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# Characterization of Freshly Isolated and Cultured Cells Derived From the Fatty and Fluid Portions of Liposuction Aspirates

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Liposuction aspirates (primarily saline solution, blood, and adipose tissue fragments) separate into fatty and fluid portions. Cells isolated from the fatty portion are termed *processed lipoaspirate (PLA)* cells and contain adipose-derived adherent stromal cells (ASCs). Here we define cells isolated from the fluid portion of liposuction aspirates as *liposuction aspirate fluid (LAF)* cells. Stromal vascular fractions (SVF) were isolated separately from both portions and characterized under cultured and non-cultured conditions. A comparable number of LAF and PLA cells were freshly isolated, but fewer LAF cells were adherent. $CD34^+CD45^-$ cells from fresh LAF isolates were expanded by adherent culture, suggesting that LAF cells contain ASCs. Although freshly isolated PLA and LAF cells have distinct cell surface marker profiles, adherent PLA and LAF cells have quite similar characteristics with regard to growth kinetics, morphology, capacity for differentiation, and surface marker profiles. After plating, both PLA and LAF cells showed significant increased expression of CD29, CD44, CD49d, CD73, CD90, CD105, and CD151 and decreased expression of CD31 and CD45. Multicolor FACS analysis revealed that SVF are composed of heterogeneous cell populations including blood-derived cells ($CD45^+$), ASCs ($CD31^-CD34^+CD45^-CD90^+CD105^-CD146^-$), endothelial (progenitor) cells ($CD31^+CD34^+CD45^-$ $CD90^+CD105^{low}CD146^+$), pericytes ($CD31^-CD34^-CD45^-CD90^+CD105^-CD146^+$), and other cells. After plating, ASCs showed a dramatic increase in CD105 expression. Although some adherent ASCs lost CD34 expression with increasing culture time, our culture method maintained CD34 expression in ASCs for at least 10–20 weeks. These results suggest that liposuction-derived cells may be useful and valuable for cell-based therapies. J. Cell. Physiol. 208: 64–76, 2006.


The stromal vascular fraction (SVF) isolated from adipose tissue contains adipogenic progenitors with fibroblast-like morphologies (Van et al., 1976). These cells have been referred to by various names including preadipocytes and vascular stromal cells. In this study, we refer to the adherent stromal cells isolated from adipose tissue as *adipose-derived stromal (or stem) cells (*ASCs). Monoclonal (Zuk et al., 2002) and polyclonal (Zuk et al., 2001) culture studies have shown that human ASCs can be obtained from liposuction aspirates and can differentiate into multiple lineages of mesodermal or ectodermal origin. In both in vitro and in vivo studies, human ASCs have been shown to differentiate not only into mesenchymal lineages, such as adipogenic, chondrogenic (Erickson et al., 2002; Awad et al., 2003; Huang et al., 2004), osteogenic (Dragoo et al., 2003; Cowan et al., 2004; Halvorsen et al., 2004; Hicok et al., 2004; Peterson et al., 2005), myogenic (Mizuno et al., 2002; Rodriguez et al., 2005), and cardiomyogenic (Planat-Benard et al., 2004a; Strem et al., 2005) lines, but also into neurogenic (Safford et al., 2002; Ashjian et al., 2003; Kang et al., 2003), angiogenic (Miranville et al., 2004; Rehman et al., 2004; Planat-Benard et al., 2004b; Cao et al., 2005), and hepatic (Seo et al., 2005) lineages.

Liposuction is one of the most popular cosmetic surgical procedures; worldwide, an estimated one million liposuctions are performed annually. Although liposuction yields a large volume (e.g., 1 L) of adipose tissue and is considered the typical method for clinically harvesting ASCs, liposuction aspirates have not been well researched for use in clinical situations. Liposuction aspirates are comprised of fatty and fluid portions (Fig. 1a). The fatty portion consists of suctioned adipose tissue that has been "shredded" by the reciprocal movement of a metal canulla and vacuum pressure (500–700 mm Hg), while the fluid portion is the liquid aspirated along with the fatty portion. The fluid portion is primarily composed of (1) a saline solution preoperatively injected into the site to prevent nerve and blood vessel damage, (2) peripheral blood, and (3) cells or tissue fractions derived from adipose tissue. Although

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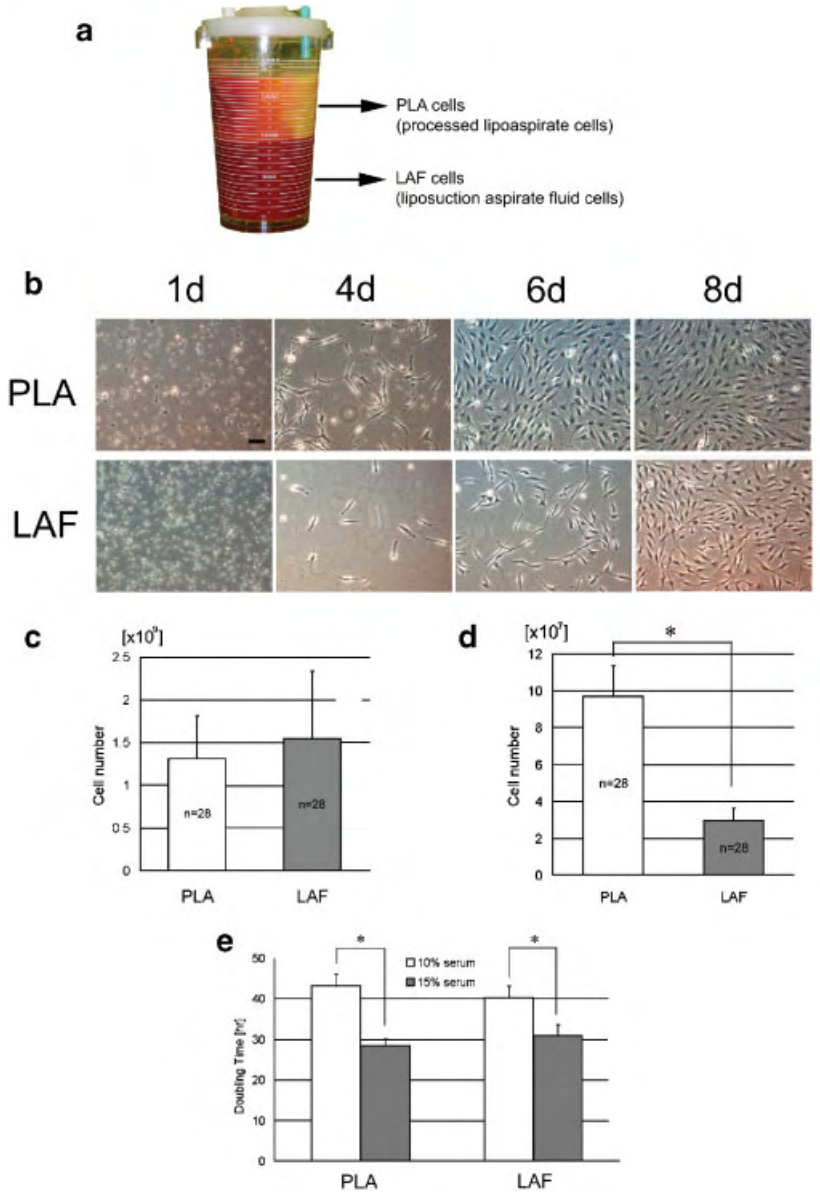


Fig. 1. Isolation of ASCs from the fatty and fluid portions of liposuction aspirates. **a**: The two portions of liposuction aspirates in a bottle. The fatty portion floats above the denser fluid portion. Cells isolated from the fatty portion were named PLA cells; those isolated from the liquid portion were termed LAF cells. **b**: Primary cultures of freshly isolated PLA and LAF cells. Scale bar = 100 μm. **c**: Cell yields of freshly isolated PLA and LAF cells. No statistically significant difference in yield was detected. **d**: Adherent cell yields of cultured PLA and LAF cells at 1 week. A significantly higher yield of adherent PLA cells was detected. $^*P < 0.05$. **e**: Doubling times of adherent PLA and LAF cells. A statistically significant difference was seen between the two serum concentrations (10% and 15%) in both PLA and LAF cells, but not between PLA and LAF cells at either serum concentration, $^*P < 0.05$.

only the fatty portion of liposuction aspirates has been investigated to date, we recognized through a preliminary survey that a significant amount of progenitor cells can be isolated from the liquid portion as well. Cells isolated from the fatty portion have been termed *processed lipoaspirate (PLA)* cells (Zuk et al., 2001, 2002), and here, we refer to cells isolated from the fluid portion as *liposuction aspirate fluid (LAF)* cells.

ASCs are currently being used in clinical trials including studies investigating bone defect (Lendeckel et al., 2004) and rectovaginal fistula (Garcia-Olmo et al., 2005) treatments and soft tissue augmentation (our unpublished data). ASCs can be used clinically without cell expansion because a sufficient number can be obtained directly by processing liposuction aspirates, which are usually of a large volume. Furthermore, the use of minimally manipulated fresh cells may lead to higher safety and efficacy in actual treatments. Thus, because freshly isolated ASCs are preferable to cultured ones for cell-based therapies, especially in the initial stage of regenerative medicine, minimally manipulated SVF from adipose tissue must be characterized in more detail. The SVF is known to be comprised of a heterogeneous cell population, but the exact cell composition remains to be determined.

In this study, the fatty and fluid portions of liposuction aspirates were investigated as sources for ASCs. Cells isolated from both portions were characterized under fresh and cultured conditions. In addition, long-term changes in cell surface marker expression profiles were determined for both cell populations.

## MATERIALS AND METHODS

### Human tissue sampling

After informed consent, we obtained liposuction aspirates using an IRB-approved protocol from healthy female donors aged 21 to 59 years who underwent liposuction of the abdomen or thighs. Liposuction aspirates were divided into two portions: a floating adipose portion (also called lipoaspirate) and a denser fluid portion (Fig. 1a). Both portions were used as sources for PLA and LAF cells. For harvesting human vascular endothelial cells (HUVEC) and dermal fibroblasts, umbilical cords and skin were obtained from separate donors under informed consent. Human mesenchymal stem cells derived from bone marrow (frozen at Passage 2) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and cultured with the same medium used for adipose-derived cells.

### Cell isolation

All chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), unless otherwise stated.

PLA cells were separated from the fatty portions of liposuction aspirates using a procedure modified from Zuk et al. (2001). Briefly, the suctioned fat was digested with 0.075% collagenase in PBS for 30 min on a shaker at 37°C. Mature adipocytes and connective tissues were separated from pellets by centrifugation (800*g*, 10 min). Pellets were resuspended in erythrocyte lysis buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA) and incubated for 5 min at room temperature. The pellets were resuspended and passed through a 100-μm mesh filter (Millipore, Billerica, MA).

LAF cells were harvested from the fluid portions of liposuction aspirates. The suctioned fluid was centrifuged (400*g*, 10 min), and the pellets were resuspended in erythrocyte lysis buffer. After 5 min at room temperature, lysates were passed through a 100-μm mesh filter. The pellets were then processed for density gradient centrifugation with Ficoll (GE Healthcare Bio-Sciences, Piscataway, NJ). After centrifugation (800*g*, 20 min), cells at the gradient interface were collected, washed with PBS, and passed through a 100-μm mesh filter. For flow cytometry of freshly isolated LAF cells, density gradient centrifugation was not conducted. Nucleated cell counts were performed using a NucleoCounter (Chemometec, Allerod, Denmark).

### Cell culture of adherent cells from adipose tissue

Freshly isolated PLA or LAF cells were plated in medium at a density of $5 \times 10^6$ nucleated cells/100-mm gelatin-coated dish. Cells were cultured at 37°C, 5% $CO_2$, in humid air. The culture medium was M-199 containing 10% FBS, 100 IU penicillin, 100 mg/ml streptomycin, 5 ng/ml heparin, and 2 ng/ml acidic FGF. For measurement of doubling time, the same medium was used with a serum concentration of 10% or 15%. Primary cells were cultured for 7 days and were defined as "Passage 0." The medium was replaced every 3 days, and cells were passaged every week. After primary culture for 7 days, attached cells were passaged by trypsinization and plated in the same medium at a density of 2,000 cells/$cm^2$.

### Induced differentiation of cultured PLA and LAF cells

Capacities to differentiate along adipogenic, chondrogenic, and osteogenic lineages were examined. Seven days after seeding PLA or LAF cells at Passage 3–5, cell differentiation was initiated by replacing the M-199 culture medium. Cells cultured in control medium (DMEM plus 100 IU penicillin and 100 mg/ml streptomycin) containing 10% FBS were used as negative controls. For adipogenic differentiation, confluent cultures were incubated for 4 weeks in the control medium containing 10% FBS supplemented with 0.5 mM isobutylmethylxanthine (Sigma, St. Louis, MO), 1 μM dexamethasone, 10 μM insulin (Sigma), and 200 μM indomethacin. Fixed cells (4% paraformaldehyde for 10 min) were washed with 60% isopropanol and incubated for 15 min with Oil-Red O to visualize lipid droplets. Cells were then washed with isopropanol and counterstained with hematoxylin.

For chondrogenic differentiation, two types of evaluations were performed. First, confluent cultures were incubated for 4 weeks with the control medium containing 1% FBS supplemented with 6.25 μg/ml insulin, 10 ng/ml TGFβ1, and 50 nM ascorbate-2-phosphate. Fixed cells (4% paraformaldehyde for 10 min) were washed with 3% acetic acid and incubated for 30 min with 1% Alcian Blue 8 GX (Sigma), 3% acetic acid to visualize the extracellular matrix. Cells were then washed with 3% acetic acid and counterstained with 0.1% Nuclear Fast Red, 5% $Al_2(SO_4)_3$ solution. Second, a micromass culture system was used as previously reported (Johnstone et al., 1998). Cells were pelleted in a 15-ml tube and cultured with the chondrogenic medium for 3 weeks.

For osteogenic differentiation, cells were incubated for 4 weeks in the control medium containing 10% FBS supplemented with 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, and 10 mM β-glycerophosphate (Nacalai Tesque, Kyoto, Japan). Fixed cells (4% paraformaldehyde for 10 min) were washed and incubated with 2.5% silver nitrate for 20 min in the dark. The cells were then washed, placed in the light for 15 min, and incubated for 2 min with 0.5% hydroquinone. Cells were incubated for 2 min with 5% sodium thiosulphate to visualize calcified deposits. The cells were then washed and counterstained with 0.1% Nuclear Fast Red, 5% $Al_2(SO_4)_3$ solution.

For adipogenic differentiation, colony-forming unit analysis was also performed to quantify colonies with intracellular lipids. One hundred fifty cells were plated on a 100-mm culture disk and cultured for 10–14 days, followed by incubation for 2 weeks with adipogenic medium and staining with Oil-Red O as described above. The numbers of colonies positive or negative for staining were counted under a microscope.

### Flow cytometry and sorting

Freshly isolated PLA and LAF cells were examined for surface and intracellular molecule expression using flow cytometry. In addition, adherent PLA and LAF cells were examined at weeks 1, 2, 4, 6, 8, 10, and 20 of cell culture. The following monoclonal antibodies (MAbs) conjugated to fluorochromes were used: anti-CD4-FITC, CD10-PE, CD13-PE, CD16-PE, CD29-PE, CD31-PE, CD34-PE, CD34-FITC, CD34-PE Cy7, CD36-PE, CD44-PE, CD45-PE, CD45-FITC, CD49d-PE, CD49e-PE, CD54-PE, CD56-PE, CD57-FITC, CD62E-PE, CD62P-PE, CD69-FITC, CD73-PE, CD90-PE, CD106-FITC, CD117-PE, CD135-PE, CD146-PE, CD-151-PE, HLA-A,B,C-PE, Tie-2-PE (BD Biosciences, San Diego, CA), CD31-APC (eBioscience, San Diego, CA), CD144-PE (Beckman Coulter, Fullerton, CA), CD59-PE (Ancell, Bayport, MN), CD71-PE, CD105-PE (Serotec, Oxford, UK), CD133-PE, and Flk-1-PE (Techne, Minneapolis, MN). Irrelevant control MAbs were included for all fluorochromes. Cells were incubated with directly conjugated MAbs for 30 min, then washed and fixed in 1% paraformaldehyde. Cells were analyzed using a LSR II (Becton Dickinson, San Jose, CA) or FACS Vantage SE (Becton Dickinson) flow cytometry system. Data acquisition and analysis were then performed (Cell Quest software, Becton Dickinson). Gates were set based on staining with combinations of relevant and irrelevant MAbs so that no more than 0.1% of cells were positive using irrelevant antibodies. Cell sorting and subsequent analyses were performed using a FACSAria cell sorter (Becton Dickinson).

### Statistical analyses

Results were expressed as mean ± SEM. Welch's *t*-test was used to compare each parameter.

## RESULTS

### Isolation and expansion of stromal cells from fatty and fluid portions of liposuction aspirates

Compared to the fatty portion of liposuction aspirates, the fluid portion contains more peripheral blood discharged from the suctioned site during liposuction. The volume of peripheral blood varied among patients. The adipose portion was subjected to collagenase digestion followed by filtration for exclusion of extracellular

matrix (ECM) fragments and debris, while the fluid portion was centrifuged and processed for lysis of contaminating erythrocytes. After plating on culture dishes, non-adherent cells were discarded by changing the culture medium. Both adherent PLA and LAF cells had fibroblast-like morphologies and proliferated with similar doubling times, although a smaller number of adherent cells were harvested from LAF cells than from PLA cells (Fig. 1b).

Cell yields were normalized by dividing the isolated cell number by the volume (in liters) of the fatty portion of the liposuction aspirate. Normalized numbers of nucleate cells in SVF from the adipose (fresh PLA cells; $n = 28$) and fluid (fresh LAF cells; $n = 28$) portions were $1.31 \pm 0.50 \times 10^9$ and $1.55 \pm 0.79 \times 10^9$ per 1 L of adipose portion ($P = 0.401$), respectively (Fig. 1c). However, the cell number varied considerably among patients. Erythrocyte contamination was seen in both fresh PLA and LAF cells, although LAF cells apparently contained a much greater number of cells derived from peripheral blood. After 1 week of cell culture, normalized numbers of adherent PLA ($n = 28$) and LAF ($n = 28$) cells were $9.7 \pm 1.7 \times 10^7$ and $3.0 \pm 0.6 \times 10^7$ ($P < 0.001$), respectively (Fig. 1d). Thus, there was no significant difference in cell yield between freshly isolated PLA and LAF cells, but there was a difference between adherent PLA and LAF cells cultured for 1 week.

PLA and LAF cells were cultured in medium with 10% ($n = 14$) or 15% FBS ($n = 15$), and doubling times were measured using cells at Passage 0. Doubling times of PLA and LAF cells were $28.5 \pm 1.7$ h and $31.0 \pm 2.6$ h, respectively, when cultured with 15% FBS, and $43.3 \pm 2.8$ h and $40.2 \pm 3.0$ h, respectively, when cultured with 10% FBS. A statistically significant difference in doubling time was observed between the two serum concentrations in both PLA ($P < 0.001$) and LAF ($P < 0.05$) cells, but not between PLA and LAF cells at either serum concentration (Fig. 1e).

### In vitro differentiation of PLA and LAF cells

To compare the multipotency of PLA and LAF cells, cell differentiation was induced using cells at Passage 3–5 by culturing the cells for 4 weeks with adipogenic, chondrogenic, or osteogenic medium. The results showed that both cell populations have similar capacities to differentiate along the adipogenic (Fig. 2a), chondrogenic (Fig. 2b), and osteogenic lineages (Fig. 2c). In addition, similar cartilage formation was observed by the micromass system (Fig. 2d). Colony-forming unit analysis showed that the percentage of cells staining positive for Oil-Red O was $29.0 \pm 7.6\%$ for PLA cells and $24.1 \pm 4.3\%$ for LAF cells ($P = 0.12$) (Fig. 2d).

### Flow cytometric analysis of PLA and LAF cells

Flow cytometric analysis revealed that freshly isolated LAF cells differ significantly in cell surface marker expression from freshly isolated PLA cells. Compared to fresh LAF cells, fresh PLA cells contained higher percentages of cells positive for CD29 (β1-integrin), CD34, and CD90 (Thy-1) expression and a decreased percentage of $CD45^+$ cells of hematopoietic origin. Conversely, in fresh LAF cells, there were higher percentages of CD31 $(PECAM\text{-}1)^+$ cells and $CD45^+$ cells, suggesting that fresh LAF cells contained a larger number of blood-derived cells than fresh PLA cells. In the FACS plot of forward and side scatter characteristics (FSC and SSC) of fresh LAF cells, there were three $CD45^+$ cell clusters corresponding to granulocytes, monocytes/macrophages, and lymphocytes (Figs. 3a, S1). $CD34^+$ cells and $CD45^-$ cells were located in the cluster corresponding to monocytes/macrophages (Figs. 3a, S1). Double color analysis of fresh LAF cells for CD34 and CD45 indicated that most of the $CD34^+$ cells were $CD45^-$, suggesting that most of the $CD34^+$ cells were not derived from peripheral blood but from adipose tissue (Fig. 3b). In addition, $CD34^+CD45^-$ cells, but not $CD34^+CD45^+$ cells, proliferated on a culture dish, suggesting that the freshly isolated LAF cells contained ASCs derived from adipose tissue (Fig. 3b). $CD34^+$ cells (P2) were sorted and cultured using the protocol described above, but after 2 weeks in culture, approximately half of the cells were negative for CD34 (Fig. 3b), suggesting that some $CD34^+$ cells lost CD34 expression with increased culture time. The $CD34^-$ cells did not reacquire CD34 expression by further culturing. The doubling time of $CD34^-$ cells was significantly shorter than that of $CD34^+$ cells (Fig. 3c).

Subsequently, we performed multicolor FACS assays to investigate the SVF (fresh PLA and LAF cells) in more detail, especially with regard to cell composition. Even after processing with hypotonic erythrocyte lysis buffer, the SVF contained a large number of erythrocytes, so erythrocytes and platelets were excluded from analysis by gating them out by cell size. Consequently, only nucleated cells were analyzed (Figs. 4 and 5). The results are summarized in Table 1. We classified freshly isolated cells derived from liposuction aspirates into 11 cell populations [4 blood-derived ($CD45^+$) and 7 adipose-derived ($CD45^-$)] as follows. There were three major (>1%) and one minor (<1%) cell populations derived from blood with regard to expression patterns of CD31, CD34, CD45, CD105 (Endoglin), CD14, and CD15 (Figs. 5a, S2): (1) $CD31^{low}CD34^-CD45^+CD105^{low}CD14^-CD15^+$ cells (corresponding to granulocytes), (2) $CD31^{low}CD34^-CD45^+CD105^{low}CD14^+CD15^-$ cells (corresponding to monocytes/macrophages), (3) $CD31^-CD34^-CD45^+CD105^-$ cells (corresponding to lymphocytes), and (4) $CD31^-CD34^+CD45^+CD105^-$ cells (corresponding to hematopoietic stem cells). Cell compositions varied among samples, with the percentages of the first three populations dependent on the composition of the peripheral blood of each sample. Furthermore, the total percentage of blood-derived populations in SVF depends on the intraoperative hemorrhage volume of each sample.

There were seven adipose-derived populations with regard to expression patterns of CD31, CD34, CD45, CD90, CD105, and CD146: (1) $CD31^-CD34^+CD45^-CD90^+CD105^-CD146^-$ cells (corresponding to ASCs), (2) $CD31^+CD34^+CD45^-CD90^+CD105^{low}CD146^+$ cells, (3) $CD31^-CD34^+CD45^-CD90^+CD105^-CD146^+$ cells, (4) $CD31^-CD34^-CD45^-CD90^+CD105^-CD146^+$ cells, (5) $CD31^-CD34^+CD45^-CD90^+CD105^{low}$ cells, (6) $CD31^{low}CD34^+CD45^-CD90^+CD105^{low}CD146^+$ cells, and (7) $CD31^-CD34^-CD45^-CD90^+CD105^-CD146^-$ cells. The first population corresponded to ASCs and comprised 70–90% of the total adipose-derived ($CD45^-$) cells. Most ASCs were $CD29^+$, CD117 (c-kit)$^-$, CD133/AC133$^-$, CD144 (VE-cadherin)$^-$, and Flk-1(VEGFR-2)$^-$ (Fig. 4a). The second population was positive for both CD31 and CD146 (identified as P1 in Fig. 5a; also identified in Figs. 4b and 5b), suggesting that it is composed of vascular endothelial cells or endothelial progenitor cells derived from adipose. The third ($CD34^+$) and fourth ($CD34^-$) populations were $CD31^-CD146^+$ (Fig. 4a,b), suggesting that they may correspond to pericyte progenitors ($CD34^+$) and pericytes ($CD34^-$), respectively. The fifth population was very small and seemed

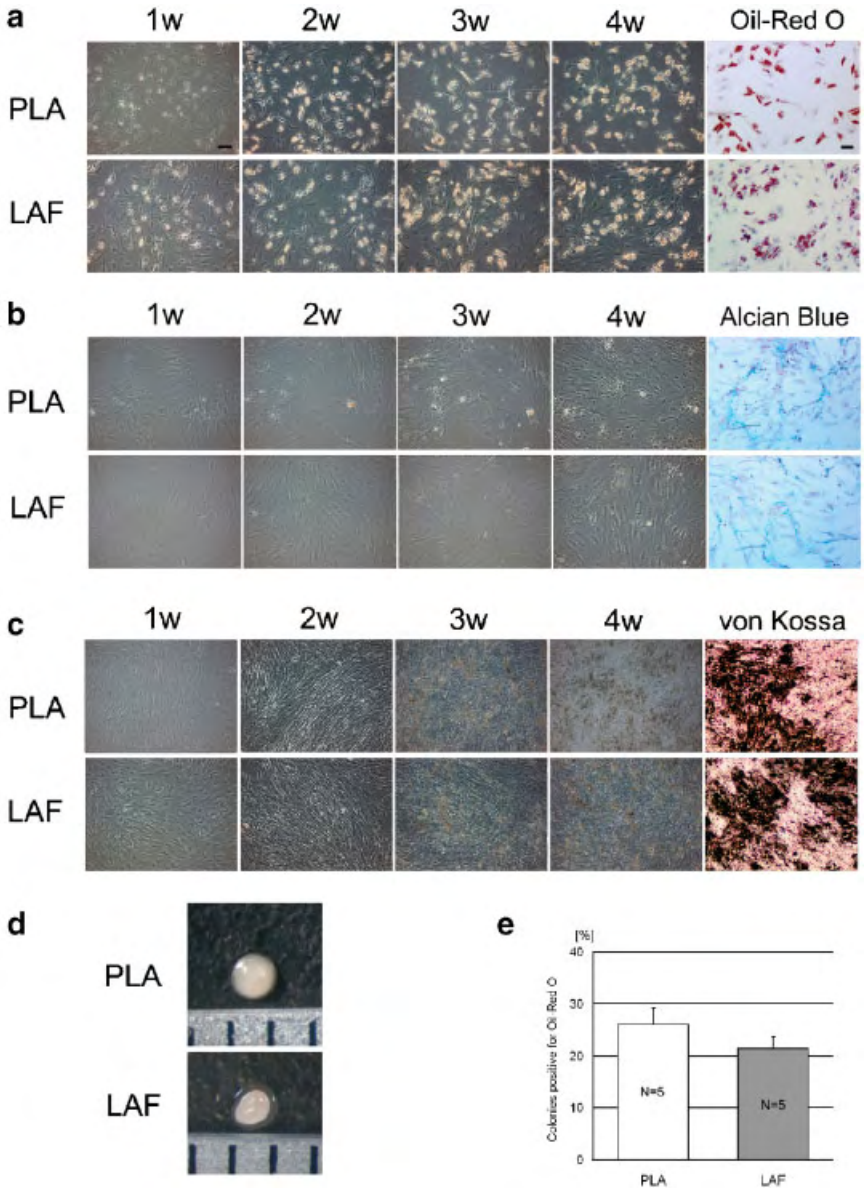


Fig. 2. Cell differentiation analysis of adherent PLA and LAF cells. Representative results are shown in **a–d:** PLA and LAF cells at Passage 3–5 were similarly induced to differentiate into adipogenic, chondrogenic, and osteogenic lineages. a: Cultures under adipogenic conditions. Adipogenic differentiation was induced, and lipid droplets were visualized with Oil-Red O staining at 4 weeks. b: Cultures under chondrogenic conditions. Chondrogenic differentiation was induced and visualized with Alcian blue staining at 4 weeks. c: Cultures under osteogenic conditions. Osteogenic differentiation was induced and visualized with von Kossa staining at 4 weeks. Scale bar = 100 μm. d: Micromass culture of PLA and LAF cells. Cartilage formation was similarly observed using the micromass system. **e**: Colony-forming unit analysis under adipogenic conditions. Between adherent PLA and LAF cells, the difference in percentages of colonies that differentiated into an adipogenic lineage was not statistically significant.

to correspond to CD105-positive ASCs (P1 in Fig. 5b). The sixth population was also small, with moderate expression of CD31 (P2 in Fig. 5a). Finally, the seventh population was positive only for CD90, suggesting that it may be fibroblasts or ASCs that lost CD34 expression (P3 in Fig. 5a).

Additionally, a multi-color FACS assay was performed for cultured PLA cells (at 5 and 10 days; Fig. 6a,b) and cultured BM-MSCs (Passage 3; Fig. 6c) for comparison. Most PLA cells cultured for 5 days were $CD31^-CD34^+CD45^-CD90^+CD105^+CD146^-$, but small percentages of other cell populations, such as $CD31^+CD34^+CD45^-CD90^+CD105^+CD146^+$ cells (endothelial cells or endothelial progenitors), $CD31^-CD34^-CD45^-CD90^+CD105^+CD146^+$ cells (possibly pericyte progenitors), and $CD31^-CD34^-CD45^-CD90^+CD105^+CD146^+$ cells (pericytes) were also observed. Unlike freshly isolated ASCs, the major ASC population expressed CD105. Compared to PLA cells cultured for 5 days, the 10-day cultures showed decreased percentages of $CD31^+$ endothelial cells and $CD34^+$ cells and increased percentages of $CD31^-CD34^+CD146^+$ cells and $CD31^-CD34^-CD146^+$ cells. Cultured BM-MSCs showed similar surface marker expression patterns. Most cultured BM-MSCs were identified as $CD31^-CD34^-CD45^-CD90^+CD105^+$ cells with variable expression of CD146. BM-MSCs contained only a very small percentage (<1%) of $CD34^+$ cells, which was the only difference from ASCs clearly detected in this assay.

Representative single-color FACS data for freshly isolated and cultured (at 2 weeks) PLA and LAF cells are shown in Figure 7 and sequential changes in cell surface

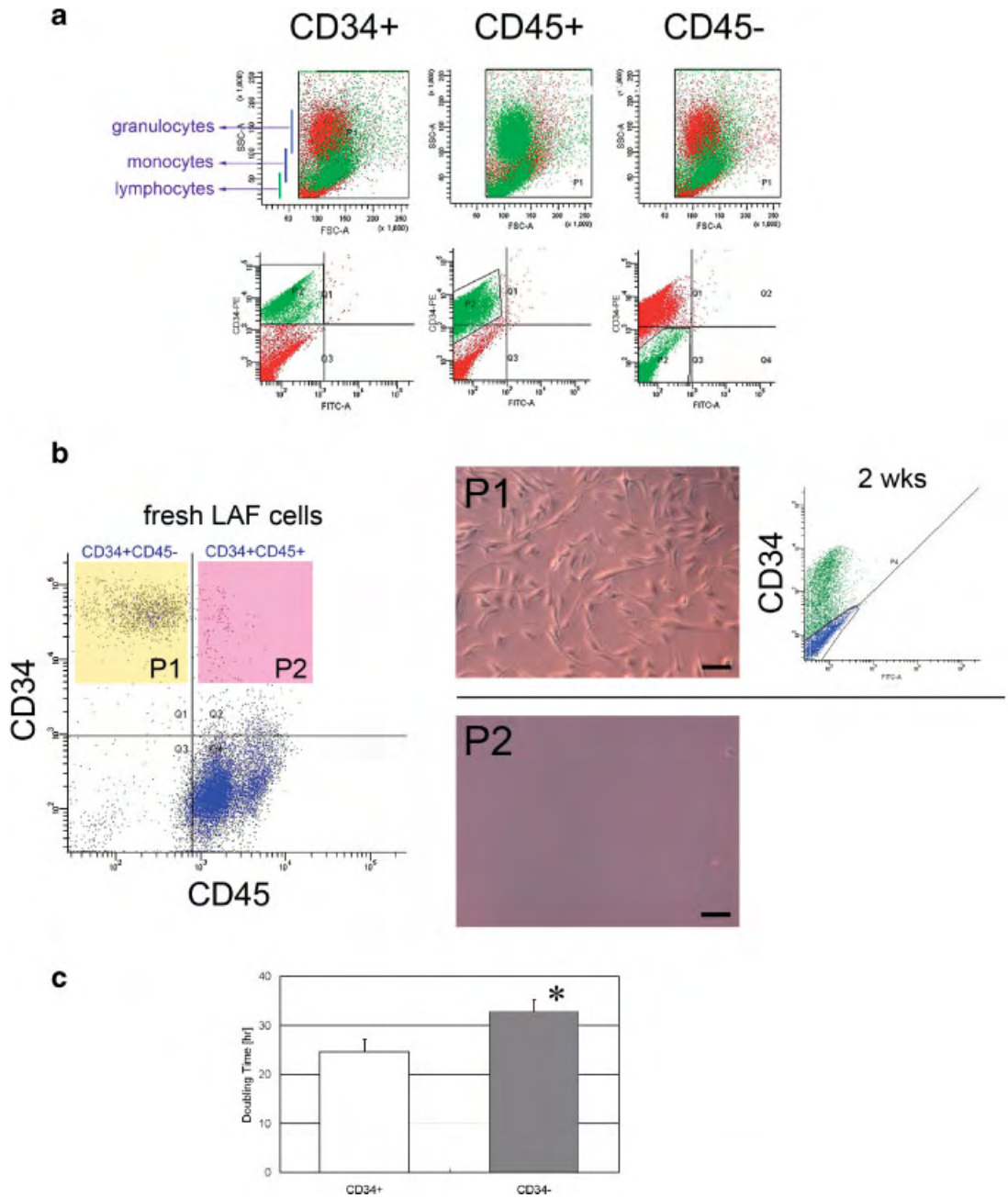


Fig. 3. CD34 expression in freshly isolated and adherent LAF cells. **a**: Flow cytometric analysis of freshly isolated LAF cells for cell size, granularity, and expression of CD34 or CD45. In the FACS plot of SSC (granularity) versus FSC (cell size) for freshly isolated LAF cells, $CD34^+$ cells (left), and $CD45^-$ cells (right) are shown in green. Both were located in the same area as monocytes/macrophages, while $CD45^+$ cells (middle) were located in the areas typical for blood cells such as granulocytes, monocytes/macrophages, and lymphocytes (see Fig. S1). **b**: Double color flow cytometric cell sorting of fresh LAF cells by CD34 and CD45 expression, and the fates of sorted cells after plating. Left: Most $CD34^+$ cells from fresh LAF cells were $CD45^-$. Middle: The $CD34^+CD45^-$ (P1) and $CD34^+CD45^+$ (P2) cell populations were sorted and plated; only $CD34^+CD45^-$ cells grew on the dish. Scale bar = 100 μm. Right: After 2 weeks' culture, approximately half of the $CD34^+CD45^-$ cells had lost CD34 expression. **c**: Comparison of doubling time between $CD34^+$ and $CD34^-$ adherent LAF cells. The doubling time of $CD34^+$ adherent LAF cells was significantly shorter than that of $CD34^-$ cells, $^*P < 0.05$.

marker expression are shown in Figure 8. After plating, the surface marker expression profiles for both PLA and LAF cells changed markedly, and adherent cells of the two cell populations showed quite similar expression profiles. The percentage of $CD34^+$ cells increased in PLA cells, which uniformly expressed mesenchymal markers, such as CD13, CD29, CD44, CD73, and CD90. In PLA cells cultured for more than 1 week, CD10, CD49e, CD59, and CD151 were also uniformly expressed. One-week culture of freshly isolated PLA or LAF cells resulted in a dramatic enrichment in CD105 from $1.2 \pm 0.6$ to $64.1 \pm 9.7$ ($P < 0.001$) or from $1.4 \pm 0.7$ to $74.6 \pm 7.6$ ($P < 0.001$), respectively. No statistically significant difference in CD105 expression was observed between adherent PLA and LAF cells. After 1 week in culture, CD45, Flk-1, Tie-2, CD31, CD117, and CD133/AC133 expression had decreased significantly in PLA and LAF cells. Both cell populations were negative for CD4, CD45, CD62E (E selectin), CD62P (P selectin), CD69, CD135, and CD144 at 1 week and were negative for CD16, CD31, CD57, CD106 (VCAM-1), CD133, Flk-1, and Tie-2 after culture for more than 2 weeks.

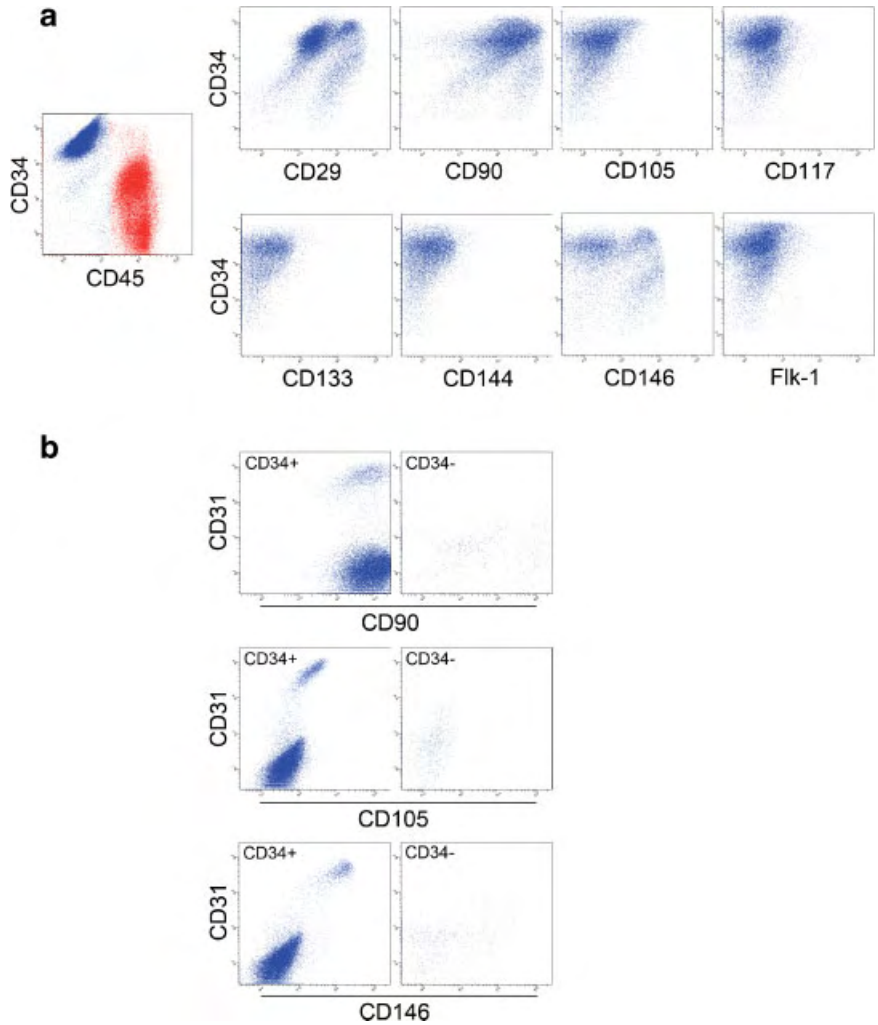


Fig. 4. Multicolor FACS analysis of SVF (1). **a**: SVF from the adipose portion of liposuction aspirates was analyzed for CD34, CD45, and one of the following markers: CD29, CD90, CD105, CD117, CD133, CD144, CD146, or Flk-1. CD34 and CD45 expression is shown in the graph at left, with blood-derived $CD45^+$ cells in red and adipose-derived $CD45^-$ cells in blue. Only adipose-derived $CD45^-$ cells (blue dots) were plotted in each of the eight graphs at right. Most adipose-derived $CD34^+$ cells were $CD29^+$, $CD90^+$, $CD105^-$, $CD117^-$, $CD133^-$, $CD144^-$, and $Flk\text{-}1^-$. It should be noted that there are two populations of adipose-derived $CD34^+$ cells with regard to CD146 expression. **b**: SVF from the adipose portion was analyzed for CD31, CD34, CD45, and one of the following markers: CD90, CD105, or CD146. Only adipose-derived CD45-cells were plotted. There are two major and one minor populations of $CD34^+$ cells. One major population (endothelial cells or endothelial progenitors) was $CD31^+CD34^+CD90^+CD105^{low}CD146^+$ and comprised 2–8% of $CD34^+$ cells. The largest population (ASCs) was $CD31^-CD34^+CD90^+CD105^-CD146^-$. The minor population was $CD31^-CD34^+CD90^+CD105^-CD146^+$. There were two small populations of $CD34^-$ cells; one was $CD31^-CD34^-CD90^+CD105^-CD146^+$, and the other was $CD31^-CD34^-CD90^+CD105^-CD146^-$.

CD34 expression decreased with increased culture time, but 10–20% of cells maintained CD34 expression up to 20 and 10 weeks' culture in PLA and LAF cells, respectively. In addition, consistent expression of the mesenchymal markers (CD13, CD29, CD44, CD73, and CD90) as well as other markers (CD 49d, CD59, CD105, and CD151) was observed in adherent PLA and LAF cells up to 20 and 10 weeks, respectively. After the initial 2 weeks, no remarkable changes in surface marker expression were seen between adherent PLA and LAF cells throughout the culture periods. Taken together, these data suggest that adherent PLA and LAF cells can be expanded using our culture method without losing stem cell-associated surface markers. Our studies further revealed that after the initial 1–2 weeks in culture, adherent PLA and LAF cells have quite similar surface marker expression profiles throughout the culture periods, suggesting that both cell populations can be considered ASCs.

## DISCUSSION

Adipose tissue is comprised predominantly of mature adipocytes, connective tissue, ASCs, blood-derived cells, vascular cells, such as endothelial (progenitor) cells, smooth muscle cells, and pericytes. Adipocytes represent roughly two-thirds of the total cell number and more than 90% of the tissue volume (van Harmelen et al., 2005). The ratio of adipocytes to ASCs is constant in humans, independent of body mass index (BMI) and age (van Harmelen et al., 2003). In the present study, a correlation between ASC cell yield and age or BMI was not detected (data not shown). ASC cell yield varies among patients and is affected by many factors including donor site and storage duration. It also depends

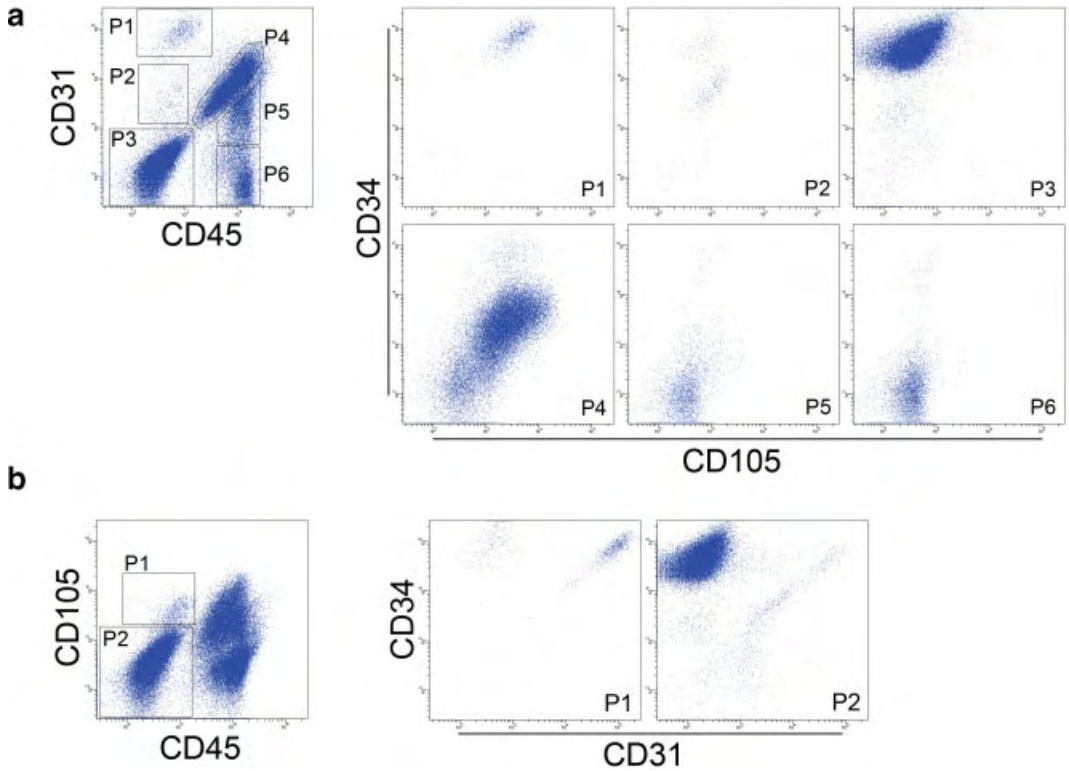


Fig. 5. Multicolor flow cytometric analysis of SVF (2). Freshly isolated SVF from the adipose portion was analyzed with multicolor flow cytometry for CD31, CD34, CD45, and CD105. **a**: CD31 and CD45 expression is shown in the graph at left, with six major populations (P1–P6) identified. Each population was individually plotted for CD34 and CD105 expression in the six graphs at right. P1 was composed of $CD31^{+}CD34^{+}CD45^{-}CD105^{low}$ cells, which are fresh endothelial cells or their progenitors. P2 was an unknown minor population ($CD31^{low}CD34^{-}CD45^{-}CD105^{low}$ cells; possibly $CD31^{low}$ endothelial cells). P3 contained ASCs, which are $CD31^{-}CD34^{+}CD45^{-}CD105^{-}$. P4 consisted predominantly of granulocytes and monocytes/macrophages, with a small number of lymphocytes. P5 and P6 consisted of lymphocytes (Fig. S2). **b**: CD45 and CD105 expression is shown in the graph at left, and two adipose-derived ($CD45^{-}$) populations (P1: $CD34^{+}$, P2: $CD34^{-}$) are identified. Each population was individually plotted for CD31 and CD34 expression in the graphs at right. P1 was comprised of $CD31^{+}CD34^{+}CD45^{-}CD105^{low}$ cells (fresh endothelial cells or progenitors) and $CD31^{-}CD34^{+}CD45^{-}CD105^{low}$ cells. P2 was composed of other adipose-derived cells including ASCs.

TABLE 1. Summary of cell composition in SVF derived from liposuction aspirates

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **blood-derived cell populations ($CD45^{+}$)** | | | | | | | |
| CD45 | + | + | + | + | | | |
| CD31 | low | low | − | − | | | |
| CD34 | − | − | − | + | | | |
| CD105 | low | low | − | − | | | |
| CD14 | − | + | − | − | | | |
| CD15 | + | − | − | − | | | |
| composition [%] in SVF from adipose / fluid portion | | 20–40/30–60 | 3–15/5–20 | 3–15/5–25 | <1/<1 | | |
| suspected cell type | | granulocytes | monocytes/ macrophages | lymphocytes | HSC | | |
| **adipose-derived cell populations ($CD45^{-}$)** | | | | | | | |
| CD45 | − | − | − | − | − | − | − |
| CD31 | − | + | − | − | − | low | − |
| CD34 | + | + | + | − | + | + | − |
| CD90 | + | + | + | + | + | + | + |
| CD105 | − | low | − | − | low | low | − |
| CD146 | − | + | + | + | N/A | + | − |
| composition [%] in SVF from adipose / fluid portion | | 15–40/3–10 | 1–6/0–3 | 0–4/0–2 | 1–4/0–2 | <1/<1 | <1/<1 | <2/<1 |
| suspected cell type | | ASC | fEC(EPC) | PPC | PC | | | fibroblasts |

HSC, hematopoietic stem cell; ASC, adipose-derived stromal cell; fEC, fresh endothelial cell; EPC, endothelial progenitor cell; PC, pericytes; PPC, pericyte progenitor cell.
Results of multi-color FACS assays are summarized. We classified freshly isolated cells from liposuction aspirates into 11 populations according to surface marker expression profiles; 4 blood-derived ($CD45^{+}$; above) and 7 adipose-derived ($CD45^{-}$; below) cell populations. Cell composition percentages varied among samples, so a range of values is presented.

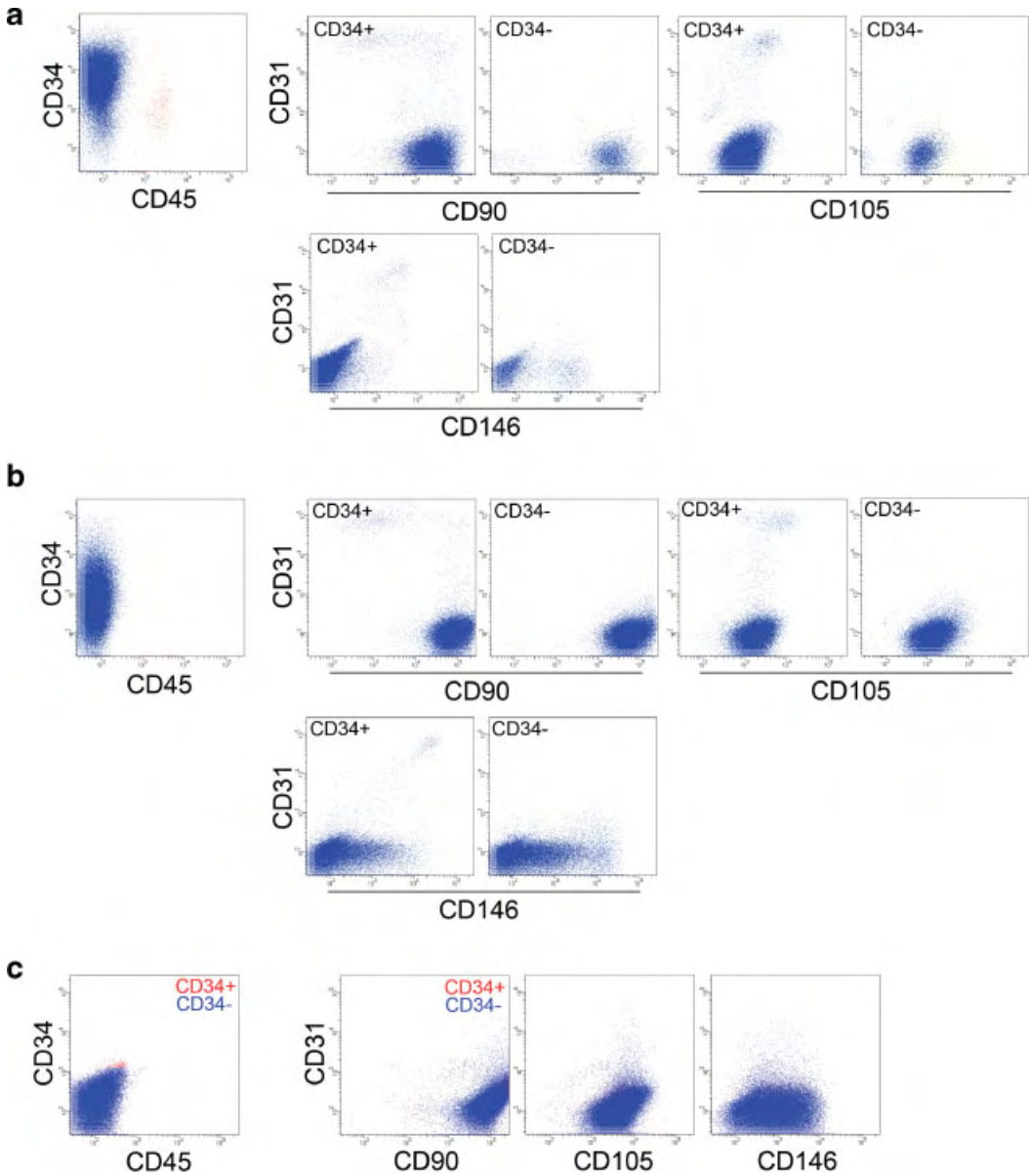


Fig. 6. Multicolor FACS analysis of cultured PLA cells and cultured BM-MSCs. PLA cells cultured for 5 days (**a**) and 10 days (**b**) and BM-MSC (Passage 3) (**c**) were analyzed for CD31, CD34, CD45, and one of following markers: CD90, CD105, or CD146. a, b: In the graphs at far left, all cells were plotted, while only $CD45^-$ cells (blue dots) are shown in the six graphs at right. There are several differences in surface marker expressions between freshly isolated and cultured PLA cells; Almost all ASCs started expressing CD105 after plating, and some ASCs lost CD34 expression with increased culture time. $CD31^+$ endothelial cells decreased in percentage with culture time, while $CD31^-CD34^-CD146^+$ cells (possibly pericyte progenitors) and $CD31^-CD34^-CD146^+$ cells (pericytes) increased. $CD31^+CD90^+$ endothelial cells observed in fresh SVF lost CD90 expression with increased culture time, as seen in HUVEC (Fig. S3a, b). **c**: Nearly all BM-MSCs were $CD34^-$. Red and blue dots are $CD34^+$ and $CD34^-$ cells, respectively. Cultured BM-MSCs and ASCs showed similar expression of CD31, CD45, CD90, CD105, and CD146, with the only detectable difference being in CD34 expression.

strongly on the isolation method, for example, duration of collagenase digestion (Aust et al., 2004; Bakker et al., 2004; von Heimburg et al., 2004).

Although only the adipose portion of liposuction aspirates has been used as a source of ASCs, we also isolated cells from the fluid portion of liposuction aspirates and found that a comparable amount of ASCs can be harvested. By flow cytometric analysis, fresh PLA and LAF cells showed distinct surface marker profiles. Higher percentages of $CD31^+$ and $CD45^+$ cells were observed in fresh LAF cells than in PLA cells, suggesting that fresh LAF cells contain a larger amount of blood-derived cells. However, adherent LAF cells cultured for 1 week showed surface marker profiles quite similar to cultured PLA cells, suggesting that the fluid portion of liposuction aspirates also contains ASCs. In addition, our results showed that most of the $CD34^+$ cells in freshly isolated LAF cells were derived from adipose tissue ($CD45^-$), and these $CD34^+CD45^-$ cells expanded in culture dishes, suggesting that they correspond to the same ASC population derived from PLA cells. Why a significant amount of ASCs are isolated from the fluid portion of liposuction aspirates has yet to be determined. Nor is the location of ASCs in adipose tissue clearly understood. Some ASCs are thought to be located in the adipose connective tissue and others between adipocytes or around micro- or macro-vasculature. ASCs located between adipocytes might be released into the fluid by mechanical injury during liposuction procedures, and other ASCs might be released by endogenous

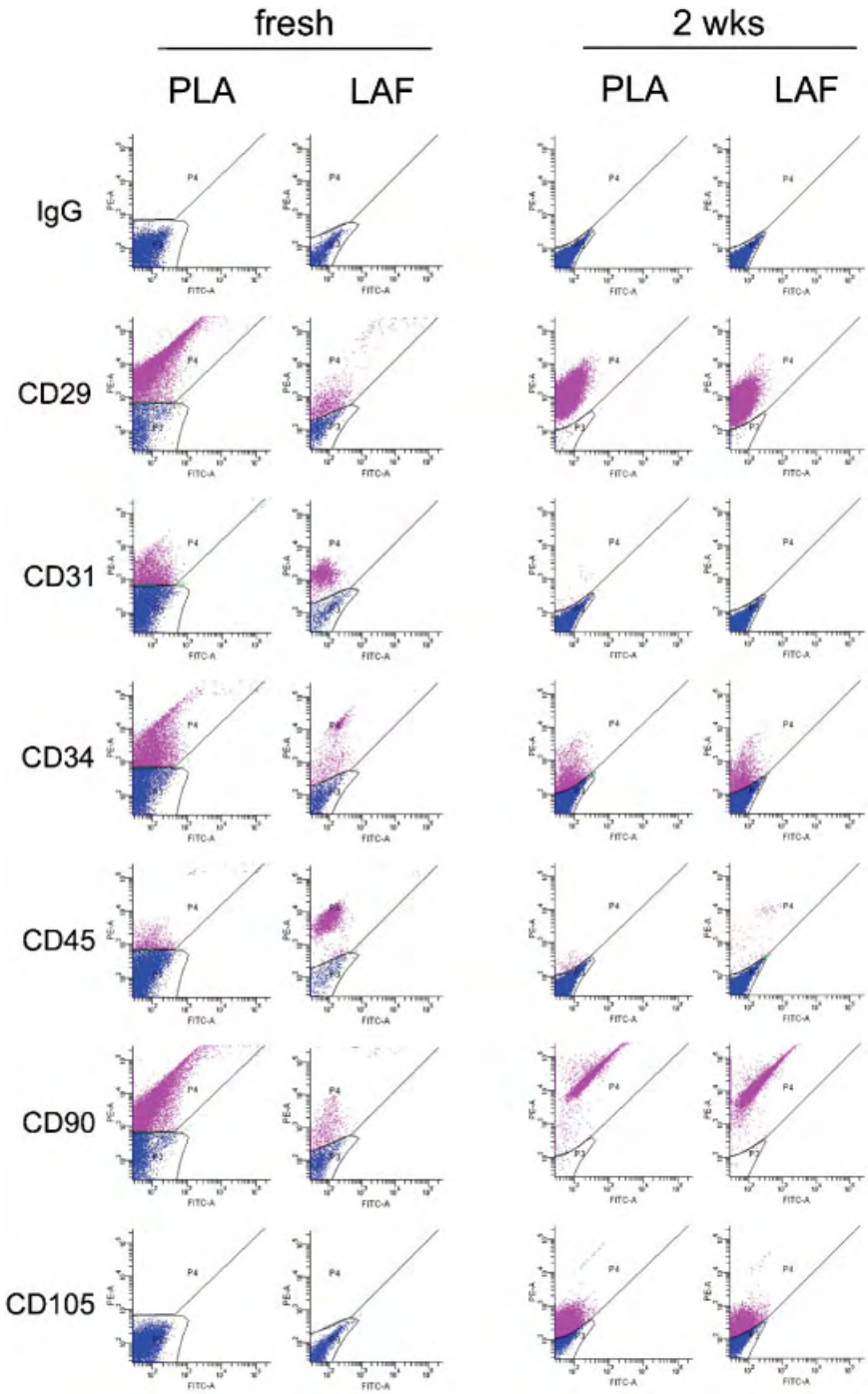


Fig. 7. Comparison of flow cytometric data for freshly isolated and 2-week-cultured PLA and LAF cells. Freshly isolated PLA and LAF cells displayed distinct cell surface marker profiles, while PLA and LAF cells cultured for 2 weeks showed quite similar expression profiles. Adherent, but not freshly isolated, PLA, and LAF cells expressed CD105. Results are representative of samples from five patients.

proteases during surgery or subsequent storage periods. The extent of mechanical injury may vary among patients because it can be affected by the size of the suction canulla, vacuum pressure strength during liposuction, suction procedure (manual or powered), and other factors.

One of the reasons why adipose tissue is thought to be a promising source of stem cells is that a large volume of adipose tissue can be harvested with minimal morbidity. Thus, ASCs can be used clinically after minimal manipulation (without cell culture). Therefore, examination and characterization of freshly isolated PLA and LAF cells have significant clinical implications. Some recent studies examined freshly isolated SVF from human adipose tissue using magnetic cell sorting (Boquest et al., 2005; Sengenès et al., 2005), and the investigators partly characterized some cell populations in the SVF. $CD31^{-}CD34^{+}CD45^{-}CD105^{+}$ cells were proposed as typical proliferating ASCs (Boquest et al., 2005). Other studies showed that $CD34^{+}CD31^{-}$ cells differentiated into endothelial cells and contributed to neovascularization (Miranville et al., 2004;

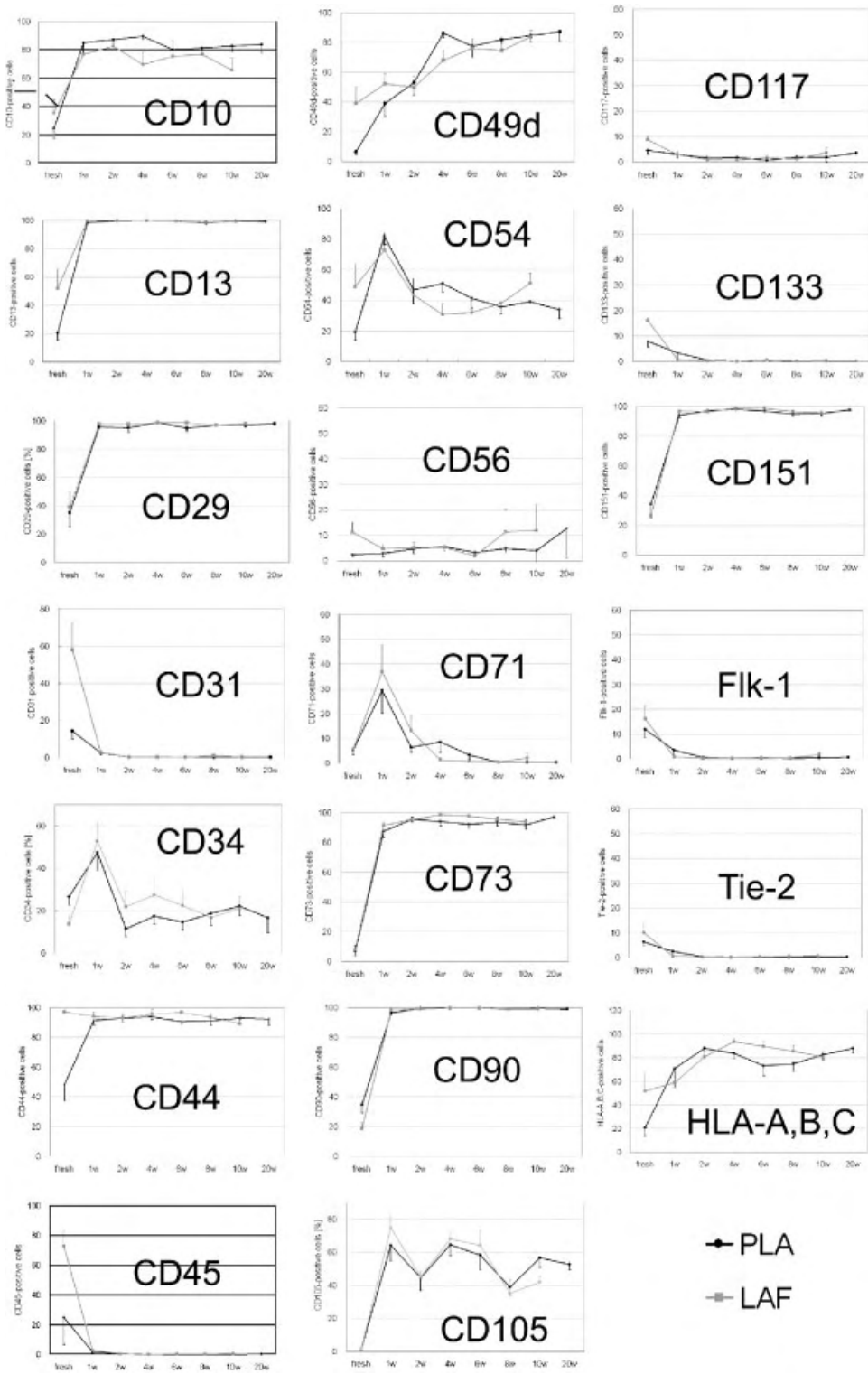


Fig. 8. Sequential changes in representative cell surface marker expression in fresh and cultured PLA and LAF cells. Statistically significant differences in expression of some cell surface markers (data not shown for CD16 and CD49e) were observed between freshly isolated PLA and LAF cells. These differences were not apparent after 1–2 weeks in culture. Data are shown as mean + (LAF) or − (PLA) standard error.

Planat-Benard et al., 2004b). For analysis of SVF, we used multicolor (four or five colors) flow cytometry assays and showed that there are several major and minor cell populations derived from blood and adipose. The typical surface marker expression pattern of fresh ASCs was $CD31^{-}CD34^{+}CD45^{-}CD90^{+}CD105^{-}CD146^{-}$, which was distinct from that of cultured ASCs in CD105 expression only. This profile of fresh ASCs differs from a previous study (Boquest et al., 2005), possibly because the magnetic cell sorting procedure used in that study might have affected CD105 expression. Our results also showed that most fresh ASCs are $CD29^{+}$, $CD90^{+}$,

$CD117^-$, $CD133^-$, $CD144^-$, and $Flk\text{-}1^-$. It should be noted that there are significant numbers of $CD146^+$ and $CD146^-$ cells in both $CD34^+$ and $CD34^-$ cell populations in SVF. Given that CD31 is an endothelial cell marker and that CD146 is a known marker of endothelial cells and pericytes, $CD31^-CD34^-CD146^+$ cells in SVF are likely pericytes, and $CD31^-CD34^+CD146^+$ cells may function as pericyte progenitors. Nearly all adipose-derived $CD31^+$ cells in SVF were found to be $CD34^+$, and this population was previously reported as endothelial cells (Sengenès et al., 2005). Our results showed that this population was $CD90^+CD105^{low}CD146^+$. However, a major proportion of cultured HUVECs (Passage 2) were $CD34^-$ and $CD90^-$ (Fig. S3b), suggesting that fresh but not cultured endothelial cells express CD34 and CD90. Indeed, freshly isolated HUVEC cells were $CD34^+$ with variable expression of CD90 (Fig. S3a). $CD31^-CD34^-CD45^-CD90^+CD105^-CD146^-$ cells in SVF may be fibroblasts, as suggested by comparison with cultured dermal fibroblasts (Fig. S3c).

PLA and LAF cells showed similar surface marker profiles after being cultured for 1 week. Notable changes between fresh and cultured states include decreased expression of CD31 and CD45 and increased expression of CD29 and CD105. These changes suggest that cells other than ASCs, such as vascular endothelial cells and blood-derived cells, are selectively excluded during culturing on plastic plates. Even when cultured in endothelial cell growth medium, ASCs quickly outgrow $CD31^+$ endothelial cells (Hutley et al., 2001). As noted above, a high percentage of ASCs began to express CD105 after plating. Our results showed that the expression of most surface markers is sustained throughout the culture period (up to 20 weeks for PLA and 10 weeks for LAF cells), and that significant changes seen after the initial 2 weeks are limited to a gradual increase in CD49d and a decrease in CD71.

Because CD34 is one of the most well established stem cell markers, CD34 expression may indicate ASC clinical usefulness. CD105, known as a mesenchymal stem cell-associated marker, was highly expressed in cultured ASCs and may reflect the capacity of ASCs to differentiate into lineages of mesenchymal origin such as adipose, cartilage, and bone. Flk-1, known to be expressed in hemangioblasts, was recently shown to be expressed in ASCs under certain culture conditions (Cao et al., 2005; Martinez-Estrada et al., 2005). These factors suggest that ASCs may have clinical potential for cell-based therapies. A number of studies characterizing human ASCs have reported that ASCs (freshly isolated or cultured for less than 2 weeks) are $CD34^+$ (Gronthos et al., 2001; Miranville et al., 2004; Rehman et al., 2004; Planat-Benard et al., 2004b; Boquest et al., 2005). However, CD34 expression was not found in human ASCs cultured for more than 2 weeks with conventional culture methods (Gronthos et al., 2001; Zuk et al., 2002; De Ugarte et al., 2003; Lee et al., 2004; Rehman et al., 2004; Boquest et al., 2005; Katz et al., 2005). In contrast, our results showed that $CD34^+$ ASCs were present at 10–20% of the cell population even after 10–20 weeks of culture (Fig. 7). $CD34^+$ cells were sorted and cultured, but about half of the cells became $CD34^-$ after 2 weeks in culture (Fig. 3b), suggesting that cultured ASCs may exist in a variety of stages ranging from $CD34^+$ undifferentiated cells to $CD34^-$ partially differentiated cells. The result that $CD34^+$ cells proliferated more quickly than $CD34^-$ cells may explain the sustained percentage of $CD34^+$ cells in cultured PLA or LAF cells. Thus, using our culture protocol, it is possible to expand $CD34^+$ ASCs taken from liposuction aspirates up to $10^4$–$10^7$ times after 4 weeks' culture. Together with the fact that $Flk\text{-}1^+$ ASCs can be obtained in high percentages using another culture protocol (Cao et al., 2005; Martinez-Estrada et al., 2005), ASCs may dramatically change their surface marker profiles depending on the culture media and methods.

It was reported that human ASCs differ from human BM-MSCs in expression of CD49d (expressed only in ASCs) and CD106 (expressed only in BM-MSCs) (Zuk et al., 2002; De Ugarte et al., 2003). In our study using multicolor assays with limited surface markers, the only difference between cultured ASCs and cultured BM-MSCs was CD34 expression (positive in ASCs and negative in BM-MSCs). Human dermal fibroblasts have a surface marker expression profile similar to ASCs but lack expression of CD34, CD105, and CD146 (Fig. S3c). Because BM-MSCs (Zuk et al., 2002; De Ugarte et al., 2003) and other stromal progenitors cultured for long periods do not express CD34, ASCs may constitute a unique mesenchymal cell population in view of their CD34 and CD146 expression. This characteristic may contribute to potentialities of ASCs other than mesenchymal progenitors, such as endothelial (Miranville et al., 2004; Rehman et al., 2004; Planat-Benard et al., 2004b) or pericyte progenitors.

In summary, adherent LAF cells have quite similar characteristics with respect to growth kinetics, morphology, surface marker profiles, and capacity for differentiation to adherent PLA cells. A significant amount of ASCs can be isolated from the fluid portion of liposuction aspirates, although in smaller amounts than from the fatty portion. In addition, we found that SVF are composed of heterogeneous cell populations including blood-derived cells, ASCs, endothelial (progenitor) cells, pericytes (and progenitors), and other unknown progenitors. A major population of ASCs in SVF was identified as $CD31^-CD34^+CD45^-CD90^+$ $CD105^-CD146^-$ cells but began to express CD105 after plating. Adipose-derived $CD34^+$ ASCs can be expanded for at least 20 weeks using our culture method. These results suggest that liposuction-derived human ASCs may have significant clinical utility for cell-based therapies.

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# Characterization of Freshly Isolated and Cultured Cells Derived From the Fatty and Fluid Portions of Liposuction Aspirates

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COLOR

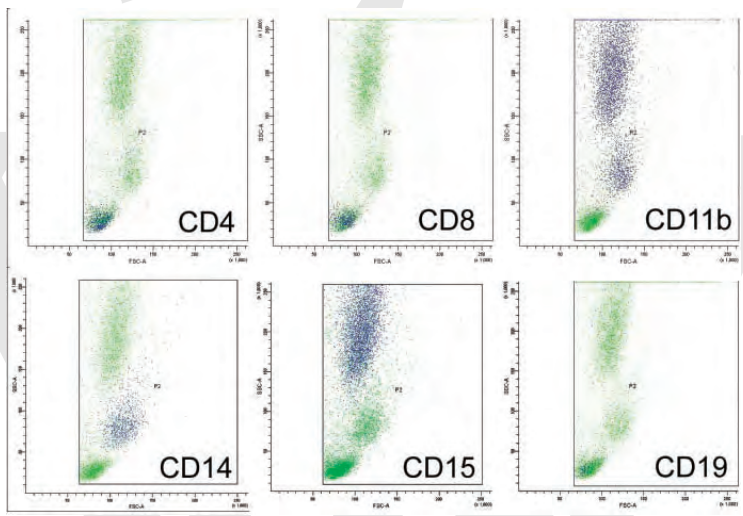


Fig. S1. FSC and SSC analysis of peripheral blood. FACS analysis of peripheral blood by FSC (cell size) and SSC (granularity). Blue dots are cells positive for each CD antigen: CD4 (helper T lymphocytes), CD8 (cytotoxic T lymphocytes), CD11b (both granulocytes and monocytes/macrophages), CD14 (monocytes/macrophages), CD15 (granulocytes), and CD19 (B lymphocytes). Granulocytes, monocytes/macrophages, and lymphocytes are located predominantly in the upper, middle, and lower populations, respectively.

COLOR

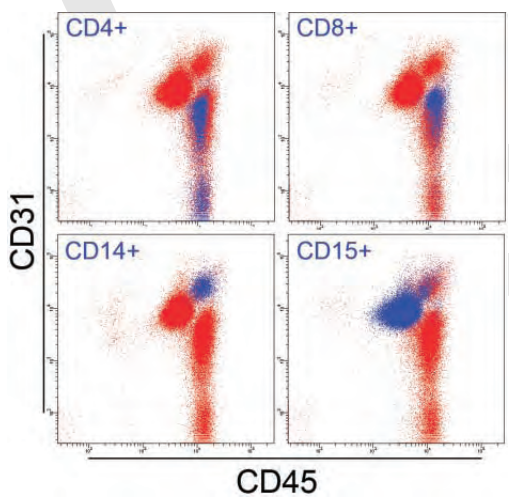


Fig. S2. Multicolor FACS analysis of peripheral blood. Blue dots are cells positive for CD4 (helper T lymphocytes), CD8 (cytotoxic T lymphocytes), CD14 (monocytes/macrophages), and CD15 (granulocytes). All blood-derived cells were $CD45^+$. Granulocytes and monocytes/macrophages were $CD31^+$, while lymphocytes were $CD31^-$.



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COLOR

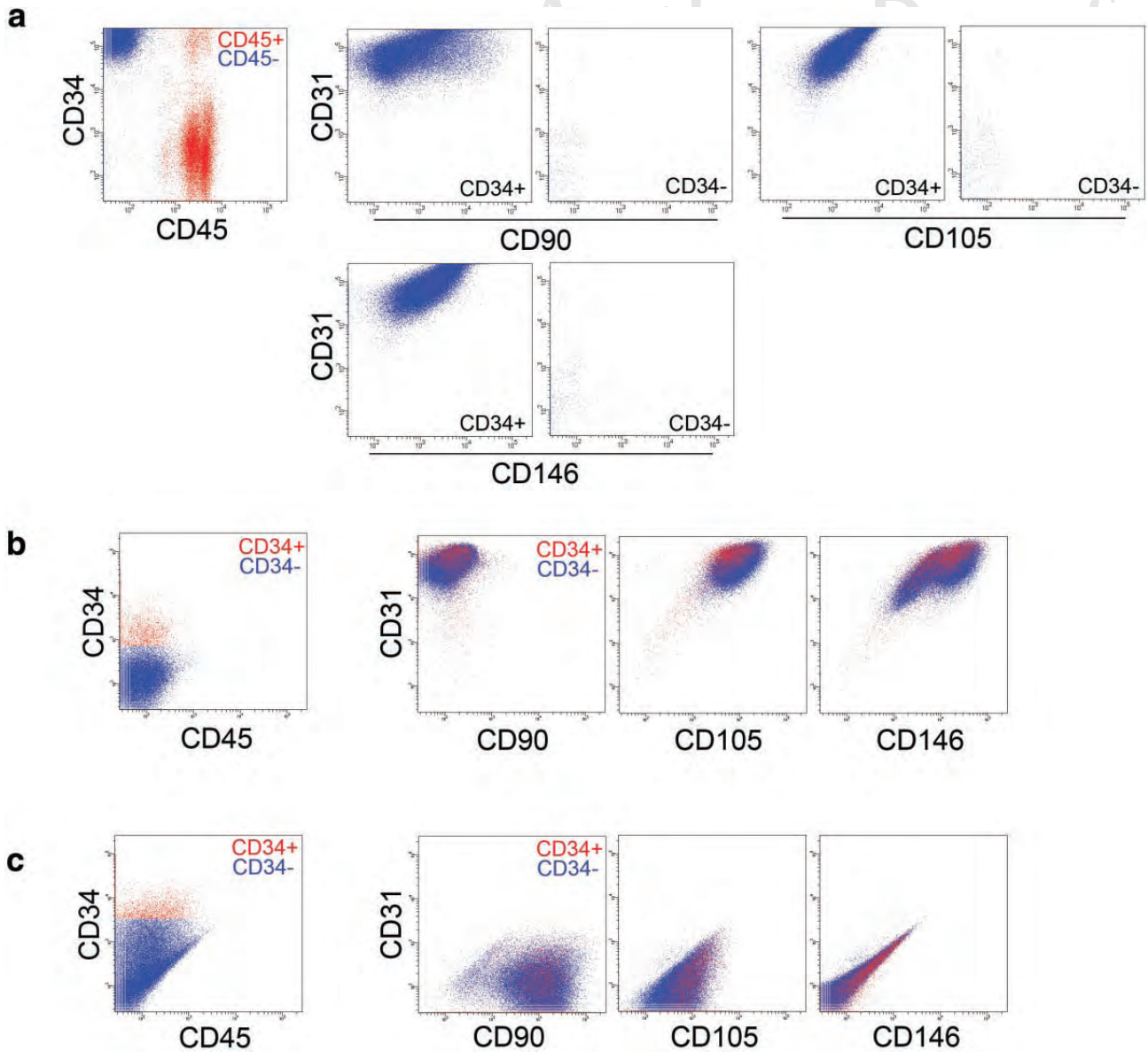


Fig. S3. Multicolor FACS analysis of freshly isolated HUVEC, cultured HUVEC, and cultured dermal fibroblasts. Freshly isolated HUVEC (**a**), cultured HUVEC (EGM-2; Passage 2) (**b**), and cultured dermal fibroblasts (DMEM+10%FBS, Passage 4) (**c**) were analyzed for CD31, CD34, CD45, and one of following markers: CD90, CD105, or CD146. a: All freshly isolated cells from human umbilical cord vein were plotted in the graph at far left, showing contamination by blood-derived $CD45^+$ cells (red dots). Only adipose-derived $CD45^-$ cells (blue dots) were shown in the six graphs at right. Most freshly isolated HUVEC were $CD31^+CD34^+CD45^-CD105^+CD146^+$, while CD90 expression varied. b: Most cultured HUVEC (Passage 2) were $CD31^+CD34^-CD45^-CD90^-CD105^+CD146^+$. Small percentages of $CD31^+CD34^+CD45^-CD90^-CD105^+CD146^+$ and $CD31^+CD34^-CD45^-CD90^+CD105^+CD146^+$ cells were also detected. Red and blue dots are $CD34^+$ and $CD34^-$ cells, respectively. c: Most of the cultured dermal fibroblasts were $CD31^-CD34^-CD45^-CD90^+CD105^-CD146^-$, and a small percentage was $CD31^-CD34^+CD45^-CD90^+CD105^-CD146^-$. Red and blue dots are $CD34^+$ and $CD34^-$ cells, respectively, gated in the graph at far left.