Clonal analysis of nestin$^-$ vimentin$^+$ multipotent fibroblasts isolated from human dermis

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Summary

Although several studies have shown that dermal fibroblasts possess adipogenic, osteogenic or chondrogenic differentiation potential, no study has characterized this cell population in detail, and there is as yet no evidence that a single dermal fibroblast can differentiate into all these types of cells. In this study, dermal fibroblasts were isolated from human foreskin using a regular dermal fibroblast culture system. These cells could be expended in adherent culture for over 40 cell doublings. In addition, dermal fibroblasts exhibited adipogenic, osteogenic and chondrogenic phenotypes when they were cultured in the presence of certain inducers. Importantly, clonal analysis showed that 6.4% (3/47) of the single-cell-derived clones were tripotent, 19.1% (9/47) of the clones were bipotent, and 10.6% (5/47) of the clones were unipotent. Furthermore, one of the three tested tripotent clones exhibited neurogenic and hepatogenic differentiation potential. Phenotypic analyses showed that the tripotent fibroblasts were nestin$^-$ vimentin$^+$, which is different from the dermis-derived stem cells reported by others. These results indicate that dermal fibroblasts are a heterogeneous population containing progenitors with various levels of differentiation potential, and the nestin$^-$ vimentin$^+$ fibroblasts may represent a novel type of multipotent adult stem cells in human dermis.

Key words: Multipotency, Dermal fibroblasts, Foreskin, Single cell cloning

Introduction

Multipotent adult stem cells isolated from postnatal tissues have attracted much attention in the past few years. The most obvious therapeutic use of these precursors is for cell transplantation and replacement. Multipotent adult stem cells seem to be almost comparable to embryonic stem cells in respect of their ability to differentiate into various cell types in vitro and in vivo. Adult mesenchymal stem cells have been identified in bone marrow (Jiang et al., 2002; Mackay et al., 1998; Muraglia et al., 2000; Pittenger et al., 1999), skeletal muscle (Asakura et al., 2001; Lee et al., 2000) and adipose tissue (De Ugarte et al., 2003; Zuk et al., 2002), as well as in cord blood (Erices et al., 2003; Lee et al., 2005). Cells isolated from those tissues are usually a heterogeneous mixture of different subpopulations. Meanwhile, studies have also demonstrated that a single mesenchymal stem cell can differentiate into multiple lineages, such as osteoblasts, chondrocytes, myocytes or even neuronal cells (Jiang et al., 2002; Muraglia et al., 2000).

Skin is the largest organ in the human or animal body. Adult skin consists of epidermis, dermis and appendages. Multipotent cell populations have been identified from different skin locations, such as epidermal stem cells from epidermis (Papini et al., 2003) and hair follicle stem cells from appendages (Rochat et al., 1994). For dermis-derived stem cells, several multipotent cell populations have been identified in rodents as well as in human beings using different culture systems (Bartsch et al., 2005; Shi and Cheng, 2004; Toma et al., 2001; Toma et al., 2005; Young et al., 1995). Toma et al. have isolated multipotent precursor cells, termed skin-derived precursors (SKPs), from mammalian dermis in a suspension culture (Toma et al., 2001; Toma et al., 2005). The characteristics of SKPs are very similar to those of embryonic neural-crest stem cells. SKPs express nestin, and can differentiate into both neural and mesodermal cell types. Recently, Bartsch et al. have isolated another multipotent population, termed dermal mesenchymal stem cells (MSC), from low-temperature preserved human foreskin biopsies by adherent culture (Bartsch et al., 2005). The isolated cells could differentiate into mesodermal lineages including adipocytes, osteocytes and myocytes. Obviously, various cell populations can be obtained from dermis with different culture systems.

Dermal fibroblasts are the major cell type in dermis and are generally thought as nearly terminally differentiated cells. Interestingly, some researchers found that dermal fibroblasts also present adipogenic (Jeney et al., 2000), osteogenic (Jeney et al., 2000; Rutherford et al., 2002; Xu et al., 1993) or chondrogenic (Mizuno and Glowacki, 2005; Yates and Glowacki, 2003) potential when they were induced by certain factors. However, no groups have characterized this cell population in detail, and no study has demonstrated that a single dermal fibroblast can differentiate into all these types of cells. In the current study, dermal fibroblasts were isolated from human foreskin dermis using a regular dermal fibroblast culture system. The subpopulations of dermal fibroblasts have been dissected in detail based on the clonal analysis of their differentiation potential.

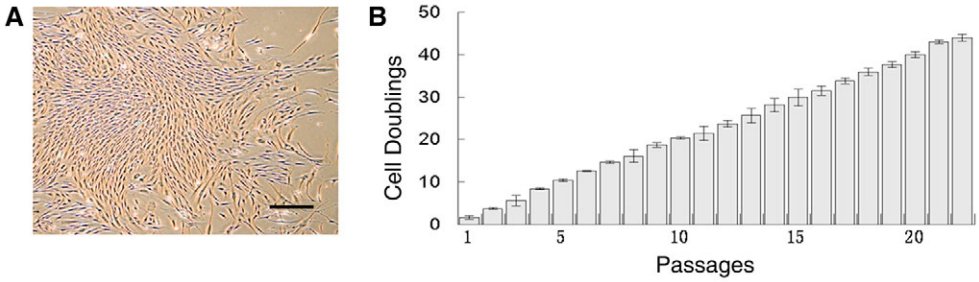

Fig. 1. Characterization of dermal fibroblasts. (A) Morphology of dermal fibroblasts under light microscopy. (B) Cumulative population doubling curve generated from cell counting after each passage (n=5, see Materials and Methods). Bar, 250 μm.

Results

Isolation and characterization of dermal fibroblasts

Human foreskin samples from patients aged from 6 to 12 years were digested by collagenase and cultured on tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). After 24 hours, few cells attached to the plates, the non-adherent cells were removed by medium change. After 7 days, the adherent cells reached 70-80% confluence with spindle-shape morphology (Fig. 1A). Cells could be continuously passaged every 4 days for over 20 passages. The cumulative population doubling curve achieved from cell counting showed that cells divided approximately twice during each passage. The population doubling ratio was stable even after 20 passages (Fig. 1B). Cells isolated from donors with different ages did not seem to have significant differences in growth rate and proliferation capacity (data not shown).

To further characterize the dermal fibroblasts, the expressions of a spectrum of cell surface markers, which have been utilized to identify mesenchymal progenitors from bone marrow or other connective tissues, were analyzed by flow cytometry at passage three. As shown in Fig. 2, cells expressed CD13 (62.1±7.26%), CD29 (53.2±14.68%), CD49d (38.4±7.44%), CD105 (30.9±7.12%), Stro-1 (8±2.14%) and a low level of CD34 (1.7±0.67%), but did not express CD45 (0.7±0.23%), CD106 (0.8±0.18%) or CD133 (0.5±0.3%). Interestingly, the expression profile was very similar to what has been reported in human processed lipoaspirate (PLA) cells (Zuk et al., 2002).

Multilineage differentiation of dermal fibroblasts

Because dermal fibroblasts present a surface marker expression profile similar to that of PLA cells, we therefore further investigated whether dermal fibroblasts also contain cells with multilineage differentiation potential as PLA cells do. Cells at passage three were examined for their adipogenic, osteogenic and chondrogenic differentiation potential.

Adipogenic differentiation

After being cultured in adipogenic medium for 3 weeks, approximately 20% of the dermal fibroblasts began to form small intracellular translucent vacuoles, which then gradually filled the cytoplasm along the cell membrane. Oil Red O staining demonstrated that they exhibited the adipogenic phenotype (Fig. 3A). When cells were cultured in regular culture medium, very few cells showed phenotypic changes with weak staining of Oil Red O (Fig. 3A). In addition, the adipogenic differentiation of dermal fibroblasts was also confirmed at mRNA level by reverse transcription (RT)-PCR analysis. After 2 weeks of adipogenic induction, mRNA expression of peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) and leptin were observed in the induced group (Fig. 3B), suggesting that some of the dermal fibroblasts have the ability to differentiate into adipocyte-like cells.

Osteogenic differentiation

After 1 week of osteogenic induction, over 30% of the cells were stained positive for alkaline phosphatase (ALP). By contrast, cells grown in regular culture medium did not show obvious ALP staining (Fig. 4A). The induced osteogenic phenotype was further confirmed by Alizarin Red (AR) staining, which detected the intracellular calcium deposition. As shown in Fig. 4B, a high level of calcium deposition was observed by the strong staining after 4 weeks of osteogenic induction. However, cells without induction stained very weakly for Alizarin Red. RT-PCR analysis showed that ALP and osteocalcin expression increased after induction (Fig. 4C).

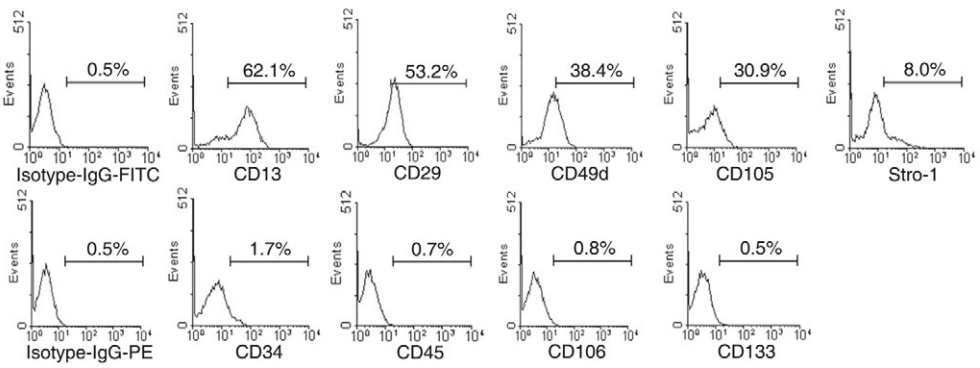

Fig. 2. Immunophenotyping of dermal fibroblasts. Dermal fibroblasts (passage three, n=3) were characterized using flow cytometry analysis for the expression of the following markers: CD13 (62.1±7.26%), CD29 (53.2±14.68%), CD49d (38.4±7.44%), CD105 (30.9±7.12%), Stro-1 (8±2.14%), CD34 (1.7±0.67%), CD45 (0.7±0.23%), CD106 (0.8±0.18%) and CD133 (0.5±0.3%). Isotype-matching IgG-FITC and IgG-PE were used to determine non-specific signals.

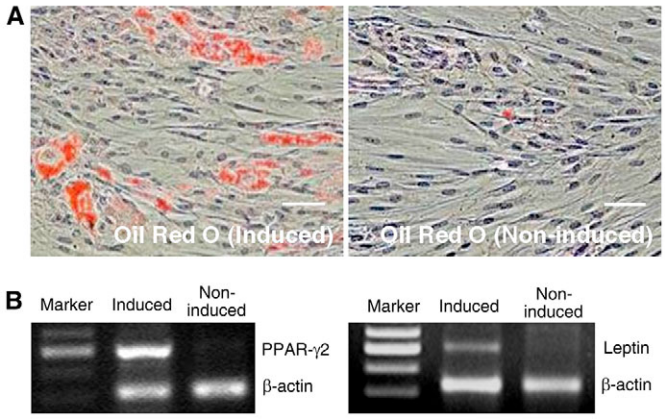

Fig. 3. Adipogenic induction of dermal fibroblasts. (A) Dermal fibroblasts were cultured in adipogenic-inducing (left panel) medium or in regular culture medium (right panel) for 3 weeks. Oil Red O staining is positive in the adipogenic-induced cells. (B) RT-PCR shows PPAR-γ2 and leptin mRNA expression in cells with adipogenic induction for 3 weeks. No expression was found in non-induced cells. Bars, 100 μm.

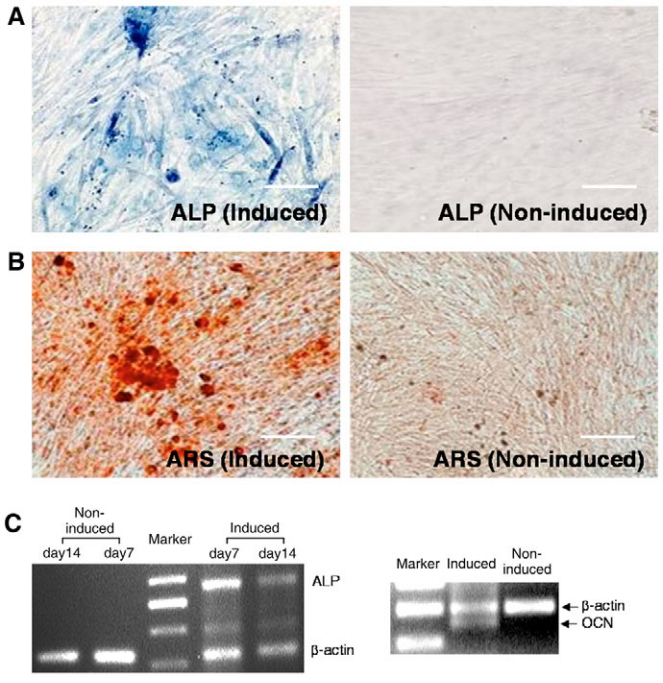

Fig. 4. Osteogenic induction of dermal fibroblasts. (A) After 1 week of osteogenic induction, positive staining of alkaline phosphatase (ALP) was observed in the induced group (left panel) but not in the non-induced group (right panel). (B) After 4 weeks of osteogenic induction, intensive calcium deposition was detected by Alizarin Red staining (ARS) in the induced group (left panel), and only very weak staining was observed in the non-induced group (right panel). (C) RT-PCR detects ALP mRNA expression after 1 and 2 weeks of induction, and osteocalcin (OCN) mRNA expression after 2 weeks of induction. No expression of ALP or OCN was observed in non-induced cells. Bars, 50 μm (A); 100 μm (B).

These results indicate that some of the dermal fibroblasts have the osteogenic differentiation potential.

Chondrogenic differentiation

Approximately 20% of the fibroblasts dramatically changed their morphology from spindle shape to paving stone-like shape and grew in a compact cluster after being treated with transforming growth factor-β1 (TGF-β1) and insulin-like growth factor-1 (IGF-1) (data not shown). To characterize the cells after induction, Safranin O staining was performed to detect the expression of proteoglycans, the extracellular matrix synthesized by chondrocytes. As shown in Fig. 5A, an intensive staining of Safranin O was observed after 2 weeks of induction. By contrast, no positive Safranin O staining could be found in non-induced cells. The chondrogenic differentiation was further confirmed by RT-PCR analysis. Aggrecan and type II collagen, which are expressed in mature chondrocytes, could be detected in the cells after 2 weeks of induction (Fig. 5C).

Studies have shown that high-density three-dimensional cell culture is more efficient than monolayer culture for chondrogenic differentiation. Therefore, cell-pellet culture was performed. Cells were pelleted by centrifugation and then cultured in chondrogenic medium or regular medium for 3 weeks. Both groups formed micromass finally. However, immunohistochemical staining showed that only the induced group and not the control group expressed type II collagen (Fig. 5B). These results demonstrate that dermal fibroblasts

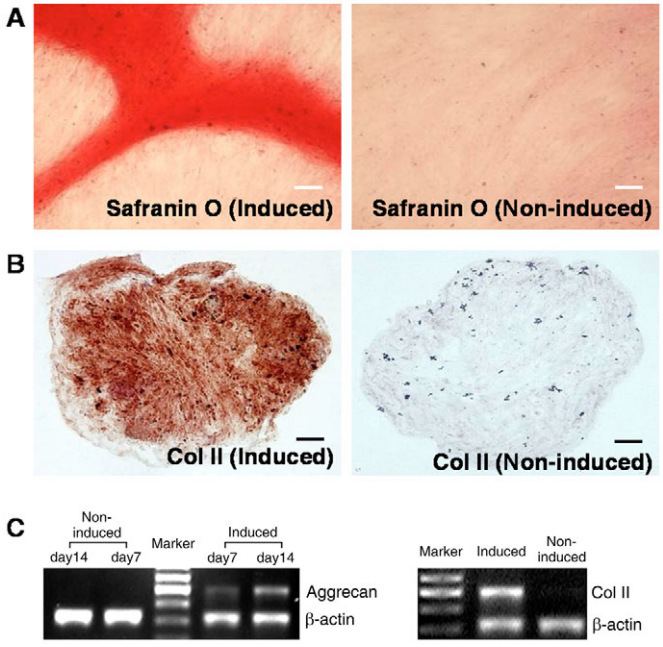

Fig. 5. Chondrogenic induction of dermal fibroblasts. (A) After 2 weeks of chondrogenic induction in plate culture, positive staining of Safranin O was observed in the induced group (left panel) but not in the non-induced group (right panel). (B) After 3 weeks of pellet culture, immunohistochemical staining showed positive staining of collagen type II (Col II) in the induced groups (left panel) but not in the non-induced group (right panel). (C) RT-PCR shows the mRNA expression of aggrecan after 1 week of induction and collagen type II mRNA expression after 2 weeks of induction. Bars, 250 μm.

Phenotype A/O/C	No. of Clones	% of total	Adipogenesis	Osteogenesis		Chondrogenesis	
			Oil Red O	ALP	ARS	Col II	Safranin O
+/+/+	3	6.4					
+/+/-	6	12.8					
-/+/+	3	6.4					
-/+/-	4	8.5					
+/-/-	1	2.1					
-/-/-	30	63.8					

Fig. 6. Differentiation potential of dermal fibroblast clones. Single-cell-derived clones were expanded for over 25 doublings and then induced in differentiation media for different time intervals as described in the Materials and Methods. Adipogenesis was evaluated by Oil Red O staining. Osteogenesis was revealed by alkaline phosphatase staining (ALP) and Alizarin Red staining (ARS). Chondrogenesis was revealed by Safranin O staining and immunostaining of type II collagen (Col II). Forty-seven clones were analyzed and numbers and percentages of different lineage types are listed. A/O/C, adipogenic/osteogenic/chondrogenic potential.

also have the chondrogenic differentiation potential.

Clonal analysis

To ask whether a single dermal fibroblast can differentiate into all cell types tested, single-cell-derived clones were established by limited dilution in a 96-well plate. From 80 plates (10 plates/donor) seeded, around 1000 wells were found to contain a single cell. After 2-3 weeks of culture, 47 single-cell-derived clones were observed and continuously expanded for over 25 cell doublings. The established clones (around 25-30 cell doublings) were then subjected to the test for their adipogenic, osteogenic and chondrogenic differentiation potential. The results were evaluated by staining with Oil Red O, AR and Safranin O, and staining for type II collagen and ALP. As shown in Fig. 6, only 6.4% (3/47) of the clones exhibited three lineages of adipogenic, osteogenic and chondrogenic differentiation potential, whereas 12.8% (6/47) of the clones exhibited two lineages of adipogenic and osteogenic potential, and 6.4% (3/47) clones exhibited osteogenic and chondrogenic potential. Interestingly, no clone was observed with both adipogenic and chondrogenic potential. Lineage-committed clones, including osteogenic (8.5%, 4/47) and adipogenic (2.1%, 1/47) clones, were also observed. However, no chondrogenically committed clone was found. In addition, 63.8% (30/47) of the clones did not show any differentiation potential that had been tested.

Three clones, which were able to differentiate into all three types of cells, are presented in Fig. 7. After 3 weeks of adipogenic induction, cells were positive for Oil Red O staining. When cells were pelleted and cultured in chondrogenic medium for 3 weeks, cartilage-like micromasses

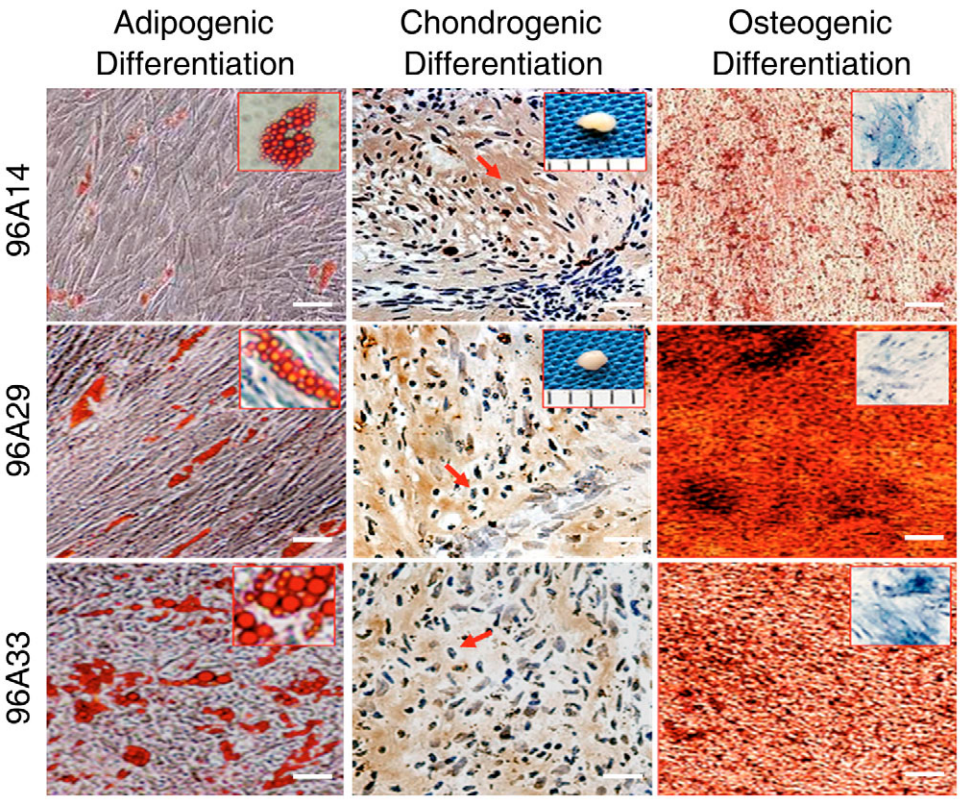

Fig. 7. Three multipotent dermal fibroblast clones exhibit trilineage potential: Clone 96A14, 96A29 and 96A33. (Left panel) Oil Red O staining after 3 weeks of adipogenic induction (upper-right corner, high magnification of one positive cell). (Middle panel) Type II collagen staining after 3 weeks of chondrogenic induction (upper-right corner, cell pellet). Red arrow shows the typical lacuna structure. (Right panel) Alizarin Red staining after 4 weeks of osteogenic induction (upper-right corner, alkaline phosphatase staining). Bars, 100 μm.

were obtained. Immunohistochemical staining showed a positive staining of type II collagen with clear lacuna structure, which is the typical morphology of chondrocytes. When cells were cultured in osteogenic medium for 4 weeks, they became positive for Alizarin Red and alkaline phosphatase staining, indicating an osteogenic differentiation.

To examine whether the tripotent clonal cells possess ectodermal and/or endodermal lineage differentiation potential, three tripotent clones were investigated for their neurogenic and hepatogenic differentiation potential. Interestingly, only one of them exhibited both neurogenic and hepatogenic differentiation potential. As shown in Fig. 8, cells switched their morphology from spindle shape to neuron-like shape and expressed neuronal markers including NF-M, NTR3 and βIII-tubulin in Clone 96A14 after neurogenic induction (Fig. 8A, upper panel), whereas no morphological change and positive staining for neuronal markers were observed in Clone 96A33 (Fig. 8A, lower panel) and Clone 96A29 (data not shown). Meanwhile hepatocyte-like cells with hepatocyte markers including ALB, CK18 and HNF-3β were observed after hepatogenic induction in Clone 96A14 (Fig. 8B, upper panel). No hepatocyte markers were positive in Clone 96A33, even though a slight morphological change could be observed after induction (Fig. 8B, lower panel). Neither morphological change nor hepatocyte markers were observed in Clone 96A29 (data not shown).

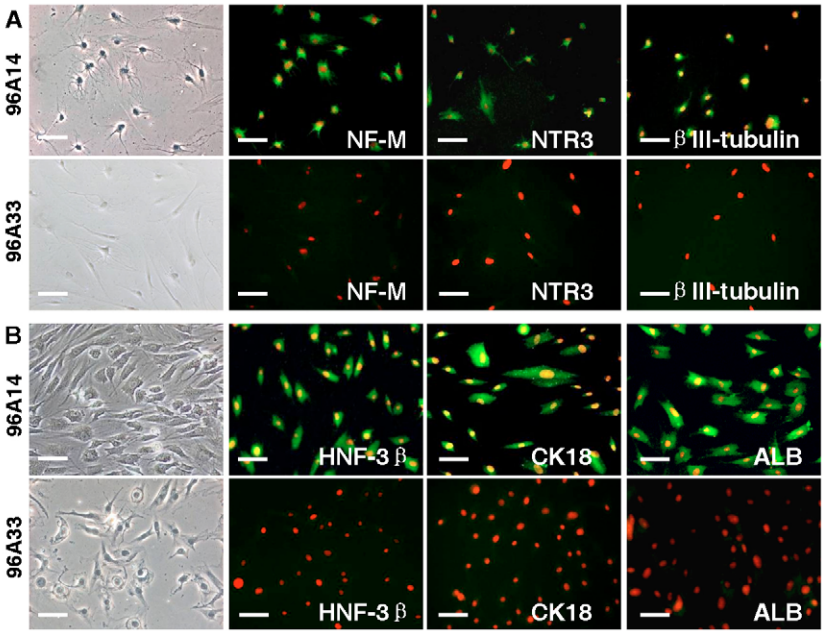

Fig. 8. One multipotent dermal fibroblast clone exhibits neurogenic and hepatogenic differentiation potentials. (A) After 10 days of neurogenic induction, cells change to a neuron-like shape and express neurofilament-M (NF-M), neurotensin receptor 3 (NTR3) and neuron-specific class III-tubulin (βIII-tubulin) in Clone 96A14, but not in Clone 96A33 or Clone 96A29 (data not shown). (B) After 3 weeks of hepatogenic differentiation, cells express albumin (ALB), CK18 and hepatocyte nuclear factor-3 beta (HNF-3β) in Clone 96A14. No hepatocyte markers are positive in Clone 96A33, even though a slight morphological change is observed. Neither morphological change nor hepatocyte markers are observed in Clone 96A29 (data not shown). Bars, 100 μm.

Once the cells have committed to a certain lineage, it is of great interest to know whether they have the ability to revert to a non-committed stage and differentiate to a new cell type. We cultured the tripotent clones in osteogenic medium for 3 weeks, and then incubated the cells with adipogenic medium for another 3 weeks. As shown in Fig. 9, parent cells could be differentiated into osteocytes (Fig. 9A) and adipocytes (Fig. 9B), respectively, whereas no Oil Red O-positive staining could be observed if the cells had been pre-induced in osteogenic medium (Fig. 9C).

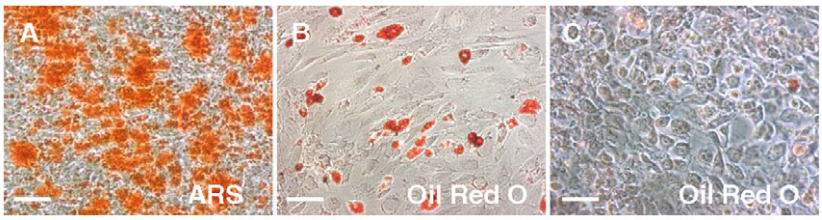

Fig. 9. Differentiation potential of lineage-committed cells. Tripotent clones were osteogenically induced for 3 weeks, followed by adipogenic induction for another 3 weeks. Parent cells show positive staining for Alizarin Red (ARS) after osteogenic induction (A), and positive staining for Oil Red O after adipogenic induction (B), whereas osteogenically induced cells fail to exhibit Oil Red O staining after switching to adipogenic medium for 3 weeks (C). Bars, 100 μm.

To compare with other multipotent cells isolated from dermis, such as the SKPs reported by Toma et al. (Toma et al., 2005), the expression of vimentin and nestin was analyzed in the tripotent clones. Immunofluorescent staining showed that all three clones were positive for vimentin but not for nestin (Fig. 10).

Taken together, the clonal analysis data indicate that dermal fibroblasts are a heterogeneous population containing progenitors with various levels of differentiation potential.

Discussion

Dermal fibroblasts are commonly accepted as terminally differentiated cells, which are routinely used as a negative control for evaluation of cell multipotency in many studies (Brendel et al., 2005; Pittenger et al., 1999). However, in the current study, we found that dermal fibroblasts isolated from human foreskin could differentiate into adipogenic, osteogenic or chondrogenic lineages in the presence of certain factors, which is consistent with what other groups have reported (Jeney et al., 2000; Mizuno and Glowacki, 2005; Rutherford et al., 2002; Xu et al., 1993; Yates and Glowacki, 2003). More importantly, by a large number of clonal analyses, we have demonstrated that dermal fibroblasts are a heterogeneous cell population that contains progenitors with various levels of differentiation potentials. These findings have never been

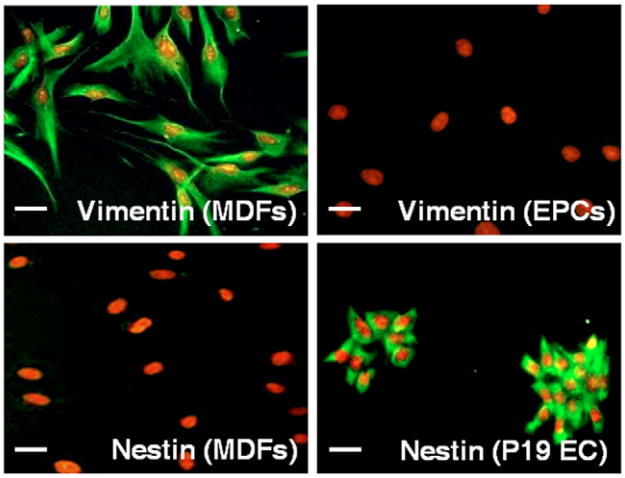

Fig. 10. Immunofluorescent staining of single-cell-derived multipotent dermal fibroblasts (MDFs). Cells were stained for vimentin (top panels) and nestin (bottom panels). Epidermal cells (EPCs) and P19 embryonal carcinoma cells (mouse neuron cell line) were used as negative and positive controls, respectively. Nuclei of all the cells are red because of the propidium iodide counterstain. Bars, 20 μm.

reported by other groups. In addition, we found that once the multipotent fibroblasts were committed to a certain lineage, these cells might be difficult to revert to a non-committed stage and then differentiate to a new cell type in currently used culture conditions. It is worth noting that the differentiation potential of dermal fibroblasts was decreased in later passages (passage 15, data not shown), indicating that regular dermal fibroblast culture conditions were not optimal for maintaining the multipotency during cell expansion. This might be the reason why in many studies, no differentiation potential of dermal fibroblasts could be observed if aged fibroblasts were used.

Previous reports have demonstrated the presence of several types of multipotent stem cells in adult dermis (Bartsch et al., 2005; Shi and Cheng, 2004; Toma et al., 2001; Toma et al., 2005; Young et al., 1995). Most of the populations have the mesodermal differentiation potential, whereas some populations can even differentiate into cells of ectoderm (neural cells) (Toma et al., 2001; Toma et al., 2005) or endoderm (insulin-producing cells) origin (Shi and Cheng, 2004). In this study, we examined the differentiation potential of clonal cells to all three germ-layer lineages. From three tripotent (mesodermal lineages) clones, only one clone exhibited both neurogenic and hepatogenic differentiation potentials, suggesting that dermal fibroblasts might contain a pluripotent subpopulation, although the frequency is extremely low. Further investigation of pluripotent potential from more tripotent cell clones and their in vivo differentiation potential should be our future aims.

The characteristics of the multipotent dermal fibroblasts isolated in this study are very different from those of SKPs that are also isolated from dermis (Toma et al., 2001; Toma et al., 2005). SKPs are cultured in suspension with medium containing epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2) and B27, which is the commonly used culture condition for neural progenitors. SKPs express nestin, fibronectin and vimentin. In addition, a single SKP can differentiate into both neural and mesodermal cell types, indicating that SKPs are very similar to embryonic neural crest stem cells. By contrast, the multipotent dermal fibroblasts are grown in adherent culture. Cells express vimentin but not nestin. In addition, some of the clonal cells could also differentiate into endodermal lineage (hepatocyte), besides the ectodermal and mesodermal lineages. The relationship between multipotent dermal fibroblasts and SKPs remains unclear and deserves further investigation.

Recently, Bartsch et al. have isolated a multipotent cell population termed dermal mesenchymal stem cells (MSC) from human foreskin biopsies (Bartsch et al., 2005). The isolated cells could be expanded for over 100 population doublings with retention of their chromosomal complement and multipotency. The group reported that a single dermal MSC could differentiate into adipogenic, osteogenic and myogenic lineages. Surprisingly, they found that all single-cell-derived clones (four clones) were multipotent. When comparing the isolation and culture condition for dermal MSCs with those for our dermal fibroblasts, we found that there are two major differences. One is that the foreskins Bartsch used had been preserved at 4°C for 6 days after harvest, whereas fresh samples were used in our study. It is possible that only very robust cell types can survive under their conditions. The other is that they cultured cells on untreated Petri dishes, whereas we cultured cells on tissue-culture dishes. It is known that only very sticky cells can attach to Petri dishes. With those two selections, it is possible that only stem cells could survive and be expanded, whereas differentiated cells would be eliminated in their culture conditions. However, our procedure would allow more cells to grow out. Therefore, a heterogeneous cell population with various levels of differentiation potential could be detected. Whether dermal MSCs are also nestin$^-$ and vimentin$^+$ is not clear. Based on their differentiation potential, we could speculate that dermal MSCs might be a subpopulation of the multipotent dermal fibroblasts found in this study.

A cell population from hair follicle, which could differentiate into adipogenic and osteogenic lineages (Jahoda et al., 2003), was isolated and recently confirmed (Hoogduijn et al., 2006). It cannot be ruled out that the dermal fibroblasts we obtained contain cells from hair follicles that are located in the dermis of foreskin. However, we have also isolated multipotent fibroblasts from the skins of foot palm, which do not have hair follicles (our unpublished data), indicating that the multipotent dermal fibroblasts are not originated from hair follicles.

The major questions raised by our findings concern the origin of the multipotent dermal fibroblasts and their physiological role in dermis. It has been reported that bone marrow stromal cells (BMSCs) can be mobilized into peripheral blood, but the frequency is extremely low (Roufosse et al., 2004; Tondreau et al., 2005). Considering that approximately 5% of dermal fibroblasts form colonies in vitro and 6% of them are multipotent, it is unlikely that the multipotent dermal fibroblasts are contaminated cells of circulating BMSCs. Zuk et al. reported that subcutaneous adipose tissue contains CD49d$^+$ and CD106$^-$ stromal cells with osteogenic, chondrogenic and adipogenic ability (Zuk et al.,

2002). However, in this study, the adipose tissue was completely removed from dermis during cell preparation, which was confirmed by histological study and RT-PCR analysis of adipocyte-specific genes (data not shown). Thus, this can rule out that the multipotent dermal fibroblasts are of adipose origin. We propose that the multipotent dermal fibroblasts originate from dermis, although no direct evidence can be provided at this moment. Despite the fact that the multipotent dermal fibroblasts were nestin$^-$ vimentin$^+$, these markers are still not specific enough to trace the cells in dermis. Further identification of unique markers for this population is under investigation. This would also be beneficial for further enrichment of progenitors from a pooled population. The physiological role of the multipotent dermal fibroblasts in dermis remains to be defined. Obviously, the osteogenic, chondrogenic and hepatogenic differentiation potentials are not necessary for repairing skin injury. It is likely that skin may serve as an adult stem cell reservoir, as other researchers have proposed (Bartsch et al., 2005; Chunmeng and Tianmin, 2004; Shi and Cheng, 2004).

In summary, this is the first study demonstrating that dermal fibroblasts contain heterogeneous progenitors with different levels of differentiation potential, and that the nestin$^-$ vimentin$^+$ fibroblasts may represent a novel type of multipotent adult stem cells in human dermis. Although the potential physiological role of the multipotent dermal fibroblasts in dermis is unclear, the multipotency of these cells suggests that they might be a useful cell source for therapeutic purposes.

Materials and Methods

Cell preparation and culture

Fresh human foreskin specimens were obtained from 8 donors aged from 6 to 12 years, who received a routine circumcision procedure at Shanghai Children's Hospital, China. The specimens were washed with sterile phosphate-buffered saline (PBS), and the subcutaneous tissues were removed carefully. The skins were then cut into small pieces (1-2 mm^3). After being digested with 0.1% dispase (Worthington Biochemical Corporation, Lakewood, NJ) at 4°C overnight, the epidermal layers were removed, and the remaining dermal parts were further digested with 0.1% collagenase I (Worthington) at 37°C for another 4 hours. The digested cells were then passed through a 70-μm cell strainer (BD Biosciences, Mississauga, ON, Canada), centrifuged, and resuspended in high-glucose DMEM (Invitrogen Corporation, Carlsbad, CA), supplemented with 10% FBS (HyClone, Logan, UT), 300 μg/m1 L-glutamine, 50 μg/ml vitamin C, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma, St Louis, MO). Cells were seeded on tissue culture plates (Falcon Labware; Becton-Dickinson, Franklin Lakes, NJ) at 1×10^3 cells/cm^2, and maintained at 37°C with 5% CO_2. After 24 hours, the plates were washed with PBS to remove residual non-adherent cells. The resulting adherent cells were grown to confluency within 7 days. Cells were subcultured every 4 days using 0.25% trypsin/EDTA (Invitrogen).

For cell proliferation analysis, cells were seeded at 3000 cells/cm^2 in six-well plates, trypsinized and counted with trypan blue on day 4. Cell doublings were calculated as follows (Jahoda et al., 2003): number of divisions=$\log_2$ (number of cells at subculture/number of cells seeded).

Cell cloning

To establish single-cell-derived clones, cells from eight different donors were seeded in 96-well tissue culture plates (Falcon) at a concentration of 10 cells/ml (200 μl/well, 10 plates/donor). After 24 hours the plates were scored under the microscope. Wells containing only one cell were marked for further analysis. Cells were kept in the original culture medium at 37°C with 5% CO_2. After 2-3 weeks, the single-cell-derived clones were then harvested and transferred to 24-well tissue culture plates (Falcon) for expansion. When cultures reached confluence in the 24-well plates, cells were then passaged to six-well tissue culture plates (Falcon) and further expanded in the original medium. In total, 47 clones were established, and their differentiation potential was then analyzed in detail.

Flow cytometry analysis

Cells at passage three were trypsinized, centrifuged and half-a-million cells were resuspended in 100 μl of wash buffer (PBS containing 4% FBS). Freshly diluted fluorescein isothiocyanate (FITC)-conjugated anti-CD13 (1:100), anti-CD29 (1:100), anti-CD45 (1:100; all from BD Pharmingen, San Diego, CA), anti-CD34 (1:100; Dako, Carpentaria, CA), anti-CD133 (1:100; R&D Systems, Minneapolis, MN), and phycoerythrin (PE)-conjugated anti-CD49d (1:100; BD Pharmingen), anti-CD105 (1:100; R&D Systems) and anti-CD106 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal antibodies were added into each of the tubes and incubated on ice for 20 minutes. After three washes, cells were resuspended in wash buffer for analysis. When cells were stained with non-labeled anti-Stro-1 antibody (1:100; R&D Systems), FITC-conjugated goat anti-rat IgM (1:200; MilleGen Prologue Biotech, Labege Cedex, France) was used to amplify the signals. FITC- or PE-conjugated isotype-matching immunoglobulins were used to determine non-specific staining. Cells were analyzed on a FACS Caliber (Becton-Dickinson, San Jose, CA), and the data were analyzed with CellQuest software (Becton-Dickinson).

Multilineage differentiation of dermal fibroblasts

Pooled dermal fibroblasts at passage three or single-cell-derived clones were analyzed for their capacity to differentiate toward adipogenic, osteogenic or chondrogenic lineages. In addition, tripotent clones were tested for their neurogenic and hepatogenic potentials. Each experiment was repeated at least three times for cells from each donor and once for each clone.

Adipogenic differentiation

Adipogenic differentiation was induced by culturing cells on 22-mm microscopy cover slips at a density of 3000 cells/cm^2 in adipogenic medium (low-glucose DMEM supplemented with 10% FBS, 1% antibiotics, 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μM insulin and 200 μM indomethacin; all from Sigma) for 3 weeks. Cells cultured in the regular culture medium were used as a control. The medium was changed every 3 days.

Osteogenic differentiation

For osteogenic differentiation, cells were seeded at a density of 3000 cells/cm^2 and were cultured in osteogenic medium (low-glucose DMEM supplemented with 10% FBS, 1% antibiotics, 0.01 μM 1,25-dihydroxyvitamin D3, 50 μM ascorbate-2-phosphate and 10 mM β-glycerophosphate; all from Sigma) for a maximal period of 4 weeks. The medium was changed every 3 days. As a control, dermal fibroblasts were cultured in the regular culture medium.

Chondrogenic differentiation

To acquire chondrogenic differentiation, dermal fibroblasts were grown in both monolayer and pelleted micromass culture. The cultures were maintained within a chondrogenic medium [low-glucose DMEM supplemented with 10% FBS, 1% antibiotics, 50 μM scorbate-2-phosphate (Sigma), 10 ng/ml TGF-β1 (R&D Systems) and 500 ng/ml IGF (R&D Systems)] for 3 weeks. Medium change was performed every 3 days. Cells maintained within the regular culture medium were used as a control.

Neurogenic differentiation

Neurogenic differentiation was performed as previously described. Briefly, cells were seeded at 1×10^3 cells/cm^2 on fibronectin-coated dishes, incubated with sequential changes of neurogenic medium for up to 10 days (D'Ippolito et al., 2004). Cells maintained within the regular culture medium were used as a control.

Hepatogenic differentiation

Hepatogenic differentiation was performed as in the method described by Schwartz et al. (Schwartz et al., 2002). Cells were seeded at 4×10^3 cells/cm^2 on fibronectin-coated dishes, pre-induced for 10 hours followed by culturing with hepatogenic medium for up to 3 weeks. Medium change was performed every 2 days (Schwartz et al., 2002). Cells maintained within the regular culture medium were used as a control.

To examine whether the cells once committed to a certain lineage have the ability to revert to a non-committed stage and differentiate to a new cell type, cells were cultured in osteogenic medium for 3 weeks, followed by incubating with adipogenic medium for another 3 weeks.

Immunofluorescent staining

Single-cell-derived clones and neurogenic or hepatogenic differentiated cells were fixed with 4% cold paraformaldehyde (Sigma) in PBS for 15 minutes on chamber slides, and permeabilized with 0.25% Triton X-100 (Sigma) in PBS for 10 minutes. After three washes with PBS, cells were blocked with blocking buffer containing 5% bovine serum albumin (BSA, Invitrogen) and 10% FBS in PBS for 1 hour, followed by incubating with primary antibodies diluted in PBS at 4°C overnight. After removal of primary antibodies, cells were washed three times with PBS, and the appropriate secondary antibodies conjugated to FITC were added and incubated for 1 hour at room temperature. Cells were washed with PBS, and mounted with a solution containing propidium iodide to detect nuclei (VectaShield; Vector Labs, Burlingame, CA). Anti-vimentin (1:100), anti-nestin (1:100), anti-neurofilament-M (NF-M, 1:100), anti-neurotensin receptor 3 (NTR3, 1:100), anti-albumin (ALB, 1:100), anti-CK18 (1:100) and anti-hepatocyte nuclear factor-3 beta (HNF-3β, 1:100) monoclonal antibodies and secondary antibodies (1:200) were all purchased

Table 1. Primers used in RT-PCR analysis

Gene	Primer sequence	Number of cycles	Annealing temperature (°C)	Product size (bp)
PPAR-γ2	5′-GGTCAGCGGGAAGGACTTTA-3′ 5′-GATCCAGTGGTTGCAGATTA-3′	35	58	516
Leptin	5′-GCCAGAGTTCCTTCCCTTAA-3′ 5′-CAAGCTGTGCCCATCCAAAA-3′	35	62	508
ALP	5′-GGCGGCAGACTTTGGTTT-3′ 5′-CCCGTGGCAACTCTATCTT-3′	35	59	552
OCN	5′-ATGAGAGCCCTCACACTCCTC-3′ 5′-CGTAGAAGCGCCGATAGGC-3′	35	56	292
Aggrecan	5′-TCCTGGAAGCTCTTCTCAGT-3′ 5′-ATGCCCAAGACTACCAGTGG-3′	35	60	500
Col II	5′-CTTGGGCACCTCGGGCTCCTTTAG-3′ 5′-TCCCCGGCACTCCTGGCACTGAT-3′	35	63	510
β-actin	5′-CATCTCTTGCTCGAAGTCCA-3′ 5′-ATCATGTTTGAGACCTTCAA-3′	35	56-63	310

ALP, alkaline phosphatase; Col II, collagen type II; OCN, osteocalcin; PPAR-γ2, peroxisome proliferator-activated receptor-γ2.

from Santa Cruz. Anti-neuron-specific class III-tubulin (βIII-tubulin, 1:100) monoclonal antibody was purchased from Chemicon (Temecula, CA). Human epidermal cells and mouse neuron cell line P19EC cells (a gift from Nai He Jin, Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Shanghai, China) were used as negative and positive controls, respectively.

Histochemical and immunohistochemical analyses

For Oil Red O staining, cells cultured on the cover glass were rinsed with PBS, fixed in 4% cold paraformaldehyde (Sigma) for 10 minutes and washed with 60% isopropyl alcohol (Sigma). Cells were then incubated in 2% (w/v) Oil Red O reagent (Sigma) for 5 minutes at room temperature. Excess stain was removed by washing with 60% isopropyl alcohol, followed by several washes with distilled water. Cells were then counter-stained with hematoxylin for 2 minutes. The adipogenic differentiation percentage was determined by counting Oil Red O-positive and -negative cells under a microscope. At least three fields (around 300 cells) were counted in each sample.

To detect alkaline phosphatase (ALP) activity, cells cultured on the cover glass were fixed with 0.4% cold paraformaldehyde for 10 minutes, rinsed with ALP solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$) and stained with a 1% BM Purple solution (Roche Molecular Biochemicals, Indianapolis, IN) at 37°C for 30 minutes. For ARS, cells were fixed with 4% cold paraformaldehyde for 10 minutes, washed with distilled water and then stained with Alizarin Red solution (40 mM Alizarin Red-Tris-HCL, pH 4.1; Sigma) at room temperature for 10 minutes. After three washes with distilled water, cells were mounted with glycerol. The osteogenic differentiation percentage was determined by counting ALP-positive and -negative cells under a microscope. At least three fields (around 300 cells) were counted in each sample.

The chondrogenic differentiation percentage was estimated by counting paving stone-like cells and spindle-shaped cells under a microscope without specific staining. At least three fields (around 300 cells) were counted in each sample. For Safranin O staining, cells were fixed in 10% formalin for 10 minutes, rinsed with distilled water and stained with 6% Safranin O (Sigma) for 2 minutes. To detect the expression of type II collagen, the induced micromasses were frozen in OCT gel, sliced into 5-μm-thick sections and subjected to immunofluorescent staining as described above. Cells were incubated with anti-human type II collagen antibody (1:100; ICN Biomedical, Costa Mesa, CA) at 4°C overnight. After three washes with PBS, the type II collagen was detected using the mouse EnVision$^+$ system (Dako).

RT-PCR

Total RNA was extracted with Tryzol (Invitrogen) and reverse transcribed into cDNA with an RT-PCR kit (TaKaRa, Shiga, Japan). The primer sequences (all from Bao Technology Co., Shanghai, China), reaction conditions and the sizes of each product are listed in Table 1. The amplified products were separated on 1.2% agarose gel and visualized with ethidium bromide (Sigma).

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