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Hair growth stimulated by conditioned medium of adipose-derived stem cells is enhanced by hypoxia: evidence of increased growth factor secretion

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Abstract

Adipose-derived stem cells (ADSCs) and their secretomes mediate diverse skin-regeneration effects, such as wound-healing and antioxidant protection, that are enhanced by hypoxia. We investigated the hair-growth-promoting effect of conditioned medium (CM) of ADSCs to determine if ADSCs and their secretomes regenerate hair and if hypoxia enhances hair regeneration. If so, we wanted to identify the factors responsible for hypoxia-enhanced hair-regeneration. We found that ADSC-CM administrated subcutaneously induced the anagen phase and increased hair regeneration in C_3H/NeH mice. In addition, ADSC-CM increased the proliferation of human follicle dermal papilla cells (HFDPCs) and human epithelial keratinocytes (HEKs), which are derived from two major cell types present in hair follicles. We investigated the effect of hypoxia on ADSC function using the same animal model in which hypoxia increased hair regrowth. Forty-one growth factors in ADSC-CM from cells cultured under hypoxic or normoxic conditions were analyzed. The secretion of insulin-like growth factor binding protein (IGFBP)-1, IGFBP-2, macrophage colony-stimulating factor (M-CSF), M-CSF receptor, platelet-derived growth factor receptor-β, and vascular endothelial growth factor was significantly increased by hypoxia, while the secretion of epithelial growth factor production was decreased. It is reasonable to conclude that ADSCs promote hair growth via a paracrine mechanism that is enhanced by hypoxia.

Adipose-derived stem cells (ADSCs) are mesenchymal stem cells within the stromal-vascular fraction of subcutaneous adipose tissue that self-renew and display multi-lineage developmental plasticity. ADSCs and bone marrow-derived stem cells share similar surface markers, gene profiles, and functions (8, 11, 31, 32). An essential function of ADSCs is the production and secretion of growth factors that activate neighboring cells. These growth factors include vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) (16, 18, 24). ADSCs and their secretomes mediate diverse skin-regenerative effects, such as wound-healing, antioxidant protection, anti-wrinkling and whitening effects (12–15, 21).

Oxygen deficiency, *i.e.*, hypoxia, may impair cell function. However, cellular responses to hypoxic stress are highly dependent on cell type, maturity and environment. ADSCs are thought to reside in

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hypoxic regions of the human body. When ADSCs are cultured under hypoxic conditions *in vitro,* their proliferative and self-renewal capacities are significantly improved (7, 19, 23, 25). Hypoxia amplifies the paracrine effects of ADSCs by enhancing the secretion of certain growth factors (17). In an animal model of hindlimb ischemia, ADSCs improved blood perfusion, and the hypoxia-induced up-regulation of growth factors enhanced ADSC function (17). In addition, the condition medium (CM) from ADSCs cultured in hypoxic conditions significantly reduced the wound area in hairless mice as compared to the CM from ADSCs grown in normoxic conditions (4), and CM from hypoxic cultures of ADSCs significantly increased the migration of dermal fibroblasts *in vitro*.

Although biologically plausible, hypoxia-induced enhancement of hair regeneration has not been investigated. The growth activity of hair follicles, which are composed of an outer root sheath, an inner root sheath and a hair shaft, is controlled by a highly regenerative neuroectoderm-mesodermal interaction (10). Hair follicles are the only component of the human body that undergo a cyclic transformation from a rapid growth phase (anagen) to apoptosis-driven regression (catagen) via an interspersed period of relative quiescence (telogen). These cyclic changes involve rapid remodeling of both epithelial and dermal components and are mediated by several molecules that control epithelial morphogenesis and growth. Growth factors stimulate hair growth in both *ex vivo* and *in vivo* animal models. In a VEGF-transgenic mouse model, VEGF controlled hair growth and follicle size by the modulation of angiogenesis (30). PDGF isoforms induce and maintain the anagen phase in murine hair follicles (27). HGF and IGF also up-regulate hair follicle growth in various systems (9, 20, 26, 29). Considering that ADSCs secrete growth factors that activate neighboring cells, we hypothesized that factors secreted by ADSCs stimulate hair follicles and promote hair growth and that both processes are enhanced by hypoxia.

MATERIALS AND METHODS

Cell culture. ADSCs were isolated from human subcutaneous adipose tissue acquired from healthy women who underwent elective liposuction. Informed consent was obtained from each subject, and all procedures were approved by the institutional review board. The adipose tissue samples were digested with 0.075% type II collagenase (Sigma-Aldrich, St. Louis, MO) under gentle agitation for 45 min at 37°C; then, they were centrifuged at $300 \times g$ for 10 min to obtain the stromal cell fraction. The pellet was resuspended in phosphate buffered saline (PBS) and filtered through 70-μm nylon mesh. The cell suspension was layered onto histopaque-1077 (Sigma-Aldrich) and centrifuged at $840 \times g$ for 10 min. The supernatant was discarded, and the cell band floating above the histopaque was collected. The retrieved cell fraction was cultured at 37°C and 5% CO_2 in control culture medium (Dulbecco's modified Eagle media (DMEM) that contained 10% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 μg/mL of streptomycin). Cells were cultured in the control media until they became confluent (3 to 5 days), and this was considered passage 1. ADSCs in passages 4 and 5 were used in the experiments. ADSCs were characterized as previously reported (12, 14).

Human follicle dermal papilla cells (HFDPCs) were purchased from PromoCell GmbH (Heidelberg, Germany) and maintained in Follicle Dermal Papilla Cell Growth Medium (PromoCell GmbH). Human epidermal keratinocytes (HEKs) were purchased from Cascade Biologics (Portland, OR) and cultured in EpiLife Medium (Cascade Biologics) supplemented with Human Keratinocyte Growth Supplement (Cascade Biologics). All cells were cultured at 37°C and 5% CO_2.

Preparation and concentration of ADSC-CM. ADSCs were cultured in control medium containing 10% FBS in normoxic conditions. After 4 passages, ADSCs (4×10^5 cells) were seeded in 100-mm culture dishes. When the ADSCs became confluent, the medium was replaced with DMEM/F12 serum-free medium (Invitrogen-Gibco-BRL, Grand Island, NY), and the cells were cultured in hypoxic conditions (2% O_2, 5% CO_2, and balanced N_2) for 72 h. Control cultures were incubated in 95% room air and 5% CO_2. Subsequently, the conditioned medium from ADSCs grown under normoxic (norCM) or hypoxic conditions (hypoCM) was collected, centrifuged at $300 \times g$ for 5 min, and filtered through a 0.22-μm syringe filter. The filtrate was centrifuged in 3-kDa molecular-weight-cut-off Centricon tubes (Millipore Corp, Bedford, MA) and concentrated.

Proliferation of HFDPCs and HEKs. HFDPCs were plated in supplement-free media at a density of 1,000 cells per well in a 96-well plate or 4,000 cells per well in a 48-well plate. HEKs were plated in supplement-free media at a density of 3,000 cells

per well in a 96-well plate or 100,000 cells per cell in a 6-well plate coated with rat type I collagen. After attachment, cells were incubated in medium containing 50 or 100% hypoCM or norCM for 24, 48, or 72 h. The cell proliferation was measured using a Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Kumamoto, Japan), which is nonradioactive and uses a sensitive colorimetric method to determine the number of viable cells. The absorbance at 450 nm was measured with a microplate reader (TECAN, Grödig, Austria). Optical density values of each well to relative cell numbers were calculated using authentic standard curves. All experiments were performed in triplicate.

Animal experiment. Female C_3H/HeN nude mice (Orient Bio, Sungnam, Korea) were used in the animal experiment. The experimental protocol was reviewed by the Animal Care and Use Committee of Inha University School of Medicine, according to the NIH Principles of Laboratory Animal Care (NIH publication number 85-23, revised 1985). Induction of hair growth was studied in 7-week-old C_3H/HeN mice (n = 21); hair follicles were synchronously matched in the telogen stage. Mice were shaved with clippers, after which they appeared pink due to bare skin. Mice received 3 subcutaneous injections of 100 μL of hypoCM or norCM into the dorsal skin at 3-day intervals, and darkening of the skin was monitored for 12 weeks.

Membrane-based human growth factor antibody array. Human Growth Factor Antibody Array 1® was purchased from Ray Biotech (Norcross, GA). The microarray assay was performed according to the manufacturer's instructions. Briefly, membranes were placed in an 8-well tissue culture tray and incubated with 2 mL of 1 × blocking buffer at room temperature for 30 min. The membrane was then incubated with 1 mL of concentrated hypoCM or norCM (100 μg) at 4°C overnight. After decanting the samples, 1 mL of biotin-conjugated antibodies (1 : 250 dilution) was incubated with the membrane for 2 h at room temperature. After incubation with 2 mL of a horseradish peroxidase-conjugated streptavidin solution at room temperature for 1 h, the signal was detected using a chemiluminescent substrate system (Immunobilon Western reagent; Millipore) and quantified using a densitometer and GeneTools software (Syngene, Cambridge, UK). The background intensity was subtracted for analysis. The data were normalized to the positive control values provided by the manufacturer.

Statistical analysis. The Student's *t*-test was used for the data analysis. *P* values less than 0.05 or 0.01 were considered to be statistically significant.

RESULTS

Hair growth stimulation effect of ADSC-CM

Because ADSC-CM (hypoCM) contains growth factors involved in hair growth, we investigated the regenerative effects of ADSC-CM using a telogen-matched animal model. Eight weeks after the subcutaneous injection of ADSC-CM into the dorsal skin, dark spots and hair regeneration were observed in the ADSC-CM-treated mice (Fig. 1B). After 12 weeks of ADSC-CM treatment, the hair was nearly regrown (n = 3, data not shown). However, the control group did not develop dark spots or hair sheaths (n = 3) (Fig. 1A). This result indicates that ADSC-CM induces progression from the telogen phase to the anagen phase.

To further study ADSC-CM-induced hair growth, we used a CCK-8 kit to measure the proliferation of HFDPCs and HEKs, which are cell lines derived from two major cell types present in hair follicles. As shown in Figs. 1C and 1D, ADSC-CM promoted the proliferation of HFDPCs and HEKs in a dose-dependent manner. When HFDPCs were treated with either 50 or 100% ADSC-CM, their proliferation increased by 20 and 37% at 24 h, and by 12 and 27% at 48 h, respectively ($P < 0.01$). Treatment of HEKs with either 50 or 100% ADSC-CM increased their proliferation by 25 and 79% on the second day, and by 58 and 146% on the third day, respectively, compared to supplement-free medium.

Hypoxia-enhanced hair growth promotion of ADSCs

Since hypoxic conditions amplify the secretion of growth factors by ADSCs, we investigated the effects of hypoxia on the promotion of hair growth by ADSC-CM. ADSC-CM was obtained from ADSCs cultured in hypoxic (hypCM) or normoxic conditions (norCM), and the hair regeneration induced by the two ADSC-CMs was compared. First, we examined anagen induction in telogen-matched mice and found that hypoCM treatment induced the anagen phase and the development of dark spots more rapidly than norCM injection (Fig. 2A). Four of the five hypoCM-treated mice developed dark spots; dark spots appeared in one mouse at 7 weeks, in two mice at 8 weeks, and in one mouse at 11 weeks (Fig. 2A). However, only two of the five norCM mice developed dark spots; one dark spot appeared 9 weeks after norCM injection, and one appeared at

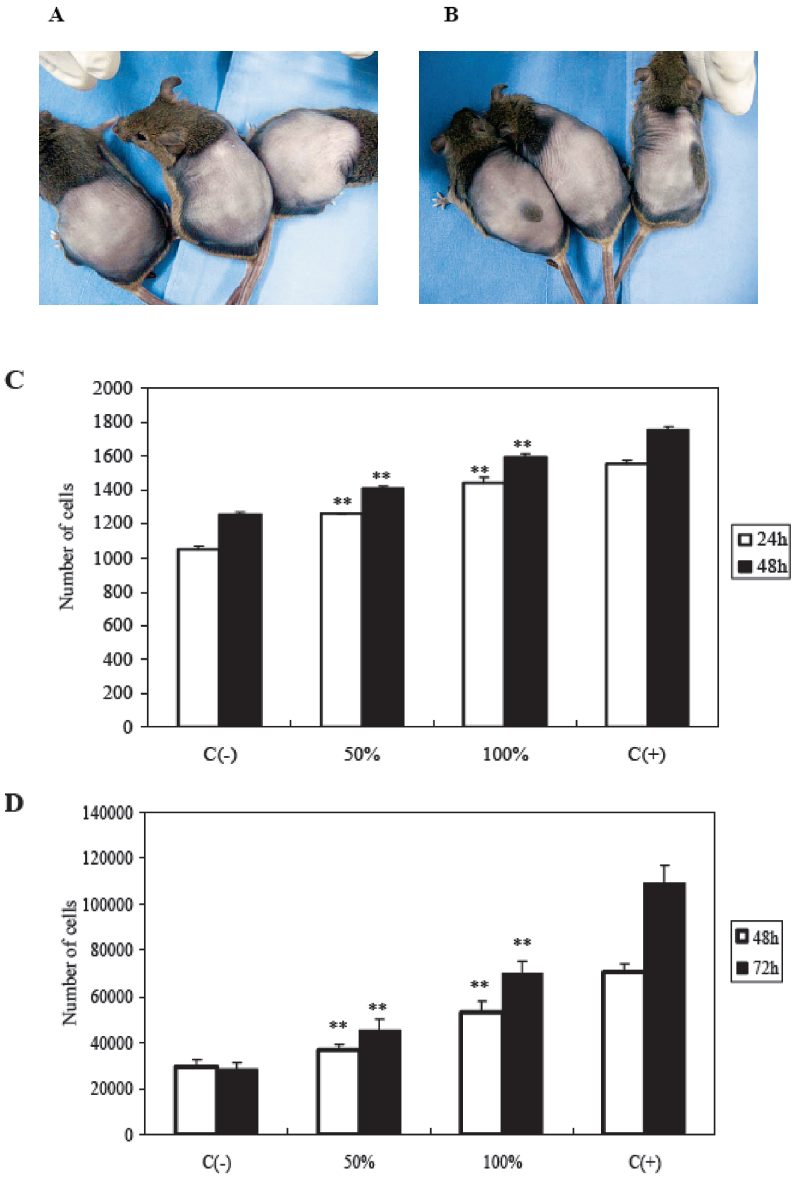

Fig. 1 Hair-growth-promoting effect of ADSC-CM. Telogen-matched, 7-week-old C_3H/NeH mice were shaved and subcutaneously injected three times with F12/DMEM medium (control, A, n = 3) or ADSC-CM (B, n = 3), and photographs were taken 8 weeks after the injections. The proliferation of human follicle dermal papilla cells (HFDPCs) (C) and human epithelial keratinocytes (HEKs) (D) was increased by ADSC-CM treatment in a dose-dependent manner. C(–) indicates that the cells were cultured in serum-free medium as negative control and C(+) indicates that the cells were cultured in medium that contained 10% fetal bovine serum as a positive control. $^{**}P < 0.01$

11 weeks. Next, we studied HFDPCs and HEKs treated with either hypoCM or norCM. Both types of conditioned media increased the proliferation of HFDPCs and HEKs. There was no significant difference in the proliferation of HFDPCs treated with hypoCM or norCM (Fig. 2B). By contrast, HEK proliferation was significantly increased by hypoCM as compared to norCM ($P < 0.01$, Fig. 2C).

Increased secretion of growth factors in hypoCM

In a preliminary study, we used the Bradford method to measure the total protein concentration in hypoCM and norCM and found that there was no difference in the total amount of secreted proteins (1.02 ± 0.04 vs 1.01 ± 0.01 μg/mL, respectively). However, it is reasonable to hypothesize that the secretion of potent growth factors might be altered, explaining the difference in hair growth between

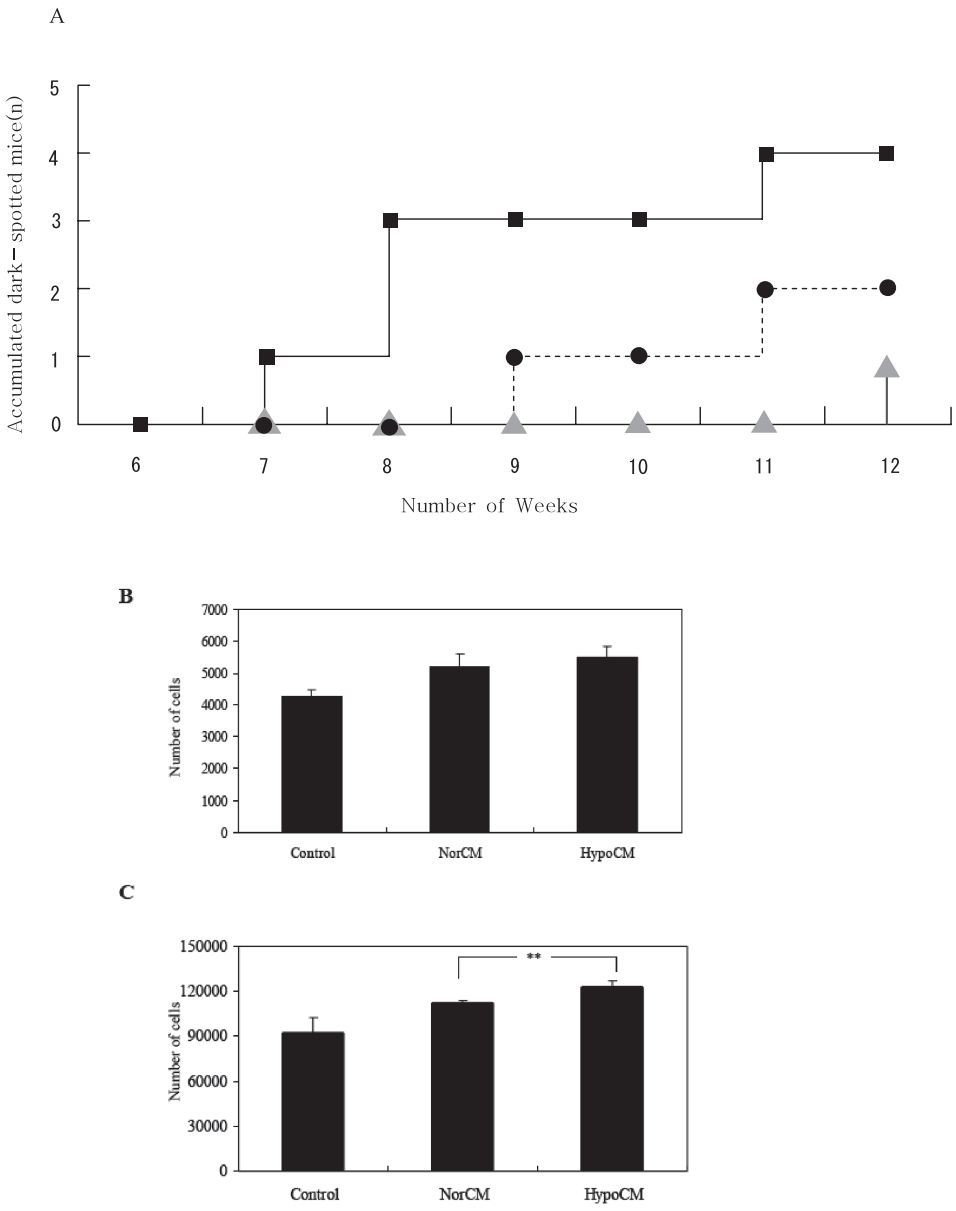

Fig. 2 Hypoxia enhances the ADSC-CM-promoted hair growth. In the animal experiment (A), dark spots appeared earlier in the hypoCM-treated group (closed squares, n = 5) than in the norCM (closed circles, n = 5) and F12/DMEM (closed triangles, n = 5) groups. Proliferation of HFDPCs was increased by both hypoCM and norCM as compared to the control, but there was no statistically significant difference between the hypoCM and norCM groups (B). However, the proliferation of HEKs was significantly increased by hypoCM treatment as compared to norCM (C). $^{**}P < 0.01$

hypoCM and norCM. Therefore, we used a commercially available antibody array to identify the growth factors that are enhanced by hypoxia. As shown in Fig. 3, most growth factors were up-regulated in hypoCM, with some exceptions. The 12 growth factors that appeared to be up-regulated were analyzed, and spot density was measured using a densitometer. The density in hypoxic and normoxic conditions was compared, and $P < 0.05$ was considered statistically significant (Table 1). The signal intensities of insulin-like growth factor binding protein-1 (IGFBP-1), IGFBP-2, macrophage colony-stimulating factor (M-CSF), M-CSF receptor (M-CSF R), platelet-derived growth factor receptor-β (PDGF R-β), and vascular endothelial growth factor (VEGF) were significantly greater in hypoCM than in norCM. By contrast, epithelial growth factor (EGF) was reduced by 70% in hypoCM relative to norCM.

A

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
2	POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
3	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
4	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
5	IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF Rα	PDGF Rβ	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
6	IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF Rα	PDGF Rβ	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
7	SCF R	TGF-α	TGF-β	TGF-β2	TGF-β3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS
8	SCF R	TGF-α	TGF-β	TGF-β2	TGF-β3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS

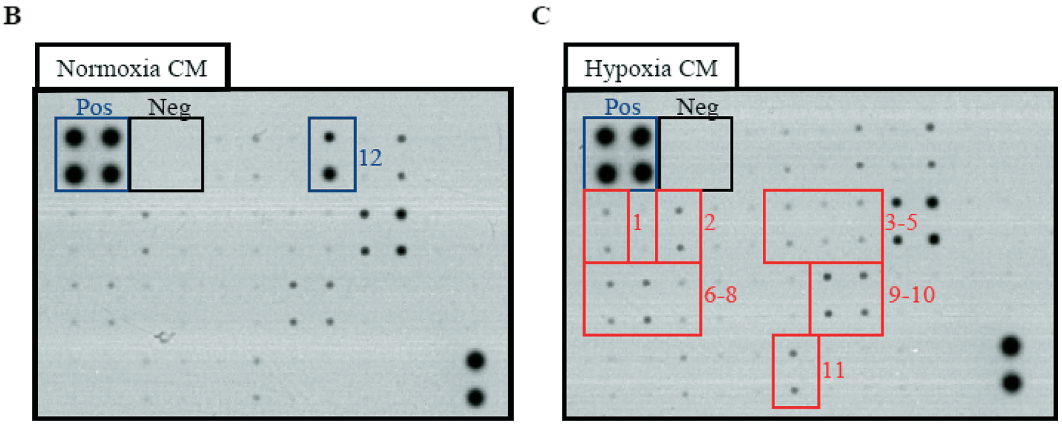

Fig. 3 Growth factor antibody array. The antibody array measured 41 growth factors (A). norCM (B) and hypoCM (C) were concentrated, and 100 μg of the concentrated CM was incubated with a membrane containing 41 antibodies against 41 growth factors. The amounts of most growth factors were increased in hypoCM. Spot densities of 11 growth factors were selected, analyzed, and summarized in Table 1.

Table 1 *Relative spot density of growth factors altered in antibody array*

#	Cytokine	Normoxia	Hypoxia	Hypoxia/Normoxia	p value	ratio
1	GCSF	10.13 ± 4.21	14.07 ± 3.84	1.43 ± 0.21	0.430	↑
2	GM-CSF	10.21 ± 1.44	13.53 ± 1.26	1.33 ± 0.06	0.133	↑
3	IGFBP-1*	5.56 ± 0.44	9.48 ± 0.44	1.70 ± 0.05	0.012	↑
4	IGFBP-2**	6.73 ± 0.31	8.91 ± 0.02	1.33 ± 0.06	0.010	↑
5	IGF-II	4.61 ± 0.93	10.62 ± 0.85	2.48 ± 0.85	0.056	↑
6	M-CSF*	7.46 ± 1.69	14.06 ± 0.13	1.93 ± 0.45	0.031	↑
7	M-CSF R*	3.31 ± 1.75	9.09 ± 0.20	3.20 ± 1.76	0.043	↑
8	PDGF Rβ*	11.47 ± 1.40	17.67 ± 1.32	1.54 ± 0.07	0.044	↑
9	PDGF-AA	12.14 ± 2.12	16.63 ± 1.33	1.38 ± 0.13	0.126	↑
11	VEGF*	5.59 ± 1.22	13.47 ± 1.26	2.44 ± 0.30	0.023	↑
12	EGF*	34.14 ± 6.75	11.06 ± 2.45	0.32 ± 0.007	0.045	↓

*$P < 0.05$ **$P < 0.01$

DISCUSSION

We investigated the ability of ADSC secretomes to promote hair growth and the effect of hypoxia on ADSC function. In an animal model, CM from hypoxic cultures of ADSCs increased hair regrowth. The proliferation of human follicle dermal papilla cells (HFDPCs) and human epidermal keratinocytes (HEKs), which are cell lines derived from the two major cell types present in hair follicles, was also increased by ADSC-CM treatment. To identify the mechanism by which hypoxia enhances ADSC-stimulated hair growth, we used a growth factor antibody array to analyze 41 growth factors. IGFBP-1, IGFBP-2, M-CSF, M-CSF R, PDGF R-β, and VEGF were significantly up-regulated ($P < 0.05$), while EGF was significantly down-regulated. Based on the results of the animal experiments and growth factor arrays, it is reasonable to conclude that secretomes stimulate hair growth and that the induced secretion

of growth factors accounts for the hypoxia-enhanced hair regrowth.

The growth factors released by ADSCs stimulate hair growth in *ex vivo* and *in vivo* animal models. VEGF controls hair growth and follicle size by angiogenesis in a VEGF transgenic mouse model (30). In addition, PDGF and its receptors are important in follicular development, and PDGF isoforms induce and maintain the anagen phase in murine hair follicles (27). IGF-I also up-regulates hair follicle growth in various systems (26, 29). IGFBP mRNA and protein are present in the dermal sheath surrounding the hair follicle, suggesting that IGFBPs modulate the actions of IGF-I in hair regeneration. Co-treatment of IGF-I with IGFBPs increases both hair cell survival and hair regeneration (1, 22, 28, 29). Thus, various growth factors in ADSC-CM might be involved in the stimulation of hair growth by ADSC-CM in the present experiment.

The proliferation of mesenchymal stem cells is increased under hypoxic conditions as compared to normoxic conditions (3,7). In addition, hypoxia amplifies the paracrine effects of mesenchymal stem cells by enhancing the secretion of certain growth factors. For example, in hairless mice, hypoCM significantly accelerates wound healing as compared to norCM (6). Expression of VEGF and bFGF in hypoCM is significantly increased by hypoxia, and neutralizing antibodies against VEGF and bFGF significantly reduced the wound area, suggesting a mechanism by which ADSCs promote wound healing (6). Potier *et al.* demonstrated that temporary hypoxia leads to a 2-fold increase in VEGF mRNA and protein expression in bone marrow-derived stem cells while the production of bFGF, TGF-β1 and IL-8 is not affected (23). Crisostomo *et al.* also reported that hypoxia activates bone marrow-derived stem cells and significantly increases the production of VEGF, FGF2, HGF, and IGF-I via the NFκB pathway (5). The present study is the first investigation to reveal the effect of hypoxia on ADSC-stimulated hair growth. We found that hypoxia enhances ADSC-promoted hair growth and significantly increases the secretion of IGFBP-1, IGFBP-2, M-CSF, M-CSF R, PDGF R-β and VEGF.

The dermal papilla, which is situated at the base of the hair follicle, is thought to induce new hair follicles. Activation signals emanating from the dermal papilla direct epithelial cell regeneration. Multiple signaling pathways, including Wnts, Sonic hedgehog (shh), and diverse growth factors secreted from dermal papilla, play roles in this process. Thus, the epithelial-mesenchymal interaction plays a critical role in organogenesis (2). Because hair-producing functions of the epithelium are maintained by the activity of mesenchymal follicular papilla cells, we studied the effects of ADSC-CM on the epithelial-mesenchymal interaction using a co-culture system of mesenchymal-derived HFDPCs and epithelium-derived HEKs. In this experiment, HFDPCs and HEKs were directly co-cultured such that the two cell types interacted, and we measured the proliferation of HEKs in the absence or presence of ADSC-CM. As expected, the proliferation of HEK cells was significantly increased in the presence of ADSC-CM (our unpublished data). Based on this result, we deduced that ADSC-CM is involved in both the epithelial-mesenchymal interaction and the activation of HFDPCs. In addition, in a preliminary study, we found that ADSC-CM-induced activation of HFDPCs was associated with the up-regulation of downstream signaling in the β-catenin pathway of HFDPCs.

In summary, we found that ADSC-CM stimulates hair growth and that this effect is enhanced by hypoxia. The secretion of growth factors such as IGFBP-1, IGFBP-2, M-CSF, M-CSF R, PDGF R-β, and VEGF was significantly increased by hypoxia. Although we have not yet performed an inhibition study with a neutralizing antibody or siRNA, we can conclude that the increased secretion of potent growth factors may partially account for the enhanced ADSC-induced hair growth resulting from hypoxia.

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