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# Generation of trichogenic adipose-derived stem cells by expression of three factors

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ABSTRACT

*Background:* Previous studies demonstrated that adipose-derived stem cells (ASCs) can promote hair growth, but unmet needs exist for enhancing ASC hair inductivity.
*Objective:* Therefore, we introduced three trichogenic factors platelet-derived growth factor-A, SOX2, and β-catenin to ASCs (tfASCs) and evaluated whether tfASCs have similar characteristics as dermal papilla (DP) cells.
*Method:* Global gene expression was examined using NGS analysis. Telogen-to-anagen induction, vibrissae hair follicle organ culture and patch assay were used.
*Results:* tfASC cell size is smaller than that of ASCs, and they exhibit short doubling time. tfASCs also resist aging and can be expanded until passage 12. Cell proportion in S and $G_2$/M increases in tfASCs, and tfASCs express high mRNA levels of cell cycle related genes. The mRNA expression of DP markers was notably higher in tfASCs. Moreover, NGS analysis revealed that the global gene expression of tfASCs is similar to that of DP cells. The injection of tfASCs accelerated the telogen-to-anagen transition and conditioned medium of tfASCs increased the anagen phase of vibrissal hair follicles. Finally, we found that the injection of 3D-cultured tfASCs at p 9 generated new hair follicles in nude mice.
*Conclusion:* Collectively, these results indicate that 1) tfASCs have similar characteristics as DP cells, 2) tfASCs have enhanced hair-regenerative potential compared with ASCs, and 3) tfASCs even at late passage can make new hair follicles in a hair reconstitution assay. Because DP cells are difficult to isolate/expand and ASCs have low hair inductivity, tfASCs and tfASC-CM are clinically good candidates for hair regeneration.


## 1. Introduction

Previous studies demonstrated the hair-growth-promoting effect of adipose-derived stem cells (ASCs) in an animal model and in dermal papilla (DP) cells by secreting hair-regenerative proteins [1,2]. Therefore, the conditioned medium of ASCs (ASC-CM) was clinically used for treating female- and male-pattern alopecia, which increased hair density in patients with hair loss [3,4]. To increase the hair-regenerative potential of ASCs, we developed preconditioning methods such as the use of vitamin C and platelet-derived growth factor-D (PDGF-D) for enhancing the telogen-to-anagen transition in animal experiments [5–7]. Preconditioning of ASCs using LL-37 also enhanced the hair-regenerative potential of ASCs in an animal model [8]. However, Seo et al. reported that three-dimensional (3D)-cultured human ASCs were inferior at inducing hair follicles compared with 3D-cultured human DP cells [9]. In addition, there is minimal evidence regarding a method of differentiating ASCs into DP cells. As ASCs can be obtained in large quantities compared with DP cells, human ASCs (hASCs) may have an advantage over hDP cells in cell therapy for patients with hair loss.

As ASCs have poor ability to induce hair follicles compared with DP cells, we established a strategy to increase the trichogenicity by overexpressing three genes (PDGF-A, Sox2, β-catenin) in ASCs, which reportedly play pivotal roles in hair biology. PDGF-A is a well-known hair growth inducer and its receptor is highly expressed in follicular sheath [10–13]. For example, PDGF isoforms

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induce and maintain the anagen phase of murine hair follicles [10]. Adipocyte lineage cells (i.e., ASCs) contribute to the skin stem cell niche to drive hair cycling by the secretion of PDGF-A [11]. It was also found that PDGF-A expression is reduced in aged ASCs, and is required for ASC proliferation and maintenance in the dermis [12]. In contrast, SOX2 is expressed in the DP region, determines the heterogeneity of DP cells, and SOX2-positive cells specify particular hair follicle types [14]. Skin-derived precursor cells are SOX2-positive cells of hair follicles; they self-renew, maintain their multipotency, and can reconstitute hair follicles [15]. Clavel et al. reported that Sox2 controls hair growth by fine-tuning BMP signaling in differentiating hair shaft progenitors [16]. In addition, β-catenin is a key trichogenic factor, and its activity in the dermal papilla regulates hair morphogenesis and regeneration [17]. Wnt signaling through the β-catenin pathway has been reported to be sufficient to maintain the anagen phase of DP cells [18]. Wnt signaling in DP cells is regulated by androgen, which plays a pivotal role in androgen's action in patients with hair loss [19]. Therefore, numerous trials have been performed to increase the β-catenin activity in DP cells to promote hair growth [20–22].

ASCs act as an epidermal stem cell niche, and PDGF-A secreted from ASCs drives hair cycling. In addition, SOX2 and β-catenin play key roles in DP growth and maintenance. Therefore, we generated trichogenic ASCs by overexpressing three factors in ASCs (tfASCs), and examined whether tfASCs have similar characteristics to DP cells. In addition, we investigated whether tfASCs have superior hair-regenerative potential to ASCs and whether tfASCs at late passage can make new hair in a hair reconstitution assay.

## 2. Materials and methods

### 2.1. Plasmid construction

To generate lentiviral plasmids, pLVX-EF1α-IRES-Puro (hereafter pLVX-EIP) was purchased from Clontech. Puromycin resistance gene was replaced by blasticidin S resistance gene and by neomycin resistance gene to generate pLVX-EIBla and pLVX-EIN, respectively. Coding regions of human SOX2, β-catenin, and PDGF-A were PCR-amplified and inserted at EcoRI/XbaI site of pLVX-EIP, pLVX-EIBla, and pLVX-EIN, respectively. The PCR products were digested with EcoRI and XbaI for subcloning except for β-catenin which was cut with MfeI and XbaI. pOTB7-SOX2 and pCMV-SPORT6-β-catenin were provided from Korea Human Gene Bank, Medical Genomics Research center, KRIBB, Korea and Open Biosystem, respectively.

### 2.2. Lentivirus production and transduction

One day before transfection, HEK293 T cells were plated on 6-well plates at a density of $5 \times 10^5$ cells/well without antibiotics. Transfections were performed with total 3 μg of plasmids that consist of lentiviral plasmid (pLVX-EIP-SOX2, pLVX-EIBla-β-catenin, or pLVX-EIN-PDGF-A), psPAX2, and pMD2.G at a ratio of 4:3:1 by using Lipofectamine2000 (Invitrogen) in accordance with the manufacturer's instruction for 12 h. Then cells were refreshed with growth medium and incubated for 36–48 h before collection of lentivirus-containing media. The media were cleared by centrifugation at 3000 rpm for 3 min and were stored at −80 °C before use. Viral supernatants used to infect with polybrene into the pre-seeded $2 \times 10^4$ ASCs at 60 mm plate with approximately 50% confluence for 3 sequential days. Puromycin for SOX2, blasticidin S for β-catenin and G418 for PDGF-A were added to select drug-resistant pools at 48 h post-infection. For tfASCs, alpha-MEM medium was changed to the partial DP culture medium (alpha-MEM; follicle DP cell medium = 1;1) after virus transfection and colony formation. The cells were used for further biochemical assays as specified in each experiment.

### 2.3. Cell culture

Human ASCs were isolated via liposuction of subcutaneous fat as described previously [23,24], after informed consent was obtained (Yonsei University college of medicine, 4-2018-0141). ASCs were cultured in alpha-MEM medium (Hyclone, Logan, UT) with 10% fetal bovine serum (Gibco, CA, USA) and 1% penicillin/streptomycin (Gibco). We purchased human DP cells from PromoCell (#C-12071). Human DP cells were cultured in follicle DP cell medium with supplementMix (PromoCell, Heidelberg, Germany) and 0.1% anti-antibiotics (Gibco). DP cells for all experiment were used at passages 2–3, but used at passages 5–6 for analysis of alkaline phosphatase activity. ASCs and tfASCs were used at passages 5–8 and 7–10, respectively. For tfASCs were cultured in the partial DP culture medium (alpha-MEM; follicle DP cell medium = 1;1). ASCs, DP cells and tfASCs were maintained at 37 °C in a humidified 5% $CO_2$ incubator.

### 2.4. Measuring the population doublings

For measuring the population doublings of ASCs and tfASCs, cells were seeded in 12-well plates at $1 \times 10^4$ cells/well, incubated for 4 days. Cells were trypsinized, stained with trypan blue (Sigma-Aldrich, MO, USA), and counted using a hemocytometer. This cycle was repeated up to seven times (until 13 passages). Cell numbers seeded at the start of each passage and harvested at the end were used to calculate the number of population doublings that had occurred, using the following formula, where PD = population doublings and ln = natural log [25].

PD = ln (harvested cells / seeded cells) / ln 2

### 2.5. RNA extraction, quantitative RT-PCR, QPCR array and RT-PCR

Total RNA was extracted from ASCs, tfASCS and DP cells using Trizol reagent (Invitrogen, NY, USA) and subjected to cDNA synthesis using oligodT and the HelixCript™ Thermo Reverse Transcription System (NANOHELIX, WI, USA) according to the manufacturer's instructions. BrightGreen qPCR master mix-ROX (abm, NY, USA) was used for QPCR reactions. For QPCR array, total RNA was extracted from ASCs or tfASCs, subjected to cDNA synthesis as same manner mentioned above. QPCR reaction for growth factors was conducted using RT$^2$ First Strand cDNA Synthesis Kit (QUAZEN, MD, USA). For RT-PCR of human *ALU* and mouse *c-MOS* genes, genomic DNA was isolated from cells and skin tissues using genomic DNA isolation kit (Bioneer, Daejeon, Korea). The amplification conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and then final extension at 72 °C for 10 min. All used primers are described in Supplementary table 1.

### 2.6. X-gal staining for cellular senescence

For cellular senescence, β-galactosidase staining kit was used (Sigma-Aldrich) according to the manufacturer's instruction. In brief, cells were fixed with fixation buffer (20% formaldehyde and 2% glutaraldehyde in PBS) for 10 min and washed with PBS. The fixed cell were incubated at 37 °C overnight after the addition of freshly prepared cell staining working solution containing potassium ferricyanide, potassium ferrocyanide and X-gal. The next day, the cell-staining solution was discarded, and the cells

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