

Chemokine-Mediated Migration of Skin-Derived Stem Cells: Predominant Role for CCL5/RANTES

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The ability of stem cells to self-renew as well as their multilineage differentiation potential makes them ideal candidates for skin regeneration strategies. Mesenchymal stem cells residing in human adult dermis, in contrast to adipose tissue, have not yet been described. The objective of this study was to determine the stemness and chemokine-mediated homing potential of dermal stromal cells (DSC) and to compare this with adipose stem cells (ASC). DSC have a less stellate form than ASC, confirming that DSC and ASC are two different types of mesenchymal cell populations. However, DSC display a mesenchymal stem cell phenotype (CD31⁻, CD34⁺, CD45⁻, CD54⁺, CD90⁺, CD105⁺, and CD166⁺) similar to ASC and are also multipotent in their ability to differentiate into adipocytes, chondrocytes, and osteoblasts. Both ASC and DSC display a similar set of chemokine receptors (CCR3, CCR4, CCR6, CCR10, CXCR1, and CXCR2). Several ligands for these receptors, with CCL5/RANTES being the most potent, can induce migration of ASC and DSC in an *in vitro* wound-healing assay. Taken together, these results show that a population of mesenchymal stem cells resides in the dermis of human adult skin and these dermal-derived stem cells have a phenotypic and chemokine-mediated homing potential similar to adipose stem cells, which to our knowledge is previously unreported.

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INTRODUCTION

Recently, increasing focus is being placed on the use of stem cell (SC) applications for the treatment of difficult to heal cutaneous wounds, such as deep burns and trauma-induced wounds (Falanga *et al.*, 2007; Prichard *et al.*, 2007). Currently these wounds heal slowly and result in excessive scar formation. SCs represent a population of multipotent cells capable of differentiating into a number of mesodermal lineages when triggered by the appropriate microenvironment. SCs were first isolated from bone marrow, but have also been reported to be present in other tissues such as adipose tissue, postnatal dermis, umbilical cord blood, peripheral blood, skeletal muscle, and brain

(Bartsch *et al.*, 2005; Chamberlain *et al.*, 2007; Oedayr-ajsingh-Varma *et al.*, 2007). The ability of SCs to self-renew as well as their transdifferentiation potential makes them ideal candidates for skin regeneration strategies.

During normal cutaneous wound-healing, growth and directed migration of skin residential and regenerative SCs into the affected area is thought to be essential for reepithelialization, fibroblast repopulation of the dermal matrix, and angiogenesis. These processes are most probably regulated by chemokine (CK) and chemokine receptor (CKR) interactions. This is supported by the differential and sequential expression of CKs during skin injury (Gillitzer and Goebeler, 2001) and several other findings: CXCL8 and CXCL1 enhance keratinocyte growth (Steude *et al.*, 2002); CXCR2^{-/-} mice show delayed reepithelialization and decreased vasculogenesis (Devalaraja *et al.*, 2000) and CXCR3^{-/-} mice show a delayed dermal maturation (Yates *et al.*, 2007).

Homing of bone marrow-derived SCs into the epidermis of mice skin, followed by transdifferentiation into keratinocytes has very recently been demonstrated by Inokuma *et al.* (2006). These results support the hypothesis that SCs, once in an appropriate microenvironment, will differentiate and in doing so repair the damaged tissue. This homing into the epidermis was shown to be regulated by specific CK/CKR interactions, in particular CCL27/CCR10 (Inokuma *et al.*, 2006). Several groups have recently been studying the expression of the CKR profile on human mesenchymal SCs derived from the bone marrow (Honczarenko *et al.*, 2006;

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Abbreviations: ASC, adipose stem cells; ASVF, adipose stromal vascular fraction; CK, chemokine; CKR, chemokine receptor; DSC, dermal stromal cells; DSVF, dermal stromal vascular fraction; EGF, epidermal growth factor; PBS, phosphate-buffered saline; P/S, penicillin-streptomycin; SC, stem cell; SVF, stromal vascular fraction

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Ponte *et al.*, 2007; Ringe *et al.*, 2007). The CKR profile of mesenchymal SCs derived from other human tissues has not yet been reported.

In deep skin wounds, the underlying adipose tissue is exposed in the wound bed and the dermis at the wound margins. Optimal tissue regeneration strategies aimed at scar-free healing in the future might be able to target SCs residing in the adipose tissue and dermis. A number of reports describe the presence of SCs within the adipose tissue of human adult skin. These cells exhibit a CD31⁻CD34⁺CD45⁻CD54⁺CD90⁺CD105⁺CD166⁺ mesenchymal SC phenotype and have been shown to be multipotent by their ability to differentiate into chondrocytes and osteoblasts (Zuk *et al.*, 2002; Ponte *et al.*, 2007; Oedayrajsingh-Varma *et al.*, 2007). With regard to dermal tissue only a single report describes the presence of multipotent SCs in human postnatal foreskin (Prichard *et al.*, 2007). It is unknown whether adult human dermis also contains mesenchymal SCs. Optimal skin regeneration strategies also depend on the homing capacities of SCs to the site of injury. The CKR CXCR4, which is reported to be present in adipose stem cells (ASCs; Cho *et al.*, 2006; Chamberlain *et al.*, 2007), may play a role in this homing, but no comprehensive study on ASC CKR profiles has been reported. Also, no information is available on CKR expression on dermis-derived stromal cells.

The aim of the present study was to determine whether or not mesenchymal SCs are present in adult human dermis and to determine the CK-mediated homing potential of these cells compared to adipose-derived SCs. Therefore, dermis-derived stromal cells (DSC) and ASC were isolated from human adult abdominal skin. Both cell populations were first compared with regard to phenotypic parameters such as SC marker expression profile and their multilineage differentiation potential. Next, the CKR profile was studied using flow cytometry. Whether receptor expression facilitated cell migration was determined in an *in vivo* scratch assay, a well-established wound-healing model (Entschladen *et al.*, 2005). Knowledge on the presence of SCs in the skin and the effect of CKs on migration of these cells is of importance for future CK-based therapies. These therapies are aimed at homing SCs into difficult to heal wounds to achieve optimal tissue regeneration.

RESULTS

Phenotypic characterization of ASC and DSC

Adipose tissue of human adult skin contains a stromal vascular fraction (SVF) with a rich source of SCs, but also adipocytes, endothelial cells and infiltrating cells. In this manuscript the uncultured cell population isolated from adipose tissue is referred to as the adipose stromal vascular fraction (ASVF) and the cultured cells at passage 3 are referred to as ASC. The mesenchymal cell population isolated and cultured from the dermis of human adult skin is studied for its SC characteristics. In this manuscript the uncultured cell population isolated from human adult dermis is referred to as the dermal stromal vascular fraction (DSVF), containing predominantly fibroblasts and endothelial cells, but also

infiltrating cells such as macrophages. The cultured cells at passage 3 are referred to as DSC.

Both ASC and DSC, when cultured up to passage 3, exhibited a spindle-shaped morphology. DSC adopted a less stellate form when compared to ASC (Figure 1). It should be noted that DSC are isolated and cultured using identical protocols as those previously described for dermal fibroblasts, indicating that these refer to the same cell population.

To determine whether stromal cells isolated from human dermis exhibit a similar SC marker profile to that described for cells isolated from human adipose tissue (Zuk *et al.*, 2002; Oedayrajsingh-Varma *et al.*, 2007), we analyzed surface expression of CD31, CD34, CD45, CD54, CD90, CD105, CD166, HLA-ABC, and HLA-DR. The analyses were performed for uncultured SVF and cultured SCs derived from adipose tissue and dermis of human skin (Figure 2; Table 1). Both ASVF and DSVF contained a small percentage of cells expressing the common leukocyte marker CD45 (LCA), the cell-surface glycoprotein CD105 (endoglin) and the adhesion molecule CD166 (ALCAM). Both ASVF and DSVF contained large percentages of cells expressing the common endothelial marker CD31 (PECAM-1), the common hematopoietic SC marker CD34, the adhesion molecule CD54 (ICAM-1), the mesenchymal marker CD90 (thy-1), HLA-ABC, and HLA-DR. The observed CD marker profile for DSVF is similar to the profile of ASVF, although CD34 and CD90 were significantly lower, and both classes of HLA molecules were higher on DSVF when compared to ASVF.

Next, cultured ASC and DSC (passage 3), derived from the same three donors as the SVF, were characterized (Figure 2, Table 1). Upon culturing expression of CD31, CD45 and HLA-DR was lost on both cell types. The CD marker profile observed for DSC is comparable to that of ASC, although CD34 and CD166 expression were slightly decreased on DSC when compared to ASC. In summary, the phenotype of DSC is essentially similar to that of ASC.

Differentiation potential of ASC and DSC

The capacity of cultured (passage 3) DSC to differentiate into lineage-specific pathways was next studied and compared to the well-known multipotency of ASC. Similar to ASC, DSC did show trilineage differentiation capacity toward

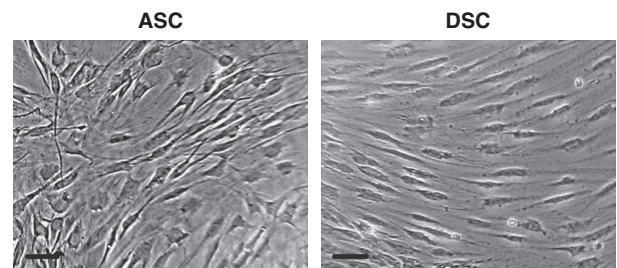


Figure 1. ASC and DSC differ in morphology. Morphology of adipose-derived stem cells (ASC) and dermal-derived stromal cells (DSC) of human abdominal skin cultured at passage 3 is shown. Note the more stellate form of ASC and the spindle shaped form of DSC. Data shown are from one individual donor (adipose and dermis matched) and are representative of experiments performed with three donors in duplicate; scale bar = 25 µm.

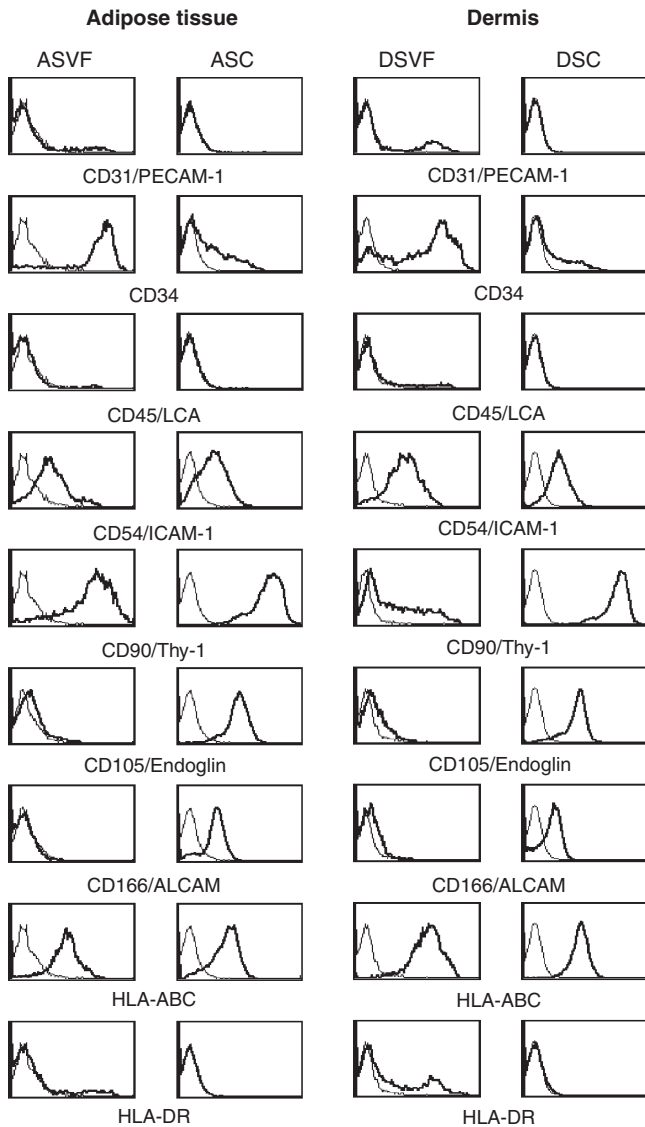


Figure 2. The phenotype of DSC is essentially similar to that of ASC.

Phenotypic characterization of the stromal vascular fraction and stem cells derived from adipose tissue (ASVF/ASC) or dermis (DSVF/DSC) of human skin. Cells were stained with mAb against the indicated PE-labeled CD markers (thick line). Each histogram plot contains a PE-labeled isotype-matched mAb control (thin line). Data shown are from one individual donor (adipose tissue and dermis matched) and are representative of experiments performed with three donors in duplicate. Corresponding percentages of positive cells are given in Table 1.

adipogenic, chondrogenic, and osteogenic pathways, although to a somewhat lesser degree than ASC (Figure 3). When culturing the cells in chondrogenic medium, chondrogenic pellets were observed as shown by collagen type II staining and Alcian blue staining of proteoglycans (Figure 3a). Also, SOX-9 gene expression was observed by reverse transcription-PCR (ASC compared to DSC: $P=0.81$; Figure 3d). Osteogenic induction of ASC and DSC resulted in osteogenic matrix formation. Calcium phosphate mineralization, typical for mature osteoblasts, was visualized by Von Kossa staining (black nodules; Figure 3b). The late osteogenic

marker COL1a was observed by reverse transcription-PCR (ASC compared to DSC: $P=0.27$; Figure 3e). Culturing ASC and DSC with adipogenic medium resulted in intracellular Oil red O staining of lipid vacuoles (Figure 3c). In agreement, PPAR γ was detected by quantitative RT-PCR (DSC compared to ASC: $P=0.035$; Figure 3f). Therefore, in summary, multipotent SC populations do reside in the dermis of human adult skin in addition to the adipose tissue.

Chemokine receptor expression on ASC and DSC

Having observed that cells residing in dermis of human skin exhibit SC characteristics similar to that previously reported for ASC, we next determined the CKR profiles on both ASC and DSC (Figure 4, Table 2). First, CKR profiles of the uncultured ASVF and DSVF were determined. Both cell populations demonstrated a similar heterogeneous expression of CCR3, CCR4, CCR6, CCR10, CXCR1, CXCR2, CXCR3, and CXCR4. However, a minor but significantly lower expression of CCR10 was observed on DSVF compared to ASVF. Neither ASVF nor DSVF showed expression for CCR1, CCR2, CCR5, CCR7, and CCR8.

The CKR profile on cultured ASC and DSC (passage 3), derived from the same 3 donors as the SVF, was next determined (Figure 4, Table 2). Both cultured cell populations demonstrated a similar homogeneous expression of CCR3, CCR4, CCR6, CCR10, CXCR1, and CXCR2. However, a minor but significantly lower expression of CCR3, CCR6, and CCR10 was observed on DSC compared to ASC. Neither ASC nor DSC showed expression for CCR1, CCR2, CCR5, CCR7, CCR8, and CXCR3.

Receptor protein expression on cultured ASC and DSC (passage 3) was confirmed by western blot analysis using the same antibodies as for FACS analysis. Western blot profiles for CCR1, CCR3, CCR4, CCR6, CCR10, and CXCR1 demonstrated a single band of approximately ~ 38 kDa, which is in agreement with the theoretical size (~ 40 kDa), calculated from the known amino-acid sequence of the CKRs. In contrast, both Western blot profiles for CXCR2 and CXCR4 showed two separate bands each of approximately ~ 38 kDa, most likely indicating two isoforms for these receptors, which is in agreement with results from others (Ludwig *et al.*, 2000; Wynn *et al.*, 2004). All receptors detected by FACS analysis on ASC and DSC were detectable using Western blot analysis, with the exception of CCR1, which was only detected by Western blot analysis. CCR2, CCR5, CCR7, CCR8, and CXCR3 were not detectable on cultured ASC and DSC, neither by FACS analysis nor western blot analysis. No bands were observed on the western blots when the isotype (negative) control antibodies (IgG1 and IgG2b) were used.

In summary, both uncultured and cultured cells, isolated from the adipose tissue and dermis of human skin have essentially similar CKR profiles.

Effect of chemokines on migration of ASC and DSC

An *in vitro* wound-healing scratch assay was used for a functional analysis of receptor expression on the ASC and DSC (Figure 5a). The ability of ASC and DSC to migrate upon exposure to CK ligands, corresponding to receptors shown to be present on the cell surfaces, was compared.

Table 1. Surface marker expression in the stromal vascular fraction and cultured stem cells derived from adipose tissue or dermis of human skin

CD marker	Percentage of positive cells		Percentage of positive cells		Significance	
	ASVF	ASC	DSVF	DSC	DSVF versus ASVF	DSC versus ASC
CD31/PECAM-1	39.95 ± 8.64	0.00 ± 0.00	47.06 ± 5.16	0.05 ± 0.02	NS	NS
CD34	84.89 ± 5.84	46.33 ± 9.68	62.32 ± 9.65	28.06 ± 9.65	↓	↓
CD45/LCA	2.02 ± 2.80	0.00 ± 0.00	9.09 ± 2.95	0.06 ± 0.02	NS	NS
CD54/ICAM-1	74.33 ± 4.23	76.58 ± 1.53	77.52 ± 7.89	79.52 ± 5.69	NS	NS
CD90/Thy-1	85.98 ± 7.05	99.5 ± 0.73	63.61 ± 2.43	99.74 ± 0.46	↓	NS
CD105/endoglin	14.21 ± 8.53	96.05 ± 3.24	15.28 ± 9.69	92.64 ± 6.21	NS	NS
CD166/ALCAM	5.11 ± 1.05	89.09 ± 3.03	7.94 ± 3.24	77.5 ± 4.32	NS	↓
HLA-ABC	76.37 ± 6.93	95.38 ± 2.83	95.09 ± 5.63	83.06 ± 13.93	↑	NS
HLA-DR	20.94 ± 8.33	0.00 ± 0.00	41.07 ± 5.96	0.00 ± 0.00	↑	NS

ASC, adipose-derived stem cells; ASVF, adipose-derived stromal vascular fraction; DSC, dermal-derived stromal cells; DSVF, dermal-derived stromal vascular fraction; NS, not significant.

Flow cytometry dot-plot data from three donors (all cell populations were donor matched) were collected and the mean number of positive cells for each CD marker is expressed as a percentage of total cell number ± standard deviation (mean ± SD). Statistical significant difference between DSVF versus ASVF and DSC (p3) versus ASC (p3) was calculated using a paired Student's *t*-test, significant lower/higher expression in dermal cell populations are indicated with an arrow pointing down/up (*P* < 0.05).

Results were divided into three categories based on the ability of a CK to increase cell migration (Figure 5). In agreement with the similar CKR profile of ASC and DSC, no significant differences were observed between migration of ASC and DSC for all CKs tested. CKs that increased the migration of the cells (indicated with a + or ++ in Table 3) all showed dose-dependent activities, with dose-effect maxima at 250 ng ml⁻¹ recombinant CK (Figure 5c-e). Notably the CK ligand resulting in the highest degree of migration above control was CCL5.

When comparing CKR expression and migration after corresponding CK ligand exposure the following could be observed:

- (1) For CCR3, CCR4, CCR6, CCR10, CXCR2, and CXCR4 surface-protein expression was observed on ASC and DSC, both by FACS and western blot analysis. In agreement, CK ligands corresponding to only these receptors (namely, CCL18 for CCR3; CCL17 and CCL22 for CCR4; CCL20 for CCR6; CCL27 for CCR10; CXCL1 for CXCR2, CXCL12 for CXCR4) were able to increase migration of ASC and DSC compared to the medium control;
- (2) For CXCR1 surface-protein expression was observed by both FACS and western blot analysis. However, as its only ligand, CXCL8 was also able to bind to CXCR2, it cannot be concluded from these results whether CXCR1 is functional in inducing cell migration;
- (3) For CCR1, protein expression was detected on ASC and DSC only by western blot analysis, which detects both extracellular and intracellular proteins in contrast to FACS analysis that detects surface marker expression. Since ligands for CCR1 (CCL4, CCL14, and CCL16) were able to induce cell migration while other receptors (CCR2, CCR5, and CCR8) to which they are able to bind to are absent, we can conclude that the CCR1 receptor is functional in inducing cell migration. It is possible that the protein for CCR1 was present at a level below the detection limit of

our FACS analysis, or that the receptor is expressed only intracellular and translocated to the cell surface upon introducing the wound in the scratch assay;

- (4) For CCR7 no protein expression was found. CCL19 and CCL21, which can only bind to CCR7, do however induce cell migration. As mRNA was detected with the aid of a PCR array (SuperArray, Bioscience Corporation (APHS 022A, USA) data not shown), it is possible that the protein for CCR7 was present at a level below the detection limit of our FACS and western blot analyses, or that the receptor was upregulated locally at the site of the wound in the scratch assay;
- (5) CCR3 is expressed on ASC and DSC. However, only CCL5, CCL7, CCL13, CCL15, and CCL18 stimulated migration whereas other ligands (CCL8, CCL11, CCL24, CCL26) did not stimulate cell migration. It is possible that these ligands induce other cellular processes upon receptor binding;
- (6) In the wound-healing scratch assay, CK CCL5 had the most potent effect in mobilizing both ASC and DSC into the area of the scratch.

Figure 5a shows cells elongating, aligning, and migrating in a directional rather than random manner into the area of the scratch. This is suggestive of chemotaxis. However, as the wound-healing scratch assay measures the degree of cell motility without distinguishing between chemotaxis and chemokinesis, we next confirmed that chemotaxis was involved using a transwell filter assay (Figure 6). Indeed, a strong dose-dependent chemotaxis response of ASC and DSC toward CCL5/RANTES occurred. CCL5/RANTES has four receptors (CCR1, 3, 4, 5; Appay and Rowland-Jones, 2001; Juremalm *et al.*, 2002) of which three (CCR1, CCR3, and CCR4) were detected on the protein level. Notably, the present results show that CCR5, which plays a pivotal role in mediating CCL5-induced migration of leukocytes, is not expressed on ASC and DSC.

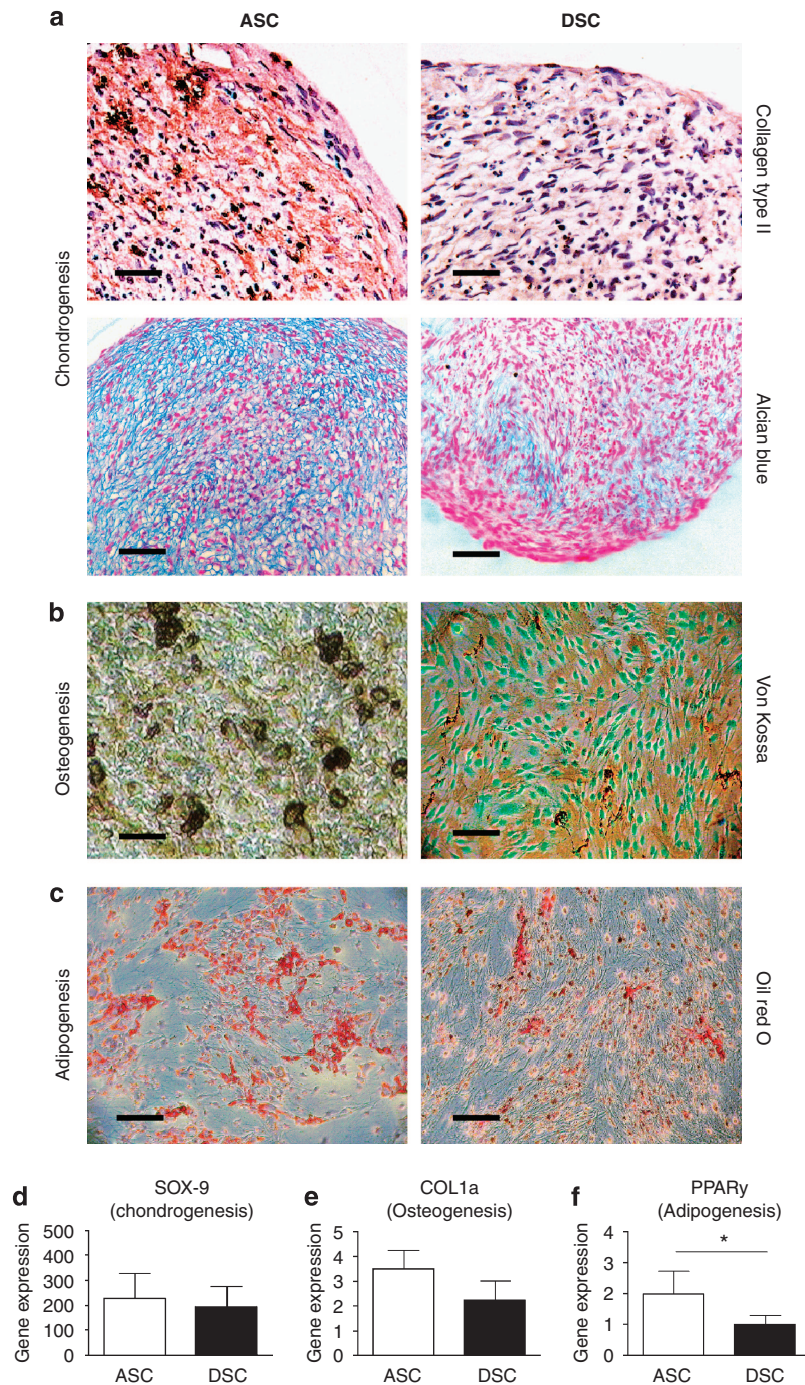


Figure 3. Multilineage differentiation potential of ASC and DSC. Induction of ASC and DSC toward lineage specific pathways was studied by (immuno)histochemistry. **(a)** Chondrogenic differentiation—histology. Representative pellets stained for collagen with collagen type II immunostaining (scale bar = 25 μ m) and for proteoglycans with Alcian blue staining (scale bar = 50 μ m). After 2 weeks of culture. **(b)** Osteogenic differentiation—histology. Representative image of 3 weeks osteogenic cultures stained for calcium phosphate matrix production by Von Kossa staining; scale bar = 100 μ m. **(c)** Adipogenic differentiation—histology. Representative images of 3 weeks adipogenic cultures stained for lipid vacuoles by Oil red O visualize histology of adipogenesis. Data shown are from one individual donor (adipose tissue and dermis matched) and are representative of experiments performed with three donors in duplicate; scale bar = 1 mm. **(d)** Chondrogenic differentiation—relative SOX-9 (marker for early differentiation) expression normalized for means of housekeeping gene expression. **(e)** Osteogenic differentiation—relative COL1 α (marker for late differentiation) expression normalized for means of housekeeping gene expression. **(f)** Adipogenic differentiation—relative PPAR γ expression normalized for means of housekeeping gene expression. Significant differences between ASC and DSC were calculated using a paired Student's *t*-test. **P*<0.05. In the graphs, the mean \pm SEM is presented of three individual experiments performed with three donors (adipose tissue and dermis matched) in duplicate.

To demonstrate that the cellular activity as observed in the wound-healing scratch assay is because of CK-mediated migration and not proliferation, a BrdU assay was performed using similar culture conditions as for the wound-healing scratch assay but with subconfluent cultures. In this experi-

mental setup, none of the CKs tested affected the proliferation rate of ASC and DSC. No difference in proliferation was observed between control- (medium without CK) and CK-stimulated cells whereas epidermal growth factor (EGF) significantly increased the proliferation rate of ASC and DSC (data not shown). To determine whether local enhanced proliferation of cells occurred at the wound margins a Ki67 staining was performed using an identical experimental setup as the wound-healing scratch assay. As CCL5 has the most significant effect on cell mobilization, this CK was used for this experiment as representative of all CKs tested. Figure 5b shows that CCL5/RANTES does not enhance proliferation of cells near and at the wound margins in the wound-healing scratch assay, although addition of EGF does increase proliferation of these cells.

DISCUSSION

Results from this study show that multipotent SCs are present in dermis of human adult skin, which to our knowledge is previously unreported. Moreover, it is demonstrated that SCs residing in the dermis and the adipose tissue of human skin have a similar SC phenotype and both are able to undergo multilineage differentiation. Interestingly, it should be noted that DSC and ASC are the same cell population, which have in the past been called dermal, and adipose-derived fibroblasts by us and others (Van den Bogaerd *et al.*, 2002; Gibbs *et al.*, 2006). This indicates that the previously named fibroblast has clearly defined, and not yet described, SC characteristics. Of note, ASC and DSC described in this study should be distinguished from fibrocytes. Although fibrocytes show some resemblance to ASC and DSC in their morphology and in the expression of CD34, CD54, CD90, and CCR3, they are unique in expressing CD45, linking these cells to the hematopoietic system (Metz, 2003).

Besides showing a similarity in SC characteristics, ASC and DSC were also found to express a similar functional CKR profile. Higher CKR expression is observed on the SVF isolated from adipose tissue and dermis, when compared to cultured ASC and DSC. This is most probably because of the heterogeneity of the cell population (for example, SCs, endothelial cells, and infiltrating cells such as macrophages). This study describes the repertoire of CKR expressed on ASC and DSC at the protein level coupled to an *in vitro* functional migration assay, which to our knowledge is previously unreported. Low, but distinct levels of CCR1, CCR3, CCR4,

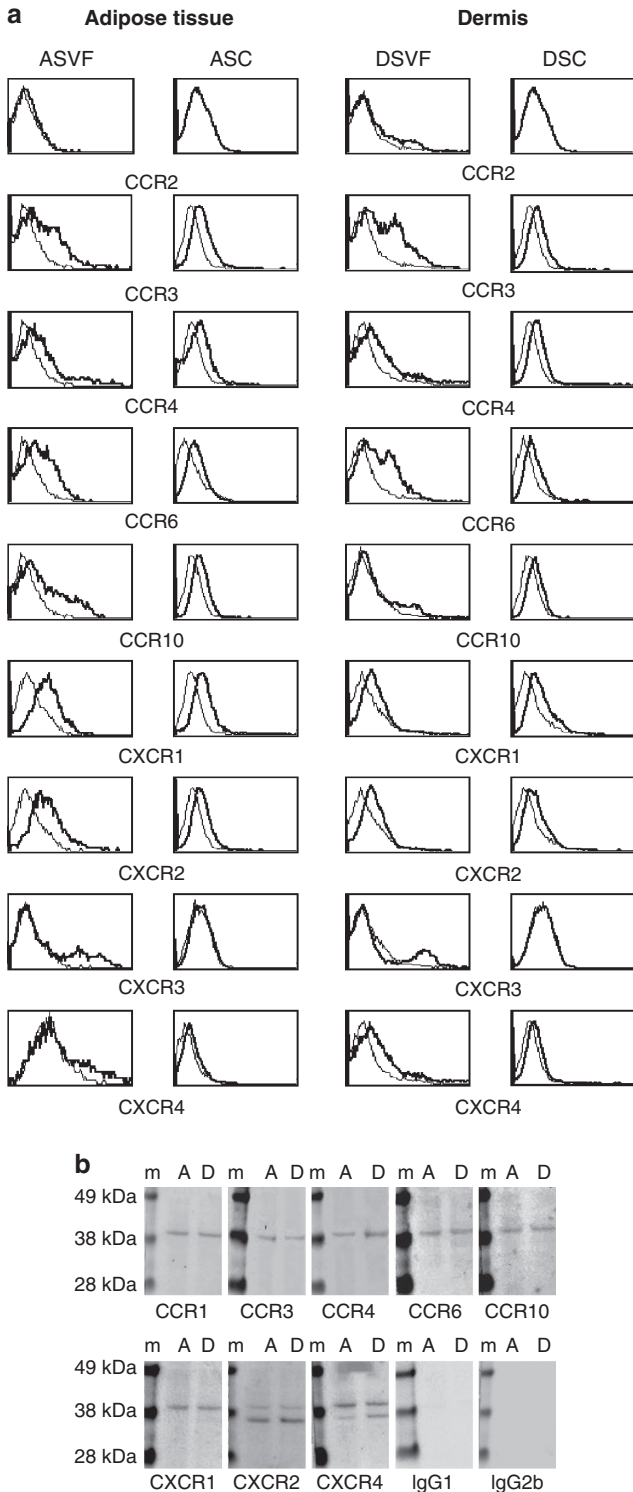


Figure 4. Chemokine receptor expression on ASC and DSC. (a) Characterization of chemokine receptor expression on the stromal vascular fraction and stem cells derived from adipose tissue (ASVF/ASC) or dermis (DSVF/DSC) of human skin using flow cytometry. Cells were stained with mAb against the indicated PE-labeled chemokine receptors (thick line). Each histogram plot contains a PE-labeled isotype-matched mAb control (thin line). Corresponding percentages of positive cells are given in Table 2. (b) Western blot analysis of intracellular and surface-expressed chemokine receptors on cultured adipose stem cells (A) and dermal stem cells (D) at passage 3 identified using a size marker (m). All data shown are from one individual donor (adipose tissue and dermis matched) and are representative of experiments performed with three donors in duplicate.

Table 2. Chemokine receptors in the stromal vascular fraction and cultured stem cells derived from adipose tissue or dermis of human skin

Chemokine receptor	Percentage of positive cells		Percentage of positive cells		Significance	
	ASVF	ASC	DSVF	DSC	DSVF versus ASVF	DSC versus ASC
CCR1	0.66 ± 0.58	0.09 ± 0.05	1.01 ± 0.89	0.06 ± 0.05	NS	NS
CCR2	0.33 ± 0.13	0.33 ± 0.21	6.39 ± 3.15	0.26 ± 0.15	NS	NS
CCR3	11.14 ± 1.09	5.88 ± 0.28	12.04 ± 2.66	4.78 ± 0.50	NS	↓
CCR4	9.98 ± 1.55	4.17 ± 1.25	9.17 ± 3.33	4.69 ± 0.29	NS	NS
CCR5	0.35 ± 0.13	0.00 ± 0.02	0.53 ± 0.81	0.25 ± 0.39	NS	NS
CCR6	7.62 ± 1.99	7.03 ± 0.78	7.48 ± 2.4	5.15 ± 0.89	NS	↓
CCR7	0.09 ± 0.16	0.05 ± 0.08	0.27 ± 0.13	0.01 ± 0.03	NS	NS
CCR8	0.99 ± 0.42	0.08 ± 0.14	1.02 ± 0.57	0.06 ± 0.02	NS	NS
CCR10	14.67 ± 1.09	5.15 ± 0.25	11.57 ± 1.32	4.48 ± 0.05	↓	↓
CXCR1	10.01 ± 2.37	5.18 ± 0.72	7.11 ± 0.64	4.28 ± 0.87	NS	NS
CXCR2	11.28 ± 3.16	5.45 ± 1.02	8.37 ± 0.56	5.49 ± 0.59	NS	NS
CXCR3	14.76 ± 6.96	2.33 ± 0.99	7.35 ± 0.53	1.38 ± 0.45	NS	NS
CXCR4	1.66 ± 0.9	1.21 ± 0.23	2.57 ± 1.2	1.31 ± 0.30	NS	NS

ASC, adipose-derived stem cells; ASVF, adipose-derived stromal vascular fraction; DSC, dermal-derived stromal cells; DSVF, dermal-derived stromal vascular fraction; NS, not significant.

Flow cytometry dot-plot data from three donors (all cell populations were donor matched) were collected and the mean number of positive cells for each chemokine receptor is expressed as a percentage of total cell number ± standard deviation (mean ± SD). Statistical significant difference between DSVF versus ASVF and DSC (p3) versus ASC (p3) was calculated using a paired Student's *t*-test, significant lower expression in dermal cell populations are indicated with an arrow pointing down ($P < 0.05$).

CCR6, CCR10, CXCR1, CXCR2, and CXCR4 were found on cultured ASC and DSC. This is in agreement with the levels of CKR expression described for cultured bone marrow-derived SCs (Wynn *et al.*, 2004; Honczarenko *et al.*, 2006; Ponte *et al.*, 2007; Ringe *et al.*, 2007). CKR expression is required to allow SCs to be directly responsive to CK gradients created by wounded tissue.

In accordance with expressing CKR, CKs efficiently induced migration responses of ASC and DSC. In a functional wound-healing assay, it was shown that exposure of ASC and DSC to CKs stimulated an equal migratory effect in both cell types. We showed that several CXC- and CC-CKs, with CCL5/RANTES being clearly the most potent CK, could induce migration of ASC and DSC in an *in vitro* wound-healing assay. The scratch wound-healing assay was chosen for our studies to complement CKR profile analysis as this enables high throughput analysis in a dose-dependent manner of a large panel of CKs. One limitation of this assay is that it does not distinguish chemotaxis from chemokinesis. On the one hand, the equal distribution of the CK throughout the medium may result in chemokinesis. On the other hand, the introduction of the scratch introduces the possibility that a CK gradient may exist with respect to CK bioavailability being highest on cell surfaces exposed to the scratch and lowest on cell surfaces protected by the confluent layer of neighboring cells. This may result in chemotaxis into the area of the scratch. This hypothesis is supported by the observation that cell elongation occurs in a directional manner into the area of the scratch rather than in random directions. The fact that indeed chemotaxis rather than chemokinesis is respon-

sible for cell mobility was confirmed by studying CCL5/RANTES in the chemotaxis transwell filter assay. In addition, assuming that bioavailability is highest on cell surfaces exposed to the scratch for all CKs tested, the significant effect of CCL5/RANTES compared to other CKs may be explained by the ability of this CK to bind to multiple receptors of which three were shown to be present on ASC and DSC (CCR1, CCR3, and CCR4). Notably, the experimental setup of our scratch assay was selected so that CKs tested had no effect on SC proliferation and therefore our results are entirely because of the effect of CKs on migration and not a combined effect of proliferation and migration. However, mitogenic effects of CXCL1, CXCL8, and CCL5 on keratinocytes and fibroblasts have been reported (Steude *et al.*, 2002; Devalaraja *et al.*, 2000). Most likely, these different results may relate to differences in experimental design in which we investigated the influence of CKs on ASC and DSC under serum-free conditions thus avoiding eventual synergism with serum components. The ability of CCL5/RANTES to stimulate migration of ASC and DSC more than other agonistic CKs can be best explained by the ability of CCL5/RANTES to bind to and activate several CKR present on ASC and DSC (CCR1, CCR3, CCR4). In addition, CCL5/RANTES also activates a CKR-independent pathway. Induction of this pathway appears to be mediated by protein tyrosine kinases and induces hyperphosphorylation and generalized cell activation, triggering a variety of biologic effects such as proliferation, cytokine release, and apoptosis (Appay and Rowland-Jones, 2001). The role of this pathway in migration is still unknown.

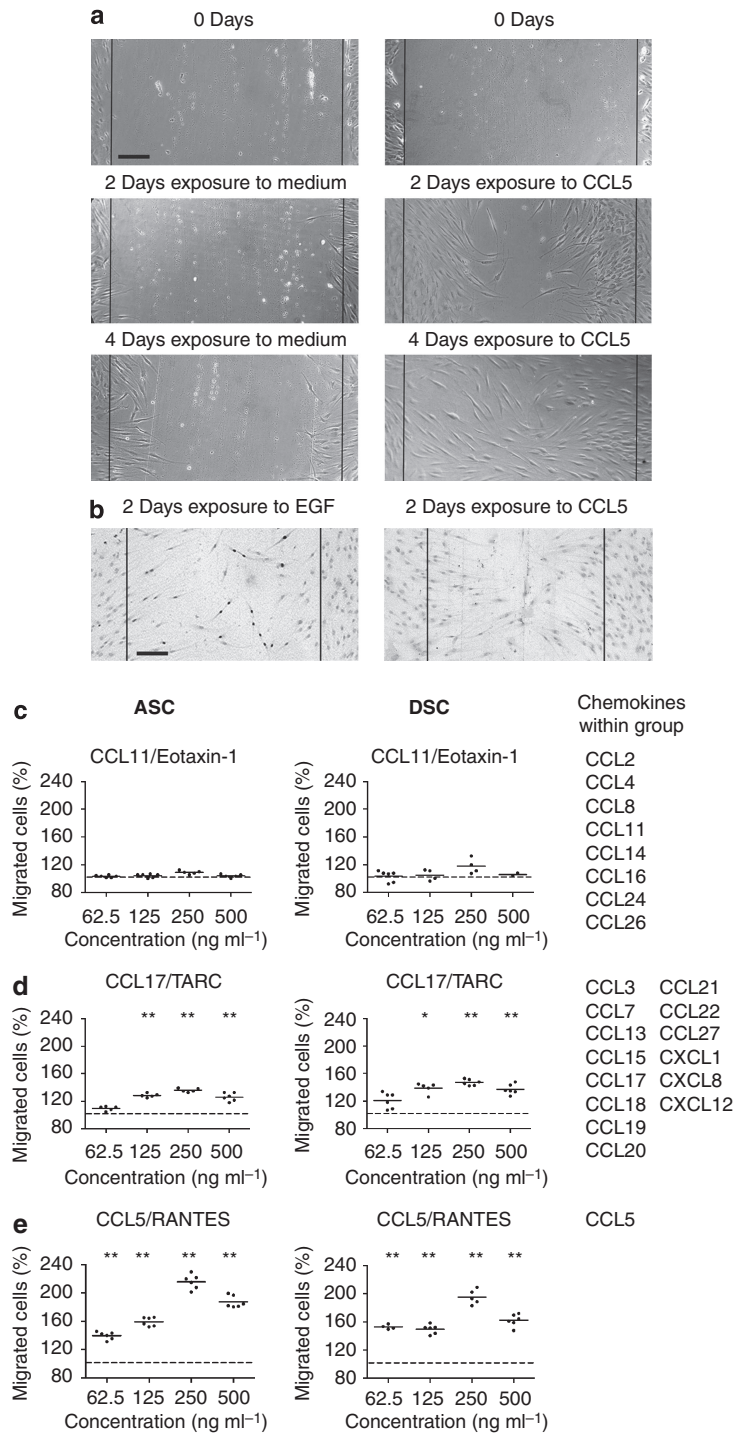


Figure 5. Wound-healing scratch assay shows CCL5/RANTES to be most potent stimulator of SC migration. (a) A representative example of the wound-healing scratch assay. Cells migrating to cover the cell-free area when cultured in medium alone and in medium supplemented with CCL5/RANTES are shown at days 2 and 4; scale bar = 100 μ m. (b) Ki67 staining of cells in the neighborhood of the wound after supplementation of culture medium with EGF or CCL5/RANTES. Ki67 stains proliferating cells (black nuclei); scale bar = 100 μ m. (c-e) Three groups of chemokines could be distinguished based upon their differential effect on cell migration after 2 and 4 days exposure (see also Table 3). A representative dose-response graph for one CK from each group for both ASC and DSC at day 4 is shown (left); a list of all chemokines within the same category is shown (right). Quantifying migration at 2 days gave similar results as the quantification of migration at 4 days. Group (c): No effect on migration. (Table 3; indicated with -). Group (d): Migration in presence of chemokine $>$ control, $\Delta \leq$ maxima 1.5-fold (Table 3; indicated with +). Group (e): Migration in presence of chemokine $>>$ control, $\Delta \geq$ maxima twofold (Table 3; indicated with ++). Statistical significant differences between cultured cells (p3) exposed to chemokine and cells cultured in absence of CK (dotted line at 100%) were calculated using a paired Student's *t*-test. * $P < 0.05$, ** $P < 0.001$ relative to control. All chemokines were tested with the same set of donors at the same passage. In the graph, the dots represent the values of three individual experiments performed with three donors (adipose tissue and dermis matched) in duplicate.

Table 3. Chemokine receptor expression on ASC and DSC and the effect of corresponding chemokine ligands on migration

Chemokine receptor	Protein expression		Chemokine ligand	Migratory effect of				
	FACS	Western blot		ASC	DSC			
CCR1	No	Yes	CCL3	+	+			
			CCL4	-	-			
			CCL5	++	++			
			CCL7	+	+			
			CCL8	-	-			
			CCL13	+	+			
			CCL14	-	-			
			CCL15	+	+			
			CCL16	-	-			
			CCR3	Yes	Yes	CCL5	++	++
						CCL7	+	+
						CCL8	-	-
CCL11	-	-						
CCL13	+	+						
CCL15	+	+						
CCL18	+	+						
CCL24	-	-						
CCR4	Yes	Yes	CCL3	+	+			
			CCL5	++	++			
			CCL17	+	+			
			CCL22	+	+			
CCR6	Yes	Yes	CCL20	+	+			
CCR7	No	No	CCL19	+	+			
			CCL21	+	+			
CCR10	Yes	Yes	CCL27	+	+			
CXCR1	Yes	Yes	CXCL8	+	+			
			CXCL8	+	+			
CXCR3	No	No	CXCL9	ND	ND			
			CXCL10	ND	ND			
			CXCL11	ND	ND			
CXCR4	No	Yes	CXCL12	+	+			

ASC, adipose-derived stem cells; control, culture medium without chemokine ligand; DSC, dermal-derived stromal cells; ND, not done. Chemokine receptor expression was determined by flow cytometry for protein expression (Figure 4). Absence of a chemokine receptor at protein level is indicated with No, presence of the receptor with Yes. For each chemokine receptor its corresponding agonistic chemokine ligands are shown in combination with the effect they had on the migration of ASC and DSC in a scratch assay. No migration is indicated with a - (migration in presence of chemokine=control, Δ=0); a moderate effect is indicated with a + (migration in presence of chemokine > control, Δ≤1.5-fold), a large effect is indicated with a ++ (migration in presence of chemokine >> control, Δ≥twofold).

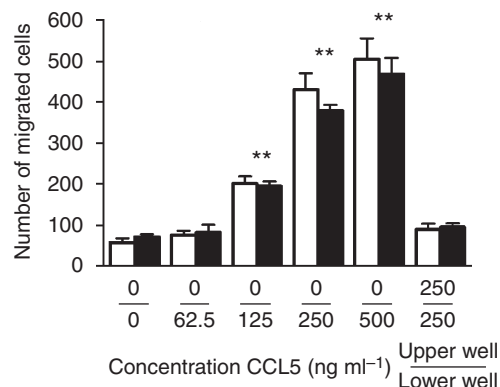


Figure 6. Transwell assay shows CCL5/RANTES to be chemoattractant for ASC and DSC. Cultured ASC (white bars) and DSC (black bars) at passage 3 were incorporated into a chemotaxis transwell assay in the presence of CCL5/RANTES supplemented in the lower well. Placing an equal concentration of CCL5/RANTES (250 ng ml⁻¹) in upper and lower well blocks the migratory response indicating chemotaxis rather than chemokinesis. Data are presented as the mean (± SEM) number of cells migrating to the underside of the transwell filter. **P<0.001 relative to control. The experiments are performed with three donors (adipose and dermis matched) in duplicate.

Beside CCL5 being the most potent stimulator of migration, other CKs also had a clear dose-dependent significant effect on migration. These results indicate that ASC and DSC can home, in a similar manner to leukocytes, to the wound bed via a CK gradient. This has been previously suggested for bone marrow SCs, in which CKs and their receptors are reported to be involved in cell migration into, for example, the brain and the epidermis (Ji *et al.*, 2004; Inokuma *et al.*, 2006). Gillitzer and Goebeler showed that skin injury is associated with a differential and sequential expression of CKs (Groα/CXCL1, IL8/CXCL8, SDFα/CXCL12, MIP1α/CCL3, and RANTES/CCL5) in the wound bed (Gillitzer and Goebeler, 2001). Their findings correlate with our results in which we show that these five CKs increase migration of ASC and DSC. Expression of CCL5 during early wound repair has been shown in murine wound repair (Frank *et al.*, 2000). Furthermore, increased CCL5 production by human endothelial cells occurs after exposure to the proinflammatory cytokines IFN-γ and tumor necrosis factor-α, which are secreted upon skin damage (Marfaing-Koka *et al.*, 1995). In line with our results on ASC and DSC, CCL5 has been reported to stimulate directional migration of bone marrow-derived SCs (Honczarenko *et al.*, 2006; Ponte *et al.*, 2007).

Notably CCL19 and CCL21 (ligands for CCR7) did induce ASC and DSC migration, whereas CCR7 could only be detected on mRNA level (data not shown). For this receptor, it might be postulated that it is upregulated upon introducing the wound in the scratch assay. This would be in agreement with CKR expression on bone marrow-derived SCs to be upregulated by cytokines such as tumor necrosis factor-α and IFN-γ (Croitoru-Lamoury *et al.*, 2007). This upregulation suggests that proinflammatory signals, released upon tissue damage, increase CKR expression on SCs, thereby further stimulating migration of SCs toward CKs.

Interestingly, some CKs (CCL8, 11, 24, and 26) did not induce cell migration, whereas their corresponding receptors

were found to be expressed on protein level. This suggests a function other than migration for these CK ligand/receptors pairs, in line with findings elsewhere. CCL11, for example, has been reported to increase MMP2 activity of lung fibroblasts (Puxeddu *et al.*, 2006). Most MMPs are known to be secreted *in vivo* upon skin damage and are therefore most probably secreted in our *in vitro* scratch wound assay. For gingival fibroblasts, it has been demonstrated that MMP1 and MMP3 expression is stimulated by cytokines such as tumor necrosis factor- α and IFN- γ , suggesting that, upon tissue damage, fibroblasts increase MMP1 and MMP3 production (Domeij *et al.*, 2002). In turn, McQuibban *et al.* reported that CKs such as CCL8 are susceptible to cleavage by MMP1 and MMP3. These cleaved CKs may then act as antagonist (McQuibban *et al.*, 2002). Cleavage of CCL8, by MMP1 and MMP3 could be a possible explanation for the nonmigratory effect of CCL8 in our *in vitro* wound-healing assay. CKs not inducing ASC and DSC migration could also be inhibitors of cell migration, as has been reported for CXCL10, which is an inhibitor of keratinocyte migration (Shiraha *et al.*, 1999). Thus CCL8, CCL11, CCL24, and CCL26 may mediate multiple effects other than affecting cell motility.

In contrast to the similarities observed in the SC phenotype, CKR profile and CK-mediated migration; ASC and DSC did differ in their morphology and their degree of multilineage differentiation. To be noted is that both cell types were donor matched and were isolated and cultured using identical protocols. The difference in morphology between ASC and DSC has previously been reported by Van den Bogaerd *et al.* who showed that adipose-derived mesenchymal cells (ASCs) had a higher α -smooth muscle expression and were more contractile than DSC (Van den Bogaerd *et al.*, 2002). Here, we show that even though DSC do have trilineage differentiation potential, it is to a slightly lesser degree than that observed for ASC. One explanation for this may be that here we show that DSC contain a smaller population of CD34⁺ cells than ASC and it has been suggested by others that CD34⁺ cells from SVF of adipose tissue are the precursors of the multipotent cells in culture (Miranville *et al.*, 2004; Boquest *et al.*, 2005). Therefore, even though ASC and DSC do exhibit many similar SC and migratory characteristics they are clearly two different cell populations.

The homing of autologous SCs into difficult to heal wounds improves wound healing (Falanga *et al.*, 2007; Prichard *et al.*, 2007). Our results indicate that beside the adipose tissue of adult human skin, the dermis is also a source of SCs, which to our knowledge is previously unreported. The presence of SCs in both the dermis and adipose tissue of human adult skin underlines the intrinsic repair capacity of human skin, which if correctly guided may lead to optimal tissue repair and regeneration. SCs residing in the adipose tissue and dermis of human skin are the most likely candidates to be directly involved in healing of deep skin wounds as adipose tissue is generally exposed in the wound bed and dermal tissue is exposed at the wound bed margins. Establishing optimal CK gradients within the wound bed, for instance with the aid of recombinant CKs, may form a basis for SC therapies in the future for healing a broad range of

difficult to heal wounds ranging from deep burns, where the quality of the scar is of major importance to chronic ulcers where obtaining wound closure is of major importance.

MATERIALS AND METHODS

Isolation and culturing of mesenchymal cells from human skin

Human adult skin was obtained from healthy donors (with written informed consent) undergoing abdominal dermolipectomy and was used directly after surgery. The VU University medical center approved the experiments described in this paper. The study was conducted according to Declaration of Helsinki Principles.

Adipose stromal vascular fraction (ASVF)/ASC: Adipose tissue was carefully dissected out from skin and large vessels. The fat was washed with phosphate-buffered saline (PBS; B. Braun, Melsungen, Germany), cut into small pieces, and incubated in collagenase type II (Gibco, Invitrogen, Paisley, UK)/dispase II (Roche Diagnostics, Mannheim, Germany) in Hanks-buffered salt solution (Gibco, Invitrogen) at 37 °C for 2 hours. Enzymatic activity was then neutralized by adding PBS supplemented with 0.1% BSA (Sigma-Aldrich, Steinheim, Germany) to the tissue-enzyme solution. The adipose-derived cells were separated from tissue debris in a filter chamber (NPI, Emmer-Compascuum, the Netherlands) after which the cell suspension was centrifuged (400 g, 6 minutes). The cell pellet was then resuspended in DMEM (ICN Biomedicals, Verviers, Belgium), containing 1% ultrosorG (Pall, Cergy-Saint-Christopher, France) and 1% penicillin-streptomycin (P/S; Gibco, Invitrogen) and passed through a 40- μ m-cell strainer (Becton Dickinson Falcon, Eremodegem, Belgium). Next, nucleated cells were separated by a Ficoll-paque PLUS (GE Healthcare Biosciences, Uppsala, Sweden) density filtration step and viability of the cells was assessed using Trypan blue. A total of 5×10^6 of the freshly isolated ASVF cells were resuspended in 1 ml DMEM, 1% P/S, 1% ultrosorG, and 10% DMSO (Sigma-Aldrich) and stored in the vapor phase of liquid nitrogen. Single cell suspensions of ASVF were seeded at 1×10^5 cells/cm² in DMEM containing 1% P/S and 1% ultrosorG and maintained in a 5% CO₂ incubator at 37 °C in a humidified atmosphere. When these cells are cultured they are called ASC.

Dermal stromal vascular fraction (DSVF)/DSC: Care was taken that all adipose tissue was removed before separating epidermis from dermis. Epidermal sheets and dermis were separated from human adult skin by incubation in dispase II overnight at 4 °C. The dermis was treated as described above for adipose tissue. A total of 5×10^6 of the freshly isolated DSVF cells were resuspended in 1 ml DMEM, 1% P/S, 1% ultrosorG, and 10% DMSO and stored in the vapor phase of liquid nitrogen. Single cell suspensions of DSVF were seeded at 1×10^5 cells/cm² in DMEM containing 1% P/S and 1% ultrosorG and maintained in a 5% CO₂ incubator at 37 °C in a humidified atmosphere. Upon culturing these cells are called DSC. Cells derived from the dermis and adipose tissue of the skin were cultured identically and medium was changed twice a week. Cultures were passaged when 90% confluent using 0.5 mM EDTA/0.05% trypsin (Gibco, Invitrogen).

Flow cytometric analysis

Freshly isolated and cultured (passage 3) cells from the adipose tissue and dermis of human skin were examined for cell-surface marker expression using flow cytometry. Cells were incubated with antibodies for 30 minutes, washed in PBS supplemented with 0.1%

BSA, and 0.1% sodium azide and resuspended in the same buffer for FACS analysis. Cells were measured on a FACScan and analyzed with Cell Quest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). PE-labeled antibodies and corresponding isotypes used are from BD Pharming, San Diego, CA, generated in mouse and reactive with human unless stated otherwise. CD31 (WM59, IgG1); CD34 (581, IgG1); CD54 (HA58, IgG1); CD90 (5E10, IgG1); CD105 (SN6, IgG1, Caltag); CD166 (3A6, IgG1); HLA-DR (L243, IgG2a); HLA-ABC (G46-2.6, IgG1); CCR1 (53504, IgG2b); CCR2 (48607, IgG2b); CCR3 (5E8, IgG2b); CCR4 (1G1, IgG1); CCR5 (2D7, IgG2a); CCR6 (11A9, IgG1); rat anti-human CCR7 (3D12, IgG2a); rat anti-human CCR8 (191704, IgG2b, R&D systems, Oxon, UK); rat anti-human CCR10 (314305, IgG2a, R&D systems); CXCR1 (5A12, IgG1); CXCR2 (CDw128b, IgG2b); CXCR3 (1C6/cxcr3, IgG1); CXCR4 (12G5, IgG2a, R&D systems).

Protein preparation and western blot analysis

ASC and DSC at passage 3 were rinsed with PBS and lysed in ice-cold NET (20 mM Tris, 100 mM NaCl, 1 mM EDTA) with 0.5% Triton X-100 and one tablet per 50 ml lysis buffer of Complete Protease and phosphatase inhibitors (Roche Diagnostics). Lysates were centrifuged and the protein content in the supernatant was quantified using protein assay as described by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Thereafter, 1 of 4 final volume of four times NuPAGE sample buffer and 1 of 10 volume reducing agent (stabilized DTT) were added to protein samples. Next, protein samples were heated for 5 minutes at 95 °C before loading (5–10 µg) and size separation on precast 1 mm thick Bis-Tris (10%) NuPAGE gels with MES as running buffer according to manufacturer's instructions (Invitrogen). After size separation, the proteins were transferred to nitrocellulose membranes with the iBlot Dry-blotting System (Invitrogen) according to program number two of iBlot device 97 minutes of transfer).

For Western blot analysis, the blots were incubated in blocking buffer (DPBS (Gibco, Invitrogen), containing 1% BSA (Roche Diagnostics) and 0.1% Tween (Merck, Whitehouse Station, NJ) for at least 30 minutes. Blots were then incubated with primary antibodies at room temperature for 1 hour followed by o/n incubation at 4 °C. All primary antibodies and isotype (negative) controls used (1:250 dilution) are listed in the FACS analysis section. Only for CCR10, another primary antibody was used (1:250 dilution; goat anti-human CKR10, N20, IgG2a, Santa Cruz Biotechnology, California). Membranes were washed three times for 5 minutes in blocking buffer. Blots were incubated with rabbit anti-mouse AP-conjugated antibody (Dako, Glostrup, Denmark; 1:2,000 dilution) or rabbit anti-goat AP conjugated antibody (Santa Cruz Biotechnology; 1:2,000 dilution) for 1 hour at room temperature. Membranes were washed in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.5), followed by AP buffer (0.2 M Tris, 0.1 M MgCl₂, 0.2 M NaCl₂, 1 mM levamisole, pH 9.7) for 5 minutes. Specific protein detection was achieved using chromogen NBT/BCIP substrate of AP (1:100 dilution; Roche Diagnostics) as described by manufacturer's instructions. Blots were imaged using HP scanjet 5590.

Differentiation potential ASC and DSC

Adipogenic, chondrogenic, and osteogenic differentiation capacities of cultured (passage 3) ASC and DSC ($n = 3$) were studied within the same donor.

Adipogenesis was induced by culturing ASC and DSC for 3 weeks in monolayer at a concentration of 2.0×10^5 cells/well in adipogenic medium (DMEM, supplemented with 2% ultrosorG, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 2 mM L-glutamine (Gibco, Invitrogen), 10 µM insulin (Sigma-Aldrich, Zwijndrecht, the Netherlands), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich), and 200 µM indomethacin (Merck) for 3 weeks.

Chondrogenic differentiation was induced for 2 weeks using a micromass culture method, as previously described (Oedayrajsingh-Varma *et al.*, 2006). Chondrogenic medium (DMEM, containing ITS + premix (final concentration in medium when diluted 1:100 insulin, 6.25 µg ml⁻¹, transferrin, 6.25 µg ml⁻¹, selenous acid, 6.25 ng ml⁻¹, BSA, 1.25 mg ml⁻¹, linoleic acid, 5.35 µg ml⁻¹; Becton Dickinson), 10 ng ml⁻¹ transforming growth factor-β1 (Biovision, ITK-diagnostics, Mountain View, CA), 0.2% ultrosorG, 25 µM ascorbate-2-phosphate (Sigma, St Louis, MO, USA), 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 2 mM L-glutamine was overlaid gently. Cells were maintained in a 5% CO₂/1% oxygen custom-designed hypoxia workstation (T.C.P.S. Rotselaar, Belgium) at 37 °C in a humidified atmosphere.

For osteogenic differentiation, ASC and DSC were seeded at 5,000 cells/cm² and cultured for 3 weeks in monolayers in osteogenic medium, consisting of normal culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerol phosphate, 50 µg ml⁻¹ ascorbate-2-phosphate, and 100 ng ml⁻¹ bone morphogenetic protein 2 (Peprotech EC Ltd, London, UK).

(Immuno)histochemistry

(Immuno)histochemistry was performed as described previously (Oedayrajsingh-Varma *et al.*, 2007). Cell nodules formed under chondrogenic culture conditions were fixed in 4% paraformaldehyde and paraffin sections were stained. Sections were stained with Alcian blue (Sigma-Aldrich) at acidic pH for detection of proteoglycans. For the detection of collagen type II, sections were incubated with mouse monoclonal antibody II-II6B3 (1:50; Developmental Studies Hybridoma Bank, IA) against human collagen type II in PBS containing 1% BSA.

Cells that were induced into the osteogenic lineage for 21 days were examined by Von Kossa staining to demonstrate the formation of a calcified matrix that is typical for mature osteoblasts. The protocol used was described previously (Oedayrajsingh-Varma *et al.*, 2007). Calcified extracellular matrix was visualized as black spots.

Adipogenesis was confirmed by staining the cells with Oil red O after 21 days to visualize lipid vacuoles formed after adipogenic induction as described previously (Zuk *et al.*, 2002).

Real-time PCR

RNA isolation and reverse transcription were performed as previously described (Oedayrajsingh-Varma *et al.*, 2007). Real-time PCR reactions were performed using the SYBRGreen reaction kit according to the manufacturer's instructions (Roche Diagnostics) in a LightCycler 480 (Roche Diagnostics). Relative housekeeping gene expressions (18S, HPRT, and YWHAZ), and relative target gene expressions (SOX-9 for early chondrogenic differentiation at 4 days; COL1a for late osteogenic differentiation at 2 weeks; PPARγ for

adipogenic differentiation at 2 weeks) were determined. Primers (Invitrogen) used for real-time PCR were 18S forward: 5' GTAACCC GTTGAACCCATT-3', reverse: 5' CCATCCAATCGGTAGTAGCG-3'; HPRT, YWHAZ; COL1 α 1, forward: 5'-AAGCCGAATTCCTGGTC T-3', reverse: 5'-TCCAACGAGATCGAGATCC-3'; PPAR γ ; SOX-9 and were designed using Clone Manager Suite software program version 6 (Scientific & Educational Software, Cary, NC, USA). For real-time PCR, the values of relative target gene expression were normalized for means of relative 18S, HPRT, and YWHAZ house-keeping gene expression.

Wound-healing scratch assay for quantifying cell migration

Wound-healing scratch assay was performed essentially as described by Entschladen *et al.* (2005). Confluent cell monolayers in a 48-well plate were incubated with serum-free medium (DMEM, 1% P/S, 0.1% BSA (Sigma-Aldrich) for 4 days. After 4 days, a scratch was drawn with a plastic disposable pipette tip. Cultures were washed with PBS to remove detached cells and then exposed to different concentrations of CKs (62.5, 125, 250, and 500 ng CK per ml DMEM, 1% P/S, 0.1% BSA; R&D Systems). Media was changed after 2 days of exposure. The wound area was photographed (when the monolayer was damaged ($t=0$) and at 2 days ($t=2$) and 4 days ($t=4$) after scratching), using phase contrast microscopy coupled to a digital camera (Coolpix 5400, Nikon Corporation, Japan). In the photograph of $t=0$, the wound is marked by black lines using Microsoft Digital Image Pro 7.0. This defined wound area of $t=0$ is copied to photographs taken at $t=2$ and $t=4$ days, for each individual condition. Cells that have migrated into the defined wound area at $t=2$ and $t=4$ days are counted. The number of cells, which migrated into the wound area of the control (without CK) after 2 and 4 days, is set at 100%. Cell migration in CK-supplemented medium was compared to control medium and given as a percentage relative to control (100%). All experiments were performed in duplicate using three different donors. Each donor (cultured to p3) was used to test the complete panel of CKs in ASC and DSC.

Assessment of cell proliferation

BrdU. Before the BrdU assay, cells were cultured for 4 days in Serum free medium. Subconfluent cell monolayers (75%), in a 96-well plate, were wounded as described above for the wound-healing scratch assay. Cultures were exposed to different concentrations of CKs (0, 7.5, 15, 30, 62.5, 125, 250, and 500 ng CK per ml medium) for 2 and 4 days. Incorporation of BrdU (10 μ l 100 μ M BrdU per well) during the final 16 hours of incubation was assessed in triplicate following the standard protocol described by the manufacturer's (Amersham, Biosciences, UK). All experiments were performed in at least three different donors in duplicate. EGF (10 ng ml⁻¹) was used as positive control.

Ki67 immunostaining. ASC and DSC were seeded onto sterile glass chamber slides (Nalge Nunc International, Rochester, NY, USA). Cells were grown to confluence and incubated with serum-free medium (DMEM, 1% P/S, 0.1% BSA; Sigma-Aldrich) for 4 days. After 4 days a scratch was introduced and cells were exposed to CCL5/RANTES (250 ng ml⁻¹) as previously described for the wound-healing scratch assay. EGF (10 ng ml⁻¹) was used as positive control. After 2 and 4 days, slides were fixed with ice-cold ethanol for

10 minutes, incubated in PBS-containing goat serum (Dako; 1:50) for 15 minutes, before incubation with Ki67 (clone MIB-1, Dako; 1:50) for 60 minutes. Thereafter, slides were incubated with goat anti-mouse biotin-conjugated secondary antibody (Dako; 1:400) for 30 minutes. Specific protein detection was achieved by incubation in streptavidine-HRP (Dako; 1:500) for 30 minutes and lightly counter-staining with hematoxylin.

Chemotaxis assay

Chemotactic responses of ASC and DSC to CCL5/Rantes were assessed by the Boyden well chamber technique using a 24-transwell system and 8 μ m poresize (Costar, Corning, NY). Before chemotaxis cells were incubated for 4 days in DMEM, 1% P/S, 0.1% BSA. Cell suspension (200 μ l; 2.5×10^5 /ml in DMEM, 1% P/S, 0.1% BSA) was loaded into the upper well of the chamber and allowed to attach overnight. After overnight attachment of the cells CCL5/RANTES (0–500 ng ml⁻¹) diluted in DMEM was placed in the lower chamber. Additionally to discriminate between chemotaxis and chemokinesis, 250 ng ml⁻¹ CCL5/RANTES was placed in both upper and lower well. Chambers were incubated for 24 hours, after which the cells that did not migrate were wiped from the upper surface of the transwell with a cotton swab. Transwells were then washed in PBS, fixed using 4% formaldehyde (Klinipath, Duiven, the Netherlands), washed again in PBS, and stained with 10 μ M propidium iodide (Invitrogen). The number of cells on the lower surface of the filter was determined by counting the number of nuclei in $\times 40$ magnification under a computer-assisted fluorescence microscope (Nikon, Düsseldorf, Germany). Three independent experiments were performed where duplicate wells were prepared in each experiment for each condition.

Experimental setup

All experiments were carried out using freshly isolated ASVF and DSVF, or ASC and DSC at passage 3, cultured for the same number of days and from the same donor. Three different donors were used and experiments with each donor were performed in duplicate.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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