Adipose-Derived Stem Cell Coculturing Stimulates Integrin-Mediated Extracellular Matrix Adhesion of Melanocytes by Upregulating Growth Factors

Hyangmi Kim[1], Nayoung Yi[1], Byung-Rok Do[2] and Ai-Young Lee[1],*

[1]*Department of Dermatology, Dongguk University Ilsan Hospital, Goyang 10326,*
[2]*Biotechnology Research Institute, Hurim BioCell Inc., Seoul 07531, Republic of Korea*

Abstract

Coculture with adipose-derived stem cells (ADSCs) can stimulate proliferation and migration of melanocytes. To enhance outcomes of skin disorders caused by melanocyte loss or death, mixed transplantation with ADSCs has been suggested. However, role of cocultured ADSCs in proliferation and migration of melanocytes remains unclear. This study determined the effect of ADSCs on production of growth factors and expression levels of intergrins in primary culture of adult human melanocytes with or without ADSCs and in nude mice grafted with such melanocytes. Higher amounts of growth factors for melanocytes, such as bFGF and SCF were produced and released from ADSCs by coculturing with melanocytes. Relative levels of integrins β1, α5, and α6 as well as adhesion to fibronectin and laminin were increased in melanocytes cocultured with ADSCs. Such increases were inhibited by neutralization of bFGF or SCF. Relative levels of bFGF, SCF and integrins were increased in nude mice skin after grafting with melanocyte+ADSC cocultures. Collectively, these results indicate that ADSCs can stimulate proliferation and migration of melanocytes by increasing expression of integrins in melanocytes through upregulation of production/release of melanocyte growth factors such as bFGF and SCF.

Key Words: Adipose-derived stem cell coculturing, Melanocytes, Growth factors, Integrins

INTRODUCTION

Melanocytes will undergo rare mitosis without the help of growth factors or mitogenic factors (Jimbow *et al*., 1975; Pawelek, 1979). Therefore, various ways of melanocyte transplantation such as skin grafting and cell transplantation have been used to treat skin disorders induced by melanocyte loss or death including vitiligo. Transplantation of cultured melanocytes provides greater size ratios between donor and recipient skin (Hong *et al*., 2010). However, long-term results of cultured melanocyte transplantation have been poorer compared to those of epidermal graft (Olsson and Juhlin, 2002). To overcome such poorer results, transplantations of non-cultured skin cells, melanocyte+keratinocyte cocultures, and melanocytes cultured on different scaffolds have been tried.

Due to their ready availability, growth factors secretion, immunomodulatory effects, and multilineage differentiation capacity, adipose-derived stem cells (ADSCs) have been clinically used in many different skin conditions, including wound repair, skin rejuvenation, scar remodeling, cell therapy, and tissue engineering (Hong *et al*., 2010; Shingyochi *et al*., 2015). Although ADSCs cannot differentiate into melanocytes, *in vitro* and *in vivo* studies have shown that ADSCs can stimulate proliferation and migration of normal human melanocytes by coculturing, suggesting that ADSCs could be a potential substitute for keratinocytes in cocultures with melanocytes (Kim *et al*., 2012; Lim *et al*., 2014). However, how ADSCs stimulate the proliferation and migration of melanocytes remains unclear.

For melanocyte proliferation, growth factors are required. In fact, grafting melanocyte+keratinocyte cocultures to treat skin disorders due to melanocyte loss is based on the fact that keratinocytes are main sources of growth factors for melanocytes, including basic fibroblast growth factor (bFGF) and stem cell factor (SCF) (Sviderskaya *et al*., 1995; Hirobe, 2005; Lee *et al*., 2005). Results in our previous study have shown that ADSCs can release bFGF and SCF proteins in amounts not less than keratinocytes (Kim *et al*., 2012), suggested that

www.biomolther.org

ADSCs might also be sources of growth factors for melanocytes. However, the effect of ADSCs on production and release of growth factors for melanocytes by coculturing has not been reported yet.

It has been identified that transplantation of melanocytes with scaffold can enhance treatment outcome of skin disorders due to melanocyte loss. In fact, attachment of melanocytes to extracellular matrix (ECM) can trigger their survival, proliferation, and migration (Scott *et al*., 1997; Kumar *et al*., 2011; Pinon and Wehrle-Haller, 2011; Ricard *et al*., 2012; Bin *et al*., 2016). Integrins are the best characterized adhesion receptors that can mediate cellular interaction with ECM components such as type IV collagen, laminin, and fibronectin. In normal human skin melanocytes, $\beta 1$ integrins mainly mediate adhesion to defined ECM. Integrin $\alpha 5\beta 1$ and $\alpha 6\beta 1$ are involved in attachment to fibronectin and laminin, respectively (Zambruno *et al*., 1993; Hara *et al*., 1994; Akiyama, 1996; Scott *et al*., 1997). However, little research has been done about the role of ADSCs or ADSC-derived growth factors in integrin expression of melanocytes.

Therefore, the objective of this study was to determine the effect of coculturing with ADSCs on the generation of growth factors and expression levels of integrins to understand how ADSCs could stimulate proliferation and migration of cocultured melanocytes. The result showed that ADSCs increased levels of integrins ($\beta 1$, $\alpha 5$, and $\alpha 6$) by upregulating bFGF and/or SCF in cocultured melanocytes compared to those in melanocyte monocultures.

MATERIALS AND METHODS

Normal human epidermal melanocyte culture

Adult skin specimens obtained from repeat Cesarean section and circumcisions were used for cultures after obtaining approval from the Institutional Review Board of the Dongguk University Ilsan Hospital (Approval number: 2012-69). The epidermis was separated from the dermis following treatment with 2.4 U/mL of dispase (Roche, Penzberg, Germany) for 1 hour. The epidermal sheets were treated with 0.05% trypsin for 10 minutes to generate a suspension of individual epidermal cells. The cells were suspended in Medium 254 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with fetal bovine serum (FBS), bovine pituitary extract, bovine insulin, hydrocortisone, bFGF, bovine transferrin, heparin, and phorbol 12-myristate 13-acetate (Thermo Fisher Scientific). When cells reached 80% confluency, they were prepared for this study by replacing the culture medium with Medium 254 contained 20% of each supplement (called supplement-starved medium in this study). For neutralization of growth factors, cells were treated with anti-bFGF antibody (4 μg/ml; Merck-millipore, Darmstadt, Germany) or anti-SCF (0.02 μg/ml; Abcam, Cambridge, UK) diluted in supplement-free Medium 254 for one day. For all experiments, melanocytes at a density of 2.5×10^4 cells/well were seeded into a 6-well plate. These cells were cultured with supplement-starved Medium 254 for six days and then cultured with Medium 254 without supplements for another day.

ADSCs preparation

Human ADSCs were provided by one of the authors, Dr. Byungrok Do (Hurim BioCell, Seoul, Korea). The cells were obtained from human lipoaspirates of volunteers after obtaining informed consent. This study was approved by the Institutional Review Board (IRB number 700069-201407-BR-002-01) of Hurim BioCell. ADSCs were isolated and cultured as reported previously (Zuk *et al*., 2002). Lipoaspirated fat was digested with 0.1% collagenase (Sigma-Aldrich, St. Louis, MO, USA) in saline and collected after centrifugation. After cell counting, cells were transferred into culture flask in low-glucose Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific), 100 U/mL of penicillin (Thermo Fisher Scientific), and 0.1 mg/mL of streptomycin (Thermo Fisher Scientific). For all experiments, ADSCs at a density of 5×10^4 cells/well were seeded into a 6-well plate. These cells were cultured with supplement-starved Medium 254 for six days and then cultured with Medium 254 without supplements for another day.

Coculture of ADSCs with melanocytes

ADSCs at a density of 5×10^4 cells/well were seeded into a 6-well plate. Melanocytes were then added to the 6-well plate at a density of 2.5×10^4 cells/well. These cells were cultured with supplement-starved Medium 254 for six days and then cultured with Medium 254 without supplements for another day. For cocultures using inserts, ADSCs were seeded onto the lower culture dish while melanocytes were seeded onto Collagen-coated Transwell® Insert (6-well, 0.4 μm; CORNING, Lowell, MA, USA). For neutralization of growth factors, cells were treated with anti-SCF (0.02 μg/ml) or anti-bFGF antibody (4 μg/ml) diluted in supplement-free Medium 254 for one day.

Grafting of cultured cells to animal skin

Female BALB/c nude mice at 8-week-old (Orient Bio Inc., Seongnam, Korea) were used for this study. After acclimatization for one week, these animals were anesthetized through intraperitoneal injection of Zoletyl and Lumpen. Four rectangular areas of approximately 1×1 cm^2 in size were marked on the dorsal lateral back skin of each mouse for dermabrasion. Melanocytes alone, ADSCs alone, or cocultures of these cells were grafted to these areas with dermabrasion.

Real-time PCR

cDNA was synthesized from total RNA using cDNA Synthesis Kit for RT-PCR (Promega, Fitchburg, WI, USA). The amount of target mRNA was quantified by real-time PCR using a Light Cycler real-time PCR machine (Roche). Relative mRNA expression level was calculated as the ratio of each target gene relative to the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. Primer sequences used were for real-time PCR were as follows: bFGF forward (5'-TCC TGCCAGTGGTAATACGAT-3') and bFGF reverse (5'-CCCTAAGAGTATAAAGGT ATCCACA AG-3'); SCF forward (5'-AAC CATTTATGTTACCCCCTGTT-3') and SCF reverse (5'-AGTCTCCAGGGGGATTTT TG-3'); GAPDH forward (5'-TCCACTGGC GTC TTCACC-3') and GAPDH reverse (5'-GGCAGAGATGATGACCCTTT-3').

Western blot analysis

Cells were homogenized in standard RIPA buffer (Thermo Fisher Scientific) supplemented with a cocktail of protease and phosphatase inhibitors (Thermo Fisher Scientific). After determining protein concentrations of extracts using a PierceTM BCA Protein Assay kit (Thermo Fisher Scientific), equal
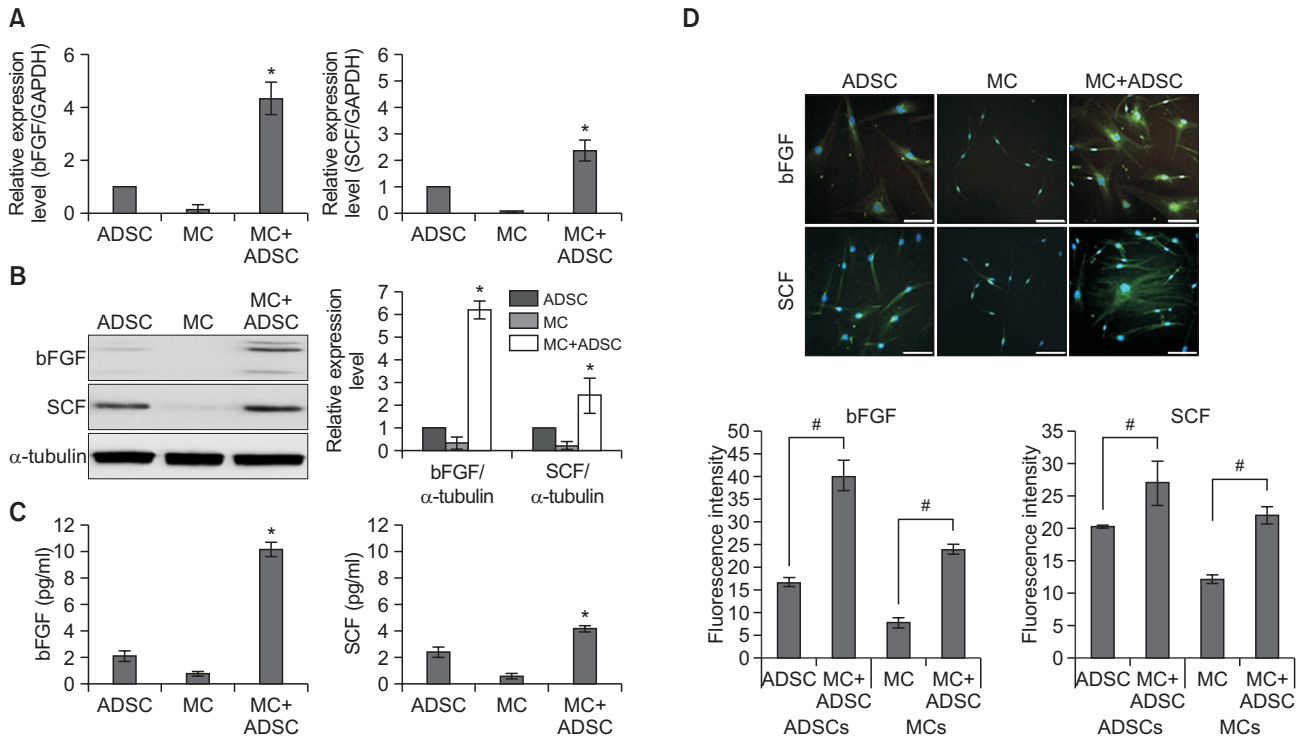
Fig. 1. ADSC coculturing increased levels of bFGF and SCF. (A, B) Real-time PCR (A) and Western blot analysis (B) results for relative ratios of bFGF and SCF mRNA and protein levels in cell lysates. (C) ELISA results for concentrations of bFGF and SCF proteins in culture supernatants. (D) Representative immunofluorescent staining using anti-bFGF or anti-SCF antibody. All these studies were performed using ADSC monocultures (ADSC), primary cultured normal adult human melanocyte monocultures (MC), and MC+ADSC cocultures (MC+ADSC). GAPDH and α-tubulin were used as internal controls for real-time PCR and Western blot analysis, respectively. In immunofluorescence staining, nuclei were counter-stained with Hoechst 33258 (Bar=0.1 mm) and intensities in randomly selected total 20 ADSCs and/or melanocytes were measured using Wright Cell Imaging Facility ImageJ software and compared between monocultures and cocultures. Data in each graph represent mean ± SD of three or four independent experiments. *$p<0.05$ vs ADSC, #$p<0.05$ vs. ADSC or MC.

amounts of extracted proteins were resolved and transferred to nitrocellulose membranes. These membranes were incubated with antibodies specific for bFGF (mouse monoclonal, BD Biosciences, San Jose, CA, USA), SCF (mouse monoclonal, Santa Cruz Biotechnology Inc., CA, USA), integrin $\beta1$ and integrin $\alpha6$ (rabbit polyclonal, Bethyl laboratories Inc., TX, USA), and integrin $\alpha5$ and α-tubulin (mouse monoclonal, Santa Cruz Biotechnology Inc.). These membranes were further incubated with anti-rabbit horseradish peroxidase–conjugated antibody (Thermo Fisher Scientific) or anti-mouse horseradish peroxidase-conjugated antibody (Pierce Biotechnology, Rockford, IL, USA). They were then developed with an enhanced chemiluminescence solution (ECL kit; Amersham Life Sciences, Buckinghamshire, UK). Signals were captured with an Image Reader (LAS-3,000; Fuji Photo Film, Tokyo, Japan). Protein bands were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of cultured cells were collected during steps of previous experiments. Concentrations of human bFGF (#DFB50, R&D Systems, Minneapolis, MN, USA), whose sensitivity was less than 2 pg/mL, and SCF (#ab100636, Abcam), with a sensitivity of 3 pg/mL, in these supernatants were measured using ELISA kit according to the manufacturer's instructions. Whenever the supernatant concentration declined

below the detection threshold of each ELISA kit, the loading volume of the supernatants double, and the concentration was calculated by dividing into two parts.

Immunofluorescence staining

Cultured cells were fixed in 4% (w/v) paraformaldehyde, treated with 0.05% Triton X-100, and blocked with 3% bovine serum albumin (BSA). These cells were then reacted with anti-bFGF, anti-SCF, anti-integrin $\beta1$, anti-integrin $\alpha5$, or anti-integrin $\alpha6$ antibody and then stained with Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) or Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes). To stain paraffin-embedded skin specimens, after deparaffinization and blocking, 4-μm-thickness sections were sequentially reacted with corresponding primary antibody and Alexa Fluor® 594 goat anti-mouse IgG (Molecular Probes) or Alexa Fluor®594 goat anti-rabbit IgG (Molecular Probes). Nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich). Images were obtained using a fluorescence microscope (Dp Manager 2.1; Olympus Optical Co., Tokyo, Japan). Fluorescence intensities were then analyzed using ImageJ software (National Institutes of Health).

Adhesion assay

Melanocyte monocultures and cocultures using inserts for seven days were reacted with anti-integrin $\beta1$ (10 ug/ml; Ab-
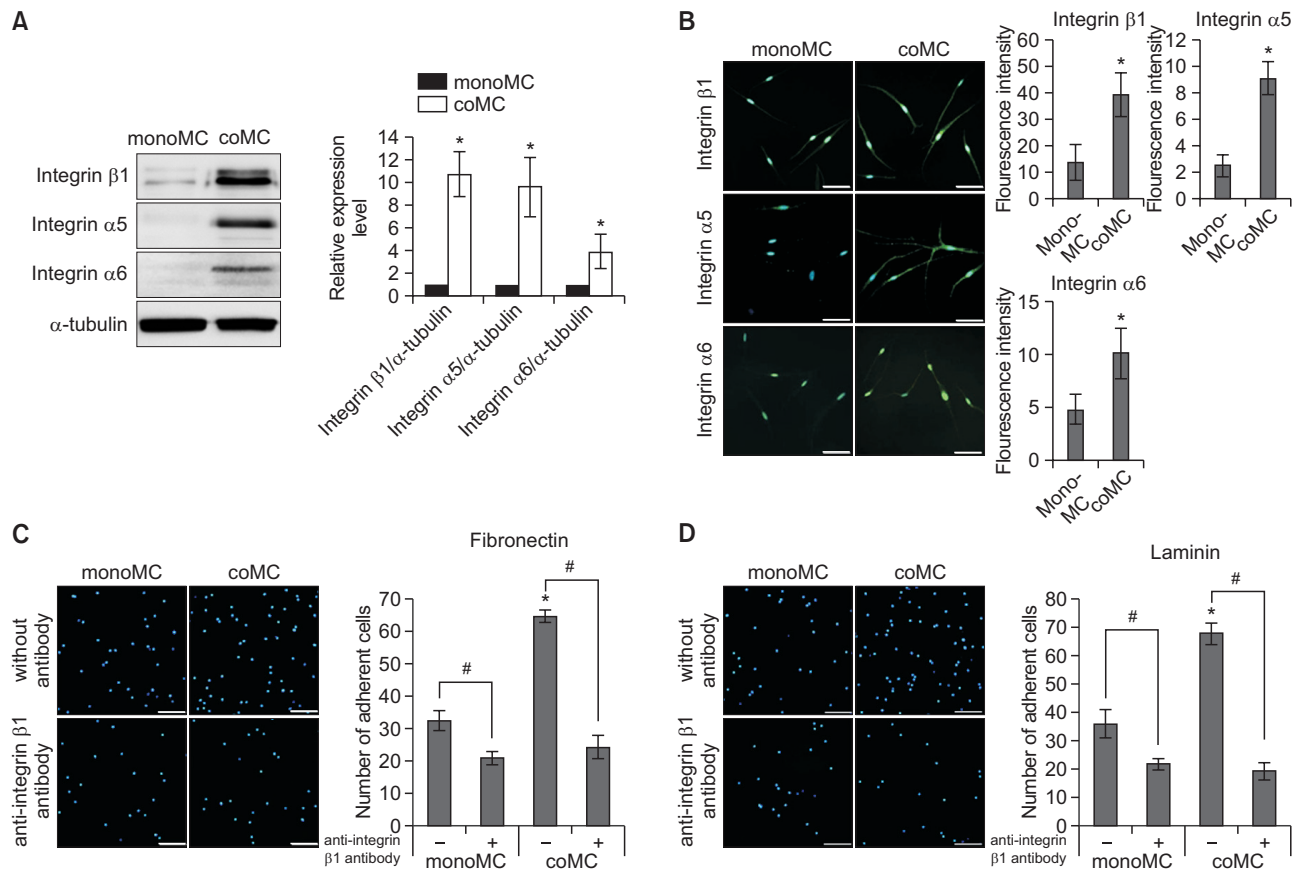
Fig. 2. ADSC coculturing enhanced melanocyte adhesion to ECM through β1 integrin upregulation. (A) Western blot analysis for relative levels of integrins β1, α5, and α6 proteins. α-tubulin was used as an internal control. (B) Representative immunofluorescent staining using anti-integrin β1, anti-integrin α5, or anti-integrin α6 antibody. Nuclei were count-stained with Hoechst 33258 (Bar=0.05 mm) and intensities were measured using Wright Cell Imaging Facility ImageJ software. (C, D) Adhesion assay on fibronectin- (C) or laminin-coated culture dishes (D) treated with or without anti-integrin β1 antibody. Nuclei were stained with Hoechst 33258 (Bar=0.2 mm). These studies were done using melanocyte monocultures (monoMC) and melanocytes taken from upper insert after coculturing with ADSCs in lower chamber (coMC). Data in each graph represent mean ± SD of three independent experiments. *$p<0.05$ vs monoMC, #$p<0.05$ vs MC without anti-integrin β1 antibody.

cam), anti-bFGF, or anti-SCF antibody for another day. These cells were transferred to each well (3×10^4 cells in 400 μl DMEM medium without FBS) of a 24-well tissue culture plate coated with fibronectin or laminin (CORNING), incubated for 30 minutes, and then washed with sterile PBS (pH 7.4). After fixing in 4% paraformaldehyde and staining nuclei with Hoechst 33258, the number of stained cells was counted in a fixed area using a standard light microscope (DM LB microscope; Leica Microsystems, Wetzla, Germany).

Statistical analysis

Statistical analysis of the experimental data was performed using Student's *t*-test. Results are expressed as mean ± SD. A *p*-value <0.05 was considered significant.

RESULTS

ADSC coculturing increased levels of bFGF and SCF

Our previous study has shown that the relative number of melanocytes is increased by coculturing with ADSCs at a ratio of 1:2 for one week (Lim *et al*., 2014). Therefore, expression levels of growth factors, bFGF and SCF, were examined and compared between melanocyte monoculture and melanocyte+ADSC coculture under the same condition. Since melanocytes culture media already contained bFGF, cells were cultured using media containing 1/5 supplements (supplement-starved media) for six days followed by culturing with supplement-free media for one day before examination of growth factors. Real-time PCR and Western blot analysis showed that levels of bFGF and SCF mRNAs and proteins were barely detectable in melanocyte monocultures (Fig. 1A, 1B). Although bFGF and SCF mRNAs and proteins were produced by ADSC monocultures, relative levels of bFGF and SCF mRNAs and proteins were higher in melanocytes+ADCSs cocultures than the sum of those in melanocyte monocultures and ADSC monocultures (*p*<0.05; Fig. 1A, 1B). ELISA results using culture supernatants also showed that concentrations of bFGF and SCF that were very low in melanocyte monocultures were increased in melanocytes+ADCSs cocultures than the sum of their concentrations from melanocyte monocultures and ADSC monocultures (*p*<0.05; Fig. 1C). Immuno-
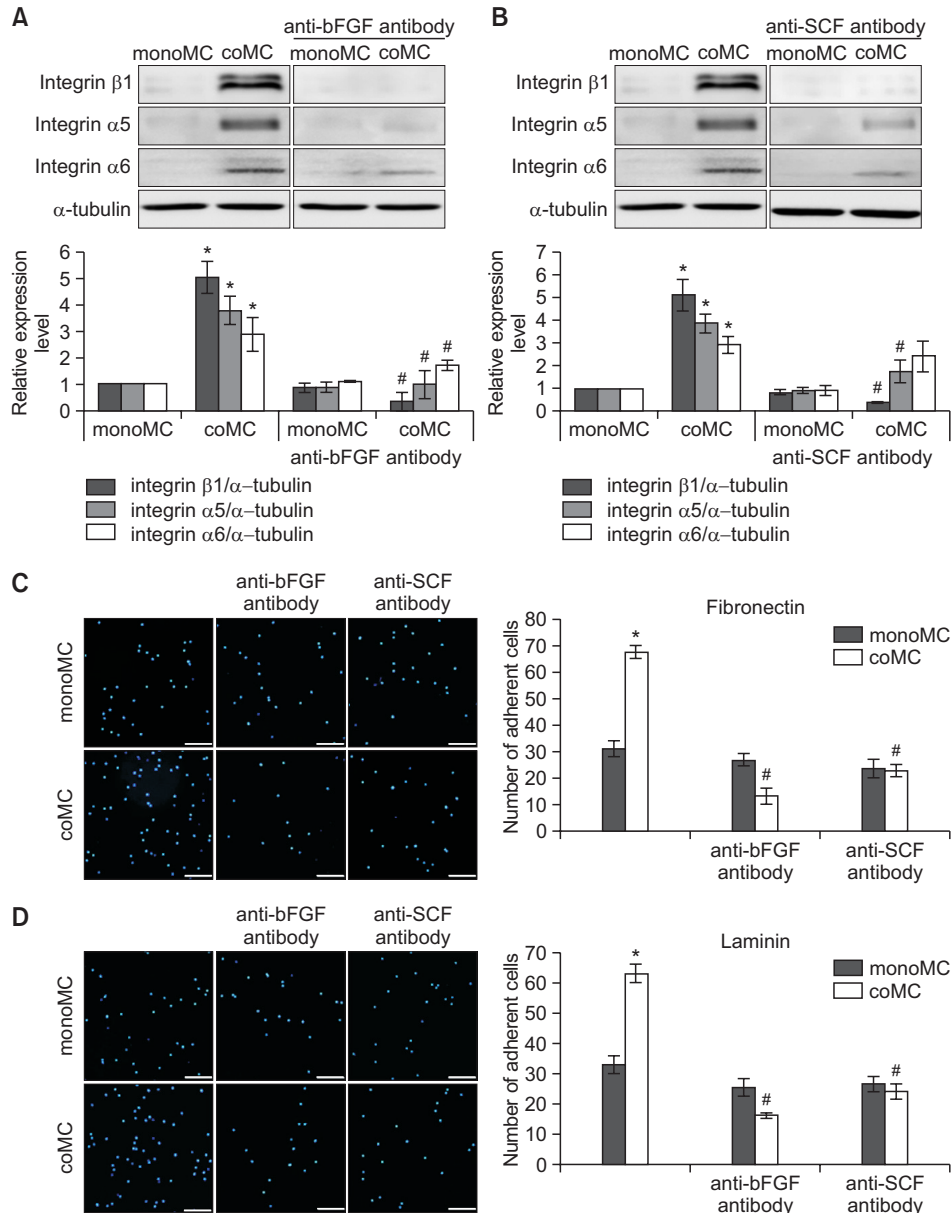
Fig. 3. Upregulation of bFGF and SCF by ADSCs was involved in integrin-mediated melanocyte adhesion to ECM. (A, B) Western blot analysis for relative levels of integrins $\beta1$, $\alpha5$, and $\alpha6$ proteins in melanocytes treated with or without anti-bFGF (A) or anti-SCF antibody (B). (C, D) Adhesion assay using fibronectin- (C) or laminin-coated culture dishes (D) treated with or without anti-bFGF or anti-SCF antibody. Nuclei were stained with Hoechst 33258 (Bar=0.2 mm). These experiments were performed using melanocyte monocultures (monoMC) and melanocytes taken from upper insert after coculturing with ADSCs in lower chamber (coMC). Data in each graph represent mean ± SD of three independent experiments. *$p<0.05$ vs monoMC, #$p<0.05$ vs coMC.

fluorescence staining using anti-SCF or anti-bFGF antibody revealed that the staining intensity in either ADSCs or melanocytes (measured in randomly selected total 20 cells for each type of cells by ImageJ software) was stronger under coculture condition ($p<0.05$; Fig. 1D).

ADSC coculturing enhanced melanocyte adhesion to ECM through $\beta1$ integrin upregulation

Integrins are known to trigger cell proliferation and survival (Pinon and Wehrle-Haller, 2011). They are expected to be present in both melanocytes and ADSCs. Among integrins,

integrins $\alpha5\beta1$ and $\alpha6\beta1$ are involved in attachment of melanocytes to fibronectin and laminin, respectively (Zambruno et al., 1993; Hara et al., 1994; Akiyama, 1996; Scott et al., 1997). Therefore, melanocytes were cultured using inserts with or without ADSCs in lower chambers to compare expression levels of integrins $\beta1$, $\alpha5$, and $\alpha6$ in melanocytes between cocultures and monocultures. Ratio of melanocyte adhesion to fibronectin and laminin was also compared between cocultures and monocultures in the presence or absence of anti-integrins $\beta1$ antibody. Western blot analysis showed that relative levels of integrins $\beta1$, $\alpha5$, and $\alpha6$ proteins were significantly in-

creased in melanocytes after coculturing with ADSCs (*p*<0.05; Fig. 2A). Immunofluorescence staining using anti-integrin β1, anti-integrin α5, and anti-integrin α6 antibody revealed that their staining intensities in melanocytes (measured in randomly selected total 20 cells by ImageJ software) were stronger after coculturing with ADSCs (*p*<0.05; Fig. 2B). Adhesion assay showed that higher number of melanocytes attached to fibronectin and laminin under coculture condition (*p*<0.05; Fig. 2C and 2D, respectively). These attached cells were reduced by treatment with anti-intergrin β1 antibody (*p*<0.05; Fig. 2C, 2D).

Upregulation of bFGF and SCF by ADSCs was involved in integrin-mediated melanocyte adhesion to ECM

Regulatory role of SCF in integrin expression has been reported previously (Scott *et al*., 1994). Thus, the effect of bFGF or SCF derived from ADSCs on expression levels of integrins in melanocytes and their adhesion to fibronectin or laminin was examined. Since relative levels of integrins β1, α5, and α6 were increased in melanocytes by coculturing with ADSCs (Fig. 2A, 2B), Western blot analysis was performed after melanocytes, which were cultured using inserts with or without ADSCs in lower chambers, were neutralized with anti-bFGF or anti-SCF antibody. Levels of integrins β1, α5, and α6 in melanocytes increased by coculturing were decreased by neutralization using anti-bFGF (*p*<0.05; Fig. 3A). Levels of integrins β1 and α5, but not those of integrin α6, in melanocytes were decreased by neutralization using anti-SCF antibody (*p*<0.05; Fig. 3B). Adhesion assay showed that the increase in the number of attached melanocytes to fibronectin and laminin by coculturing with ADSCs was inhibited by anti-bFGF antibody or anti-SCF antibody (*p*<0.05; Fig. 3C, 3D, respectively).

Grafting of melanocyte-ADSC cocultures increased levels of growth factors and integrins in nude mice

To confirm these *in vitro* results described above, immunofluorescence staining was performed using biopsied skin specimens obtained from nude mice grafted same number of cells (melanocyte+ADSC cocultured at a ratio of 1:2 ratio, melanocyte monocultures, or ADSC monocultures) after culturing under the same condition as *in vitro* with using anti-bFGF, anti-SCF, anti-integrin β1, anti-integrin α5, or anti-integrin α6 antibody. Because grafted melanocytes were mostly identified in the dermis of nude mice (Lim *et al*., 2014), staining intensities were measured in five randomly selected high-power fields (×400) of the dermis using ImageJ software. Staining intensity against anti-bFGF or anti-SCF antibody was much stronger in skin specimens of mice grafted with melanocyte+ADSC cocultures than that in skin specimens of mice grafted with ADSC monocultures or melanocyte monocultures (*p*<0.05; Fig. 4A). The intensity against anti-integrin β1, anti-integrin α5, or anti-integrin α6 antibody was also higher in skin specimens from mice grafted with cocultures than that in specimens from mice grafted with ADSC monocultures or melanocyte monocultures (*p*<0.05; Fig. 4B).

DISCUSSION

Proliferation of melanocytes by coculturing with ADSCs in our previous study (Kim *et al*., 2012) suggests a direct or indirect cell-cell interaction between melanocytes and ADSCs. Melanocytes require growth factors for their proliferation (Jim-
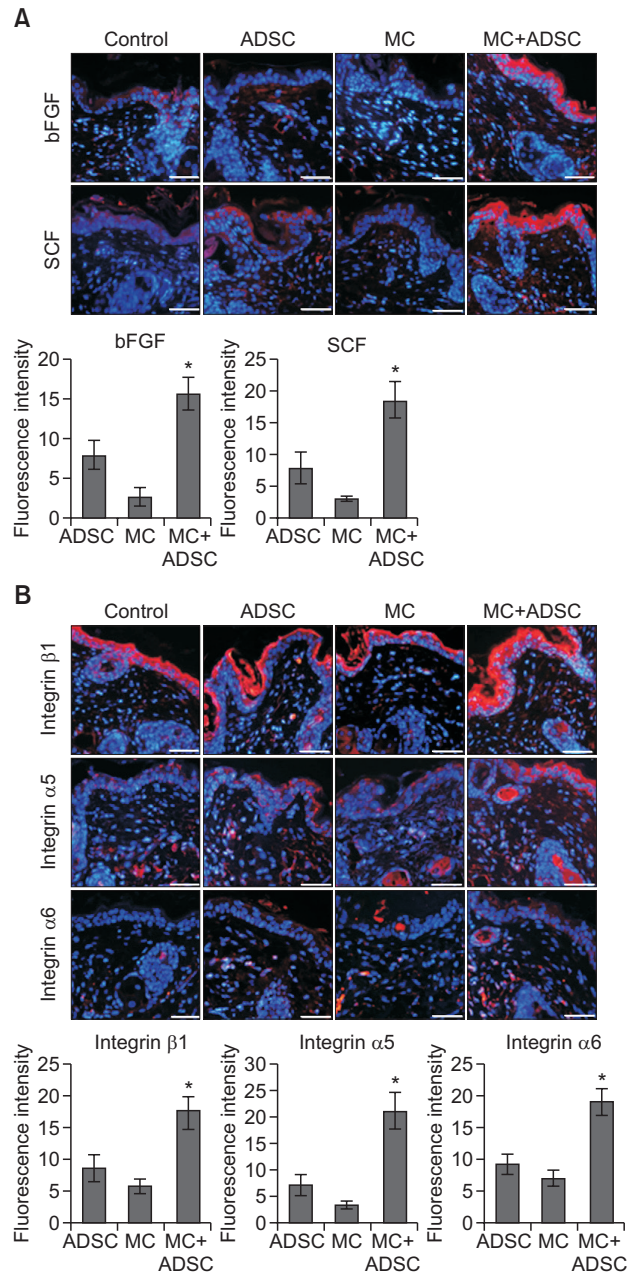
Fig. 4. Grafting of melanocyte-ADSC cocultures increased levels of growth factors and integrins in nude mice. Representative immunofluorescent staining using anti-bFGF or anti-SCF (A) and anti-integrin β1, anti-integrin α5, or anti-integrin α6 (B) in skin specimens of nude mice grafted with ADSC monocultures (ADSC), melanocyte monocultures (MC), or melanocyte+ADSC cocultures (MC+ADSC). Nuclei were count-stained with Hoechst 33258 (Bar=0.05 mm). Intensities were measured in five randomly selected high-power fields (x400) of the dermis using Wright Cell Imaging Facility ImageJ software. Data in each graph represent mean ± SD from five nude mice. *p<0.05 vs ADSC.

bow *et al*., 1975; Pawelek, 1979). bFGF and SCF are well-known growth factors for melanocytes. This study showed that relative levels of bFGF and SCF in melanocyte+ADSC cocultures were higher than those in ADSC monocultures as

well as the sum of those in ADSC monocultures and melanocyte monocultures (Fig. 1A, 1B). Melanocyte monoculture did not produce or release detectable amount of these growth factors (Fig. 1A-1C), suggesting that the cell-cell interaction between melanocytes and ADSCs might have enhanced the production and release of growth factors from ADSCs. Melanocytes have receptors for these growth factors (Halaban *et al.*, 1992). Growth factors released extracellularly could play a critical role in cell-cell interaction between melanocytes and ADSCs, leading to melanocyte growth and proliferation. Stronger staining intensities against anti-SCF or anti-bFGF antibody in melanocytes by coculturing with ADSCs (Fig. 1D) might indicate an uptake of increased growth factors by receptors in melanocytes. In addition, nude mice skin specimens grafted with melanocyte+ADSC cocultures showed stronger staining intensities against anti-bFGF and anti-SCF antibodies compared to those grafted with ADSC or melanocyte monocultures (Fig. 4A), consistent with the *in vitro* result.

Cells can attach to ECM through a family of receptors called integrins. Attachment of melanocytes from ECM is necessary for survival of melanocytes (Scott *et al.*, 1997; Zhang *et al.*, 2013). β1 integrins mainly mediate adhesion to ECM in normal human skin melanocytes (Zambruno *et al.*, 1993; Hara *et al.*, 1994). β1 integrin is also known to be an indicator of attachment in ADSCs (Ahn *et al.*, 2014; Liu *et al.*, 2014). Although levels of integrin β1 protein have not been compared between melanocytes and ADSCs, results of Western blotting in this study showed much higher levels of integrin β1 in ADSCs than those in melanocytes (Fig. 2A). Nonetheless, coculturing with ADSCs using culture dish equipped with insert without mixing melanocytes and ADSCs increased expression levels of integrin β1 in melanocytes compared to those in ADSCs (Fig. 2A). Levels of integrins α5 and α6 were also remarkably increased in melanocytes by coculturing with ADSCs (Fig. 2A, 2B). Increases in these integrins by coculturing with ADSCs were also found in nude mice skin (Fig. 4B). This result suggests that coculturing with ADSCs may facilitate adhesion of melanocyte to ECM such as fibronectin and laminin through upregulation of integrins α5β1 and α6β1, respectively (Zambruno *et al.*, 1993; Hara *et al.*, 1994; Akiyama, 1996; Scott *et al.*, 1997). In fact, adhesion assay showed that higher number of melanocytes attached in fibronectin-coated and laminin-coated culture dishes by coculturing with ADSCs. Such increase was inhibited by integrin β1 antibody (Fig. 2C). Therefore, coculturing with ADSCs could play a critical role in melanocyte adhesion to these ECMs through regulation of integrin β1 levels known to affect survival, proliferation, and migration of melanocytes (Scott *et al.*, 1997; Kumar *et al.*, 2011; Pinon and Wehrle-Haller, 2011; Ricard *et al.*, 2012; Bin *et al.*, 2016).

Cells will attach to the ECM in order to respond to soluble growth factors (Giancotti and Tarone, 2003). Regulatory role of SCF in integrin expression has been reported (Scott *et al.*, 1994). This study showed that increased levels of integrins β1, α5, and α6 in melanocytes along with cell adhesion to fibronectin and laminin by coculturing with ADSCs were reduced by anti-bFGF or anti-SCF antibody, although the effect of SCF on integrin α6 level was not significant (Fig. 3A-3D). These results indicate that these growth factors released from cocultured ADSCs play a role in integrin expression in melanocytes.

Collectively, although other factors contribute to melanocyte proliferation by coculturing with ADSCs, our results demonstrate that coculturing with ADSCs can stimulate adhesion of melanocytes to ECM by upregulating integrin levels through production/release of growth factors for melanocytes, such as SCF and bFGF.

REFERENCES

Ahn, H. H., Lee, I. W., Lee, H. B. and Kim, M. S. (2014) Cellular behavior of human adipose-derived stem cells on wettable gradient polyethylene surfaces. *Int. J. Mol. Sci.* **15**, 2075-2086.

Akiyama, S. K. (1996) Integrins in cell adhesion and signaling. *Hum. Cell* **9**, 181-186.

Bin, B. H., Kim, D. K., Kim, N. H., Choi, E. J., Bhin, J., Kim, S. T., Gho, Y. S., Lee, A. Y., Lee, T. R. and Cho, E. G. (2016) Fibronectin-containing extracellular vesicles protect melanocytes against ultraviolet radiation-induced cytotoxicity. *J. Invest. Dermatol.* **136**, 957-966.

Giancotti, F. G. and Tarone, G. (2003) Positional control of cell fate through joint integrin/receptor protein kinase signaling. *Annu. Rev. Cell Dev. Biol.* **19**, 173-206.

Halaban, R., Fan, B., Ahn, J., Funasaka, Y., Gitay-Goren, H. and Neufeld, G. (1992) Growth factors, receptor kinases, and protein tyrosine phosphatases in normal and malignant melanocytes. *J. Immunother.* **12**, 154-161.

Hara, M., Yaar, M., Tang, A., Eller, M. S., Reenstra, W. and Gilchrest, B. A. (1994) Role of integrins in melanocyte attachment and dendricity. *J. Cell Sci.* **107**, 2739-2748.

Hirobe, T. (2005) Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes. *Pigment Cell Res.* **18**, 2-12.

Hong, S. J., Traktuev, D. O. and March, K. L. (2010) Therapeutic potential of adipose-derived stem cells in vascular growth and tissue repair. *Curr. Opin. Organ. Transplant.* **15**, 86-91.

Jimbow, K., Roth, S. I., Fitzpatrick, T. B. and Szabo, G. (1975) Mitotic activity in non-neoplastic melanocytes *in vivo* as determined by histochemical, autoradiographic, and electron microscope studies. *J. Cell Biol.* **66**, 663-670.

Kim, J. Y., Park, C. D., Lee, J. H., Lee, C. H., Do, B. R. and Lee, A. Y. (2012) Co-culture of melanocytes with adipose-derived stem cells as a potential substitute for co-culture with keratinocytes. *Acta Derm. Venereol.* **92**, 16-23.

Kumar, R., Parsad, D. and Kanwar, A. J. (2011) Role of apoptosis and melanocytorrhagy: a comparative study of melanocyte adhesion in stable and unstablevitiligo. *Br. J. Dermatol.* **164**, 187-191.

Lee, A. Y., Kim, N. H., Choi, W. I. and Youm, Y. H. (2005) Less keratinocyte-derived factors related to more keratinocyte apoptosis in depigmented than normally pigmented suction-blistered epidermis may cause passive melanocyte death in vitiligo. *J. Invest. Dermatol.* **124**, 976-983.

Lim, W. S., Kim, C. H., Kim, J. Y., Do, B. R., Kim, E. J. and Lee, A. Y. (2014) Adipose-derived stem cells improve efficacy of melanocyte transplantation in animal skin. *Biomol. Ther. (Seoul)* **22**, 328-333.

Liu, J., Wang, H., Wang, Y., Yin, Y., Wang, L., Liu, Z., Yang, J., Chen, Y. and Wang, C. (2014) Exendin-4 pretreated adipose derived stem cells are resistant to oxidative stress and improve cardiac performance via enhanced adhesion in the infarcted heart. *PLoS ONE* **9**, e99756.

Olsson, M. J. and Juhlin, L. (2002) Long-term follow-up of leucoderma patients treated with transplants of autologous cultured melanocytes, ultrathin epidermal sheets and basal cell layer suspension. *Br. J. Dermatol.* **147**, 893-904.

Pawelek, J. M. (1979) Evidence suggesting that a cyclic AMP-dependent protein kinase is a positive regulator of proliferation in Cloudman S91 melanoma cells. *J. Cell Physiol.* **98**, 619-625.

Pinon, P. and Wehrle-Haller, B. (2011) Integrins: versatile receptors controlling melanocyte adhesion, migration and proliferation. *Pigment Cell Melanoma Res.* **24**, 282-294.

Ricard, A. S., Pain, C., Daubos, A., Ezzedine, K., Lamrissi-Garcia, I., Bibeyran, A., Guyonnet-Dupérat, V., Taieb, A. and Cario-André, M. (2012) Study of CCN3 (NOV) and DDR1 in normal melanocytes and vitiligo skin. *Exp. Dermatol.* **21**, 411-416.

Scott, G., Ewing, J., Ryan, D. and Abboud, C. (1994) Stem cell factor regulates human melanocyte-matrix interactions. *Pigment Cell Res.* **7**, 44-51.

Scott, G., Cassidy, L. and Busacco, A. (1997) Fibronectin suppresses apoptosis in normal human melanocytes through an integrin-dependent mechanism. *J. Invest. Dermatol.* **108**, 147-153.

Shingyochi, Y., Orbay, H. and Mizuno, H. (2015) Adipose-derived stem cells for wound repair and regeneration. *Expert Opin. Biol. Ther.* **15**, 1285-1292.

Sviderskaya, E. V., Wakeling, W. F. and Bennett, D. C. (1995) A cloned, immortal line of murine melanoblasts inducible to differentiate to melanocytes. *Development* **121**, 1547-1557.

Zambruno, G., Marchisio, P. C., Melchiori, A., Bondanza, S., Cancedda, R., De Luca, M. (1993) Expression of integrin receptors and their role in adhesion, spreading and migration of normal human melanocytes. *J. Cell Sci.* **105**, 179-190.

Zhang, R., Premi, S., Kilic, S. S., Bacchiocchi. A., Halaban, R. and Brash, D. E. (2013) Clonal growth of human melanocytes using cell-free extracellular matrix. *Pigment Cell Melanoma Res.* **26**, 925-927.

Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., Alfonso, Z. C., Fraser, J. K., Benhaim, P. and Hedrick, M. H. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* **13**, 4279-4295.