의학석사 학위논문

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In Vitro Comparison of Early and Late Phase Endothelial Progenitor Cells Induced from Adipose-derived Stem Cells

by

WANG FANG

A thesis submitted to the Department of Plastic and Reconstructive Surgery in partial fulfillment of the requirements for the Degree of Master of Science in Department of Plastic and Reconstructive Surgery at Seoul National University College of Medicine

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Approved by Thesis Committee

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ABSTRACT

In Vitro Comparison of Early and Late Phase Endothelial Progenitor Cells Induced from Adipose-derived Stem Cells

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Adipose-derived stem cells (ADSCs) are multipotent cells that can differentiate into endothelial progenitor cells (EPCs), which are essential for new vessel formation. EPCs can also be incorporated into blood vessels to promote neovascularization. This study aims to investigate the characteristics of the EPCs differentiated from ADSCs and identifying early and late phase EPCs-distinguishing characteristics for our future in vivo neovascularization study. ADSCs were isolated from adipose tissue and cultured to passage 1 (ADSCs P1) in complete DMEM. ADSCs P1 were then induced to differentiate into EPCs by culturing in EGM-2 BulletKit for 9 weeks. A microscopic examination of ADSCs P1 and EPCs collected at the 3-week (EPCs-3w) and 9-week (EPCs-9w) time points revealed differences in morphology between these 3 subsets. The gene expression levels of CD31, vWF, VE-cadherin, and VEGFR-2 were also different. The proliferation rate of EPCs was higher than that of ADSCs P1. Notably, EPCs-3w showed the

highest proliferation rate, which then decreased with time of differentiation. Based on these comparisons, EPCs-3w and EPCs-9w were defined as the early phase EPCs and the late phase EPCs, respectively. Fluorescence staining with 1, 1′-Dioctadecyl-3, 3, 3′, 3′-Tetramethylindocarbocyanine (Dil) was also performed. Cells were observed 1-and 3-d after Dil labeling to evaluate their ability to take up Dil. In conclusion, we compared EPCs from successive weeks of differentiation from ADSCs, and identified two phases (early phase and late phase) EPCs with distinct differences.

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Key words: adipose derived stem cells (ADSCs), endothelial progenitor cells (EPCs), early phase EPCs, late phase EPCs, neovascularization.

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INTRODUCTION

Neovascularization can be divided into three distinct physiological processes: angiogenesis, vasculogenesis, and arteriogenesis (1). Vasculogenesis refers to the formation of blood vessels induced by circulating endothelial progenitor cells. Angiogenesis and arteriogenesis refer to the sprouting and subsequent stabilization of these sprouts by endothelial cells, and collateral growth denotes the expansive growth of pre-existing vessels, forming collateral bridges between arterial networks (2).

The existence of adipose derived stem cells (ADSCs) in adipose tissue was reported in 1984 (3). Since then, many researchers started to explore the isolation, purification, expansion, differentiation, and extensive characterization of ADSCs. ADSCs were found to be similar to bone marrow derived stem cells (BMSCs). Both of these are multipotent stem cell subsets that can differentiate into hepatocytes, osteoblasts, chondrocytes, neurons, endothelial cells and other cell types (4-7). ADSCs present many advantages. They can be harvested, handled, and induced to proliferate easily, effectively, and noninvasively. Their pluripotency and proliferative efficiency is identical to that of BMSC. Finally, donor morbidity is lower than that in the case of mesenchymal stem cells (MSCs) harvested from other sites (8). The possibility of ADSCs enhancing neovascularization has been described (9). Many researchers have demonstrated the neovascular potential of ADSCs, in particular, their ability to release many potent angiogenic factors that promote endothelial cells proliferation and migration into the perivascular stroma and induce tubule formation by endothelial cells in vitro (10).

Endothelial progenitor cells (EPCs) have been isolated from human peripheral blood, and were

found to be incorporated into sites of active angiogenesis andincrease collateral vessel growth in ischemic tissues (11). EPCs were also reported to circulate from bone marrow to incorporate into and thus contribute to the postnatal physiological and pathological neovascularization consistent with postnatal vasculogenesis (12). In addition, an animal study showed that local BMSCs implantation induces a neovascular response resulting in a significant increase of blood flow to the ischemic limb (13). However, the relatively low abundance, small tissue volume, difficult accessibility, and disease-related malfunction of BMSCs make their clinical application difficult.

Studies have shown that transplanted ADSCs can differentiate into endothelial cells that are incorporated into blood vessels to promote neovascularization of hindlimb ischemia in murine models (14-16). ADSCs were also shown to proliferate at a faster rate in EGM-2 than in standard DMEM, expressthe endothelial markers CD31, vWF, and eNOS, form tube-like structures in Matrigel, and endocytose Ac-LDL (17). In addition, Hur et al (18) cultured two different types of EPCs (early EPCs and late EPCs) from peripheral blood according to their time-dependent appearance. Although these subsets showed different morphology, proliferation rates, survival features, and gene expression profiles, they shared some endothelial phenotypes and similarly contributed to neovasculogenesis in vivo. Early EPCs secreted angiogenic cytokines, whereas late EPCs supplied a sufficient number of endothelial cells (18). However, the number of mononuclear cells isolated from peripheral blood was very small.

In our study, EPCs were differentiated from ADSCs, which can be isolated in high numbers from adipose tissue. Cell morphology, expression of endothelial markers, and proliferation rate of ADSCs P1 and EPCs were evaluated to select early and late phase EPCs and to compare their

neovascularization potential with that of ADSCs. ADSCs were isolated from transverse rectus abdominal musculocutaneous (TRAM) flap adipose tissue discarded during reconstructive surgery and cultured to P1 in complete DMEM. ADSCs P1 were then induced to differentiate into EPCs for 9 weeks (EPCs-1w to EPCs-9w) in the EGM-2 BulletKit. Next, the morphology of ADSCs P1 and EPCs-1w to EPCs-9w was examined by microscopic analysis. Gene expression of endothelial markers of CD31, vWF, VE-cadherin, and VEGFR-2 was also determined by PCR. In addition, their proliferation rate was indirectly evaluated using CCK-8. Based on these comparisons, EPCs-3w and EPCs-9w were defined as the early phase EPCs and the late phase EPCs respectively. Lastly, ADSCs P1, EPCs-3w, and EPCs-9w populations were labeled with 1, 1′-Dioctadecyl-3, 3, 3′, 3′-Tetramethylindocarbocyanine (Dil) staining to evaluate their Dil uptake ability.

MATERIALS AND METHODS

ADSCs isolation and culture

All protocols were approved by the institutional review board (IRB) of Seoul National University Hospital. After obtaining informed consent from patients, ADSCs were harvested from adipose tissue discarded during reconstructive surgery, such as the TRAM flap used for breast reconstruction after mastectomy. The adipose tissue was rinsed extensively with 1×Phosphate Buffered Saline (PBS, Gibco-BRL, Grand Island, NY, USA) containing a 1% antibiotic-antimycotic (AA, WelgeneInc, Daegu, Republic of Korea) to remove contaminating hematopoietic cells. It was then finely minced, digested with 0.1% collagenase type I (Worthington, Lakewood, NJ, USA) and incubated for 60min in 37℃, applying constant gentle rotation. Next, an equal volume of complete DMEM (Dulbecco's modified Eagle medium (DMEM, Welgene Inc, Daegu, Republic of Korea) with 10% fetal bovine serum (FBS, Invitrogen-Gibco, Grand Island, NY, USA) and 1% AA) was added and mixed well. The cell suspension was then filtered through a 100-nm cell strainer (BD Biosciences, Bedford, MA, USA) and centrifuged at 1200 rpm for 5min at room temperature. The supernatant was removed, and 10ml of PBS containing 1% AA was added and the cell suspension was centrifuged again at 1200 rpm for 5min at room temperature. The supernatant was removed and 10ml of complete DMEM was added to the cell pellet. The cells were resuspended by mixing with the help of a pipette and transferred to a 100-mm dish (BD Biosciences, Bedford, MA, USA). The dish was maintained in a humidified tissue culture incubator at 37℃ with 5% CO_2. The medium was replaced every 2-3 d and the non-adherent cells were discarded. Cultures were observed under a microscope to assess expansion and cell morphology. To prevent differentiation, cells were harvested at 80 to 90% confluence and then were either subcultured or stocked. For subculture,

the cells were trypsinized as follows: the cells were washed twice by adding 5ml sterile and warm PBS to the dish. After the second wash, the PBS was replaced with 1ml of 0.05% trypsin-ethylene diaminetetraacetic acid (Trypsin-EDTA, Invitrogen-Gibco, Grand Island, NY, USA). The dish was placed in the incubator at 37°C with 5% CO_2 for 5min. The cells were then observed under a microscope to assess that they have detached from the dish. Four milliliters of medium with serum were then added to neutralize the trypsin reaction. The resulting cell suspension was then diluted 1-5 fold for subculture. For stock, the same trypsinization procedure was used, but afterwards, the cells were collected into a sterile 15-ml tube with 5ml PBS. They were then centrifuged at 1200 rpm for 5min. The supernatant was aspirated and 1 ml of freezing media (DMEM, 50% FBS, and 10% DMSO) was added. After mixing, the cell suspension was transferred into a 1.5-ml tube and kept at -20°C for 1h, then at -80°C overnight, lastly, stored in liquid nitrogen. Cells were thawed and re-cultured as needed. Only ADSCs P1 was used in this study.

Endothelial differentiation of ADSCs

To stimulate endothelial differentiation, ADSCs P1 were cultured in EGM-2 BulletKit (Cat. No. CC-3162, Lonza, Republic of Korea) containing FBS, hydrocortisone, human basic fibroblast growth factor (hFGF-B), vascular endothelial growth factor (VEGF), R3-IGF-1, ascorbic acid, human epidermal growth factor (hEGF), GA-1000, heparin. The dish was maintained in a humidified tissue culture incubator at 37°C with 5% CO_2. The medium was exchanged every 2-3 d and the non-adherent cells were discarded. For subcultures and stocks, the same procedure described for ADSCs was used. Cells were thawed and re-cultured as needed.

Human Umbilical Vein Endothelial Cells (HUVEC) culture

HUVEC cells were used as a positive control. Cryopreserved HUVEC cells (Cat. No.CC-2517,

Lonza, Republic of Korea) were thawed and cultured in a 100-mm dish with EGM-2 BulletKit. The dish was maintained in a humidified tissue culture incubator at 37℃ with 5% CO_2. The medium was exchanged every 2-3 d and the non-adherent cells were discarded. The cells were subcultured or stocked as ADSCs when 80 to 90% confluence was reached. Cells were thawed and re-cultured as needed.

Determination of the cell proliferation rate

Ten thousand HUVECs, ADSCs P1, or EPCs at 1-9 weeks were transferred into 8 wells of a 96-well plate and labeled using the Cell Counting Kit-8 (Dojindo Molecular Technology Inc., Maryland, USA) following the manufacturer recommendations. After 72h of incubation at 37℃ with 5% CO_2, the medium was aspirated. After two washes using PBS, 200 µl EGM-2 BulletKit mixed with 10% CCK-8 were added to each well. After 2h of incubation at 37°Cwith 5% CO_2, the absorbance was measured at 450nm using a microplate reader.

Evaluation of molecular markers

In order to confirm the endothelial phenotype of ADSCs P1, and EPCs at 1-9 weeks, expression of messenger RNA for endothelial-specific markers vWF, platelet/ endothelial cell adhesion molecule (CD31), VE-cadherin, and VEGFR2 were determined by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using RNeasy mini columns (Qiagen, Valencia, Spain). Reverse transcription was performed using the RNA to cDNAEcoDry^{TM} Premix (OligodT, Takara Korea Biomedical Inc, Republic of Korea). Template RNA (1 µg) and DEPC-treated water were added to the premix to a final volume of 20µl. The reverse transcription conditions were 42℃ for 60min, then 70℃ for 10min, lastly 14℃. To detect the expression of specific genes, PCR was performed using the following primers: gene

CD31 forward primer 5′-ACTGGACAAGAAAGAGGCCATCCA-3′ and gene CD31 reverse primer 5′-TCCTTCTGGATGGTGAAGTTGGCT-3′; gene vWF forward primer 5′-ACTCAGTGCATTGGTGAGGATGGA-3′ and gene vWF reverse primer 5′-TCGGACACACTCATTGATGAGGCA-3′; gene VE-cadherin forward primer 5′-AAGACATCAATGACAACTTCC-3′ and reverse 5′-CCTCCACAGTCAGGTTATACC-3′; VEGFR2 forward primer 5′-CTGGCATGGTCTTCTGTG-3′ and reverse primer, 5′-AATGGGATTGGTAAGGATG-3′. The PCR conditions were 94℃ for 5min, followed by 35 cycles of 94℃ for 20s, 63℃ for 30s, and 72℃ for 90s, then, 72℃ for 10min, and lastly, 15℃. Finally, agarose gel electrophoresis was used to assess whether the anticipated DNA fragments were generated.

1, 1′-Dioctadecyl-3, 3, 3′, 3′-Tetramethylindocarbocyanine (Dil) labeling

The neovascularization potential of ADSCs P1, EPCs-3w and EPCs-9w will be determined in future in vivo studies. In order to evaluate whether flap survival area capillaries originate from preexisting capillaries or if they are newly formed, cells are labeled using fluorescent-positive Dil dye before being injected into the flaps. CellTracker^{TM} CM-Dil (Cat. NO.C7000, Invitrogen-Gibco, Grand Island, NY, USA) was dissolved in DMSO (50 µl of DMSO for one vial containing 50µg of Dil) immediately before labeling to obtain a 1mg/ml Dil solution. Five microliters of the Dil solution was then added to ADSCs P1, EPCs-3w, and EPCs-9w cell suspensions in 100µl PBS. The cells were then incubated at 37℃ for 5min, and then at 4℃ for an additional 15min. After two washes with PBS, labeled ADSCs P1, EPCs-3w, and EPCs-9w cells were transferred into dishes. After 1- and 3-d of culture, pictures of ADSCs P1, EPCs-3w and EPCs-9w cells were captured using a fluorescence microscope.

Statistical analysis

Data are expressed as mean ± SEM (standard error of the mean). Intergroup comparison was performed by paired student t test. Probability values of $p < 0.05$ were interpreted to denote statistical significance.

RESULTS

Morphologic analysis

The morphology of HUVEC, ADSCs P1, and EPCs at 1-9 weeks was observed under the 100× objective of the microscope, and pictures were taken. As shown in our results (Figure 1), ADSCs presented a spindle shape and the cell body was long and narrow. The EPCs-1w to EPCs-3w also presented an elongated spindle shape. However, as EPCs were cultured longer (the 4-9 week period), they became longer and irregularly arranged. Taken together, our data indicated that the morphology of EPCs-9w differed from that of the parental ADSCs and EPCs-3w.

Endothelial cell marker study

As presented in figure 2, RT-PCR indicated that ADSCs P1 did not express any of the endothelial cell markers (CD31, vWF, VE-cadherin, and VEGFR-2) while our positive control (HUVEC cells) expressed all four genes. CD31 was expressed in EPCs-1w but was undetectable in EPCs-2w. CD31 expression was again detected in EPCs-3w and then, gradually decreased to become undetectable in EPCs-7w, EPCs-8w and EPCs-9w subsets. vWF was expressed in the EPCs-1w and EPCs-3w subsets. vWF was undetectable in EPCs-2w, EPCs-4w to EPCs-6w, and then was observed again in EPCs-7w to EPCs-9w. A low level of VE-cadherin expression was detected in EPCs-1w, EPCs-3w, and EPCs-6w. VEGFR-2 expression was detected in EPCs-1w, EPCs-3w, and EPCs-7 to EPCs-9w (Figure 2).

Proliferation rate

After changing the medium from DMEM to EGM-2 BulletKit, the cells proliferation rate was determined indirectly by measuring the absorbance at 450nm, increased (Figure 3). Notably, the

absorbancereached a peak at the EPCs-3w cultures, then gradually decreased (Figure 3). The absorbance at 450nm is proportional to the number of viable cells. Since the same number of each cell type was seeded in eachwell (1×10^4), our results indicated that the proliferation rate increased from the first to the third week of EPCs culture, and then slowed down from the fourth to the ninth weeks.

Dil staining

Dil is a red fluorescent dye, which was detected in all cell types 1- and 3-d after labeling. For all cell types, no significant change was observed over the 1- and 3-d post-labeling period, indicating that the cells were able to take up CM-Dil (Figure 4).

DISCUSSION

In plastic and reconstructive surgery, skin flaps play an important role in reconstructing defects resulting from trauma, tumor excision, congenital disease, etc. However, partial or complete necrosis of skin flaps remains a great challenge for plastic surgeons. Inadequate blood perfusion and ischemia-reperfusion injury are believed to be the two major factors that cause several damages to the tissue and vasculature, resulting in flap necrosis (20). Many studies that reported the use of sympatholytic drugs, vasodilators, and calcium channel blockers designed to reduce or overcome the necrosis presented conflicting or inconsistent results in the prevention of flap necrosis (21-23). Growth factors related to angiogenesis, such as basic fibroblast growth factor (bFGF) and vascular endothelium growth factor (VEGF), seem promising but are limited by their short half-life and potential side effects (24, 25). Therefore, the development of better therapies to prevent flap necrosis is urgently needed.

Cell based therapy is a promising approach. In recent years, MSCs have generated interest due to their potential in improving flap survival. MSCs were first identified by Friedenstein et al in bone marrow and subsequently found to be multipotent and able to differentiate into osteocytes, adipocytes, chondrocytes, endothelial cells, and so forth (26). MSCs can be obtained from a variety of sources, including amniotic fluid, the umbilical cord, umbilical cord blood, bone marrow, and adipose tissue (5, 27-29). BMSCs have been studied by many researchers. However, the procedure to obtain BMSCs is painful, and sometimes requires general anesthesia or spinal anesthesia. Moreover, this procedure may yield low numbers of MSCs (4). In contrast, large volumes of adipose tissue can be obtained and high yields of ADSCs are easily achieved. This procedure results in minimal patient discomfort, and can obviate extensive expansion in

culture (4). In our study, ADSCs were isolated from adipose tissue. Through this simple method, ADSCs are obtained in large amounts and can be quickly expanded in vitro, then further differentiated into EPCs.

EPCs were first identified in human peripheral blood (PB) and characterized by the expression of CD34, KDR (VEGFR-2), and CD133 markers (11, 30, 31). Later they were isolated from umbilical cord blood, bone marrow and adipose tissue (7, 11, 32). ADSCs have been reported to have a faster proliferation rate in EGM-2 than in DMEM, and to express endothelial markers (17). This suggests that ADSCs can differentiate into EPCs in EGM-2. In our study, after ADSCs were cultured to P1, complete DMEM was replaced with EGM-2 BulletKit to induce the differentiation of ADSCs into EPCs. Then, EPCs in EGM-2 BulletKit were cultured successively from EPCs-1w to EPCs-9w. Microscopic observation allowed us to observe a change in ADSCs morphology after the DMEM was replaced by EGM-2 BulletKit. Our data, which indicated that EPCs retained their spindle shapes are in agreement with a recent study (33). In this study, the morphology of endothelial-like cells derived from human adipose mesenchymal stem cells and umbilical cord blood-derived endothelial cells were compared. The results showed that after 3 weeks of culture in endothelial media, MSCs retained their spindle-shaped morphology, which could be easily distinguished from the cobblestone morphology of endothelial cells derived from umbilical cord blood (33).

Two types of EPCs isolated from adult peripheral blood were characterized by Hur et al (18). Mononuclear cells were first isolated from peripheral blood and suspended in EGM-2 BulletKit. Six days after plating, the medium was changed for the first time and early EPCs were derived and cultured for 8 weeks. After about a 2-week culture of early EPCs, late EPCs were harvested

from the early EPCs-derived colonies that consisted of cells whose morphology differed from that of early EPCs, and re-plated on a 100-mm plate and were cultured for 12 weeks. Early EPCs were found to be elongated with a spindle shape. They gradually lost their CD31 expression and showed low-level expression of VE-cadherin. However, the low level of VE-cadherin expression was lost at 3 weeks of culture. In contrast, late EPCs presented a cobblestone appearance with strong expression of CD31, vWF, and VE-cadherin. Our study results are consistent with those of Hur et al on early EPCs differentiation (18). EPCs differentiation was induced from ADSCs and cells could be cultured successively from EPCs-1w to EPCs-9w, similar to the early EPCs differentiation process described by this group. Morphologic analysis indicated that EPCs-3w were elongated spindle shaped cells similar to ADSCs. As the culture progressed, EPCs gradually became longer and irregularly arranged. This morphology was more obvious in EPCs-9w.

Four endothelial markers, KDR, VE-cadherin, CD31, and von Willebrand factor (vWF) were used to detect the EPCs differentiated from cord blood mononuclear cells, and these cells expressed all four molecules (34). Dimmeler et al also showed that endothelial phenotype of EPCs can be confirmed by documenting the expression of well-established cell surface markers such as vWF, VEGF-receptor 2 (KDR-receptor), VE-cadherin, and CD31 (19). Therefore, the expression of the endothelial cell markers CD31, vWF, VE-cadherin, and VEGFR-2 were detected by PCR. CD31 was expressed in EPCs-1w and then gradually decreased to finally become undetectable. These results are similar with the report by Hur et al, showing that early EPCs gradually lost CD31 expression (18). However, in our study, vWF, VE-cadherin, and VEGFR-2 expression differed from that in the Hur et al study. As mentioned by this group, EPCs represent a heterogeneous group of cells that differentiate from hemangioblasts to mature

cells (18). Therefore, it is not surprising to observe different gene expression patterns.

It was previously reported that ADSCs proliferate faster in EGM-2 than in DMEM (17). Using the CCK-8 assay to evaluate the proliferation rate of ADSCs P1 and EPCs-1w to EPCs-9w cells confirmed that, indeed, the proliferation rate of EPCs cultured in EGM-2 BulletKit was higher than that of ADSCs. Interestingly, the proliferation rate of EPC-3w was the highest, which then decreased as the culture was continued until the ninth week. Hur et al determined the growth curve of early EPCs, late EPCs and gastroepiploic artery endothelial cells (GEAEC) (18).The growth curve of early EPCs was mostly similar to that of our EPCs.

Based on the differences in morphology, endothelial marker gene expression, and proliferation rate, EPCs-3w and EPCs-9w were selected as the early and the late phase EPCs, respectively. The success of cell-based therapies depends on whether the engrafted cells can differentiate into functional vascular cells and whether those cells can produce paracrine signals that encourage cell survival in the ischemic environment (1). Therefore, in future in vivo studies, we plan to compare the neovascularization potential of ADSCs and early and late phase EPCs. In order to detect the origin of the endothelial cells forming the vessels in the flap, ADSCs P1, EPCs-3w, and EPCs-9w will be labeled with Dil before being injected into the flap. To evaluate their Dil uptake ability, ADSCs P1, EPCs-3w, and EPCs-9w cells were labeled with Dil and observed under a fluorescent microscope 1- and 3-d after labeling. Our results indicated that all 3 cell types presented a clear red fluorescence signal which did not change with time, showing that the cells were able to take up Dil, and this ability will play a pivotal role in in vivo studies.

In summary, we successfully induced the differentiation of ADSCs into EPCs which were

maintained in culture for 9 consecutive weeks. Although there have been reports about early EPCs and late EPCs differentiated from peripheral blood and cord umbilical blood, in their studies, as mentioned above, late EPCs were harvested from the early EPCs-derived colonies that consisted of cells whose morphology differed from that of early EPCs and re-plated on a 100-mm plate and were cultured, which were different from our consecutive culture. Therefore, our study demonstrated a new classification of early and late phase EPCs differentiated from ADSCs and evaluated their characteristics. The results showed that they differ with respect to morphology, endothelial marker gene expression, and the proliferation rate. Future studies are planned to determine the contribution of these early and late phase EPCs to neovascularization in vivo.

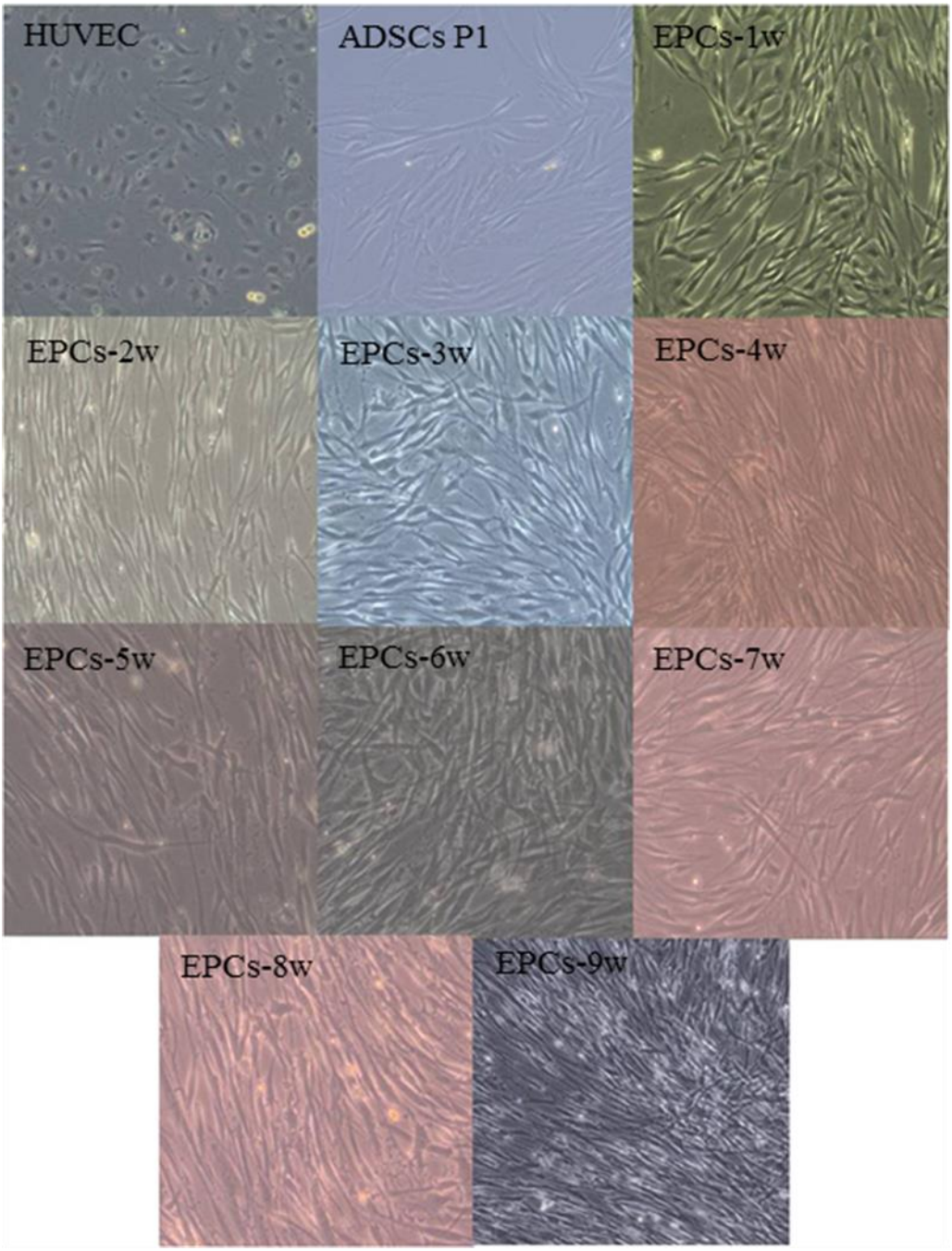

Figure 1. Morphologic analyses of HUVEC, ADSCs P1,and EPCs at 1-9 weeks (100×).

ADSCs were isolated from TRAM flap adipose tissue, and cultured in complete DMEM to ADSCs P1. DMEM was then replaced by EGM-2 BulletKit. Cryopreserved HUVECs (the positive control) were cultured in EGM-2 BulletKit. Images of HUVECs, ADSCs P1, and EPCs at 1-9 weeks (EPCs-1w to EPCs-9w) were captured by microscopy. ADSCs presented a spindle shape with a long and narrow cell body. The EPCs-1w to EPCs-3w also presented an elongated spindle shape, but cell bodies became longer and irregularly arranged from EPCs-4w to EPCs-9w.

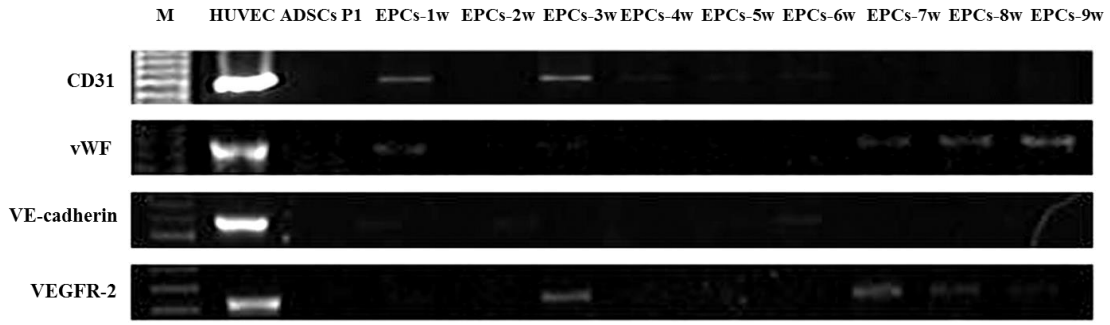

Figure2. mRNA expression level of CD31, vWF, VE-caderin, and VEGFR-2 in HUVECs, ADSCs P1, and EPCs at 1-9 weeks. Total RNA were extracted from HUVEC, ADSCs P1, and EPCs at 1-9 weeks, and reverse transcription was performed to obtain cDNAs. cDNAs were then amplified by PCR to determine CD31, vWF, VE-caderin and VEGFR-2 gene expression. CD31 was expressed in EPCs-1w and EPCs-3w, and gradually decreased to become undetectable from EPCs-7w. vWF was expressed in the EPCs-1w, EPCs-3w and EPCs-7w to EPCs-9w. A low level of VE-cadherin expression was detected in EPCs-1w, EPCs-3w, and EPCs-6w. VEGFR-2 expression was detected in EPCs-1w, EPCs-3w, and EPCs-7 to EPCs-9w.

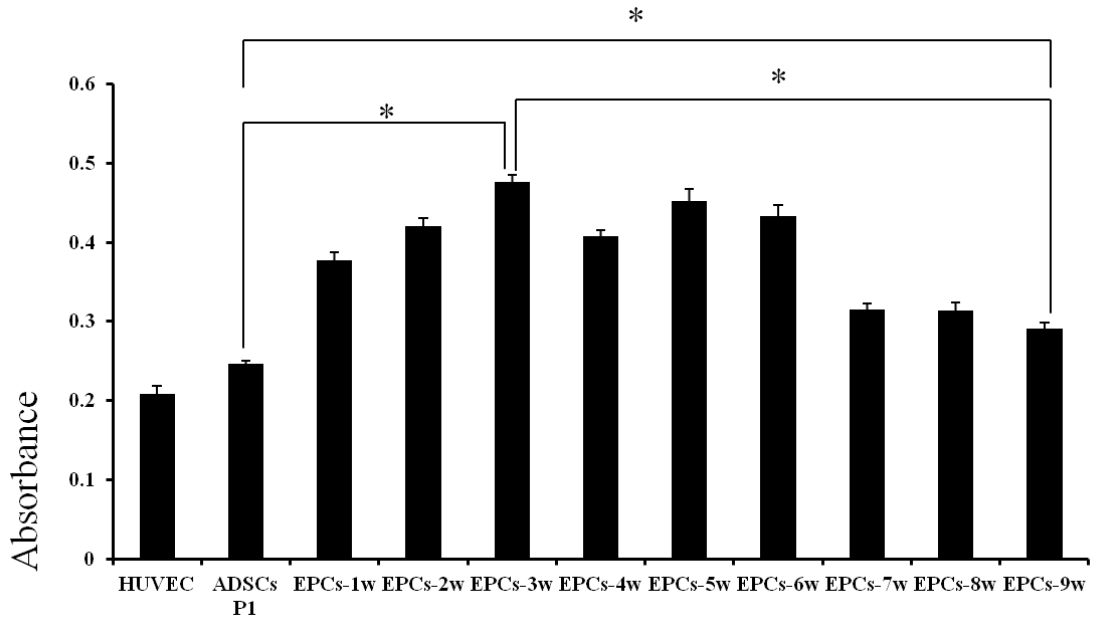

Figure 3. Proliferation rate of HUVECs, ADSCs P1, and EPCs at 1-9 weeks. Ten thousand HUVECs, ADSCs P1 and EPCs-1w to EPCs-9w cells were culured in 96 well plate for 72h. CCK-8 (10%) was then added to each well and incubated for 2h and absorbance was measured at 450nm using a microplate reader. The absorbance increased until the peak at the EPCs-3w cultures, then gradually decreased. The results are presented as mean ± SEM. Experiments were run in duplicate (n=8 each). * represents $p < 0.05$.

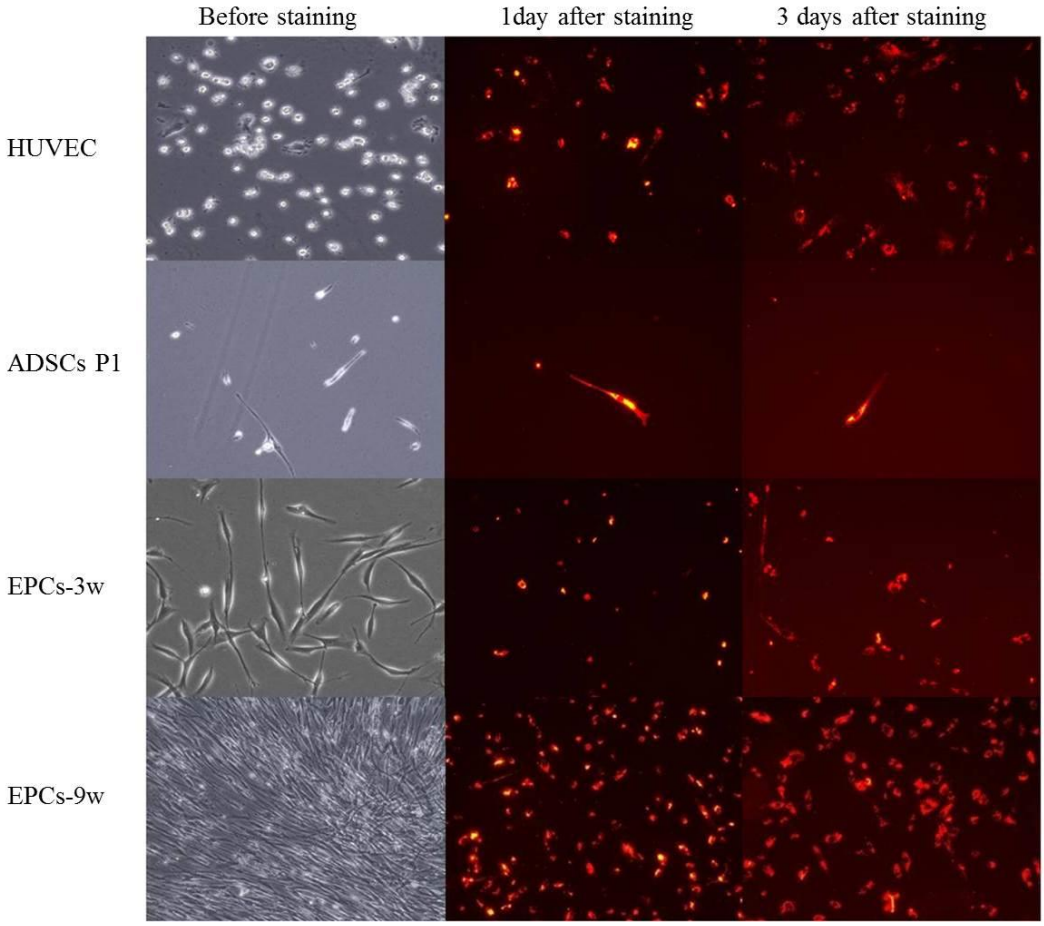

Figure 4. Dil staining images of HUVECs, ADSCs P1, EPCs-3w, and EPCs-9w cells (100×). HUVECs, ADSCs P1, EPCs-3w, and EPCs-9w cells were stained with Dil at 37℃ for 5 min, and 4℃ for 15 min. Images of the cells were captured using a fluoresence microscope 1 and 3 d after labeling, which indicated that all the cells were able to take up CM-Dil.

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국문초록

지방유래줄기세포에서 유도한 조기와 후기 혈관내피전구세포의 생체외 비교연구

왕팡

의학과 성형외과학 전공

서울대학교 대학원

지방유래줄기세포는 다양한 분화능력을 가지고 있는 세포로서 혈관내피전구세포로 도분화가 가능하며 혈관내피전구세포는 새로운 혈관형성에서 중요한 작용을 한다. 또한혈관의일부분으로통합되여신혈관 형성을촉진한다. 본연구는지방유래줄기세포로부터분화된혈관내피전구세포의특성을조사하고나아가신혈관 형성의생체 내연구를 하기 위해조기및후기혈관내피전구세포의구별되는특성을식별하는것을목표로하고있다. 인체의지방조직에서분리한지방유래줄기세포는 DMEM 배양액을이용하여 passage 1 까지배양한후 EGM-2 BulletKit 를이용하여혈관내피전구세포로분화시켰으며 9 주동안배양하였다. 지방유래줄기세포 passage 1 과혈관내피전구세포-3 주및혈관내피전구세포-9 주배양시간점으로하여각각현미경으로관찰하여형태학적구별점을확인하였다. 이세가지세포에서 CD31, vWF, VE-cadherin, 하고 VEGFR-2 등유전자의발현수준도서로달랐다. 그리고혈관내피전구세포의증식속도는지방유래줄기세포 passage 1 보다빠른것을확인하였으며특히혈관내피전구세포-3 주는 분화과정에서차별화된높은

증식속도를나타냈다. 이러한비교를바탕으로혈관내피전구세포-3 주, 혈관내피전구세포-9 주를포인트로잡아각각조기 혈관내피전구세포, 후기 혈관내피전구세포라고 정의를 하였다. 또한 1, 1′-Dioctadecyl-3, 3, 3′, 3′-Tetramethylindocarbocyanine (Dil)를 이용한 형광염색을 실시하였다. 세포에 Dil 표기를 하고 1~3 일 후에 세포를 관찰 및 분석하였다. 결론적으로이 실험은지방유래줄기세포로부터혈관내피전구세포로분화하는과정에서혈관내피전구세포의차별화된특징을가지는조기와 후기 단계를확인하였다.

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주요어: 지방유래줄기세포, 혈관내피전구세포, 조기 혈관내피전구세포, 후기 혈관내피전구세포, 신혈관 형성.

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의학석사 학위논문

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In Vitro Comparison of Early and Late Phase Endothelial Progenitor Cells Induced from Adipose-derived Stem Cells

by

WANG FANG

A thesis submitted to the Department of Plastic and Reconstructive Surgery in partial fulfillment of the requirements for the Degree of Master of Science in Department of Plastic and Reconstructive Surgery at Seoul National University College of Medicine

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Approved by Thesis Committee

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ABSTRACT

In Vitro Comparison of Early and Late Phase Endothelial Progenitor Cells Induced from Adipose-derived Stem Cells

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Adipose-derived stem cells (ADSCs) are multipotent cells that can differentiate into endothelial progenitor cells (EPCs), which are essential for new vessel formation. EPCs can also be incorporated into blood vessels to promote neovascularization. This study aims to investigate the characteristics of the EPCs differentiated from ADSCs and identifying early and late phase EPCs-distinguishing characteristics for our future in vivo neovascularization study. ADSCs were isolated from adipose tissue and cultured to passage 1 (ADSCs P1) in complete DMEM. ADSCs P1 were then induced to differentiate into EPCs by culturing in EGM-2 BulletKit for 9 weeks. A microscopic examination of ADSCs P1 and EPCs collected at the 3-week (EPCs-3w) and 9-week (EPCs-9w) time points revealed differences in morphology between these 3 subsets. The gene expression levels of CD31, vWF, VE-cadherin, and VEGFR-2 were also different. The proliferation rate of EPCs was higher than that of ADSCs P1. Notably, EPCs-3w showed the

highest proliferation rate, which then decreased with time of differentiation. Based on these comparisons, EPCs-3w and EPCs-9w were defined as the early phase EPCs and the late phase EPCs, respectively. Fluorescence staining with 1, 1′-Dioctadecyl-3, 3, 3′, 3′-Tetramethylindocarbocyanine (Dil) was also performed. Cells were observed 1-and 3-d after Dil labeling to evaluate their ability to take up Dil. In conclusion, we compared EPCs from successive weeks of differentiation from ADSCs, and identified two phases (early phase and late phase) EPCs with distinct differences.

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Key words: adipose derived stem cells (ADSCs), endothelial progenitor cells (EPCs), early phase EPCs, late phase EPCs, neovascularization.

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INTRODUCTION

Neovascularization can be divided into three distinct physiological processes: angiogenesis, vasculogenesis, and arteriogenesis (1). Vasculogenesis refers to the formation of blood vessels induced by circulating endothelial progenitor cells. Angiogenesis and arteriogenesis refer to the sprouting and subsequent stabilization of these sprouts by endothelial cells, and collateral growth denotes the expansive growth of pre-existing vessels, forming collateral bridges between arterial networks (2).

The existence of adipose derived stem cells (ADSCs) in adipose tissue was reported in 1984 (3). Since then, many researchers started to explore the isolation, purification, expansion, differentiation, and extensive characterization of ADSCs. ADSCs were found to be similar to bone marrow derived stem cells (BMSCs). Both of these are multipotent stem cell subsets that can differentiate into hepatocytes, osteoblasts, chondrocytes, neurons, endothelial cells and other cell types (4-7). ADSCs present many advantages. They can be harvested, handled, and induced to proliferate easily, effectively, and noninvasively. Their pluripotency and proliferative efficiency is identical to that of BMSC. Finally, donor morbidity is lower than that in the case of mesenchymal stem cells (MSCs) harvested from other sites (8). The possibility of ADSCs enhancing neovascularization has been described (9). Many researchers have demonstrated the neovascular potential of ADSCs, in particular, their ability to release many potent angiogenic factors that promote endothelial cells proliferation and migration into the perivascular stroma and induce tubule formation by endothelial cells in vitro (10).

Endothelial progenitor cells (EPCs) have been isolated from human peripheral blood, and were

found to be incorporated into sites of active angiogenesis andincrease collateral vessel growth in ischemic tissues (11). EPCs were also reported to circulate from bone marrow to incorporate into and thus contribute to the postnatal physiological and pathological neovascularization consistent with postnatal vasculogenesis (12). In addition, an animal study showed that local BMSCs implantation induces a neovascular response resulting in a significant increase of blood flow to the ischemic limb (13). However, the relatively low abundance, small tissue volume, difficult accessibility, and disease-related malfunction of BMSCs make their clinical application difficult.

Studies have shown that transplanted ADSCs can differentiate into endothelial cells that are incorporated into blood vessels to promote neovascularization of hindlimb ischemia in murine models (14-16). ADSCs were also shown to proliferate at a faster rate in EGM-2 than in standard DMEM, expressthe endothelial markers CD31, vWF, and eNOS, form tube-like structures in Matrigel, and endocytose Ac-LDL (17). In addition, Hur et al (18) cultured two different types of EPCs (early EPCs and late EPCs) from peripheral blood according to their time-dependent appearance. Although these subsets showed different morphology, proliferation rates, survival features, and gene expression profiles, they shared some endothelial phenotypes and similarly contributed to neovasculogenesis in vivo. Early EPCs secreted angiogenic cytokines, whereas late EPCs supplied a sufficient number of endothelial cells (18). However, the number of mononuclear cells isolated from peripheral blood was very small.

In our study, EPCs were differentiated from ADSCs, which can be isolated in high numbers from adipose tissue. Cell morphology, expression of endothelial markers, and proliferation rate of ADSCs P1 and EPCs were evaluated to select early and late phase EPCs and to compare their

neovascularization potential with that of ADSCs. ADSCs were isolated from transverse rectus abdominal musculocutaneous (TRAM) flap adipose tissue discarded during reconstructive surgery and cultured to P1 in complete DMEM. ADSCs P1 were then induced to differentiate into EPCs for 9 weeks (EPCs-1w to EPCs-9w) in the EGM-2 BulletKit. Next, the morphology of ADSCs P1 and EPCs-1w to EPCs-9w was examined by microscopic analysis. Gene expression of endothelial markers of CD31, vWF, VE-cadherin, and VEGFR-2 was also determined by PCR. In addition, their proliferation rate was indirectly evaluated using CCK-8. Based on these comparisons, EPCs-3w and EPCs-9w were defined as the early phase EPCs and the late phase EPCs respectively. Lastly, ADSCs P1, EPCs-3w, and EPCs-9w populations were labeled with 1, 1′-Dioctadecyl-3, 3, 3′, 3′-Tetramethylindocarbocyanine (Dil) staining to evaluate their Dil uptake ability.

MATERIALS AND METHODS

ADSCs isolation and culture

All protocols were approved by the institutional review board (IRB) of Seoul National University Hospital. After obtaining informed consent from patients, ADSCs were harvested from adipose tissue discarded during reconstructive surgery, such as the TRAM flap used for breast reconstruction after mastectomy. The adipose tissue was rinsed extensively with 1×Phosphate Buffered Saline (PBS, Gibco-BRL, Grand Island, NY, USA) containing a 1% antibiotic-antimycotic (AA, WelgeneInc, Daegu, Republic of Korea) to remove contaminating hematopoietic cells. It was then finely minced, digested with 0.1% collagenase type I (Worthington, Lakewood, NJ, USA) and incubated for 60min in 37℃, applying constant gentle rotation. Next, an equal volume of complete DMEM (Dulbecco's modified Eagle medium (DMEM, Welgene Inc, Daegu, Republic of Korea) with 10% fetal bovine serum (FBS, Invitrogen-Gibco, Grand Island, NY, USA) and 1% AA) was added and mixed well. The cell suspension was then filtered through a 100-nm cell strainer (BD Biosciences, Bedford, MA, USA) and centrifuged at 1200 rpm for 5min at room temperature. The supernatant was removed, and 10ml of PBS containing 1% AA was added and the cell suspension was centrifuged again at 1200 rpm for 5min at room temperature. The supernatant was removed and 10ml of complete DMEM was added to the cell pellet. The cells were resuspended by mixing with the help of a pipette and transferred to a 100-mm dish (BD Biosciences, Bedford, MA, USA). The dish was maintained in a humidified tissue culture incubator at 37℃ with 5% CO_2. The medium was replaced every 2-3 d and the non-adherent cells were discarded. Cultures were observed under a microscope to assess expansion and cell morphology. To prevent differentiation, cells were harvested at 80 to 90% confluence and then were either subcultured or stocked. For subculture,

the cells were trypsinized as follows: the cells were washed twice by adding 5ml sterile and warm PBS to the dish. After the second wash, the PBS was replaced with 1ml of 0.05% trypsin-ethylene diaminetetraacetic acid (Trypsin-EDTA, Invitrogen-Gibco, Grand Island, NY, USA). The dish was placed in the incubator at 37°C with 5% CO_2 for 5min. The cells were then observed under a microscope to assess that they have detached from the dish. Four milliliters of medium with serum were then added to neutralize the trypsin reaction. The resulting cell suspension was then diluted 1-5 fold for subculture. For stock, the same trypsinization procedure was used, but afterwards, the cells were collected into a sterile 15-ml tube with 5ml PBS. They were then centrifuged at 1200 rpm for 5min. The supernatant was aspirated and 1 ml of freezing media (DMEM, 50% FBS, and 10% DMSO) was added. After mixing, the cell suspension was transferred into a 1.5-ml tube and kept at -20°C for 1h, then at -80°C overnight, lastly, stored in liquid nitrogen. Cells were thawed and re-cultured as needed. Only ADSCs P1 was used in this study.

Endothelial differentiation of ADSCs

To stimulate endothelial differentiation, ADSCs P1 were cultured in EGM-2 BulletKit (Cat. No. CC-3162, Lonza, Republic of Korea) containing FBS, hydrocortisone, human basic fibroblast growth factor (hFGF-B), vascular endothelial growth factor (VEGF), R3-IGF-1, ascorbic acid, human epidermal growth factor (hEGF), GA-1000, heparin. The dish was maintained in a humidified tissue culture incubator at 37°C with 5% CO_2. The medium was exchanged every 2-3 d and the non-adherent cells were discarded. For subcultures and stocks, the same procedure described for ADSCs was used. Cells were thawed and re-cultured as needed.

Human Umbilical Vein Endothelial Cells (HUVEC) culture

HUVEC cells were used as a positive control. Cryopreserved HUVEC cells (Cat. No.CC-2517,

Lonza, Republic of Korea) were thawed and cultured in a 100-mm dish with EGM-2 BulletKit. The dish was maintained in a humidified tissue culture incubator at 37℃ with 5% CO_2. The medium was exchanged every 2-3 d and the non-adherent cells were discarded. The cells were subcultured or stocked as ADSCs when 80 to 90% confluence was reached. Cells were thawed and re-cultured as needed.

Determination of the cell proliferation rate

Ten thousand HUVECs, ADSCs P1, or EPCs at 1-9 weeks were transferred into 8 wells of a 96-well plate and labeled using the Cell Counting Kit-8 (Dojindo Molecular Technology Inc., Maryland, USA) following the manufacturer recommendations. After 72h of incubation at 37℃ with 5% CO_2, the medium was aspirated. After two washes using PBS, 200 µl EGM-2 BulletKit mixed with 10% CCK-8 were added to each well. After 2h of incubation at 37°Cwith 5% CO_2, the absorbance was measured at 450nm using a microplate reader.

Evaluation of molecular markers

In order to confirm the endothelial phenotype of ADSCs P1, and EPCs at 1-9 weeks, expression of messenger RNA for endothelial-specific markers vWF, platelet/ endothelial cell adhesion molecule (CD31), VE-cadherin, and VEGFR2 were determined by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using RNeasy mini columns (Qiagen, Valencia, Spain). Reverse transcription was performed using the RNA to cDNAEcoDry^{TM} Premix (OligodT, Takara Korea Biomedical Inc, Republic of Korea). Template RNA (1 µg) and DEPC-treated water were added to the premix to a final volume of 20µl. The reverse transcription conditions were 42℃ for 60min, then 70℃ for 10min, lastly 14℃. To detect the expression of specific genes, PCR was performed using the following primers: gene

CD31 forward primer 5′-ACTGGACAAGAAAGAGGCCATCCA-3′ and gene CD31 reverse primer 5′-TCCTTCTGGATGGTGAAGTTGGCT-3′; gene vWF forward primer 5′-ACTCAGTGCATTGGTGAGGATGGA-3′ and gene vWF reverse primer 5′-TCGGACACACTCATTGATGAGGCA-3′; gene VE-cadherin forward primer 5′-AAGACATCAATGACAACTTCC-3′ and reverse 5′-CCTCCACAGTCAGGTTATACC-3′; VEGFR2 forward primer 5′-CTGGCATGGTCTTCTGTG-3′ and reverse primer, 5′-AATGGGATTGGTAAGGATG-3′. The PCR conditions were 94℃ for 5min, followed by 35 cycles of 94℃ for 20s, 63℃ for 30s, and 72℃ for 90s, then, 72℃ for 10min, and lastly, 15℃. Finally, agarose gel electrophoresis was used to assess whether the anticipated DNA fragments were generated.

1, 1′-Dioctadecyl-3, 3, 3′, 3′-Tetramethylindocarbocyanine (Dil) labeling

The neovascularization potential of ADSCs P1, EPCs-3w and EPCs-9w will be determined in future in vivo studies. In order to evaluate whether flap survival area capillaries originate from preexisting capillaries or if they are newly formed, cells are labeled using fluorescent-positive Dil dye before being injected into the flaps. CellTracker^{TM} CM-Dil (Cat. NO.C7000, Invitrogen-Gibco, Grand Island, NY, USA) was dissolved in DMSO (50 µl of DMSO for one vial containing 50µg of Dil) immediately before labeling to obtain a 1mg/ml Dil solution. Five microliters of the Dil solution was then added to ADSCs P1, EPCs-3w, and EPCs-9w cell suspensions in 100µl PBS. The cells were then incubated at 37℃ for 5min, and then at 4℃ for an additional 15min. After two washes with PBS, labeled ADSCs P1, EPCs-3w, and EPCs-9w cells were transferred into dishes. After 1- and 3-d of culture, pictures of ADSCs P1, EPCs-3w and EPCs-9w cells were captured using a fluorescence microscope.

Statistical analysis

Data are expressed as mean ± SEM (standard error of the mean). Intergroup comparison was performed by paired student t test. Probability values of $p < 0.05$ were interpreted to denote statistical significance.

RESULTS

Morphologic analysis

The morphology of HUVEC, ADSCs P1, and EPCs at 1-9 weeks was observed under the 100× objective of the microscope, and pictures were taken. As shown in our results (Figure 1), ADSCs presented a spindle shape and the cell body was long and narrow. The EPCs-1w to EPCs-3w also presented an elongated spindle shape. However, as EPCs were cultured longer (the 4-9 week period), they became longer and irregularly arranged. Taken together, our data indicated that the morphology of EPCs-9w differed from that of the parental ADSCs and EPCs-3w.

Endothelial cell marker study

As presented in figure 2, RT-PCR indicated that ADSCs P1 did not express any of the endothelial cell markers (CD31, vWF, VE-cadherin, and VEGFR-2) while our positive control (HUVEC cells) expressed all four genes. CD31 was expressed in EPCs-1w but was undetectable in EPCs-2w. CD31 expression was again detected in EPCs-3w and then, gradually decreased to become undetectable in EPCs-7w, EPCs-8w and EPCs-9w subsets. vWF was expressed in the EPCs-1w and EPCs-3w subsets. vWF was undetectable in EPCs-2w, EPCs-4w to EPCs-6w, and then was observed again in EPCs-7w to EPCs-9w. A low level of VE-cadherin expression was detected in EPCs-1w, EPCs-3w, and EPCs-6w. VEGFR-2 expression was detected in EPCs-1w, EPCs-3w, and EPCs-7 to EPCs-9w (Figure 2).

Proliferation rate

After changing the medium from DMEM to EGM-2 BulletKit, the cells proliferation rate was determined indirectly by measuring the absorbance at 450nm, increased (Figure 3). Notably, the

absorbancereached a peak at the EPCs-3w cultures, then gradually decreased (Figure 3). The absorbance at 450nm is proportional to the number of viable cells. Since the same number of each cell type was seeded in eachwell (1×10^4), our results indicated that the proliferation rate increased from the first to the third week of EPCs culture, and then slowed down from the fourth to the ninth weeks.

Dil staining

Dil is a red fluorescent dye, which was detected in all cell types 1- and 3-d after labeling. For all cell types, no significant change was observed over the 1- and 3-d post-labeling period, indicating that the cells were able to take up CM-Dil (Figure 4).

DISCUSSION

In plastic and reconstructive surgery, skin flaps play an important role in reconstructing defects resulting from trauma, tumor excision, congenital disease, etc. However, partial or complete necrosis of skin flaps remains a great challenge for plastic surgeons. Inadequate blood perfusion and ischemia-reperfusion injury are believed to be the two major factors that cause several damages to the tissue and vasculature, resulting in flap necrosis (20). Many studies that reported the use of sympatholytic drugs, vasodilators, and calcium channel blockers designed to reduce or overcome the necrosis presented conflicting or inconsistent results in the prevention of flap necrosis (21-23). Growth factors related to angiogenesis, such as basic fibroblast growth factor (bFGF) and vascular endothelium growth factor (VEGF), seem promising but are limited by their short half-life and potential side effects (24, 25). Therefore, the development of better therapies to prevent flap necrosis is urgently needed.

Cell based therapy is a promising approach. In recent years, MSCs have generated interest due to their potential in improving flap survival. MSCs were first identified by Friedenstein et al in bone marrow and subsequently found to be multipotent and able to differentiate into osteocytes, adipocytes, chondrocytes, endothelial cells, and so forth (26). MSCs can be obtained from a variety of sources, including amniotic fluid, the umbilical cord, umbilical cord blood, bone marrow, and adipose tissue (5, 27-29). BMSCs have been studied by many researchers. However, the procedure to obtain BMSCs is painful, and sometimes requires general anesthesia or spinal anesthesia. Moreover, this procedure may yield low numbers of MSCs (4). In contrast, large volumes of adipose tissue can be obtained and high yields of ADSCs are easily achieved. This procedure results in minimal patient discomfort, and can obviate extensive expansion in

culture (4). In our study, ADSCs were isolated from adipose tissue. Through this simple method, ADSCs are obtained in large amounts and can be quickly expanded in vitro, then further differentiated into EPCs.

EPCs were first identified in human peripheral blood (PB) and characterized by the expression of CD34, KDR (VEGFR-2), and CD133 markers (11, 30, 31). Later they were isolated from umbilical cord blood, bone marrow and adipose tissue (7, 11, 32). ADSCs have been reported to have a faster proliferation rate in EGM-2 than in DMEM, and to express endothelial markers (17). This suggests that ADSCs can differentiate into EPCs in EGM-2. In our study, after ADSCs were cultured to P1, complete DMEM was replaced with EGM-2 BulletKit to induce the differentiation of ADSCs into EPCs. Then, EPCs in EGM-2 BulletKit were cultured successively from EPCs-1w to EPCs-9w. Microscopic observation allowed us to observe a change in ADSCs morphology after the DMEM was replaced by EGM-2 BulletKit. Our data, which indicated that EPCs retained their spindle shapes are in agreement with a recent study (33). In this study, the morphology of endothelial-like cells derived from human adipose mesenchymal stem cells and umbilical cord blood-derived endothelial cells were compared. The results showed that after 3 weeks of culture in endothelial media, MSCs retained their spindle-shaped morphology, which could be easily distinguished from the cobblestone morphology of endothelial cells derived from umbilical cord blood (33).

Two types of EPCs isolated from adult peripheral blood were characterized by Hur et al (18). Mononuclear cells were first isolated from peripheral blood and suspended in EGM-2 BulletKit. Six days after plating, the medium was changed for the first time and early EPCs were derived and cultured for 8 weeks. After about a 2-week culture of early EPCs, late EPCs were harvested

from the early EPCs-derived colonies that consisted of cells whose morphology differed from that of early EPCs, and re-plated on a 100-mm plate and were cultured for 12 weeks. Early EPCs were found to be elongated with a spindle shape. They gradually lost their CD31 expression and showed low-level expression of VE-cadherin. However, the low level of VE-cadherin expression was lost at 3 weeks of culture. In contrast, late EPCs presented a cobblestone appearance with strong expression of CD31, vWF, and VE-cadherin. Our study results are consistent with those of Hur et al on early EPCs differentiation (18). EPCs differentiation was induced from ADSCs and cells could be cultured successively from EPCs-1w to EPCs-9w, similar to the early EPCs differentiation process described by this group. Morphologic analysis indicated that EPCs-3w were elongated spindle shaped cells similar to ADSCs. As the culture progressed, EPCs gradually became longer and irregularly arranged. This morphology was more obvious in EPCs-9w.

Four endothelial markers, KDR, VE-cadherin, CD31, and von Willebrand factor (vWF) were used to detect the EPCs differentiated from cord blood mononuclear cells, and these cells expressed all four molecules (34). Dimmeler et al also showed that endothelial phenotype of EPCs can be confirmed by documenting the expression of well-established cell surface markers such as vWF, VEGF-receptor 2 (KDR-receptor), VE-cadherin, and CD31 (19). Therefore, the expression of the endothelial cell markers CD31, vWF, VE-cadherin, and VEGFR-2 were detected by PCR. CD31 was expressed in EPCs-1w and then gradually decreased to finally become undetectable. These results are similar with the report by Hur et al, showing that early EPCs gradually lost CD31 expression (18). However, in our study, vWF, VE-cadherin, and VEGFR-2 expression differed from that in the Hur et al study. As mentioned by this group, EPCs represent a heterogeneous group of cells that differentiate from hemangioblasts to mature

cells (18). Therefore, it is not surprising to observe different gene expression patterns.

It was previously reported that ADSCs proliferate faster in EGM-2 than in DMEM (17). Using the CCK-8 assay to evaluate the proliferation rate of ADSCs P1 and EPCs-1w to EPCs-9w cells confirmed that, indeed, the proliferation rate of EPCs cultured in EGM-2 BulletKit was higher than that of ADSCs. Interestingly, the proliferation rate of EPC-3w was the highest, which then decreased as the culture was continued until the ninth week. Hur et al determined the growth curve of early EPCs, late EPCs and gastroepiploic artery endothelial cells (GEAEC) (18).The growth curve of early EPCs was mostly similar to that of our EPCs.

Based on the differences in morphology, endothelial marker gene expression, and proliferation rate, EPCs-3w and EPCs-9w were selected as the early and the late phase EPCs, respectively. The success of cell-based therapies depends on whether the engrafted cells can differentiate into functional vascular cells and whether those cells can produce paracrine signals that encourage cell survival in the ischemic environment (1). Therefore, in future in vivo studies, we plan to compare the neovascularization potential of ADSCs and early and late phase EPCs. In order to detect the origin of the endothelial cells forming the vessels in the flap, ADSCs P1, EPCs-3w, and EPCs-9w will be labeled with Dil before being injected into the flap. To evaluate their Dil uptake ability, ADSCs P1, EPCs-3w, and EPCs-9w cells were labeled with Dil and observed under a fluorescent microscope 1- and 3-d after labeling. Our results indicated that all 3 cell types presented a clear red fluorescence signal which did not change with time, showing that the cells were able to take up Dil, and this ability will play a pivotal role in in vivo studies.

In summary, we successfully induced the differentiation of ADSCs into EPCs which were

maintained in culture for 9 consecutive weeks. Although there have been reports about early EPCs and late EPCs differentiated from peripheral blood and cord umbilical blood, in their studies, as mentioned above, late EPCs were harvested from the early EPCs-derived colonies that consisted of cells whose morphology differed from that of early EPCs and re-plated on a 100-mm plate and were cultured, which were different from our consecutive culture. Therefore, our study demonstrated a new classification of early and late phase EPCs differentiated from ADSCs and evaluated their characteristics. The results showed that they differ with respect to morphology, endothelial marker gene expression, and the proliferation rate. Future studies are planned to determine the contribution of these early and late phase EPCs to neovascularization in vivo.

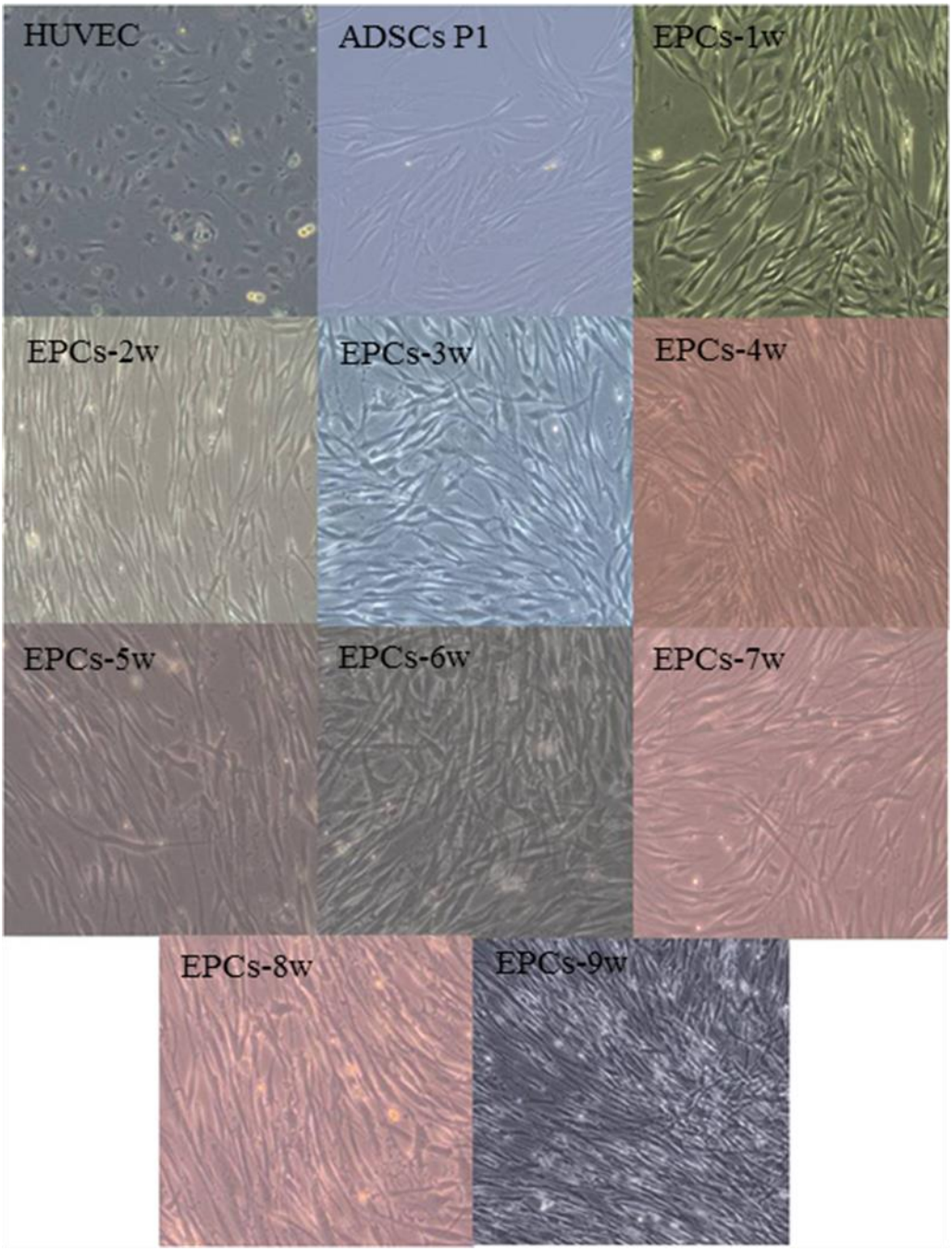

Figure 1. Morphologic analyses of HUVEC, ADSCs P1,and EPCs at 1-9 weeks (100×).

ADSCs were isolated from TRAM flap adipose tissue, and cultured in complete DMEM to ADSCs P1. DMEM was then replaced by EGM-2 BulletKit. Cryopreserved HUVECs (the positive control) were cultured in EGM-2 BulletKit. Images of HUVECs, ADSCs P1, and EPCs at 1-9 weeks (EPCs-1w to EPCs-9w) were captured by microscopy. ADSCs presented a spindle shape with a long and narrow cell body. The EPCs-1w to EPCs-3w also presented an elongated spindle shape, but cell bodies became longer and irregularly arranged from EPCs-4w to EPCs-9w.

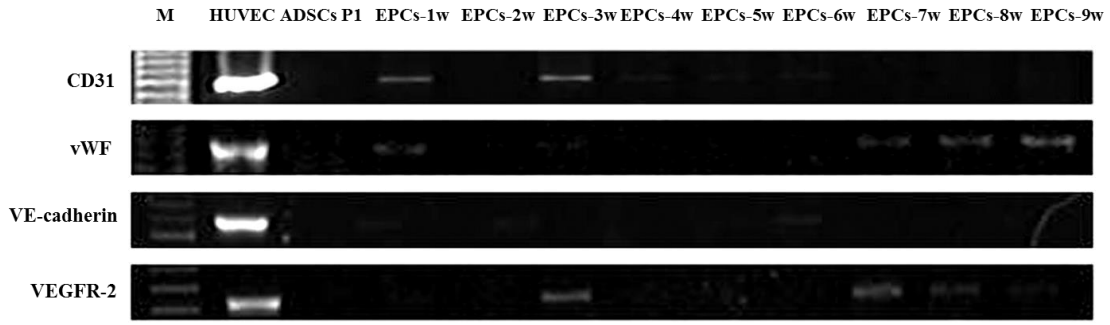

Figure2. mRNA expression level of CD31, vWF, VE-caderin, and VEGFR-2 in HUVECs, ADSCs P1, and EPCs at 1-9 weeks. Total RNA were extracted from HUVEC, ADSCs P1, and EPCs at 1-9 weeks, and reverse transcription was performed to obtain cDNAs. cDNAs were then amplified by PCR to determine CD31, vWF, VE-caderin and VEGFR-2 gene expression. CD31 was expressed in EPCs-1w and EPCs-3w, and gradually decreased to become undetectable from EPCs-7w. vWF was expressed in the EPCs-1w, EPCs-3w and EPCs-7w to EPCs-9w. A low level of VE-cadherin expression was detected in EPCs-1w, EPCs-3w, and EPCs-6w. VEGFR-2 expression was detected in EPCs-1w, EPCs-3w, and EPCs-7 to EPCs-9w.

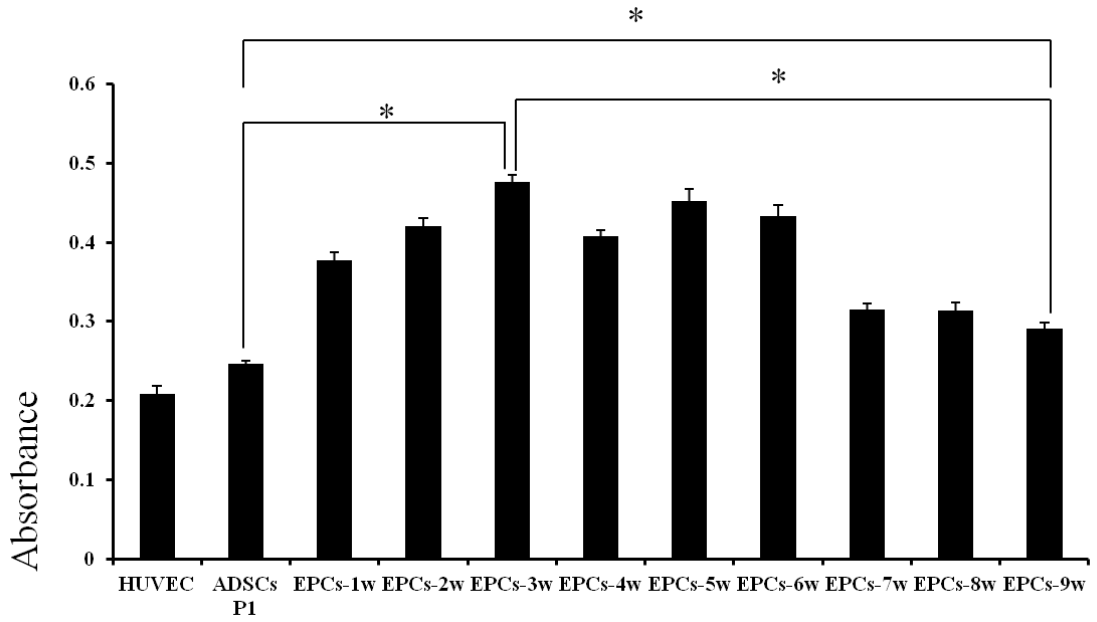

Figure 3. Proliferation rate of HUVECs, ADSCs P1, and EPCs at 1-9 weeks. Ten thousand HUVECs, ADSCs P1 and EPCs-1w to EPCs-9w cells were culured in 96 well plate for 72h. CCK-8 (10%) was then added to each well and incubated for 2h and absorbance was measured at 450nm using a microplate reader. The absorbance increased until the peak at the EPCs-3w cultures, then gradually decreased. The results are presented as mean ± SEM. Experiments were run in duplicate (n=8 each). * represents $p < 0.05$.

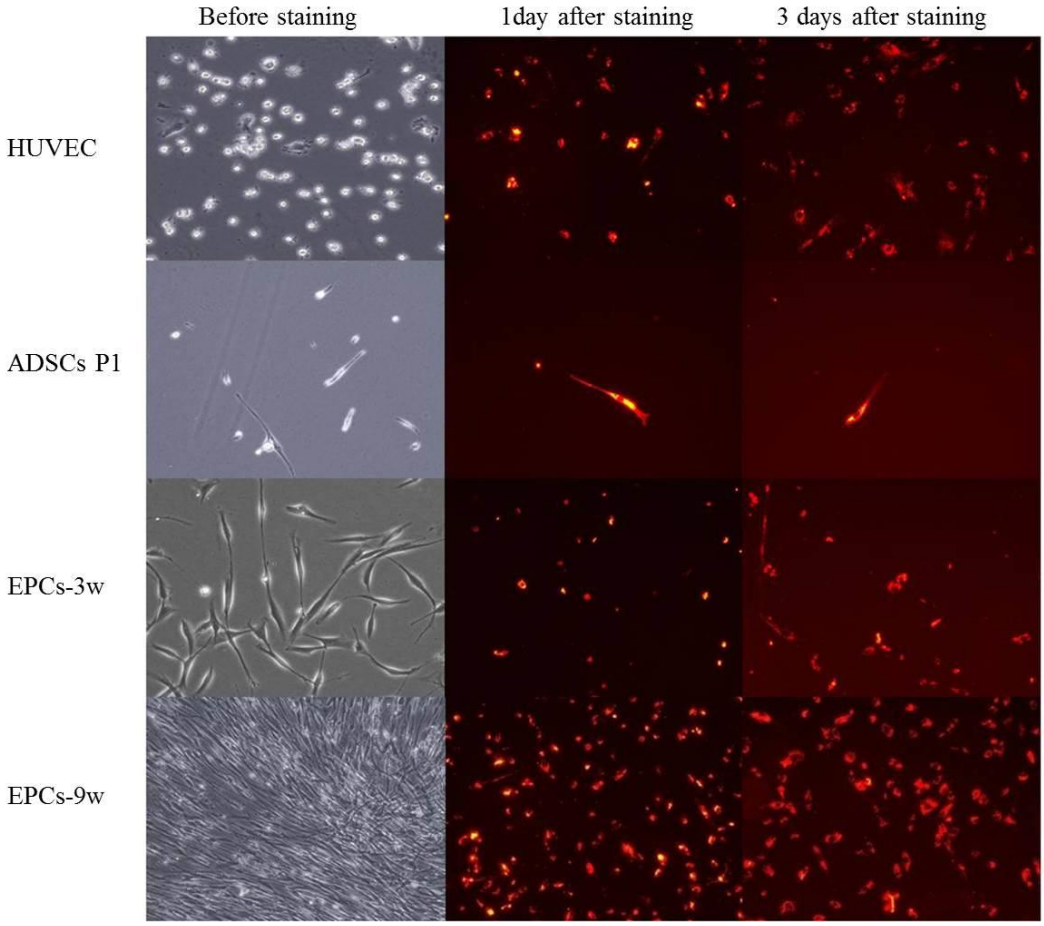

Figure 4. Dil staining images of HUVECs, ADSCs P1, EPCs-3w, and EPCs-9w cells (100×). HUVECs, ADSCs P1, EPCs-3w, and EPCs-9w cells were stained with Dil at 37℃ for 5 min, and 4℃ for 15 min. Images of the cells were captured using a fluoresence microscope 1 and 3 d after labeling, which indicated that all the cells were able to take up CM-Dil.

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국문초록

지방유래줄기세포에서 유도한 조기와 후기 혈관내피전구세포의 생체외 비교연구

왕팡

의학과 성형외과학 전공

서울대학교 대학원

지방유래줄기세포는 다양한 분화능력을 가지고 있는 세포로서 혈관내피전구세포로 도분화가 가능하며 혈관내피전구세포는 새로운 혈관형성에서 중요한 작용을 한다. 또한혈관의일부분으로통합되여신혈관 형성을촉진한다. 본연구는지방유래줄기세포로부터분화된혈관내피전구세포의특성을조사하고나아가신혈관 형성의생체 내연구를 하기 위해조기및후기혈관내피전구세포의구별되는특성을식별하는것을목표로하고있다. 인체의지방조직에서분리한지방유래줄기세포는 DMEM 배양액을이용하여 passage 1 까지배양한후 EGM-2 BulletKit 를이용하여혈관내피전구세포로분화시켰으며 9 주동안배양하였다. 지방유래줄기세포 passage 1 과혈관내피전구세포-3 주및혈관내피전구세포-9 주배양시간점으로하여각각현미경으로관찰하여형태학적구별점을확인하였다. 이세가지세포에서 CD31, vWF, VE-cadherin, 하고 VEGFR-2 등유전자의발현수준도서로달랐다. 그리고혈관내피전구세포의증식속도는지방유래줄기세포 passage 1 보다빠른것을확인하였으며특히혈관내피전구세포-3 주는 분화과정에서차별화된높은

증식속도를나타냈다. 이러한비교를바탕으로혈관내피전구세포-3 주, 혈관내피전구세포-9 주를포인트로잡아각각조기 혈관내피전구세포, 후기 혈관내피전구세포라고 정의를 하였다. 또한 1, 1′-Dioctadecyl-3, 3, 3′, 3′-Tetramethylindocarbocyanine (Dil)를 이용한 형광염색을 실시하였다. 세포에 Dil 표기를 하고 1~3 일 후에 세포를 관찰 및 분석하였다. 결론적으로이 실험은지방유래줄기세포로부터혈관내피전구세포로분화하는과정에서혈관내피전구세포의차별화된특징을가지는조기와 후기 단계를확인하였다.

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주요어: 지방유래줄기세포, 혈관내피전구세포, 조기 혈관내피전구세포, 후기 혈관내피전구세포, 신혈관 형성.

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