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ORIGINAL RESEARCH REPORT

Functional Implications of CD34 Expression in Human Adipose–Derived Stem/Progenitor Cells

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CD34 is frequently used as a marker of adipose-derived stem/stromal/progenitor cells (ASCs). However, CD34 expression in human ASCs (hASCs) decreases over time in culture, and the implications of this remain unclear. In this study, we sorted shortly-cultured hASCs into CD34+ and CD34− fractions and compared their biological functions. Results indicated that CD34+ hASCs were more proliferative and had a greater ability to form colonies. In contrast, CD34− cells showed a greater ability for differentiation into adipogenic and osteogenic lineages. Both CD34+ and CD34− cells showed similar abilities in migration and capillary formation in response to growth factors. Expression levels of endothelial progenitor markers (Flk-1, FLT1, and Tie-2) were higher in CD34+ cells, whereas those of pericyte markers (CD146, NG2, and α-smooth muscle actin) were higher in CD34− cells. Both CD34+ and CD34− cells showed similar expression levels of vascular endothelial growth factor and hepatocyte growth factor, although hypoxia affected the expression levels. Together these results suggest that CD34 expression in hASCs may correlate with replicative capacity, differentiation potentials, expression profiles of angiogenesis-related genes, and immaturity or stemness of the cells. Loss of CD34 expression may be related to the physiological process of commitment and/or differentiation from immature status into specific lineages such as adipose, bone, or smooth muscle.

Introduction

In humans [1,2] and mice [3], the stromal vascular fraction (SVF), a heterogeneous mixture of cells isolated by enzymatic dissociation of adipose tissue, has been shown to contain multipotent cells, which are referred to as adipose-derived stem/stromal/progenitor cells (ASCs). In addition to ASCs, the SVF also contains blood-derived cells, such as erythrocytes and leukocytes, and other adipose-derived cells, such as vascular endothelial and mural cells. Adipose tissue can be harvested in a large quantity (>500 mL) by a minimally invasive procedure. Human ASCs (hASCs) have biological capacities similar to bone marrow–derived mesenchymal stem cells; thus, hASCs are considered to be a promising tool for clinical use in regenerative medicine [4,5]. Freshly isolated hASCs are identified as CD31−/CD34+/CD45−/CD90+/CD105−/CD146− cells in the SVF, but become CD105+ when plated [6]. To distinguish hASCs from other cells in the SVF, CD34 is frequently used as a marker for hASCs [6–9]; vascular endothelial cells also express CD34, but are easily discriminated from hASCs by positive expression of CD31 [6]. Recently, human adipose tissue was shown to turn over [10], and adipocyte progenitor cells in mice have been identified in the adipose vasculature [11] and characterized as Lin−/CD29+/CD34+/Sca-1+/CD24+ cells [12].

CD34 expression by hASCs decreases over time in culture [6,8,13,14], although the extent and time course of the decrease in CD34 expression strongly depends on culture conditions, such as plating density and culture medium. Dulbecco's modified Eagle's medium (DMEM) and DMEM/F12 are widely used for culturing hASCs; notably, most hASCs cultured in DMEM or DMEM/F12 lose CD34 expression within 2 weeks; thus, culture-expanded hASCs have been described as CD34− cells in many publications [1,15]. Along with the decline of CD34 expression, stromal cell–associated markers, such as CD90 and CD105, increase with successive passages [6,8]. Despite these phenotypic changes during culture, hASCs retain proliferative capacity and multipotency even after several passages and loss of CD34 expression [6,13]. Under these circumstances, the

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functional implications of CD34 expression in ASCs remain unclear.

In this study, we collected CD34+ and CD34− cell fractions from early passages of cultured hASCs using fluorescence activated cell sorting (FACS). Comparison of the two cell populations in multiple assays provided new insights into the functional implications of CD34 expression in hASCs.

Materials and Methods

Cell isolation and culture

Liposuction aspirates were obtained from healthy female donors (mean age, 31.8 ± 3.1 years; mean body mass index, 21.3 ± 0.4; $n = 12$) undergoing liposuction of the abdomen or thighs. Each patient provided informed consent using an institutional review board–approved protocol before the procedure. ASCs were isolated from the aspirated fat tissue as described previously [6]. Briefly, the aspirated fat was digested in phosphate-buffered saline (PBS) containing 0.075% collagenase (Wako Chemicals, Osaka, Japan) for 30 min on a shaker at 37°C. After digestion, mature adipocytes and connective tissue were separated as floating tissues from sedimented cell pellets (corresponding to the SVF) by centrifugation (800*g*, 10 min). The SVF cells were resuspended, filtered through 100-μm mesh, plated at a density of 5×10^5 nucleated cells/100-mm dish, and cultured at 37°C in an atmosphere of 5% CO_2 in humid air. The culture medium was Medium 199 (GIBCO Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), 100 U penicillin, 100 mg/mL streptomycin, 5 μg/mL heparin, and 2 ng/mL fibroblast growth factor-1 (FGF-1). Primary cells were cultured until confluence (~7 days) and were defined as "Passage 0."

Flow cytometry and sorting

The cells at Passage 0 were examined or sorted using a flow cytometer (BD LSR II; BD Biosciences, San Diego, CA) or a cell sorter (BD FACSAria; BD Biosciences), respectively. For cell sorting, the cells were incubated with a phycoerythrin (PE)-labeled anti-CD34 antibody (clone 563, class III epitope; BD Biosciences), and CD34+ and CD34− cells were then separately collected, and immediately used for experiments as below. Control gates were set based on staining with an irrelevant (matched labeled isotype control IgG) antibody so that no more than 0.1% of cells were positive using the irrelevant antibody. The sorting gate for collecting CD34+ cells was set sufficiently away from the control gate to avoid contamination of CD34− cells (as shown in Fig. 1B). In each cell sorting run, more than 3×10^6 events (mean 4.1×10^6) were counted. For double-fluorescent flow-cytometric analysis, allophycocyanin (APC)-labeled anti-CD31 (eBioscience, San Diego, CA) and phycoerythrin and cyanine dye (PE-Cy7)-labeled anti-CD34 (BD Biosciences) were used.

Cell doubling assay

Each sorted cell population (CD34+ and CD34−) was seeded at 3,000 cells/cm² in two dishes. After the cells reached the logarithmic growth phase, they were separately trypsinized with a 48-h interval (4 and 6 days after plating) and counted using a cell counter (NucleoCounter; Chemometec, Allerod, Denmark). The doubling time was calculated according to the following formula: doubling time = $48/\log_2(N_2/N_1)$ hours, where N_1 is the first cell count and N_2 is the cell count 48 h later.

Colony-forming unit assay

Each sorted cell population (CD34+ and CD34−) was seeded at 150 cells/cm² and cultured for 14 days. The cells were then fixed in 100% methanol for 15 min and stained with 0.1% Giemsa solution for 10 min. The dishes were rinsed with water and allowed to air dry. A colony was included in the total number of colonies only if it contained more than 50 cells.

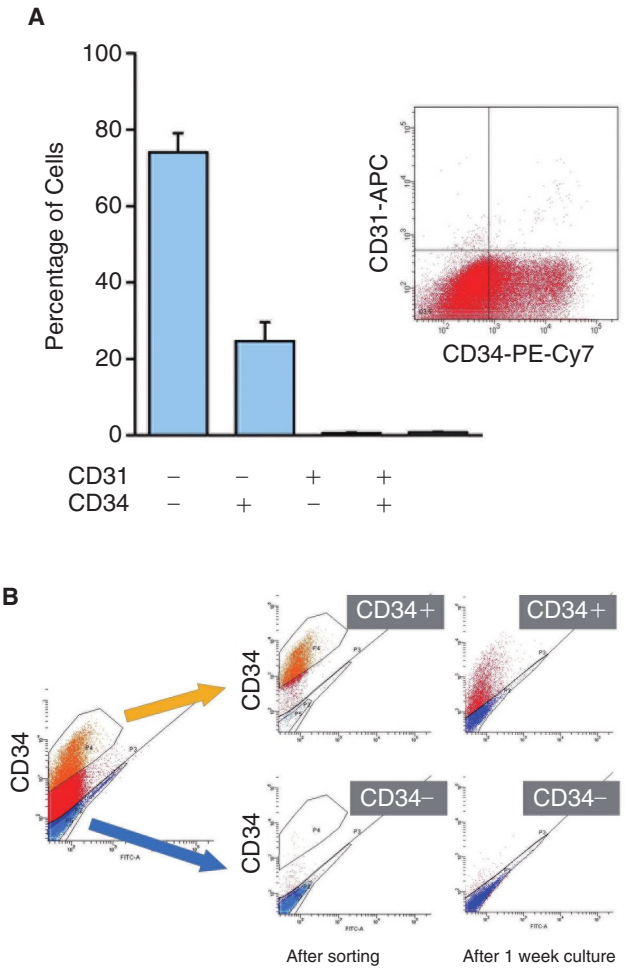

FIG. 1. Flow cytometric analyses and cell sorting of human adipose–derived stromal vascular fraction (SVF). (**A**) Flow cytometric analysis of the SVF cells cultured for 1 week. Most CD45+ nucleate cells (leukocytes) and CD31+ cells (vascular endothelial cells) were removed by adherent culture, and remaining cells were predominantly composed of CD31−/CD34− cells (74%) and CD31−/CD34+ cells (25%). (**B**) Flow cytometric analysis of sorted populations before and after further culture. One-week-cultured cells, most of which were ASCs, were successfully sorted into CD34+ and CD34− populations. Both populations were further cultured for 1 week; a portion of the CD34+ cells lost CD34 expression, while CD34− cells did not regain CD34 expression.

Adipogenic differentiation assay

Each sorted cell population (CD34+ and CD34−) was seeded at 3,000 cells/cm^2 and cultured until confluence (~5 days). The cells were then incubated for 21 days in DMEM (Nissui Pharmaceutical, Tokyo, Japan) or adipogenic medium (DMEM containing 10% FBS, 0.5 mM isobutylmethylxanthine, 1 µM dexamethasone, 10 µM insulin, and 200 µM indomethacin). Adipogenic differentiation was visualized with Oil Red O staining. For quantitative analysis of lipid droplets, Nile Red fluorescence (AdipoRed; Cambrex, Walkersville, MD) was measured with excitation at 485 nm and emission at 535 nm using a fluorescent plate reader (DTX 880 Multimode Detector; Beckman Coulter, Fullerton, CA).

Osteogenic differentiation assay

Each sorted cell population (CD34+ and CD34−) was seeded at 3,000 cells/cm^2 and cultured until confluence (~5 days). The cells were then incubated for 21 days in DMEM or osteogenic medium (DMEM containing 10% FBS, 0.1 µM dexamethasone, 50 µM ascorbic acid), and 10 mM β-glycerophosphate. Osteogenic differentiation was visualized with von Kossa staining. Calcium deposition was quantified with a commercial kit (Calcium C-Test Wako; Wako Chemicals) according to the manufacturer's instructions.

Migration assay

Each sorted cell population (CD34+ and CD34−) was resuspended in endothelial basal medium (EBM; Cambrex). Cells (2×10^5 cells in 200 µL of EBM) were seeded onto the upper chamber with 8-µm pores (Transwell; Corning Life Sciences, Lowell, MA), while 600 µL of EBM with or without growth factors (10 ng/mL FGF-2, 2.5 ng/mL platelet-derived growth factor [PDGF], 2 ng/mL epidermal growth factor [EGF], and 1 ng/mL transforming growth factor [TGF]-β) were added to the lower chamber. The cells were then allowed to migrate for 4 h at 37°C, after which they were fixed in 100% methanol for 15 min and stained with 0.1% Giemsa solution for 10 min. Cells remaining on the bottom of the upper chamber were removed using a cotton swab. Cells that migrated through the pores were counted under a light microscope (×200).

Capillary network formation assay

Each sorted cell population (CD34+ and CD34−) was seeded onto Matrigel (BD Biosciences) in a 96-well plate (5×10^3 cells per well) after pretreatment for 24 h in EBM with or without growth factors (10 ng/mL FGF-2, 2.5 ng/mL PDGF, 2 ng/mL EGF, and 1 ng/mL TGF-β). The cells were incubated for 6 h at 37°C; formation of a capillary-like network was evaluated under a light microscope (×50), and total network length per field was calculated. By using an image editing software (Photoshop 7.0; Adobe Systems Inc., San Jose, CA), the network was traced on a histological image and the length (pixel) of each trace was measured using a scale image.

Polymerase chain reaction (PCR) array and quantitative real-time PCR

For the PCR array assay, total RNA (2 µg) was isolated from each noncultured sorted cell population (CD34+ and CD34−) using an RNeasy Mini kit (Qiagen, Hilden, Germany), followed by reverse transcription. A commercially available kit (Human Angiogenesis RT^2 Profiler PCR Array; SABiosciences, Frederick, MD) was used to examine the expression of 84 key genes involved in modulating angiogenesis. For quantitative real-time PCR, each sorted cell population (CD34+ and CD34−) was seeded at 3,000 cells/cm^2 and cultured until confluence (~5 days). Cells were then placed in normoxic (20% O_2) or hypoxic (2% O_2) conditions for 24 h, after which RNA was isolated and reverse transcribed. We amplified cDNA for 40 cycles with a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) using an ABI7700 sequence detection system (Applied Biosystems). The primer sequences used in real-time PCR analysis are shown in Supplementary Table 1. (supplementary table is available online at http://www.liebertpub.com) Expression levels were calculated by the comparative C_T method using GAPDH as an endogenous reference gene.

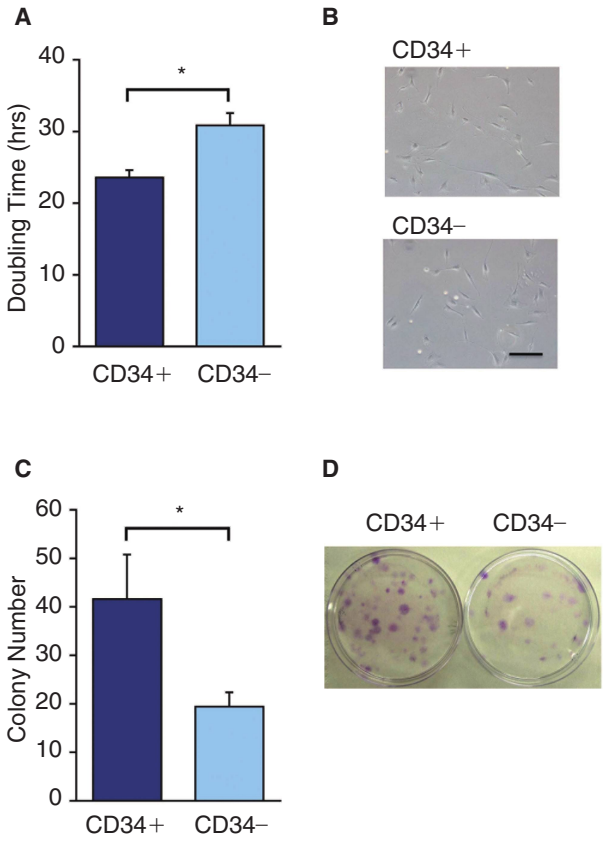

FIG. 2. CD34+ cells exhibit shorter doubling time and increased colony formation. (**A**) Doubling time of CD34+ and CD34− cells. CD34+ cells exhibited a significantly shorter doubling time ($n = 8$, $^*P < 0.05$). (**B**) Microphotographs of cultured CD34+ and CD34− cells. No morphological differences were observed. Scale bar = 200 µm. (**C**) Number of CD34+ and CD34− colonies per culture dish. CD34+ cells formed a significantly higher number of colonies ($n = 5$, $^*P < 0.05$). (**D**) Representative photos of the cultured CD34+ and CD34− colonies.

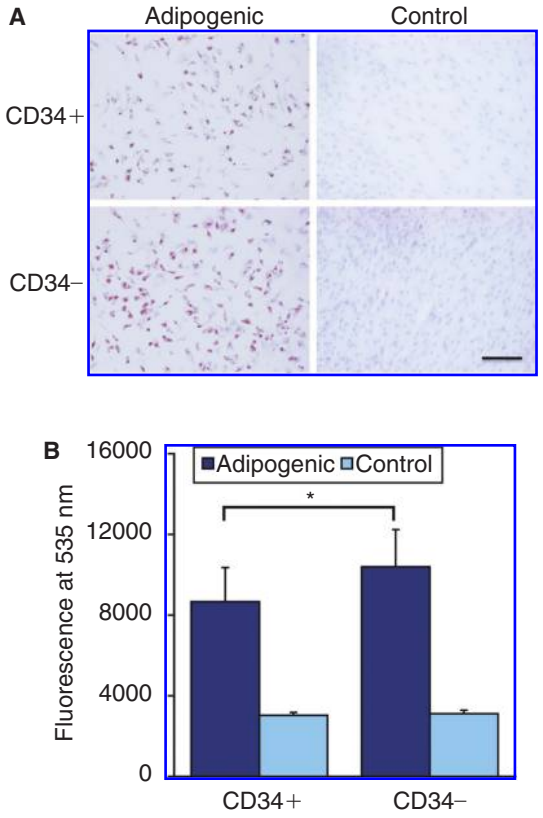

FIG. 3. CD34− cells exhibit higher adipogenic differentiation capacity. (**A**) Oil Red O staining of the differentiated and undifferentiated CD34+ and CD34− cells cultured with adipogenic and control medium, respectively. Scale bar = 200 μm. (**B**) Quantitative analysis of lipid droplets. CD34− cells showed significantly higher lipid content than CD34+ cells ($n = 12$, $^*P < 0.05$).

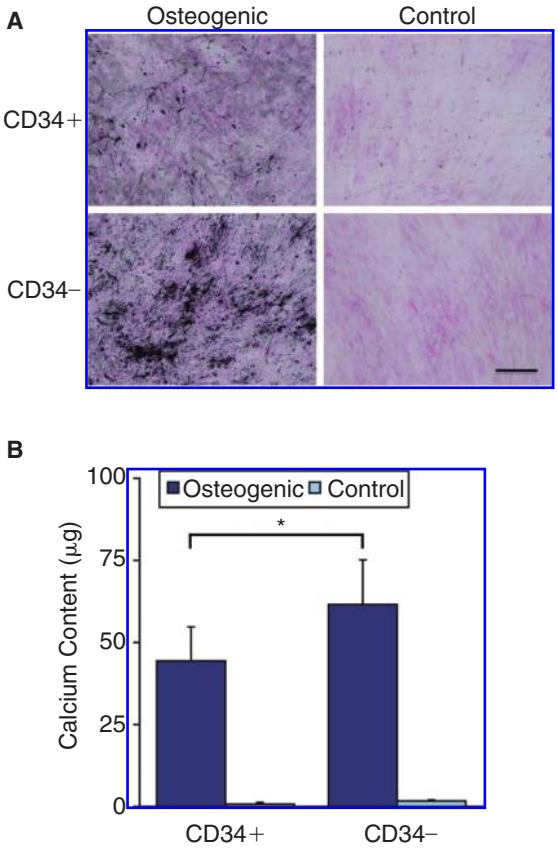

FIG. 4. CD34− cells exhibit higher osteogenic differentiation capacity. (**A**) von Kossa staining of the differentiated and undifferentiated CD34+ and CD34− cells cultured with osteogenic and control medium, respectively. Scale bar = 200 μm. (**B**) Quantitative analysis of calcium deposition. CD34− cells showed significantly higher calcium content than CD34+ cells ($n = 10$, $^*P < 0.05$).

Statistical analysis

Results were expressed as mean ± standard error. Comparisons between CD34+ and CD34− cells were performed with a paired *t*-test. A value of $P < 0.05$ was considered significant.

Results

Flow cytometry and cell sorting

After adherent culture of SVF cells for 1 week (Passage 0), flow cytometry analysis demonstrated only few CD31+/CD34+ vascular endothelial cells (0.71 ± 0.18%), with the rest of the cells as hASCs. Approximately one-quarter of the hASCs were CD31−/CD34+, and the remaining hASCs were CD31−/CD34− (Fig. 1A). Although CD34+ and CD34− hASCs did not represent populations clearly separated from one another, each subpopulation of hASCs was successfully sorted and collected. The sorted cells were reanalyzed for CD34 expression, and the efficiency of sorting was confirmed as successful (Fig. 1B). Flow cytometry analysis of the two populations after another 1 week of culture revealed that CD34+ cells partly lost CD34 expression during culture and the percentage of CD34+ cells was 51.3 ± 5.4% at 1 week, while CD34− cells did not regain CD34 expression (Fig. 1B).

Proliferative and colony-forming capacity

The cell doubling assay showed that doubling time was shorter in CD34+ hASCs compared to CD34− hASCs (Fig. 2A), although no morphological differences were observed between CD34+ and CD34− hASCs (Fig. 2B). CD34+ ASCs contained a significantly larger number of colony-forming cells compared to CD34− ASCs (Fig. 2C and 2D). These results suggested that CD34 expression in hASCs correlates with their replicative potential.

Differentiative capacity

Although adipogenic differentiation was induced in both CD34+ and CD34− hASCs, CD34− hASCs differentiated into adipocytes more readily than CD34+ hASCs (Fig. 3A and 3B). Regarding osteogenesis, CD34− ASCs also showed a significantly greater ability for differentiation compared

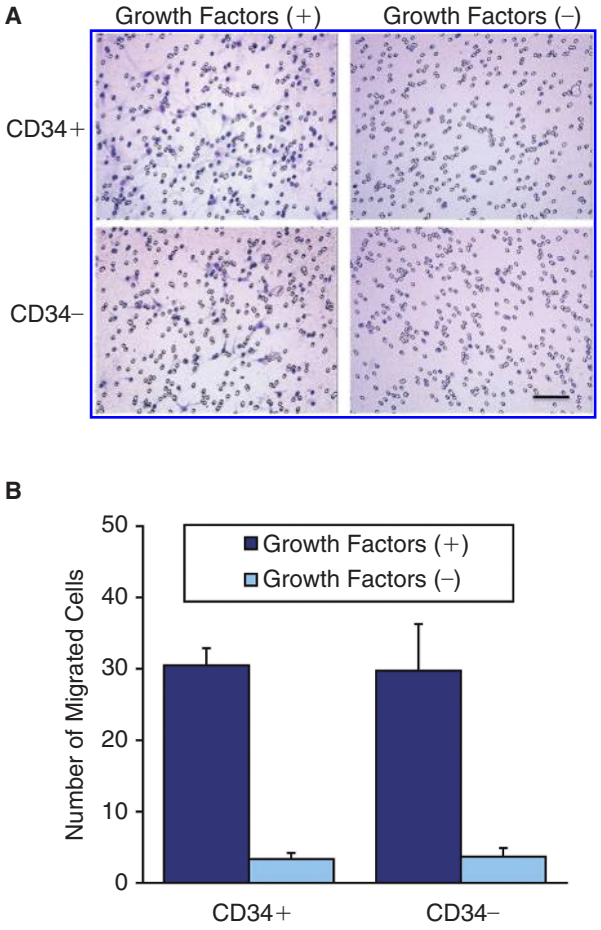

FIG. 5. CD34+ and CD34− cells exhibit similar migration capacity. (**A**) Representative photos of the migration assay. Scale bar = 100 μm. (**B**) Number of migrated cells. Both CD34+ and CD34− cells migrated in response to growth factors and no significant difference in migrated cell number was observed between the two groups ($n = 4$).

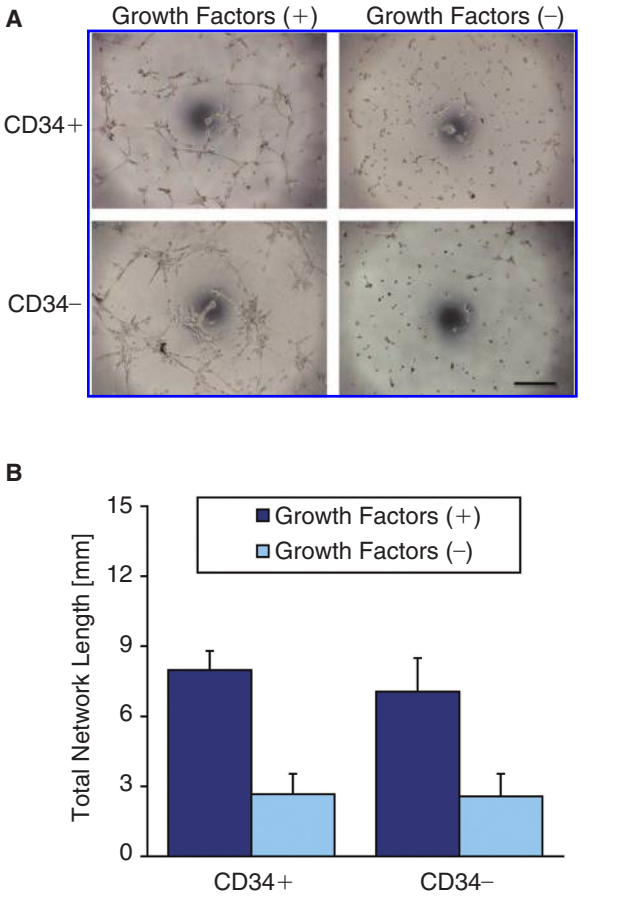

FIG. 6. CD34+ and CD34− cells exhibit similar capillary network forming capacity. (**A**) Representative photos of the network-forming assay. Scale bar = 400 μm. (**B**) Total capillary-like network length. Both CD34+ and CD34− cells showed substantial formation of a capillary-like network in response to pretreatment with growth factors, but no significant differences in length were observed between the two groups ($n = 4$).

to CD34+ counterparts (Fig. 4A and 4B). These results suggested that CD34− hASCs have increased capacity for differentiation into mesenchymal lineages compared to CD34+ hASCs.

Migrating and network-forming capacity

A migration assay confirmed that both CD34+ and CD34− hASCs migrated in response to stimulation by growth factors, but no differences in the number of migrated cells were observed between the two populations (Fig. 5A and 5B). In a capillary network formation assay, both CD34+ and CD34− cells formed a capillary network to a similar extent in response to pretreatment with growth factors. No significant difference in total network length was detected, although formation of networks without pretreatment was scarcely observed with both populations (Fig. 6A and 6B). These results indicated that both CD34+ and CD34− hASCs retain similar capabilities for migration and capillary-like network formation in response to growth factor stimulation.

Expression of angiogenesis-related genes

We used a PCR array to analyze the expression of 84 angiogenesis-related genes in both cell populations immediately after cell sorting (Supplemental Table 2). A total of 10 genes were induced by more than fourfold in CD34+ hASCs, while four genes were upregulated in CD34− hASCs. Most of the genes upregulated in CD34+ cells were vascular endothelial (progenitor) cell markers, such as Flk-1, VE cadherin, CD31, FLT1, and Tie-2 (Table 1). Real-time PCR confirmed significantly higher expression of CD31 and Flk-1 in CD34+ hASCs (Fig. 7). In contrast, expression levels of pericyte markers, such as CD146, NG2, and α-smooth muscle actin (SMA), were higher in CD34− cells. CD34+ and CD34− cells showed similar levels of expression of vascular endothelial growth factor (VEGF) and hepatocyte growth

Table 1. Upregulated Genes in CD34+ and CD34− Cells (Fold Change >4) Using a PCR Array

Gene symbol	*Gene name*	*Fold change*
Genes upregulated in CD34+ cells		
NOTCH4	Notch homolog 4	77.2
KDR (Flk-1)	Kinase insert domain receptor, vascular endothelial growth factor receptor-2	45.9
CDH5 (CD144, VE-cadherin)	Cadherin 5, type 2	45.6
EFNA1	Ephrin-A1	44.6
PECAM1 (CD31)	Platelet/endothelial cell adhesion molecule	27.5
EFNB2	Ephrin-B2	20.7
FLT1	fms-related tyrosine kinase 1, vascular endothelial growth factor receptor-2	11.7
PGF	Placental growth factor	11.2
TEK (Tie-2)	TEK tyrosine kinase, endothelial	7.2
EDG1	Sphingosine-1-phosphate receptor 1	5.7
Genes upregulated in CD34− cells		
EREG	Epiregulin	18.3
INFG	Interferon-γ	11.4
TGFA	Transforming growth factor-α	5.9
IL8	Interleukin 8	4.3

factor (HGF). In both populations, VEGF was upregulated by hypoxia, while HGF was downregulated (Fig. 7). These results suggested that CD34 expression in hASCs correlates positively with endothelial characteristics and negatively with pericytic characteristics.

Discussion

CD34 is a cell surface glycoprotein that was first discovered in a small fraction of human bone marrow cells [16]. Since the successful hematopoietic reconstitution of baboons with selected CD34+ bone marrow cells [17], CD34 has become the hallmark of hematopoietic stem cells. CD34 is also expressed in a wide variety of nonhematopoietic cells, such as vascular endothelial cells and soft tissue neoplasms [18,19]. Although a role for CD34 in cell adhesion and hematopoietic differentiation has been described, the precise function(s) of CD34 remain unclear [18,20]. Interestingly, reversibility of CD34 expression has been reported in murine [21] and human [22] hematopoietic stem cells; a CD34− stem cell population can be generated from CD34+ cells, and these CD34− cells can then regain CD34 expression upon treatment with 5-fluorouracil [21] or secondary transplantation [22], suggesting that CD34 may be a marker of an activated state of stem cells but not necessarily a marker for all stem cells [23]. The expression of CD34 in hASCs is also reversible; hASCs lost CD34 expression upon culture with DMEM, but regained expression after culture with EBM (data not shown). In this study, CD34+ and CD34− cells were collected from adipose-derived SVF cells cultured for 1 week, which contained both populations at similar proportions. We cultured the sorted cells for 5 days before use in differentiation assays and PCR assay under hypoxia, while cells were used immediately after cell sorting in other assays. Possible loss of CD34 expression after cell sorting and further culture in the downstream assays may decrease and underscore the difference between CD34+ and CD34− populations and this may be a limitation in assessing cells with dynamic nature.

The results from this study demonstrate that CD34+ hASCs have a greater replicative capacity compared with CD34− hASCs. Taking into consideration that hASCs express CD34 in histological sections of human adipose tissue and almost all hASCs in the SVF are CD34+ [6,9], it was suggested that hASCs physiologically express CD34 when localized in their niche and may lose CD34 expression in response to microenvironment changes, such as injury, inflammation, or upon culture. We recently reported that CD34+ hASCs proliferate in adipose tissue remodeling after ischemia-reperfusion injury and contribute to the regeneration of adipose tissue [24]. Thus, ASCs seem to first undergo proliferation before functioning in their physiological roles, such as in migration and differentiation.

Although many factors, such as FGF-2 or hypoxia, affect the differentiation capacity or direction of hASCs [25–27], the correlation between CD34 expression and the differentiation potential of hASCs has not been studied previously. Our results also indicated that CD34− cells could differentiate into mesenchymal lineages more readily than CD34+ cells. In hematopoietic cells, CD34 is expressed at high levels on the earliest hematopoietic cells, and expression decreases with their maturation, suggesting that CD34 expression indicates immature cell status [18]. The same interpretation may be applied to hASCs; loss of CD34 expression may reflect the differentiation (or commitment) of ASCs. Human ASCs do not express several stem cell markers, including telomerase, CD133, and the membrane transporter ABCG2 [28]. A recent study identified a CD24+ subpopulation in murine SVF as physiological adipocyte progenitors [12]. As we confirmed a small fraction of CD24+/CD34+ cells in freshly isolated

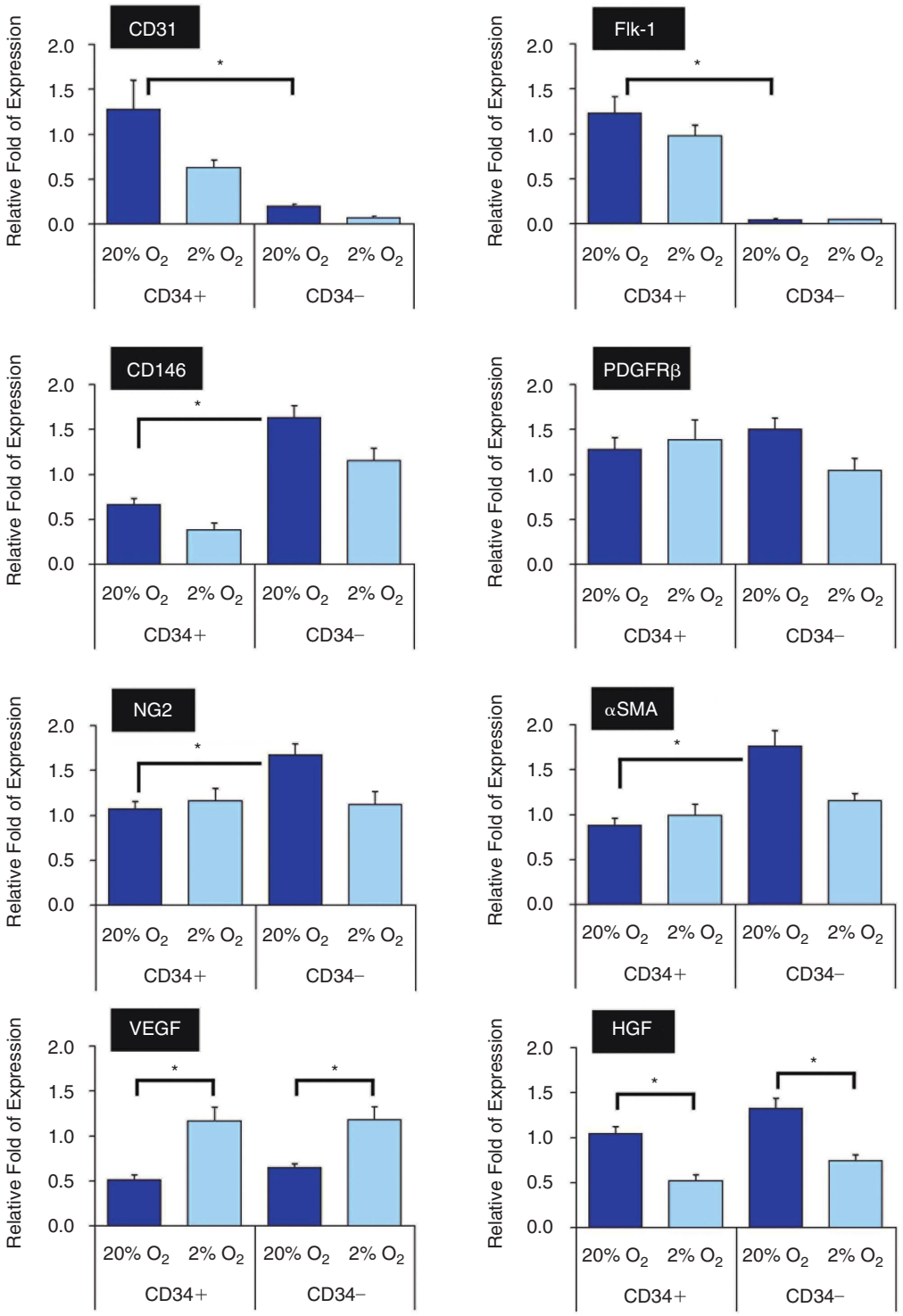

FIG. 7. Real-time PCR of angiogenesis-related genes under hypoxic and normal conditions. Expression levels of CD31 and Flk-1 were much higher in CD34+ cells, while those of pericyte markers (CD146, NG2, and α-SMA) were higher in CD34− cells. In both cell types, VEGF was upregulated under hypoxic conditions, while HGF was downregulated ($n = 3$, $^*P < 0.05$).

human SVF (data not shown), CD24 may also be a specific marker for hASCs with particular potentials such as stemness or commitment to adipocytes.

CD34+ and CD34− hASCs showed similar capabilities of migration and capillary network formation in this study. Many studies have described the migration assay and Matrigel assay using hASCs [9,14], and various growth factors have been used as activators. We selected a combination of FGF-2, PDGF, EGF, and TGF-β, all of which were highly released in the early phase of wound healing after adipose

tissue injury [29]. FGF-2 and PDGF were reported to synergistically promote migration of vascular endothelial and mural cells as well as angiogenesis [30]. Although direct and undirected migration were not discriminated in our migration assay, no apparent difference in migrating capacity was detected between CD34+ and CD34− hASCs. Capillary formation was also induced to the similar extent to each other population between the two populations upon treatment with the combination of growth factors.

ASCs have been reported to promote angiogenesis by their endothelial differentiation [31,32] and/or secretion of angiogenic growth factors [33]. Furthermore, recent reports showed pericytic characteristics and localization of ASCs [9,11]. Our results showed that expression levels of endothelial progenitor cell markers such as Flk-1, FLT1, and Tie-2 were higher in CD34+ hASCs, while those of pericyte markers such as CD146, NG2, and α-SMA were higher in CD34− hASCs. One possibility is that the higher expression of CD31 in the CD34+ population could be the result of contamination by CD31+/CD34+ endothelial cells, although the percentage of endothelial cells after 7 days of culture was <1%. CD34 is also a marker of endothelial progenitor cells (EPCs) in peripheral blood or bone marrow [34,35]. Recently, EPC-like cells were identified in a distinct zone of the vascular wall, between the smooth muscle and adventitial layers [36]; these vascular wall resident EPCs also express CD34 and may constitute a part of the ASCs in the adipose tissue. Human ASCs in tissue and those freshly isolated in the SVF express CD34 but not pericyte markers, such as CD146 [6] and NG2 (data not shown). After culture, some hASCs lost CD34 expression but expressed higher levels of the pericyte markers; the CD34 expression in hASCs may correlate with original and physiological characteristics and functions. However, even after the loss of CD34 expression, hASCs seem to retain angiogenic potential and capacity for the secretion of angiogenic growth factors, such as VEGF and HGF. The secretion of angiogenic growth factors can be influenced by hypoxia [33,37], although downregulation of HGF by hypoxia in our study is inconsistent with a previous report [33].

In conclusion, our findings suggest that CD34 expression by hASCs may correlate with replicative capacity, differentiation potentials, specific expression profiles of angiogenesis-related genes, and immaturity or stemness of the cells, while no apparent effects on response to and secretion of growth factors were observed. Loss of CD34 expression may be a physiological process of commitment and/or differentiation from immature status into specific lineages, such as adipose, bone, and smooth muscle. Further studies on CD34 in ASCs will be needed to elucidate other functions, reversibility of expression, and correlation to localization in the niche.

Acknowledgments

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