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Human Dermis Harbors Distinct Mesenchymal Stromal Cell Subsets

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Multipotent mesenchymal stromal cells (MSCs) are found in a variety of adult tissues including human dermis. These MSCs are morphologically similar to bone marrow–derived MSCs, but are of unclear phenotype. To shed light on the characteristics of human dermal MSCs, this study was designed to identify and isolate dermal MSCs by a specific marker expression profile, and subsequently rate their mesenchymal differentiation potential. Immunohistochemical staining showed that MSC markers CD73/CD90/CD105, as well as CD271 and SSEA-4, are expressed on dermal cells *in situ*. Flow cytometric analysis revealed a phenotype similar to bone marrow–derived MSCs. Human dermal cells isolated by plastic adherence had a lower differentiation capacity as compared with bone marrow–derived MSCs. To distinguish dermal MSCs from differentiated fibroblasts, we immunoselected $CD271^+$ and $SSEA\text{-}4^+$ cells from adherent dermal cells and investigated their mesenchymal differentiation capacity. This revealed that cells with increased adipogenic, osteogenic, and chondrogenic potential were enriched in the dermal $CD271^+$ population. The differentiation potential of dermal $SSEA\text{-}4^+$ cells, in contrast, appeared to be limited to adipogenesis. These results indicate that specific cell populations with variable mesenchymal differentiation potential can be isolated from human dermis. Moreover, we identified three different subsets of dermal mesenchymal progenitor cells.

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INTRODUCTION

The major obstacle for the identification of multipotent mesenchymal stromal cells (MSC) is their morphological and phenotypical similarity to fibroblasts. Even 40 years after their first description, no definite cell surface marker is available that reliably identifies MSCs and enables their isolation (Friedenstein *et al.*, 1966). Rather, a variety of markers are used for the identification of MSCs. Recently, the multitude of cell surface markers used in various studies has been limited to a marker panel representing, in addition to plastic adherence and differentiation capacity, the minimal criteria for the identification of MSCs (Dominici *et al.*, 2006). These markers, however, do not discriminate between MSCs and fibroblasts (Haniffa *et al.*, 2009) and partially overlap with other cell lineages. Nevertheless, the prevailing method for the isolation of MSCs irrespective of the original tissue is plastic adherence, which in turn leads to the selection of a very heterogeneous starting population (Ho *et al.*, 2008). Therefore, a number of approaches have been undertaken to identify cell surface markers that hold promise for a specific isolation of MSCs from various tissues that include CD49a (Deschaseaux *et al.*, 2003), CD105 (Conconi *et al.*, 2006), CD271 (Quirici *et al.*, 2002; Bühring *et al.*, 2007), GD2 (Martinez *et al.*, 2007), SSEA-4 (Gang *et al.*, 2007), and STRO-1 (Simmons and Torok-Storb, 1991). Apart from bone marrow, MSCs have been isolated from a variety of other tissues, such as umbilical cord blood (Erices *et al.*, 2000; Mareschi *et al.*, 2001), adipose tissue (Miyazaki *et al.*, 2005; Gimble *et al.*, 2007), placenta (Fukuchi *et al.*, 2004; Miao *et al.*, 2006; Battula *et al.*, 2007), synovial fluid (Jones *et al.*, 2004), fibrous and adipose synovium (Mochizuki *et al.*, 2006), pancreas (Seeberger *et al.*, 2006; Gallo *et al.*, 2007), and dermis (Yates *et al.*, 2001; Young *et al.*, 2001; Bartsch *et al.*, 2005; Chen *et al.*, 2007; Lysy *et al.*, 2007).

The main focus of cutaneous stem cell research is on epidermal stem cells and stem cells associated with the hair follicle. However, various studies proved the presence of mesenchymal progenitors also in the dermis. Skin-derived precursors with the capacity of neural and mesodermal

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Abbreviations: AP, alkaline phosphatase; MSC, multipotent mesenchymal stromal cell

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differentiation were obtained from nonadherent dermal cells (Yates *et al.*, 2001; Young *et al.*, 2001; Bartsch *et al.*, 2005; Toma *et al.*, 2005; Chen *et al.*, 2007; Lysy *et al.*, 2007). Other studies, in contrast, identified mesenchymal progenitor cells within the fraction of plastic-adherent dermal cells (Bartsch *et al.*, 2005; Chen *et al.*, 2007; Lysy *et al.*, 2007; Lorenz *et al.*, 2008). Among other cell types, adherent dermal cells include fibroblasts, which are the prevailing cell type in the dermis and are of a yet not fully understood physiological heterogeneity (Sorrell *et al.*, 2007). In addition, adherent dermal cells contain subsets that are capable of differentiating into osteoblasts, adipocytes, chondrocytes (Lorenz *et al.*, 2008), and myocytes (Bartsch *et al.*, 2005). Even ectodermal and endodermal potential has been assigned to these cells (Chen *et al.*, 2007; Lysy *et al.*, 2007). When using cell surface markers commonly used for the characterization of MSCs, the phenotype of dermal fibroblasts was largely similar to that of bone marrow–derived MSCs (Ishii *et al.*, 2005; Wagner *et al.*, 2005; Chen *et al.*, 2007). There are, however, approaches to identify phenotypically distinct progenitor cells from overall fibroblasts. Chen *et al.* (2007) identified a nestin^{-}vimentin^{+} multipotent dermal fibroblast and thereby provide evidence for the possible isolation of progenitor subsets from dermal adherent cells.

The goal of our study was to characterize MSCs in human dermis and isolate progenitor cell subsets based on a specific phenotype for further investigation.

RESULTS

MSC markers are expressed on various dermal cell types

The presence of mesenchymal stem/progenitor cells in human dermis has been reported previously (Toma *et al.*, 2001; Yates *et al.*, 2001; Young *et al.*, 2001; Bartsch *et al.*, 2005; Chen *et al.*, 2007; Lysy *et al.*, 2007). As their phenotype is still poorly characterized, we analyzed the expression of MSC markers in human dermis. *In situ* analysis of skin cryosections by immunohistochemical staining revealed that MSC markers (CD73, CD90, CD105, CD271, SSEA-4) are expressed on different dermal cell types (Figure 1a, upper panel). CD73, CD90, and CD105 show similar single-cell, vascular and perivascular expression patterns (Figure 1a, inset, lower panel). It is noteworthy that CD90 is also faintly expressed on some basal keratinocytes, thus confirming previous results (Nakamura *et al.*, 2006). In contrast, CD271 is present on cutaneous nerve fibers, Schwann cells, dermal single cells, and, faintly, on clusters of basal keratinocytes as reported (Figure 1a, inset, lower panel; Liang *et al.*, 1999; Nakamura *et al.*, 2007). The dermal expression pattern of SSEA-4 is almost analogous to CD271. As SSEA-4 expression has been demonstrated on neural cells (Dodd and Jessell, 1986; Holford *et al.*, 1994; Barraud *et al.*, 2007), we conclude that SSEA-4 is also expressed on neural structures in human dermis and, to a lesser extent, on dermal single cells. These observations confirm the shared expression of markers used for the identification of MSCs by a variety of cell types (Kolf *et al.*, 2007). For a more detailed analysis of dermal cell populations expressing MSC markers, we double- and triple-stained dermal single-cell suspensions and analyzed them by flow cytometry. The marker combinations were selected to define cell types typically found in human dermis, such as endothelial cells (CD31^{+}, CD34^{+}) and leukocytes (CD45^{+}). As a marker for ''contaminating'' keratinocytes, we used CD318 (Brown *et al.*, 2004). Apart from CD73^{+}, CD90^{+}, and CD105^{+} cells co-expressing CD31 and CD34 or CD45, we identified CD73^{+}, CD90^{+}, and CD105^{+} cells lacking endothelial and leukocyte

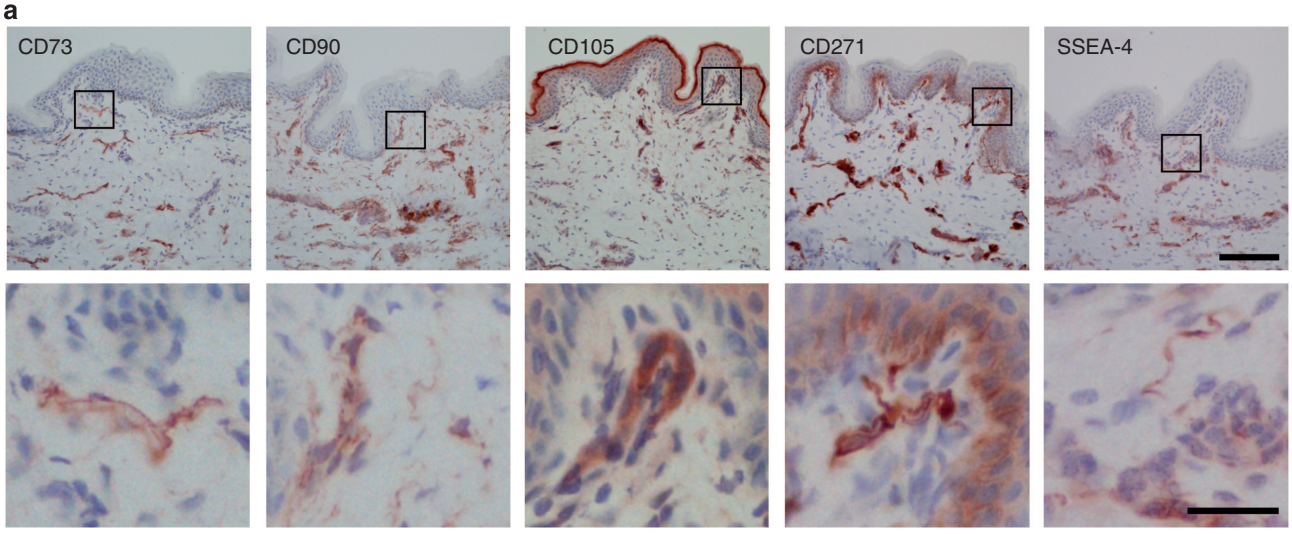

Figure 1. Expression of MSC markers in human dermis. (**a**) Immunohistochemistry staining for the indicated markers (brown) of human foreskin. Nuclei were counterstained with hematoxylin (blue). (**b**) Evaluation of expression of multipotent mesenchymal stromal cell (MSC) markers on CD31^{+} endothelial cells, CD45^{+} leukocytes, and CD318^{+} keratinocytes by multiparameter flow cytometry of freshly isolated cells of human foreskin was performed by incubation with mAb against the indicated cell surface markers. Dot plots display 2.5×10^{4} cells (donor age: 3–8 years, $n = 6$). Histograms display CD34 expression of gated double-positive cells (black rectangles). Dead cells were excluded by 7-amino-actinomycin-D uptake. Quadrants in dot plots were set according to isotype-matched control staining. *Y*-axis label applies to all dot plots in a row. Bar (**a**, upper panel) = 200 μm, (**a**, lower panel) = 50 μm.

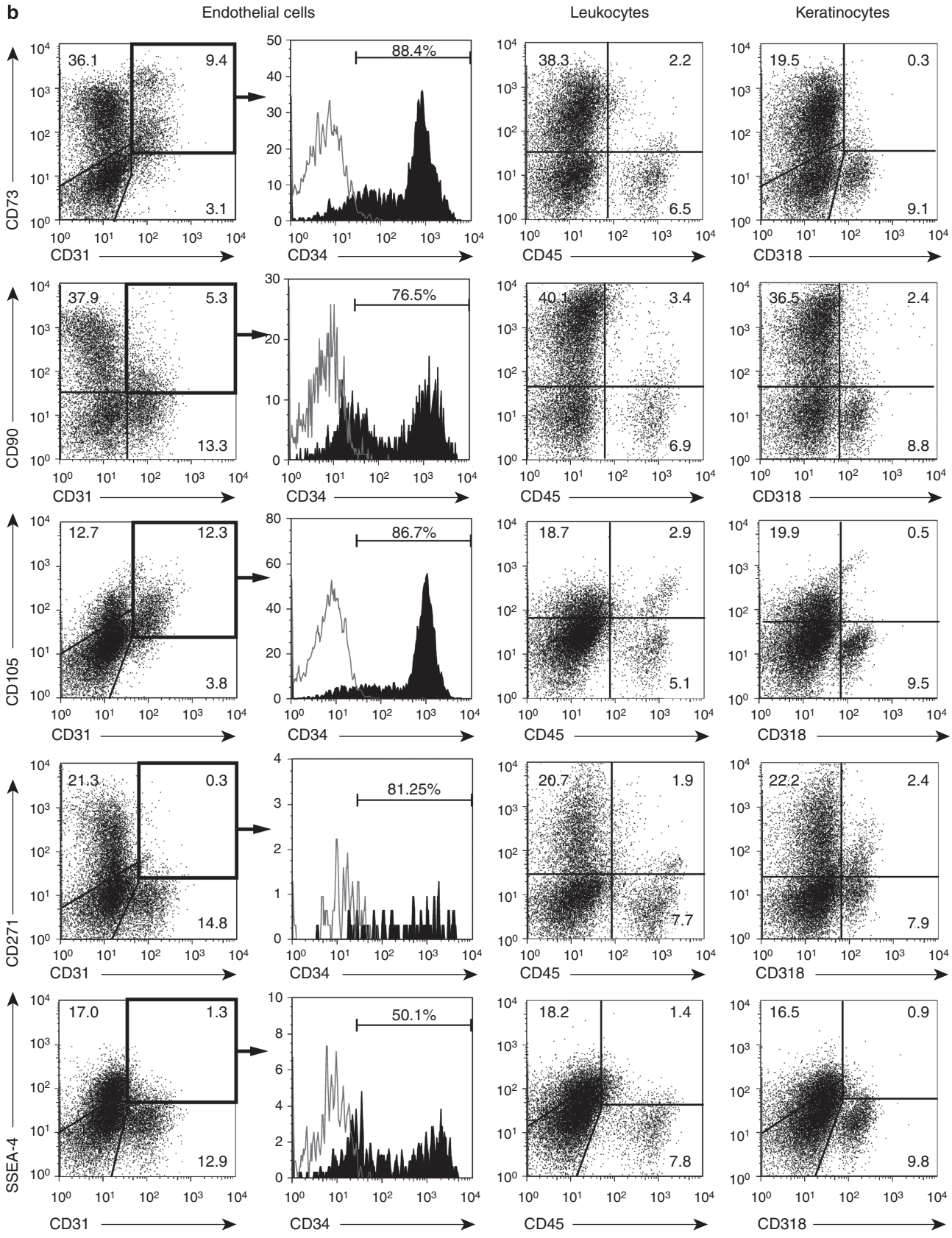

Figure 1. Continued.

markers, thus rendering these cells potential mesenchymal progenitor cells (Figure 1b; Dominici *et al.*, 2006). CD271 and SSEA-4, in contrast, were only negligibly expressed on endothelial cells in human dermis, but we detected small populations of $CD271^+CD45^+$ cells and $SSEA\text{-}4^+CD45^+$ cells. Corroborating our immunohistochemistry staining, we found expression of CD318 only on a small population of $CD271^+$ and $CD90^+$ cells, which are most probably keratinocyte stem/progenitor cells (Figure 1b; Liang *et al.*, 1999). $CD318^+$ cells occur in variable numbers in dermal single-cell suspensions and represent epithelial cells derived from skin appendages and epidermal cells (Brown *et al.*, 2004) as contaminants of the incomplete removal of the epidermis during the skin preparation procedure.

Adherent dermal cells have MSC characteristics

To test whether adherent dermal cells harbor mesenchymal progenitors, they were subjected to mesenchymal differentiation assays. Similar to previous observations (Yates *et al.*, 2001; Young *et al.*, 2001; Bartsch *et al.*, 2005; Chen *et al.*, 2007; Lysy *et al.*, 2007; Lorenz *et al.*, 2008), we found that human dermis contains cells capable of differentiating into osteoblasts and adipocytes (Figure 2a), whereas we could never observe chondrogenic differentiation of adherent dermal cells without further enrichment (data not shown). Adipogenic and osteogenic differentiation was confirmed by molecular analysis in comparison with negative controls using peroxisome proliferator–activated receptor-γ-2 or alkaline phosphatase (AP) and osteocalcin, respectively (Supplementary Figure S1 online). Assuming that phenotypic analysis of adherent dermal cells is informative regarding the nature of cells capable of mesenchymal differentiation, they were subjected to analysis by flow cytometry with a selection of MSC markers. We found that dermis-derived cells express all mesenchymal markers such as CD10, CD13, CD26, CD29, CD44, CD71, CD73, CD90, CD105, CD146, and to a lesser extent CD106, CD271, STRO-1, and SSEA-4, thus exhibiting the phenotype reported for mesenchymal progenitor cells (Figure 2b; Minguell *et al.*, 2001; Dominici *et al.*, 2006; Chamberlain *et al.*, 2007). We next compared the phenotype of adherent dermal cells and bone marrow–derived cells, both cultured under the same conditions (Figure 2c), and thereby confirmed the intriguing phenotypic similarity of dermis– and bone marrow–derived cells. The expression of CD29, CD73, CD90, CD105, CD106, and SSEA-4 was significantly higher on bone marrow–derived cells (Figure 2c). Dermis– and bone marrow–derived cells showed either no expression or only negligible expression of endothelial cell and hematopoietic stem cell markers (CD34, CD133), co-stimulatory molecules for T-cell activation (CD80, CD86), leukocyte maturation markers (CD83), and HLA-DR. Only the leukocyte common antigen CD45 was expressed on significantly more dermis-derived cells. Virtually all cells expressed HLA-ABC, whereas HLA-DR expression was hardly detectable on either cell type. Half of the dermis– and bone marrow–derived cells were $CD318^+$ (Figure 2c). The fact that CD318 can be detected on certain subsets of mesenchymal stromal cells has been reported previously (Battula *et al.*, 2008). Interestingly, under adipogenic culture conditions, virtually every adherent bone marrow–derived cell differentiated, whereas only a proportion of adherent dermal cells formed adipocytes (Figure 2d).

$CD73^+$ cells show a significantly higher adipogenic differentiation capacity than $CD90^+$ cells

Multicolor flow cytometry analysis of the expression of MSC markers on adherent dermal cells revealed that CD73 and CD105 are co-expressed. Co-expression of CD73 and CD105 was also observed in freshly isolated dermal cells (Supplementary Figure S2 online). CD90, in contrast, was not expressed on all adherent dermal cells, as a minor population of the $CD73^+$ cells is $CD90^-$ (Figure 3a). $CD271^+$ cells were $CD73^+$ and $CD105^+$, whereas the majority of $CD271^+$ cells were $CD90^-$ (Figure 3b, upper panel). Visualization of $CD271^+$ cells clearly showed their localization within the $CD73^+CD105^+$ cells, whereas only a small subset of $CD73^+CD90^+$ cells expressed CD271 (Figure 3b, lower panel). This difference between $CD73^+$ and $CD90^+$ dermal cells with respect to expression of the MSC marker CD271 might reflect different stem/progenitor cell properties. We therefore directly compared the adipogenic differentiation capacity of $CD73^+$ and $CD90^+$ cells (purity $>85\%$) isolated from freshly prepared dermal cell suspensions of the same skin sample to exclude donor-dependent variability and included the respective unfractionated dermal single-cell suspensions. We found that the differentiation capacity of $CD73^+$ cells was significantly higher when compared with $CD90^+$ cells (896.4 ± 309.1 vs. 323.9 ± 132.8 adipocytes per well; $P<0.006$; $n=5$) and total dermal cells (520.4 ± 139.4 adipocytes per well; $P<0.02$; Figure 3c, left panel). Interestingly, the differentiation capacity of $CD90^+$ cells is even significantly lower when compared with total dermal cells ($P<0.04$). For control purposes, we also investigated the differentiation potential of the $CD73^-$ (260.4 ± 380.2 adipocytes per well) and $CD90^-$ (594 ± 554.9 adipocytes per well) fractions of the respective experiments (Figure 3c, right panel). Negative fractions diverged from the respective positive fractions regarding adipogenic differentiation. However, the fact that the negative fractions still contained considerable numbers of positive cells (8–22%) potentially influences the results.

$CD271^+$ and $SSEA\text{-}4^+$ dermal cells reveal high adipogenic differentiation potential

We showed that the MSC markers CD271 and SSEA-4 are only expressed on a small subset of adherent dermal cells (Figure 2b, inset) implying a progenitor cell nature. To test this hypothesis, the adherent dermal cell fraction was magnetic-activated cell sorted for $CD271^+$ or $SSEA\text{-}4^+$ cells, which, together with the respective negative and total dermal cell fraction of the same skin sample, were subjected to differentiation assays (Figure 4c and b). We found that $CD271^+$ cells possess a 3- or 2-fold higher adipogenic differentiation capacity than $CD271^-$ ($P=0.003$, $n=5$) or total dermal cells ($P=0.003$; Figure 4c and e). As expected, the number of adipocytes in the $CD271^-$ fractions was

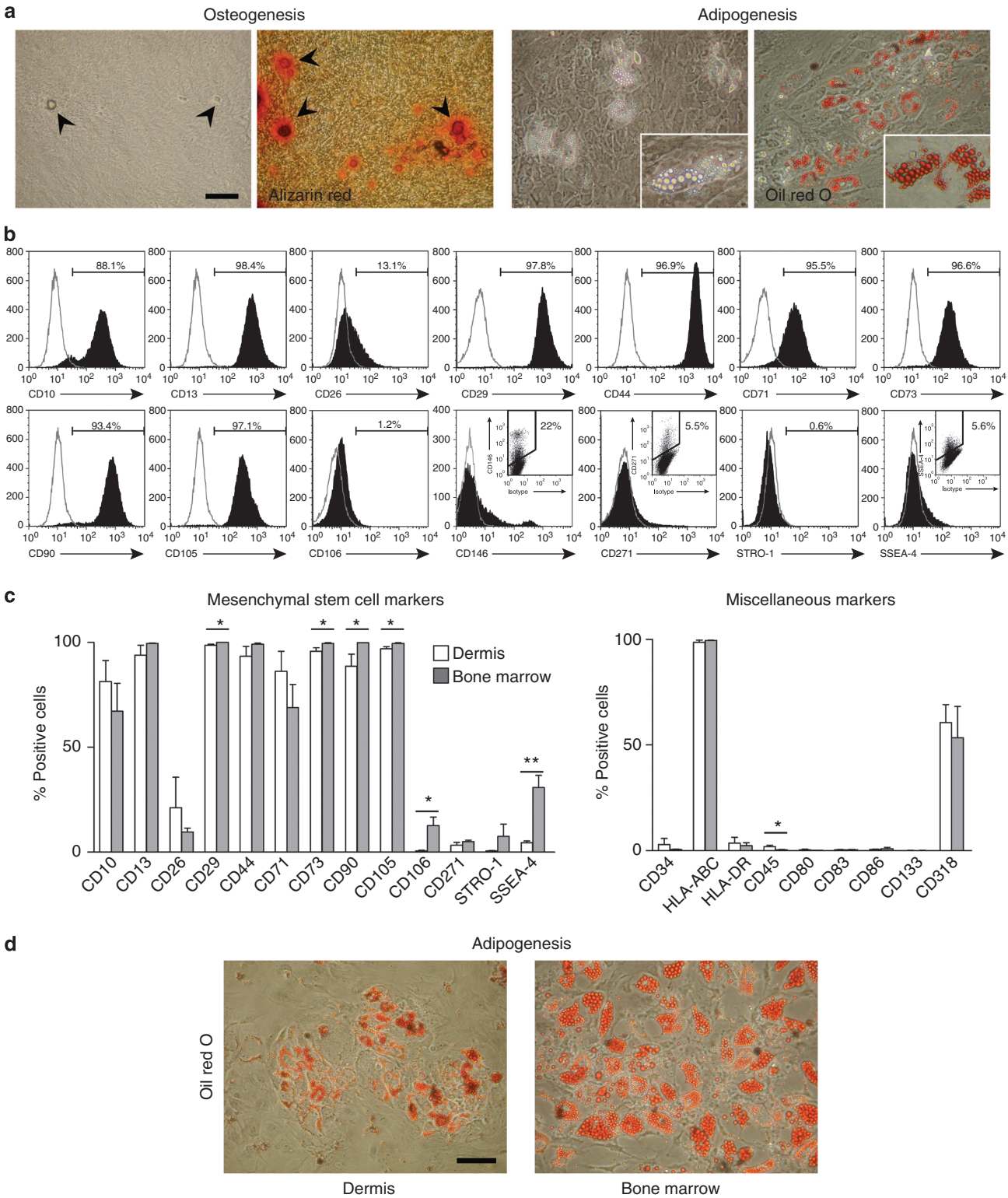

Figure 2. Adherent dermal cells display mesenchymal progenitor cell characteristics. (**a**) Adherent dermal cells after osteogenic and adipogenic differentiation stained with Alizarin red and Oil red O, respectively. Photomicrographs of unstained and stained cells were taken (arrowheads indicate osteogenic nodules). (**b**) Analysis of adherent dermal cells by flow cytometry (closed profiles: specific staining, open profiles: isotype-matched controls, 2×10^4 cells displayed, donor age: 3–9 years). Dead cells were excluded by 7-amino-actinomycin-D uptake. (**c**) Marker expression profiles of dermal cells (white bars) and bone marrow–derived cells (gray bars). Error bars indicate mean ± SD ($n = 4$). Significance: $^*P < 0.05$, $^{**}P < 0.01$ tested by Mann–Whitney *U*-test. (**d**) Oil red O staining of dermal cells and bone marrow–derived cells after adipogenic differentiation. Bar = 200 μm.

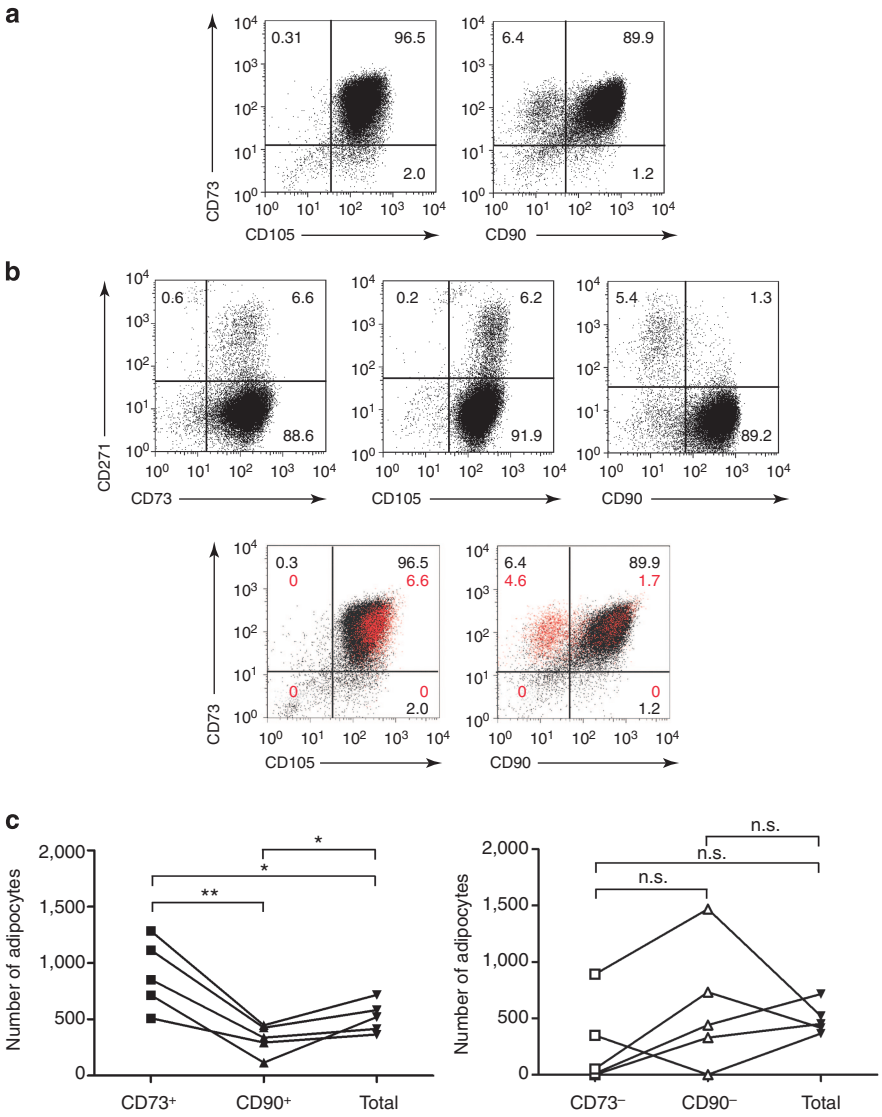

Figure 3. CD73$^+$ dermal cells express higher levels of CD271 and have a higher adipogenic differentiation capacity as compared with CD90$^+$ cells. (**a**, **b**) Adherent dermal cells were analyzed by flow cytometry (1×10^4 cells displayed, donor age: 10 months–7 years, $n = 4$). Red dots (**b**, lower panel) represent CD271$^+$ cells. (**c**) Equal numbers (2×10^4 cells per well, duplicates) of CD73$^+$, CD90$^+$, CD73$^-$, and CD90$^-$ cells isolated from the same skin sample and the respective total dermal cells were subjected to adipogenesis and stained with Oil red O. Cells containing lipid vacuoles were counted. Each symbol indicates the mean of one of five independent experiments (donor age: 5–12 years). Significance: $^*P<0.05$, $^{**}P<0.01$ tested by a paired *t*-test. n.s., nonsignificant.

significantly lower than in total adherent dermal cells ($P = 0.011$). Similar to CD271$^+$ cells, SSEA-4$^+$ cells exhibited a significantly higher adipogenic differentiation potential than the SSEA-4$^-$ fractions ($P = 0.049$) and total dermal cells (Figure 4d and f).

CD271$^+$ but not SSEA-4$^+$ dermal cells exhibit osteogenic and chondrogenic differentiation potential

To evaluate the MSC nature of dermal subpopulations, we also investigated the osteogenic and chondrogenic differentiation potential of dermal CD271$^+$ and SSEA-4$^+$ cells. The osteogenic potential was assessed qualitatively by Alizarin red staining (Figure 5a and b), which revealed strong staining of the CD271$^+$ fraction in comparison with the negative and total dermal cell fraction. Comparison of SSEA-4$^+$ cells with the respective cell fractions, however, did not show any apparent differences with regard to Alizarin red staining. Quantitatively we determined osteogenic differentiation by measurement of AP levels (Figure 5c and d). Significantly higher AP levels in the CD271$^+$ fraction than in the respective negative ($P = 0.0278$; $n = 5$) and total dermal cell fractions ($P = 0.0129$) reflect the high osteogenic potential of CD271$^+$ cells (Figure 5c). In contrast, we could not detect any differences in the AP levels when comparing SSEA-4$^+$, SSEA-4$^-$, and total adherent dermal cells (Figure 5d). Similar to osteogenic differentiation, only CD271$^+$ dermal cells were able to give rise to typical chondrocytes (Figure 5e, arrow), whereas they have not been found in the

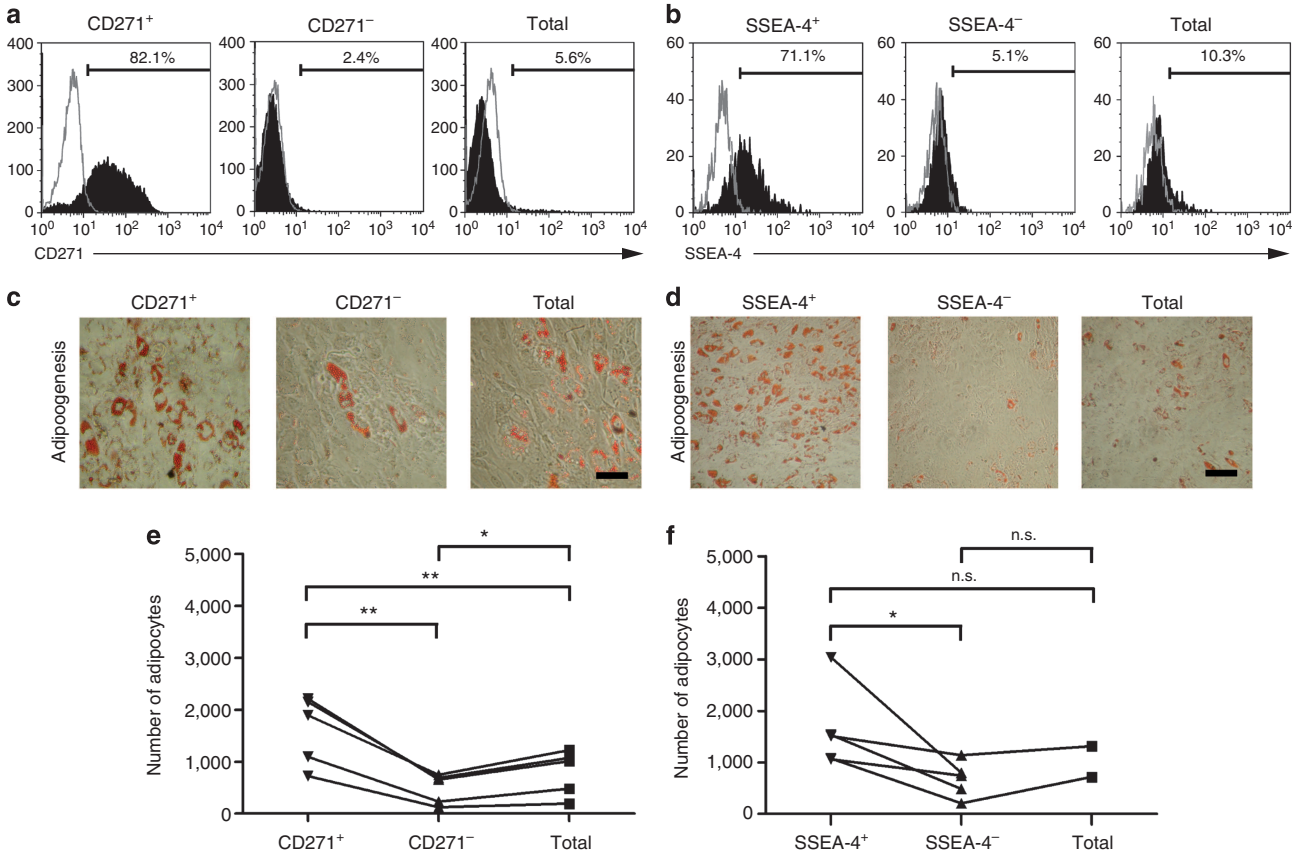

Figure 4. Dermal CD271$^+$ and SSEA-4$^+$ cells have a high adipogenic differentiation capacity. (**a**, **b**) Percentage of CD271$^+$ and SSEA-4$^+$ cells in positive, negative, and total cell fractions analyzed by flow cytometry after sorting (closed profiles: specific staining, open profiles: isotype-matched controls; 1×10^4 cells (CD271) or 1×10^3 cells (SSEA-4) displayed, donor age: 3–9 years, $n = 5$). Equal numbers (2×10^4 cells per well, duplicates) of CD271$^+$, CD271$^-$, and total adherent cells, (**c**) or of SSEA-4$^+$, SSEA-4$^-$, and total adherent cells, (**d**) were subjected to adipogenesis and stained with Oil red O. (**e**, **f**) Cells containing lipid vacuoles were counted. Each symbol indicates the mean of one of five independent experiments (donor age: 3–9 years). Significance: $^*P<0.05$, $^{**}P<0.01$ tested by a paired *t*-test. Bar = 200 μm. n.s., nonsignificant.

other investigated cell fractions including SSEA-4$^+$ cells (Figure 5e). All cell fractions formed a typical chondrogenic micromass. These differences in the osteogenic and chondrogenic potential between CD271$^+$ and SSEA-4$^+$ dermal cells provide evidence that these populations represent distinct dermal progenitor cells. Analyses of adherent dermal cells confirmed that dermal CD271$^+$ and SSEA-4$^+$ cells mutually exclude each other and seem to represent two separate populations (Figure 5g).

Our results clearly show that cell populations that possess divergent mesenchymal differentiation potential can be isolated from fresh dermal cell suspensions, as well as from dermal cells preselected by plastic adherence. Among the cell populations investigated in our study, however, only CD271$^+$ cells show significantly elevated differentiation potential in comparison with CD271$^-$, as well as total dermal cells in all differentiation assays performed.

DISCUSSION

We have shown for the first time that distinct populations of cells expressing MSC markers can be isolated from freshly separated and adherent cultured dermal cells, which to our knowledge is previously unreported. On the basis of comprehensive analysis by immunohistochemistry and flow cytometry, we isolated three cell populations that exhibit either significantly elevated mesenchymal differentiation potential in all three differentiation assays performed compared with the negative fractions and total dermal cells (CD271) or show elevated, however, in some respects, restricted differentiation potential (CD73 and SSEA-4).

MSCs have been isolated from a wide range of tissues (Erices *et al.*, 2000; Mareschi *et al.*, 2001; Yates *et al.*, 2001; Young *et al.*, 2001; Fukuchi *et al.*, 2004; Jones *et al.*, 2004; Bartsch *et al.*, 2005; Miyazaki *et al.*, 2005; Miao *et al.*, 2006; Mochizuki *et al.*, 2006; Seeberger *et al.*, 2006; Battula *et al.*, 2007, 2008; Chen *et al.*, 2007; Gallo *et al.*, 2007; Gimble *et al.*, 2007; Lysy *et al.*, 2007). Assessment of the expression of MSC markers in widely hair follicle–free skin revealed that antigens frequently used for the characterization of MSCs in various tissues are also expressed in human dermis, and that their expression evidently overlaps with other lineages such as endothelial cells, leukocytes, keratinocytes, and neural

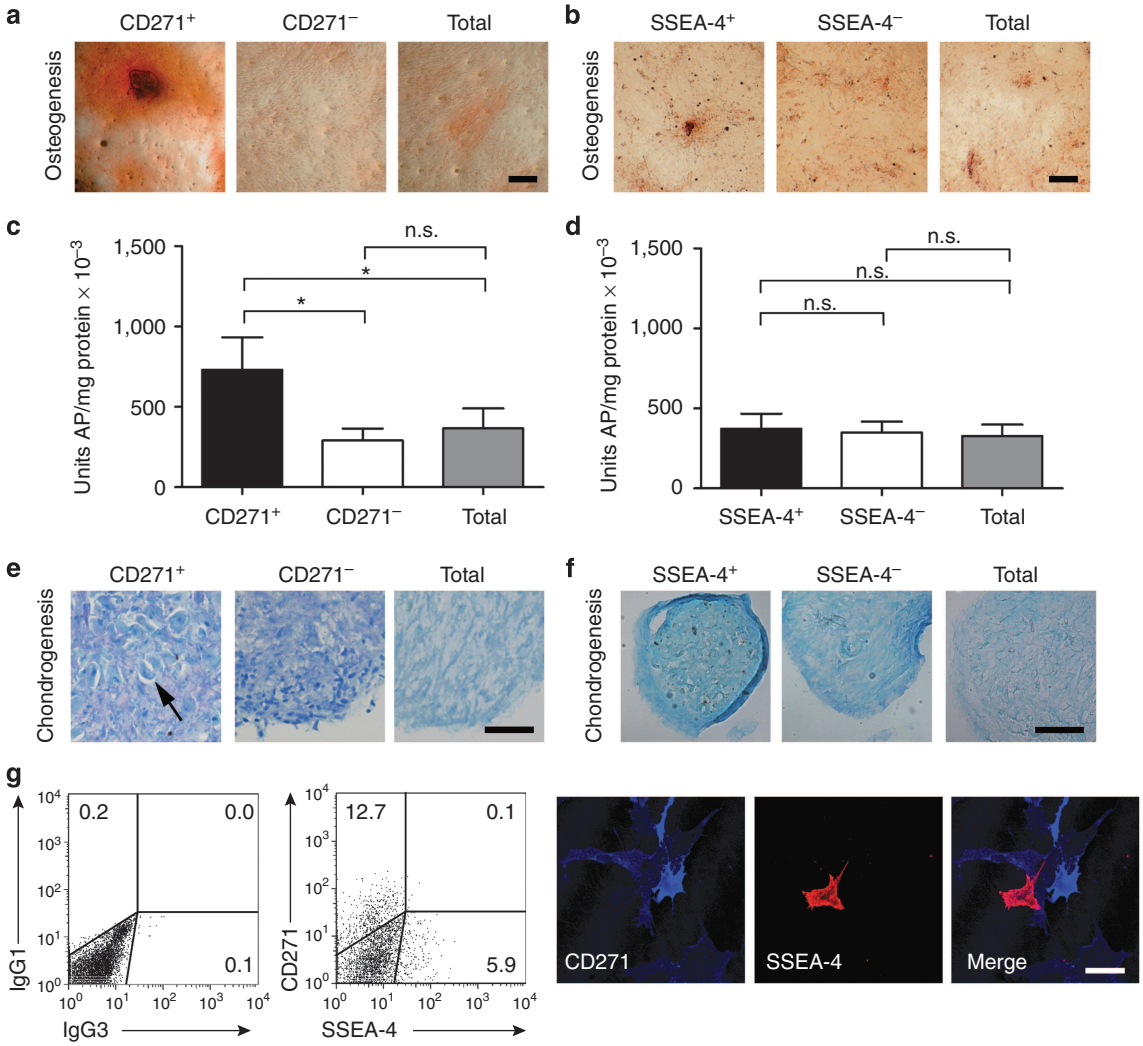

Figure 5. Dermal CD271$^+$ and SSEA-4$^+$ cells differ in osteogenic and chondrogenic capacity. (**a**) Osteogenic differentiation of CD271$^+$, CD271$^-$, and total adherent cells, or of SSEA-4$^+$, SSEA-4$^-$, and total adherent cells (**b**) (2×10^4 cells per well, duplicates, Alizarin red). (**c**, **d**) Osteogenic differentiation expressed in units of alkaline phosphatase (AP). Error bars indicate mean ± SD (donor age: 3–10 years, $n = 5$). Significance: $^*P < 0.05$, tested by a paired *t*-test. Chondrogenesis assays of CD271$^+$, CD271$^-$, and total adherent cells (**e**, arrow: chondrocytes), or of SSEA-4$^+$, SSEA-4$^-$, and total adherent cells (**f**; toluidine blue, donor age: 2–12 years, $n = 3$). (**g**) Adherent dermal cells analyzed by flow cytometry (1×10^4 cells displayed, donor age: 3–9 years, $n = 4$). Immunofluorescence of cultured dermal cells. Bar (**a**, **b**) = 200 µm, (**e**–**g**) = 50 µm. n.s., nonsignificant.

structures. These findings emphasize the difficulty of identifying MSCs in freshly prepared tissues and imply the necessity of preselecting the dermal stromal fraction by plastic adherence. There exist concerns that the typical properties of MSCs are culture phenomena, most importantly because only few studies use noncultured MSCs as reviewed by Javazon *et al.* (2004). Our experiments investigating the differentiation capacity of CD73$^+$ cells isolated from freshly prepared dermal single-cell suspensions indicate that this cell fraction is enriched for MSCs in comparison with CD90$^+$ cells. This demonstrates that cells with MSC characteristics can directly be isolated from the dermis using specific antibodies without preselection by plastic adherence. Similarly, CD105$^+$ cells were isolated from noncultured bone marrow aspirates, showing that CD105$^+$ cells form significantly higher numbers of colony-forming unit-F in comparison with unseparated bone marrow mononuclear cells (Majumdar *et al.*, 2000; Aslan *et al.*, 2006; Kastrinaki *et al.*, 2008) supporting our results attributing MSC characteristics to freshly isolated dermal CD73$^+$ cells, which are also CD105$^+$.

A comparison of the cell surface makers of adherent dermal– and bone marrow–derived MSCs revealed that these two cell types have very similar marker expression profiles (Ishii *et al.*, 2005; Wagner *et al.*, 2005). CD271 and SSEA-4 expression might point toward a progenitor nature compared with terminally differentiated fibroblasts, particularly because both molecules have been previously reported as promising markers for the isolation of MSCs from bone marrow (Jarocha *et al.*, 2008).

We found that isolated CD271$^+$ and SSEA-4$^+$ dermal adherent cells were significantly more prone to undergo

adipogenesis than their respective negative fractions. Moreover, a correlation between the percentage of CD271$^+$ and SSEA-4$^+$ cells and their differentiation capacity was identified. Interestingly, CD271$^+$ cells exhibited elevated osteogenic and chondrogenic differentiation potential as compared with the negative and total adherent cell fractions. SSEA-4$^+$ cells did not differ from the related cell fractions, indicating the existence of distinct subtypes of MSCs in human dermis. Only cells within the adherent CD271$^+$ dermal cell population fulfil the proposed criteria of multipotent MSCs (Dominici *et al.*, 2006) with (i) adherence to plastic and (ii) differentiation potential *in vitro* into osteoblasts, adipocytes, and chondrocytes. The observation that CD271$^+$ cells exhibiting a stem cell character exist, apart from bone marrow, also in other tissues is corroborated by a report about MSCs derived from human synovium (Jones *et al.*, 2008). In line with our results, CD271$^+$ cells isolated from human synovium have a clearly elevated mesenchymal differentiation capacity.

The anatomical niche for cutaneous MSCs is not entirely clear. The follicular connective sheath and the papilla have been considered to represent the site of dermal MSCs (Lako *et al.*, 2002; Richardson *et al.*, 2005; Hoogduijn *et al.*, 2006; Kruse *et al*, 2006). Less is known about the distribution of MSCs in hair follicle–free dermis. This study and others provide evidence for the occurrence of dermal stem cells in human foreskin independent of hair follicles (Toma *et al.*, 2005; Chen *et al.*, 2007; Lorenz *et al.*, 2008). It is conceivable that dermal MSCs are located in a perivascular pattern, which has been shown for a variety of organs (Crisan *et al.*, 2008; Morikawa *et al*, 2009). Owing to their localization and their expression of CD146, these cells might be related to pericytes. CD146 is expressed on pericytes (Crisan *et al.*, 2008) and all types of human endothelial cells (Bardin *et al.*, 2001). It may be speculated that also the dermal MSCs characterized in this study are of perivascular origin, because ~20% of adherent dermal cells expressed CD146 while lacking endothelial markers CD31 and CD34 (not shown).

It is conceivable that because of their morphological similarity, dermal MSCs are present among already well-studied fibroblast subsets. The functional heterogeneity of morphologically homogenous fibroblast cultures has already been described (Goldring *et al.*, 1990; Sorrell *et al.*, 2007). Various studies suggest a role of fibroblasts in angiogenesis after injury and support of vascular endothelial cell differentiation and basement membrane formation (Sorrell *et al.*, 2007, 2008; Salvolini *et al.*, 2010). The regenerative role of fibroblasts has also been shown in a comprehensive study using a skin reconstruction model (Aoki *et al.*, 2004). It remains to be determined whether these regenerative characteristics are carried by all fibroblasts, or whether they are reserved to different subsets of progenitor cells residing in human dermis.

Our study makes an attempt to phenotype, isolate, and differentiate subpopulations of cells in the human dermis expressing MSC markers, which to our knowledge is previously unreported. The results might also have an impact on our understanding of certain aspects in dermatopathology as dermal MSCs potentially have a role in the evolution of skin neoplasms (Sellheyer and Krahl, 2010).

MATERIALS AND METHODS

Skin samples

Foreskin samples (donor age: 10 months–12 years) were obtained as discarded material from routine circumcisions according to the Declaration of Helsinki principles, and following approval by the local ethics committee and informed written consent by the parents.

Preparation and culture of dermal single-cell suspensions and bone marrow–derived MSCs

Skin samples were depleted of subcutaneous tissue and incubated on 1.2 U ml^{-1} Dispase II (Roche Applied Science, Penzberg, Germany) in phosphate-buffered saline (Gibco, Eugene, OR) overnight at 4 °C. The dermis was separated from the epidermis and digested in 0.71 U ml^{-1} Liberase Blendzyme 3 (Roche Applied Science) for 90 minutes at 37 °C in a shaking water bath. Cells were passed through a 40-μm cell strainer and either directly processed for flow cytometric analysis or magnetic-activated cell sorting or plated in 225-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) containing mesenchymal growth medium (α-MEM (Gibco) supplemented with 10% fetal calf serum (PromoCell, Heidelberg, Germany), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Gibco)). Nonadherent cells were removed by medium changes every 48 hours. Adherent cells were grown until they reached 80% confluence, trypsinized, and subjected to magnetic-activated cell sorting and/or flow cytometry.

Bone marrow–derived MSCs were prepared as described (Schallmoser *et al.*, 2008). For flow cytometry and differentiation experiments, cryopreserved cells were thawed, plated in tissue culture flasks containing mesenchymal growth medium, and grown to 80% confluence.

Magnetic-activated cell sorting

Freshly prepared dermal cells were labeled with phycoerythrin (PE)-anti-CD73 (AD2; BD Biosciences, San Jose, CA) and PE-anti-CD90 (5E10; BD Biosciences), incubated with anti-PE-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and further processed according to the manufacturer's instructions. In another series of experiments, cultured cells were trypsinized, labeled with biotinylated anti-CD271 (BD Biosciences), or purified anti-SSEA-4 (MC813-70; Millipore, Billerica, MA) and biotinylated goat anti-mouse IgG (H + L) (Caltag, Buckingham, UK), followed by incubation with anti-biotin microbeads.

Flow cytometry

Freshly prepared or cultured cells were analyzed using the following mAbs: PE-anti-CD26 (M-A261), allophycocyanin (APC)-anti-CD29 (MAR4), PE-anti-CD44 (515), APC-anti-CD71 (M-A712), PE-anti-CD73 (AD2), FITC-anti-CD80 (L307.4), FITC-anti-CD83 (HB15e), FITC-anti-CD86 (2331), FITC- and PE-anti-CD90 (5E10), APC-anti-CD106 (51-10C9), PE-anti-CD146 (P1H12), APC- and PE-anti-CD271 (C40-1457), APC-anti-CD318 (CUB1), FITC-anti-HLA-ABC (G46-2.6), APC-anti-HLA-DR (L243) (all from BD Biosciences), FITC-anti-CD10 (SS2/36; Dako, Glostrup, Denmark), FITC-anti-CD13 (5-390; provided by Dr Otto Majdic, Institute of Immunology, Medical University of Vienna, Austria), APC- and PE-Cy7-anti-CD45

(J33; Immunotech, Fullerton, CA), APC- and FITC-anti-CD34 (AC136), PE-anti-CD133 (AC141; both Miltenyi Biotec), FITC- and PE-anti-CD105 (166707), PE-anti-SSEA-4 (MC813-70; R&D Systems, Minneapolis, MN), FITC-anti-CD31 (WM59), and PE-anti-STRO-1 (STRO-1; Serotec, Kidlington, UK). Appropriate isotype controls were included and gates were set according to isotype-matched controls. Dead cells were excluded with 7-amino-actinomycin-D (Calbiochem, Darmstadt, Germany). Analysis of cells was performed on a LSR-II (BD Biosciences) and data were evaluated using the FlowJo software (Tree Star, Ashland, OR).

In vitro differentiation assays

For adipogenic differentiation, cells (2×10^4 cells per well) were seeded in 48-well plates in duplicates and cultured in mesenchymal growth medium. Upon reaching confluence, adipogenic induction medium (mesenchymal growth medium supplemented with 0.2 mM indomethacin, 0.1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine, and 10 μg ml^{-1} recombinant human insulin (all from Sigma-Aldrich, St Louis, MO)) was applied for 21 days. Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) and stained with Oil red O (Sigma-Aldrich). The differentiated cells were counted and statistically analyzed.

For osteogenic differentiation, confluent cells (48-well plate, duplicates) were stimulated with osteogenic induction medium (mesenchymal growth medium supplemented with 0.1 μM dexamethasone, 50 μg ml^{-1} ascorbic acid and 10 mM glycerol 2-phosphate (all from Sigma-Aldrich)) for 21 days and then either stained with Alizarin red (Sigma-Aldrich) or subjected to an AP assay. For this, cells were lysed (CelLytic M; Sigma-Aldrich) and the supernatant was subjected to analysis by a fluorometric AP Detection Kit (Sigma-Aldrich) according to the manufacturer's instructions.

For chondrogenic differentiation, cells (2.5×10^5) were placed in chondrogenic medium (D-MEM/F-12 medium containing 2 mM L-glutamine, 100 μg ml^{-1} sodium pyruvate, 0.1 mM nonessential amino acids (all from Gibco), 1% insulin transferrin sodium selenite plus, 50 μg ml^{-1} ascorbic acid 2-phosphate, 0.1 μM dexamethasone (all from Sigma-Aldrich), 10 ng ml^{-1} TGFβ3 (R&D Systems)), centrifuged at 450*g* for 5 minutes, and pellets were cultivated for 21 days. Micromasses were fixed in 4% paraformaldehyde, embedded in O.C.T. (Tissue-Tek, Sakura Finetek, Zoeterwoude, The Netherlands), cut into 5 μm sections, stained with toluidine blue (Sigma-Aldrich), and analyzed.

Negative controls, in which media supplements inducing differentiation were omitted, were included for each differentiation assay and did not show spontaneous differentiation. Moreover, adipogenic and osteogenic differentiation of adherent dermal cells in contrast to negative controls was confirmed by molecular analysis of the expression of peroxisome proliferator–activated receptor-γ-2 or AP and osteocalcin, respectively (Supplementary Figure S1 online).

Immunohistochemistry

Skin sections (4 μm) were fixed in ice-cold acetone for 10 minutes and air-dried. Purified primary antibodies (anti-CD73 (AD2), anti-CD90 (5E10), anti-CD271 (C40-1457) (all from BD Biosciences)), anti-CD105 (166707), anti-SSEA-4 (MC813-70; R&D Systems), and isotype controls were diluted in 2% BSA/phosphate-buffered saline, applied to the sections, and incubated overnight at 4 °C. Endogenous peroxidase was blocked by incubation with 0.03% hydrogen peroxide/methanol (room temperature, 10 minutes). Subsequently, sections were incubated with biotin-conjugated goat anti-mouse IgG (room temperature, 60 minutes) using the Elite mouse IgG Vectastain Kit (Vector Laboratories, Burlingame, CA). Biotinylated antibodies were detected with horseradish peroxidase–streptavidin and visualized with amino-ethyl-carbazole (Dako). Sections were counterstained with hematoxylin. Photomicrographs were taken using an Eclipse 80 microscope (Nikon, Tokyo, Japan).

Immunofluorescence

Freshly isolated dermal cells were cultured in eight-well-chamber slides (Lab-Tek; Nunc) for 5 days. Adherent cells were fixed with methanol and incubated with APC-anti-CD271 (C40-1457; BD Biosciences), PE-anti-SSEA-4 (MC813-70; R&D Systems), and isotype controls diluted in 2% BSA/phosphate-buffered saline overnight at 4 °C. Slides were mounted using Vectashield (Vector Laboratories) and images were recorded using a confocal laser scanning microscope (LSM 410; Carl Zeiss, Jena, Germany).

Statistical analysis

Differences between groups were assessed using the Mann–Whitney *U*-test or a paired *t*-test for related groups, respectively (GraphPad Software, La Jolla, CA). The reported *P*-value is a result of a two-sided test. A *P*-value below 5% was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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