Cell Transplantation, Vol. 25, pp. 1439–1451, 2016

0963-6897/16 $90.00 + .00
DOI: http://dx.doi.org/10.3727/096368916X691466
E-ISSN 1555-3892
www.cognizantcommunication.com

Neural Stem Cells Restore Hair Growth Through Activation of the Hair Follicle Niche

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Several types of hair loss result from the inability of hair follicles to initiate the anagen phase of the hair regeneration cycle. Modulating signaling pathways in the hair follicle niche can stimulate entry into the anagen phase. Despite much effort, stem cell-based or pharmacological therapies to activate the hair follicle niche have not been successful. Here, we set out to test the effect of neural stem cell (NSC) extract on the hair follicle niche for hair regrowth. NSC extracts were applied to the immortalized cell lines HaCaT keratinocytes and dermal papilla cells (DPCs) and the shaven dorsal skin of mice. Treatment with NSC extract dramatically improved the growth of HaCaT keratinocytes and DPCs. In addition, NSC extract enhanced the hair growth of the shaven dorsal skin of mice. In order to determine the molecular signaling pathways regulated by NSCs, we evaluated the expression levels of multiple growth and signaling factors, such as insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and bone morphogenetic protein (BMP) family members. We found that treatment with an NSC extract enhanced hair growth by activating hair follicle niches via coregulation of TGF-β and BMP signaling pathways in the telogen phase. We also observed activation and differentiation of intrafollicular hair follicle stem cells, matrix cells, and extrafollicular DPCs in vivo and in vitro. We tested whether activation of growth factor pathways is a major effect of NSC treatment on hair growth by applying the growth factors to mouse skin. Combined growth factors, including TGF-β, significantly increased the hair shaft length and growth rate. DNA damage and cell death were not observed in skin cells of mice treated with the NSC extract for a prolonged period. Overall, our data demonstrate that NSC extract provides an effective approach for promoting hair growth by directly regulating hair follicle niches through TGF-β and BMP signaling pathways as well as induction of core growth factors.

Key words: Hair growth; Neural stem cell extract; Hair follicle niche; Transforming growth factor-β (TGF-β)

INTRODUCTION

The niche of the mammalian hair follicle contains a heterogeneous cell population, including hair follicle stem cells (HFSCs) and epithelial cells such as keratinocytes and melanocytes[1–3]. HFSCs and epithelial cells closely interact with mesenchymal lineage dermal papilla cells (DPCs) embedded in the hair bulb as well as with various dermal cells such as fibroblasts, immune cells, and adipocytes[4–6]. Hair follicles undergo regeneration and resting cycles that are driven by stem cells residing in the niche known as the bulge[7–9]. During the hair cycle, multiple signaling interactions among the hair follicle cells increase the proliferation of matrix keratinocytes and differentiation of HFSCs or their progenitor cells into mature hair cells[10,11]. DPCs and HFSCs as well as cells in the dermal layer secrete paracrine factors such as growth factors[12] and multiple signaling factors, including Wnt/β-catenin, sonic hedgehog (SHH), bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β), and Notch[13–17]. The secreted factors mediate the cross talk

Received February 9, 2016; final acceptance May 10, 2016. Online prepub date: April 22, 2016.
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among multiple signaling pathways in regulating the initiation and growth of new hair follicles. Indeed, the transition from the telogen (rest) phase to the anagen (growth) phase is regulated by growth factors[18], Wnt/β-catenin[19,20], BMP[21–23], and TGF-β[8,24] signaling. In addition, introduction of either DPCs or HFSCs, or both of them, into the dermal layers of mouse skin triggers the formation and growth of hair follicles[8,13,25]. These data suggest that DPCs and HFSCs can stimulate the arrested telogen phase to the anagen phase. Although the direct injection of HFSCs or DPCs into the skin is one promising approach for hair growth, difficulties in acquiring sufficient numbers of cells having a hair growth-inducing capacity limit the practical utility of the method[26]. Thus, identifying materials or factors that trigger activation of HFSCs and DPCs in vivo is critical for hair regeneration.

We hypothesized that activation of microenvironments in the epidermal and dermal layers of skin would enhance hair growth by activation, proliferation, and differentiation of stem and mature cells. The activation of the microenvironment can be achieved through regulating multiple signaling pathways and growth factors. In the current study, we found that a neural stem cell (NSC) extract promoted hair growth by activating the niches of hair follicles via core signaling and subsequently converting cells from the telogen phase to the anagen phase. Our data demonstrate that an NSC extract contains multiple factors regulating the signaling pathways that stimulate entry into the anagen phase. NSC extract can be developed as a potential therapeutic agent for patients with hair loss such as patients with alopecia areata, which is caused by an autoimmune disease that attacks the patient's own hair follicles.

MATERIALS AND METHODS

Materials

Water-soluble tetrazolium salt (WST-1) was purchased from Daeil Lab Service Co. Ltd (Seoul, Korea). 4′6-Diamidino-2-phenylindole (DAPI) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies used were against cytokeratin 15 (K15), hair cortex cytokeratin (AE13), CD34, TGF-β2 (Abcam, Cambridge, MA, USA), (sex-determining region Y)-box 2 (SOX2; Millipore, Billerica, MA, USA), Ki-67 (Dako, Glostrup, Denmark), γH2AX (Millipore), caspase 3 (Cell Signaling Technology, Danvers, MA, USA), p-Smad1/5/8, SMAD1, transmembrane protein with epidermal growth factor (EGF)-like and two follistatin-like domains 1 (TMEFF1), and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Recombinant human insulin-like growth factor-1 (IGF-1), TGF-β2, and keratinocyte growth factor (KGF) were purchased from R&D Systems (Minneapolis, MN, USA). Hepatocyte growth factor (HGF) was purchased from BioLegend (San Diego, CA, USA).

Isolation and Characterization of NSCs

Isolation, propagation, and characterization of immortalized NSCs were performed as described previously[27–29]. In brief, $CD45^+/CD133^+/CD34^-$ NSCs were isolated from the ventricular zone of 14-week gestational age aborted human fetus by fluorescence-activated cell sorting (FACS). NSCs were grown as neurospheres in culture with Dulbecco's modified Eagle's medium (DMEM)/F12 containing N2 supplement (Gibco, Carlsbad, CA, USA), heparin (0.2 mg/ml; Gibco), basic fibroblast growth factor (bFGF; 20 ng/ml; R&D Systems), and leukemia inhibitory factor (LIF; 10 ng/ml; Millipore). After about 2 weeks of cell culture, the NSCs were transduced with a retroviral vector containing v-myc. NSCs were characterized by the expression of various NSC-specific markers including *SRY box 1* (*SOX1)* and *NESTIN* (Millipore) (data not shown). The immortalized NSCs were cultured in DMEM (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone), 1% penicillin, and 1% streptomycin (Gibco) at 37°C with 5% CO_2. This study was approved by the Korea University Institutional Review Board (IRB; Approval No. KU-IRB-12-140-A-1). We obtained a written informed consent from the donor's mother, who was not compensated for donation.

Preparation of NSC-Conditioned Medium and the NSC Extract

In order to obtain NSC-conditioned medium (CM), a culture of 5×10^5 NSCs per 150-mm culture dish was initiated in phenol red-free DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO_2. NSC-CM was harvested at 96 h after cell culture and centrifuged at $450\times g$ for 5 min. In preparing the NSC extract, cultured NSCs were washed with phosphate-buffered saline (PBS; Hyclone) twice, treated with trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco), and then collected by centrifugation at $450\times g$ for 5 min. The cell pellets were homogenized in 100 ml of distilled water containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF) (Santa Cruz Biotechnology) and cOmplete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA) and homogenized at $3{,}370\times g$ by a WiseTis Digital/Analog Homogenizer (DAIHAN Scientific, Seoul, Korea) for 5 min. The homogenate was centrifuged at $9{,}358\times g$ for 15 min, and then the supernatant was collected. NSC-CM and NSC extract were filter sterilized with a 0.2-μm pore size membrane, aliquoted, and stored at −80°C.

Cell Culture

Immortalized human DPCs were a gift from Dr. Y. K. Sung (Department of Immunology and Hair Research Center, Kyungpook National University, Daegu, Korea)[30].

HaCaT keratinocytes (an immortalized keratinocyte line) were purchased from Thermo Fisher Scientific. DPCs and HaCaT keratinocytes were cultured in DMEM (Hyclone) and supplemented with 10% FBS (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C with 5% CO_2.

Cell Proliferation and Viability Assays

The proliferation of human DPCs and HaCaT keratinocytes was examined by WST-1 cell proliferation assay reagent. Human DPCs and HaCaT keratinocytes (1×10^3 cells/well in 96-well plates) were treated with various concentrations of NSC-CM (10–100%), NSC extract (5–50%), IGF-1, and TGF-β2. After 24, 48, and 72 h of incubation, 10 μl of WST-1 reagent was added to each well. The absorbance was measured at 450 nm using a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 37°C.

Quantitative Real-Time PCR

Total RNAs of human DPCs and HaCaT keratinocytes and skin tissues of mice were extracted with TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions and were used to make cDNA with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Thermo Fisher Scientific) and an oligo(dT) primer. Quantitative real-time polymerase chain reaction (RT-qPCR) was performed using a StepOnePlus and QuantStudio 6 Flex Real-Time PCR machine (Applied Biosystems, Carlsbad, CA, USA) and KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA). All RT-qPCR assays were carried out under the following conditions: 40 cycles of denaturation at 95°C for 20 s, annealing at 56°C (or 62°C) for 20 s, and extension at 72°C for 20 s. Samples were run in triplicate, and relative expression was determined by normalizing the data to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. PCR primers are listed in Supplementary Table 1 (available at: https://drive.google.com/file/d/0B37gUn0qrQc3NWViMGRLVjE3bzA/view).

Western Blot Analysis

Cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% Triton X-100] (Thermo Fisher Scientific) containing cOmplete Mini Protease Inhibitor Cocktail (Roche) and PMSF (Santa Cruz Biotechnology). Cells were incubated on ice for 15 min and then centrifuged for 10 min at $450 \times g$ at 4°C. The supernatant was collected, and the protein concentration was determined using Bradford Dye Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of cell lysates were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene fluoride (PVDF) (Bio-Rad Laboratories) membrane. The membrane was blocked with 5% dry skim milk (BD Biosciences, San Jose, CA, USA) in PBS containing 0.1% Tween 20 for 1 h and then incubated with appropriate primary antibodies: anti-SOX2 (1:250), anti-TGF-β2 (1:1,000), p-Smad1/5/8 (1:500), SMAD1 (1:500), TMEFF1 (1:400), or anti-α-tubulin (1:1,000). Secondary antibodies were horseradish peroxide-conjugated goat anti-mouse and anti-rabbit immunoglobulin G (IgG) (Thermo Fisher Scientific) used at dilutions of 1:10,000. Antibody complexes were incubated with Clarity Western ECL Substrate (Bio-Rad Laboratories). Protein bands were detected using the FluorChem E system (ProteinSimple, San Jose, CA, USA).

Immunohistochemistry

Skin biopsies were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO, USA) for immunohistochemistry. Paraffin-embedded 3-μm-thick sections were cut, mounted on POLYSINE Slides (Thermo Fisher Scientific), dewaxed in xylene, and then dehydrated in an ethanol series. Antigen retrieval was performed by boiling in sodium citrate (Santa Cruz Biotechnology) buffer (10 mM sodium citrate and 0.05% Tween 20, pH 6.0). Sections were blocked for 30 min with PBS containing 1% horse serum (Sigma-Aldrich) and then incubated overnight at 4°C with anti-AE13 (1:100), anti-K15 (1:200), anti-γH2AX (1:500), anti-caspase 3 (1:200), anti-CD34 (1:100), or anti-Ki-67 (1:50) antibodies. The sections were rinsed three times with PBS and then incubated for 1 h at room temperature with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (1:1,000 in PBS; Invitrogen, Carlsbad, CA, USA). After further rinsing, nuclei were counterstained with DAPI (1:10,000; Thermo Fisher Scientific) in PBS (Hyclone). Images were captured using an EVOS fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA) and a confocal microscope with an FV1000 FluoView camera (Olympus, Tokyo, Japan).

Hair Growth Analysis In Vivo

Six-week-old male C57BL/6 mice were purchased from Orient Bio (Seoul, Korea) and housed under a 12-h light/dark cycle. Mice were divided into four groups ($n = 5$ each). Progression to the anagen phase was induced by depilation of the skin on the dorsal aspect of mice[31]. The dorsal side of the mice was shaved, and hair removal cream (Reckitt Benckiser, Parsippany-Troy Hills, NJ, USA) was applied. Prior to topical application of NSC-CM, NSC extract, and minoxidil (Hyundai Pharm, Seoul, Korea), we used a 0.5-mm mesoroller (Moohan, Seoul, Korea) to increase skin absorption. One hundred percent NSC-CM (0.5 ml, v/v), 20% NSC extract (0.5 ml, v/v), or 5% minoxidil (0.5 ml, v/v) was

topically applied daily to the dorsal skin with a small brush for 16 days once a day. Minoxidil was used as a positive control. Three independent experiments were performed. The hair length was determined from 30 hair follicles plucked from three different areas of the dorsal skin using a digital caliper (World Precision Instruments Inc., Sarasota, FL, USA). The hair weight was quantitatively evaluated after shaving the hair shafts of dorsal skin at day 17. Recombinant human proteins were used at concentrations of 100 ng of IGF-1, 1 pM TGF-β2, 100 ng of KGF, and 10 ng of HGF. The growth factor mixture was topically applied daily to shaved mice ($n = 5$). To increase skin absorption, a 0.5-mm mesoroller (Moohan) was used prior to topical application. The formula to calculate the hair growth rate was average rate (v) = hair length (mm)/growth time (day), where v indicates velocity. Three independent experiments were performed.

Quantitation of Hair Follicle Numbers

The number of hair follicles was determined in 10-μm-thick, paraffin-embedded longitudinal and transverse skin sections as described previously[32]. In brief, calculations were based on the average number of hair follicles in five randomly chosen areas of each group at ×100 magnification in brightfield microscopy. At least 300 hair follicles were counted to calculate the percentage of hair follicles. Three independent experiments were performed.

Statistical Analysis

Results are presented as mean ± standard deviation (SD). To determine the number of cells and hair follicles, Ki-67$^+$ and CD34$^+$ cells and AE13$^+$ hair follicles were counted at five randomly selected fields at ×100 magnification using a confocal microscope with a FV1000 FluoView camera (Olympus). Three independent experiments were performed for cell viability, proliferation, and RT-qPCR analyses. Statistical significance was determined by using independent and paired Student's *t*-tests with SPSS software (SPSS 12.0; IBM, Armonk, NY, USA) in the unpaired and paired samples. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

NSC Extract Increases Cell Proliferation and Induction of Growth Factors in Human DPCs and Hair Follicle Keratinocytes

Previous studies showed that the CM and cell extracts of various types of stem cells have prominent effects on depigmentation, hair growth, various cancers, and other diseases[29,33–35]. These findings prompted us to investigate whether NSC-CM or an NSC extract affects hair growth. Before testing the efficacy of hair growth, we examined the cytotoxicity of NSC-CM and the NSC extract in human DPCs. Human DPCs were applied with NSC-CM ranging from 5% to 100% or NSC extract ranging from 5% to 50% for 72 h. The cell viability was over 95% without any apparent cytotoxic effects at all concentrations of NSC-CM. However, cytotoxicity was observed in the treatment with NSC extract at over 30% (Fig. 1A). Human DPC proliferation was significantly increased by both 100% NSC-CM and 20% NSC extract after 72 h of culture, while proliferation was more effective in the NSC extract (Fig. 1B). Cytotoxicity of NSC-CM and NSC extract on HaCaT keratinocytes, a main cell type in the hair shaft[36], was negligible and only found to be >50% in the NSC extract (Fig. 1C). NSC extract induced proliferation of HaCaT keratinocytes when applied at 10–30% (Fig. 1D).

Next, in order to identify key growth factors that positively influence cell proliferation, we performed gene expression analysis on human DPCs that were treated with 100% NSC-CM or 20% NSC extract. We examined the expression of growth factor genes, including *IGF-1*, *KGF*, *HGF*, and vascular endothelial growth factor (*VEGF*), all of which are known to play pivotal roles in hair growth[11,37]. Interestingly, the gene expression of *IGF-1*, *HGF*, *KGF*, and *VEGF* was significantly increased in DPCs treated with 20% NSC extract, but this was not observed in DPCs treated with 100% NSC-CM. Notably, the gene expression of TGF-β2 was robustly induced by the 20% NSC extract treatment (Fig. 1E). Because TGF-β2 is a core signaling factor that regulates HFSC activation and differentiation[38], our data imply that the 20% NSC treatment affects HFSCs by inducing TGF-β2 expression. While 20% NSC extract induced TGF-β2, 100% NSC-CM suppressed the expression of TGF-β2. However, 100% NSC-CM induced the expression of *BMP4* and *BMP6*, which are key factors regulating HFSC self-renewal and quiescence[38] (Fig. 1E). These results indicated that the NSC extract rather than NSC-CM was more effective for the activation of hair growth. In addition, our data provide new evidence of a mechanism by which NSC extract promotes hair growth and that is through counterbalancing BMP and TGF-β signaling pathways as well as inducing growth factor expression such as HGF, IGF-1, KGF, and VEGF.

NSC Extract Enhances Hair Growth In Vivo

Prompted by the promising in vitro effect of NSC extract on hair follicle cells, we next examined whether NSC extract or NSC-CM enhances hair growth in mice. We used the shaven dorsal skin of 6-week-old male C57BL/6 mice, which is synchronized to the telogen phase, a resting or quiescent phase of hair follicles. The shaven dorsal skin was treated with 20% NSC extract or 100% NSC-CM for 16 days; 5% minoxidil was used as the positive control. At 10–14 days after treatment, hair growth was distinctly increased in mice treated with 20% NSCs. At 17 days after application, most mice treated

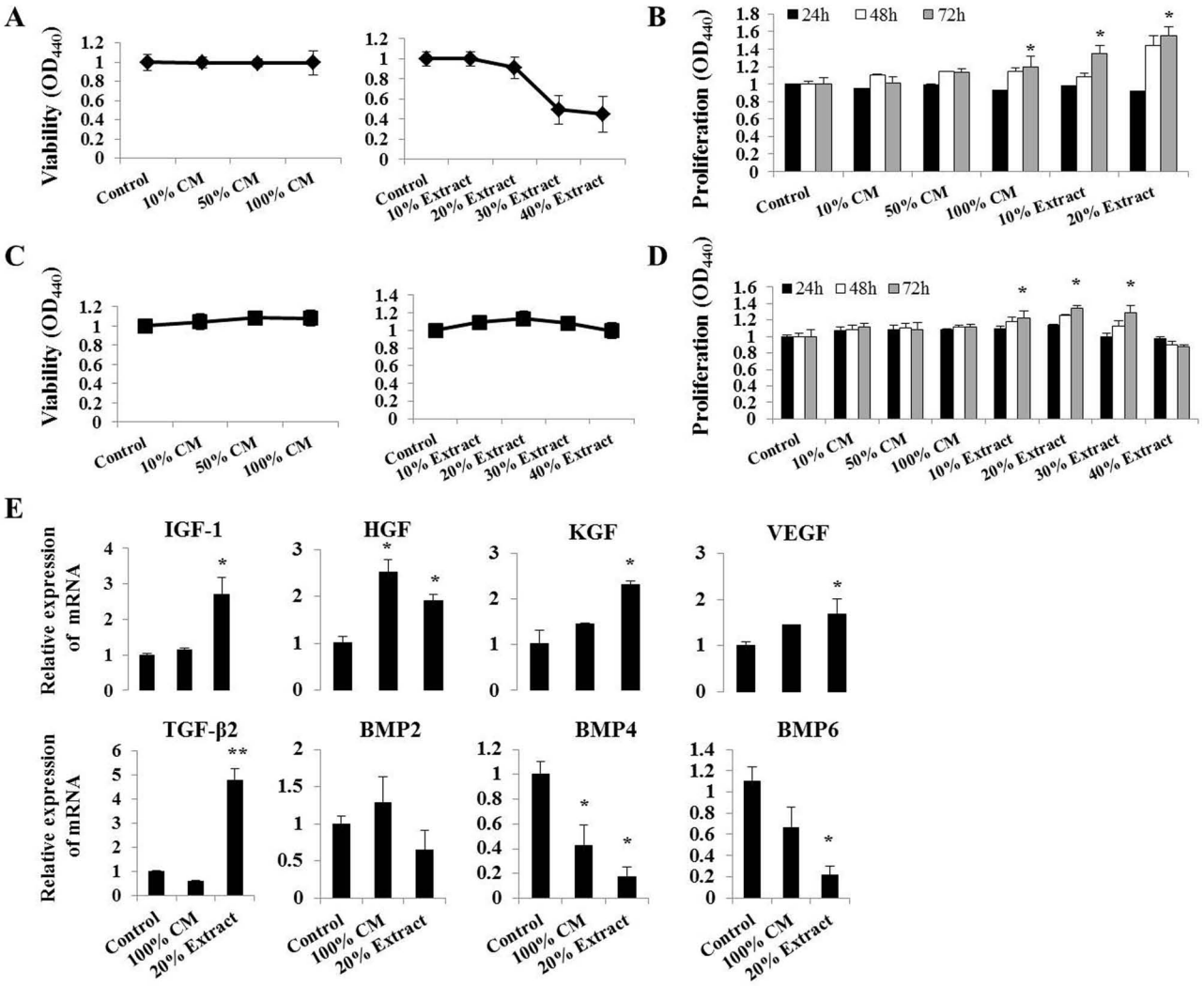

Figure 1. Analysis of cytotoxicity, cell proliferation, and gene expression of growth factors in human dermal papilla cells (DPCs) and HaCaT keratinocytes treated with the neural stem cell-conditioned medium (NSC-CM) and NSC extract. (A) Cytotoxicity of human DPCs and (C) HaCaT keratinocytes treated with NSC-CM (10–100%, v/v) or the NSC extract (10–40%, v/v) for 72 h. (B) Cell proliferation of DPCs and (D) HaCaT keratinocytes treated with NSC-CM or NSC extract as determined by WST-1 proliferation assays. (E) Gene expression levels of growth factors and *BMP-2*, *BMP-4*, and *BMP-6* in the human DPCs treated with NSC-CM or the NSC extract as measured by quantitative real-time polymerase chain reaction (RT-qPCR). Data represent the mean ± SEM ($n = 3$). *$p < 0.05$, **$p < 0.01$, compared to control.

with 20% NSC extract were covered with a significant amount of hair, but not those in other groups (Fig. 2A). These results showed that the NSC extract significantly stimulated and promoted hair growth on the dorsal skin of mice, indicating that the resting phase of the hair follicle can be converted to the active growth phase by daily treatments with NSC extract.

Next, to quantify the effect of the NSC extract on hair growth, we examined the hair shaft length and growth rate of hairs plucked from six areas of the dorsal skin. At 17 days after topical application, the growth rate and shaft length of hair were significantly increased in mice treated with the 20% NSC extract (Fig. 2B and C). Minoxidil treatments significantly increased hair shaft length at day 16, which is consistent with other studies[33,34,39,40]. Consistent with the increase in hair length, we found that the 20% NSC extract also significantly increased hair weight by more than 1.6-fold compared with the control, 100% NSC-CM, and 5% minoxidil groups (Fig. 2D).

Histological analysis of hair follicles in longitudinal and transverse skin tissue sections showed that the number of hair follicles was significantly increased by the 20% NSC extract, but not by other treatments (Fig. 3A and B). In addition, we examined whether new hair shafts formed in the hair follicles. We stained the skin tissue sections with an antibody against AE13 cytokeratin, a hair cortex

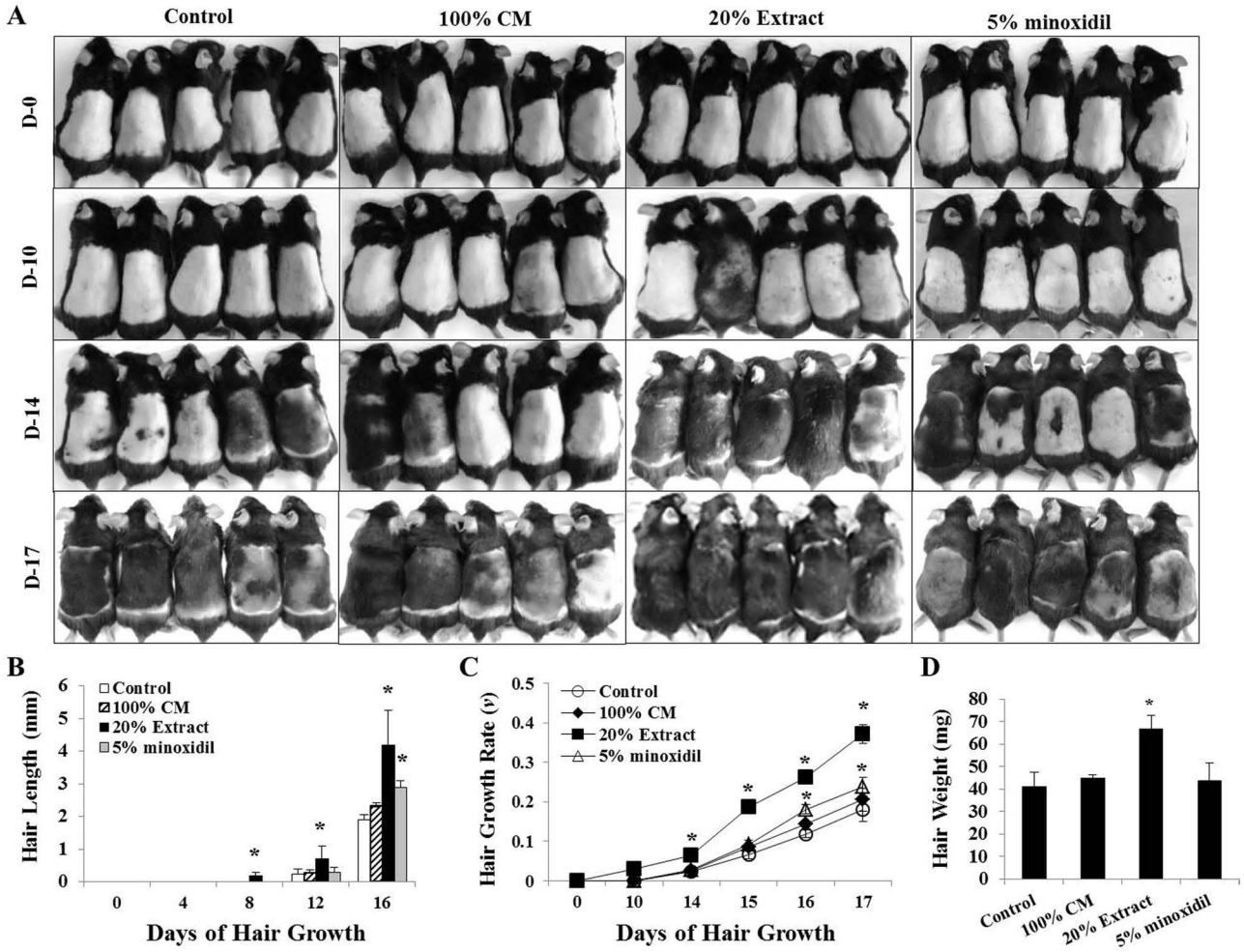

Figure 2. Promotion of hair growth in the dorsal skin of mice treated with the NSC extract. (A) Hair growth images of control (DMEM + 10% FBS), 100% NSC-CM, 20% NSC extract, and minoxidil groups at day 10, 14, and 17 ($n=5$ each). (B) Length, (C) growth rate, and (D) weight of hair shafts in control (DMEM + 10% FBS), 100% NSC-CM (v/v), 20% NSC extract (v/v), and 5% minoxidil groups. The formula to calculate the hair growth rate was average rate (v) = hair length (mm)/growth time (day); v indicates velocity. Three independent experiments were performed. Data represent the mean ± SEM ($n=5$). *$p<0.05$, **$p<0.01$, compared to control.

and cuticle marker. As shown in Figure 3C and D, AE13$^+$ hair shafts significantly increased in mice treated with the 20% NSC extract. Moreover, we found that the number of Ki-67$^+$ proliferative cells increased in the hair follicular matrix of mice treated with the 20% NSC extract (Fig. 3E and F). These data strongly suggest that the NSC extract may influence HFSC activation and differentiation by providing growth factors that activate the niches of hair follicles. These results were consistent with our in vitro data (Fig. 1B, D, and E) showing increases in various growth factors and cell proliferation. Because safety is critical when using cell-based biomaterials as therapeutics, we examine the long-term impact of the NSC extract and NSC-CM on mouse skin. We immunostained skin sections for caspase 3 or γH2AX that mark cell death and DNA double strand breakages, respectively. The results showed no significant increase of caspase 3$^+$ or γH2AX$^+$ cells in any of the analyzed skin tissues (Fig. 3E and G), indicating that NSC-CM and the NSC extract could be agents safe for long-term treatment in skin. Taken together, these results demonstrated that hair growth increased in mice treated with NSC extract but not with 100% NSC-CM or 5% minoxidil, suggesting that the NSC extract may be a safe and valuable biomaterial for enhancement of hair growth.

NSC Extract Activates HFSCs and DPCs In Vivo and In Vitro

Next, in order to determine which cell types are directly regulated by the NSC extract, we examined the proliferation and activation of HFSCs and hair germ cells in skin tissue. We immunostained skin tissue sections with K15 and CD34 antibodies for epithelial stem cells

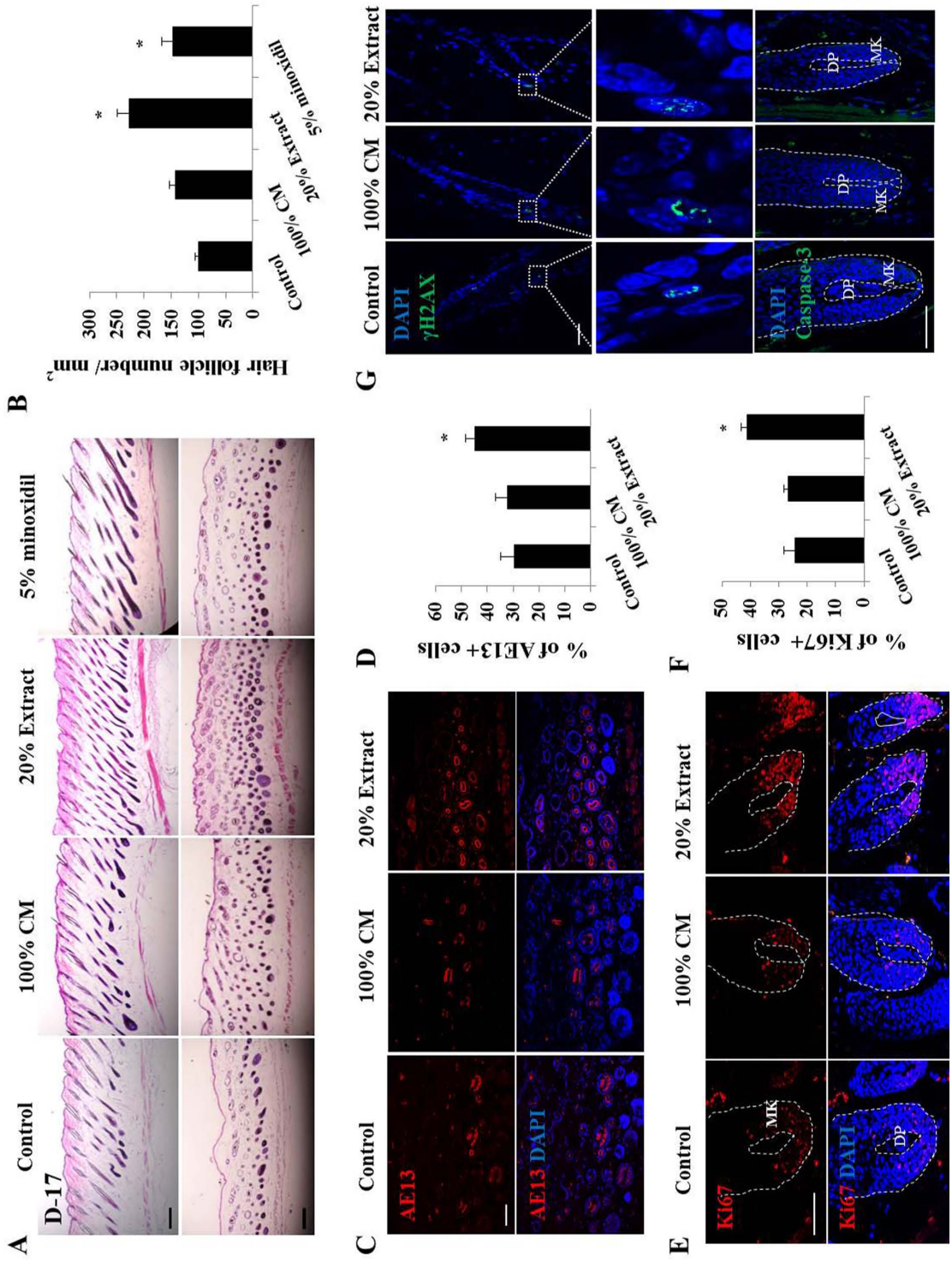

Figure 3. Hair follicles and matrix cells were increased in mice treated with the NSC extract. (A) Histological analysis of longitudinal and transverse sections of dorsal skin by hematoxylin and eosin staining (×100, scale bar: 50 μm) at day 17 ($n=5$). (B) Relative comparison of the number of hair follicles between groups ($n=5$). (C) Analysis of the hair shaft by expression of hair cortex marker AE13 in mice ($n=5$) treated with the NSC extract at day 17 (×100, scale bar: 50 μm). (D) Relative comparison of the number of AE13$^+$ cells between groups. (E) Cell proliferation of hair matrix keratinocytes within hair follicles determined by immunofluorescence for Ki-67 at day 17 (×200, scale bar: 20 μm). (F) Relative comparison of the number of Ki-67$^+$ cells between groups. (G) Safety analysis of DNA and cells within hair follicles by immunofluorescence for the DNA damage marker γH2AX and apoptosis marker caspase 3 ($n=5$) at day 17 (×200, scale bar: 20 μm). Data represent the mean ± SEM ($n=5$). $^*p<0.05$, $^{**}p<0.01$, compared to control.

and hair germ cells, respectively. Interestingly, the 20% NSC extract dramatically increased the K15 expression in the interfollicular epidermis and outer root sheath of the hair follicles (Fig. 4A and B), while the number of $CD34^+$ cells significantly increased in bulge regions of hair follicles (Fig. 4C and D). In addition to K15 protein expression, *K10* (a nonproliferative and intermediate differentiation marker), *K14* (a proliferative progenitor marker), and *K36* (a hair cortex and cuticle marker) were increased significantly compared with control (Fig. 4B). These results suggest that the 20% NSC extract activated HFSCs ($K15^+$ cells), which subsequently proliferated and differentiated into $K14^+$ and $CD34^+$ undifferentiated hair progenitor cells. $K14^+$ and $CD34^+$ hair progenitor cells further differentiated into $K10^+$ hair cells, such as hair follicle epidermal cells including keratinocytes. Overall, our data suggest that the NSC extract activated niches within the hair follicle.

DPCs are mesenchymal dermal cells that are important for hair follicle growth during the anagen phase. To examine whether the NSC extract affected activation of DPCs in mice, we examined the proliferation and activation of DPCs. Interestingly, the expression levels of the DPC marker SOX2 protein and mRNA significantly increased in mice treated with the NSC extract (Fig. 5A). In addition, the mRNA expression levels of other DPC makers *CD133* and *Akp* increased significantly (Fig. 5B and C). In particular, *Versican*, which encodes a proteoglycan component of the extracellular matrix (ECM), was highly expressed (Fig. 5B). This result suggests that the

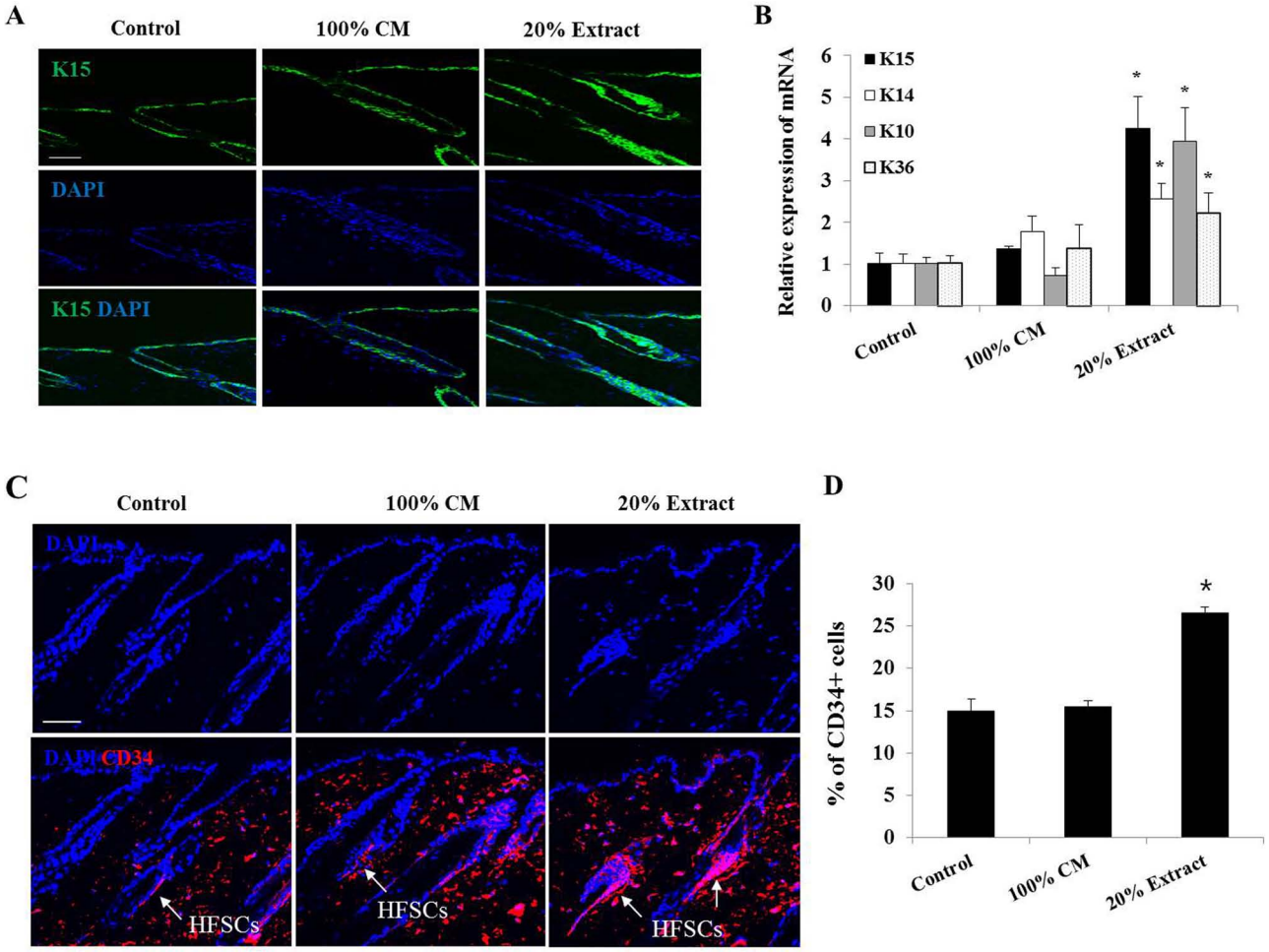

Figure 4. Activation, proliferation, and differentiation of hair follicle stem cells (HFSCs) in mice treated with the NSC extract. (A) Activation analysis of HFSCs by immunohistochemistry for HFSC marker cytokeratin 15 (K15) at 17 days (×200, scale bar: 20 μm). (B) Analysis of gene expression levels of *K15* and hair-specific keratin genes *K10* (nonproliferative and intermediate differentiation marker), *K14* (proliferative progenitor marker), and *K36* (hair cortex and cuticle marker) in mouse skin tissue treated with the NSC extract for 17 days. (C) Activation analysis of bulge stem cells by immunohistochemistry for the bulge stem cells marker CD34 in mice treated with the NSC extract for 17 days (×200, scale bar: 20 μm). (D) Relative comparison of the number of $CD34^+$ cells between groups. Data represent the mean ± SEM ($n = 5$). *$p < 0.05$, **$p < 0.01$, compared to control.

NSC extract may affect cell signaling pathways by inducing ECM components, including integrin receptors, which links HFSCs to various ECM components that regulate activation, proliferation, and differentiation of HFSCs and DPCs through the basement membrane.

In addition, to test whether the effects of the NSC extract on skin tissues are cell autonomous in DPCs, we analyzed the expression levels of *Sox2* and *Akp* in cultured human DPCs. When treated with the NSC extract, the expression of *Sox2* and *Akp* increased significantly compared with control and 100% NSC-CM groups (Fig. 5C). These results indicated that the 20% NSC extract directly activated human DPCs in skin tissues and the cultured DPC line. Overall, our data demonstrate that the NSC extract promoted hair growth through activation and differentiation of HFSCs as well as activation of human DPCs. In addition, the activation of interfollicular and outer follicular niches by the NSC extract is mediated by various growth factors and signaling pathways as well as ECM components present internally and externally at the hair follicle.

Mechanism of Hair Growth Induced by the NSC Extract

TGF-β2 has been known as an essential factor for the activation and differentiation of stem cells in hair follicles as well as activation of DPCs, and thus is crucial for hair follicle development and regeneration[24,41], while BMP signaling is essential to sustain HFSC quiescence[38]. In this study, we found that the NSC extract stimulated

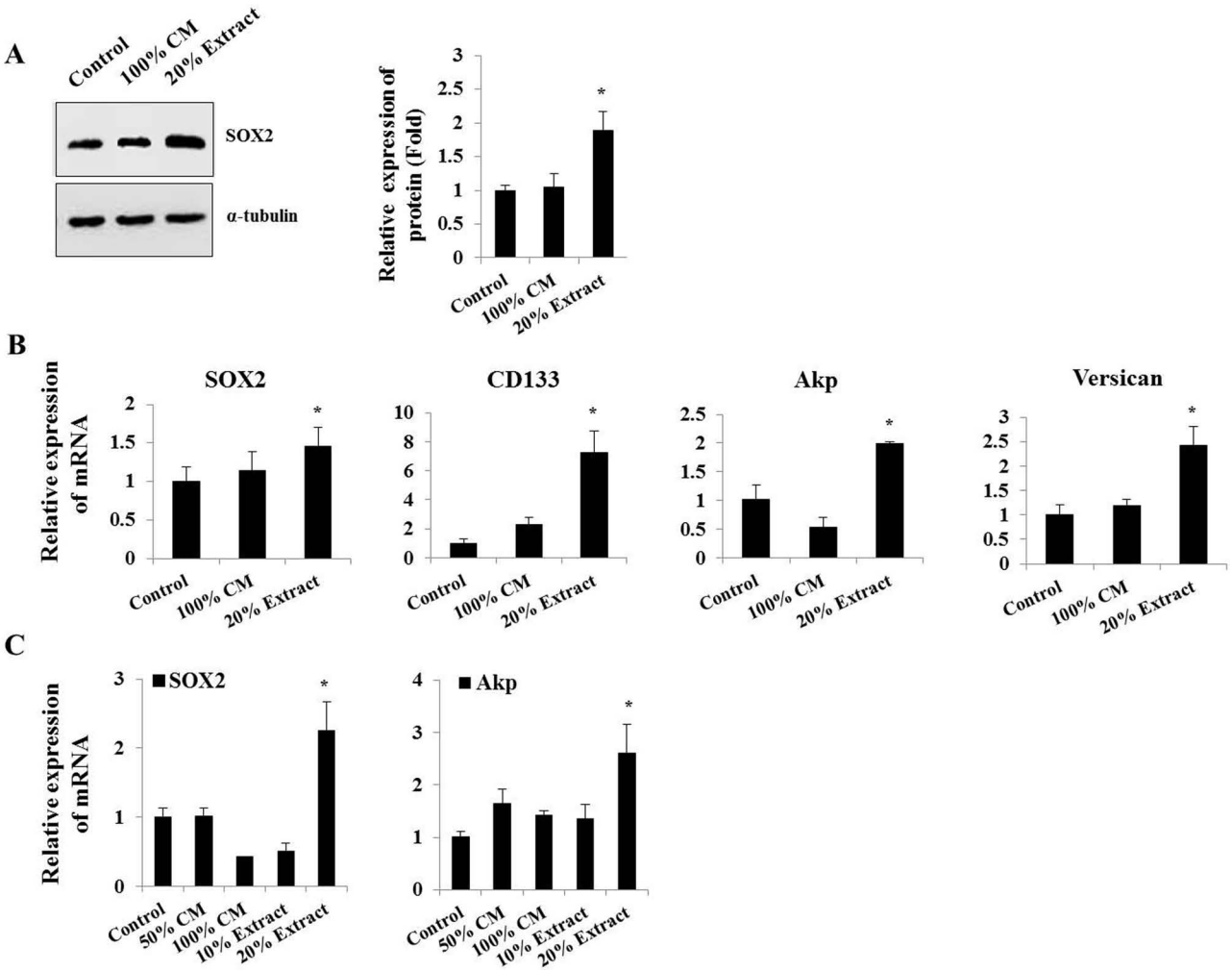

Figure 5. Activation of DPCs in mice and the DPC line treated with the NSC extract. (A) Relative protein expression levels of SOX2 as measured by Western blot. Protein levels were analyzed using ImageJ software. (B) Gene expression levels of DPC markers *SOX2*, *CD133*, alkaline phosphatase (*AKP*), and *Versican* as measured by RT-qPCR in skin from mice treated with the NSC extract for 17 days. (C) Gene expression levels of *SOX2* and *AKP* as measured by RT-qPCR in human DPCs of control, NSC-CM, and NSC extract groups (×200, scale bar: 20 μm). Data represent the mean ± SEM ($n = 5$). *$p < 0.05$, **$p < 0.01$, compared to control.

these signals for hair follicle formation and hair growth in mice. However, it was unclear how these signals initiate the coordinated regulation of HFSC activation and quiescence as well as activation of DPCs. To determine the mechanism by which the NSC extract stimulated and promoted hair growth, we first analyzed the expression of TGF-β family genes in mice treated with the NSC extract, because we found distinct expression of the *TGF-β2* gene in DPCs (Fig. 1E). Consistent with our in vitro data, the expression of TGF-β2, but neither TGF-β1 nor TGF-β3, remarkably increased in mice treated with the 20% NSC extract (Fig. 6A and B). In addition, we found that the gene expression of the TGF-β2 receptor was only significantly increased by the NSC extract (Fig. 6C).

Next, we examined the expression levels of downstream targets of TGF-β signaling in dorsal skin. Interestingly, the expression of *TMEFF1*, an important mediator of HFSC activation during the telogen-to-anagen transition[24], was robustly increased by the 20% NSC extract and not by the other treatments (Fig. 6D and F). We next examined the expression of BMP family genes in mice treated with the NSC extract. Interestingly, expression of *BMP4* and *BMP6* genes decreased dramatically, while no significant change was found in *BMP2* expression (Fig. 6E). In addition, decrease of p-Smad1/5/8 by only the 20% NSC extract indicated the suppression of BMP signaling pathway by the NSC extract treatment (Fig. 6F). These results of the 20% NSC extract in mice were consistent with our in vitro data showing the increase of TGF-β2 in human DPCs (Fig. 6G).

These results indicated that the NSC extract directly activated TGF-β signaling in epidermal and dermal layers

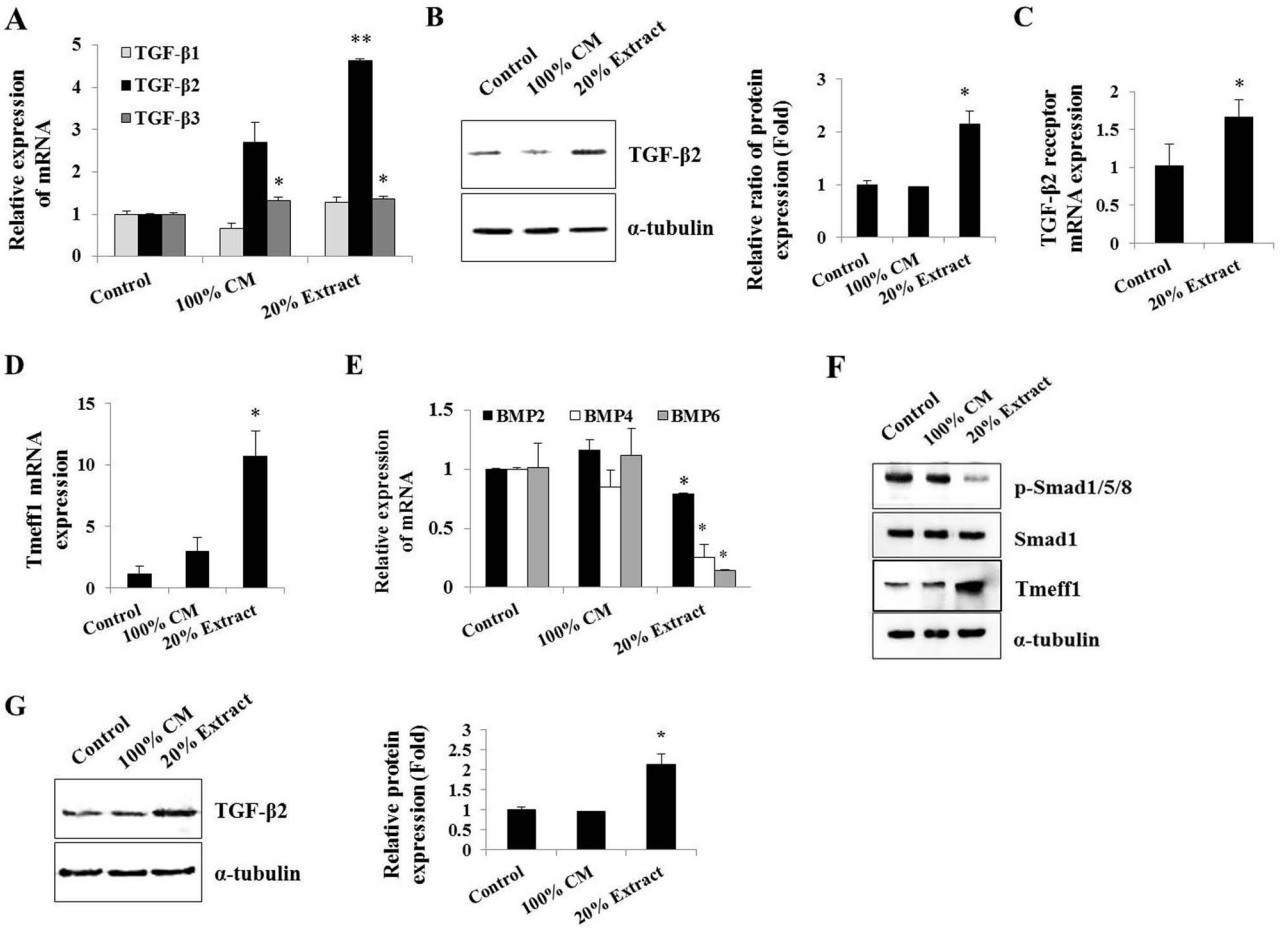

Figure 6. Counterbalancing of TGF-β2 and BMP signaling pathways in vitro and in vivo. (A) Gene expression levels of TGF-β1, TGF-β2, and TGF-β3 measured by RT-qPCR in skin from control, NSC-CM, and NSC extract groups at day 17. (B) Relative protein expression levels of TGF-β2 measured by Western blot. Protein levels were analyzed using ImageJ software. (C–E) Gene expression levels of TGF-β2 receptor (C), *TMEFF1* (D), and *BMP2, BMP4*, and *BMP6* (E) measured by RT-qPCR in skin from mice of control, NSC-CM, and NSC extract groups at day 17. (F) Analysis of BMP signaling effectors by Western blot of skin from mice of control, NSC-CM, and NSC extract groups. (G) Relative protein expression levels of TGF-β2 measured by Western blot in human DPCs of control, NSC-CM, and NSC extract groups at day 17. Protein levels were analyzed using ImageJ software (×200, scale bar: 20 μm). Data represent the mean ± SEM ($n = 3$ in vitro and $n = 5$ in vivo). *$p < 0.05$, **$p < 0.01$, compared to control.

of hair follicle progressing into the anagen phase during treatments of mice dorsal skin, while it suppressed BMP signaling.

Combined Growth Factors Promote Hair Growth in Mice

Next, we examined whether a combination of growth factors including TGF-β2 directly increases hair growth in mice. Because the human DPCs treated with 20% NSC extract showed increases in the expression of *IGF-1*, *KGF*, *TGF-β2*, and *HGF*, we first examined the proliferation of human DPCs and HaCaT keratinocytes treated with each of recombinant human IGF-1, KGF, TGF-β2, and HGF. TGF-β2 significantly increased the proliferation of DPCs and HaCaT keratinocytes (data not shown). Next, to examine whether combined growth factors, including TGF-β2, IGF-1, KGF, and HGF, enhanced hair growth in mice, we topically applied the combined growth factors to the dorsal skin of mice every day for 16 days. Surprisingly, the hair shaft length and growth rate were increased by the combined growth factors at 17 days after treatment (Fig. 7A and B), while hair weights did not increase when treated with the combined growth factors. These results indicated that the combined growth factors including TGF-β2 enhanced hair shaft elongation and the growth rate in the dorsal skin of mice, suggesting that optimal combinations of growth factors may be good candidates to promote hair growth. In future studies, the treatment conditions, including the concentration of each growth factor, the addition of other growth factors, and the treatment time and duration, should be experimentally evaluated and optimized for clinical application.

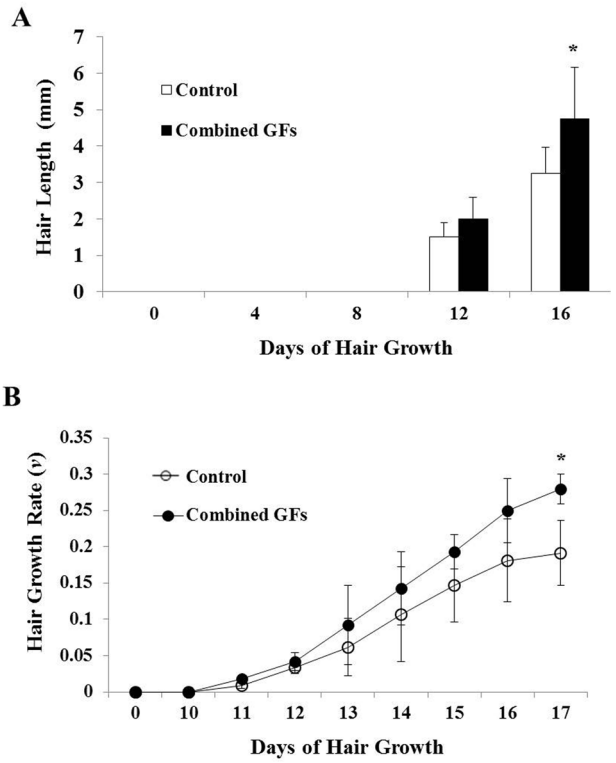

Figure 7. Enhancement of the hair shaft length and growth rate in mice treated with combined growth factors. (A) Analysis of hair length in mice treated with the combined growth factors, including recombinant human TGF-β2 (1 pM), for 17 days. A 0.5-mm mesoroller was used to increase skin absorption for topical treatment. (B) Analysis of the hair growth rate induced by the combined growth factors. The formula to calculate the hair growth rate was average rate (v) = hair length (mm)/growth time (day); v indicates velocity. Data represent the mean ± SEM (n = 5 in vivo). *p < 0.05, **p < 0.01, compared to control.

DISCUSSION

We found that an NSC extract stimulates and enhances hair growth during the telogen phase of mouse skin by regulating the niches within and outside of hair follicles. In addition to inducing the production of various growth factors, the NSC extract showed the enhancement of hair growth by activating TGF-β2 and repressing BMP4/6 signaling pathways. In terms of cellular targets, the NSC extract directly induced activation, proliferation, and differentiation of HFSCs, and it also induced activation and proliferation of DPCs. Moreover, treatment of mice with combined growth factors including IGF-1, HGF, KGF, and TGF-β2 significantly increased the hair shaft length. Furthermore, treating the shaven skin of mice with the NSC extract did not induce any DNA damage or cell death. These results indicate that the NSC extract activated various growth factors and core signaling pathways, converting the telogen phase to the anagen phase. The NSC extract represents an innovative therapeutic biomaterial for treating patients with hair loss.

DPCs regulate the growth and activity of various cells to regenerate hair follicles by secreting autocrine and paracrine factors[42]. Treating DPCs with keratinocyte- or adipose stem cell-derived CM or regulators of a specific signaling pathway was shown to induce the production of several specific growth factors and maintained the hair-inducing capacity of DPCs[33,43]. However, these studies did not present data directly addressing the effect on the niche of hair follicles. The hair-inducing effect of the NSC extract on DPCs seems most likely through induction of HFSC fate-determining core signaling pathways and growth factors, suggesting that our NSC extract may activate the niche in hair follicles through the regulation of specific signaling pathways, such as TGF-β and BMP, and growth factors.

Indeed, daily treatments of mice during the telogen phase with the NSC extract distinctly enhanced hair growth in terms of the hair shaft length, hair growth rate, hair weight, and number of hair follicles compared

with the minoxidil treatment (Fig. 2). Minoxidil is currently used for clinical treatment of androgenic alopecia as reported in the UK Drug Tariff on April 11, 2009[44]. Our results suggest that the NSC extract could be used as a potential therapeutic stimulator to restore hair loss in alopecia patients. Importantly, hair follicle cells of mice treated with the NSC extract or NSC-CM for long periods of time did not present any signs of cell death or DNA damage (Fig. 3).

In our mechanistic experiments, NSC extract treatments not only induced TGF-β2 and its target gene *TMEFF1* but also suppressed *BMP4* and *BMP6* genes in vitro and in vivo (Figs. 1 and 6). These results indicate that the NSC extract played dual roles in activating and differentiating HFSCs by activating TGF-β and in suppressing the senescence by repressing BMP signaling pathways in hair follicle niches. Interestingly, the hair growth enhancement by our NSC extract was achieved via a similar fashion to a mechanism reported by Oshimori and Fuchs[24].

As shown in Figures 4 and 5, induction of HFSC and DPC markers, including K15, CD34, *SOX2*, *CD133*, *AKP*, and *Versican*, and the differentiation marker *K10* by the HSC extract strongly supports that the NSC extract directly influenced activation and differentiation of HFSCs and DPCs within niches internally and externally of hair follicles in vivo and in vitro. Furthermore, the increase in the number of cells within the hair matrix indicated that HFSCs and hair germ cells may differentiate into mature cells, such as keratinocytes, for hair development. Therefore, our comprehensive and systematic experiments show that the NSC extract treatment during the telogen phase in mice induces activation of the TGF-β signaling pathway and its downstream targets, and subsequently or simultaneously suppresses the BMP signaling pathway in specific microenvironments consisting of a variety of cells including HFSCs, DPCs, keratinocytes, fibroblasts, and adipocytes under the skin.

Interestingly, topical treatment with the combined growth factors significantly increased the hair shaft length and growth rate, while no significant increase was found in the number of hair follicles and the hair weight as compared with the NSC extract treatment. These results indicate that the combined growth factors enhanced hair growth to a certain extent, suggesting that successful hair growth can be achieved by combined growth factors if the optimal growth factors are identified and optimized through further study.

In summary, we demonstrated that an NSC extract enhanced hair growth in mice through proliferation, activation, and differentiation of HFSCs, as well as proliferation and activation of DPCs and hair matrix cells. A key mechanism of action by the NSC extract that enhanced hair growth is the regulation of both TGF-β2 and BMP4/6 signaling pathways. In addition, we show that the NSC extract did not induce DNA damage or cell death that might occur in skin cells of mice treated for long periods of time. Interestingly, topical treatment with the combined growth factors enhanced the hair shaft length and growth rate by daily application to the dorsal skin of mice. These results suggest that the NSC extract might be a safe and effective therapeutic agent for hair loss patients by activating certain growth factor-linked core signaling pathways and subsequently activating niches within and outside of hair follicles.

ACKNOWLEDGMENTS: This work was supported by grants from the Ministry of Science, ICT and Future planning (2012M3A9C6050131) and the Ministry of Health & Welfare (HR14C0007) of the Government of the Republic of Korea. I.-H.P. was supported in part by National Institutes of Health (NIH) (GM0099130-01, GM111667-01), and CSCRF (12-SCB-YALE-11, 13-SCB-YALE-06). The authors declare no conflicts of interest.

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