Biol. Cell (2011) **103**, 197–208 (Printed in Great Britain) doi:10.1042/BC20100117

Research article

Fibroblasts share mesenchymal phenotypes with stem cells, but lack their differentiation and colony-forming potential

Eckhard Alt*[1], Yasheng Yan*†[1], Sebastian Gehmert*, Yao-Hua Song*, Andrew Altman*, Sanga Gehmert*, Daynene Vykoukal* and Xiaowen Bai*†[2]

*Department of Molecular Pathology, The University of Texas M. D. Anderson Cancer Center, Unit 951, 7435 Fannin Street, Houston, TX 77054, U.S.A., and †Department of Anesthesiology, The Medical College of Wisconsin. Milwaukee, 8701 Watertown Plank Road, Milwaukee, WI 53226, U.S.A.

Background information. Although MSCs (mesenchymal stem cells) and fibroblasts have been well studied, differences between these two cell types are not fully understood. We therefore comparatively analysed antigen and gene profiles, colony-forming ability and differentiation potential of four human cell types *in vitro*: commercially available skin-derived fibroblasts [hSDFs (human skin-derived fibroblasts)], adipose tissue-derived stem cells [hASCs (human adipose tissue-derived stem cells)], embryonic lung fibroblasts (WI38) and dermal microvascular endothelial cells [hECs (human dermal microvascular endothelial cells)].

Results. hSDFs, hASCs and WI38 exhibited a similar spindle-like morphology and expressed same antigen profiles: positive for MSC markers (CD44, CD73 and CD105) and fibroblastic markers [collagen I, HSP47 (heat shock protein 47), vimentin, FSP (fibroblast surface protein) and αSMA (α smooth muscle actin)], and negative for endothelial cell marker CD31 and haemopoietic lineage markers (CD14 and CD45). We further analysed 90 stem cell-associated gene expressions by performing real-time PCR and found a more similar gene expression pattern between hASCs and hSDFs than between hSDFs and WI38. The expression of embryonic stem cell markers [OCT4, KLF4, NANOG, LIN28, FGF4 (fibroblast growth factor 4) and REST] in hASCs and hSDFs was observed to differ more than 2.5-fold as compared with WI38. In addition, hSDFs and hASCs were able to form colonies and differentiate into adipocytes, osteoblasts and chondrocytes *in vitro*, but not WI38. Moreover, single cell-derived hSDFs and hASCs obtained by clonal expansion were able to differentiate into adipocytes and osteoblasts. However, CD31 positive hECs did not show differentiation potential.

Conclusions. These findings suggest that (i) so-called commercially available fibroblast preparations from skin (hSDFs) consist of a significant number of cells with differentiation potential apart from terminally differentiated fibroblasts; (ii) colony-forming capacity and differentiation potential are specific important properties that discriminate MSCs from fibroblasts (WI38), while conventional stem cell properties such as plastic adherence and the expression of CD44, CD90 and CD105 are unspecific for stem cells.

Introduction

MSCs (mesenchymal stem cells) are currently defined as plastic adherent fibroblast-like cells with extensive proliferative capacity and differentiation potential.

[1]These authors contributed equally to this work.

[2]To whom correspondence should be addressed (email baixw01@yahoo.com).

Key words: stem cells, fibroblasts, differentiation, colony-forming unit (CFU), proliferation.

Abbreviations used: αSMA, α smooth muscle actin; CFU, colony-forming unit; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FSP, fibroblast surface protein; hASC, human adipose tissue-derived stem cell; hEC, human dermal microvascular endothelial cell; HGF, hepatocyte growth factor; hSDF, human skin-derived fibroblast; HSP47, heat shock protein 47; MSC, mesenchymal stem cell; VEGFA, vascular endothelial growth factor A; VEGFB, vascular endothelial growth factor B; VEGFC, vascular endothelial growth factor C.

They have been identified in many organs and tissues, including bone marrow (Pittenger et al., 1999), adipose tissue (Bai et al., 2007) and skin (Richardson et al., 2005). Experimental evidence *in vivo* demonstrated the benefit of MSCs in stem cell-based therapies (Altman et al., 2008). We and others have recently shown that hASCs (human adipose tissue-derived stem cells) improved cardiac function and enhanced wound repair (Altman et al., 2009; Bai et al., 2010).

Fibroblasts are plastic adherent mesenchymal cells that play a significant role during tissue development, maintenance and repair (Flavell et al., 2008). In contrast to MSCs, fibroblasts are widely considered to be terminally differentiated cells that are primarily responsible for the synthesis and remodelling of extracellular matrix in tissues and do not convert into other types of cells. Traditionally, differentiation capacity has been used as a line of demarcation to distinguish fibroblasts from MSCs. Therefore, fibroblasts are routinely used as a negative control for evaluation of cell differentiation capacity in many studies. It was reported that dermal fibroblasts did not have differentiation potential (Brendel et al., 2005). However, recent studies tend to obscure this line of demarcation. Several reports have demonstrated that fibroblasts were able to differentiate into various lineages. Human foreskin dermal fibroblasts gave rise to adipocytes, osteocytes and chondrocytes (Chen et al., 2007). Fibroblasts isolated from human bronchial and uterine tissues also could differentiate into adipocytes and osteocytes (Kim et al., 2008; Strakova et al., 2008).

These conflicting results regarding the differentiation capacity of fibroblasts raise the following questions: how are fibroblasts defined, how do fibroblasts differ from MSCs and are the beneficial effects obtained from so-called fibroblasts (that have been described nearly for a decade) mediated by stem cells or by fibroblasts? Answering these questions is important for understanding the biology of fibroblasts and stem cells and explaining the related experimental results, and evaluating stem cell- and fibroblast-based clinical therapeutic effect. Therefore, the aim of this study was to compare the differentiation capacity, antigen profiles, stem cell-associated gene expression, angiogenic growth factor expression and the ability to form colonies of both commercially available and prepared hSDFs (human skin-derived fibroblasts), a known human embryonic lung fibroblast cell line (WI38) and hECs (human dermal microvascular endothelial cells) to those of well-studied and -defined populations of hASCs.

Results

Morphology and expansion characteristics

hSDFs, hASCs and WI38 exhibit a similar spindle-like morphology *in vitro* (Figure 1A) when cultured in their respective culture media as described in the Materials and methods section. The cell numbers of hSDFs, hASCs and WI38 expanded to approx. 600 000 from 250 000 in 24-cm^2 flasks 10 days after seeding. There were no significant differences in the proliferation capability between hSDFs, hASCs and WI38 (Figure 1B).

CFU (colony-forming unit) assay

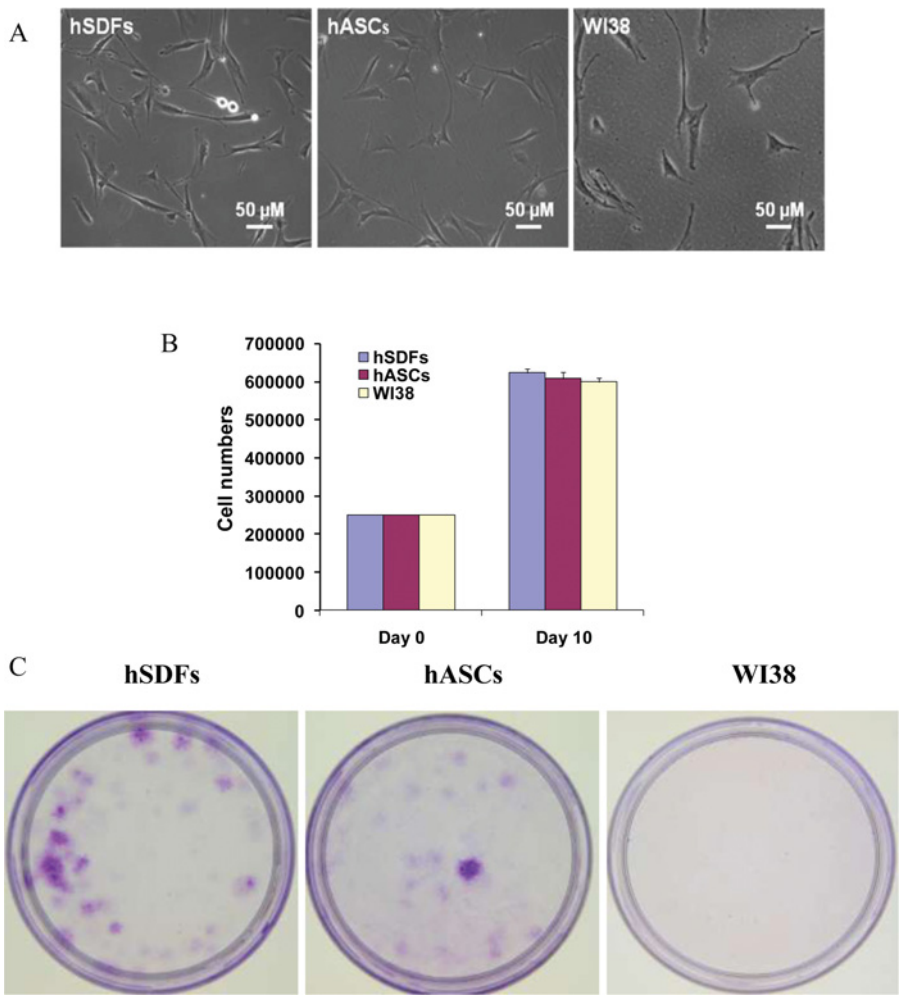
The efficiency of forming CFU was significantly higher in hSDF ($5 \pm 0.2\,\%$) and hASC ($4.4 \pm 0.4\,\%$) culture than in WI38 ($P < 0.01$). We did not observe any colonies in WI38 culture (Figure 1C).

Expression of cell surface markers

The hSDFs were negative for MSC markers (CD44, $95.92 \pm 2.23\,\%$; CD73, $99.35 \pm 0.72\,\%$; CD90, $97.33 \pm 3.51\,\%$; CD105, $99.61 \pm 0.10\,\%$). Both haemopoietic cell markers (CD14, $0.19 \pm 0.39\,\%$; CD45, $0.12 \pm 0.34\,\%$) and endothelial cell marker (CD31, $0.23 \pm 0.25\,\%$) were absent in hSDFs (Figure 2A). This result precludes contamination by haemopoietic cells and endothelial cells (Etheridge et al., 2004). Cell surface proteins expressed in hASCs were highly comparable to those in hSDFs. hASCs were also positive for CD44 ($91.74 \pm 5.20\,\%$), CD73 (98.12 ± 1.62) and CD105 ($93.37 \pm 7.22\,\%$) and negative for CD14 ($0.11 \pm 0.11\,\%$), CD45 ($0.30 \pm 0.37\,\%$) and CD31 ($0.11 \pm 0.33\,\%$) (Figure 2B). The fibroblast cell line WI38 expressed the same cell surface antigens as did hSDFs and hASCs. They were positive for CD44 ($97.9 \pm 0.20\,\%$), CD73 ($98.08 \pm 0.05\,\%$) and CD105 ($98.33 \pm 2.11\,\%$) and negative for CD14 ($0.28 \pm 0.33\,\%$), CD31 ($0.18 \pm 0.06\,\%$) and CD45 ($0.52 \pm 0.47\,\%$) (Figure 2C). The expression pattern of cell surface markers in hSDFs, hASCs and WI38 was consistent with the pattern of cell surface markers in MSCs reported by others (Izadpanah et al., 2006).

Figure 1 | Morphology, proliferation and colony-forming ability of hSDFs, hASCs and human embryonic lung fibroblasts (WI38) *in vitro*

(**A**) The three cell types exhibit a similar spindle-like shape. (**B**) Ten days after seeding, hSDFs, hASCs and WI38 expanded *in vitro* to approx. 600 000 cells from 250 000 cells. There was no significant difference in the proliferation potential between hSDFs, hASCs and WI38 ($P > 0.05$). (**C**) CFU assay. hSDFs, hASCs and WI38 (500 cells each) were cultured in 60 mm dishes and incubated for 2 weeks. hSDFs and hASCs show a similar ability to form colonies ($P > 0.05$). No colonies were observed in WI38 culture, $n = 3$.



Collagen I, HSP47 (heat shock protein 47), vimentin, FSP (fibroblast surface protein) and αSMA (α smooth muscle actin) expression

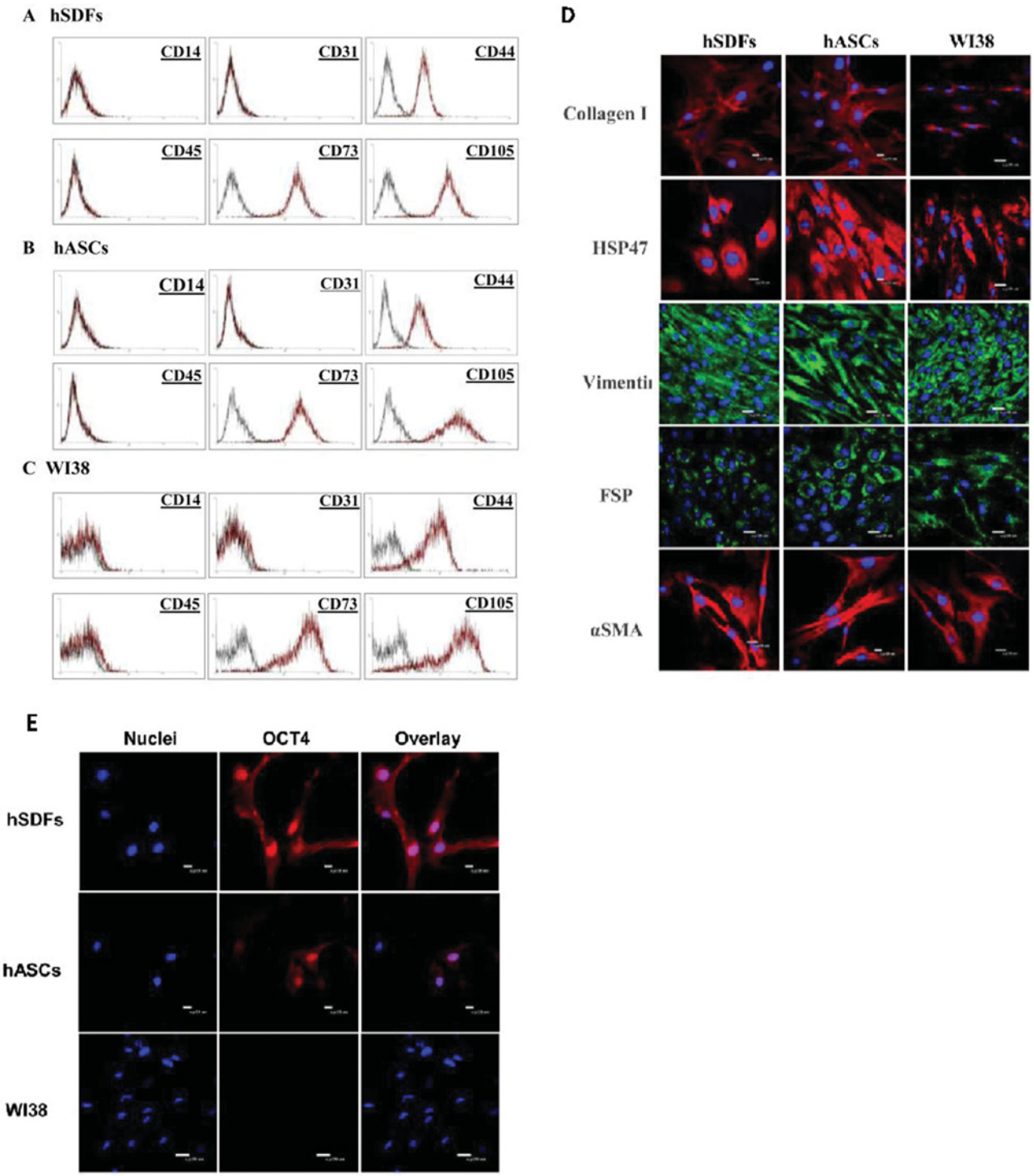
Collagen I, HSP47, vimentin and FSP are commonly regarded as the markers of fibroblasts (Strutz et al., 1995; Kalluri and Zeisberg, 2006). αSMA is the marker of myofibroblasts (McAnulty, 2007). hSDFs, hASCs and WI38 showed expression of collagen I, HSP47, vimentin, FSP and αSMA (Figure 2D). As a control, we did not find any signals in cells stained with isotype-matched controls instead of primary antibodies (results not shown).

OCT4 expression

Transcription factor OCT4 are highly associated with pluripotency of stem cells (Choo et al., 2004; Buitrago and Roop, 2007). Immunofluorescence staining analysis displayed OCT4 expression in both hSDFs and hASCs. However, the signals of OCT4 were not found in WI38 (Figure 2E).

Figure 2 | Cell surface antigen profiles in hSDFs (A), hASCs (B) and WI38 (C) by flow cytometric analysis

(**A**–**C**) Black traces indicate isotype controls and red traces show surface antigen expression level. hSDFs, hASCs and WI38 were positive for conventional MSC markers CD44, CD73 and CD105 and negative for CD14, CD31 and CD45. Traces shown here were from a representative result of three similar results. (**D**) The expression of collagen I, HSP47, vimentin, FSP and αSMA in hSDFs (left column), hASCs (middle column) and WI38 (right column) analysed by immunofluorescence staining.



All three cell types showed uniform expression of commonly accepted fibroblast markers (collagen I, HSP47, vimentin and FSP) and myofibroblast marker (αSMA). Blue indicates nuclei stained with DAPI. Red or green indicates the signals of collagen I, HSP47, vimentin, FSP and αSMA. (**E**) Pluripotent cell marker OCT4 expressed in both in hSDFs and hASCs but not in WI38. Immunofluorescence staining analysis displayed OCT4 expression both in hSDFs and hASCs (upper and middle panels). However, the signals of OCT4 were not found in WI38 (bottom panels). Scale bar in (**D**) and (**E**) = 20 μm.

Stem cell associated gene expression in hASCs, hSDFs and WI38

The expression of 90 human stem cell-related genes in hASCs, hSDFs and WI38 was analysed by performing real-time PCR. Differentiated cell markers such as COL2A (collagen II, chondrocyte marker), AFP (alpha-fetoprotein, hepatocyte marker) and insulin (pancreatic cell marker) were not expressed in all three of these cell lines. Gene expression heat map demonstrated that 70 genes were commonly expressed by hASCs, hSDFs and WI38 (Figure 3A). There was a more similar gene expression pattern between hASCs and hSDFs than between hSDFs and WI38, as evident from the higher expression level of more than 70 % or 40 % detectable genes in hASCs and hSDFs compared with WI38 respectively. The expression of embryonic stem cell markers [OCT4, KLF4, NANOG, LIN28, FGF4 (fibroblast growth factor 4), REST, SALL2 and CER1] in hASCs and hSDFs was observed to differ more than 2.5-fold as compared with WI38 (Figure 3B). In addition, other mesoderm markers (FN1, SFRP2 and NPPA) and endoderm marker (SERPINA1) also showed higher expression level (>3-fold) in hASCs and hSDFs than in WI38 (Figure 3C).

Multi-lineage differentiation potential of hSDFs, hASCs and WI38

To evaluate the differentiation capacity of hSDFs, hASCs and WI38, cells were cultured in adipogenic (Figure 4A), osteogenic (Figure 4B) and chondrogenic (Figure 4C) induction media for 3 weeks. hSDFs and hASCs cultured in adipogenic media displayed multiple intracellular bright white oil droplets. These oil droplets showed red vesicles when stained with Oil Red O. There were more Oil Red O-positive adipocytes differentiated from hASCs (59.98 ± 7.62 %) than from hSDFs (44.26 ± 6.09 %) ($P < 0.05$). No lipid droplets could be observed in WI38 cultured in adiogenic media or control media (Figure 4A). hSDFs and hASCs cultured in osteogenic media showed black regions within the monolayer, indicating calcium deposits derived from the osteoblasts differentiated from hSDFs or hASCs. There were no obvious differences in osteogenic capacity between hSDFs and hASCs as determined by measuring the intensity of calcium deposition after Alizarin Red S staining. However, WI38 cultured in osteogenic media or control medium did not show any red signals after Alizarin Red S staining (Figure 4B). Cell pellet sections from hSDFs and hASCs cultured for 3 weeks in chondrogenic media showed brown signals after immunohistochemical staining. The brown signals represent the expression of collagen II (a specific marker of chondrocytes). There were no obvious differences of chondrogenic potency between hSDFs (64.40 ± 4.96 %) and hASCs (65.87 ± 3.54 %), as calculated by measuring collagen II-positive brown areas (Figure 4C). The corresponding brown signals were absent in hASC and hSDF pellet sections cultured in control media. WI38 formed cell pellets 24 h after culturing in chondrogenic or control media. However, the cell pellets disaggregated after 4 days of culture; therefore the chondrogenic potential of WI38 could not be analysed further.

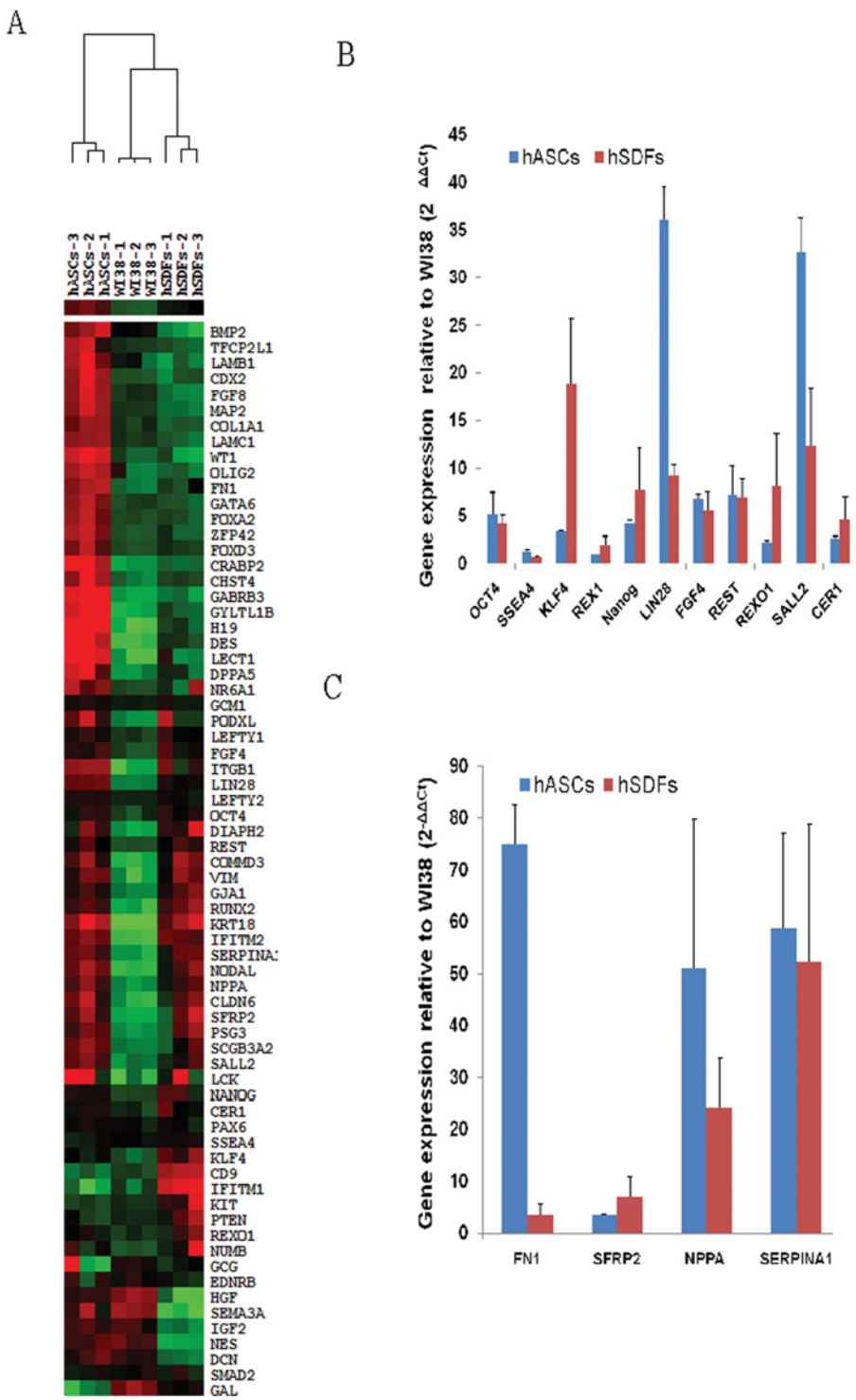
hECs are terminally differentiated cells and distinctly different from WI38, hSDFs and hASCs in morphology, cell surface marker expression and differentiation potential. These cells show a cobblestone-like shape and expressed CD31 but not CD45. hECs did not form cell pellets after 24 h of culture in chondrogenic media. In addition, most hECs died after 4 days of culture in hASCs, hSDFs, WI38 culture media or induction media. The remaining few surviving cells did not differentiate into adipocytes and osteoblasts.

Clonal expansion and differentiation of single cell-derived hASCs and hSDFs

In order to further investigate the differentiation potential of hSDFs and hASCs, single cell-derived hASCs and hSDFs were obtained by clonal expansion.

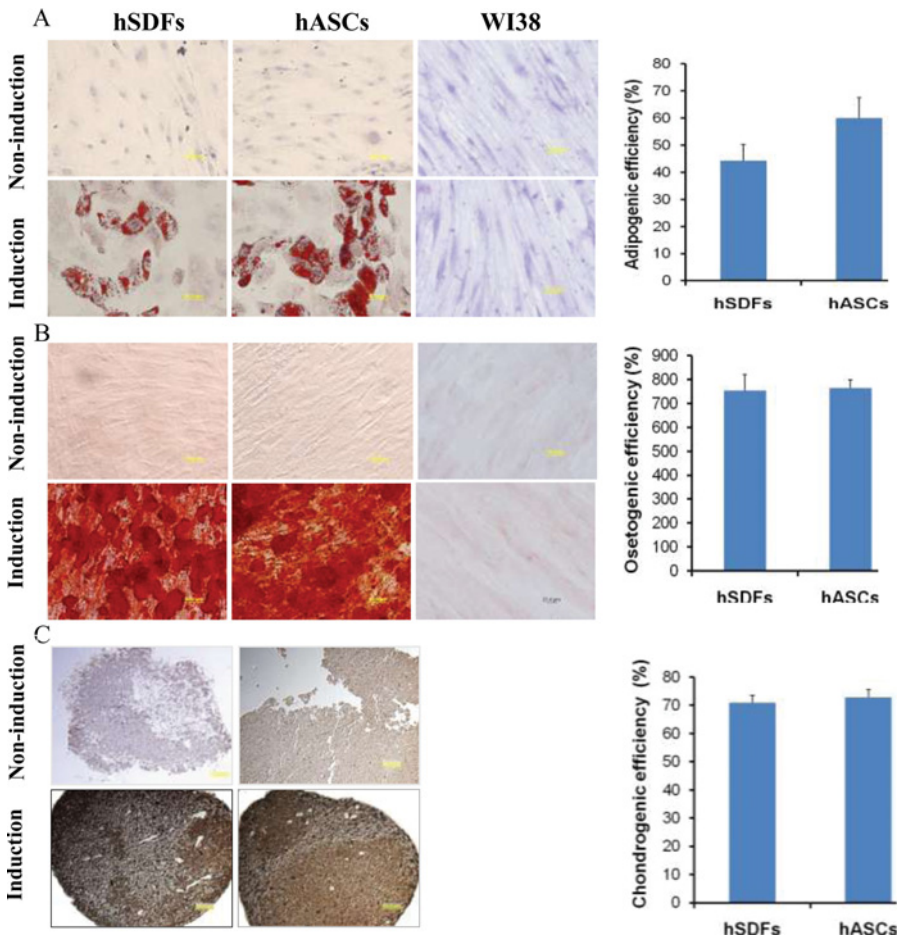
Figure 3 | Real-time PCR analysis of stem cell-associated mRNA expression in hASCs, hSDFs and WI38

(**A**) Heat map demonstrates relative gene expression values in hASCs, hSDFs and WI38. The colour represents the relative expression levels of each gene in different cell lines, with red indicating higher expression, green indicating low expression and black representing middle expression of genes. (**B**) Real-time PCR analysis demonstrates the fold changes in embryonic



stem cells marker expression of hASCs and hSDFs compared with WI38. (**C**) Real-time PCR results demonstrate the fold changes in the expression of other stem cell-associated genes in three cell lines. These genes were expressed at more than 3-fold in hASCs, hSDFs than in WI38, $n = 3$.

Figure 4 | Adipogenesis (A), osteogenesis (B) and chondrogenesis (C) of hSDFs, hASCs and WI38 *in vitro*
Both hSDFs (left column) and hASCs (middle column) cultured in respective induction media differentiated into adipocytes, as verified by the existence of lipid drops in cytoplasm positively stained red with Oil Red O (**A**), osteoblasts, as confirmed by calcium deposits on monolayer positively stained red by Alizarin Red S dye (**B**), chondrocytes expressing collagen II (a specific marker of chondrocytes) showing brown signals after immunohistochemistry staining (black signals represent nuclei stained by haematoxylin) (**C**). However, WI38 cultured in any induction media and all cell types cultured in control media did not show any differentiation signals. Scale bar = 50 μm (**A–C**). hASCs showed higher adipogenic efficiency than did hSDFs ($P < 0.05$). No significant differences of osteogenenic and chondrogenenic potential between hSDFs and hASCs were observed.



These single cell-derived hASCs and hSDFs could differentiate into adipocytes and osteoblasts (Figure 5).

Secretion of angiogenic growth factors

The expression of five angiogenic growth factors in hSDFs, hASCs and WI38 was analysed using real-time PCR. These growth factors are VEGFA (vascular endothelial growth factor A), VEGFB (vascular endothelial growth factor B), VEGFC (vascular endothelial growth factor C), HGF (hepatocyte growth factor) and FGF4. The expression of VEGFA, VEGFB, VEGFC and FGF4 was

Figure 5 | Adipogenesis and osteogenesis of single cell-derived hSDFs and hASCs *in vitro*

Single cell-derived hSDFs and hASCs differentiated into adipocytes and osteoblasts. Scale bar = 50 μm.

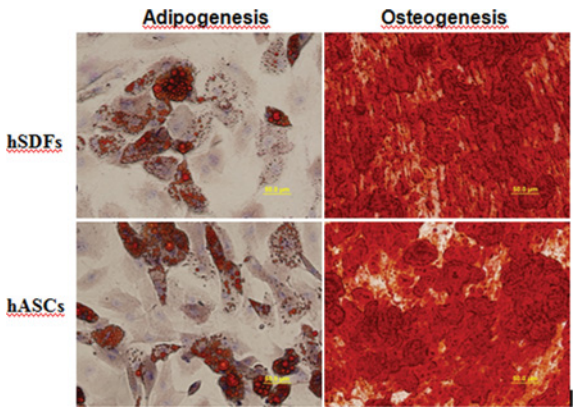
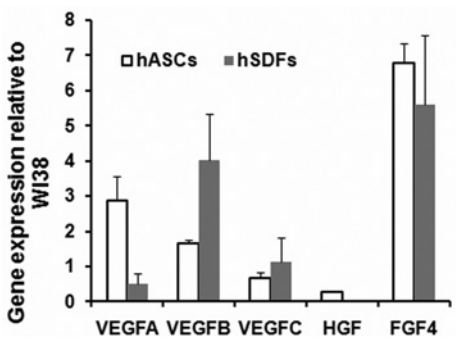


Figure 6 | Secretion of angiogenic growth factors in hSDFs, hASCs and WI38 *in vitro* by real-time PCR analysis

The expression of VEGFA, VEGFB, VEGFC and FGF4 is higher in hASCs and hSDFs than in WI38. However, there is no difference between the HGF expression in hASCs, hSDFs and WI38, $n = 3$.



higher in hASCs and hSDF than in WI38 (Figure 6).

Discussion

In this study, we comparatively analysed for the first time stem cell-associated antigen and gene profiles, proliferation capacity, colony-forming ability, differentiation potential and growth factor secretion of different cell populations. The major findings are: (i) skin fibroblast preparation (hSDFs), WI38 and hASCs showed a similar spindle-like morphology and expressed not only commonly accepted MSC surface markers (CD44, CD73 and CD105) (Sadat et al., 2007) but also fibroblast markers (collagen I, HSP47, vimentin, FSP and αSMA); (ii) there was a similar gene expression pattern between hASCs and hSDFs, however, only hSDFs and hASCs were able to form colonies and differentiate into tissue-specific lineages; and (iii) fibroblasts (WI38) and endothelial cells (hECs) were terminally differentiated cells, as widely accepted, and did not convert into other cell types.

As described above, hASCs expressed fibroblast makers (collagen I, HSP47, vimentin, FSP and αSMA). Collagen I is one of the primary collagens in skin. It provides structure, strength and integrity of skin and is involved in stimulating cell proliferation and differentiation, guiding cell migration and modulating cellular responses (Midwood et al., 2004). HSP47 is translated in the endoplasmic reticulum and specifically recognizes the triple helical region of collagen and is required for folding and maturation of collagen. HSP47 knockout embryos died just after 10.5 days due to the absence of functional collagen (Kubota and Nagata, 2004). Vimentin, belongs to type III intermediate filaments, play a fundamental role in maintaining mesenchymal cell shape and integrity and in stabilizing cytoskeletal interactions and cell substrate adhesion (Goldman et al., 1996). The expression of fibroblast antigens in WI38, hASCs and hSDFs might reflect their common function of collagen secretion and cell adhesion in tissue maintenance and wound healing.

Although pure fibroblasts (WI38) shared similar phenotypic features with hASCs, they did not undergo differentiation and had no colony-forming capacity as did hASCs and hSDFs. PCR array data further demonstrated the higher expression of embryonic stem cell markers in hASCs and hSDFs than in WI38. Embryonic stem cell markers such as OCT4, KLF4 and NANOG are of importance for maintaining self-renewal and pluripoteny of embryonic stem cells (Xu et al., 2009). The expression of stem cell markers and the capacity of both hSDFs and hASCs to differentiate into different types of cells indicate that both adipose tissue and skin contain multipotent stem cells. Accordingly, OCT4 positive

stem cells were also identified recently in adult human bone marrow and hair follicles (Yu et al., 2006). These cells are probably deposited in peripheral tissues during embryogenesis and sustain cell turnover in their respective tissues.

In order to evaluate the heterogenity of cells within fibroblast preparation, a commercially available skin fibroblast cell preparation and two additional samples of hSDFs prepared via the specific laboratory procedure commonly used for skin fibroblast preparation were analysed in this study. Our data indicate that there are no specific antigens that distinguish cells obtained from fibroblast preparations from WI38. However, the following experimental evidence presented in this study and other reports indicate that hSDFs contain a heterogeneous cell population, including stem cells with various levels of differentiation potential and fibroblasts with no capacity to convert into other cell types. First, the dermis is composed of various tissues including blood vessels, lymph vessels, hair follicles and sweat glands. Secondly, dermis contains WI38-like fibroblast population that are routinely accepted terminated differentiated cells with specific functions such as synthesizing extracellular matrix during development, wound healing and fibrosis processes. Thirdly, dermis also contains stem cells. Hair follicle and dermis-derived stem cells have demonstrated the capacity to differentiate into adipocytes, osteocytes or chondrocytes (Jahoda et al., 2003). Fourthly, fibroblasts are usually acquired via two different methods: (i) fibroblasts are isolated through the collagenase digestion procedure as used in this study and cultured in FBS (fetal bovine serum)-containing media (Johnen et al., 2006); (ii) dermis is cut into small pieces and cultured in FBS-containing media. A few days later, the spindle-like cells grow out of the explants and grow as a monolayer. These cells are considered to be fibroblasts (Villegas and McPhaul, 2005). Obviously, either of these two fibroblast preparation procedures leads to a heterogeneous cell population (including stem cells and fibroblasts) within the so-called fibroblast preparation. Furthermore, CFU assays in hASCs and hSDFs indicate that both hASCs and hSDFs preparations are not homogeneous. Pittenger et al. reported that skin fibroblasts did not differentiate into adipocytes, osteocytes and chondrocytes as did bone marrow-derived MSCs (Pittenger et al., 1999; Brendel et al., 2005). In their study, they used foreskin fibroblasts at passage 14 for differentiation assay. The high number of passages and different sources might explain their inability to differentiate into other cell types.

Previously published data have shown that hASCs could improve cardiac function and enhance wound repair partially via the angiogenesis pathway (Altman et al., 2010; Bai et al., 2010). Injected stem cells might secret angiogenic growth factors to promote angiogenesis in host injured tissues. In order to analyse the secretion capacity of stem cells and fibroblasts, we analysed the expression of angiogenic growth factors in different cell populations. There is higher expression of VEGF and FGF4 in hSDFs and hASCs than in WI38. In addition, previously published data by our group demonstrated that hASCs secreted CCL5 (CC chemokine ligand 5) after stimulation with breast cancer cell line MBA231 conditioned medium and promoted MBA231 invasion (Pinilla et al., 2009), suggesting that the secretion capacity of specific proteins might also be an important characteristic for stem cells. The secretion capacity of stem cells might contribute to stem cell-mediated tissue regeneration or tumorigenesis.

Li et al. investigated the relationship between MSCs and fibroblasts (Li et al., 2007). They found that bone marrow-derived MSCs differentiated into fibroblasts and the number of bone marrow-derived myofibroblasts coincided with the development of fibrosis in a mouse doxorubicin-induced nephrosis model of chronic, progressive renal fibrosis, suggesting that MSCs are able to differentiate into terminally differentiated fibroblasts.

In summary, our results indicate that commonly used parameters such as spindle-like morphology, plastic adherence or cell surface markers such as CD14, CD31, CD44, CD45, CD73 and CD105 are not capable of allowing us to discriminate between stem cells and fibroblasts. Only colony-forming capacity and differentiation potential of cells could be used to distinguish between stem cells and fibroblasts. These main findings shed new light on the commonly used definition of fibroblasts. In addition, this study provides novel insights into many past studies performed with fibroblast preparations, since the reported effects might be attributable to a great extent to stem cell content within these cell preparations.

Materials and methods

Cell isolation and culture

hASCs were a donation from InGeneron (Houston, TX, U.S.A.). Adipose tissue from three donors aged 32, 36 and 40 years old was obtained with informed consent under a tissue acquisition protocol approved by the Institutional Review Board. hASCs were prepared from lipoaspirate acquired from donors undergoing elective lipoplasty. hASCs were isolated as described previously, with minor modifications (Sadat et al., 2007). Briefly, fat tissue was minced and incubated for 90 min at 37°C on a shaker with Liberase Blendzyme 3 (Roche) at a concentration of 4 units per gram of fat tissue in PBS. The digested tissue was sequentially filtered through 100 μm and 40 μm filters (Fisher Scientific) and centrifuged at 450 g for 10 min. The supernatant containing adipocytes and debris was discarded, and the pelleted cells were washed twice with Hanks' balanced salt solution (Cellgro) and finally resuspended in growth media. Growth media contained alpha-modification of Eagle's medium (Cellgro), 20% FBS (Atlanta Biologicals), 2 mM glutamine, 100 units/ml penicillin with 100 μg/ml streptomycin (Cellgro). Adherent cells were designated hASCs and grown in culture flasks at 37°C in a humidified atmosphere containing 5% CO_2 followed by daily washes to remove red blood cells and non-attached cells. After confluence of hASCs, cells were seeded at a density of 3000 cells/cm^2 (passage 1).

hSDFs were from three samples. One was purchased from Sciencell (San Diego, CA, U.S.A.) and another two skin fibroblast preparations from the donors aged 32 and 40 years old were provided by InGeneron and isolated from human dermis according to the procedure described previously (Sorrell et al., 2007) with minor modifications. Skin was clearly separated from subcutaneous adipose, and dermis was then separated from epidermis by treatment with 50 units/ml dispase (Roche) overnight at 4°C. The dermal component was minced and incubated for 60 min at 37°C on a shaker with Liberase Blendzyme 3 (Roche) at a concentration of 4 units/g of skin tissue in PBS. The digested tissue was sequentially filtered through 100 μm and 40 μm filters and centrifuged at 450 g for 5 min. The supernatant containing debris was discarded; the pelleted cells were washed twice with Hanks balanced salt solution and finally resuspended in fibroblast medium (Sciencell). WI38, a well-defined human embryonic lung fibroblast line, was a gift from Dr Hung Mien-Chie (Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, U.S.A.) (Chen et al., 2004). WI38 were cultured in DMEM (Dulbecco's modified Eagle's medium)/F12 (Invitrogen) supplemented with 10% FBS, 2 mM glutamine and 100 units/ml penicillin with 100 μg/ml streptomycin. hECs (Cambrex) were cultured in EGM-2 medium and used as negative control for differentiation experiments. Isolated hASCs and hSDFs from passage 4 and WI38 were used in the experiments as indicated. Skin fibroblasts from Sciencell and hECs from Cambrex were passaged 4 times after they were received, and then used for the indicated experiments.

Proliferation

hASCs, hSDFs and WI38 were cultured in 25-cm^2 flasks at a density of 10000/cm^2, with the cells counted 10 days after seeding.

CFU assay

Cells were plated at a density of 500 cells in a 60-mm culture dish (17.69 cells/cm^2). The culture medium was changed every 3 days. After 14 days' culture, the cells were washed with PBS, fixed with 4% (w/v) paraformaldehyde, and then stained with saturated Methyl Violet (Sigma–Aldrich, St. Louis, MO, U.S.A.) solution at room temperature (22°C). After 20 min of staining, cells were washed with distilled water. The colonies that were greater than 50 cells were then counted. The colony-forming efficiency of cells was calculated by dividing the number of colonies per dish by the number of cells (500) seeded per dish. The CFU assay was performed in hASCs and hSDFs from three donors and performed in triplicate for WI38.

Flow cytometry

hASCs, hSDFs, WI38 and hECs were harvested by treatment with trypsin and washed twice with PBS. Cell aliquots were stained with primary antibodies or isotype-matched normal mouse IgGs (Chemicon International, Temecula, CA, U.S.A.) at room temperature for 30 min. The primary antibodies were FITC-conjugated anti-human CD31, CD44 (Chemicon), or PE (phycoerythrin)-conjugated anti-human CD73, CD105 (eBioscience, San Diego, CA, U.S.A.), CD14 and CD45 (US Biological, Swampscott, MA, U.S.A.). hECs were stained with CD45 and CD31. Flow cytometry was performed using a fluorescence-activated cell sorter (FACSCalibur, BD Biosciences, San Jose, CA, U.S.A.), and the results were analysed with Cell Quest software (Becton-Dickinson).

Immunofluorescence staining

hASCs, hSDFs and WI38 cultured on glass coverslips were washed thrice with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were then washed thrice with PBS alone or PBS containing 0.3% Triton X-100 (Sigma) and blocked with 10% donkey serum for 30 min at room temperature. After that, cells were incubated with primary antibodies in a moist chamber for 1 h at 37°C. The primary antibodies were anti-collagen I (Santa Cruz Biotechnology), OCT4 (Chemicon), αSMA, HSP47 (Abcam), vimentin, FSP (Sigma) or isotype-matched control (Chemicon). After three washes, cells were incubated with an Alexa Fluor® 594 donkey anti-mouse (or anti-rabbit, anti-goat) IgG (Invitrogen) for 1 h at room temperature. DAPI (4′,6-diamidino-2-phenylindole; Sigma) was used to stain nuclei. The images of the cells were taken under a Nikon confocal microscope.

Real-time PCR analysis

Human stem cell array (real-time primer) containing 88 primer sets was used for analysing the expression of human embryonic stem cell markers, three different germ layers (ectoderm, endoderm and mesoderm) markers, and trophoblast markers in hASCs, hSDFs and WI38. Two other embryonic stem cell markers SSEA4 (forward: 5′-TGGACGGGCACAACTTCATC-3′; reverse: 5′-GGGCAGGTTCTTGGCACTCT-3′) and KLF4 (forward: 5′-TGCTGATTGTCTATTTTTGCGTTTA-3′; reverse: 5′-GAGAAGAAACGAAGCCAAAACC-3′) were also analysed. Total RNA was isolated from hASCs, hSDFs and WI38 using an RNAqueous-micro scale RNA isolation kit (Ambion). cDNA was synthesized from 1 μg of total RNA using an iScript™

cDNA synthesis kit (Bio-Rad) in 20 μl following the manufacturer's instructions. cDNA were then subjected to PCR amplification using iQ^{TM} SYBR green supermix (Bio-Rad) in a final volume of 25 μl with each specific primer set at a final concentration of 0.1 μM using the Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories). A no cDNA template reaction served as a negative control. PCR reaction cycles were programmed as follows: 95°C for 3 min, 40 cycles of 10 s denaturation at 95°C, 30 s at 60°C, with a final melting curve analysis at the beginning of 55°C till 95°C with 0.50°C interval. Melting curve analysis was performed to confirm the occurrence of specific amplification peaks and the absence of primer–dimer formation. Three hASC or hSDF samples and WI38 in triplicate were analysed by real-time PCR. The threshold cycle (Ct) of each well was determined. ΔCt and $\Delta\Delta$Ct values of each gene were then calculated. ΔCt = Ct of gene of interest − Ct of housekeeping gene beta actin. Gene expression values were median centred and a hierarchical clustering heat map was generated by using Cluster 2.51 with Java TreeView. The expression of five angiogenic growth factors (VEGFA, VEGFB, VEGFC, HGF and FGF4) in hASCs or hSDFs versus WI38 were also analysed. The primers used were purchased from real-time primer. The relative gene expression ratio was presented by the $2^{-\Delta\Delta CT}$ formula in which $\Delta\Delta$Ct = ΔCt of hASCs (or hSDFs) – ΔCt of WI38.

Adipogenic differentiation

The cells were cultured in adipogenic media containing low-glucose DMEM supplemented with 10% FBS, 100 μM L-ascorbate acid (Sigma), 1 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine, 100 μM indomethacin and 10 μg/ml human recombinant insulin (Sigma) for 21 days. Cells of the control group were cultured in low-glucose DMEM plus 10% FBS (control medium). The media were changed every 3 days. Adipogenesis was assessed by staining with Oil Red O (Sigma).

Osteogenic differentiation

The cells were cultured in high-glucose DMEM supplemented with 10% FBS, 0.1 μM dexamethasone, 200 μM L-ascorbic acid and 10 mM β-glycerol phosphate (Sigma). Media were changed every 3 days for 3 weeks. Cells of the control group were cultured in high-glucose DMEM plus 10% FBS for 3 weeks. To assess mineralization, calcium deposits in cultures were stained with Alizarin Red S (Sigma).

Chondrogenic differentiation

Cells (5×10^5) suspended in a 15-ml tube were centrifuged at 800 *g* for 5 min, and the cell pellets were then incubated with chondrogenic media for 3 weeks. The media were changed every 3 days. Chondrogenic media contained high-glucose DMEM plus 10% FBS, 0.1 μM dexamethasone, 25 μg/ml ascorbate-2-phosphate, 10 ng/ml TGF-β3 (transforming growth factor-β 3; R&D), 1% ITS (Sigma). As controls, cells were cultured as pellets in 10% FBS contained DMEM. Chondrogenesis of cells was assessed by the expression of collagen II, a specific marker of chondrocytes, using primary antibody against collagen II (Chemicon) by immunohistochemical staining as described previously (Bai et al., 2004). The nuclei of the cell pellet section were counterstained with haematoxylin. The brown signal represents the signals of collagen II secreted by the differentiated chondrocytes.

Differentiation efficiency

After the cells were cultured in induction or culture media for 3 weeks, one slide was prepared from each sample and three fields per slide were photographed. Adipogenic efficiency was determined by counting Oil Red O-positive adipocytes against overall nuclei stained with haematoxylin. Osteogenic capacity of cells was quantified by evaluation of the intensity of Alizarin Red S staining images with an image analysis program (Image J). Results are presented as a percentage of control. Chondrogenic efficiency was from the ratio between the areas with brown signals and the whole area of the pellet sections.

Clonal expansion

We were not able to perform a clonal expansion assay of WI38 due to the incapacity of forming any colonies in WI38 culture. hASCs and hSDFs were plated at 10 cells/cm^2 in a 35-mm culture plate. The cells adhered within 30 min. Six hours after plating, single cells were marked by circling the underside of the plate with a diamond marker (4 mm diameter). Fifty percent of the culture medium was exchanged every other day. When a colony was evident, it was prepared by removing the culture medium and placing a clone ring around the clone. The colony within the ring was then rinsed with PBS and subsequently detached by trypsinization for primary explants. The single cell-derived cell suspension consisting of 20–50 cells was subsequently placed into a well of a 24 well plate for expansion used for future differentiation experiment.

Data analysis

Reported values are expressed as means ± S.D.. The statistical significance of differences between groups was tested by using one-way ANOVA or Student's *t* test using SPSS software version 15. A level of $P \leqslant 0.05$ was considered to be statistically significant.

Author contribution

Xiawen Bai conceived and designed the study, acquired, analysed and interpreted data, and wrote the paper. Yasheng Yan was involved in experimental design, acquisition of data, analysis and interpretation of data, and manuscript writing. Yao-Hua Song, Andrew Altman, Sebastian Gehmert, Sanga Gehmert and Daynene Vykoukal analysed and interpreted data. Eckhard Alt conceived and designed the study, and wrote the paper.

Funding

This work was partially supported by the Alliance of Cardiovascular Researchers [grant number 543102 (to E.A.)].

References

Altman, A.M., Matthias, N., Yan, Y., Song, Y.H., Bai, X., Chiu, E.S., Slakey, D.P. and Alt, E.U. (2008) Dermal matrix as a carrier for *in vivo* delivery of human adipose-derived stem cells. Biomaterials **29**, 1431–1442

Altman, A.M., Yan, Y., Matthias, N., Bai, X., Rios, C., Mathur, A.B., Song, Y.H. and Alt, E.U. (2009) IFATS Series: Human adipose-derived stem cells seeded on a silk fibroin-chitosan scaffold enhance wound repair in a murine soft tissue injury model. Stem Cells **27**, 250–258

Altman, A.M., Gupta, V., Rios, C.N., Alt, E.U. and Mathur, A.B. (2010) Adhesion, migration and mechanics of human adipose-tissue-derived stem cells on silk fibroin-chitosan matrix. Acta Biomater. **6**, 1388–1397

Bai, X., Xiao, Z., Pan, Y., Hu, J., Pohl, J., Wen, J. and Li, L. (2004) Cartilage-derived morphogenetic protein-1 promotes the differentiation of mesenchymal stem cells into chondrocytes. Biochem. Biophys. Res. Commun. **325**, 453–460

Bai, X., Sadat, S., Gehmert, S., Alt, E. and Song, Y.H. (2007) VEGF receptor Flk-1 plays an important role in c-kit expression in adipose tissue derived stem cells. FEBS Lett. **581**, 4681–4684

Bai, X., Yan, Y., Song, Y.H., Seidensticker, M., Rabinovich, B., Metzele, R., Bankson, J.A., Vykoukal, D. and Alt, E. (2010) Both cultured and freshly isolated adipose tissue-derived stem cells enhance cardiac function after acute myocardial infarction. Eur. Heart J. **31**, 489–501

Brendel, C., Kuklick, L., Hartmann, O., Kim, T.D., Boudriot, U., Schwell, D. and Neubauer, A. (2005) Distinct gene expression profile of human mesenchymal stem cells in comparison to skin fibroblasts employing cDNA microarray analysis of 9600 genes. Gene Expr. **12**, 245–257

Buitrago, W. and Roop, D.R. (2007) Oct-4: the almighty POUripotent regulator? J. Invest. Dermatol. **127**, 260–262

Chen, J.S., Liu, J.C., Shen, L., Rau, K.M., Kuo, H.P., Li, Y.M., Shi, D., Lee, Y.C., Chang, K.J. and Hung, M.C. (2004) Cancer-specific activation of the survivin promoter and its potential use in gene therapy. Cancer Gene Ther. **11**, 740–747

Chen, F.G., Zhang, W.J., Bi, D., Liu, W., Wei, X., Chen, F.F., Zhu, L., Cui, L. and Cao, Y. (2007) Clonal analysis of nestin(−) vimentin(+) multipotent fibroblasts isolated from human dermis. J. Cell Sci. **120**, 2875–2883

Choo, A.B., Padmanabhan, J., Chin, A.C. and Oh, S.K. (2004) Expansion of pluripotent human embryonic stem cells on human feeders. Biotechnol. Bioeng. **88**, 321–331

Etheridge, S.L., Spencer, G.J., Heath, D.J. and Genever, P.G. (2004) Expression profiling and functional analysis of wnt signaling mechanisms in mesenchymal stem cells. Stem Cells **22**, 849–860

Flavell, S.J., Hou, T.Z., Lax, S., Filer, A.D., Salmon, M. and Buckley, C.D. (2008) Fibroblasts as novel therapeutic targets in chronic inflammation. Br. J. Pharmacol. **153** Suppl. 1, S241–S246

Goldman, R.D., Khuon, S., Chou, Y.H., Opal, P. and Steinert, P.M. (1996) The function of intermediate filaments in cell shape and cytoskeletal integrity. J. Cell Biol. **134**, 971–983

Izadpanah, R., Trygg, C., Patel, B., Kriedt, C., Dufour, J., Gimble, J.M. and Bunnell, B.A. (2006) Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. J. Cell Biochem. **99**, 1285–1297

Jahoda, C.A., Whitehouse, J., Reynolds, A.J. and Hole, N. (2003) Hair follicle dermal cells differentiate into adipogenic and osteogenic lineages. Exp. Dermatol. **12**, 849–859

Johnen, C., Hartmann, B., Steffen, I., Brautigam, K., Witascheck, T., Toman, N., Kuntscher, M.V. and Gerlach, J.C. (2006) Skin cell isolation and expansion for cell transplantation is limited in patients using tobacco, alcohol, or are exhibiting diabetes mellitus. Burns **32**, 194–200

Kalluri, R. and Zeisberg, M. (2006) Fibroblasts in cancer. Nat. Rev. Cancer **6**, 392–401

Kim, S.W., Cho, J.H., Hong, M.W., Rhie, J.W. and Yoon, H.R. (2008) Induction of chondrogenic differentiation in cultured fibroblasts isolated from the inferior turbinate. Otolaryngol. Head Neck Surg. **139**, 143–148

Kubota, H. and Nagata, K. (2004) Roles of collagen fibers and its specific molecular chaperone: analysis using HSP47-knockout mice. Biol. Sci. Space **18**, 118–119

Li, J., Deane, J.A., Campanale, N.V., Bertram, J.F. and Ricardo, S.D. (2007) The contribution of bone marrow-derived cells to the development of renal interstitial fibrosis. Stem Cells **25**, 697–706

McAnulty, R.J. (2007) Fibroblasts and myofibroblasts: their source, function and role in disease. Int. J. Biochem. Cell Biol. **39**, 666–671

Midwood, K.S., Williams, L.V. and Schwarzbauer, J.E. (2004) Tissue repair and the dynamics of the extracellular matrix. Int. J. Biochem. Cell Biol. **36**, 1031–1037

Pinilla, S., Alt, E., Abdul Khalek, F.J., Jotzu, C., Muehlberg, F., Beckmann, C. and Song, Y.H. (2009) Tissue resident stem cells produce CCL5 under the influence of cancer cells and thereby promote breast cancer cell invasion. Cancer Lett. **284**, 80–85

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S. and Marshak, D.R. (1999) Multilineage potential of adult human mesenchymal stem cells. Science **284**, 143–147

Richardson, G.D., Arnott, E.C., Whitehouse, C.J., Lawrence, C.M., Hole, N. and Johada, C.A. (2005) Cultured cells from the adult human hair follicle dermis can be directed toward adipogenic and osteogenic differentiation. J. Invest. Dermatol. **124**, 1090–1091

Sadat, S., Gehmert, S., Song, Y.H., Yen, Y., Bai, X., Gaiser, S., Klein, H. and Alt, E. (2007) The cardioprotective effect of mesenchymal stem cells is mediated by IGF-I and VEGF. Biochem. Biophys. Res. Commun. **363**, 674–679

Sorrell, J.M., Baber, M.A. and Caplan, A.I. (2007) Clonal characterization of fibroblasts in the superficial layer of the adult human dermis. Cell Tissue Res. **327**, 499–510

Strakova, Z., Livak, M., Krezalek, M. and Ihnatovych, I. (2008) Multipotent properties of myofibroblast cells derived from human placenta. Cell Tissue Res. **332**, 479–488

Strutz, F., Okada, H., Lo, C.W., Danoff, T., Carone, R.L., Tomaszewski, J.E. and Neilson, E.G. (1995) Identification and characterization of a fibroblast marker: FSP1. J. Cell Biol. **130**, 393–405

Villegas, J. and McPhaul, M. (2005) Establishment and culture of human skin fibroblasts. Curr. Protoc. Mol. Biol, **Chapter 28**, Unit 28.23

Xu, B., Zhang, K. and Huang, Y. (2009) Lin28 modulates cell growth and associates with a subset of cell cycle regulator mRNAs in mouse embryonic stem cells. RNA **15**, 357–361

Yu, H., Fang, D., Kumar, S.M., Li, L., Nguyen, T.K., Acs, G., Herlyn, M. and Xu, X. (2006) Isolation of a novel population of multipotent adult stem cells from human hair follicles. Am. J. Pathol. **168**, 1879–1888

Received 28 September 2010/31 January 2011; accepted 18 February 2011

Published as Immediate Publication 18 February 2011, doi:10.1042/BC20100117