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Impairment of Hair-Inducing Capacity of Three-Dimensionally Cultured Human Dermal Papilla Cells by the Ablation of STAT5

Chang Hoon Seo, Mi Hee Kwack[1], Moon Kyu Kim[1], Jung Chul Kim[1], Young Kwan Sung[1]

New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation, [1]Department of Immunology, School of Medicine, Kyungpook National University, Daegu, Korea

Dear Editor:

The dermal papilla (DP) of hair follicle is essential for hair morphogenesis, growth, and regeneration. Therefore, DP cells are considered to be an optimal cell source for genesis of new hair follicles[1,2]. However, there remains an experimental challenge resulting from DP cells gradually losing their hair-inductive capacity when cultured two-dimensionally (2D)[3]. Three-dimensional (3D) spheroid culturing was successfully employed to overcome the loss of hair inductivity of 2D-cultured human DP cells[4-6].

STAT5 is a signal transducer and activator of transcription (STAT). Recently, Legrand et al.[7] reported that the actived form of STAT5 (phospho-STAT5; P-STAT5) is restricted to the DP cells and activation of STAT5 in the DP plays an important role in hair growth phase induction. They showed that hair-inductive capacity of mouse DP-derived multi-potent stem cells, skin-derived precursors, is significantly enhanced by adenoviral overexpression of STAT5A or STAT5B[7]. In line with this, STAT5 deletion impaired formation of *de novo* hair follicles in skin-derived precursors[7]. In contrast, Harel et al.[8] reported that the hair inductivity of human DP spheres is enhanced by the treatment with tofacitinib, a STAT signaling inhibitor. These controversial reports prompted us to investigate role of STAT5 in the hair inductivity of human DP cells. We performed STAT5 knock-down in human DP spheres and the spheres were implanted into the back of the nude mice together with mouse epidermal cells.

The Medical Ethical Committee of the Kyungpook National University Hospital (Daegu, Korea) approved all of the described studies (IRB no. KNUH 2013-02-001-007). Student's t-test was used to analyze differences between groups us-
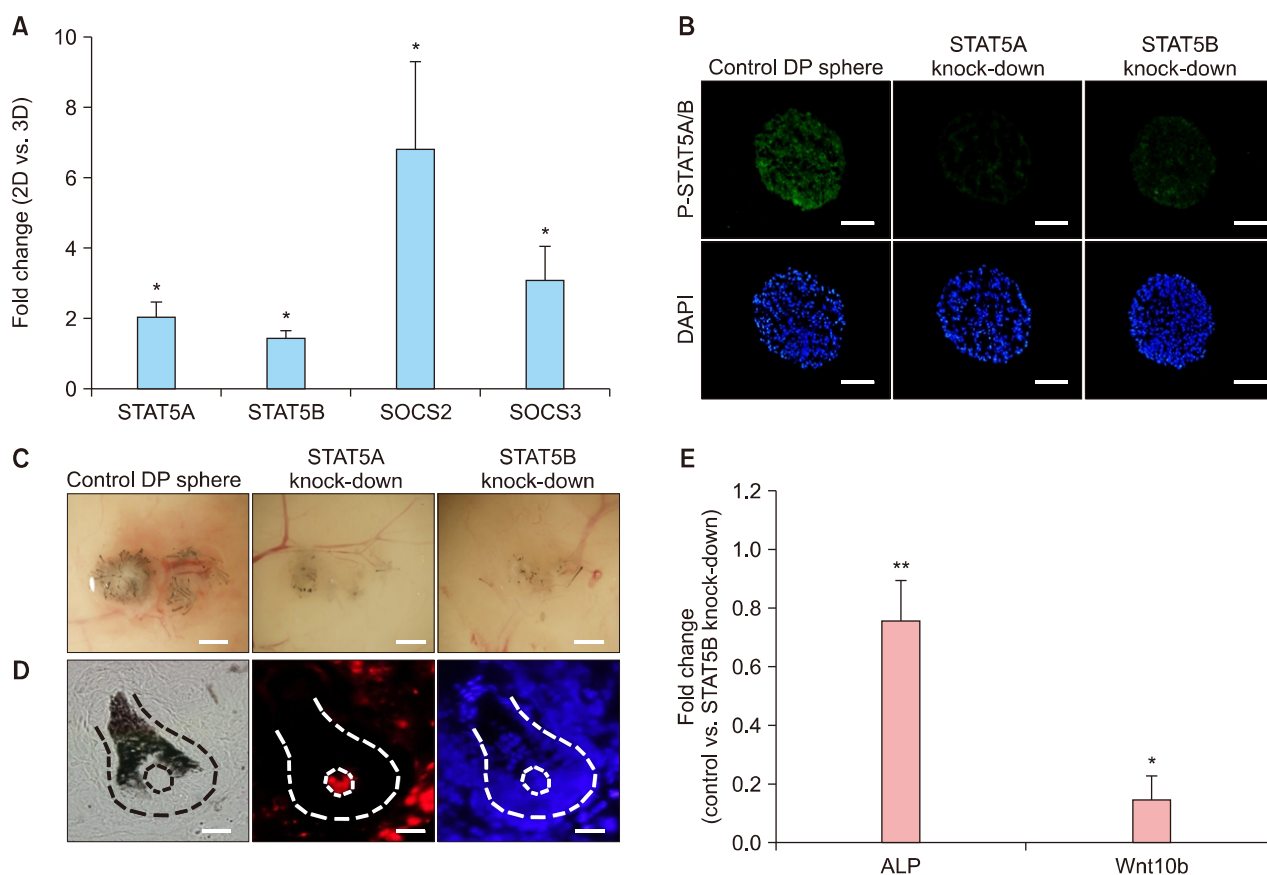
Fig. 1. Hair-inducing capacity of three-dimensionally (3D) cultured human dermal papilla cells is impaired by the knock-down of STAT5. (A) The expression of STAT5A, STAT5B, SOCS2, and SOCS3 in 3D dermal papilla (DP) spheres was quantitatively compared with that in two-dimensionally (2D) cultured DP cells by real-time polymerase chain reaction (PCR). Data are means±standard deviation (SD) of three independent experiments using DP cells of three different patients (*$p<0.01$). (B) Immunofluorescence staining of phospho-STAT5 (P-STAT5) (green; upper panels) and the corresponding 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (blue; bottom panels) in DP spheres are shown. Bar=0.1 mm. (C) Hair induction assay with three kinds of DP spheres (10^6 cells) in combination with freshly isolated mouse epidermal cells (10^6 cells). Bar=1 mm. (D) Phase-contrast and fluorescence imaging of DiI and DAPI in hair follicles induced from the DP spheres. Bar=0.1 mm. (E) The expression of ALP and Wnt10b in STAT5B knock-down DP spheres was quantitatively compared with that in control DP spheres using real-time PCR. Data are means±SD of three independent experiments using DP cells of three different patients (*$p<0.01$, **$p<0.05$). The sequences of primers used are as follows: STAT5A, 5'-GTAAGGCTGTGTACACTGACAC-3' and 5'-CATAGGGTTCACAGAGAGTCTG-3'; STAT5B, 5'-GACTCAGAAATTGGCGGCATC-3' and 5'-CGGTGTCAAGGACTGAGTCAG-3'; SOCS2, 5'-CCCGCAGGGACTCGTTTT-3' and 5'-TTCGCGTCCTTCCTTGAAGT-3'; SOCS3, 5'-CAGCTCCAAGAGCGAGTACCA-3' and 5'-AGAAGCCGCTCTCCTGCAG-3'; ALP, 5'-CTGGAACCGCACGGAACT-3' and 5'-CTGCTTG GCTTTTCCTTCATG-3'; Wnt10b, 5'-CTCTGGGATGTGTAGCCTTC-3' and 5'-GGCTCTGGAGTTGAGAAGTG-3'; and GAPDH, 5'-TGGAAATCCCATCACCATCTTC-3' and 5'-CGCCCCACTTGATTTTGG-3'.

ing Microsoft Excel. We considered *p*-values <0.05 to be statistically significant. We first verified whether STAT5 expression and that of its known downstream transcriptional targets, SOCS2 and SOCS3, is higher in the 3D human DP spheres, compared with the expression of these transcripts in the 2D-cultured human DP cells. Expression of STAT5A, STAT5B, SOCS2, and SOCS3 was significantly upregulated in the DP spheres as examined by qPCR analysis (Fig. 1A). Next, to address the functional role of STAT5 in the hair inductivity of DP spheres, 2D-cultured DP cells were transfected with STAT5A siRNA, or STAT5B

siRNA for 48 hours, before being harvested and reseeded to form STAT5 knock-down spheres. The spheroid formation was induced for 48 hours in each well of 96-well HydroCell plates (10^4 cells per well) before implantation. The sphere blocks were cut to 7-μm thick sections on glass slides. The slides were incubated with a rabbit monoclonal antibody against phosphorylated STAT5A/B (1:100 dilution; Santa Cruz, CA, USA), washed with phosphate-buffered saline, and incubated with Alexa Fluor 488-labeled donkey anti-rabbit secondary antibody (1:100 dilution; Molecular Probes, Eugene, OR, USA). The slides

Table 1. Summary of patch hair reconstitution assay results

Kinds of DP spheres	Number of DP cells	Number of mNE cells	N	Number of induced hair follicles
Control siRNA-transfected	10^6	10^6	8/8	79 ± 14
STAT5A siRNA-transfected	10^6	10^6	6/6	$38 \pm 16*$
STAT5B siRNA-transfected	10^6	10^6	9/9	$26 \pm 21*$

DP: dermal papilla, N: instances of hair follicle formation/injection time, mNE: freshly isolated neonatal mouse epidermal cells. *$p < 0.05$.

were then incubated with 4',6-diamidino-2-phenylindole for nuclear staining. The P-STAT5 expression was markedly reduced in STAT5 (A or B) knock-down DP spheres, when compared to that in control DP spheres as confirmed by immunochemical analysis (Fig. 1B).

An established patch hair reconstitution assay was employed to assess the hair-inductive capacity of three kinds of DP spheres[9]. Implantation was performed as described previously[4,6]. Briefly, freshly isolated neonatal mouse epidermal cells were combined with control or STAT5A, or STAT5B siRNA-transfected DP spheres (100 spheres) and co-transplanted subcutaneously into the skin on the back of the nude mice. To verify newly induced hair formation, skin samples were excised from the mice and examined after three weeks. Knock-down of STAT5 significantly decreased the hair inductivity of DP spheres (Fig. 1C). Number of induced hair follicles was significantly reduced in STAT5A siRNA-transfected or STAT5B siRNA-transfected DP spheres compared to control siRNA-transfected DP spheres ($p < 0.05$). Control siRNA-transfected DP spheres induced 79 ± 14 hair follicles, while STAT5A and STAT5B knock-down DP spheres induced 38 ± 16 and 26 ± 21 hair follicles, respectively. The results of patch assays are summarized in Table 1. To confirm that the DP cells of the newly induced hair follicles were originated from human, cells were labelled with the fluorescent dye. As shown in Fig. 1D, DP in the induced follicles contained dye-labelled human cells.

We further examined whether knock-down of STAT5 regulates the expression of DP signature genes, such as ALP and Wnt10b, using qPCR analysis. We found that mRNA levels of ALP and Wnt10b are significantly decreased in STAT5 siRNA-transfected DP spheres, compared to those in control DP spheres (Fig. 1E). Considering that the hair-inductive capacity of dermal mesenchymal components is closely correlated with the expression of ALP[10], significantly reduced hair inductivity in STAT5 knock-down DP

spheres is validated by these observations. How STAT5 regulates mRNA levels of ALP and Wnt10b remains to further investigated.

In summary, our study demonstrates that expression of STAT5 and its target genes is elevated when 2D-cultured human DP cells are induced to form *in vivo*-like DP spheroids. In addition, our hair reconstitution data demonstrate that enhanced hair-inductive capacity in DP spheres is at least partly due to enhanced STAT5 levels. Our data is in line with the findings of Legrand et al.[7] who reported impaired hair inductivity in STAT5-deleted skin-derived precursors.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

ORCID

Chang Hoon Seo, https://orcid.org/0000-0003-2786-8304
Mi Hee Kwack, https://orcid.org/0000-0002-7381-6532
Moon Kyu Kim, https://orcid.org/0000-0003-0081-4234
Jung Chul Kim, https://orcid.org/0000-0002-0720-0991
Young Kwan Sung, https://orcid.org/0000-0002-5122-4616

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Long-Term Prognosis of Alopecia Areata in Children and Adolescents

Yong Hyun Jang, Dong Hyuk Eun, Do Won Kim

Department of Dermatology, School of Medicine, Kyungpook National University, Daegu, Korea

Dear Editor:

The prognosis of alopecia areata (AA) during childhood may be worse in comparison to cases involving adult patients[1,2]. However, there is no concrete evidence for this assumption because it is based on a small number of studies on Asian subjects with insufficient long-term follow-up data[3,4]. Disease progression in children with AA is also more difficult to predict in comparison to adults. It is possible that prognosis is worse because disease progression in children with mild AA is not necessarily better[2]. When AA progresses to alopecia totalis (AT) or alopecia universalis (AU), long-term prognosis with respect to full recovery is generally reported at a frequency of less than 10%[5]. However, the data for this result is outdated and was reported in the 1950s, thereby making evidence unreliable.

Recently, we evaluated the long-term prognosis of patients with AT or AU and found that it is better than previously thought[6]. Long-term outcome evaluation of patients with AA is important. Because AA is associated with frequent recurrence, it is difficult to determine whether complete recovery has occurred when AA progresses to more severe conditions. Our previous study suggests that at least 5 years of follow-up observation is required to predict the long-term prognosis of patients with AA[7].

In this study, we investigated the long-term prognosis (>10 years) of children and adolescents (≤19 years old) with AA. Among 140 patients with pediatric AA from our outpatient database, 75 who were available for follow-up observations for long-term prognosis via their medical records and phone surveys were included. This study was