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Review

Hair regeneration using adipose-derived stem cells

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Summary. Adipose-derived stem cells (ASCs) have been used in tissue repair and regeneration. Recently, it was reported that ASC transplantation promotes hair growth in animal experiments, and a conditioned medium of ASCs (ASC-CM) induced the proliferation of hair-compositing cells *in vitro*. However, ASCs and their conditioned medium have shown limited effectiveness in clinical settings. ASC preconditioning is one strategy that can be used to enhance the efficacy of ASCs and ASC-CM. Therefore, we highlighted the functional role of ASCs in hair cycle progression and also the advantages and disadvantages of their application in hair regeneration. In addition, we introduced novel ASC preconditioning methods to enhance hair regeneration using ASC stimulators, such as vitamin C, platelet-derived growth factor, hypoxia, and ultraviolet B.

Key words: Adipose-derived stem cells (ASCs), Conditioned medium, Hair regeneration, ASC preconditioning

Introduction

Adipose-derived stem cells (ASCs) are mesenchymal stem cells (MSCs) derived from the natural selection of the stromal-vascular fraction (SVF) of subcutaneous adipose tissue (Zuk et al., 2001, 2002). ASCs exhibit similar profiles of surface markers with bone marrow-derived MSCs and display a multi-lineage differentiation. They produce and secrete growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and, platelet-derived growth factor (PDGF) (Kim et al., 2007; Park et al., 2008; Song et al., 2010). Their secreted factors are an essential function of ASCs, contribute to the activation of the surrounding cells, (Takahashi et al., 2010; Lee et al., 2012; Hsiao et al., 2013), and mediate the various cytoprotective effects in injury models, such as wound and hind-limb ischemia (Moon et al., 2006; Kim et al., 2007; Lee et al. 2009; Fan et al., 2012). Based on their unique regeneration potential, ASCs are fascinating therapeutic tools for the treatment of hair loss (Park et al., 2010; Won et al., 2010; Jeong et al., 2013; Kim et al., 2014a,b; Hye et al., 2015).

Drug therapies, hair transplantation, laser, and/or dietary supplementation are currently used to treat hair loss (Hong and Hart, 1990; Kaufman et al., 1998; McClellan and Markham, 1999; Avram, 2005). However, they have limitations due to side effects or lack of effectiveness. For example, topical minoxidil is associated with skin irritation (Shatalebi and Rafiei, 2014). Oral finasteride also has sexual side effects and is teratogenic (Thompson et al., 2003; Mondaini et al., 2007). In addition, hair loss is accelerated in patients who discontinue finasteride. Although hair transplantation is the current most effective therapy for hair loss, it is expensive and painful (Nusbaum, 2004). Therefore, alternative therapies using ASCs and their conditioned medium (ASC-CM) should be developed (Fukuoka and Suga, 2015; Shin et al., 2015).

Recent approaches of hair regeneration using ASCs have revealed that ASCs and ASC-CM stimulate hair

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follicle growth and modulate the hair cycle (Park et al., 2010; Won et al., 2010; Jeong et al., 2013). However, ASCs and ASC-CM have shown limited effectiveness in clinical settings (Fukuoka and Suga, 2015; Shin et al., 2015). Preconditioning of ASCs with several ASC stimulators is a promising strategy for enhancing the hair regeneration potential of ASCs (Park et al., 2010; Jeong et al., 2013; Kim et al., 2014; Hye et al., 2015). In this review, we highlight the functional role of adipose tissue and ASCs in hair regeneration and discuss the clinical applications of ASC and ASC-CM. In addition, we introduce ASC preconditioning methods to enhance hair regenerative potentials using ASC stimulators, such as vitamin C, PDGF, hypoxia, and ultraviolet B (UVB).

Adipose tissue and hair biology

Hair regeneration depends on cell-cell interaction and external signals around the hair follicles (Schmidt and Horsley, 2012; Chen et al., 2015). Communication between adipose tissue and hair follicles is particularly important, and adipocytes and ASCs play a key role in hair cycle progression.

Hair regeneration cycle

The hair follicle is a mini-organ composed of outer root sheath, inner root sheath, and hair shaft. Under normal homeostasis of the hair follicle, it is characterized by repeated cycles of regeneration (Ebling, 1988; Paus, 1998). Dermal papilla cells (DPCs) are located at the base of the hair follicle, a key element for hair regeneration, and are surrounded by epithelial matrix cells (Randall, 1996; Driskell et al., 2011). These cells support proliferation and differentiation of epithelial matrix cells during hair cycle progression and stimulate quiescent bulge stem cells to become activated during the late-telogen-to-early-anagen transition. During anagen, the cells at the base of the hair follicle start to proliferate and generate a new hair filament, while bulge stem cells give rise to progeny. In the catagen phase, the hair follicle shrinks due to disintegration, and the papilla detaches. During the telogen or resting phase, the follicle remains dormant.

Adipose tissue and hair cycle progression

Recently, a functional role of intradermal adipose tissue during hair cycle progression has been demonstrated (Driskell et al., 2014). Adipocytes and ASCs control hair cycle progression via the release of signaling molecules, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), PDGF, and Wnts (Festa et al., 2011; Schmidt and Horsley, 2012; Driskell et al., 2014; Rivera-Gonzalez et al., 2014). These signals activate stem cells in the hair follicle to differentiate, and suppress bulge stem cells during telogen. Of interest, the thickness of the intradermal adipose tissue is proportional to hair cycle progression (Schmidt and Horsley, 2012; Rivera-Gonzalez et al., 2014). During anagen, the intradermal adipocyte layer of the skin is expanded, and the skin thickness is doubled. In contrast, the thickness of the intradermal adipocyte layer is decreased during catagen and telogen, which indicates that intradermal ASCs and adipocytes act as a niche for the hair follicle, and

Table 1. Genes related to defects of adipose tissues and hair regeneration.

Gene	Expression	Phenotype	References
Apolipoprotein C-I	Overexpression	- Completely deficient of subcutaneous fat - Heterogeneous mice (+/-) have a thin hair-coat compared with wild-type mice - Homozygous mice (+/+) appear almost hairless	Jong et al., 1998
Fatty acid transport protein (FATP)-4	Knock-out	- Hyperkeratosis with a disturbed epidermal barrier - Homozygous mice (-/-) appear hairless	Herrmann et al., 2003
Dgat1	Knock-out	- Atrophic sebaceous gland and fur lipid abnormalities - Dry fur and hair loss in a 16-week-old male Dgat1–/– mouse	Chen et al., 2002
Dgat2	Knock-out	- Abnormal skin - Normal hair growth in grafts three weeks after wild-type and Dgat2(-/-) skin	Stone et al., 2004
Ebf1	Knock-out	- Defects in the generation of adipocyte lineage cells - Ebf1(–/–) and Azip mice experienced block in follicle stem cell activation	Festa et al., 2011
PDGF-A	Knock-out	- Postnatal PDGF-A(-/-) mice developed a thinner dermis, misshapen hair follicles, smaller dermal papillae, abnormal dermal sheaths and thinner hair, compared with wild-types	Karlsson et al., 1999
Leptin receptor	Knock-out	- Five-week-old leptin receptor deficient db/db mice remained in the first telogen stage and later entered the anagen stage at postnatal day 40 -Leptin acts as an anagen inducer	Sumikawa et al., 2014
Sonic hedgehog (shh)	Knock-out	- shh(-/-) mouse embryos display disrupted formation of the dermal papillae	Karlsson et al., 1999
Noggin	Overexpression	- Overexpression of noggin, a BMP antagonist, in mouse skin resulted in a markedly shortened refractory phase and faster propagation of the regenerative wave	Plikus et al., 2008
EGFR	Knock-out	- Defects in intradermal adipocytes - Egfr null and skin-targeted Egfr mutant mice exhibit disorganized hair follicles	Maklad et al., 2009

intercellular communications between (pre)adipocytes and DPCs or between (pre)adipocytes and bulge stem cells are important for regulating the hair cycle (Festa et al., 2011; Schmidt and Horsley, 2012; Driskell et al., 2014; Rivera-Gonzalez et al., 2014). For example, ASCs are adipocyte lineage cells that exert positive effects on DPC activation through growth factor secretion (i.e., PDGF-A, VEGF and bFGF) and activation of signaling pathways in DPCs (e.g., the Wnt/β-catenin pathway). On the other hand, BMP2 is highly expressed in mature intradermal adipocytes and suppresses bulge stem cell activity in late anagen and early telogen (Plikus et al., 2008; Schmidt and Horsley, 2012).

Transgenic and knock-out murine models have been studied to reveal the functional roles of adipocytes or adipose tissue in hair biology (Table 1). Although the skin structure of the mouse is different from that of a human, murine models are still useful. For example, apolipoprotein C-I overexpressing transgenic mice, FATP-4 deficient mice, and Dgat1-/- mice showed decreased intradermal adipose tissues (Jong et al., 1998; Chen et al., 2002; Herrmann et al., 2003), and defects in adipocyte or adipose tissue produce hair loss and/or skin diseases. In addition, PDGF-A-deficient mice exhibited a thinner dermis, misshapen hair follicles, smaller dermal papillae, abnormal dermal sheaths, and thinner hair compared with wild-type mice (Karlsson et al., 1999). Those animals that lacked EGFR in the skin epithelium showed defects in intradermal adipocytes and delayed entry into the anagen phase (Maklad et al., 2009). Collectively, these results support the importance of the communication between adipose tissue and hair follicles, and it is reasonable to assume that adipocytes and ASCs play a key role in hair cycle progression.

Hair regeneration by exogenous ASCs or ASC-CM

As previously described, adipocytes and ASCs secrete regenerative growth factors that participate in hair morphogenesis and hair regeneration (Festa et al., 2011; Schmidt and Horsley, 2012). Therefore, we and others have applied cultured ASCs and a conditioned medium to promote hair growth (Park et al., 2010; Won et al., 2010). Won et al. first reported that a conditioned medium of ASCs (ASC-CM) promoted hair growth both *in vitro* and *in vivo* (Won et al., 2010). ASC-CM increased the proliferation in a dose-dependent manner and phosphorylated the mitogenic signals in DPCs. The subcutaneous injection of ASC and ASC-CM also accelerated the telogen-to-anagen transition in the C_3H mouse. Park et al. further expanded the experiment to find that hypoxia enhances the hair growth promotion effects of ASCs and ASC-CM through up-regulation of growth factor secretion (Park et al., 2010). He et al. reported that CD34 positive ASCs in the SVF have superior effects in new hair generation compared with CD34 negative cells or unsorted SVF (He et al., 2013). They co-injected CD34 positive cells with dermal and epidermal cells to generate new hair follicles. Festa et al. reported that adipose lineage cells, including mature adipocytes and preadipocytes, secreted PDGF-A to regulate hair cycle progression (Festa et al., 2011). It is of interest that adipogenic cells exhibited superior effects to fresh cells of the SVF in regard to hair growth induction. In addition, conditioned medium of murine preadipocytes exhibited hair growth promotion effects in an animal experiment (Jung et al., 2015).

Recently, we developed a new isolation method of ASCs to enhance the hair regenerative potential of ASCs using a subfractionation culturing method (Yi et al., 2014). Clonal ASCs (cASCs) were isolated without using enzyme digestion or centrifugation steps. We selected three cASCs that have known high rates of proliferation. The selected cASC lines exhibited better paracrine effects than the ASCs obtained using the traditional method. In addition, the ASC-CM obtained from cASCs increased the proliferation of DPCs, and the injection of the cASCs demonstrated enhanced hair growth-promoting effects in animal experiments.

Clinical application of ASC and ASC-CM

Although cultured ASCs are effective in hair growth promotion in animal experiments, ASC therapy for hair regeneration has not been approved by the Korean Food and Drug Administration. Of note, cultured ASCs are approved only for Chrohn's disease in Korea (Lee et al., 2013; Cho et al., 2015). However, uncultured SVF or ASC-CM are commonly used for anti-aging therapy (Park et al., 2008; Charles-de-Sa et al., 2015). Recently, it was reported that treatment with ASC-CM is effective for female pattern hair loss (FPHL). In that study, the authors used microneedles to allow ASC-CM to penetrate human skin, and ASC-CM showed efficacy in treating FPHL after 12 weeks of therapy (Shin et al., 2015). For example, hair density and hair thickness significantly increased, and none of the patients reported adverse reactions. Fukuoka and Suga also reported the effectiveness of ASC-CM in hair regeneration. ASC-CM was intradermally injected in 22 patients with alopecia (Fukuoka and Suga, 2015). These patients received treatment every 3 to 5 weeks for a total of 6 sessions, and hair numbers were significantly increased after treatment in both male and female patients. However, clinical achievement was not satisfactory compared with hair transplantation. In addition, no previous clinical trials of ASC and ASC-CM have been registered or reported at ClinicalTrials.gov.

ASC preconditioning

ASC and ASC-CM can be applied to treat hair loss, but their regenerative potential should be enhanced. In other words, alternative methods to obtain better results in clinical studies need to be established. Preconditioning with ASC stimulators is one strategy that can be used to enhance the hair growth-promoting effect of ASCs (Park et al., 2010; Jeong et al., 2013; Kim

et al., 2014a; Hye Kim et al., 2015; Jung et al., 2015). Fig. 1 summarizes the preconditioning methods of ASCs to promote hair regeneration.

Vitamin C

Vitamin C is an essential nutrient that serves as a cofactor for many chemical reactions in the human body. Depending on its concentration, it also acts as an antioxidant and a pro-oxidant. At high concentrations (>mM concentration), vitamin C inhibits the proliferation of cancer cells (Osmak et al., 1997; McConnell and Herst, 2014). However, at low concentrations, vitamin C reportedly increases cell proliferation in normal cells (Jeong et al., 2013; Yu et al., 2014). Vitamin C is usually used for culture supplementation of stem cells in order to increase their proliferation. Vitamin C also induces the stemness gene expression of embryonic stem cells and pluripotent stem cells through epigenetic modification (Cao et al., 2012; Chen et al., 2013).

Kim et al. first examined whether vitamin C preconditioning in ASCs could enhance the hair regenerative potential of ASCs (Kim et al., 2014a). Sodium-dependent vitamin C transporter 2 (SVCT2) is expressed in ASCs, where it mediates vitamin C uptake. Vitamin C uptake enhances the survival and proliferation of ASCs via the dose-dependent activation of mitogen-activated protein kinase (MAPK) signaling. It also upregulates proliferation-related genes like Fos, E2F2, Ier2, Mybl1, Cdc45, Jun B, Fos B, and Cdca 5. Vitamin C increases the levels of growth factors, including HGF, IGFBP6, VEGF, bFGF, and KGF, which collectively promote the hair regenerative potential of ASCs. Therefore, the subcutaneous injection of vitamin C-preconditioned ASCs accelerated the phase transition from telogen to anagen in C_3H mice (Kim et al., 2014a,b).

PDGF

PDGF has four subtypes, PDGF-A, -B, -C, and -D. The PDGF isoforms bind PDGF receptors (PDGFR-α and -β), and exhibit diverse functions, such as cell proliferation and migration (Antoniades, 1991; Tallquist and Kazlauskas, 2004; Kim et al., 2015). PDGFRs auto-

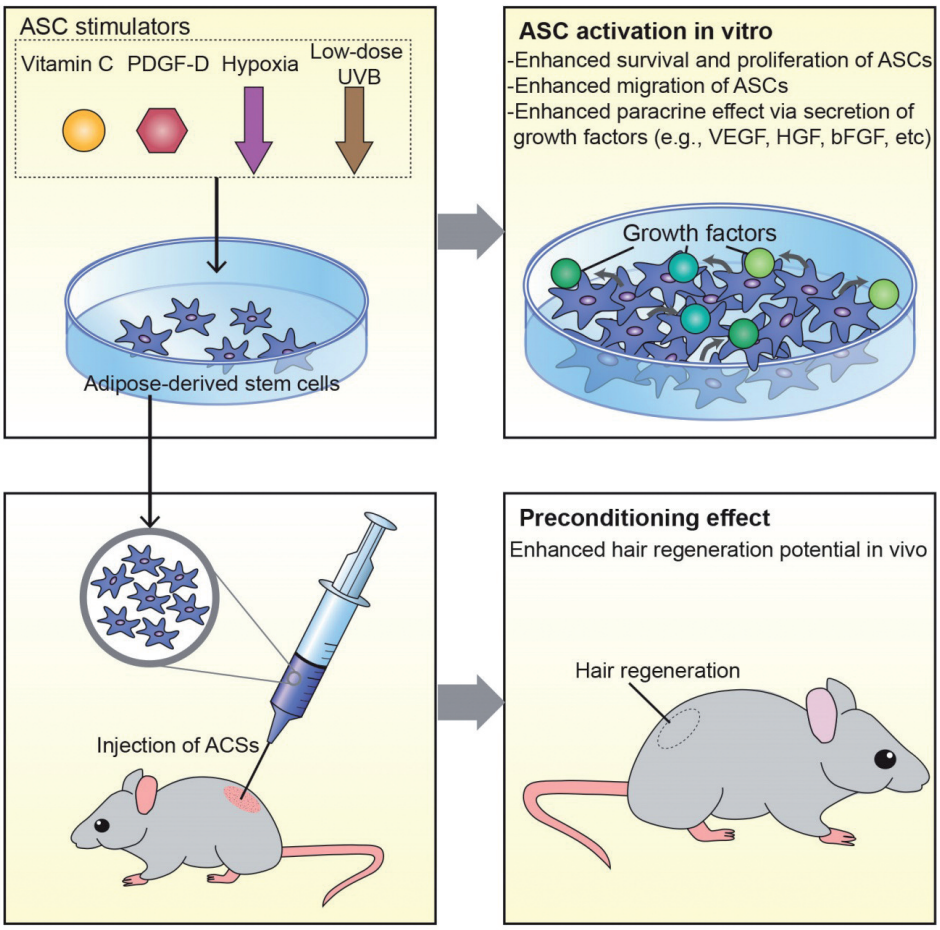

Fig. 1. ASC preconditioning methods for enhancing hair regeneration.

phosphorylate their cytosolic domains and activate the PI3K and MAPK pathways (Kim et al., 2015). The PDGF isoforms reportedly induce hair regeneration. For example, local injection of recombinant PDGF-A and PDGF-B showed hair growth-promoting effects *in vivo* (Tomita et al., 2006). In addition, the synergistic effect of PDGF and FGF2 on cell proliferation and hair inductive activity was reported in murine vibrissal dermal papillae (Kiso et al., 2015). However, the hair regenerative potential of PDGF-C and -D has not yet been reported.

Growth factors have stimulating effects on ASCs during expansion (Kaewsuwan et al., 2012; Kim et al., 2014a,b, 2015; Hye et al., 2015). Of these, PDGF-B and PDGF-D reportedly have strong effects on the proliferation and differentiation of ASCs through PDGFR-β (Kim et al., 2013, 2015; Hye Kim et al., 2015). PDGF-B increased the proliferation and migration of ASCs through reactive oxygen species (ROS) generation and miR-210 upregulation (Kim et al., 2013). However, PDGF-B is not expressed in ASCs, while PDGF-D is highly expressed (Hye Kim et al., 2015). Therefore, we examined the stimulating effects of PDGF-D. It acts as a novel ASC stimulator, and can be used to enhance the hair regenerative potential of ASCs (Hye Kim et al., 2015). PDGF-D treatment upregulated diverse growth factors such as VEGF-A, FGF1, FGF5, leukemia inhibitory factor, and heparin-binding EGF-like growth factor in ASCs, and growth factor induction was mediated through the MAPK pathway. Subcutaneous injection of PDGF-D-preconditioned ASCs accelerated anagen induction in animal experiments. In addition, we investigated the underlying mechanism of proliferation and migration by PDGF-D, and found that mitochondrial ROS generation and mitochondrial fission play key roles in these functions.

Hypoxia

Hypoxia is an oxygen deficiency, and it mediates impaired cellular responses which are highly dependent on cell type, maturity and cellular environment (Chung et al., 2009; Kim and Sung, 2012; Kang et al., 2014). For example, hypoxic conditions during culture usually harm neurons but also produce stimulation signals for stem cells (Chung et al., 2009; Wang et al., 2014). We examined the signaling pathways involved in the hypoxia (1~2% oxygen) stimulation of ASCs and found that ROS generation by NADPH oxidase phosphorylated Akt and ERK1/2 molecules to increase the proliferation and migration of ASCs (Kim et al., 2011a,b, 2012, 2013).

Culturing ASCs in hypoxic conditions also enhanced the secretion of paracrine factors due to the stabilization of HIF-1α, and hypoxia also increased the levels of growth factors, including IGFBP-1, IGFBP-2, M-CSF, PDGFR-β, and VEGF (Chung et al., 2009; Lee et al., 2009; Park et al., 2010; Kang et al., 2014). Therefore, ASC-CM harvested under hypoxic conditions (hypo-CM) increased the proliferation of DPCs, while hypo-CM accelerated the anagen transition in a C3H mice model. In addition, the direct injection of hypoxia-preconditioned ASCs accelerated hair regeneration in an animal model (Park et al., 2010).

UVB

UVB is an important mediator of ROS generation, and UVB at high-doses causes harm to cells. Human skin receives regular exposure to UV light, and UV rays can penetrate the epidermis and mid-dermis of human skin. Therefore, its adverse effects on the epidermis and dermis have been well demonstrated (Kalimo et al., 1983; Di Nuzzo et al., 2002; Kim et al., 2009). Recently, it was reported that the thickness of subcutaneous fat tissue in chronically sun-damaged skin is decreased compared with that in naturally aged skin due to the effects of UVB on subcutaneous adipose tissue (Kim et al., 2011a). Even a single UV exposure reduces lipid synthesis in the subcutaneous fat tissue through transcriptional regulation of lipogenic enzymes in human skin (Kim et al., 2011a,b).

Of interest, we identified an alternative role of UVB in ASCs. As a preconditioning agent, UVB exposure at a low dose (10 or 20 mJ/cm^2) increased ASC survival, migration, and tube-forming activity *in vitro*. Low-dose UVB treatment also amplified ROS generation through NADPH oxidase 4, which increases the proliferation, migration, and paracrine effect of ASCs (Jeong et al., 2013). Low-dose UVB upregulated the expression of hair-related growth factors, and a conditioned medium of UVB-irradiated ASCs increased the proliferation of DPCs and outer root sheet cells. In addition, subcutaneous injection of ASCs after UVB preconditioning promoted the anagen induction in an animal experiment (Jeong et al., 2013).

Conclusion

ASCs and ASC-CM promoted hair growth in an animal model, and ASC-CM induced the proliferation of hair-compositing cells *in vitro*. Although ASCs and ASC-CM have hair growth-promoting effects, they have shown limited effects in clinical applications. Therefore, ASC preconditioning can be used to enhance the hair growth-promoting effect of ASC. Vitamin C, PDGF, hypoxia, and UVB are key ASC stimulators that can enhance proliferation or hair regenerative potential.

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