ORS cells of hair follicles.** **A**, Left panel, Western blot analysis of TRPV3 proteins in human outer root sheath (ORS) cells, dorsal skin of C57BL/6 mice, and HEK293/TRPV3 stably expressing the TRPV3 as positive controls, and untransfected HEK293 cells as negative control. Right panel, whole-cell patch clamp recordings of HEK293 cells over-expressing hTRPV3 in response to carvacrol alone (300  $\mu$ M, red bar) or co-application of 100  $\mu$ M forsythoside B (FB, blue bar). **B**, Immunohistochemistry staining of TRPV3 proteins in mouse skin keratinocytes and hair follicles. Scale bar = 100  $\mu$ m. **C**, TRPV3 current in ORS cells was activated by 300  $\mu$ M carvacrol (red bar) and inhibited by co-application of 100  $\mu$ M forsythoside B (FB, blue bar). The right panel shows current-voltage curves of hTRPV3 channel in response to voltage ramps from -100 mV to +100 mV before (1), and after 300  $\mu$ M carvacrol (2), and co-addition of 300  $\mu$ M carvacrol and 100  $\mu$ M forsythoside B (3) and washout (4).

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**Figure 2. Inhibition of TRPV3 by forsythoside B attenuates carvacrol-induced ORS cell death.** **A**, Measurement of cell death for ORS cells before and after treatment of agonist carvacrol in different concentrations for 12 h in cell death assay. Scale bar = 100  $\mu\text{m}$ . **B**, Summary from (A) for cell death ratio before and after treatment of carvacrol in different concentrations (N.S., no significance,  $**p < 0.01$  and  $****p < 0.0001$ , by one-way ANOVA, followed by Dunnett's test). **C**, Measurement of cell death for ORS cells before and after treatment of carvacrol (300  $\mu\text{M}$ ) and different concentrations of forsythoside B for 12 h in cell death assay. Scale bar = 100  $\mu\text{m}$ . **D**, Summary of (C) for cell death ratio after treatment of carvacrol (300  $\mu\text{M}$ ) and forsythoside B in different concentrations. Data are presented as the mean  $\pm$  S.D. N.S., no significance,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ , by one-way ANOVA, followed by Dunnett's test. **E**, Western blot analysis of TRPV3 proteins in ORS cells and ORS cells transfected with *TRPV3* shRNA. **F**, ORS cells and ORS cells transfected with *TRPV3* shRNA were treated with 300  $\mu\text{M}$  carvacrol for 24 h. Cell viability was determined by colorimetric MTT cell viability assay. Data are presented as the mean  $\pm$  S.D.  $n=6$ ,  $****p < 0.0001$ , by Student's *t* test (unpaired).

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**Figure 3. Pharmacological activation of TRPV3 by carvacrol inhibits hair growth**

**A**, Top panel, a schematic drawing for generation of TRPV3 agonist carvacrol-induced hair growth inhibition in C57BL/6 mice. Bottom panel, mice (7-8 weeks) were shaved and topically given to different compounds once a day until hair growth out, images of hair growth were recorded every 3 days. Scale bar = 500  $\mu\text{m}$  **B**, Summary from (A) for hair length randomly plucked and measured (n=8) at different time intervals (9, 12, 15, 18 and 21 d) after topical application of different concentrations of TRPV3 agonist carvacrol (Car) and antagonist forsythoside B (FB). Data are presented as the mean  $\pm$  S.D. \*\*\*\* $p < 0.0001$ , by two-way ANOVA, followed by Dunnett's test. **C**, A self-comparison group between the left and right side of the skin after topical application of 191.1 mM carvacrol and vehicle (50% ethanol), respectively, once a day, and images for hair growth were recorded every 3 days. Scale bar = 500  $\mu\text{m}$  **D**, Summary from (C) for comparison of hair length randomly plucked (n=8) between left (L) and right (R) side of the skin. Data are presented as the mean  $\pm$  S.D. \*\*\*\* $p < 0.0001$ , by two-way ANOVA, followed by Sidak's test.

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**Figure 4. Inhibition of TRPV3 by forsythoside B attenuates carvacrol-induced hair growth inhibition in mice.** **A**, Top panel, a schematic drawing for generation of TRPV3 agonist carvacrol-induced hair growth inhibition and treatment with forsythoside B (FB) in C57BL/6 mice. In bottom panel, mice (7-8 weeks) were shaved and intradermal injections of forsythoside B in different concentrations (0.3, 1, 3 mM in 50  $\mu$ l) into the dorsal area of skin 30 min before topical application of carvacrol (63.7 mM) once a day until hair grew out, images were recorded in every 3 days. Scale bar = 500  $\mu$ m **B**, Summary from (A) for hair length randomly plucked and measured (n=8) at different time intervals (9, 12, 15, 18 and 21 d) after topical application or co-application of different concentrations of TRPV3 agonist carvacrol (Car) and antagonist forsythoside B (FB). Data are presented as the mean  $\pm$  S.D. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001, by two-way ANOVA, followed by Dunnett's test.

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**Figure 5. Activation of TRPV3 decreases hair follicles and inhibition of TRPV3 prevents loss of hair follicles induced by carvacrol. A,** Transverse sections (top two rows) and longitudinal sections (bottom row) of dorsal skin tissues were stained with H&E before and after topical application of TRPV3 inhibitor forsythoside B (FB) alone or activator carvacrol (Car) in 63.7 mM or 191.7 mM or combination of Car and FB once a day. Scale bar = 100  $\mu$ m. The skin tissue sections were prepared on day 10 after shaving. Representative images of skin tissue sections in each group were from 8 mice. **B,** Summary from (A) for the number of hair follicles before and after topical applications of TRPV3 modulators (carvacrol or forsythoside B) or combination. Data are presented as the mean  $\pm$  S.D. N.S., no significance, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , by one-way ANOVA followed by Bonferroni's test.

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**Figure. 6. A proposed mechanism underlying the death for hair follicle ORS cells and hair loss by TRPV3 activation.** TRPV3 channels are expressed in the hair follicle ORS cells. Activation of TRPV3 channel by agonist carvacrol or gain-of-function mutation causes an excessive  $\text{Ca}^{2+}$  influx that induces ORS cell death via activation of unidentified  $\text{Ca}^{2+}$ -dependent kinase. Overactive TRPV3 either by carvacrol (or gain-of-function mutations) causes a time- and dose-dependent reduction of hair shaft elongation and a decrease of hair follicles likely through the induction of ORS cell death in hair follicles, thus leading to inhibition of hair growth. Pharmacological inhibition of overactive TRPV3 by forsythoside B can reverse hair loss.



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## Tables

**Table 1.** Pharmacological activation of TRPV3 by carvacrol causes delay of hair growth in mice

Group	Time for pink skin turning to black (Day)	Time for black skin to hair growth (Day)	Time to full hair growth (Day)
Control	8.0 ± 0.6	3.0 ± 0.5	9.0 ± 0.4
50% ethanol	8.0 ± 0.6	3.0 ± 0.7	9.0 ± 0.6
0.3 mM FB	7.0 ± 0.4	2.0 ± 0.5	8.0 ± 0.6
19.1 mM Car	9.0 ± 0.9	4.0 ± 0.7	9.0 ± 0.6
63.7 mM Car	10.0 ± 1.2 (**)	4.0 ± 0.8 (*)	10.0 ± 0.9
191.1 mM Car	11.0 ± 1.8 (****)	5.0 ± 0.9 (***)	10.0 ± 1.1 (**)

*Note: (mean ± S.D. n=8)*

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**Table 2.** Reversal of TRPV3 agonist carvacrol-induced inhibition of hair growth by inhibitor forsythoside B

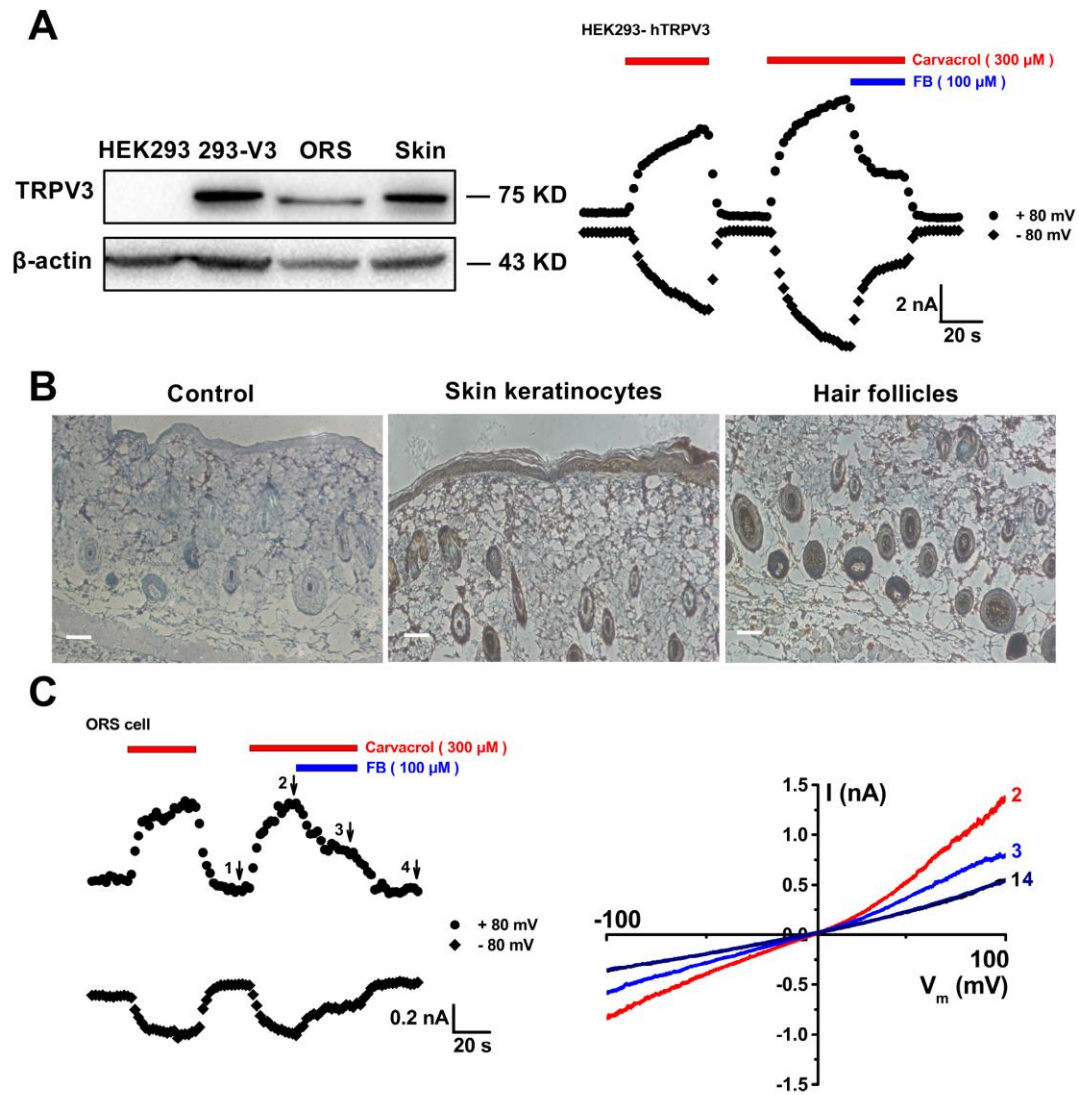
Group	Time for pink skin to black (Day)	Time for black skin to hair growth (Day)	Time to full growth of hair (Day)
63.7 mM Car	10.0 ± 0.9	4.0 ± 0.7	10.0 ± 1.0
Car + 0.3 mM FB	9.0 ± 1.8	4.0 ± 0.7	10.0 ± 1.1
Car + 1.0 mM FB	9.0 ± 1.2	4.0 ± 0.6	9.0 ± 0.8
Car + 3.0 mM FB	7.0 ± 0.7 (*)	3.0 ± 0.5 (*)	9.0 ± 0.6

*Note: (mean ± S.D. n=8)*

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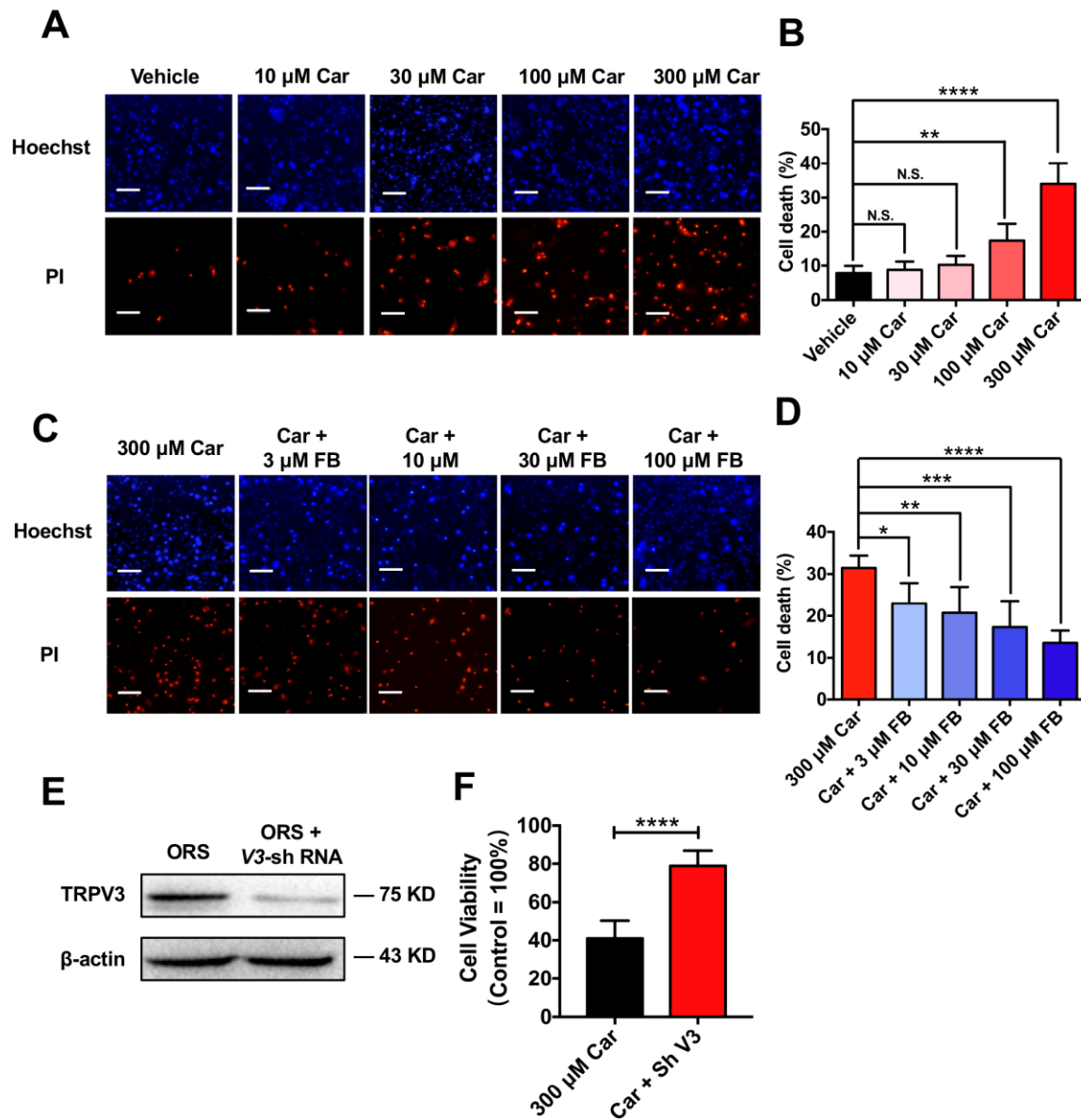
## Figures

### Figure 1



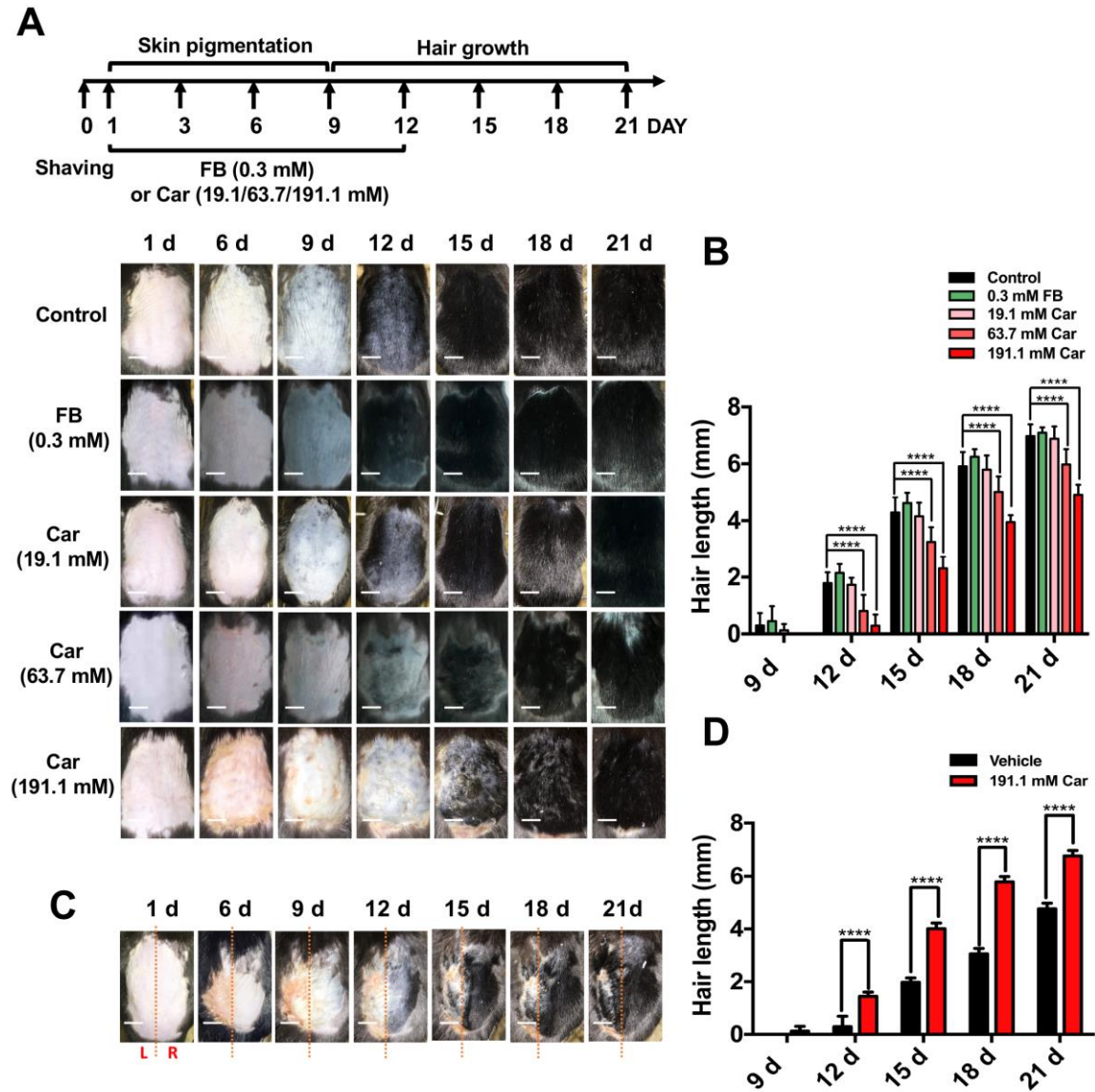
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**Figure 2**



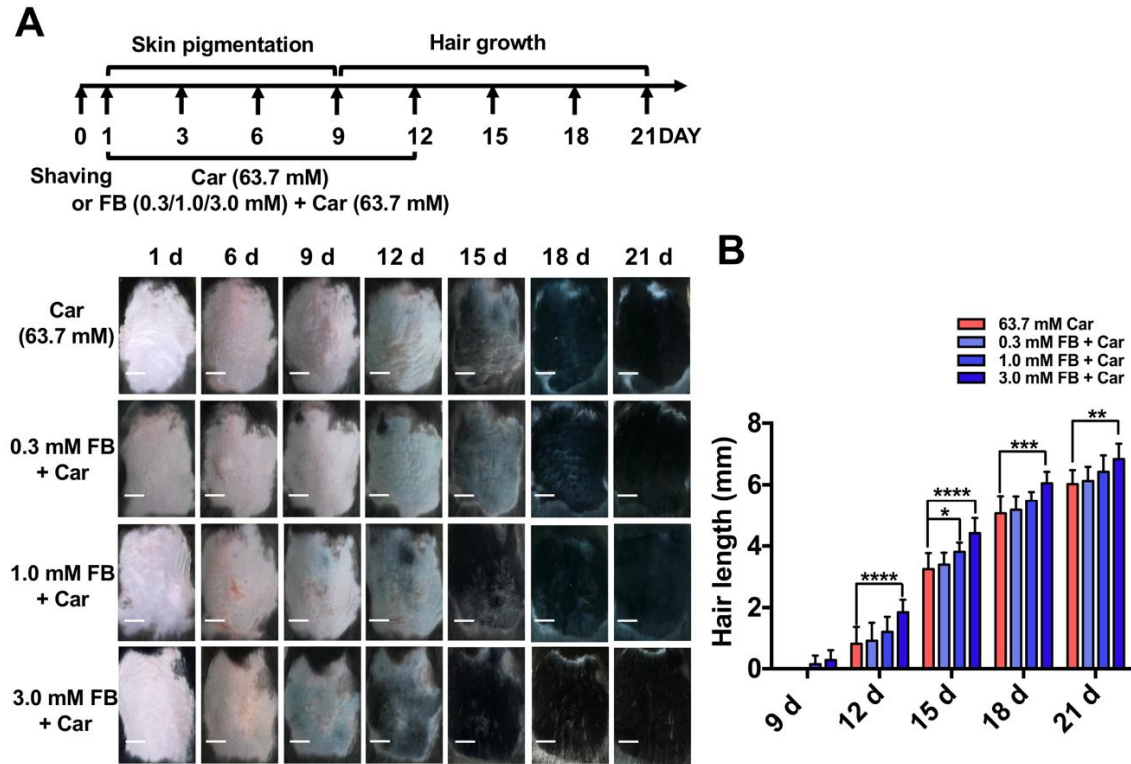
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**Figure 3**



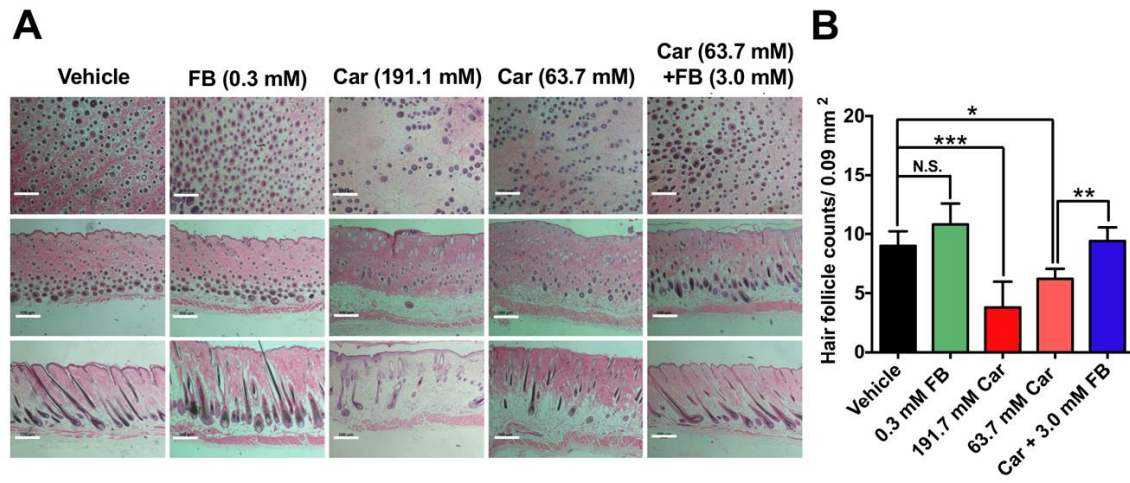
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Figure 4



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Figure 5



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Figure 6

