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Characterization of Mesenchymal Progenitor Cell Populations Directly Derived from Human Dermis

Vaughan Feisst, Anna E.S. Brooks, Chun-Jen J. Chen, and P. Rod Dunbar

Mesenchymal stem cell (MSC) and progenitor cell (MPC) populations in human dermis remain poorly characterized, despite their importance to wound repair and the pathogenesis of many skin diseases. To identify MSC/MPC populations in human dermis we developed an 11-marker flow cytometry technique that enabled sorting of mesenchymal cell populations for functional assays, using adipose-derived stem cells (ASCs) from human adipose tissue as a positive control. Two populations of dermal cells had similar phenotypes to ASCs: both were $CD34^+$ $CD73^+$ $CD105^-$/low, and lacked expression of c-kit (CD117) and hematopoietic or vascular markers (CD31, CD45, CD146, and HLA-DR). However, whereas ASCs were $CD36^{+/-}$ $CD90^+$, dermal mesenchymal progenitor cells (DMPCs) were split between a dominant $CD36^-$ $CD90^+$ population (DMPC1) and a small $CD36^+$ $CD90^-$ population (DMPC2). Both these populations were capable of differentiating into adipocytes, but only DMPC1 localized to a perivascular location, similar to that reported for ASCs. Re-gating of the flow cytometry data revealed that both DMPC1 and DMPC2 were part of $CD45^-$ $CD73^+$ $CD146^-$ populations with variable expression of CD34. This suggests that CD34 may not be a stable marker of DMPC populations in human dermis, consistent with data from MSCs in human bone marrow, and with the loss of CD34 we observed from both ASCs and DMPCs on cell culture. These data enable future study of DMPCs in health and disease, and may also explain why some mesenchymal cell lines derived from human dermis exhibit characteristics of MSCs.

Introduction

In recent years there has been an explosion of research into human adipose-derived stem cells (ASCs), mesenchymal stem cells (MSCs) found in adipose tissue, since they were first isolated and found to be capable of differentiation into multiple cell lineages [1]. ASCs have potential in tissue engineering because they are a relatively easily accessible source of cells capable of producing many different tissue types. Recently some cultured human dermal fibroblastic cell lines have been shown to have similar properties to ASCs [2–4]. They are almost indistinguishable with respect to morphology and expression of cell surface markers, and subpopulations of dermal fibroblastic cell lines have shown a surprising capacity to differentiate into similar cell lineages [2–4]. Investigation of the mesenchymal cells in dermal tissue has suggested the presence of mesenchymal cells with stem cell characteristics, such as the ability to differentiate into multiple cell lineages [4–8]. It seems likely that these populations may persist in fibroblastic cell lines obtained from dermis by adherent cell culture. However, most comparisons between ASCs and dermal fibroblastic cell lines have been limited to the use of cells purified by adherent culture. There is now abundant evidence that the cell surface phenotype of MSCs can change substantially on adherent cell culture, so we sought to isolate mesenchymal cell populations directly from fresh human dermal tissue [9,10]. We therefore developed a new 11-marker flow cytometric assay capable of separating mesenchymal cell populations from other populations in dissociated human tissue, and sorting these cell populations for functional assays. Having characterized and compared the cell populations present in human adipose tissue and dermis, and identified mesenchymal progenitor cell populations in the dermis similar to ASCs, we then used the novel combinations of cell surface molecules that marked these cells to localize them in human tissue by four-color immunofluorescence microscopy.

Materials and Methods

Cell isolation and culture

All biological materials were obtained under protocols approved by the Northern Regional Ethics Committee, New Zealand. Lipoaspirate was obtained from three female patients and one male patient. Skin was obtained from three female patients aged 39–59 undergoing either breast reduction or abdominoplasty.

School of Biological Sciences and Maurice Wilkins Centre, University of Auckland, Auckland, New Zealand.

To obtain single-cell suspensions containing ASCs, lipoaspirate was washed with DMEM/F12 1:1, 10% fetal bovine serum (FBS), and penicillin (100 U/mL)–streptomycin (100 μg/mL)–glutamine (292 μg/mL) (PSG; Life Technologies) (ASC medium) and digested in 0.075% collagenase I and phosphate-buffered saline (PBS; Life Technologies) for 45 min. Samples were centrifuged at 700 *g* for 10 min, supernatants containing mature adipocytes were discarded, and cell pellets were resuspended in ASC medium. Cell pellets containing the stromal vascular fraction (SVF) of adipose tissue were washed with ASC medium, passed through a 100-μm cell filter (BD Biosciences), and cryopreserved in 50% ASC medium, 40% FBS, and 10% DMSO (Sigma-Aldrich).

All subcutaneous adipose tissue and part of the dermis were mechanically trimmed from skin samples, to minimize contamination of dermal cell preparations by cells derived from subcutaneous adipose tissue. To obtain single-cell suspensions from human dermis, split-thickness skin was digested in 1 mg/mL dispase (Life Technologies) for 3 h at 37°C/5% CO_2. Enzyme-containing medium was removed and replaced with DMEM, 10% FBS, and 1× PSG (Life Technologies) (fibroblast medium) and incubated overnight at 37°C/5% CO_2. Epidermis was peeled away from dermis and then dermis was digested in 0.5 mg/mL collagenase type I (Life Technologies) at 37°C/5% CO_2 overnight. Dermis was mechanically disrupted, passed through a 100-μm cell filter, and then centrifuged at 700 *g* for 10 min to obtain a cell pellet. Cells were then cryopreserved in 50% fibroblast medium, 40% FBS, and 10% DMSO.

Polychromatic flow cytometry

For phenotypic analyses adherent cells were detached by incubation in PBS with 0.02% EDTA, washed, and resuspended in 100 μL of cold staining buffer (PBS with 1% FBS). Frozen cell samples were thawed, washed, and resuspended in growth medium and incubated at 37°C/5% CO_2 for 1 h prior to staining. Cells were stained with antibodies listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd) and incubated on ice for 30 min. DAPI (1:5,000) was added to determine cell viability. For cell sorting, cells were labeled with CD31, CD34, and CD45, or CD31, CD34, CD45, CD73, and CD90. Samples were run on a BD SORP FACS Aria II machine and data were analyzed using FlowJo V7.6.5. Quadrant markers to determine marker expression were set according to negative control stains, including ''fluorescence minus one'' (FMO) controls that used all antibodies except that to the marker in question.

Adipogenic differentiation and staining

Cells were seeded in ASC medium and cultured at 37°C/5% CO_2. After 4 days the medium was either replaced with fresh ASC medium, for undifferentiated samples, or adipogenesis differentiation medium [ASC medium supplemented with 0.25 mM IBMX (Sigma-Aldrich), 0.5 μM dexamethasone (Sigma-Aldrich), 5 μM insulin (Sigma-Aldrich), and 100 μM indomethacin (Sigma-Aldrich)], for differentiated samples. The medium was changed for all samples every 2–3 days for 14 days. Samples were fixed with acetone for 5 min and then washed with Tris-buffered saline [20 mM Tris and 150 mM NaCl (pH 8.0); TBS]. Samples were treated with 0.25% casein for 10 min and then washed with TBS. Samples were incubated with anti-FABP4 antibody (Cayman Chemical) in TBS supplemented with 1% FBS, for 1 h. Samples were washed with TBS and then incubated with anti-rabbit IgG Alexa 488 (1:200; Life Technologies), phalloidin Alexa 555 (1:100; Life Technologies), and DAPI nuclear stain (1:2,000; Life Technologies) for 30 min. Samples were washed with TBS and then mounted using Prolong Gold Antifade Reagent (Life Technologies) before images were obtained by microscopy. Images were processed using Cytosketch software (Cytocode.com).

Immunofluorescence microscopy

Five-micrometer sections of samples of human skin were fixed with acetone for 5 min and then washed with TBS. Samples were treated with 0.25% casein for 10 min and then washed once with TBS. Samples were incubated with antibodies listed in Supplementary Table S1 in TBS supplemented with 1% FBS, for 1 h. Samples were washed with TBS and then incubated with secondary antibodies and DAPI nuclear stain for 30 min. Samples were washed with TBS and then mounted using Prolong Gold Antifade Reagent before images were obtained by microscopy. Images were processed using Cytosketch software (Cytocode.com).

Results

Using polychromatic flow cytometry with a panel of 11 markers, including CD31, CD34, CD36, CD45, CD73, CD90, CD105, CD117, CD146, HLA-DR, and DAPI, we characterized and compared the nonhematopoietic ($CD45^-$) cell populations in the SVF of human adipose tissue, and in human dermis. $CD45^-$ cell populations were identified by flow cytometry using the strategy shown in Figs. 1A and 2A by gating on $CD45^-$ cells after exclusion of debris, doublets, and nonviable ($DAPI^+$) cells (Fig. 1A, 2A). ASCs have previously been identified within human adipose SVF as $CD31^-$ $CD34^+$ $CD45^-$[9,11]. We identified a cell population in human SVF that corresponded to this cell surface phenotype, which we labeled as ASCs ($CD31^-$ $CD34^+$ $CD36^{+/-}$ $CD73^+$ $CD90^+$ CD105 low $CD146^-$ $HLA\text{-}DR^-$) (Fig. 1B). ASCs made up the majority of $CD45^-$ cells present within SVF, ranging from 80% to 93% across four donors (Table 1). Although some research groups have reported CD117 expression on ASCs, we did not find any $CD45^-$ cells that express CD117 in SVF (data not shown) [10]. ASCs in SVF were homogeneous for the 10 markers examined, except for expression of CD36 (Fig. 1B).

Adherent-culture purification of ASCs from SVF lasted 28 days with cultures reaching passage 4. Cell population doubling time varied across the 28-day culture period. Cells proliferated slowly initially and increased their proliferation rate with culture time; reaching passage 4 in 28 days is only an indication of the average population doubling time for the 28-day period. We saw differences in the cell surface phenotype of adherent-culture purified and freshly sorted ASCs (Fig. 1). Both freshly isolated and culture-purified ASCs were $CD31^-$ $CD36^{+/-}$ $CD73^+$ $CD90^+$ $CD117^-$ $CD146^-$ $HLA\text{-}DR^-$, but freshly isolated ASCs were

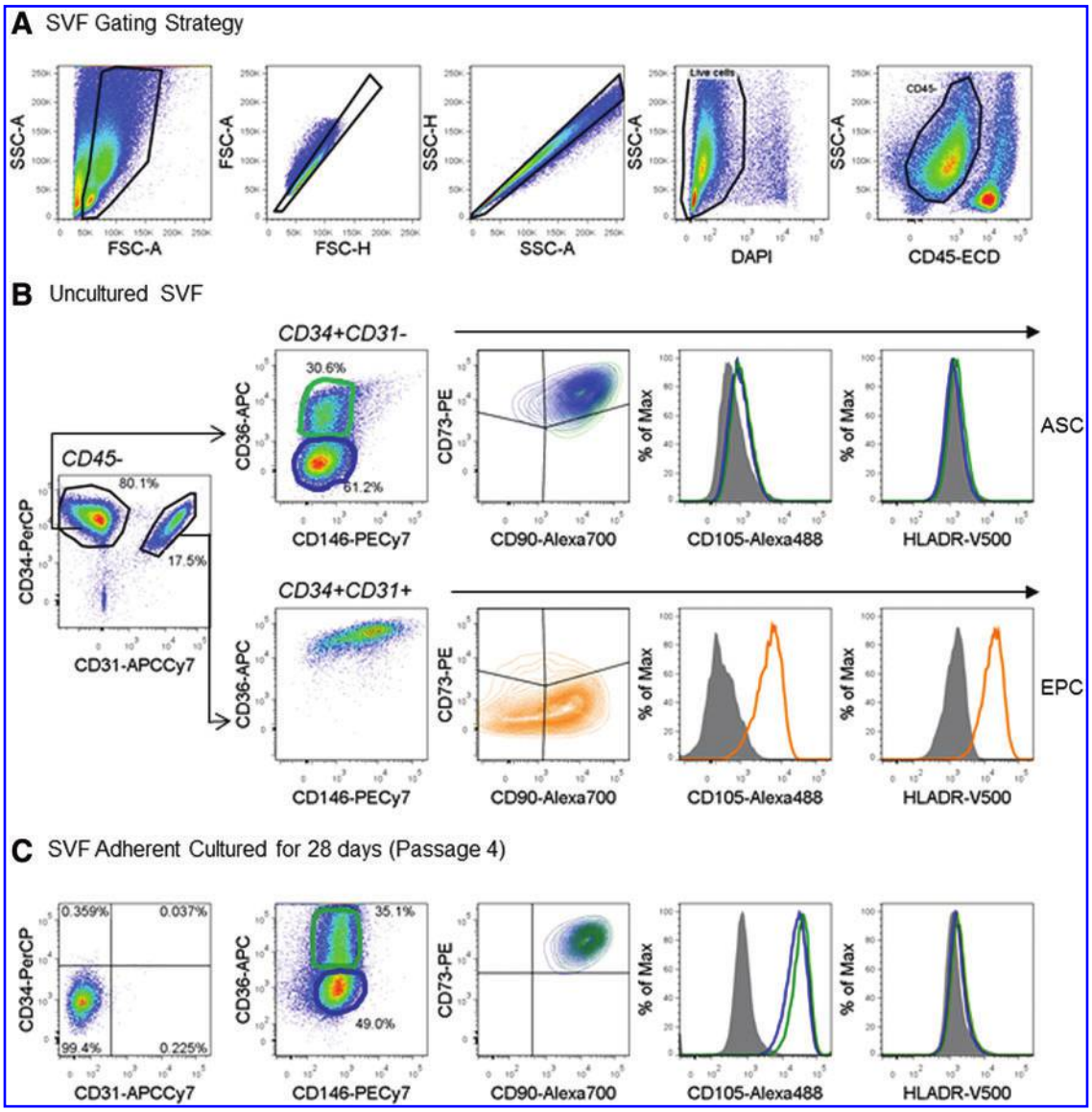

FIG. 1. Flow cytometry analysis of CD45$^-$ cell populations in stromal vascular fraction (SVF). SVF single-cell suspensions were analyzed by polychromatic flow cytometry with a panel of markers, including CD31, CD34, CD36, CD45, CD73, CD90, CD105, CD146, and HLA-DR. Nonhematopoietic cell populations in SVF were identified by the following gating strategy. Cells were first gated by FSC-A and SCC-A followed by doublet exclusion using FCS-A/FSC-H and SSC-A/SSC-H. Dead cells were excluded by DAPI staining. Nonhematopoetic cells were identified by gating on the CD45$^-$ cell population **(A)**. A CD31 versus CD34 plot of nonhematopoetic cell populations separated ASC and EPC populations **(B)**. Adipose-derived stem cells purified from SVF by adherent culture for 28 days, having reached passage 4 in that time, were analyzed by polychromatic flow cytometry **(C)**. Data shown are representative of four biological replicates. Color images available online at www.liebertpub.com/scd

CD34$^+$ CD105low and culture purified ASCs were CD34$^-$ CD105high (Fig. 1). The other major CD45$^-$ cell population we identified in human SVF was a CD31$^+$ CD34$^+$ population that ranged between 1% and 18% of CD45$^-$ cells; this population matched an endothelial progenitor cell (EPC) population previously defined in human SVF [12] and had the full surface phenotype CD31$^+$ CD34$^+$ CD36High CD73$^-$ CD90$^{+/-}$ CD105$^+$ CD146$^+$/low HLA-DR$^+$ (Fig. 1B and Table 1). EPCs did not appear in the FACS analysis of SVF that had been adherent-culture purified for 28 days, indicating that they may have been lost in the adherent culture process (Fig. 1C) [12]. Taking into account all markers and changes that occur with culture, CD31, CD73, CD146, and HLA-DR are the markers best able to differentiate between ASCs and EPCs within human SVF (Table 1).

The same panel of markers we used to characterize adipose SVF was then applied to dermal single-cell suspensions. The CD31$^-$ CD34$^+$ CD45$^-$ population in dermis contained three subpopulations, compared with the single ASC population in SVF (Figs. 1B and 2B). Unlike the ASC population in SVF, which only varied in expression of CD36, the three populations in dermis had distinct expression profiles with respect to CD36, CD73, CD90, and CD146 (Figs. 1B and 2B). The population we labeled as

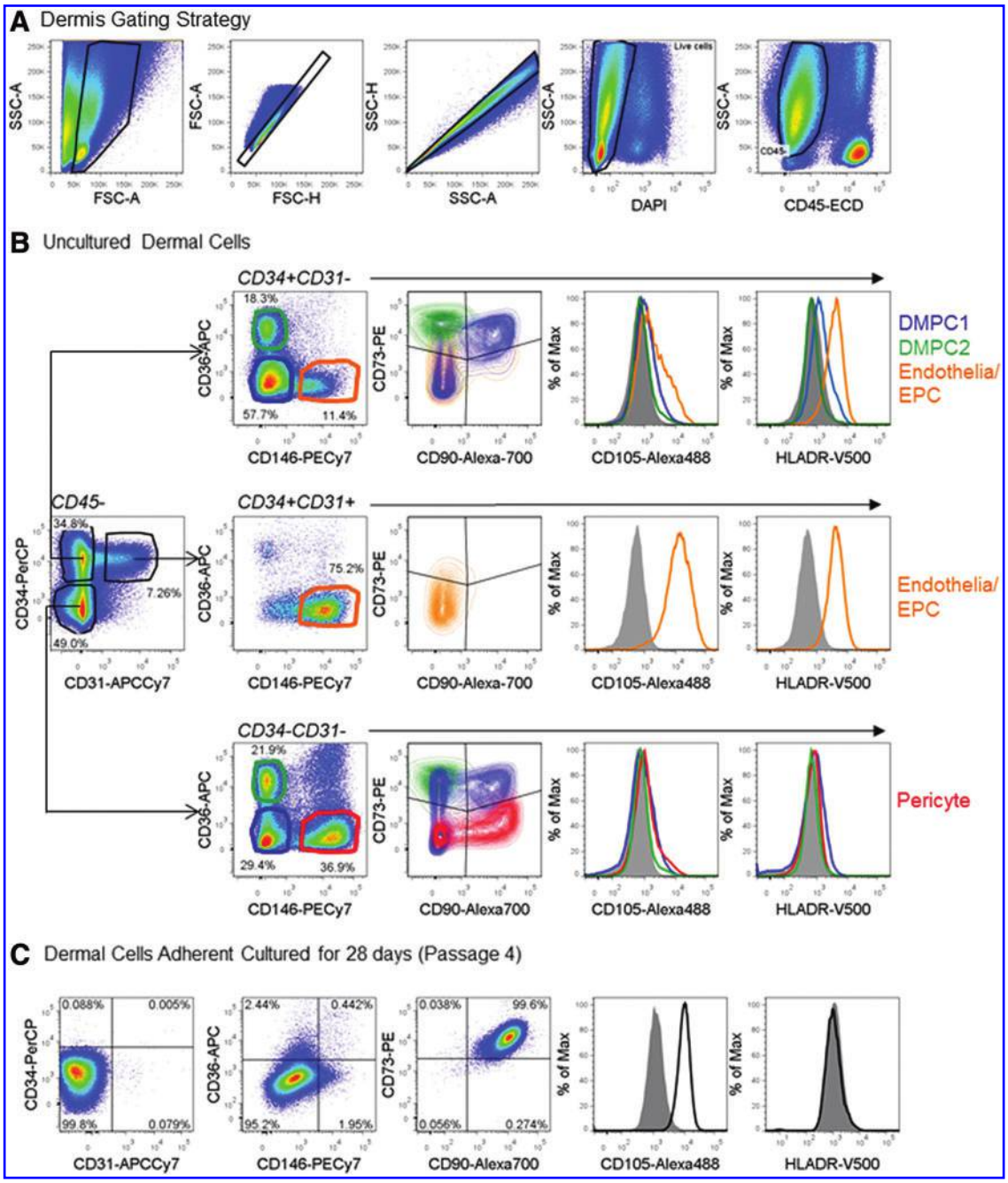

FIG. 2. Flow cytometry analysis of CD45$^-$ cell populations in dermis. Dermal single-cell suspensions were analyzed by polychromatic flow cytometry with a panel of markers, including CD31, CD34, CD36, CD45, CD73, CD90, CD105, CD146, and HLA-DR. Nonhematopoietic cell populations in dermis were identified by the following gating strategy. Cells were first gated by FSC-A and SCC-A followed by doublet exclusion using FCS-A/FSC-H and SSC-A/SSC-H. Dead cells were excluded by DAPI staining. Nonhematopoetic cells were identified by gating on the CD45$^-$ cell population **(A)**. Nonhematopoetic cell populations were separated by expression of CD31 and CD34 and then plotted as CD36 versus CD146 **(B)**. Dermal single-cell suspensions were purified by adherent culture for 28 days, having reached passage 4 in that time, and analyzed by polychromatic flow cytometry **(C)**. Data shown are representative of three biological replicates. Color images available online at www.liebertpub.com/scd

dermal mesenchymal progenitor cell 1 (DMPC1) was the most similar in phenotype to ASCs (CD31$^-$ CD34$^+$ CD36$^-$ CD73$^+$ CD90$^+$ CD105low CD117$^-$ CD146$^-$ HLA-DR$^-$) (Table 1). The only difference from ASCs was that the DMPC1 population was CD36$^-$, while ASCs in freshly isolated SVF were CD36$^{+/-}$ (Figs. 1B and 2B). The cell population that we labeled dermal mesenchymal progenitor cell 2 (DMPC2) (CD31$^-$ CD34$^+$ CD36$^+$ CD73$^+$ CD90$^-$ CD105low CD117$^-$ CD146$^-$ HLA-DR$^-$) had a similar cell surface phenotype to ASCs, but were CD36$^+$ and CD90$^-$ (Figs. 1B and 2B). Hence, both DMPC1 and DMPC2 populations had similar phenotypes but expression of CD36 was

Table 1. $CD45^-$ Cell Populations Identified in SVF and Dermis

	SVF		*Dermis*			
	ASCs	*EPCs*	*DMPC1*	*DMPC2*	*Endothelia/EPCs*	*Pericytes*
CD31	−	+	−	−	+/−	−
CD34	+	+	+	+	+	−
CD36	+/−	High	−	+	−[a]	+/−
CD73	+	−	+	+	−/low	−/low
CD90	+	+/−	+	−	−[a]	+/−
CD105	Low	+	Low	Low	+/low	−
CD117	−	−	−	−	−	−
CD146	−	+/low	−	−	+/low	+
HLA-DR	−	+	−	−	+	−
Range	80%–93%	1%–18%	14%–21%	7%–9%	7%–14%	8%–20%

Summary of cell surface phenotypes of $CD45^-$ cell populations identified in SVF and dermal single-cell suspensions by polychromatic flow cytometry. All population ranges are expressed as a percentage of $CD45^-$ cells.

[a]Majority of cells were $CD36^-$ and $CD90^-$, but a small percentage, ~7%, of EPC/endothelia was either $CD36^+$ or $CD90^+$.

ASC, adipose derived stem cell; EPC, endothelial progenitor cell; DMPC, dermal mesenchymal progenitor cell; SVF, stromal vascular fraction.

associated with a lack of expression of CD90, and vice versa (Fig. 2B). In dermis there were also two $CD31^-$ $CD34^-$ $CD45^-$ cell populations that could be distinctly separated by expression of CD36, CD73, CD90, and CD146 and had identical cell surface phenotypes to DMPC1 and DMPC2 populations, but lacked CD34 expression (Fig. 2B). The relationship between these two populations is more clearly demonstrated with an alternative plotting method that separates stromal and vascular cell populations using CD73 and CD146 (Fig. 3A). Most stromal cell populations were $CD73^+$ $CD146^-$ and most vascular cell populations were $CD73^-$/low $CD146^+$ (Fig. 3A). Plotting the stromal $CD73^+$ $CD146^-$ populations' expression of CD90 versus CD36 segregates DMPC1 and DMPC2 with their mirror $CD34^-$ populations, and demonstrates that the $CD34^+$ DMPC populations are not clearly separated from their $CD34^-$ counterparts (Fig. 3A). Hence the alternative gating strategy reveals that the two $CD73^+$ $CD146^-$ populations both have a continuum of CD34 expression, from $CD34^+$ to $CD34^-$ (Fig. 3A).

We identified two main vascular cell populations in dermal single-cell suspensions, all of which were $CD146^+$ (Figs. 2B and 3A). The $CD31^+$ $CD34^+$ cell population had a similar cell surface phenotype to the EPC population in SVF ($CD31^+$ $CD34^+$ $CD36^-$ $CD73^-$/low $CD90^-$ $CD105^+$ $CD146^+$/low HLA-DR^+), the only differences being lack of expression of CD36 and CD90 (Fig. 2B). The $CD146^+$ populations within the $CD31^-$ $CD34^+$ and $CD31^+$ $CD34^+$ cell populations only differed by expression of CD31 and our alternative plotting method demonstrates the close relationship between these two populations (Figs. 2B and 3A). The $CD34^+$ HLA-DR^+ population contained all $CD31^+$ and CD105high endothelial cells and EPCs, a small percentage of which expressed CD90 (Fig. 3A). The $CD31^-$ $CD34^-$ population contained a prominent $CD36^-$ $CD146^+$ population that also expressed varying levels of CD90, very low levels of CD73, and lacked HLA-DR (Fig. 2B). This phenotype ($CD31^-$ $CD34^-$ $CD36^{+/-}$ $CD73^-$/low $CD90^{+/-}$ $CD105^-$ $CD117^-$ $CD146^+$ HLA-DR^-) is consistent with vascular pericytes in the adventitia of blood vessels in SVF [12,13]. Replotting the data based on CD73 and CD146 expression allowed the $CD73^-$/low $CD146^+$ vascular cells to be re-examined for other markers and demonstrated that the $CD34^-$ HLA-DR^- population contained no $CD31^+$ cells and very few $CD105^+$ cells, but expressed variable CD90, consistent with pericytes (Fig. 3A).

Similar to SVF, adherent-culture purification of dermal cell populations for 28 days, up to passage 4, resulted in no detection of vascular cell populations by FACS analysis (Fig. 2C). Cell population doubling time varied across the 28-day culture period; cells proliferated slowly initially and increased their proliferation rate with culture time; reaching passage 4 in 28 days is only an indication of the average population doubling time for the 28-day period. The cell population that remained most closely resembled DMPC1, although CD105 expression was increased and CD34 expression was not detected, as observed for cultured ASCs (Figs. 1C and 2C).

The markers CD271 and SSEA4 have previously been reported to enrich for adherent dermal cells capable of differentiation [8]. To determine which of the $CD45^-$ cell populations we identified in human dermis expressed CD271 and SSEA4, we stained dermal single suspensions for CD31, CD34, CD36, CD45, CD73, CD90, CD146, CD271, SSEA4, and HLA-DR. The DMPC2 and endothelia/EPC populations were $CD271^-$ $SSEA4^-$ (Fig. 3B). The DMPC1 cell population had low expression of SSEA4, and a subpopulation was $CD271^+$ (Fig. 3B). CD271 and SSEA4 expression did not segregate to $CD34^+$ or $CD34^-$ cells within the DMPC1 cell population, with both $CD34^+$ and $CD34^-$ subpopulations capable of expressing each marker (Fig. 3B). Subpopulations of pericytes expressed CD271 and SSEA4, and some pericytes expressed both molecules; pericytes positive for CD271 and/or SSEA4 were predominantly $CD90^+$ (Fig. 3B). In summary, both CD271 and SSEA4 were expressed by subpopulations of pericytes and DMPC1 cells, but not by DMPC2 cells or endothelial cells (Fig. 3).

We sorted uncultured $CD31^-$ $CD34^+$ $CD45^-$ populations from adipose SVF and dermis (Fig. 4A) and compared their ability to differentiate into adipocytes to the remainder of the cell populations present within adipose SVF and dermis (ie, non-$CD31^-$ $CD34^+$ $CD45^-$) (Fig. 4B). Fewer cells tended to be present in the non-$CD31^-$ $CD34^+$ $CD45^-$

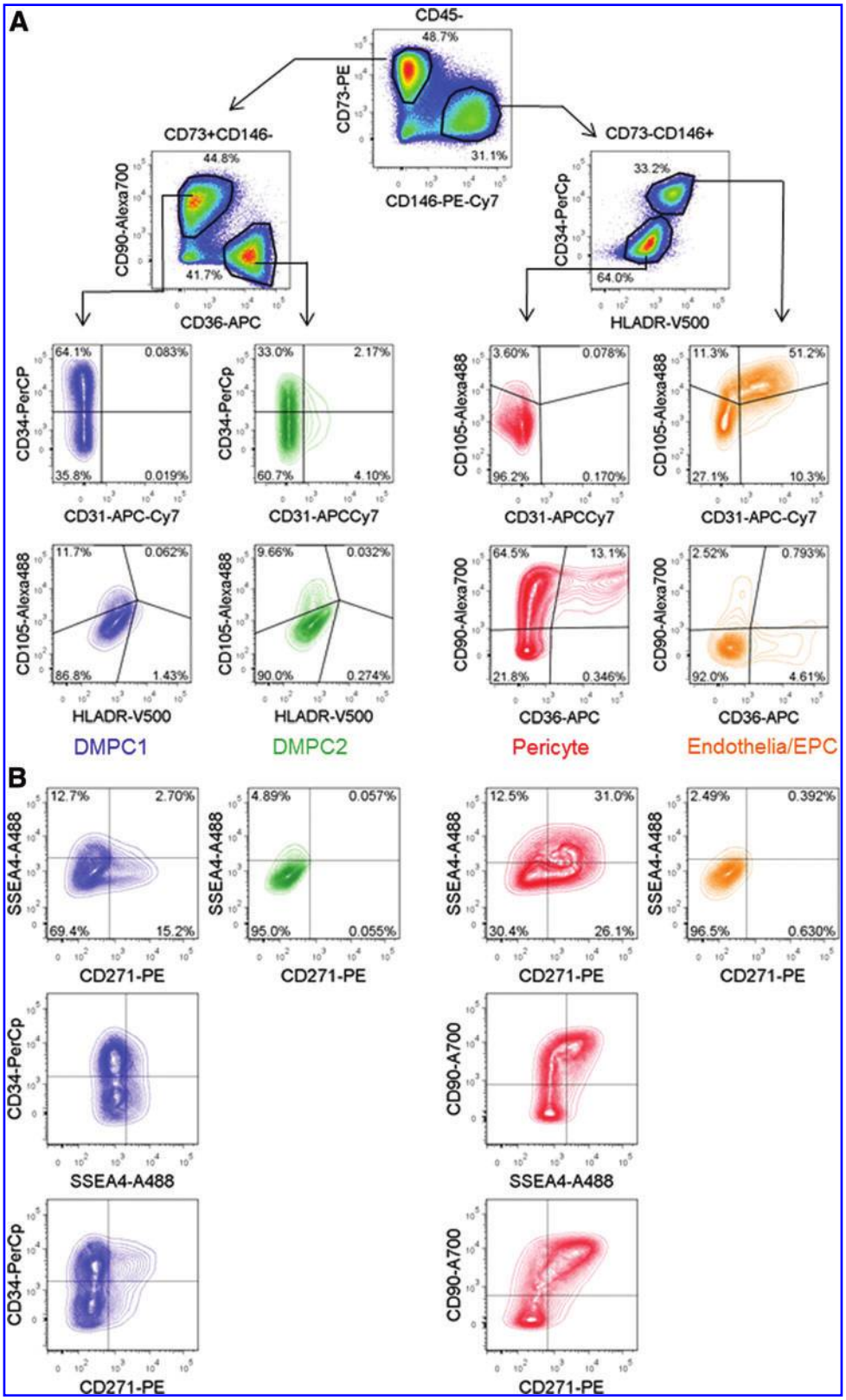

FIG. 3. Alternate dermal FACS data-plotting strategy. An alternative plotting strategy for dermal single-cell suspensions, beginning with plotting CD73 versus CD146, which separates stromal cell populations from vascular cell populations, identifies relationships between populations **(A)**. CD271 and SSEA4 flow cytometry analysis of dermal cell populations **(B)**. Data shown are representative of three biological replicates. Color images available online at www.liebertpub.com/scd

cell population cultures, most likely due to a large number of these cells being nonadherent. Analysis of differentiated and undifferentiated samples of each population for expression of FABP4, a marker of adipogenic differentiation, demonstrated that $CD31^-$ $CD34^+$ $CD45^-$ populations in SVF and dermis were the only adherent cell populations capable of adipogenic differentiation (Fig. 4) [14,15].

To determine which of the three uncultured $CD31^-$ $CD34^+$ $CD45^-$ dermal cell populations were capable of adipogenic differentiation, we sorted them based on CD73

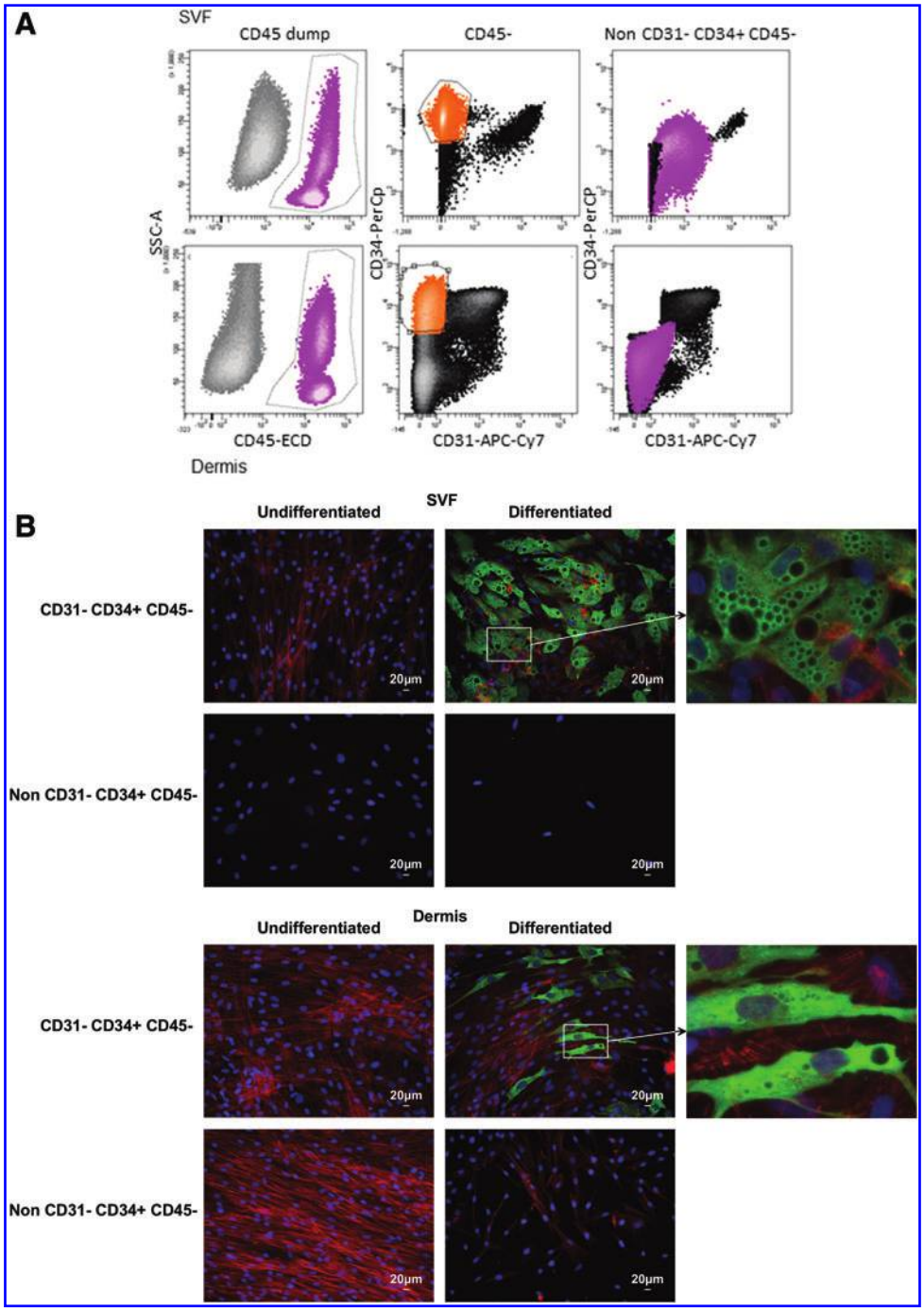

FIG. 4. Adipogenic differentiation of $CD31^-$ $CD34^+$ $CD45^-$ cell populations and non-$CD31^-$ $CD34^+$ $CD45^-$ cell populations sorted from SVF and dermis. $CD31^-$ $CD34^+$ $CD45^-$ cells were sorted from SVF and dermal single-cell suspensions by first excluding $CD45^+$ cells and then only sorting $CD31^-$ $CD34^+$ cells as shown in **(A)**. Adipogenic differentiation of $CD31^-$ $CD34^+$ $CD45^-$ cells sorted from SVF and dermal single-cell suspensions was compared with all non-$CD31^-$ $CD34^+$ $CD45^-$ cell populations of SVF and dermal single-cell suspensions **(B)**. Adipogenic differentiation was assessed by immunocytochemistry for FABP4 expression. Differentiated and undifferentiated samples were stained for FABP4 (*green*), cytoskeletal actin (*red*), and nuclei (*blue*). Data shown are representative of three biological replicates. Color images available online at www.liebertpub.com/scd

and CD90 expression (Fig. 5A). Both DMPC1 and DMPC2 cell populations contained adherent cells capable of adipogenic differentiation (Fig. 5B). The endothelia/EPC population was negative for adipogenic differentiation in two out of three biological replicates; one replicate showed a small amount of expression of FABP4, but this expression was observed in only 2 out of 15 images sampled across three technical replicates (data not shown). Our sort purities ranged from 92% to 99% raising the possibility that some cells from the DMPC1 or DMPC2 populations contaminated the sorted endothelia/EPC population in this instance.

After demonstrating that there are two adherent cell populations within human dermis that were capable of adipogenic differentiation, we wanted to localize them in tissue sections. The localization of ASCs in adipose tissue has not been well defined in the literature, but recent data suggests that they surround the adventitial layer of many vessels, outside the $CD146^+$ pericytes [12,13]. Flow cytometry analysis indicated that at least some DMPC1 cells could potentially be identified within skin sections using the marker combination of CD34, CD90, and CD146. With flow cytometry CD146 proved capable of identifying the major vascular cell populations, and was clearly not expressed by DMPC1 (Fig. 3). CD34 has traditionally been associated with ASCs, and in our data $CD31^-$ $CD34^+$ cells from dermis had functional characteristics of MSCs, although our

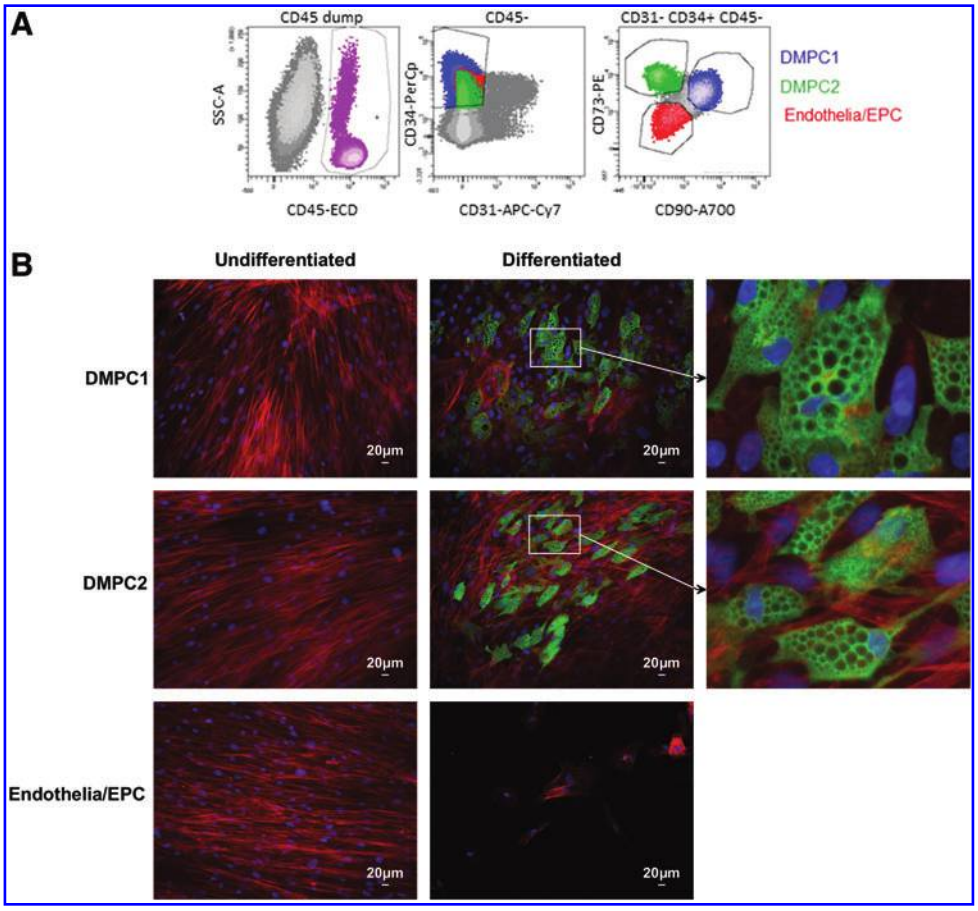

FIG. 5. Adipogenic differentiation of cell populations sorted from dermis. DMPC1 (*blue*), DMPC2 (*green*), and endothelia/EPC (*red*) cell populations were sorted from the $CD31^-$ $CD34^+$ $CD45^-$ cells in dermal single-cell suspensions based on CD73 and CD90 expression as shown in **(A)**. Adipogenic differentiation of cell populations was assessed by immunocytochemistry for FABP4 expression **(B)**. Differentiated and undifferentiated samples were stained for FABP4 (*green*), cytoskeletal actin (*red*), and nuclei (*blue*). Data shown are representative of three biological replicates. Color images available online at www.liebertpub.com/scd

regated flow cytometry plots suggested that not all DMPC1 may express CD34 brightly (Fig. 3). $CD34^+$ DMPC1 are $CD90^+$ while CD34+ endothelial cells and EPCs are usually not, so we postulated that inclusion of CD90 might allow definitive localization of DMPC1 in relation to endothelial cell populations (Fig. 3). Four-color immunofluorescence microscopy of transverse sections of human skin revealed potential DMPC1 cells ($CD34^+$ $CD90^+$ $CD146^-$) in intimate contact with, and in the areas surrounding, $CD146^+$ vascular cells (Fig. 6). There seemed to be two common $CD146^+$ structures around which we identified potential DMPC1 cells. The first was a vascular structure that consisted of an empty space lined by a thin inner layer of $CD34^+$ $CD146^-$ endothelial cells, which in turn were surrounded by a $CD146^+$ layer of pericytes (Fig. 6A–C). The second type of $CD146^+$ structure did not have an empty lumen but was made up entirely of $CD146^+$ cells, with $CD34^+CD146^+$ cells at their center, and may be a collapsed vessel or a vessel wall (Fig. 6D–F). $CD34^+$ $CD90^+$ $CD146^-$ DMPC1 cells could be seen ensheathing both structures (Fig. 6).

Flow cytometry analysis indicated that DMPC2 could be identified with the marker combination of CD31, CD34, and CD36, with the same caveat regarding CD34 expression as for DMPC1, namely, that regating of the flow cytometry data suggested not all DMPC2 necessarily express CD34 brightly (Fig. 3). Transverse sections of human skin were therefore costained for CD31, CD34, and CD36 (Fig. 6G–I). Small numbers of $CD31^-$ $CD34^+$ $CD36^+$ cells were identified in these sections, most frequently localized near groups of $CD31^+$ $CD146^+$ cells (Fig. 6G–I), but not clearly associated with the walls of blood vessels, in contrast to DMPC1. We were only able to identify 21 cells corresponding to the DMPC2 phenotype across 62 images in a total of five donor samples. The low number of DMPC2 seen was consistent with the flow cytometry data that estimated them to be only 7%–9% of $CD45^-$ cell populations, compared with DMPC1 that were estimated to be 14%–21% of $CD45^-$ cell populations (Table 1).

Discussion

Mesenchymal cells bearing stem cell markers have been reported in human dermis [7,8]. Recent data on fibroblastic cell lines derived from human dermis suggest that many of these lines may also contain MSCs [2,3]. Many of these lines can be cultured for a high number of passages before they senesce, suggesting that at least some of these cells possess proliferative potential more typical of stem cells than highly differentiated fibroblasts. But it has also become apparent that many of these cell lines have subpopulations that are multipotent, for example, possessing the capacity to differentiate into adipocytes, reminiscent of the mouse fibroblast 3T3 cell line that can differentiate into 3T3-L1 adipocytes [2,3]. We suspected that human dermis contained cell populations related to ASCs that were contributing to some of the fibroblastic cell lines derived from human skin.

Our aim was therefore to characterize the nonhematopoietic ($CD45^-$) cell populations within human dermal tissue, distinguishing the mesenchymal populations from other

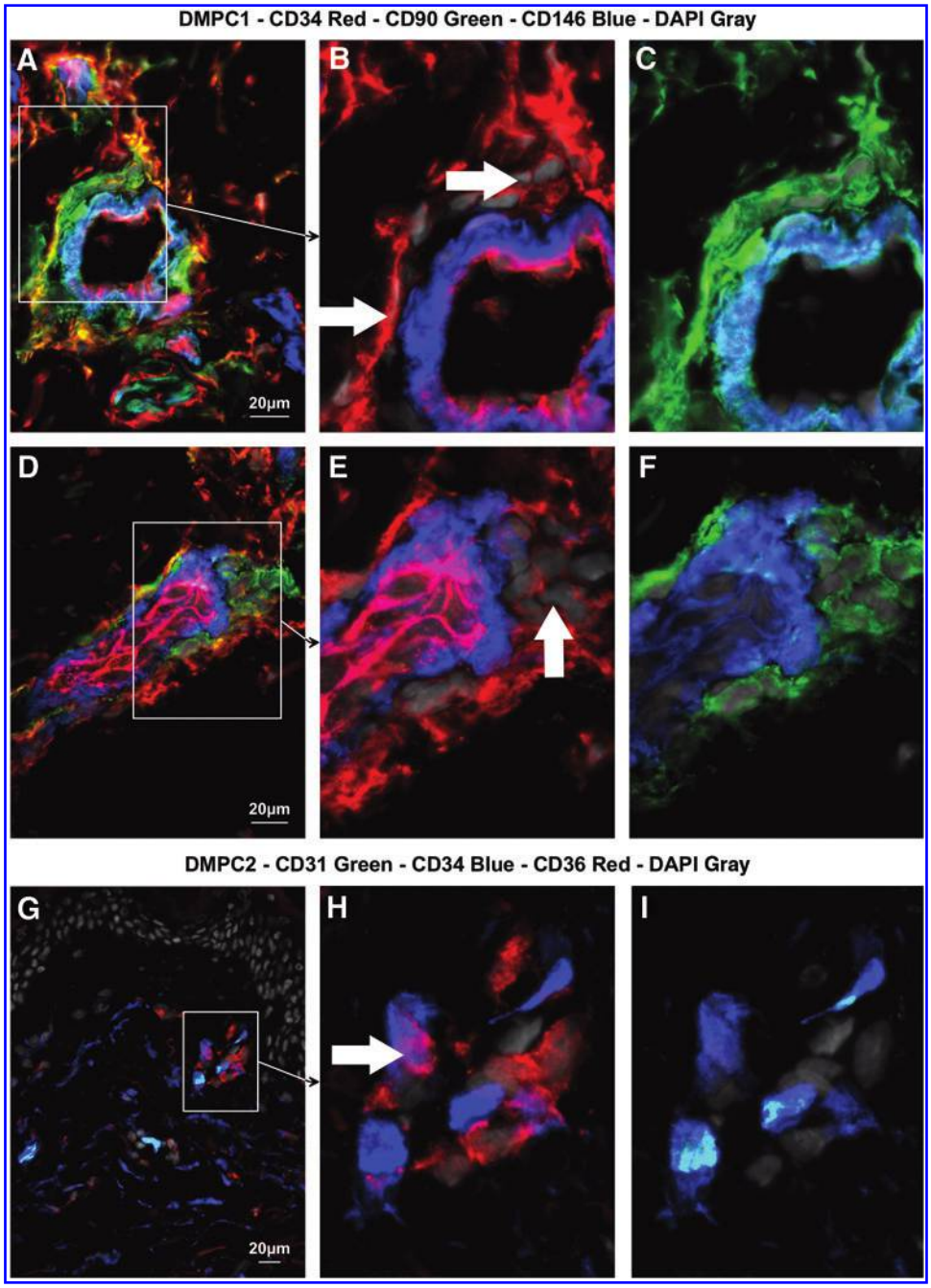

FIG. 6. CD34, C90, and CD146 and CD31, CD34, and CD36 immunohistochemistry of skin. Skin transverse sections were stained with CD34 (*red*), CD90 (*green*), CD146 (*blue*), and DAPI (*gray*) to identify DMPC1 localization **(A–F)**. Skin transverse sections were stained with CD31 (*green*), CD34 (*blue*), CD36 (*red*), and DAPI (*gray*) to identify DMPC2 localization **(G–I)**. *White markers* identify DMPC1 and DMPC2 cells. Data shown are representative of four biological replicates. Color images available online at www.liebertpub.com/scd

populations such as endothelial cells, and testing which mesenchymal populations had the ability to differentiate into adipocytes. Our ability to dissociate cell populations from dermal tissue and determine their cell surface phenotypes with 11 different markers concurrently meant that we were able to separate the complex mix of cell populations present in dermis, and sort them for functional analysis. To the best of our knowledge this is the most extensive flow cytometric analysis performed to date of nonhematopoietic cells in human dermal or adipose tissues. Cell populations in SVF of adipose tissue have been characterized with up to seven markers simultaneously previously, but this level of flow cytometry technology has not been applied to human dermis, which has a much greater complexity of cell populations [10,12,16]. Previous cell sorting experiments from human dermis have been restricted to isolating dermal stem cells with magnetic beads based on a single-cell surface marker [8]. By sorting defined populations from complex mixes of dermal cell populations, we were able to show that there were two distinct adherent cell populations within human dermis capable of mesenchymal differentiation, one of which has a cell surface phenotype and localization pattern similar to ASCs.

Uncultured SVF contained two main nonhematopoietic ($CD45^-$) cell populations, consistent with previous descriptions of ASCs and EPCs, although there were subtle differences between our 11-marker data and previous reports [9,12]. ASCs have previously been reported, and defined by the International Federation for Adipose Therapeutics and Sciences (IFATS) and International Society for Cellular Therapy (ICST), as a $CD31^-$ $CD34^+$ $CD45^-$

cell population within SVF [9,11]. Our analysis of freshly isolated SVF extends this cell surface phenotype to $CD31^-$ $CD34^+$ $CD36^{+/-}$ $CD45^-$ $CD73^+$ $CD90^+$ CD105low $CD117^-$ $CD146^-$ $HLA\text{-}DR^-$ (Table 1). Within this population there was heterogeneity of expression of CD36, with ~31% of the cells expressing CD36 on fresh isolation, consistent with a previous report (Fig. 1B) [9]. We confirmed with cell-sorting experiments that these cells in uncultured SVF were capable of adipogenic differentiation (Fig. 4). One subtle difference between our data and that previously published is that although we found ASCs to express CD105, the level of expression was very low. Hence, ASCs in fresh SVF may in some experiments appear to be $CD105^-$.

We identified two cell populations among uncultured dermal single-cell suspensions that were capable of adipogenic differentiation, which we labeled DMPC1 and DMPC2 (Fig. 2). The DMPC populations had cell surface phenotypes similar to ASCs ($CD31^-$ $CD34^+$ $CD45^-$ $CD73^+$ CD105low $CD117^-$ $CD146^-$ $HLA\text{-}DR^-$) but two markers in our panels differed from ASCs (Table 1). While ASCs were $CD36^{+/-}$ $CD90^+$, DMPC1 were $CD36^-$ $CD90^+$ and DMPC2 were $CD36^+$ $CD90^-$ (Figs. 1 and 2). Replotting of the flow cytometry data using a schema that begins with analysis of CD73 and CD146 revealed that the DMPCs were part of populations with variable expression of CD34, suggesting that the $CD34^+$ and $CD34^-$ populations among the $CD73^+$ $CD146^-$ populations are closely related (Fig. 3). CD34 is commonly considered a reliable marker of circulating hematopoietic stem cells (HSCs), although a recent critical review of the literature reveals that it does not necessarily mark HSCs in the bone marrow, since HSCs may ''cycle'' their expression of CD34 [17]. Expression of CD34 by bone-marrow-derived MSCs (BM-MSCs) has also been a source of some controversy, although once again careful review of the literature reveals that while cultured BM-MSCs are commonly $CD34^-$, most are $CD34^+$ in vivo [17]. It is clear from our data that CD34 expression is lost with culture of both ASCs (Fig. 1) and DMPCs (Fig. 2), consistent with the concept that MSCs may also alter their expression of CD34 depending on their environment, and that CD34 expression does not necessarily correlate with the functional capacity of MSCs [17,18].

Given our flow cytometry reanalysis (Fig. 3) it will now be important to conduct cell-sorting experiments of CD34 low and $CD34^-$ cells within the dermal $CD73^+$ $CD146^-$ populations to see whether these cells have the same differentiation potential as the $CD34^+$ populations we have sorted in this article. If these experiments confirm that differentiation potential in these populations does not segregate with CD34, then the new classification of MSCs shown in Figure 3 will prove a very useful alternative to a CD34-based classification, due to its clean division of the populations; both DMPC populations reside within the $CD73^+$ $CD146^-$ population of $CD45^-$ cells in human dermis, and DMPC1 and DMPC2 can be separated based on their opposing expression of CD36 and CD90, with DMPC1 $CD36^-$ $CD90^+$ and DMPC2 $CD36^+$ $CD90^-$. Consistently under this gating scheme, both populations demonstrably lack CD31 and HLA-DR, although small subpopulations express low-level CD105 (Fig. 2), as for ASCs (Fig. 1).

Adherent culture is a technique commonly used to purify ASCs from SVF, with passages 3 to 4 generally accepted as a pure population [18]. Similarly, a ''dermal fibroblast'' cell line is the result of adherent purification of dermal cells. When SVF was plated and adherent cells were cultured for 28 days, reaching passage 4, only one population was detected by FACS analysis (Fig. 1). These cells appear to be ASCs, and are consistent with the IFATS/ICST definition, although the cell surface phenotype differed substantially from that in fresh SVF, with decreased CD34 and increased CD105 expression (Fig. 1) [11]. This is consistent with previous work using flow cytometry to characterize the cell surface phenotype of ASCs, showing the same trend with CD34 and CD105 [10,18]. Our data extend knowledge of the stability of the other cell surface markers expressed by freshly isolated ASCs; in that, they continue to express consistent levels of CD73 and CD90, and consistently fail to express CD146 or HLA-DR (Fig. 1). Similarly dermal cells that were adherent-culture purified for 28 days, reaching passage 4, consisted of only a single-cell population that was detected by our FACS analysis (Fig. 2). This cell population had a similar cell surface phenotype to $CD34^-$ DMPC1, except they were $CD105^+$ (Fig. 2). Loss of CD34 and gain of CD105 expression is consistent with the changes we observed on culture of ASCs.

It is unclear whether this ''dermal fibroblast'' culture was derived from $CD34^+$ DMPC1 (with loss of CD34 and increased expression of CD105 as seen on culture of ASCs), or from $CD34^-$ DMPC1 (by increased expression of CD105 on culture). Cell sorting and adherent culture of $CD34^+$ and $CD34^-$ DMPC1 cell populations will be required to answer this question.

ASCs have been shown to be localized to the adventitia of vascular structures in adipose tissue [12,13,19]. Our data would seem to place the DMPC1 population in a similar location. However, we add to existing information about the relationship of mesenchymal populations with vessel walls by demonstrating that the DMPC1 population is $CD146^-$ (Fig. 2) and localizes to the vessel adventitia outside the ring of $CD146^+$ pericytes (Fig. 6). DMPC2 were not found associated with vessel walls, nor any other obvious structure, so their localization is distinct from DMPC1 cells (Fig. 6). This localization pattern does not support a close relationship between the DMPC1 and DMPC2 populations, opening up the possibility that they play quite distinct roles in the dermis, despite their phenotypic and functional similarities.

We identified an EPC population in both SVF and dermis, consistent with a previously published phenotype in SVF ($CD31^+$ $CD34^+$ $CD45^-$) [12]. However, this population in dermis ($CD36^-$) differed subtly from SVF ($CD36^-$ high) (Figs. 1 and 2). The EPC population in dermis also seemed to have a $CD31^-$ component, which replotting using our alternative gating strategy identified, with only expression of CD146 identifying this population as a potentially endothelial like, and may possibly be an intermediate population, somewhere between an endothelial cell and a pericyte (Fig. 3). We identified pericytes in dermis but not in SVF (Figs. 1 and 2). Pericytes were nonendothelial since they lacked expression of CD31, CD34, or HLA-DR; however, this population was $CD73^-$ $CD146^+$, the converse of the DMPC populations, suggesting it might represent cells within the middle layers of blood vessels, where CD146 is strongly expressed (Fig. 6). These $CD146^+$ cells had an interesting pattern of expression of CD36 and CD90, where CD36 was

expressed on those cells most highly expressing CD90 (Fig. 3). These features are consistent with previous reports that suggest that pericytes may express different levels of CD36 and CD90, depending on their differentiation status [13].

Within our panel, CD31, CD73, CD146, and HLA-DR are the markers that most clearly discriminate between the ASCs and EPCs in SVF, with ASC $CD31^-$ $CD73^+$ $CD146^-$ $HLA\text{-}DR^-$ and EPC $CD31^+$ $CD73^-$ $CD146^+$ $HLA\text{-}DR^+$ (Table 1). Importantly, the low-level expression of CD105 in freshly isolated ASCs, and its sensitivity to upregulation in cell culture, rules out this marker as an absolute discriminator between ASCs and EPCs; the same is true of expression of CD90 given its substantial expression by many EPCs. For dermal cell populations, CD31 and HLA-DR do not separate DMPC populations from pericyte/EPC/endothelia cell populations, but CD73 and CD146 can be reliably used to discriminate between DMPCs ($CD73^+$ $CD146^-$) and pericytes/EPCs/endothelia ($CD73^-$ $CD146^+$) (Table 1).

A number of cell populations with stem cell properties have been identified in cell lines grown from human dermis, including ''skin-derived precursors'' (SKPs), ''fibroblastic mesenchymal stem-cell-like cells,'' ''DMSCs,'' and ''Muse'' cells [4,6–8]. However, the cell culture methods used to derive these cells and the phenotypic and functional properties of these cells differ substantially and there is currently no consensus regarding MSC populations in human dermis. SKPs formed floating spheres when single-cell suspensions from de-epithelialized human foreskin were cultured in flasks, in contrast to the adherent cell MSC populations we describe here [6]. SKPs seem to have more than one localization, follicular and nonfollicular [20]. Recently nonfollicular SKPs were shown to be $CD146^+$, indicating that they may in fact be of pericytic origin, a different niche from those we have identified for DMPC1 and DMPC2, but intimately associated with DMPC1 (Fig. 6) [20].

Cells present within primary dermal fibroblastic cell lines have been shown to be capable of differentiation into mesenchymal lineages in the same manner as ASCs [2–4,21–24]. The studies by Lorenz et al., Blasi et al., and Manini et al. demonstrated that cultured dermal fibroblastic cell lines and ASCs were indistinguishable based on morphology, or the cell surface markers they each used to characterize their cell populations, and that a proportion of the cells within the culture had the capability to undergo mesenchymal differentiation [2–4]. Cell surface phenotypes of the adherent dermal cells in each of these studies have similar elements to the cells we derived from adherent culture (Fig. 2). Notably the reported cell phenotypes of these cultured dermal cell lines were $CD34^-$ and $CD105^+$, in contrast to the freshly isolated DMPC populations we report, suggesting that the cultured lines reported in these previous articles had downregulated CD34 and upregulated CD105 in the same way as we observed on culture. Therefore, it seems possible that the cell lines previously reported as capable of mesenchymal differentiation within these fibroblastic cultures may have been derived from one or both of the DMPC populations we have identified within freshly isolated dermis.

Wakao et al. identified an $SSEA3^+$ subpopulation of dermal fibroblastic cultures with MSC properties, termed ''Muse'' cells [7]. The cell surface phenotype of these cells is again $CD34^-$ $CD105^+$, differing from our freshly isolated DMPC populations, but consistent with our observations after culture; the reported lack of CD117 and CD146 is also consistent with our data on cultured dermal cells (Fig. 2) [7]. While the MSCs Wakao et al. identified in their cultures were $CD271^-$, Vaculik et al. demonstrated that $CD271^+$ and $SSEA4^+$ adherent cell populations within dermal fibroblast cell cultures had a greater capacity for mesenchymal differentiation compared with $CD271^-$ and $SSEA4^-$ cells, and that the $CD271^+$ cells were $CD73^+$ [7,8]. Our analysis showed that both DMPC1 and pericyte cell populations were capable of expressing CD271 and SSEA4, but CD73 was not expressed by pericytes (Fig. 3B). It therefore seems likely that at least some of the $CD271^+$ cell population isolated by Vaculik et al. may have been DMPC1 cells (Fig. 3B) [8], which we have shown as capable of adipogenic differentiation.

Our studies represent the most extensive flow cytometric characterization of mesenchymal cells in human dermis to date. The cell surface phenotype of freshly isolated DMPCs differs substantially from that of cultured cells, as it does for MSCs isolated from human adipose tissue, so the markers on DMPCs in cultured dermal fibroblastic lines are unreliable for identifying DMPCs in tissue. Our ability to separate and culture two distinct DMPC populations from human dermis should also allow us to determine which of these populations give rise to the MSCs present in adherent cultures, and to determine whether they fulfill distinct roles. Clearly DMPCs are likely to play roles in wound healing and tissue homeostasis. Since these two processes involve different cellular activities, it is possible that DMPC1 and DMPC2 make different contributions to each. It also seems likely that either or both of these cell populations will be involved in fibroproliferative diseases of the skin, given their lack of terminal differentiation, and likely proliferative potential. But the ability of these cells to differentiate into adipocytes suggests that they may also be capable of forming other mesenchymal structures within or adjacent to skin. This raises the possibility that these cells are involved in disease processes where mesenchymal cell behavior becomes corrupted, in the same way as ASCs are now believed to generate bone in progressive osseous heteroplasia [25]. Hence, in parallel with investigations of their normal physiology, it may be very productive to being investigation of the role of these cells in a broad range of skin diseases where there is evidence of mesenchymal cell dysfunction.

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Author Disclosure Statement

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Address correspondence to:
Professor Rod Dunbar
School of Biological Sciences and Maurice Wilkins Centre
University of Auckland
Private Bag 92019
Auckland Mail Centre
Auckland 1142
New Zealand

E-mail: r.dunbar@auckland.ac.nz

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