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Cell Biological Function of Secretome of Adipose-Derived Stem Cells on Human Dermal Fibroblasts and Keratinocytes

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The beneficial effects of adipose-derived stem cell conditioned media (ADSC-CM) for skin regeneration have previously been reported, despite the precise mechanism of how ADSC-CM promotes skin regeneration remaining unclear. ADSC-CM contains various secretomes and this may be a factor in it being a good resource for the treatment of skin conditions. It is also known that ADSC-CM produced in hypoxia conditions, in other words Advanced Adipose-Derived Stem cell Protein Extract (AAPE), has excellent skin regenerative properties. In this study, a human primary skin cell was devised to examine how AAPE affects human dermal fibroblast (HDF) and human keratinocyte (HK), which both play fundamental roles in skin regeneration. The promotion of collagen formation by HDFs was observed at 0.32 mg/ml of AAPE. AAPE treatment significantly stimulated stress fiber formation. DNA gene chips demonstrated that AAPE in HKs ($p<0.05$) affected the expression of 133 identifiable transcripts, which were associated with cell proliferation, migration, cell adhesion, and response to wounding. Twenty five identified proteins, including MMP, growth factor and cytokines such as CD54, FGF-2, GM-CSF, IL-4, IL-6, VEGF, TGF-β2, TGF-β3, MMP-1, MMP-10, and MMP-19, were contained in AAPE via antibody arrays. Thus, AAPE might activate the HK biological function and induce the collagen synthesis of HDF. These results demonstrate that AAPE has the potential to be used for clinic applications aimed at skin regeneration.

Keywords: ADSC-CM, skin regeneration, stress fiber, collagen synthesis

Introduction

Adipose-derived stem cells (ADSC) derived from human subcutaneous adipose tissue are a population of pluripotent mesenchymal cells and possess the ability to differentiate into various lineages. The secretome of ADSCs repair and replace the defective surrounding cells. Thus, secretome itself is a good resource for skin regeneration, re-epithelialization, wound healing, and wrinkling care. Conditioned medium from ADSCs (ADSC-CM) contained various growth factors secreted from ADSC [12] and has excellent advantages for the treatment of skin problems such as skin scar, replacement and regeneration. ADSC-CM can be applied for biotechnology such as skin care product like cosmetic, protein drug industries.

Oxygen deficiency, *i.e.*, hypoxia, may have negatively affected cell biological action. However, cellular functions to hypoxic stress are highly dependent in cell type, position and micro-environment. ADSCs are thought to reside in hypoxic position covered with various tissues in complicated 3-dimensional space of the human body. Therefore, when ADSCs are cultured under hypoxic conditions *in vitro* their proliferative and self-renewal capacities are significantly improved [18] and hypoxia enhanced the expression of certain secretome [7].

Therefore, we focused on Advanced Adipose-Derived Stem cell Protein Extract (AAPE), which is conditioned medium produced under a hypoxia of ADSCs. Human dermal fibroblasts (HDF) and human HKs (HK) play an important role in skin biology such as wound re-epitheliali-

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zation, the re-establishment and wound healing of the skin [15, 17, 26]. HKs with normal dermal fibroblasts must be lead to upregulation of mRNA for collagen type I and III, increased fibroblast proliferation, and extracellular matrix accumulation [27]. Thus, cell biological function of both cells is essential for performing regenerative processes on the skin surface. In this study, we examined the cell biological function of AAPE on HDF and HK *in vitro*, and the components of AAPE through antibody array analysis.

Materials and Methods

Stem cell culture and AAPE production

Human subcutaneous adipose tissue derived stem cells (ADSC) were prepared from Prostemics Research Institute (Sungnam, Korea) followed by characteristic expressions of stem cell-related surface markers were confirmed by flow cytometry [15, 16] and adipogenic, osteogenic, and chondrogenic differentiation were also checked by the conventional method [11, 13]. ADSCs were cultured and expanded in normal control medium, and used for the experiments at passages 4. Cells were finally frozen in aliquots using CellFreezer^{TM} (Genenmed, Seoul, Korea) for the future. To produce a ADSC-CM (AAPE^{TM}), a frozen vial containing 1×10^6 cells were launched onto culture medium containing 10% FBS. After repeating subcultures to reach 5×10^8 cells, the expanded ADSCs were introduced into CellFactory^{TM} CF10 (Nunc, Rochester, NY, US) in DMEM/F12 serum-free medium (Welgene, Taegu, Korea). Cultures were conducted under a hypoxia by providing 2% O_2 using N_2 gas supply in a humidified multichannel incubator during 2 weeks. The conditioned media were collected and micro-filtered, followed by quantitated total protein production. Finally, for fresh use, 4 ml vials containing equal protein concentration were freeze-dried as a single lot sample preparation of AAPE (Prostemics Research Institute, Sungnam, Korea) for this study.

Cell culture

Normal HK was purchased from ATCC cell bank (ATCC# PCS-200-011). HKs were cultured in serum-free HK media with epidermal growth factor at concentrations of 0.2% (v/v) of bovine pituitary extract, 5 g/mL bovine insulin, 0.18 g/mL hydrocortisone, 5 g/mL bovine transferrin, 0.2 ng/mL human epidermal growth factor (EGF) at 37°C in a 5% CO_2 humidified atmosphere. HDF cultured primarily from human fetal skin was grown in DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin-streptomycin at 5% CO_2 and 37°C humidified atmosphere.

Cell proliferation

Cell viability and proliferation was determined using CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, US). Briefly, cells (2×10^5) were placed in 96-well plastic culture plates and incubated at 37°C in 5% CO_2 for 24 h, at which point 100 μL of 0.5 mg/mL MTS solution was added to each well and incubated for 4h at 37°C. Formazan absorbance was read at 490 nm using a plate reader.

Collagen synthesis assay

HDF was inoculated into 48-well plate (1×10^5 cells/well) and cultivated for 24 h. After culture, the medium was changed to serum-free medium containing vitamin C as a positive control and AAPE at several concentrations, and then HDF were cultivated for 48 h. Control cells were cultivated without samples. After culture, supernatants of each well were collected, and the amount of procollagen type I C-peptide was measured by using a type I C-peptide EIA kit (TakaRa, Kyoto, Japan). Finally, samples were measured by ELISA reader at 450 nm wave length.

Fluorescence microscopy of stress fibers

HDF and HK on collagen coated chamber slides (Lab-Tek, Nalge Nunc Int. Naperville, IL, US) were cultured in serum-free DMEM and growth factor free HK media for 12 h, respectively. The cells were treated with the AAPE for 24 h, then fixed in formalin and treated with ice-cold methanol for 10 min. The cells were then stained with rhodamine phalloidin and observed by fluorescence microscopy.

DNA chip analysis

For this analysis with GeneChip Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA, US) containing 28,869 gene-level probe set, total cellular RNA was isolated from HK incubated with AAPE (1.25 μg/ml) for 24 h using Trizol reagent (Invitrogen, Foster, CA, US) according to manufacturer's directions. The RNA samples were submitted to the Advanced Medical Technology Center for Diagnosis & Prediction (School of Medicine, Kyungpook National University, Taegu, Korea) for array preparation and analysis.

The signal intensity of the gene expression level was calculated by Expression Console software, Version 1.1 (Affymetrix). The list was filtered first for the absent genes, secondly for a fold change cutoff of 2, and thirdly for p value of ≤0.05 using Welch's *t*-test.

Antibody array

The freeze dried protein sample was submitted to the Ebiogen (Kyung Hee business center, Kyung Hee University, Seoul, Korea) for array preparation and analysis. The explorer antibody microarray slide (Fullmoon biosystems, Sunnyvale, CA, US) with 656 antibodies and the cytokine-focused microarray slide with 77 antibodies were treated 30 ml of blocking solution and incubated on shaker for 45 minutes at room temperature. After blocking and coupling, the slide was washed 2 times with 30 ml of washing solution. The 30 μL of 0.5 mg/ml Cy3-streptavidin (GE Healthcare, Chalfont St. Giles, UK) was mixed in 30 ml of detection buffer. After detecting, the slide was washed 2 times with 30 ml of washing solution. The slide scanning was performed using Revolution^{TM} 4200 microarray scanner (Vidar Systems, Herndon, VA, US) and ArraySifter Express 1.3 (Vidar Systems). The slide was scanned at 10 μm resolution, optimal laser power and PMT. After got the scan image, it was grided and quantified with ArraySifter Express 1.3. The numeric data were analized using Genowiz 4.0^{TM} (Ocimum Biosolutions, India). After analizing, the data about protein informations were annotated using UniProt DB.

Results

HDF proliferation

AAPE is a component of ADSC-CM, cell culture medium for ADSC. Since AAPE had the effect of the cell growth, we first examined the effect of AAPE on HDF proliferation. However, AAPE concentrations ranging from 0 to 0.313 μg/ml did not show significant proliferative effect on HDF (Fig. 1). Instead, we observed inhibition of HDF proliferation at AAPE concentrations greater than 0.625 μg/ml (Fig. 1). This suggests that it is dependent on cell types about AAPE effect for cell proliferation.

Collagen synthesis in HDFs

Promotion of collagen synthesis was evaluated in HDF treated with AAPE. As shown in Fig. 2, the amount of type I collagen in the culture medium of HDF was increased by treatment with AAPE for 42 h, in a dose-dependent manner. Compared with untreated fibroblasts, 0.32 μg/ml AAPE increased collagen production of HDF by a maximum of 142.7%, whereas 35.2 μg/ml vitamin C increased collagen production by 161.0%.

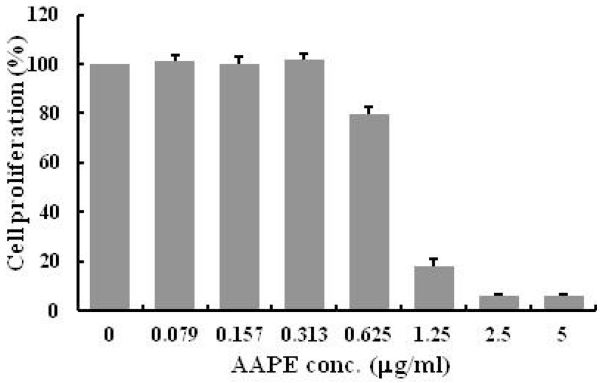

Fig. 1. Human dermal fibroblast (HDF) proliferation. The amount of HDF proliferation is represented by the cell proliferation (%) in the MTS assay (n=3). The amount HDF proliferation was significantly less in AAPE (more than 0.625 μg/ml) treated group compared to the group treated with AAPE (from 0 to 0.313 μg/ml) by Student's t-test.

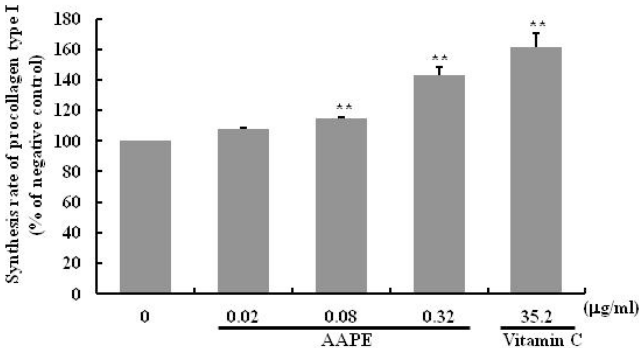

Fig. 2. Effects of AAPE and Vitamin C on synthesis rate of pro-collagen type I in normal HDF. The HDF was incubated with medium and samples. The cells were incubated for 42 h at 37°C incubator. Collagen content was measured by amount of pro-collagen type I C-peptide in the supernatant after incubation. Each value represents the mean ± S.D. of three experiments, and values containing asterisks are significantly from the nontreatment group (Cont.) at $p<0.01$(**) by Dunnett's t-test.

AAPE augments stress fiber formation in HDF and HK

Stress fibers are composed of bundles of approximately 10-30 actin filaments [6] held together by the actin-crosslinking protein such as fascin, espin and filamin [1, 4, 24, 28]. This serve as a cross-linker between the towing and

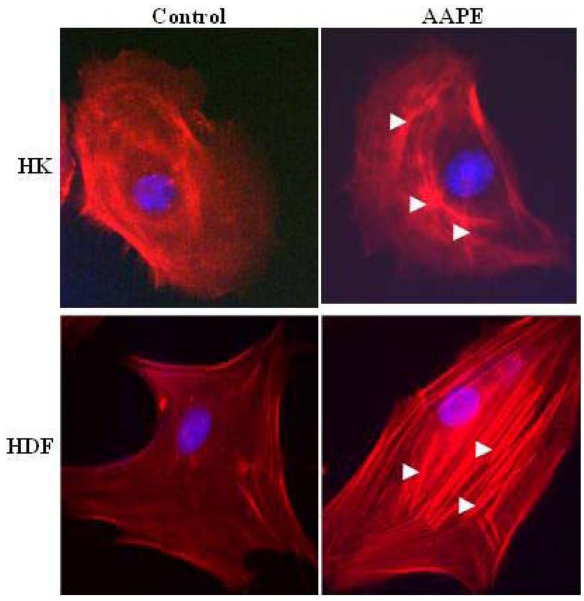

Fig. 3. AAPE induce actin stress fiber formation. HK and HDF cells were left untreated or challenged for 1 h with AAPE (1.22 μg/ml). The cells were then fixed, permeabilized, and stained with rhodamine phalloidin to visualize the actin stress fibers by fluorescence microscopy. The results are representative of two or three experiments.

trailing adhesions, and their organization reflects the direction of the traction force. In motile fibroblasts, ventral stress fibers are oriented parallel to the axis of locomotion [6], which suggests that force generated by contraction of these structures could drive tail retraction. Therefore, these structures provide mechanical contractile force for cell migration. Because stress fiber formation is a cell response characteristic of HK [28] and fibroblast [19] migration, we investigated whether stress fiber formation is induced by AAPE. Stress fiber formation was markedly enhanced by the stimulation of AAPE (Fig. 4) in both cells. We therefore propose that the induction of stress fiber requires the transduction of AAPE signals.

DNA microarray of AAPE

HKs up-regulates collagen type I and III, fibroblast proliferation, and extracellular matrix accumulation to normal dermal fibroblasts. Therefore, the activation of HK is very important about fibroblast activation. In order to address the gene alterations of HK on AAPE we compared the panel of transcripts whose expression was altered in AAPE-treated HKs compared to AAPE-untreated HKs. We screened DNA chip arrays using RNA isolated from HKs. Our results demonstrate that AAPE in HKs ($p < 0.05$) affected expression of 133 identified transcripts regulated minimally by greater than or equal to a 2-fold change. The identified transcripts were associated with 4 functional classes (Fig. 4A). Of the identified regulated genes, 94 were up-regulated (Fig. 4C, Table 1, 2, 3, 4) and 39 were down-regulated (Fig. 4B, Table 1, 2, 3, 4). Of the genes regulated, notable fractions are known to affect cell proliferation and cell adhesion.

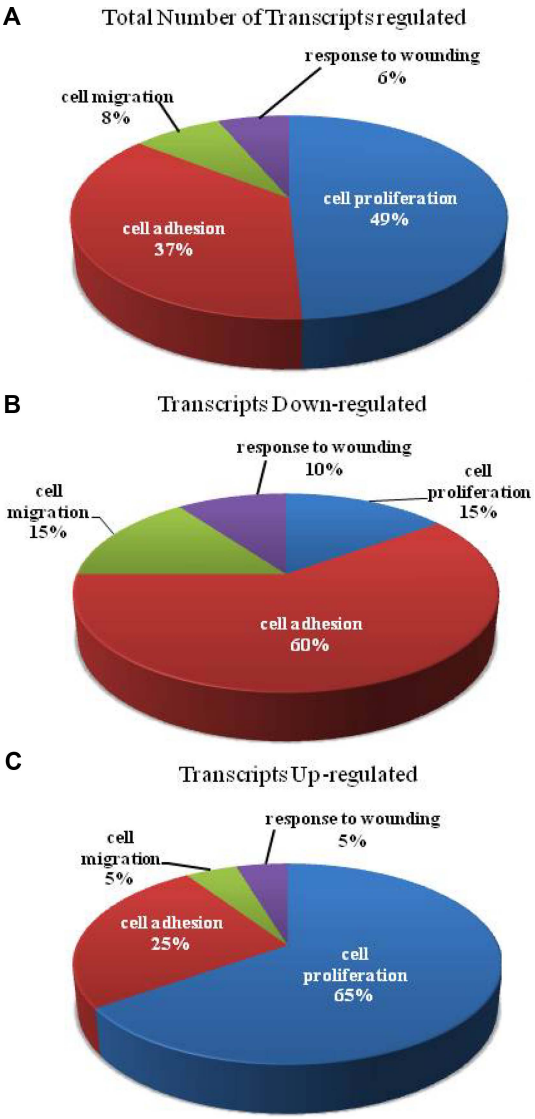

Fig. 4. DNA chip analysis. Functional classes of differentially regulated genes in HK incubated with AAPE. Regulated genes were grouped into 4 functional categories and graphed as a percentage of the total based on their GeneGo designation. 133 genes were differentially regulated based on analysis of the array data (A) Of the regulated genes, 39 were down-regulated (B) and 94 were up-regulated (C). A number of down-regulated genes (12) are associated with cell adhesion.

Table 1. Gene list related to cell proliferation.

Gene Name	Gene_info (Synonyms//chromosome//description)	Probe ID	Regulation
STIL	SIL // 1 // SCL/TAL1 interrupting locus	7915926	UP/ Positive
FGF5	HBGF-5 // 4 // fibroblast growth factor 5	8096050	UP/ Positive
TTK	MPS1 // 6 // TTK protein kinase	8120838	UP/ Positive
IL31RA	GLM-R // 5 // interleukin 31 receptor A	8105411	UP/ Positive
KIF2C	KNSL6 // 1 // kinesin family member 2C	7901010	UP/ Positive
CDCA7	JPO1 // 2 // cell division cycle associated 7	8046488	UP/ Positive
MTBP	MDM2BP // 8 // Mdm2, transformed 3T3 cell double minute 2, p53 binding protein	8148124	UP/Negative
FANCA	FAH // 16 // Fanconi anemia, complementation group A	8003503	UP/Positive
ASPM	MCPH5 // 1 // asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	7923086	UP/Positive
CYR61	CCN1 // 1 // cysteine-rich, angiogenic inducer, 61	7902687	UP/ Positive
CDC7	HsCDC7 // 1 // cell division cycle 7 homolog (S. cerevisiae)	7902913	UP/ Positive
KIF15	HKLP2// 3 // kinesin family member 15	8079237	UP/ Positive
TPX2	DIL-2 // 20 // TPX2, microtubule-associated, homolog (Xenopus laevis)	8061579	UP/ Positive
SKP2	FBL1 // 5 // S-phase kinase-associated protein 2 (p45)	8104912	UP/ Positive
PROX1	- // 1 // prospero homeobox 1	7909681	UP/ND
CDK2	p33(CDK2) // 12 // cyclin-dependent kinase 2	7956076	UP/Positive
PDCD1LG2	PD-L2 // 9 // programmed cell death 1 ligand 2	8154245	UP/Negative
PTHLH	HHM // 12 // parathyroid hormone-like hormone	7962000	UP
VEGFC	VRP // 4 // vascular endothelial growth factor C	8103822	UP
UHRF1	RNF106 // 19 // ubiquitin-like with PHD and ring finger domains 1	8024900	UP
EREG	ER // 4 // epiregulin	8095728	UP
TIMELESS	TIM // 12 // timeless homolog (Drosophila)	7964145	UP
BUB1B	BUBR1 // 15 // budding uninhibited by benzimidazoles 1 homolog beta (yeast)	7982663	UP
CXCL1	GROa// 4 // chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity)	8095697	UP
BLM	BS // 15 // Bloom syndrome, RecQ helicase-like	7986068	UP
IFITM1	CD225 // 11 // interferon induced transmembrane protein 1 (9-27)	7937335	UP
FOXM1	FKHL16 // 12 // forkhead box M1	7960340	UP
POLA1	p180 // X // polymerase (DNA directed), alpha 1, catalytic subunit	8166525	UP
TACSTD2	TROP2 // 1 // tumor-associated calcium signal transducer 2	7916584	UP
CHEK1	CHK1 // 11 // CHK1 checkpoint homolog (S. pombe)	7945014	UP
ZFP36L2	TIS11D // 2 // zinc finger protein 36, C3H type-like 2	8051814	UP
NCAPG2	MTB // 7 // non-SMC condensin II complex, subunit G2	8144153	UP
BUB1	BUB1A // 2 // budding uninhibited by benzimidazoles 1 homolog (yeast)	8054580	UP
BCL6	ZNF51 // 3 // B-cell CLL/lymphoma 6	8092691	UP/Positive
FGFBP1	HBP17 // 4 // fibroblast growth factor binding protein 1	8099467	UP
HELLS	PASG // 10 // helicase, lymphoid-specific	7929438	UP
GINS1	PSF1 // 20 // GINS complex subunit 1 (Psf1 homolog)	8061471	UP
MKI67	KIA // 10 // antigen identified by monoclonal antibody Ki-67	7937020	UP
NF2	SCH // 22 // neurofibromin 2 (merlin)	8072242	UP
DLGAP5	DLG7 // 14 // discs, large (Drosophila) homolog-associated protein 5	7979307	UP
NASP	PRO1999 // 1 // nuclear autoantigenic sperm protein (histone-binding)	7901123	UP
TGFBR2	TGFR-2 // 3 // transforming growth factor, beta receptor II (70/80kDa)	8078350	UP
CENPF	hcp-1 // 1 // centromere protein F, 350/400ka (mitosin)	7909708	UP
BRCA2	GLM3 // 13 // breast cancer 2, early onset	7968484	UP
RACGAP1	ID-GAP // 12 // Rac GTPase activating protein 1	7963157	UP
CDC25A	CDC25A2 // 3 // cell division cycle 25 homolog A (S. pombe)	8086880	UP
BRCA1	PSCP // 17 // breast cancer 1, early onset	8015769	UP
PLK1	STPK13 // 16 // polo-like kinase 1 (Drosophila)	7994109	UP
EPGN	EPG // 4 // epithelial mitogen homolog (mouse)	8095723	UP
CKS2	CKSHS2 // 9 // CDC28 protein kinase regulatory subunit 2	8156290	UP
PCNA	MGC8367 // 20 // proliferating cell nuclear antigen	8064844	UP

Table 1. Continued.

Gene Name	Gene_info (Synonyms//chromosome//description)	Probe ID	Regulation
TCF19	SC1 // 6 // transcription factor 19	8179228	UP
UTP20	DRIM // 12 // UTP20, small subunit (SSU) processome component	7957890	UP
NRP1	CD304 // 10 // neuropilin 1	7932985	DOWN
L1CAM	SPG1 // X // L1 cell adhesion molecule // protein-coding	8175871	DOWN
VIPR1	VPAC1 // 3 // vasoactive intestinal peptide receptor 1	8079060	DOWN
DDR2	TKT // 1 // discoidin domain receptor tyrosine kinase 2	7906900	DOWN
WARS	IFP53 // 14 // tryptophanyl-tRNA synthetase	7981290	DOWN
ANG	RNASE4 // 14 // angiogenin, ribonuclease, RNase A family, 5	7973084	DOWN
FGF2	HBGF-2 // 4 // fibroblast growth factor 2 (basic)	8097256	DOWN
ADM	AM // 11 // adrenomedullin	7938390	DOWN
GPNMB	HGFIN|NMB // 7 // glycoprotein (transmembrane) nmb	8131844	DOWN
LRP1	CD91// 12 // low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)	7956301	DOWN
LAMP3	CD208 // 3 // lysosomal-associated membrane protein 3	8092348	DOWN
HBEGF	HEGFL // 5 // heparin-binding EGF-like growth factor	8114572	DOWN
IGFBP4	IBP4 // 17 // insulin-like growth factor binding protein 4	8007100	DOWN

Table 2. Gene list related to cell adhesion.

Gene Name	Gene_info (Synonyms//chromosome//description)	Probe ID	Regulation
CCL2	MCP-1 // 17 // chemokine (C-C motif) ligand 2	8006433	UP
S100A4	FSP1 // 1 // S100 calcium binding protein A4	7920271	UP
FAR2	SDR10E2 // 12// fatty acyl CoA reductase 2	7954631	UP
CTGF	NOV2 // 6 // connective tissue growth factor	8129562	UP
ITGB8	- // 7 // integrin, beta 8	8131666	UP
ITGB6	- // 2 // integrin, beta 6	8056184	UP
BCL6	ZNF51 // 3 // B-cell CLL/lymphoma 6	8092691	UP
LOXL2	WS9-14 // 8 // lysyl oxidase-like 2	8149774	UP
CYR61	CCN1 // 1 // cysteine-rich, angiogenic inducer, 61	7902687	UP
HAPLN3	EXLD1 // 15 // hyaluronan and proteoglycan link protein 3	7991224	UP
NF2	SCH // 22 // neurofibromin 2 (merlin)	8072242	UP
ITGA4	CD49D// 2 // integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	8046695	UP
NID2	- // 14 // nidogen 2 (osteonidogen)	7979133	UP
THY1	CD90 // 11 // Thy-1 cell surface antigen	7952268	UP
DSG1	DG1 // 18 // desmoglein 1	8020724	UP
CLDN1	SEMP1 // 3 // claudin 1	8092726	UP
APAF1	CED4 // 12 // apoptotic peptidase activating factor 1	7957759	UP
PDZD2	PAPIN // 5 // PDZ domain containing 2	8104693	UP
CDH11	OSF-4 // 16 // cadherin 11, type 2, OB-cadherin (osteoblast)	8001800	UP
CLDN7	CEPTRL2 // 17 // claudin 7	8012126	DOWN
NRP1	CD304 // 10 // neuropilin 1	7932985	DOWN
COL21A1	COLA1L // 6 // collagen, type XXI, alpha 1	8127201	DOWN
L1CAM	SPG1 // X // L1 cell adhesion molecule	8175871	DOWN
DDR2	MIG20a // 1 // discoidin domain receptor tyrosine kinase 2	7906900	DOWN
CDH4	RCAD // 20 // cadherin 4, type 1, R-cadherin (retinal)	8063796	DOWN
CDSN	HTSS // 6 // corneodesmosin	8124862	DOWN
LAMB4	- // 7 // laminin, beta 4	8142232	DOWN
B4GALNT2	GALGT2 // 17 // beta-1,4-N-acetyl-galactosaminyl transferase 2	8008172	DOWN
GPNMB	NMB // 7 // glycoprotein (transmembrane) nmb	8131844	DOWN
THBS3	TSP3 // 1 // thrombospondin 3	7920664	DOWN
FTL	MGC71996 // 19 // ferritin, light polypeptide	8030171	DOWN
SLAMF7	CD319 // 1 // SLAM family member 7	7906613	DOWN
CERCAM	GLT25D3 // 9 // cerebral endothelial cell adhesion molecule	8158250	DOWN
RND3	ARHE // 2 // Rho family GTPase 3	8055688	DOWN

Table 3. Gene list related to cell migration.

Gene Name	Gene_info (Synonyms//chromosome//description)	Probe ID	Regulation
VEGFC	Flt4-L // 4 // vascular endothelial growth factor C	8103822	UP
CCL2	MCP-1 // 17 // chemokine (C-C motif) ligand 2	8006433	UP
NF2	BANF // 22 // neurofibromin 2 (merlin)	8072242	UP
CTGF	CCN2 // 6 // connective tissue growth factor	8129562	UP
AMOT	KIAA1071 // X // angiomotin	8174576	UP
ITGA4	CD49D// 2 // integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	8046695	UP
NEXN	NELIN // 1 // nexilin (F actin binding protein)	7902495	UP
SLIT2	SLIL3 // 4 // slit homolog 2 (Drosophila)	8094301	UP
THY1	CD90 // 11 // Thy-1 cell surface antigen	7952268	UP
ANG	RNASE5 // 14 // angiogenin, ribonuclease, RNase A family, 5	7973084	DOWN
HBEGF	HEGFL // 5 // heparin-binding EGF-like growth factor	8114572	DOWN
L1CAM	CD171 // X // L1 cell adhesion molecule	8175871	DOWN

Table 4. Gene list related to response to wounding.

Gene Name	Gene_info (Synonyms//chromosome//description)	Probe ID	Regulation
PLAT	TPA // 8 // plasminogen activator, tissue	8150509	UP
CXCL1	CYB1//4//chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity)	8095697	UP
CCL2	MCP-1 // 17 // chemokine (C-C motif) ligand 2	8006433	UP
MAP2K3	MEK3 // 17 // mitogen-activated protein kinase kinase 3	8005707	UP
ELK3	ERP // 12 // ELK3, ETS-domain protein (SRF accessory protein 2)	7957665	UP
HRH1	H1-R // 3 // histamine receptor H1	8077851	UP
EREG	ER // 4 // epiregulin	8095728	UP
PLSCR4	TRA1 // 3 // phospholipid scramblase 4	8091306	UP
CTGF	NOV2 // 6 // connective tissue growth factor	8129562	UP
SLC7A2	HCAT2 // 8 // solute carrier family 7 (cationic amino acid transporter)	8144786	UP
ITGB6	ITGB6 // 2 // integrin, beta 6	8056184	UP
KRT1	KRT1A // 12 // keratin 1	7963491	UP
BCL6	LAZ3 // 3 // B-cell CLL/lymphoma 6	8092691	UP
TFPI2	REF1 // 7 // tissue factor pathway inhibitor 2	8141016	UP
SYT7	IPCA-7 // 11 // synaptotagmin VII	7948588	DOWN
CD55	DAF // 1 // CD55 molecule, decay accelerating factor for complement	7909332	DOWN
ADM	AM // 11 // adrenomedullin	7938390	DOWN
C1RL	CLSPa // 12 // complement component 1, r subcomponent-like	7960757	DOWN
HBEGF	HEGFL // 5 // heparin-binding EGF-like growth factor	8114572	DOWN
NFE2L1	NRF1 // 17 // nuclear factor (erythroid-derived 2)-like 1	8008087	DOWN
PTX3	TSG-14 // 3 // pentraxin-related gene, rapidly induced by IL-1 beta	8083594	DOWN
BLNK	SLP65 // 10 // B-cell linker	7935270	DOWN

Proteome analysis via antibody array

In order to visualize protein expression for proteome analysis, an explorer antibody array chip coated with 656 antibodies was conducted to identify proteins in AAPE (Fig. 5). Protein level measurements were clustered by the ratio of hormone (Fig. 5A), cytokine (Fig. 5B), cell cycle (Fig. 5C), cancer marker (Fig. 5D), apoptosis (Fig. 5E), angiogenesis (Fig. 5F), stem cell (Fig. 5G), and signal transduction (Fig. 5H) level by comparing the signal intensity of soluble protein expression in AAPE to a negative control. Furthermore, a cytokine-focused antibody array with 77 antibodies was conducted to identify proteins that may be involved in mediating the effect of AAPE on proliferation, migration or regeneration (Fig. 6) by comparison with explorer antibody array data. p value < 0.05 was considered significantly. Analysis of the array results revealed high protein expression level of VEGF, TGF-β2, CD54 and TGF-β3 compared to HGF, shows low protein expression level of FGF-1, FGF-2, IL-4, IL-6, G-CSF, GM-CSF compared to HGF (Fig. 6). HGF is composed of 60 kDa alpha-chain and a 34 kDa beta-chain and induced HK in vitro scratch-wound healing in a dose-dependent manner [21].

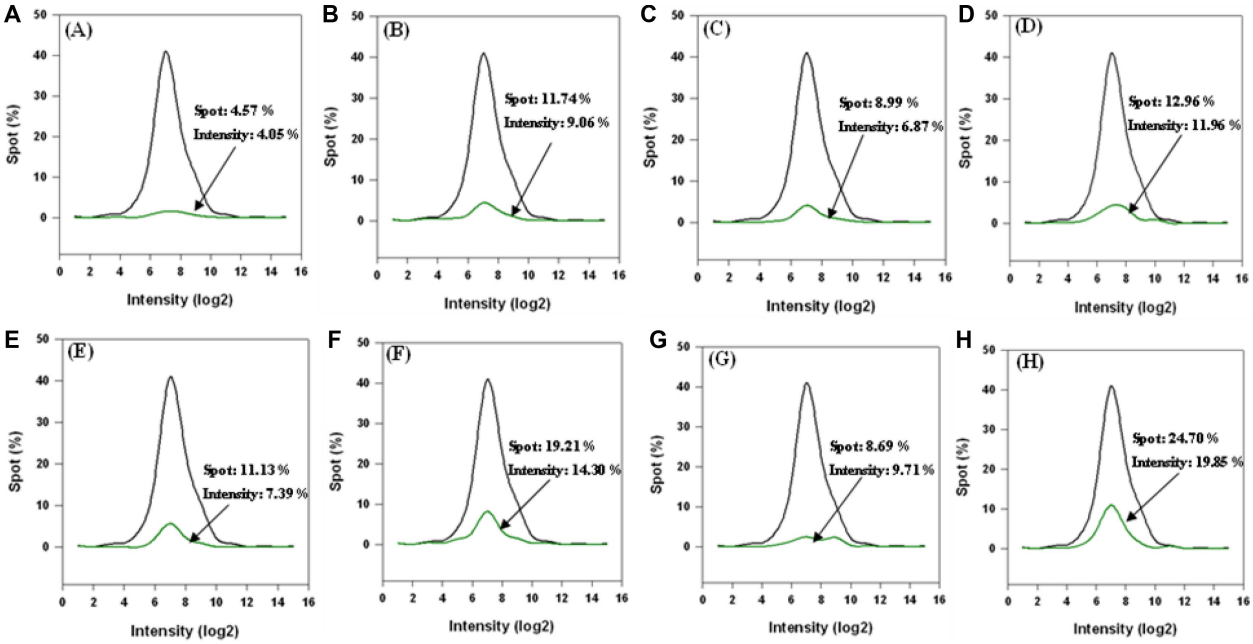

Fig. 5. Antibody array of AAPE. Result of quantile normalization with explorer array data. Total data in explorer array: black line; hormone, cytokine, cell cycle, cancer marker, apoptosis, angiogenesis, stem cell, and signal transduction data in explorer array: green line.

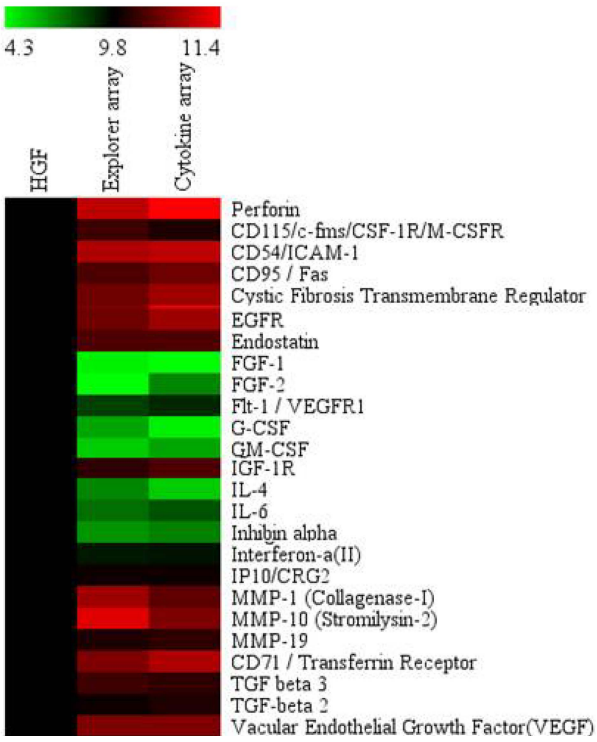

Fig. 6. Heatmap analysis of AAPE followed by antibody array. AAPE was subjected to antibody array. Portions of the array illustrating the differential expression of proteins between HGF and sample antibody are shown. Heat map were analyzed by Genowiz 4.0TM as described in Materials and Methods. The median streptavidin-Cy3 fluorescence from all microsphere subsets was exported. The heat map shows antibody reactivity intensity (*i.e.*, values above HGF) as red pixels and (values below HGF) as green pixels.

IL-6 has a proliferative and migration effect on mouse HKs [23, 8]. GM-CSF also stimulates of HK proliferation, granulation tissue formation, and vascularization [14]. TGF-β3 promotes wound healing by recruiting fibroblast to the wound site. Other protein contained AAPE. Anyway, these proteins support evidences of the application of AAPE in the skin regeneration.

Disscusion

In this study, the ability of ADSC secretomes to promote skin cell regeneration was investigated. We called condition-medium of ADSC obtained under hypoxia condition an AAPE. Skin wound healing is a complex process having combined efforts of various types and lineages of skin cells, ECMs, and soluble factors. Immune activation, inflammation, re-epithelialization, ECM reorganization and tissue remodeling are sequential events to repair skin wounds. HKs stimulated during wound repair secrete several growth factors and multiple cytokines that activate fibroblasts and endothelial cells, initiate the infiltration of immune cells, and produce systemic effects [22, 25]. They also produce extracellular matrix proteins, and adhesive molecules. This study investigated whether or not locally applied AAPE can accelerate skin regeneration process in vitro system. The collagen neo-synthesis of HDF, which is

one major cell type present in skin, was increased by AAPE treatment. AAPE are thought to trigger the proliferation, cell migration, response to wound, and adhesion of HK through DNA chip assay. Moreover, AAPE induced the stress fiber formation of HK that are able to migrate efficiently (Fig. 4). Cell mobility is generally described as a periodic process between alternating phases of protrusion and adhesion. Cell adhesion provides the direction point required for generating pulling forces, and the cell migrates forward direction by the tension driven by contraction of the cell body and retraction of the tail [20]. Because stress fiber supplies contractile force derived from the contractile nature, cell movement is dependent on reorganization of cell cytoskeleton, predominantly actin filaments. Thus, the stress fiber was formed during cell traffic, suggesting that this architecture might be important for efficient cell motility. The regeneration ability of AAPE is owed to several secretome identified by proteome analysis via an antibody array. Hepatocyte growth factor (HGF) triggers influx of neutrophils, monocytes and mast cells into wounded areas [3] and facilitates the release of pro-angiogenesis factors [5]. Scratch assays were performed in the presence and absence of HGF treatment to assess the response of HaCaT cells to HGF. PAI-1, a member of the serine protease inhibitor (serpin) superfamily, involved in wound healing [9]. *In vivo* introduction of the IL-6 gene into human HKs induces epidermal proliferation [23]. CTGF expression is increased during wound repair and is involved in inflammation, matrix deposition and remodeling in human dermal fibroblast [10]. In skin wounds, granulocyte-macrophage colony stimulating factor (GM-CSF) mediates HK proliferation at the wound edges [14]. Many other cells are involved in wound healing including macrophages, lymphocytes, fibroblasts, endothelial cells, and dendritic cells, which is target for GM-CSF. Therefore, GM-CSF is a pleiotropic cytokine controlling organized processes during wound repair. AAPE also contained TGF-β3, which promotes wound healing by facilitating HK migration of *in vivo* studies [2]. Collectively, present study provides the clues of the regenerative effect of the AAPE. However, for the proper application of AAPE into skin biology, more *in vivo* studies about the action mechanisms of AAPE on skin are needed and clinic standard application method of AAPE should be established. In conclusion, the present researches have been reported about HDF and HK biological effect of AAPE *in vitro*. AAPE activates HK proliferation and migration. Moreover, AAPE induces the collagen synthesis of HDF. Thus, AAPE is suitable for application in the treatment of skin problems.

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국문초록

인체 섬유아세포 및 케라티노사이트에 대한 지방줄기세포 분비물의 세포생물학적 기능

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피부재생에 대한 지방줄기세포 배양상등액(ADSC-CM)의 효능에 대한 연구를 진행하였다. ADSC-CM이 피부재생에 기여하는 기작은 명확하지 못하지만, ADSC-CM은 다양한 분비물을 포함하고 있고 따라서 피부트러블 처리를 위한 훌륭한 재료이다. 저 산소 상태에서 생산된 ADSC-CM, 즉 advanced adipose-derived stem cell protein extract (AAPE)는 피부재생에 보다 좋은 재료이다. 본 연구는 피부 재생에 결정적 역할을 하는 인체 primary 세포인 섬유아세포(HDF)와 케라티노사이트(HK)를 이용하여 AAPE의 효능을 검증하였다. 0.32 μg/ml AAPE에서 콜라겐 합성이 관찰 되었으며 AAPE는 stress fiber 형성을 강화하였다. DNA microarray 결과에서는 세포증식, 세포이동, 세포부착, 상처반응에 관여하는 133개의 유전자 발현이 조절되는 것을 알았다. Antibody array를 통해 CD54, FGF-2, GM-CSF, IL-4, IL-6, VEGF, TGF-β2, TGF-β3, MMP-1, MMP-10, 그리고 MMP-19와 같은 MMP, 성장인자, 사이토카인등 25개의 알려진 단백질이 포함되어 있다는 것을 알았다. 따라서, AAPE는 HK의 세포생물학적 기능을 활성화 할 수 있다고 사료되며 HDF에서는 콜라겐 합성을 유도하였다. 이러한 결과는 AAPE가 피부재생에 임상적 적용이 가능하리라는 것을 의미한다.