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ORIGINAL ARTICLE

Expression of *Sfrp2* Is Increased in Catagen of Hair Follicles and Inhibits Keratinocyte Proliferation

Bong-Kyu Kim, Sungjoo Kim Yoon

Department of Medical Lifesciences, The Catholic University of Korea, School of Medicine, Seoul, Korea

Background: Hair follicles undergo cycles of repeated growth and regression. The Wnt pathway plays an important role in the regeneration and differentiation of hair follicles. *Sfrp2*, a Wnt inhibitor, is involved in the developmental and disease processes of various cells and tissues by modulating the Wnt pathway. **Objective:** The aim of this study was to understand the role of *Sfrp2* in hair follicles through investigation of the *Sfrp2* expression pattern in the skin and its effect on keratinocytes. **Methods:** We investigated *Sfrp2* mRNA expression and the expression of the wnt target genes, *Ccnd1* and *C-myc*, at various mouse hair follicle developmental stages using Real-time polymerase chain reaction. We also investigated the effect of SFRP2 on the proliferation and differentiation of mouse keratinocyte cells by adding SFRP2 protein or overexpressing *Sfrp2* using an *in vitro* culture system. **Results:** *Sfrp2* expression peaked in the catagen phase and remained high until telogen, and then declined at the beginning of the next anagen. An inverse relationship to *Sfrp2* expression was found for the expression of the Wnt target genes, *C-myc* and *Ccnd1*. In addition, we also observed inhibited proliferation of mouse keratinocytes in the presence of SFRP2. **Conclusion:** These results suggest that *Sfrp2* may play a role in the catagen phase by inhibiting the proliferation of keratinocyte and functioning as a Wnt inhibitor in keratinocytes. **(Ann Dermatol 26(1) 79～87, 2014)**

-Keywords-
Catagen, Keratinocyte, Proliferation, SFRP2, Wnt signaling pathway

INTRODUCTION

The hair follicle (HF) is a unique mini organ in the skin of mammals that produces hair. Hair functions to thermoregulate and protect the skin, helps detect senses, and a marker for sexual development. HFs undergo a continuous cycle consisting of growth (anagen), regression (catagen), and relative quiescence (telogen) phases. Growth of new hair shafts and regression of old hair shafts are repeated through this cycle[1]. Multiple signaling pathways and genes are involved in regulating the hair cycle and HF morphogenesis[2-4].

Wnts are a family of secreted glycoproteins that play a critical role in the embryonic development and maintenance of homeostasis in mature tissues by regulating proliferation, differentiation, migration, and apoptosis of cells[5-8]. The Wnt pathway regulates the regeneration and differentiation of HFs[9-11]. Wnt signaling pathway activity is regulated by secreted inhibitors, which are divided into two functional classes. One class includes the secreted frizzled-related protein (*Sfrp*) family, Wnt inhibitory factor 1 (*Wif1*), and Cerberus which binds directly to Wnt, thus, sequestering Wnt from its receptors. The Dickkopf (*Dkk*) class binds to the LRP5/LRP6 component, thereby inhibiting binding of Wnt to its receptor[12].

The other class, sFRP, a family of glycoproteins, has five members (Sfrp1～Sfrp5) that participate in the developmental and disease processes in various cells and tissues by controlling the Wnt pathway[13,14]. The sFRP family possesses structural similarities to the cysteine-rich do-

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Corresponding author: Sungjoo Kim Yoon, Department of Medical Lifesciences, The Catholic University of Korea, School of Medicine, 222 Banpo-daero, Seocho-gu, Seoul 137-701, Korea. Tel: 82-2-2258-7476, Fax: 82-2-594-2385, E-mail: sjkyoon@catholic.ac.kr

main of frizzled receptors; thus, they interact with Wnt and form the Wnt-Sfrp complex. Therefore, the Wnt protein is sequestered from the frizzled receptor[15].

Sfrp2 has been studied in different cell types and organs to determine its role[16-18]. Although *Sfrp2* is expressed in the upper and lower matrix, outer root sheath, and dermal papilla[19,20], its role in HFs has not been investigated.

Thus, we investigated *Sfrp2* expression at various HF stages in mouse dorsal skin and the effect of SFRP2 on keratinocytes to identify the role of SFRP2 in HFs. We found that *Sfrp2* expression peaked at the catagen phase, and that this expression pattern was inversely related to that of Wnt target genes. We also observed inhibited mouse keratinocyte proliferation by SFRP2. These results suggest that *Sfrp2* may play an important role during the catagen phase by inhibiting keratinocyte proliferation in HFs.

MATERIALS AND METHODS

Animal

BALB/C mice were purchased from Orient Bio (Seongnam, Korea) and maintained in the barrier system with regulated light (700 to 1,900 h), temperature (23 ± 1^{o}C), humidity ($50\%\pm5\%$), and ventilation (10 to 12 times/h). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Korea and were carried out in accordance with the Guidelines for Animal Experimentation.

Histological study

Mouse dorsal skins of BALB/C mice at postnatal days 10 (P10), P14, P17, P21 and P28 were gathered as previously described[21]. All skin samples were harvested from the same region (2×2 cm) of the upper back skin. Six-micrometer-thick paraffin sections were prepared. Hematoxylin and eosin (H&E) staining was carried out using the standard method, and the stained sections were observed with an optical microscope (Olympus, Tokyo, Japan).

RNA isolation and reverse transcription

Total RNA was extracted from the dorsal skins of BALB/C mice at P10, P12, P14, P17, P21, P28, and P35 using TRIZOL following the manufacturer's instructions (Invitrogen, Camarillo, CA, USA). Two micrograms of RNA were utilized to synthesize single stranded cDNAs using the PrimeScript 1st strand cDNA Synthesis kit following the manufacturer's instructions (TaKaRa Bio Inc., Shiga, Japan).

Reverse transcription-polymerase chain reaction and Real-time polymerase chain reaction

Polymerase chain reaction (PCR) was performed using Thermal Cycler-100 (MJ Research Inc., Waltham, MA, USA). PCR conditions were as follows: initial denaturation for 2 minutes at 95°C followed by 28~30 cycles of 15 seconds at 94°C, 15 seconds at 62°C, and 15 seconds at

Table 1. List of gene specific primers

Gene	Accession number	Sequence	Size (bp)	Temperature (°C)
Sfrp2 (RT)	NM_009144	F: agcctgcaaaaccaagaatg R: atacggagcgttgatgtcgt	253	62
Sfrp2 CDS	NM_009144	F: atgccgcggggccctgcctc R: ctagcattgcagcttgcgga	888	60
Dkk1	NM_ 010051	F: caaaggacaagaaggctccgtc R: ggtgcacacctgaccttctt	121	62
Dkk2	NM_020265	F: catcctcaccccacatatcc R: gtaggcatgggtctccttca	155	62
Dkk4	NM_145592	F: agcctgcaaaaccaagaatg R: atacggagcgttgatgtcgt	191	62
Sfrp1	NM_013834	F: tcagaggccatcattgaaca R: atacggagcgttgatgtcgt	258	60
Wif1	NM_011915	F: caacaagtgccagtgtcgag R: gcatttgaacatccaacacg	257	62
β-actin	NM_ 007393	F: tcacccacactgtgcccatctacga R: cagcggaaccgctcattgccaatgg	295	60
Gapdh	NM_008084	F: aactttggcattgtggaagg R: acacattgggggtaggaaca	223	62

RT: real-time, CDS: coding sequence.

72°C. The final extension was performed for 10 minutes at 72°C. Real-time PCR was performed with the cDNAs prepared from the total RNAs of the skin at various time points (See the above section) using SYBR Premix Ex Taq (TaKaRa Bio Inc.) in an Mx3000P (Stratagene, La Jolla, CA, USA). Specific primer sequences for each gene are listed in Table 1. The cycling conditions were as follows; initial heating for 2 minutes at 95°C followed by 45 cycles of 15 seconds at 94°C, 15 seconds at 62°C, and 15 seconds at 72°C, and the final extension was performed for 10 minutes at 72°C. Gene expression levels were determined by the comparative $\Delta\Delta$Ct method using the expression level of *Gapdh* as control[22]. Relative mRNA expression levels were determined based on Real-time PCR performed in duplicate using various numbers of independent samples for each point (number of sample for each point; P10=3, P12=4, P14=7, P17=4, P21=5, P28=5, P35=4).

Plasmids

The 888 bp of *Sfrp2* coding sequence (CDS) were amplified by PCR using Expand High Fidelity enzyme (Roche Diagnostics, Basel, Switzerland) from skin cDNAs of BALB/C mice. Forward and reverse primer sequences are listed in Table 1. PCR products were subcloned into pcDNA 3.1 using EcoRI cloning sites (Invitrogen).

Cell culture and transfection

Mouse keratinocyte cells (PAM212 cell line) were cultured in DMEM (Invirogen) containing 10% FBS with 5% CO_2 in a 37°C incubator. Transfection experiments were performed using polyethyleneimine (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. A total of 8×10^5 cells were plated in 60 mm dishes in triplicate. Following this, either 500 ng of pcDNA 3.1/ *Sfrp2* CDS or only pcDNA 3.1 plasmid was transfected into the cells with 0.4 μg of pCMV3.1/β-gal. Transfection experiments were normalized against transfection efficiency determined by β-galactosidase activity. To ensure that *Sfrp2* overexpression was well induced, we performed Real-time PCR and found a 27118-fold increase in the *Sfrp2* mRNA level compared with that of pcDNA

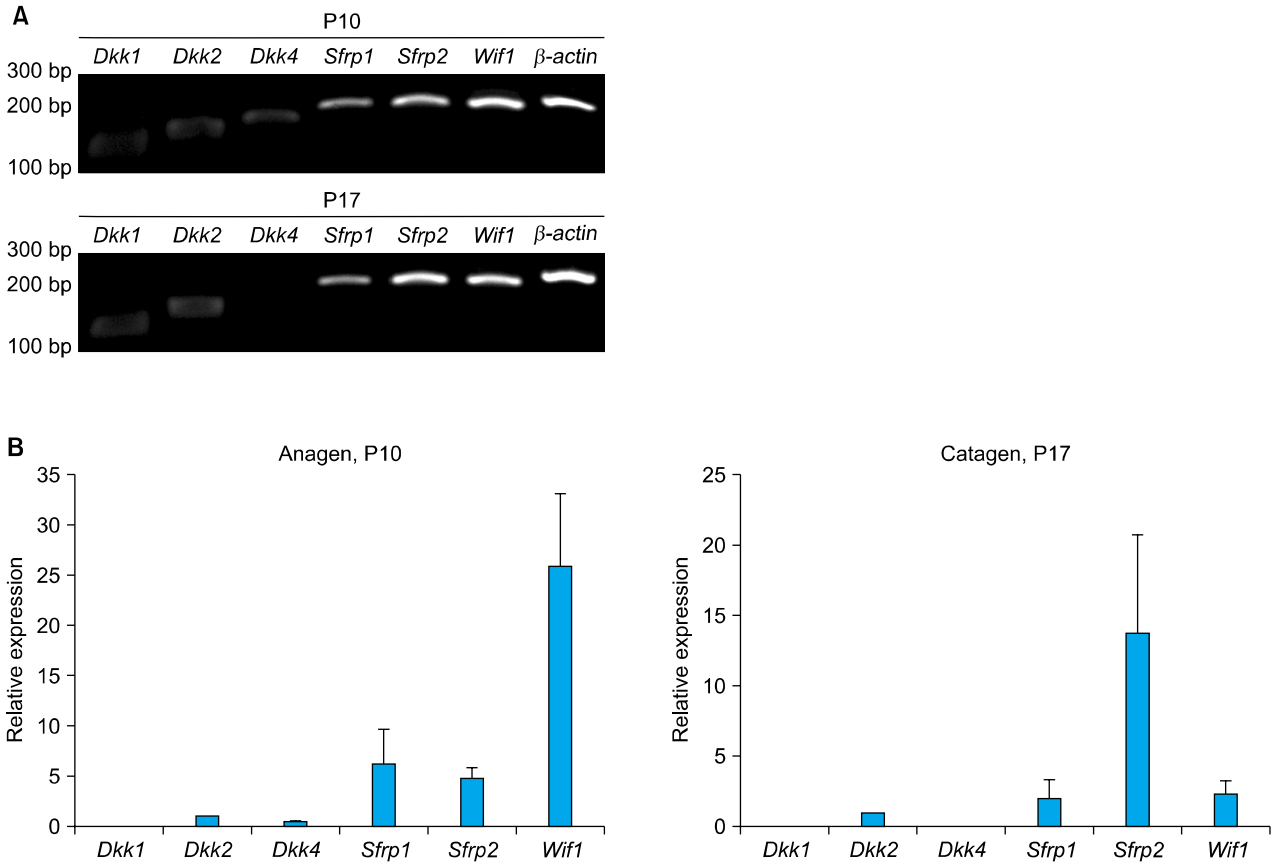

Fig. 1. Expression levels of *Sfrp2* compared with those of other Wnt inhibitors including *Dkk1*, *Dkk2*, *Dkk4*, *Sfrp1* and *Wif1*. Reverse transcription-polymerase chain reaction (A) and Real-time polymerase chain reaction (B) revealed that *Sfrp2* is the most abundantly expressed Wnt inhibitor in hair follicles, especially at the phase of catagen. P: postnatal day.

3.1 transfection. After incubation for 48 hours at 37°C, the transfected cells were observed using a microscope (Olympus) or harvested for extraction of total RNAs.

SFRP2 protein treatment

SFRP2 protein was purchased from R&D system and reconstituted in sterilized phosphate buffered saline (PBS). A total of 5×10^5 PAM212 cells were plated on a 100 mm culture dish in DMEM media and were treated with SFRP2 the following day by replacing media with the media containing SFRP2 (50 ng/ml or 100 ng/ml). The treatment was continued for 3 days by changing the media every day.

Immunocytochemistry

For immunocytochemistry, PAM212 cells either treated with SFRP2 or transfected with pcDNA 3.1/*Sfrp2* were washed three times with cold PBS and fixed using 4% paraformaldehyde for 10 minutes at room temperature. The cells were then treated with 0.5% Triton X-100 for 10 minutes and washed three times with PBS. After blocking with 3% bovine serum albumin for 1 hour, the cells were incubated with antibodies against Ki67 (Thermo Scientific, Hudson, NH, USA; 1 : 200) or *Involucrin* (Covance, Berkeley, CA, USA; 1 : 200) overnight. After washing, Alexa Fluor 488 goat anti-rabbit secondary antibody was applied for 3hr (Invitrogen, 1 : 500), and 4 g/ml of Hoechst 33342 (Sigma-Aldrich) was also applied for 1 minute to stain the nuclei. Fluorescence signal was observed with a Fluorescent microscope (Olympus).

Statistical analysis

p-values were calculated using Student's t-test. $p<0.05$ was regarded as statistically significant. Analyses were performed using SPSS for Windows version 12.00 (SPSS Inc., Chicago, IL, USA).

RESULTS

Sfrp2 mRNA expression during the hair cycle

To determine the role of *Sfrp2* in HFs, first we investigated the relative expression level of *Sfrp2* compared to those of other Wnt inhibitors using reverse transcription-polymerase chain reaction (RT-PCR) and Real-time PCR. Among the Wnt inhibitors with relatively high expression at P10, *Sfrp2* was the only one whose expression was dramatically increased at P17. Furthermore, the expression level of *Sfrp2* was 5.9-fold higher than that of the second most expressed Wnt inhibitor, *wif1* (Fig. 1). These results suggest that *Sfrp2* is the most abundantly expressed Wnt inhibitor in HFs at the catagen phase.

Relative expression level of *Sfrp2* mRNA was determined at different hair cycle stages by RT-PCR as well as Real-time PCR analyses. We found that *Sfrp2* was highly expressed during catagen, which was maintained until the early telogen phase (Fig. 2A). During anagen, *Sfrp2* expression was weaker than those of other stages. After this, the expression level increased significantly by 2.4∼3.6 fold as the stages progressed into the catagen and early telogen phases. As the next anagen phase began, *Sfrp2* expression started to decline and then decreased again to a low level that is similar to that of the first anagen phase by P35 (Fig. 2B). These *Sfrp2* mRNA expression patterns suggest that *Sfrp2* may function during the catagen phase.

Effect of SFRP2 on keratinocyte proliferation

During the catagen phase, HFs undergo degeneration through cessation of proliferation and subsequent differentiation of keratinocytes. Because *Sfrp2* expression increased at the catagen phase as shown in Fig. 2, we investigated whether *Sfrp2* is involved in the regulation of proliferation and/or differentiation using the PAM212 mouse keratinocyte cell line. Cells were treated with

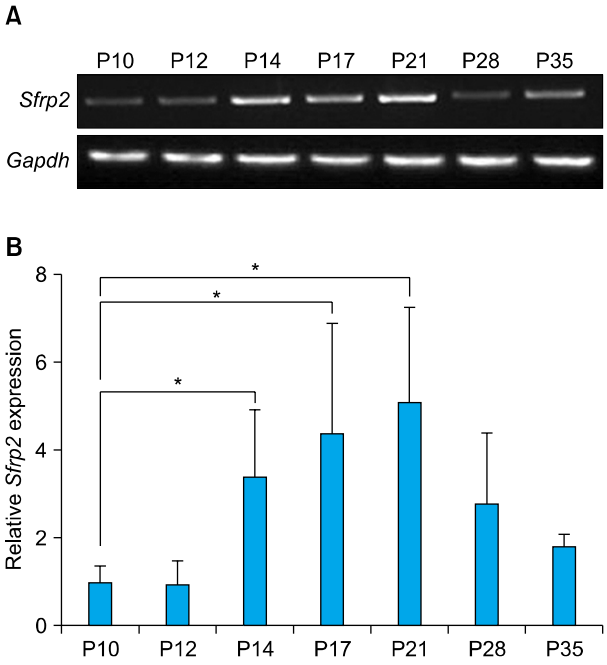

Fig. 2. *Sfrp2* is mainly expressed during the catagen phase in the hair cycle. To investigate *Sfrp2* expression, total RNA of BABL/C mice was prepared from the dorsal skin at each time point. Reverse transcription-polymerase chain reaction (A) and Real-time polymerase chain reaction (B) analyses showed expression patterns of *Sfrp2* mRNA from P10 until P35 after birth. The data are normalized against *Gapdh* mRNA expression levels. The value is the average of the relative expression levels of three independent mice cDNAs measured in duplicate polymerase chain reaction reactions. *$p<0.05$. P: postnatal day.

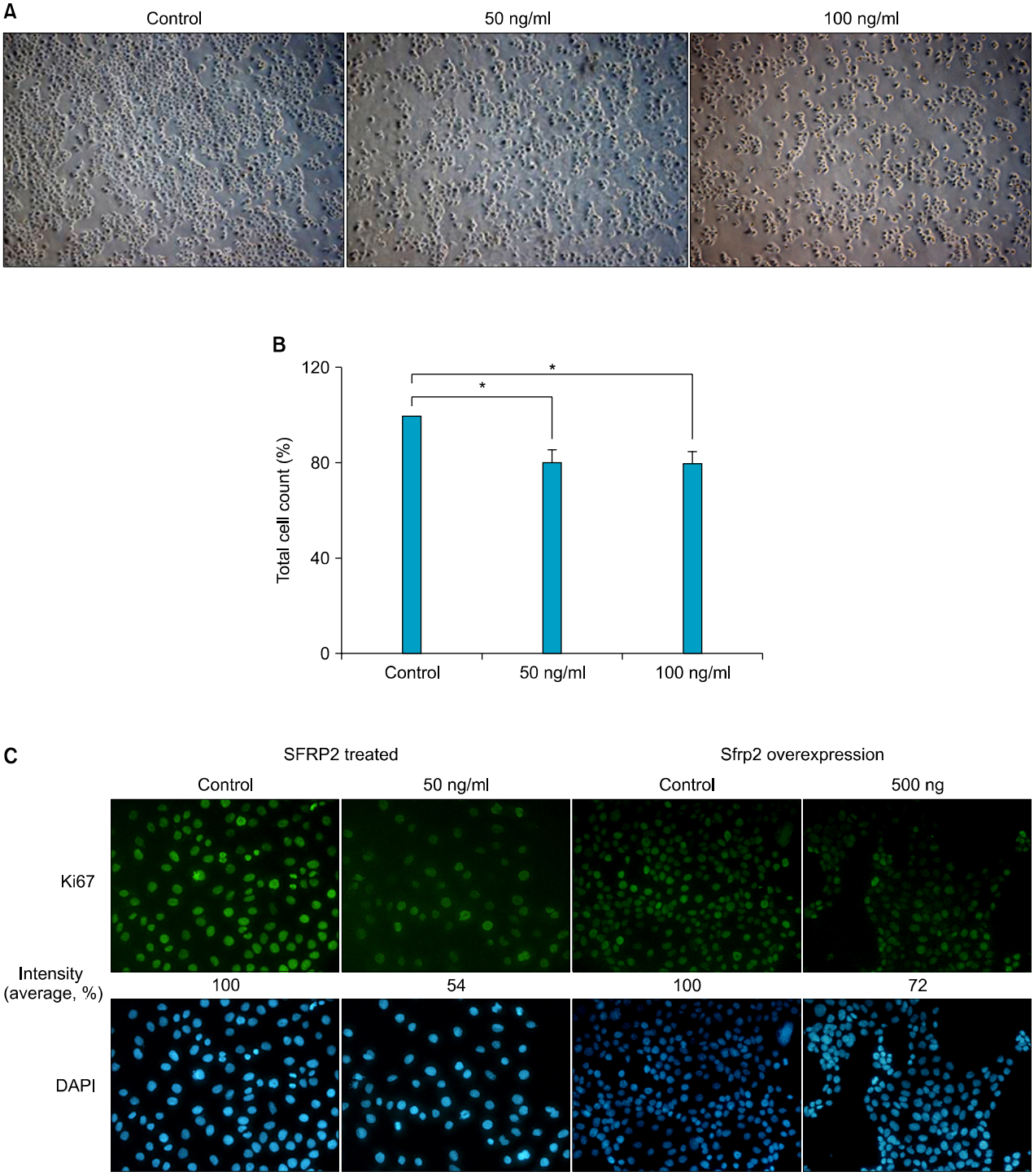

Fig. 3. Effect of SFRP2 on keratinocyte proliferation. SFRP2 treatment on PAM212 cells decreased the number of live cells compared to the mock-treatment. After 48 hour treatment with 50 ng/ml of SFRP2, the cells were observed using a microscope (A) and counted using the trypan blue exclusion method (B). Three independent experiments were carried out in duplicate for cell count. The values are mean±standard deviation. *$p<0.05$. (C) SFRP2 inhibits keratinocyte proliferation. PAM212 cells were treated with SFRP2 or transfected with 500 ng of pCDNA3.1/ *Sfrp2* CDS vector. The cells were then used for immnucytochemistry for Ki67. 4',6-diamidino-2-phenylindole (DAPI) staining (blue) indicates nuclei. The numbers under the figures represent relative intensity of florescence (A: ×10; C: ×100).

SFRP2 at 50 and 100 ng/ml for 48 hours, and the number of live cells was counted using the trypan blue exclusion method. We found that SFRP2 significantly reduced the number of cells by 20% compared to the mock-treated control at both 50 and 100 ng/ml (Fig. 3A, B).

To investigate whether this decrease was caused by inhibition of cell proliferation, we used immunofluorescent staining for the Ki67 proliferation marker[23]. Immunocytochemistry showed decreased Ki67 expression in cells treated with SFRP2 and in cells overexpressing SFRP2 (Fig. 3C), indicating that SFRP2 inhibits keratinocyte proliferation.

Effect of SFRP2 on *Involucrin* expression in keratinocytes

To investigate whether SFRP2 treatment would affect the differentiation status of keratinocytes, we performed immunocytochemistry for *Involucrin* expression, a keratinocyte differentiation marker[24], in SFRP2 treated or *Sfrp2*-overexpressed PAM212 cells. Unlike Ki67, we found no difference in *Involucrin* expression between control and SFRP2-treated cells (Fig. 4A).

RT-PCR and Real-time PCR analyses also revealed no significant difference in the *Involucrin* mRNA level between control and SFRP2-overexpressed cells (Fig. 4B, C). These results indicate that *Sfrp2* does not affect *Involucrin* expression and suggest that *Sfrp2* is not involved in

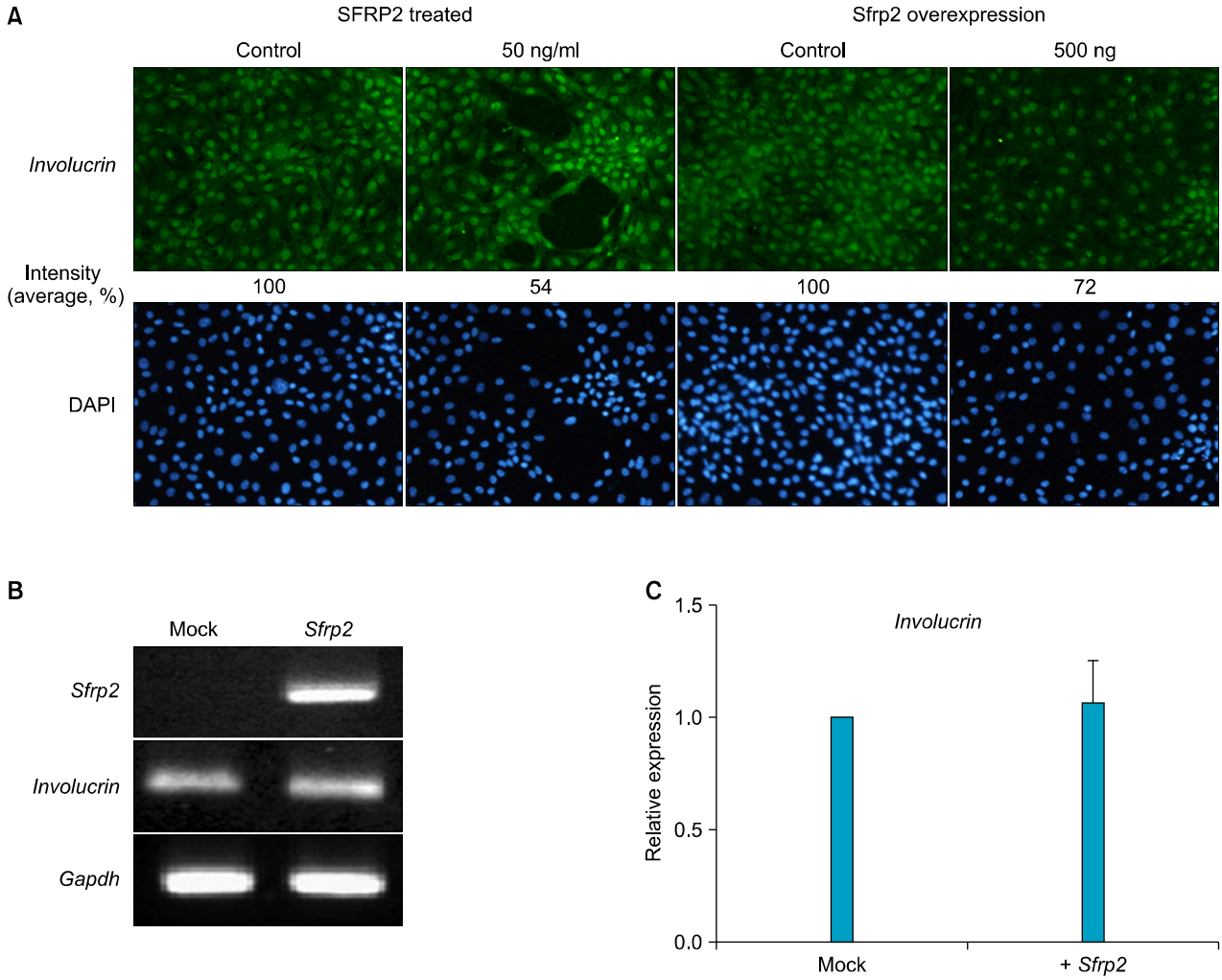

Fig. 4. Effect of SFRP2 on the expression of keratinocyte differentiation markers. (A) PAM212 cells were treated with 50 ng/ml of SFRP2 or transfected with 500 ng of pCDNA3.1/ *Sfrp2* CDS vector. The cells were then used for immnucytochemistry for involucrin. 4',6-diamidino-2-phenylindole (DAPI) staining (blue) indicates nuclei. The numbers under figures represent the relative intensity of florescence (immunohistochemistry, ×100). (B, C) Expression of *Involucrin* mRNA detected by reverse transcription-polymerase chain reaction (B) and Real-time polymerase chain reaction (C). The data are normalized against *Gapdh* mRNA expression.

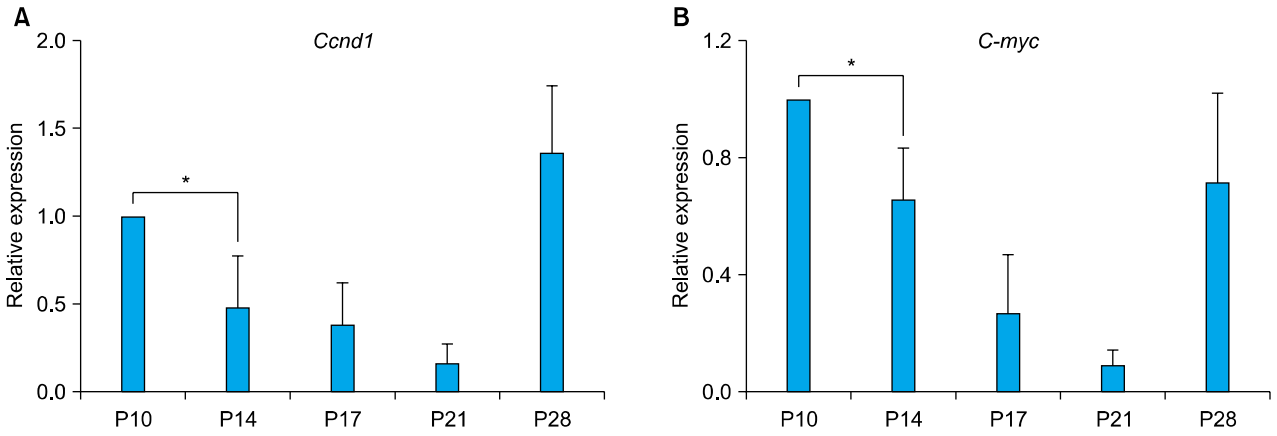

Fig. 5. Expression of Wnt target genes, *Ccnd1* (A) and *C-myc* (B) in the mouse skin. Relative expression levels of *Ccnd1* and *C-myc* were determined using Real-time polymerase chain reaction with total RNAs extracted from the dorsal skins of BALB/C at P10～P28. The expression of these genes was inversely proportional to that of *Sfrp2*. The data are normalized against *Gapdh* mRNA expression. The values are the average of the relative expression levels determined in three mice, each measured in duplicate. *$p<0.05$. P: postnatal day.

keratinocyte differentiation.

Effect of SFRP2 on the Wnt pathway during the hair cycle

SFRP2 function in the Wnt pathway is controversial. SFRP2 was reported to be inhibitory in the Wnt pathway[12], whereas it was also shown to activate Wnt signaling through the canonical activation of β-catenin in intestinal epithelial cells and canine mammary glands[25-28]. To determine SFRP2 function in the Wnt pathway of HFs, we investigated the expression of the wnt target genes *Ccnd1* and *C-myc* on the back skin of mice. Real-time PCR analysis revealed an inverse relationship between the *Ccnd1* and *C-myc* expression pattern and that of *Sfrp2* throughout the HF cycle (Fig. 5), suggesting that *Sfrp2* may function as a Wnt inhibitor in HFs.

DISCUSSION

Many signaling pathways crosstalk with each other to control HF development and cycling[29]. Among these signaling pathways, the Wnt pathway has been widely accepted to function in hair morphogenesis and HF cycling[30-33]. Excess induction of Wnt signaling causes abnormal hair cycles and abnormal formation of HFs[34]. Several mice with Wnt inhibitor mutations have been reported. For example, a *Dkk1* or *Dkk2* mutation in mice causes complete hair loss through failure to initiate development and mis-spacing of HFs[6,9], showing that *Dkk1* and *Dkk2* are essential in normal HF formation and control of the hair cycle. Although mice with the *Sfrp2* mutation have been reported and studied[35], its function in the normal hair cycle has not been documented.

In this study, we found that *Sfrp2* is mainly expressed during the catagen phase and inhibited mouse keratinocyte proliferation. This inhibition was also observed in human HaCaT keratinocytes (data not shown). These results suggest that *Sfrp2* may function in the catagen phase by inhibiting keratinocyte proliferation. The inhibition rate by SFRP2 in keratinocytes was 20%, suggesting that SFRP2 may not be the main catagen regulator. Nevertheless, SFRP2 must be considered a contributor in the catagen phase of HFs.

Several investigators have shown that the action of *Sfrp2* in the Wnt pathway differs in different cell types. These studies suggest that *Sfrp2* acts not as a Wnt inhibitor but as an activator[25,26]. However, in our study, we showed that *Sfrp2* expression is inversely related to Wnt target gene expression (Fig. 5), suggesting that *Sfrp2* is a Wnt inhibitor in HFs. In addition to *Sfrp2* expression, other Wnt inhibitors are also expressed in HFs (Fig. 1). Thus, Wnt target gene expression in HFs must be the sum of Wnt inhibitor expression. Therefore, although *Sfrp2* acts as a Wnt inhibitor in the normal hair cycle, a further study is required to understand its precise function in HFs.

SFRP2 is also known to regulate the differentiation of myoblasts and osteoblasts[18,36]. Our study found that SFRP2 treatment on keratinocytes did not affect the expression of Involucrin, a differentiation marker of keratinocytes. Furthermore, there was no significant difference between the control and SFRP2 treated human HaCaT keratinocytes (data not shown). These results suggest that SFRP2 may not play a role in the differentiation of keratinocytes.

In conclusion, our results suggest that SFRP2 plays a role in the catagen phase by inhibiting keratinocyte proliferation. A further study with microdissection of the epidermis is needed for a better understanding of SFRP2 function in hair cycle regulation.

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