

Dedifferentiation derived cells exhibit phenotypic and functional characteristics of epidermal stem cells

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Abstract

Differentiated epidermal cells can dedifferentiate into stem cells or stem cell-like cells *in vivo*. In this study, we report the isolation and characterization of dedifferentiation-derived cells. Epidermal sheets eliminated of basal stem cells were transplanted onto the skin wounds in 47 nude athymic (BALB/c-nu/nu) mice. After 5 days, cells negative for CK10 but positive for CK19 and β_1 -integrin emerged at the wound-neighbouring side of the epidermal sheets. Furthermore, the percentages of CK19 and β_1 -integrin⁺ cells detected by flow cytometric analysis were increased after grafting ($P < 0.01$) and CK10⁺ cells in grafted sheets decreased ($P < 0.01$). Then we isolated these cells on the basis of rapid adhesion to type IV collagen and found that there were 4.56% adhering cells (dedifferentiation-derived cells) in the grafting group within 10 min. The *in vitro* phenotypic assays showed that the expressions of CK19, β_1 -integrin, Oct4 and Nanog in dedifferentiation-derived cells were remarkably higher than those in the control group (differentiated epidermal cells) ($P < 0.01$). In addition, the results of the functional investigation of dedifferentiation-derived cells demonstrated: (1) the numbers of colonies consisting of 5–10 cells and greater than 10 cells were increased 5.9-fold and 6.7-fold, respectively, as compared with that in the control ($P < 0.01$); (2) more cells were in S phase and G2/M phase of the cell cycle (proliferation index values were 21.02% in control group, 45.08% in group of dedifferentiation); (3) the total days of culture (28 days *versus* 130 days), the passage number of cells (3 passages *versus* 20 passages) and assumptive total cell output (1×10^5 cells *versus* 1×10^{12} cells) were all significantly increased and (4) dedifferentiation-derived cells, as well as epidermal stem cells, were capable of regenerating a skin equivalent, but differentiated epidermal cells could not. These results suggested that the characteristics of dedifferentiation-derived cells cultured *in vitro* were similar to epidermal stem cells. This study may also offer a new approach to yield epidermal stem cells for wound repair and regeneration.

Keywords: epidermal cells • epidermal stem cells • dedifferentiation • isolation • characterization

Introduction

Epidermal stem cells, which give rise to cells of different fates including those forming hair follicles, interfollicular epidermis and associated glands such as sebaceous and sweat glands, play an important role in wound repair and tissue engineering of replacement skin [1–3]. To date, however, the limited number and capac-

ity for the isolation or purification of epidermal stem cells have hampered the widely clinical applications, which compel scientists to look for new sources of stem cells.

Studies on cellular dedifferentiation provide a new insight into the research of stem cells. Dedifferentiation means recessive differentiation, that is, the progression of cells from a more differentiated to a less differentiated state. It has been shown that dedifferentiation occurs during wound repair and regeneration of plants and various vertebrates. Especially, recent studies suggest that dedifferentiation may be also possible in mammalian system. Terminally differentiated mammalian cells are commonly thought to be incapable of undergoing a reversal of cell differentiation. However, Odelberg *et al.* [4] found that the combined effects of growth medium and ectopic *msx1* expression can cause the dedifferentiation of mammalian myotubes. Intriguingly, the succedent

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researches have shown that mammalian myotubes can dedifferentiate when stimulated with an extract prepared from newt regenerating limb tissue [5], ciliary neurotrophic factor [6] or small molecules such as reversine [7, 8]. Moreover, it was also reported that skin cells, such as fibroblasts, were reprogrammed to an embryonic-like state by the injection of a nucleus into an enucleated oocyte [9, 10] or ectopic expression of some transcription factors including Oct4, Sox2, c-myc, Klf4, Nanog and LIN28 [11, 12]. These observations indicate that differentiated mammalian cells can dedifferentiate when stimulated with the appropriate signals. So, it is conceivable that healthy, abundant stem cells could be generated with an efficient dedifferentiation process, which offers a new approach to yield stem cells for repair of damaged tissues.

Certainly, dedifferentiation of epidermal cells can offer seed cells – epidermal stem cells – for repair and regeneration of skin wound. Pivarcsi *et al.* [13] demonstrated that serum factors can induce the dedifferentiation of cultured HaCaT keratinocytes. In addition, Fu *et al.* and Li *et al.* [14–16] reported that the differentiated epidermal cells in the wound treated with recombinant human epidermal growth factor (rhEGF) dedifferentiated into stem cells or stem cell-like cells *in vivo*. However, the finding of the differentiated cell dedifferentiation was just preliminary. Moreover, the dedifferentiation-derived cells had never been isolated and the characteristics of these cells cultured *in vitro*, such as the expression of stem cell markers, the clonogenicity, the proliferative potential, and the ability to ‘regenerate itself’ – *i.e.* generate a tissue-engineered skin – remain to be fully understood. We, therefore, further investigated the dedifferentiation of differentiated epidermal cells and isolated the dedifferentiation-derived cells for identification of phenotypic and functional characteristics.

Materials and methods

Preparation of ultrathin epidermal sheets

Human foreskin specimens were digested at 4°C with 2 mg/ml protease (Sigma, St Louis, MO, USA) for 10–12 hrs and then the epidermis was isolated from dermis. The isolated epidermis, with the stratum basale downwards, was anchored to the bottom of culture dishes coated with collagen type IV (100 µg/ml; Sigma) for 10 min., and flushed with Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA), this process being repeated three to four times to eliminate the cells of the stratum basale [16].

Xenografting of ultrathin epidermal sheets

Forty-seven female nude athymic (BALB/c-nu/nu) mice from academy of military medical sciences (Beijing, China), weighting 20 ± 2 g, were anaesthetized by ethylether inhalation. After the skin was sterilized with 75% alcohol, a sterile template of 1.0 cm in diameter was placed on the side of the mid-back and a full-thickness wound (to deep fascia) corresponding to

the template was made by excising the skin. Ultrathin epidermal sheets were transplanted, with collagen type IV treated side downwards (wound-neighbouring side), onto the wounds. After 5 days, some grafted ultrathin epidermal sheets were taken away for immunohistochemical detections of CK10, CK19 and β_1 -integrin, the others were for flow cytometric analysis.

Immunohistochemistry

Formalin fixed and paraffin embedded epidermal sheet samples were cut into 5-mm-thick sections, deparaffinized in xylene, rehydrated in graded ethanol, and then incubated in 1% H₂O₂ to block endogenous peroxidase activity. After that, the sections were incubated with primary mouse monoclonal anti-human CK10, CK19 and β_1 -integrin antibodies (1:500; Chemicon, Temecula, USA) overnight at 4°C, washed three times with phosphate buffered saline (PBS) and then incubated with horseradish peroxidase conjugated goat antimouse IgG (1:100; Vector, Loerrach, Germany) for 2 hrs at room temperature. The immunoreactivity of CK10, CK19 and β_1 -integrin was visualized as a brown precipitate using a 3, 3V-diaminobenzidine procedure.

Flow cytometric analysis

Epidermal sheets were treated with 0.25% trypsinase and collected in 15 ml tubes. After centrifugation, the pellet was resuspended in ice-cold 2% paraformaldehyde buffer. Normal goat serum was added to block nonspecific protein interactions. The cell suspension was then incubated with primary mouse monoclonal anti-human CK10, CK19 and β_1 -integrin antibodies (1:500; Chemicon) in PBS for 1 hr (there were no antibodies in blank control), washed with PBST (phosphate buffered saline tween-20) and resuspended in 100 µl PBS containing fluorescein isothiocyanate conjugated goat antimouse secondary antibody (1:50; Santa Cruz Biotech, Santa Cruz, CA, USA) for 30 min. The percentage of CK10⁺, CK19⁺ and β_1 -integrin⁺ cells was detected by flow cytometric analysis (FACS scan; Beckton Dickinson, Franklin Lakes, NJ, USA).

Isolation and culture of epidermal cells

Briefly, ultrathin epidermal sheets (grafted or ungrafted) were cut into pieces, digested with 0.25% trypsinase for 20 min. at 37°C and made into single cell suspension. After centrifugation, the keratinocytes were gently resuspended in Epilife medium supplemented with 1% human keratinocyte growth supplement and 0.2% PSA (Cascade Biologics, Portland, OR, USA) and seeded on collagen IV coated culture flasks (Costar, Cambridge, MA, USA) at a density of 5×10^5 keratinocytes/cm². After 10 min., 3, 6, 12 or 24 hrs, non-adherent cells were gently removed and the percentages of adherent cells were calculated. A series of assays were performed on initial adherent cells in each group. Epidermal stem cells dispersed from full-thickness epidermal sheets by adhering to collagen IV for 10 min. [17] were used as the positive control for the phenotypic and functional assays of the dedifferentiation-derived cells.

PCR

The PCR reactions were performed in a total volume of 25 µl containing 2 µl DNA template, 10 pmol of each primer, 250 µM deoxynucleoside

triphosphates, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ and 0.5 U Super Taq DNA Polymerase (Eppendorf, Hamburg, Germany). The amplification profiles were as follows: one step at 94°C for 2 min., then 40 cycles 94°C for 30 sec., 58°C for 40 sec. and 72°C for 1 min. and one final step at 72°C for 10 min. The oligonucleotides used were as follows: X-chromosome sense 5'-AATCATCAAATGGAGATTG-3', antisense 5'-GTTGAGCTCTGTGAGTAAA-3', Y-chromosome sense 5'-ATGATA-GAAACGGAAATATG-3', antisense 5'-AGTAGAATCAAAGGGCTC-3', resulting in amplification of a 130-bp X-chromosome-specific band or a 170-bp Y-chromosome-specific band [18].

Phenotypic characteristics

Immunofluorescence

Cultured primary keratinocytes were plated onto collagen IV-coated glass cover slips, fixed with 4% paraformaldehyde, and then were incubated with primary mouse monoclonal anti-human CK19 and β_1 -integrin antibodies (1:500; Chemicon) for 1 hr at room temperature. The primary antibody was removed, and the slides were incubated for 30 min. with PE-conjugated goat antimouse IgG secondary antibody (1:1,000; Chemicon). The nuclei were counterstained with DAPI (Roche Diagnostics, Basel, Switzerland). Fluorescence images were visualized with a fluorescence microscope (Eclipse 80i; Nikon, Tokyo, Japan).

Quantitative real-time PCR

mRNA was isolated from initial adherent cells in each group by using the TRIzol kit (Invitrogen) and reverse-transcribed into cDNA with SuperScript III (Invitrogen) as per the manufacturer's instructions. Gene-specific primers for Oct4 (forward, 5'-GAAGTTGGAGAAGGTGGAACCA-3'; reverse, 5'-GCTTCAGCAGCTTGGCAA-3') and Nanog (forward, 5'-TCTTCTGGTCCACAGTTT-3'; reverse, 5'-GCAAGAATAGTTCTCGGGATGAA-3') were optimized for expression analyses by real-time PCR on the ABI 7500 thermocycler (Applied Biosystems, Foster City, CA, USA) using Absolute QPCR SYBR Green ROX mix (ABgene, Epsom, UK) for 2 min. at 50°C, 15 min. at 95°C, 40 cycles of 15 sec. at 95°C and 1 min. at 60°C, followed by dissociation-curve analysis to confirm specificity. EF-1 α (forward, 5'-ATTCGAGACCAGCAATACTATGTGA-3'; reverse, 5'-AGCCTGGGATGTGCCTGTAA-3') was used for normalization, and relative expression was calculated by using the comparative CT method [19].

Functional characteristics

Colony-forming efficiency

We plated 5×10^3 isolated cells, from cultured primary keratinocytes, into a six-well plate (Costar), cultured them for another 7 days in Epilife medium that was changed 4 days after plating. Under the microscope, colonies consisting of 5–10 cells and greater than 10 cells were counted separately. This assay was performed five times.

Cell cycle

We plated 5×10^4 isolated cells onto a 60-mm culture dish (Costar) and cultured them for another 3 days. After fixation with 75% ethanol, single cell suspensions were digested with DNase-free RNase in PBS containing 5 μ g/ml propidium iodide (Sigma) for DNA staining (45 min. at 37°C). The propidium iodide fluorescence and forward light scattering were detected

with a flow cytometer (FACS scan; Beckton Dickinson) equipped with Cellquest software (Cellquest Inc., Largo, FL, USA). The percentage of cells in S/G₂/M phases of the cell cycle was calculated. Proliferation index (PI) = (G₂M + S) / (G₀G₁ + S + G₂M).

Long-term growth potential

Harvested cells were seeded onto a culture vessel at a density of 5.0×10^3 cells/cm². When cell density reached 70–90% confluence, they were serially passaged (up to 1.5×10^6 cells seeded) until cells lost their proliferative capacity. The total days of culture, the passage number of cells, and assumptive total cell output were determined. Because up to 1.5×10^6 cells were re-plated at each passage, the cell outputs were calculated based on the assumption that all the cells from the previous passage had been re-plated.

Fabrication of skin equivalent

'Three-dimensional' cultivation of epidermal cells was performed as previously described [20]. Briefly, mixture of fibroblasts (1.5×10^3 cells/ml) and rat tail type I collagen (1.35 mg/ml) was moved in 35 mm plates (Costar) and cultured for 5 days. Epidermal cells (0.6×10^6 cells/ml) were seeded on the dermal equivalent surface and grew to fuse to form an epidermal equivalent, which was cultured at the air liquid interface a week later to promote the differentiation and cornification of the epidermis. After 11 days, skin equivalents were harvested and stained by haematoxylin and eosin staining.

Statistical analysis

All the experiments were repeated at least three times, unless otherwise indicated. Data are presented as mean \pm S.D. Statistical analysis was performed by t-test. A statistical probability of $P < 0.05$ was considered significant.

Results

Elimination of stem cells in the stratum basale of the epidermis

In normal epidermis, stem cells locating at the bottom of the epidermal rete ridges form a single layer in the basement membrane and can be identified by the examination of β_1 -integrin and CK19, which are believed to be possible specific markers for epidermal stem cells. Being consistent with prior studies, cells which were positive for CK19 and β_1 -integrin were found in the basal layer of full-thickness epidermal sheets (Fig. 1D and G). However, CK10, as the marker of terminally differentiated epidermal cells of the cornified layer, are negative in the basal layer and mostly expressed above the stratum basale (Fig. 1A). In contrast to full-thickness epidermal sheets, cells in ultrathin epidermal sheets fully exhibited positivity for CK10 and there were no cells positive for CK19 and β_1 -integrin (Fig. 1B, E and H). In addition, before grafting, the percentages of CK19 and β_1 -integrin⁺ cells were 0.05% and 0.03%, respectively, but there was no significant difference compared with blank control (Table 1). These results showed the successful preparation of ultrathin epidermal sheets eliminated of basal stem cells.

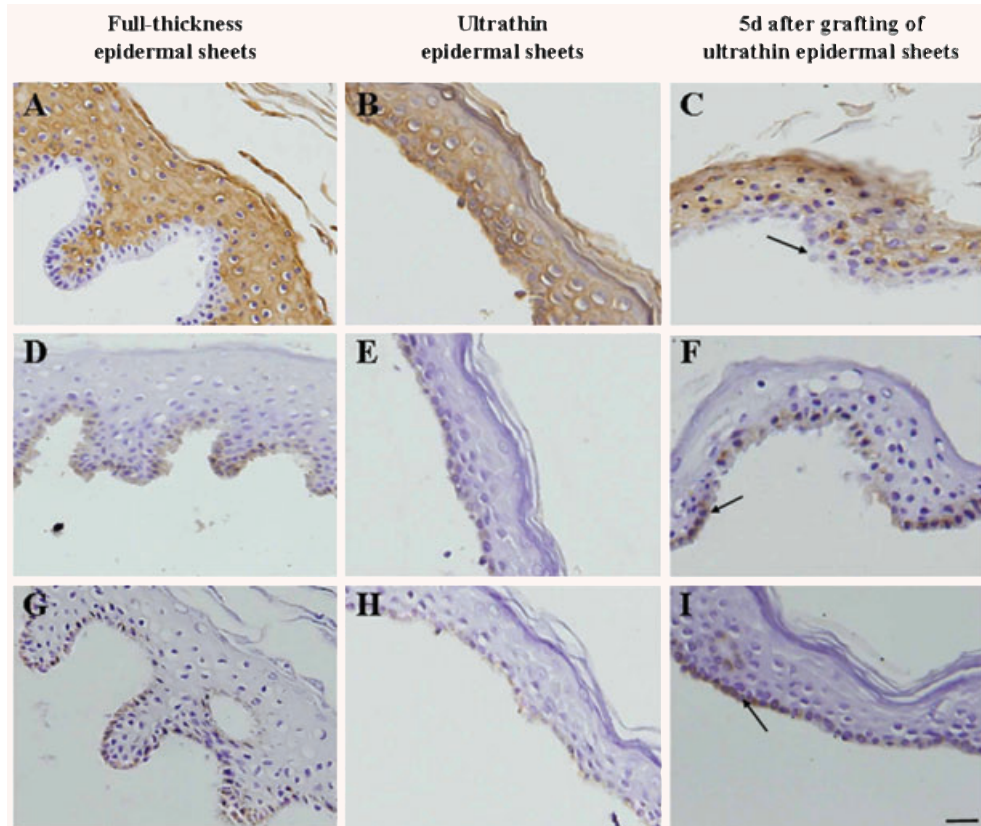


Fig. 1 Distribution of CK10, CK19 and β_1 -integrin⁺ cells in full-thickness epidermal sheets and ultrathin epidermal sheets before and after grafting. (A, B, C) Representative photographs of CK10⁺ cells; (D, E, F) Representative photographs of CK19⁺ cells; (G, H, I) Representative photographs of β_1 -integrin⁺ cells. Scale bar = 50 μ m. The arrows point to cells deriving from dedifferentiation.

Table 1 Percentages of CK19, β_1 -integrin and CK10 populations in ultrathin epidermal sheets before and after grafting (%)

Groups	CK19	β_1 -integrin	CK10
Blank control	0.04 \pm 0.01	0.04 \pm 0.02	0.06 \pm 0.03
Ungrafted sheets	0.05 \pm 0.02	0.03 \pm 0.01	98.53 \pm 2.21
Grafted sheets	7.21 \pm 2.02**	5.63 \pm 1.81**	88.57 \pm 4.65**

The results are presented as the means \pm S.D. ($n = 5$), and assessed by analysis of t-test. ** $P < 0.01$ compared with the group of ungrafted sheets.

Dedifferentiation of differentiated epidermal cells in grafted sheets

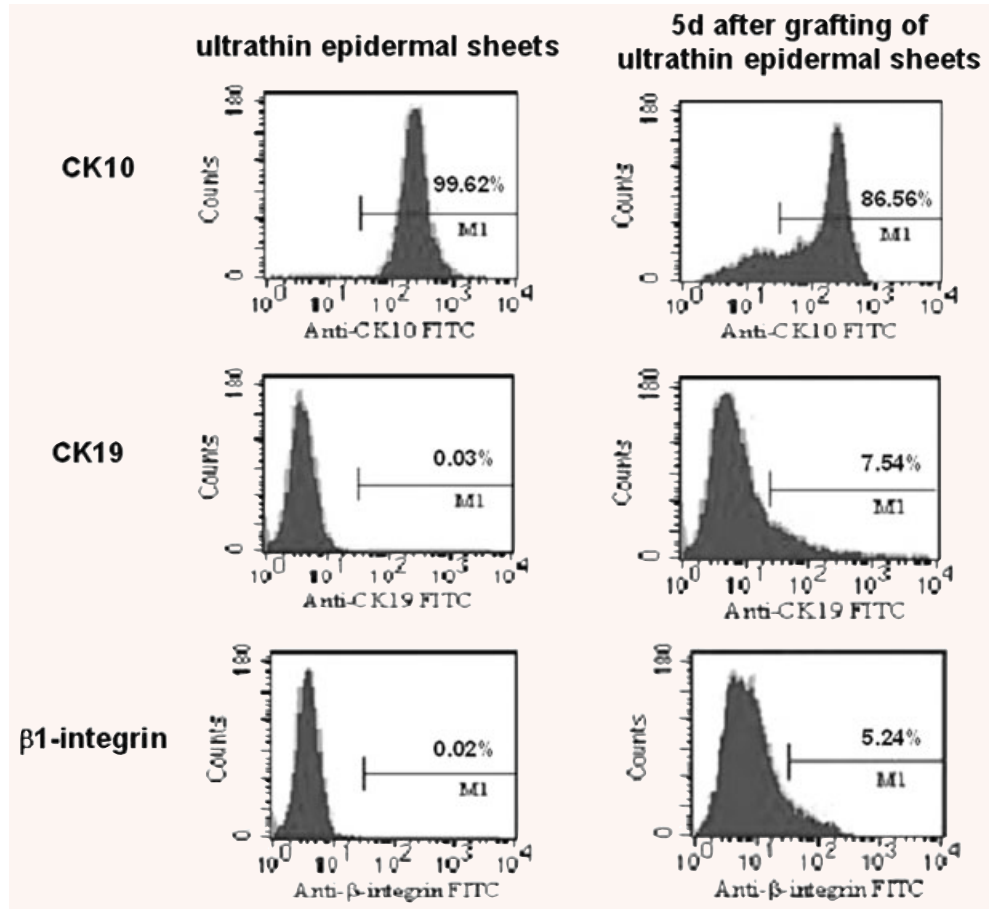
After the ultrathin epidermal sheets were transplanted for 5 days, cells negative for CK10 but positive for CK19 and β_1 -integrin emerged at the wound-neighbouring side of the skin grafts (Fig. 1C, F and I). Furthermore, the percentages of CK19 and β_1 -integrin⁺ cells detected by FACS were increased after grafting ($P < 0.01$, Fig. 2C–E, Table 1) and CK10⁺ cells in grafted sheets decreased ($P < 0.01$, Fig. 2A and B, Table 1). These data sug-

gested that some of the differentiated epidermal cells in grafted epidermal sheets dedifferentiated into stem cell-like cells, named as dedifferentiation-derived cells, *in vivo*.

Isolation and morphological features of epidermal cells

Epidermal stem cells have good adhering characteristics to type IV collagen. They can adhere rapidly to type IV collagen, fibronectin, or keratinocyte ECM within 10 min., but transit amplifying cells and post-mitotic differentiating cells adhere more slowly [21, 22]. So, we can separate dedifferentiation-derived cells from other cells by differential adhesion. The data showed that there were 4.56% adhering cells (dedifferentiation-derived cells) in the grafting group within 10 min. (Table 2). They were small round-shaped cells with large nuclear-cytoplasmic ratio. However, there were no adhering cells in the control group (ungrafted sheets) within 3 hrs, which further demonstrated that there were no stem cell-like cells in ultrathin epidermal sheets. After 6 hrs, 8.11% adhering cells (differentiated epidermal cells) emerged in control group, which were large flat-shaped cells with small nuclear-cytoplasmic ratio. A series of assays were performed on the initial adherent cells in each group and epidermal stem cells dispersed from full-thickness epidermal sheets were used as the positive control.

Fig. 2 Representative flow cytometric analysis of CK10, CK19 and β_1 -integrin⁺ cells in ultrathin epidermal sheets before and after grafting. (A, B) CK10⁺ cells; (C, D) CK19⁺ cells; (E, F) β_1 -integrin⁺ cells. (A, C, E) Ultrathin epidermal sheets before grafting; (B, D, F) Ultrathin epidermal sheets after grafting.



Detection of chromosome-specific sequences by PCR

To discriminate dedifferentiated-derived cells of human origin from mouse stem cells of the host, polymerase chain reaction was performed on DNA extracted from above initial adherent cells and from mouse skin tissue. Y-chromosome-specific sequences could be detected in the adhering cells isolated from ultrathin epidermal sheet (grafted and ungrafted) and full-thickness epidermal sheets. However, in mouse skin tissue, there were no Y-chromosome-specific sequences (Fig. 3). These results demonstrate that cells isolated from xenografts were originally from human male donors and are not mouse stem cells coming from the host that have attached to the graft.

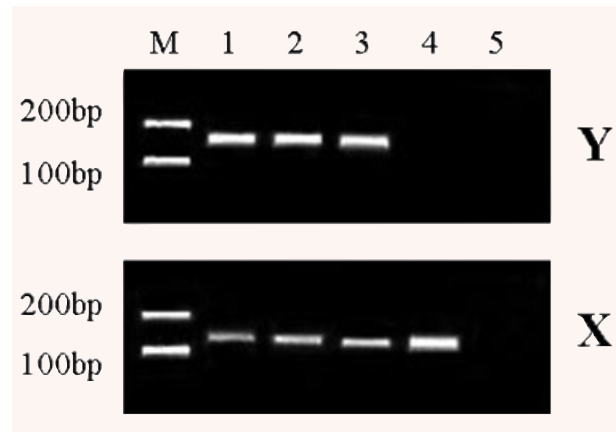


Fig. 3 Detection of X- and Y-chromosome specific sequences by PCR. M, Marker; lane 1, control (differentiated epidermal cells); lane 2, positive control (epidermal stem cells); lane 3, grafting group (dedifferentiation-derived cells); lane 4, mouse skin tissue; lane 5, no DNA (negative control).

Phenotypic characteristics

CK19 and β_1 -integrin expression

To examine whether CK19 and β_1 -integrin proteins were expressed in dedifferentiation-derived cells cultured *in vitro*,

Table 2 Percentages of adhering cells in ultrathin epidermal sheets before and after grafting (%)

Groups	10 min.	3 hrs	6 hrs	12 hrs	24 hrs
Ungrafted sheets	0	0	8.11 ± 2.09	14.27 ± 4.12	16.12 ± 5.76
Grafted sheets	4.56 ± 1.01	7.21 ± 1.23	14.32 ± 3.53	20.09 ± 7.57	22.41 ± 5.98

The results are presented as the means ± S.D. ($n = 5$).

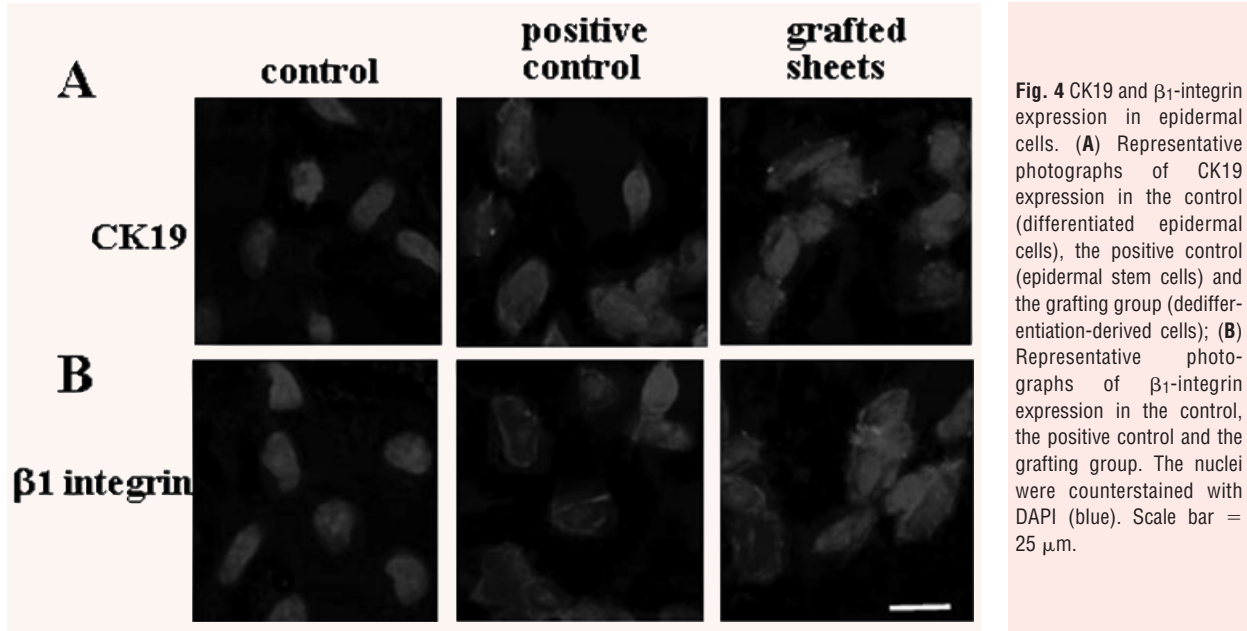


Fig. 4 CK19 and β_1 -integrin expression in epidermal cells. **(A)** Representative photographs of CK19 expression in the control (differentiated epidermal cells), the positive control (epidermal stem cells) and the grafting group (dedifferentiation-derived cells); **(B)** Representative photographs of β_1 -integrin expression in the control, the positive control and the grafting group. The nuclei were counterstained with DAPI (blue). Scale bar = 25 μ m.

immunofluorescence was performed on differentiated epidermal cells, epidermal stem cells and dedifferentiation-derived cells. Results showed that CK19 and β_1 -integrin n proteins were present in epidermal stem cells and dedifferentiation-derived cells, but not in differentiated epidermal cells. Moreover, red staining indicated very intense CK19 and β_1 -integrin expressions in the membrane and cytoplasm of epidermal stem cells and dedifferentiation-derived cells (Fig. 4).

Oct4 and Nanog expression

More recently, Oct4 and Nanog expressions have been found in stem cells from different adult human tissues. Thus their expressions have been considered general markers of self-renewal and pluripotency in stem cells. To further confirm the stem cell-like nature of the dedifferentiation-derived cells, we sought to investigate the expressions of the transcription factors Oct4 and Nanog. Real-time PCR analysis revealed that the dedifferentiation-derived cells, as well as epidermal stem cells, were >5-fold enriched for both Oct4 and Nanog (Fig. 5). This finding is consistent with observations reporting Oct4 and Nanog in epidermal stem cells cultured *in vitro* [19] and Oct4 in rare interfollicular basal cells of human epidermis *in situ* [23].

Functional characteristics

Colony-forming efficiency

It has been reported that epidermal stem cells are clonogenic and have high colony-forming ability. In order to elucidate whether dedifferentiation-derived cells also have this ability, the number of colonies consisting of 5–10 cells and greater than 10 cells were counted blindly. We found that dedifferentiation-derived cells, as well as epidermal stem cells, possessed high clonogenic capacity (Fig. 6A–C). The numbers of colonies consisting of 5–10 cells and greater than 10 cells in the grafting group were increased 5.9-fold and 6.7-fold, respectively, as compared with that in the control and there was no significant difference compared with positive control ($P > 0.05$, Fig. 6D). These data showed a highly significant capacity of dedifferentiation-derived cells to give rise to colonies.

Proliferation index

To study the growth characteristics of dedifferentiation-derived cells, three cell subpopulations (G_0/G_1 , S and G_2/M) were estimated by performing a flow cytometric measurement of DNA distributions of cells. In grafting and positive control groups, more cells were in S phase and G_2/M phase of the cell cycle as

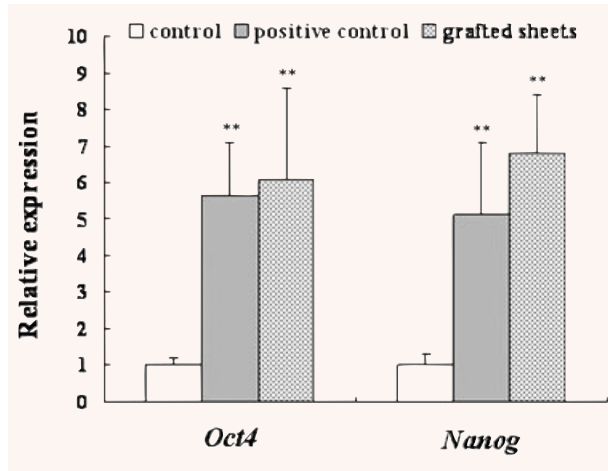


Fig. 5 Relative expression of self-renewal and pluripotency genes *Oct4* and *Nanog* in epidermal cells by quantitative real-time PCR. Results demonstrated that the dedifferentiation-derived cells, as well as epidermal stem cells, were >5-fold enriched for both *Oct4* and *Nanog*. The data are the means \pm S.D. ($n = 10$). ** $P < 0.01$, as compared with control.

compared with control group (PI values were 21.02 ± 5.01 in control group, 45.08 ± 10.11 in group of grafted sheets, and 48.00 ± 8.21 in positive control group; Fig. 7). The data here suggested that there were more dedifferentiation-derived cells in the proliferative phase.

Long-term growth potential

We further examined whether dedifferentiation-derived cells had long-term growth potential, as well as epidermal stem cells. Days in culture in control group ranged from 18 to 36, with an average of 28 ± 8 days. However, days in the grafting group ranged from 105 to 165, with an average of 130 ± 35 days and there was no significant difference compared with that in positive control group which ranged from 120 to 169, with an average of 140 ± 25 days (Fig. 8A). Numbers of passage in control group ranged from 2 to 4, with an average of 3 ± 1 ; however, that in grafting group ranged from 17 to 25, with an average of 20 ± 5 and there was no significant difference compared with that in positive control group which ranged from 18 to 26, with an average of 21 ± 4 (Fig. 8B). Total cell outputs of control, positive control, and grafting groups were 1×10^5 , 1×10^{13} and 1×10^{12} , respectively (Fig. 8C). These results demonstrate that dedifferentiation-derived cells, as well as epidermal stem cells, have long-term growth potential.

Fabrication of skin equivalent

Cultured fibroblasts were mixed with rat tail type I collagen to form a dermal equivalent. Differentiated epidermal cells, epidermal stem cells, and dedifferentiation-derived cells were seeded on the

dermal equivalent surface and grew to fuse to form an epidermal equivalent. After 11 days, we found that differentiated epidermal cells could not form an epidermal monolayer and dedifferentiation-derived cells, as well as epidermal stem cells, were capable of regenerating a highly stratified epidermal layer on the dermal equivalent surface (Figs. 9A–C).

Discussion

In the present study, we demonstrated that the differentiated epidermal cells dedifferentiated into stem cells or stem cell-like cells *in vivo* and these cells isolated and cultured *in vitro* had phenotypic and functional characteristics of epidermal stem cells: (1) some of differentiated epidermal cells in grafted epidermal sheets eliminated of basal stem cells dedifferentiated into stem cell-like cells expressing CK19 and β_1 -integrin under wound microenvironment; (2) these dedifferentiation-derived cells could be isolated by differential adhesion, cultured *in vitro*, and had morphological features of epidermal stem cells; (3) dedifferentiation-derived cells exhibited positive expression of CK19, β_1 -integrin, *Oct4* and *Nanog* and (4) dedifferentiation-derived cells, as well as epidermal stem cells, exhibited a high colony-forming efficiency, a long-term proliferative potential, and the ability to regenerate a skin equivalent. These suggest that we can generate abundant epidermal stem cells with an efficient dedifferentiation process for wound repair and regeneration.

In mammalian system, dedifferentiation is often associated with carcinogenesis [24]. In addition, it was also reported that pancreatic cells [25], renal epithelial cells, retinal cells, myoblasts [6], chondrocytes, and many other cells all have the potential of dedifferentiation in response to appropriate signals or factors [26]. In epidermis tissue, differentiated epidermal cells *in vivo* treated with rhEGF were found dedifferentiated into stem cells or stem-like cells in prickle cell and granular layers of regenerated epidermis [14–16]. These small cells exhibited positive expression of β_1 -integrin and CK19, which are thought to be special molecular markers of epidermal stem cells [27]. Here, we transplanted the human epidermal sheets eliminated of basal stem cells onto the skin wounds for 5 days without treatment of any growth factors and found that cells negative for CK10 but positive for CK19 and β_1 -integrin emerged at the wound-neighbouring side of the grafted sheets. These results suggested that some of the differentiated epidermal cells in grafted epidermal sheets dedifferentiated into stem cells or stem cells-like cells under the wound microenvironment. This finding is not surprising, because serum factors, which may be similar to the situation of wound microenvironment which contains many serum factors, can induce the dedifferentiation of HaCaT keratinocytes [13].

If these dedifferentiation-derived cells were intended to be used widely in regenerative medicine, they should be isolated for characteristic identification. Now, there are three ways of isolating epidermal stem cells. These include methods of isolating stem

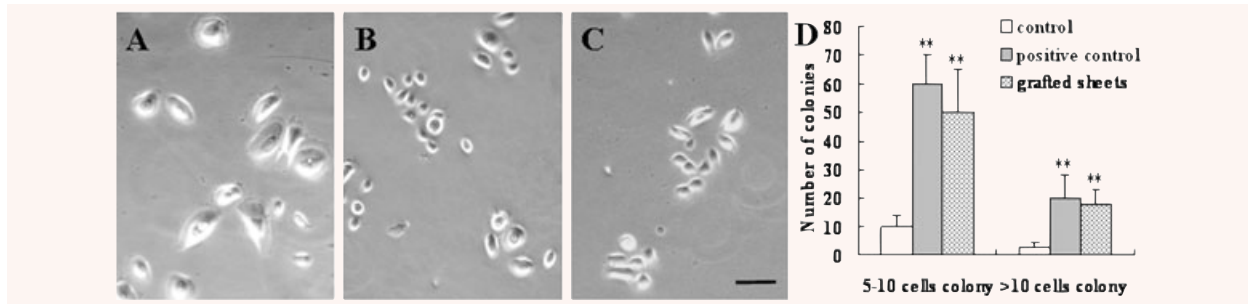


Fig. 6 Colony-forming efficiency of epidermal cells. (A) Representative photograph of cell colonies in the control (differentiated epidermal cells); (B) Representative photograph of cell colonies in the positive control (epidermal stem cells); (C) Representative photograph of cell colonies in the grafting group (dedifferentiation-derived cells); (D) Cartogram of Colony-forming efficiency. The data are the means \pm S.D. ($n = 5$). ** $P < 0.01$, as compared with control. Scale bar = 100 μ m.

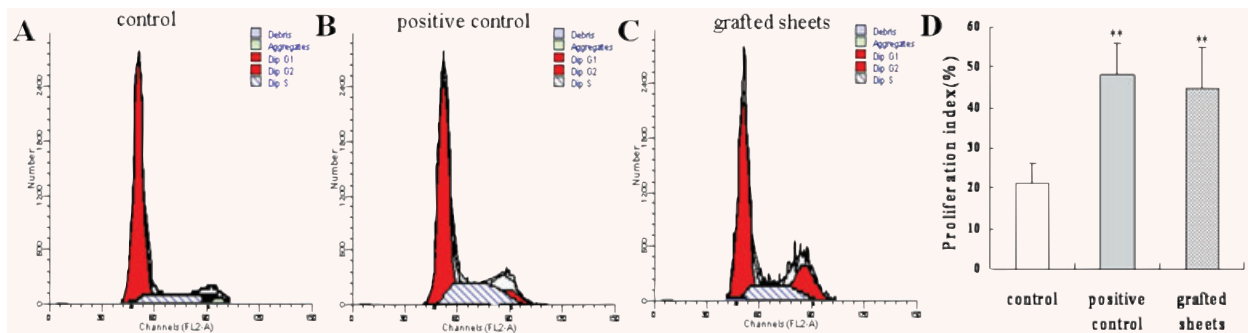


Fig. 7 Proliferation index of epidermal cells. (A) Representative flow cytometric analysis of the control (differentiated epidermal cells); (B) Representative flow cytometric analysis of the positive control (epidermal stem cells); (C) Representative flow cytometric analysis of the grafting group (dedifferentiation-derived cells) and (D) Cartogram of flow cytometric analyses. The data are the means \pm S.D. ($n = 5$). ** $P < 0.01$, as compared with control.

cells on the basis of rapid adhesion to type IV collagen [17], high surface expression of special molecular markers [28] and relative cell size [29, 30]. However, the most simply and effectively isolating method is differential adhesion. It was reported that epidermal stem cells can be isolated to greater than 90% purity on the basis of their adhesive properties [31]. In this study, we showed that there were 4.56% adhering cells in the grafted group within 10 min. These rapidly adhering cells probably are dedifferentiation-derived cells, because they have morphological features of epidermal stem cells which were small round-shaped cells with large nuclear-cytoplasmic ratio. However, there were no adhering cells in control group (ungrafted sheets) within 3 hrs, which further demonstrated no remnant basal stem cells in ultrathin epidermal sheets. After 6 hrs, 8.11% adhering cells (differentiated epidermal cells) emerged, which were large flat-shaped cells with small nuclear-cytoplasmic ratio. These slowly adhering cells may harbour the post-mitotic differentiating cells and minority transit amplifying cells which, after one to five rounds of division, undergo terminal differentiation [32]. Furthermore, Y-chromosome-specific sequences could be detected in the adhering cells

isolated from grafted ultrathin epidermal sheet, which clearly excluded the possibility that the cells isolated from xenografts were originally from recipient female mice and supported that these cells highly likely derived from the human male donors.

The phenotypic characterization of dedifferentiation-derived cells cultured *in vitro* revealed that dedifferentiation-derived cells exhibited positive expressions of CK19, β_1 -integrin, Oct4 and Nanog. It is well known that CK19 and β_1 -integrin are mainly expressed in the basal layer of epidermis as special molecular markers of epidermal stem cells [14, 27]. The self-renewal genes *Oct4* and *Nanog*, known to be highly expressed in self-renewing embryonic stem cells [33, 34], are also expressed in several adult stem cells including epidermal stem cells, but not in normal differentiated cells [19, 23, 35]. So, Oct4 and Nanog expressions have been considered general markers of self-renewal in stem cells. In addition, the *in vitro* functional assays showed that dedifferentiation-derived cells, as well as epidermal stem cells [36, 37], were able to develop significantly larger, active colonies than were differentiated epidermal cells. In differentiated epidermal cells, the proportion of cells in S and G₂M phases remained lower, because

Fig. 8 Long-term growth potential of epidermal cells. (A) Total days in culture of each group; (B) Numbers of passage in each group; (C) Assumptive total cell output in each group. The data are the means \pm S.D. ($n = 5$). $**P < 0.01$, as compared with control.

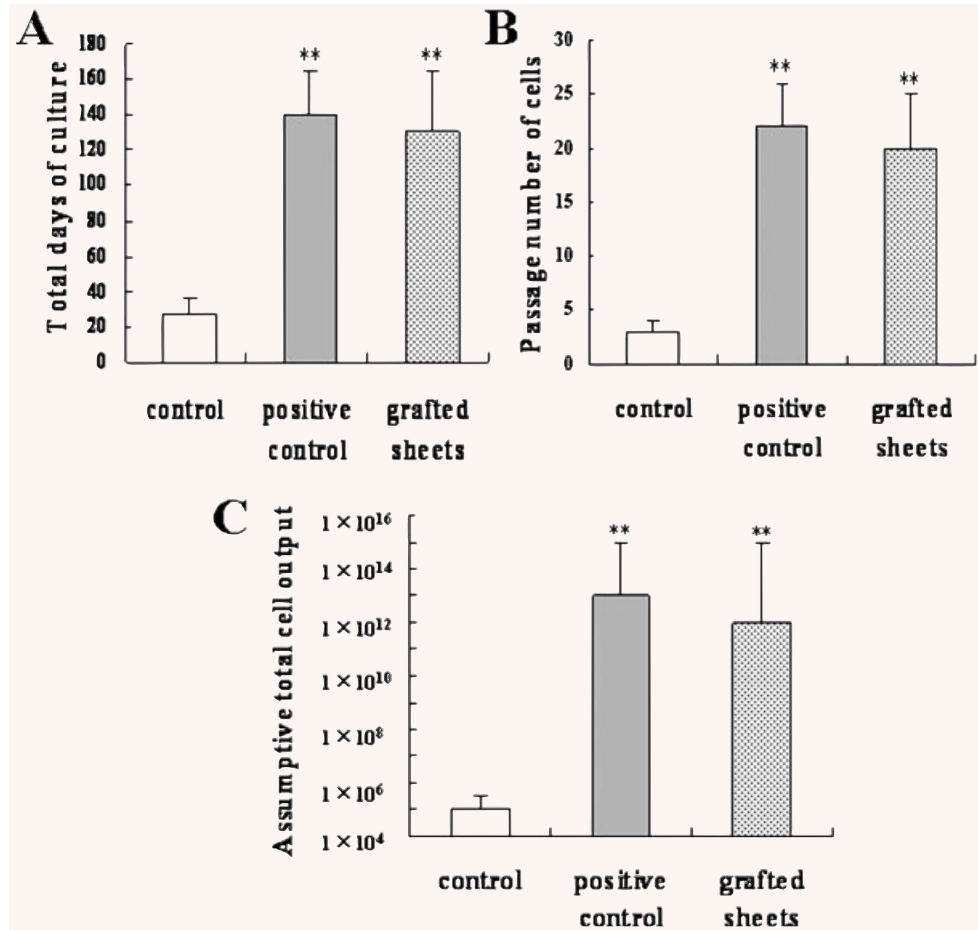
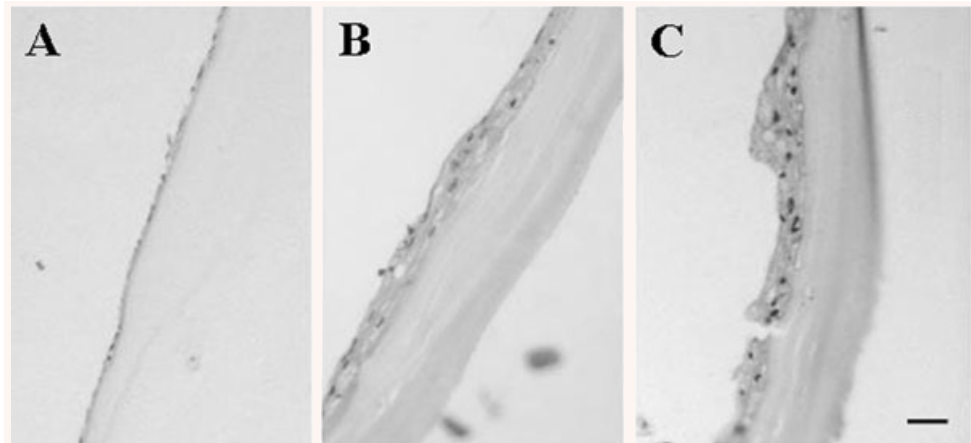


Fig. 9 Skin equivalent fabricated by epidermal cells. (A) Skin equivalent of the control (differentiated epidermal cells); (B) Skin equivalent of the positive control (epidermal stem cells); (C) Skin equivalent of the grafting group (dedifferentiation-derived cells). Scale bar = 100 μ m.



they had reached their post-mitotic stage. In contrast, dedifferentiation-derived cells consisted of the rapidly proliferating cell population (cells in S and G₂M phases). Furthermore, the total days of culture, the passage number of cells, and assumptive total cell

output all suggested that dedifferentiation-derived cells, as well as epidermal stem cells, had long-term growth potential. Intriguingly, the most significant characteristic of dedifferentiation-derived cells was their ability to regenerate a highly stratified skin

equivalents [38]. These results demonstrated that the phenotypic and functional characteristics of dedifferentiation-derived cells cultured *in vitro* were similar to epidermal stem cells. So, it is possible that dedifferentiation-derived cells will be another source of epidermal stem cells for wound repair and regeneration in the future. This fancy may resolve well the problem of a shortage of epidermal stem cells and provide us an ethically acceptable alternative treatment route without risk of genetic incompatibility and tissue rejection.

Taken together, we have reproduced the phenomenon of differentiated epidermal cell dedifferentiation, and for the first time demonstrated that dedifferentiation-derived cells isolated and cultured *in vitro* have characteristics of epidermal stem cells: rapid adhesion to type IV collagen, small round shape with large

nuclear-cytoplasmic ratio, positive expression of CK19, β_1 -integrin, Oct4 and Nanog, high colony-forming efficiency, long-term proliferative potential and ability to regenerate a skin equivalent. This provides us another promising way to yield epidermal stem cells for wound repair and regeneration.

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References

- Horch RE, Kopp J, Kneser U, *et al.* Tissue engineering of cultured skin substitutes. *J Cell Mol Med.* 2005; 9: 592–608.
- Kellouche S, Martin C, Korb G, *et al.* Tissue engineering for full-thickness burns: a dermal substitute from bench to bedside. *Biochem Biophys Res Commun.* 2007; 363: 472–8.
- Xie JL, Li TZ, Qi SH, *et al.* A study of using tissue-engineered skin reconstructed by candidate epidermal stem cells to cover the nude mice with full-thickness skin defect. *J Plast Reconstr Aesthet Surg.* 2007; 60: 983–90.
- Odelberg SJ, Kollhoff A, Keating MT. Dedifferentiation of mammalian myotubes induced by mscx1. *Cell.* 2000; 103: 1099–109.
- McGann CJ, Odelberg SJ, Keating MT. Mammalian myotube dedifferentiation induced by newt regeneration extract. *Proc Natl Acad Sci USA.* 2001; 98: 13699–704.
- Chen X, Mao Z, Liu S, *et al.* Dedifferentiation of adult human myoblasts induced by ciliary neurotrophic factor *in vitro*. *Mol Biol Cell.* 2005; 16: 3140–51.
- Chen S, Zhang Q, Wu X, *et al.* Dedifferentiation of lineage-committed cells by a small molecule. *J Am Chem Soc.* 2004; 126: 410–1.
- Duckmanton A, Kumar A, Chang YT, *et al.* A single-cell analysis of myogenic dedifferentiation induced by small molecules. *Chem Biol.* 2005; 12: 1117–26.
- Eggan K, Baldwin K, Tackett M, *et al.* Mice cloned from olfactory sensory neurons. *Nature.* 2004; 428: 44–9.
- Li J, Ishii T, Feinstein P, *et al.* Odorant receptor gene choice is reset by nuclear transfer from mouse olfactory sensory neurons. *Nature.* 2004; 428: 393–9.
- Lowry WE, Richter L, Yachechko R, *et al.* Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci USA.* 2008; 105: 2883–8.
- Yu J, Vodyanik MA, Smuga-Otto K, *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 2007; 318: 1917–20.
- Pivarcsi A, Szell M, Kemeny L, *et al.* Serum factors regulate the expression of the proliferation related genes $\alpha 5$ integrin and keratin1, but not keratin10 in HaCaT keratinocytes. *Arch Dermatol Res.* 2001; 293: 206–13.
- Fu X, Sun X, Li X, *et al.* Dedifferentiation of epidermal cells to stem cells *in vivo*. *Lancet.* 2001; 358: 1067–8.
- Fu X, Sun X, Sun T. Epidermal growth factor induce the epithelial stem cell island formation in the regenerated epidermis. *Zhonghua Yi Xue Za Zhi.* 2001; 81: 733–6.
- Li H, Fu X, Zhang L, *et al.* *In vivo* dedifferentiation of human epidermal cells. *Cell Biol Int.* 2007; 31: 1436–41.
- Liu Y, Zhou H, Gao F. Isolation and identification of stem cells from adult cashmere goat skin. *Int J Dermatol.* 2008; 47: 551–6.
- Kamolz LP, Kolbus A, Wick N, *et al.* Cultured human epithelium: human umbilical cord blood stem cells differentiate into keratinocytes under *in vitro* conditions. *Burns.* 2006; 32: 16–9.
- Redvers RP, Li A, Kaur P. Side population in adult murine epidermis exhibits phenotypic and functional characteristics of keratinocyte stem cells. *Proc Natl Acad Sci USA.* 2006; 103: 13168–73.
- Regnier M, Patwardhan A, Scheynius A, *et al.* Reconstructed human epidermis composed of keratinocytes, melanocytes and Langerhans cells. *Med Biol Eng Comput.* 1998; 36: 821–4.
- Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell.* 1993; 73: 713–24.
- Kaur P, Li A. Adhesive properties of human basal epidermal cells: an analysis of keratinocyte stem cells, transit amplifying cells, and postmitotic differentiating cells. *J Invest Dermatol.* 2000; 114: 413–20.
- Tai MH, Chang CC, Kiupel M, *et al.* Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. *Carcinogenesis.* 2005; 26: 495–502.
- Helm J, Enkemann SA, Coppola D, *et al.* Dedifferentiation precedes invasion in the progression from Barrett's metaplasia to esophageal adenocarcinoma. *Clin Cancer Res.* 2005; 11: 2478–85.
- Bouckenooghe T, Vandewalle B, Moerman E, *et al.* Expression of progenitor cell markers during expansion of sorted human pancreatic beta cells. *Gene Expr.* 2005; 12: 83–98.
- Collas P. Dedifferentiation of cells: new approaches. *Cytotherapy.* 2007; 9: 236–44.
- Larouche D, Hayward C, Cuffley K, *et al.* Keratin 19 as a stem cell marker *in vivo* and *in vitro*. *Methods Mol Biol.* 2005; 289: 103–10.

28. **Radu E, Simionescu O, Regalia T, et al.** Stem cells (p63⁺) in keratinocyte cultures from human adult skin. *J Cell Mol Med.* 2002; 6: 593–8.
29. **De Paiva CS, Pflugfelder SC, Li DQ.** Cell size correlates with phenotype and proliferative capacity in human corneal epithelial cells. *Stem Cells.* 2006; 24: 368–75.
30. **Izumi K, Tobita T, Feinberg SE.** Isolation of human oral keratinocyte progenitor/stem cells. *J Dent Res.* 2007; 86: 341–6.
31. **Jones PH, Watt FM.** Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell.* 1993; 73: 713–24.
32. **Marconi A, Dallaglio K, Lotti R, et al.** Survivin identifies keratinocyte stem cells and is downregulated by anti-beta1 integrin during anoikis. *Stem Cells.* 2007; 25: 149–55.
33. **Pesce M, Scholer HR.** Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells.* 2001; 19: 271–8.
34. **Mitsui K, Tokuzawa Y, Itoh H, et al.** The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell.* 2003; 113: 631–42.
35. **Zangrossi S, Marabese M, Brogginini M, et al.** Oct-4 expression in adult human differentiated cells challenges its role as a pure stem cell marker. *Stem Cells.* 2007; 25: 1675–80.
36. **Papini S, Cecchetti D, Campani D, et al.** Isolation and clonal analysis of human epidermal keratinocyte stem cells in long-term culture. *Stem Cells.* 2003; 21: 481–94.
37. **Islam MS, Zhou H.** Isolation and characterization of putative epidermal stem cells derived from Cashmere goat fetus. *Eur J Dermatol.* 2007; 17: 302–8.
38. **Riva F, Casasco A, Nespoli E, et al.** Generation of human epidermal constructs on a collagen layer alone. *Tissue Eng.* 2007; 13: 2769–79.