Journal of Cellular Biochemistry 98:174–184 (2006)

# Isolation of Epithelial Stem Cells From Dermis by a Three-Dimensional Culture System

**Reinhold J. Medina, Ken Kataoka, Mikiro Takaishi, Masahiro Miyazaki, and Nam-ho Huh***

Department of Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Shikata-chou, Okayama 700-8558, Japan

**Abstract** Skin is a representative self-renewing tissue containing stem cells. Although many attempts have been made to define and isolate skin-derived stem cells, establishment of a simple and reliable isolation procedure remains a goal to be achieved. Here, we report the isolation of cells having stem cell properties from mouse embryonic skin using a simple selection method based on an assumption that stem cells may grow in an anchorage-independent manner. We inoculated single cell suspensions prepared from mouse embryonic dermis into a temperature-sensitive gel and propagated the resulting colonies in a monolayer culture. The cells named dermis-derived epithelial progenitor-1 (DEEP) showed epithelial morphology and grew rapidly to a more than 200 population doubling level over a period of 250 days. When the cells were kept confluent, they spontaneously formed spheroids and continuously grew even in spheroids. Immunostaining revealed that all of the clones were positive for the expression of cytokeratin-8, −18, −19, and E-cadherin and negative for the expression of cytokeratin-1, −5, −6, −14, −20, vimentin, nestin, a ckit. Furthermore, they expressed epithelial stem cell markers such as p63, integrin β1, and S100A6. On exposure to TGFβ in culture, some of DEEP-1 cells expressed α-smooth muscle actin. When the cells were transplanted into various organs of adult SCID mice, a part of the inoculated cell population acquired neural, hepatic, and renal cell properties. These results indicate that the cells we isolated were of epithelial stem cell origin and that our new approach is useful for isolation of multipotent stem cells from skin tissues. J. Cell. Biochem. 98: 174–184, 2006. 

**Key words:** stem cells; 3-dimentional culture; multipotency; Merkel

Recently accumulating evidence indicates that many somatic organs harbor various types of stem/progenitor cells in mammals [Weiss et al., 1996; Jiang et al., 2002a,b; D'Ippolito et al., 2004]. The cells have been demonstrated to possess a potency to differentiate into multiple cell lineages even across the three-germ layers, thus comprising potentially useful cell sources for regenerative medicine [Joshi and Enver, 2002]. Skin is the largest organ in the body and relatively easily sampled with a minimal insult to the donor. It is, therefore, one of the best autologous source organs to isolate stem/progenitor cells for future therapeutic applications.

A number of different stem cells have been isolated and characterized from the skin. A stem cell population was identified in the bulge of hair follicles based on the label-retaining property in vivo and higher clonogenicity in culture [Rochat et al., 1994; Taylor et al., 2000; Oshima et al., 2001]. Jones and Watt [1993] demonstrated that integrin β1-positive cells isolated from the epidermis have a greater proliferation capacity. These skin-derived stem cells, however, are preferentially differentiated into epithelial components of skin itself such as the epidermis, sebaceous glands, and hair follicles but not into the other cell types [Jones and Watt, 1993; Rochat et al., 1994; Taylor et al., 2000; Huelsken et al., 2001; Oshima et al., 2001]. On the other hand, Toma et al. [2001] isolated

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant numbers: 14370260, 17014065.

*Correspondence to: Nam-ho Huh, Department of Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-chou, Okayama 700-8558, Japan.
E-mail: namu@md.okayama-u.ac.jp.

Received 9 November 2005; Accepted 10 November 2005
DOI 10.1002/jcb.20757

multipotent adult stem cells from mammalian skin dermis. The cells, named skin-derived precursors (SKPs), can differentiate into neurons, glial, smooth muscle cells, and adipocytes. SKPs are thought to be of neural crest origin and to reside in association with hair follicles, though their precise localization remains controversial [Fernandes et al., 2004; Amoh et al., 2005]. SKPs were isolated by serial passage of small spheres of floating cells [Toma et al., 2001]. Liang and Bickenbach [2002] have isolated another type of stem cells by sorting mouse epidermal cells based on their dye-exclusion capacity and smaller size. When injected into blastocysts, the epidermal stem cells could differentiate into not only epidermal cells but also many different cell types, including endothelial cells, hematopoietic cells, neural precursor cells, and glial cells.

In this paper, we describe a new approach to isolate stem-like cells from the dermis. The use of a synthetic thermo-sensitive bio-inactive gel enabled us to clonally establish cell lines having epithelial stem cell properties in a simple and rapid manner.

## MATERIALS AND METHODS

### Preparation of Dermal Cells

Dorsal skin tissues were dissected from embryonal (16.5 days postcoitus; dpc) and neonatal C57Bl/6 mice (Nippon SLC, Hamamatsu, Japan) and incubated overnight at 4°C in 0.05% collagenase (Wako, Osaka, Japan) in MCDB153 (Sigma, St. Louis) without growth factors. The dermis was separated from the epidermis using forceps under a dissecting microscope, and a dermal cell suspension was obtained by gently pipetting in the medium and filtrating with a #150 mesh (Ikemoto, Tokyo, Japan). The final inoculation density was $10^5$ cells/ml.

### Primary Culture in a Thermo-Reversible Gel

For three-dimensional gel culture, we used Mebiol gel, a thermo-reversible gelation polymer (Mebiol, Tokyo, Japan). It is in a fluid state at temperatures lower than the gel-sol transition point (~20°C). To prepare the gel, the powdered polymer was dissolved in Dulbecco's modified MEM (DMEM; Nissui, Tokyo, Japan), gently shaken overnight at 4°C, and then left undisturbed for 3 days to eliminate bubbles in the solution. The gel was stored at 4°C stationarily until use.

One part of the dermal cell suspension and nine parts of the Mebiol solution were gently mixed, and the mixture was plated into 4-well plates (250 μl/well; Nunc, Rochester). After gelation by incubating the plates at 37°C, 600 μl of DMEM containing 10% FBS was overlaid. The plates were incubated in a humidified atmosphere with 5% $CO_2$ at 37°C. The medium was carefully replaced every 3 days without disturbing the gel.

### Propagation of Cells in a Monolayer Culture and in a Suspension Culture

Cells isolated by primary culture in the gel were propagated in a monolayer culture on collagen I-coated cover slips (Iwaki, Tokyo, Japan) or in polystyrene culture dishes (Corning) precoated with collagen type I (Nitta Gelatin, Osaka, Japan). The cells were passaged every 3 days by trypsinization and reinoculation at a density of $\sim 6 \times 10^4$ cells/cm$^2$.

For suspension culture, $\sim 10^6$ cells were inoculated into a polystyrene bacterial dish (90 mm × 20 mm; Iwaki). The cells readily aggregated and formed small spheroids, which grew progressively. Cells were passaged by dissociating the spheroids with Accumax (ICT, San Diego, CA) every 5~6 days.

### Marking of Cells With Green Fluorescent Protein (GFP)

pEGFP-N2 provided by Dr. Masakiyo Sakaguchi was transfected to cells using Lipofectamin 2000 under the conditions recommended by the manufacturer (Invitrogen). Successfully transfected cells were selected with Neomycin sulfate (400 μg/ml; Invitrogen). Single cell clones were isolated by a limiting dilution method.

### RT-PCR

RT-PCR was performed under conventional conditions. Briefly, total RNA was isolated by the guanidine–isothiocyanate–phenol method and 1 μg each of the samples was used for cDNA synthesis. The products were amplified under the following conditions: Initial incubation at 94°C for 4 min; 20 or 30 cycles of amplification at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and final incubation at 72°C for 5 min. Forward and reverse primer sequences and the lengths of products were as follows: Oct3 (5′-AGA GGG

AAC CTC CTC TGA GC-3′, 5′-CCT GGG AAA GGT GTC CTG TA-3′, 593 bp); ABCG2 (5′-AGC AGC AAG GAA AGA TCC AA-3′, 5′-AAT CCG CAG GGT TGT TGT AG-3′, 599 bp); nestin (5′-GGG GAG CAA GCA TTC AAA TA-3′, 5′-CTT GGT GTT GGA AAC CTC GT-3′, 590 bp); CD133 (5′-TGG CCC TCT CTA CAA AAT GG-3′, 5′-GCC TTG TTC TTG GTG TTG GT-3′, 525 bp); mRIF1 (5′-GGA GCC TCT AGG GAA ATT GG-3′, 5′-TGG ACT CGT CCA TCA TTT CA-3′, 554 bp). The amplified products were subjected to electrophoresis in 1% agarose gels and stained with ethidium bromide. Mouse embryonic stem cells and freshly-isolated adult liver cells were used as controls.

### Immunostaining of Cells and Tissues

Cells cultured in a monolayer were fixed with 100% methanol for 20 min at −20°C. For immunostaining of cell surface receptors and nuclear ligands, cells were fixed with 10% formalin for 10 min at room temperature and permeabilized with 1% Triton X-100 for 10 min. For immunostaining of spheroids, paraffin sections of ~6 μm in thickness were made under conventional conditions after fixation with 10% formalin overnight at 4°C, except for immersing the dehydrated spheroids in a low-melting-point paraffin for 12 h at 60°C prior to the conventional paraffin embedding. For immunostaining of tissues, frozen sections made as described below were used.

After blocking with 5% goat serum and 0.1% Tween 20 in PBS for 60 min at room temperature, the cells were treated with a primary antibody overnight at 4°C. After washing with PBS, the cells were incubated with appropriate secondary antibodies for 1 h at room temperature and observed with a fluorescence microscope (Olympus IX71; Olympus, Tokyo, Japan) or with a confocal fluorescence scanning microscope (LSM 510; Zeiss, Germany). Omission of the first antibodies consistently gave no signals. The primary antibodies used were as follows: monoclonal antibodies against E-cadherin, M-cadherin, N-cadherin, R-cadherin, integrin β1 (CD29), and ZO-1 (BD Biosciences, San Diego, CA); polyclonal antibodies against CK 1, CK 5, and CK 6 (Covance, Princeton, NJ); monoclonal antibodies against CK 8, CK 18, CK 19, and CK 20 (Progen, Heidelberg, Germany); monoclonal antibody against CK 14 (YLEM, Rome, Italy); monoclonal antibodies against nestin, p63, glial fibrillary acidic protein (GFAP), and Tubulin βIII and polyclonal antibody against aquaporin-1 (Chemicon, Temecula, CA); monoclonal antibodies against vimentin, α-smooth muscle actin, and BrdU (DAKO, Glostrup, Denmark); anti-cKit polyclonal antibody (Santa Cruz Biotechnology, CA); monoclonal antibodies against pancytokeratin and S100A6 (Sigma); and anti-albumin polyclonal antibody (Biogenesis, Bournemouth, UK).

For monitoring DNA synthesis, cells were pre-treated with 30 μM BrdU for 1 h prior to fixation. Apoptotic cells were visualized by the TUNEL method using an in-situ cell death detection kit (Boehringer, Mannheim, Germany) following the manufacturer's instructions. Briefly, after deparaffinization and microwave treatment for antigen retrieval, nucleosomal fragmentation products were detected by 3′-end labeling with fluorescein-dUTP using terminal deoxynucleotidyl transferase.

### Transplantation of Cells into SCID Mice and Histological Analysis of Tissues

To induce differentiation in vivo, the GFP-labeled cells of clonal origin were transplanted into the liver, brain, kidney, and testicle of 5~6-week-old SCID mice (Mouse colony, Okayama U, Japan) under anesthesia with 300 μl tribromoethanol (Sigma). For transplantation into the liver, $5 \times 10^6$ cells in 200 μl PBS were directly injected using a 26-gauge syringe after median laparotomy. For transplantation into the brain, $2.5 \times 10^5$ cells in 10 μl PBS were injected using a Hamilton syringe into the right parietal region 3~5 mm backward from the bregma with the aid of a stereotaxic apparatus. Five million cells in 100 μl PBS were transplanted into the renal cortex and testicle parenchyma. All of the experiments were performed in conformity to the institutional code for animal experiments.

Transplanted mice were sacrificed 10 days after injection into the liver or brain and 6 weeks after injection into the kidney or testicle. For sampling, the mice were anesthetized and perfused intracardially first with PBS and then with 4% paraformaldehyde. The respective organs were excised and further fixed in 4% paraformaldehyde overnight at 4°C. The organs were cryoprotected by incubation in 30% sucrose for 24 h at 4°C and quickly frozen in Tissue Tek OCT compound (Sakura). Tissue sections of 5 μm in thickness were made using a cryostat (Leica, Wetzlar, Germany).

## RESULTS

### Establishment of Rapidly and Persistently Growing Cell Lines by a Clonal Three-Dimensional Culture Method

When we inoculated a single cell suspension prepared from mouse embryonic dermis at 16.5 dpc into Mebiol gel, two morphologically distinct types of colonies were formed, that is, smooth- and rough-surfaced colonies (Fig. 1A). Formation efficiencies were ~0.004% and ~0.02% for the smooth- and rough-surfaced colonies, respectively, when inoculated at $2.5 \times 10^4$ cells/250 μl/well. FBS was essential for the colonial growth. Addition of epidermal growth factor at 10 ng/ml resulted in preferential growth of the rough-surfaced colonies with no apparent effects on the growth of the smooth-surfaced colonies. Similar colonies were formed when cells from newborn mouse dermis were inoculated. Inoculation of cells from embryonic epidermis resulted in no colony formation.

We propagated the colonies in a monolayer culture after transferring them onto collagen coated-cover slips. Spindle-shaped cells migrated out of the rough-surfaced colonies. The cells readily accumulated lipid in the cytoplasm (Supporting Information Fig. S1) and stopped growing after several doublings. On the other hand, cuboidal cells with short processes migrated out of the smooth-surfaced colonies (Fig. 1B). The cells grew rapidly (doubling time: ~24 h) without any appreciable changes in cell properties such as morphology, doubling time, serum dependence, expression profile of marker proteins, and differentiation potential to a more than 200 population doubling level over a period of 250 days (Fig. 1C), indicating possession of self-renewing capacity. As shown below, these cells express marker proteins featured by positiveness in primitive epithelial cells and stem cells and we, therefore, named them dermis-derived epithelial progenitor (DEEP) cells. One line of the DEEP cells, DEEP-1, was characterized further in the present study.

### Expression in DEEP-1 Cells of Marker Proteins Featuring Distinct Cell Types

Expression of various marker proteins in DEEP-1 cells was examined by immunostaining (Fig. 2). Among the cytokeratins (CK) examined, DEEP-1 cells expressed cytokeratin (CK) 8, CK 18, CK 19 but not CK 1, CK 5, CK 6, CK 14, or CK 20 (Fig. 2A). Vimentin and nestin were not detected in DEEP-1 cells. DEEP-1 cells were positive for expression of E-cadherin but

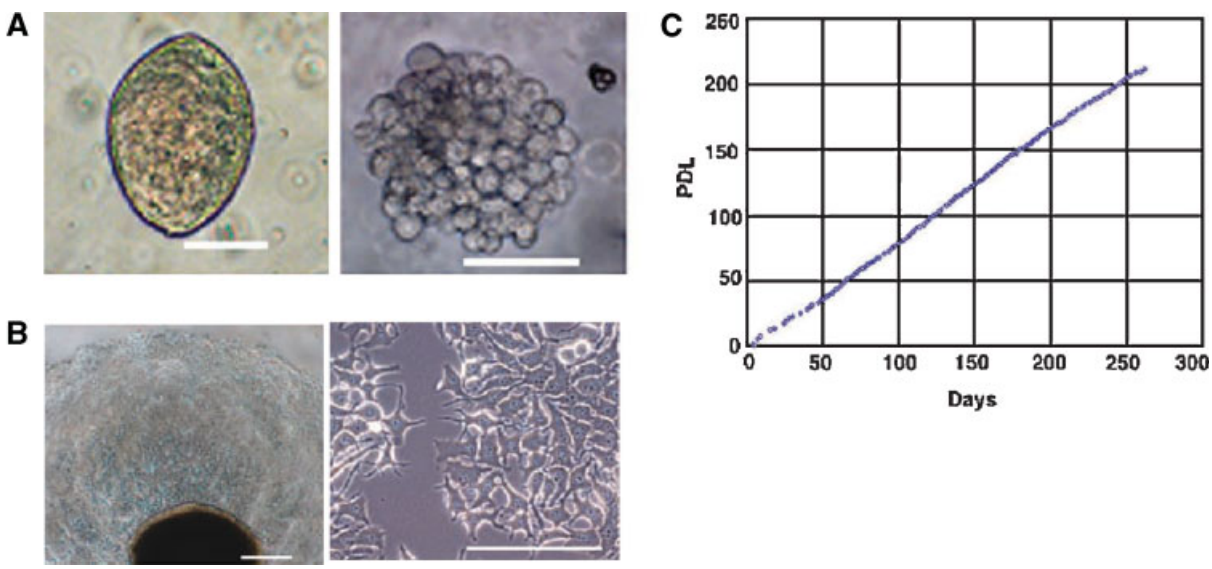


**Fig. 1.** Isolation of DEEP cells by a three-dimensional culture. **A**: Two representative types of colonies, smooth- (**left**) and rough-surfaced (**right**), were formed in Mebiol gel by cells prepared from mouse embryonic dermis of 16.5 dpc. **B**: The smooth-surfaced colony shown in (A) was transferred onto a collagen-coated cover slip to establish a permanent cell line, DEEP-1. Migration of cells from the explanted colony (**left**) and the resulting cell line showing polygonal morphology with short processes (**right**). **C**: A cumulative growth curve of DEEP-1 cells in a monolayer. Scale bars, 100 μm.

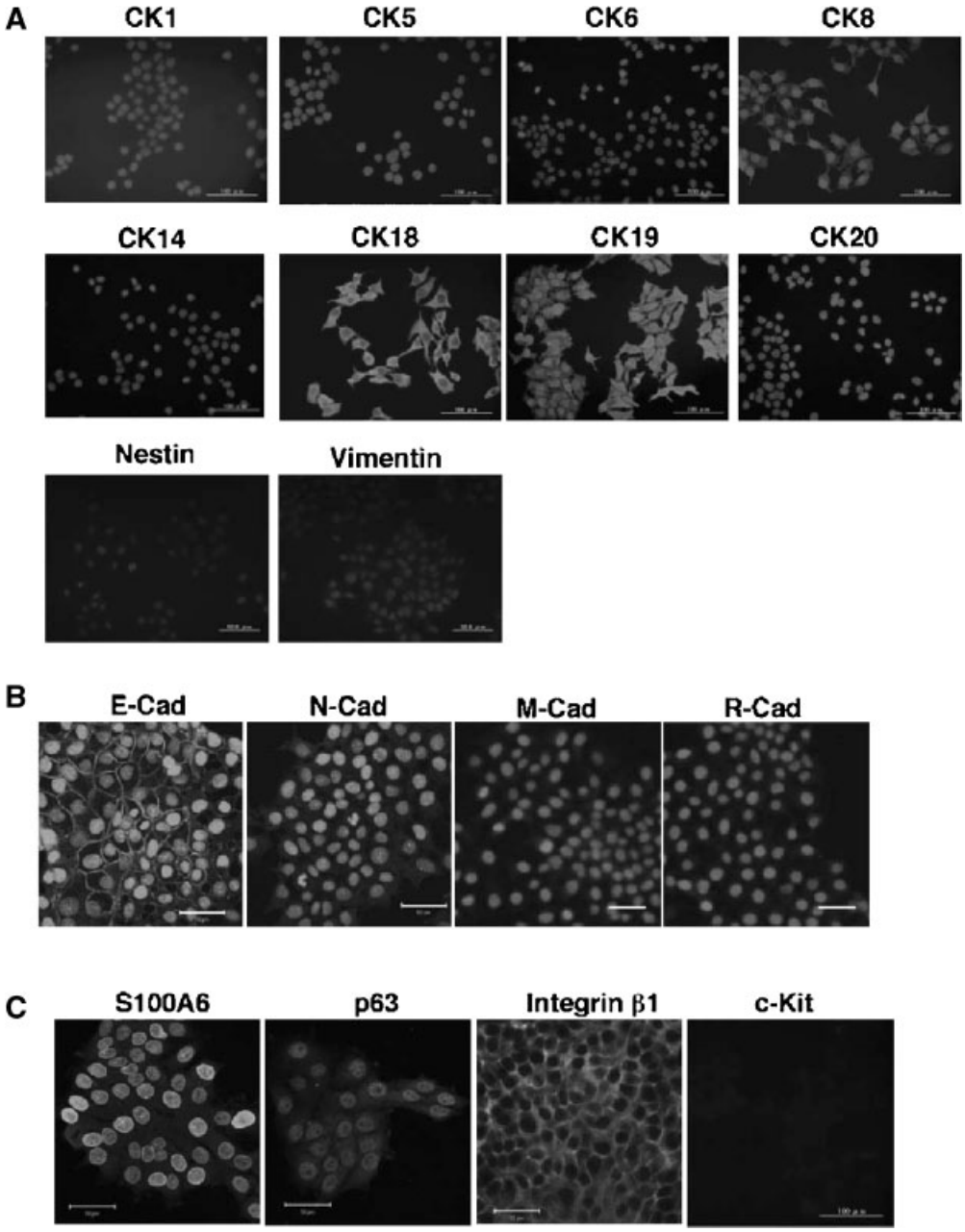


**Fig. 2.** Expression of marker proteins in DEEP-1 cells determined by immunostaining. **A**: Among intermediate filament proteins examined, CK 8, CK 18, and CK 19 were detected (shown in red). Nuclei were stained with DAPI. **B**: Expression of cadherin family proteins. **C**: Expression of marker proteins that are often detected in various stem cells. In (B), the specific signal was shown in green with nuclear staining with propiodium iodide in red. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

negative for expression of N-, R-, and M-cadherin (Fig. 2B). S100A6 is known to be expressed in bulge stem cells [Ito and Kizawa, 2001], while integrin β1 and p63 in epidermal stem cells [Jones and Watt, 1993; Pellegrini et al., 2001]. All the three proteins were detected in DEEP-1 cells (Fig. 2C). c-Kit, an authentic marker of hematopoietic stem cells [Spangrude et al., 1988; Randall and Weissman, 1998], was not expressed in DEEP-1 cells. The positive expression of CK 8, CK 18, CK 19, and E-cadherin and the lack of expression of CK 14, nestin, synaptophysin, or N-cadherin in DEEP-1 cells were confirmed by Western blot analysis

(Fig. S2). RT-PCR revealed that mRif1 [a marker of pluripotent stem cells; Adams and McLaren, 2004] transcripts were present in DEEP-1 cells, but no bands were observed for Oct3/4 [a marker of embryonic stem cells; Scholer et al., 1989], ABCG2 [a marker of the side population; Zhou et al., 2001], CD133/Prominin-1 [a marker of various stem cells, including those of the hematopoietic and nervous system; Bhatia, 2001], and nestin [a marker of neural progenitors; Lendahl et al., 1990] (Fig. 3). Altogether, the observed gene expression profile of DEEP-1 is compatible with that of an epithelial progenitor.

### Formation of Characteristic Spheroids by DEEP-1 Cells

DEEP-1 cells grew rapidly in a monolayer (Fig. 1C). When the cells were maintained in a confluent state, they continued to grow and formed piled-up cell clusters (Fig. 4A). Finally, the cell clusters spontaneously detached from the cell sheet and floated in the medium as spheroids. Alternatively, when DEEP-1 cells were inoculated into non-adherent culture vessels, they readily formed spheroids. The spheroids could be transferred to a monolayer culture by inoculating them into collagen-coated culture vessels.

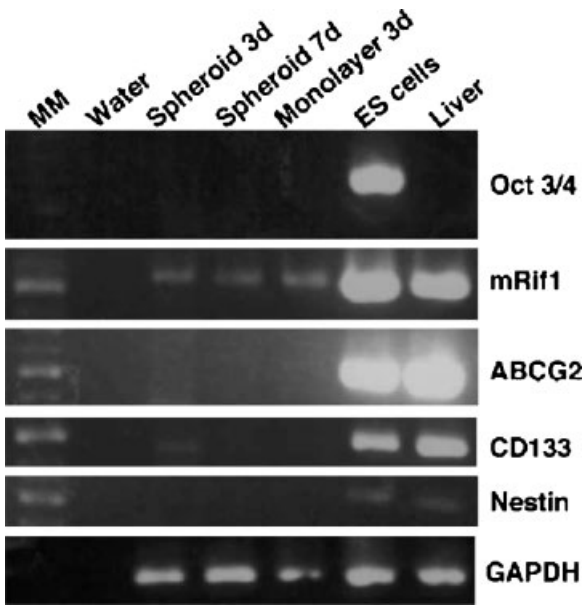


**Fig. 3.** Expression of representative stem cell marker genes in DEEP-1 cells determined by RT-PCR. DEEP-1 cells were assayed after 3-day cultivation in a monolayer or 3 and 7-day cultivation in spheroids. MM, molecular markers.

DEEP-1 cells could be serially passaged in spheroids by trypsinization. The growth rate of DEEP-1 cells in spheroids (population doubling time: ~2.6 days) was lower than that observed in a monolayer (Fig. 4B), probably because only cells located in the outer area of spheroids had capacity to grow as indicated by incorporation of BrdU (Fig. 4C).

DEEP-1 spheroids with diameters less than 150 μm were mostly solid. Mitotic figures were frequently observed in the periphery (Fig. 5A-1). As the size of spheroids increased, the central core of the spheroids underwent apoptosis as revealed by TUNEL assays (Fig. 5A-2 and A-4). At later stages, the cortical area of many spheroids also underwent apoptosis, leaving viable cells only in the inner and outer shells, thus resulting in the formation of a double-layered structure (Fig. 5A-3). CK 18 was homogeneously expressed in the smaller spheroids (Fig. 5B-1 and B-2), but its expression was gradually downregulated in the cortical area (Fig. 5B-3 and B-4). On the other hand, the expression of E-cadherin remained unchanged among viable cells of DEEP-1 spheroids (Fig. 5C).

### Differentiation Potential of DEEP-1 Cells

When DEEP-1 cells were cultivated with or without TGFβ1 (8 ng/ml) for 1 week, morphology of the cells changed from small cells in a cobble stone-like arrangement to a large flattened shape with downregulation of E-cadherin and ZO-1 and induction of α-smooth muscle actin at least in some of the cells (Fig. 6). These changes are characteristic of epithelial mesenchymal transition. Except for this response, DEEP-1 cells were refractory to a number of different conditions known to induce neuronal cells [D'Ippolito et al., 2004], hepatocytes [Miyazaki et al., 2002], osteogenic cells [Colter et al., 2001; Dennis et al., 2002], and adipocytes [Colter et al., 2001; Dennis et al., 2002] in culture. We then examined the differentiation potential of DEEP-1 cells in vivo by injecting the cells directly into various organs.

When injected, the vast majority of DEEP-1 cells remained in the injected site, but some of the cells migrated into the neighboring region and formed small nests as exemplified in the liver (Fig. 7A). Some of the cells that had migrated acquired a novel phenotype characteristic of surrounding cells. For example, GFP-labeled DEEP-1 cells were positive for albumin in the liver (Fig. 7B). Similarly, we found GFP-labeled

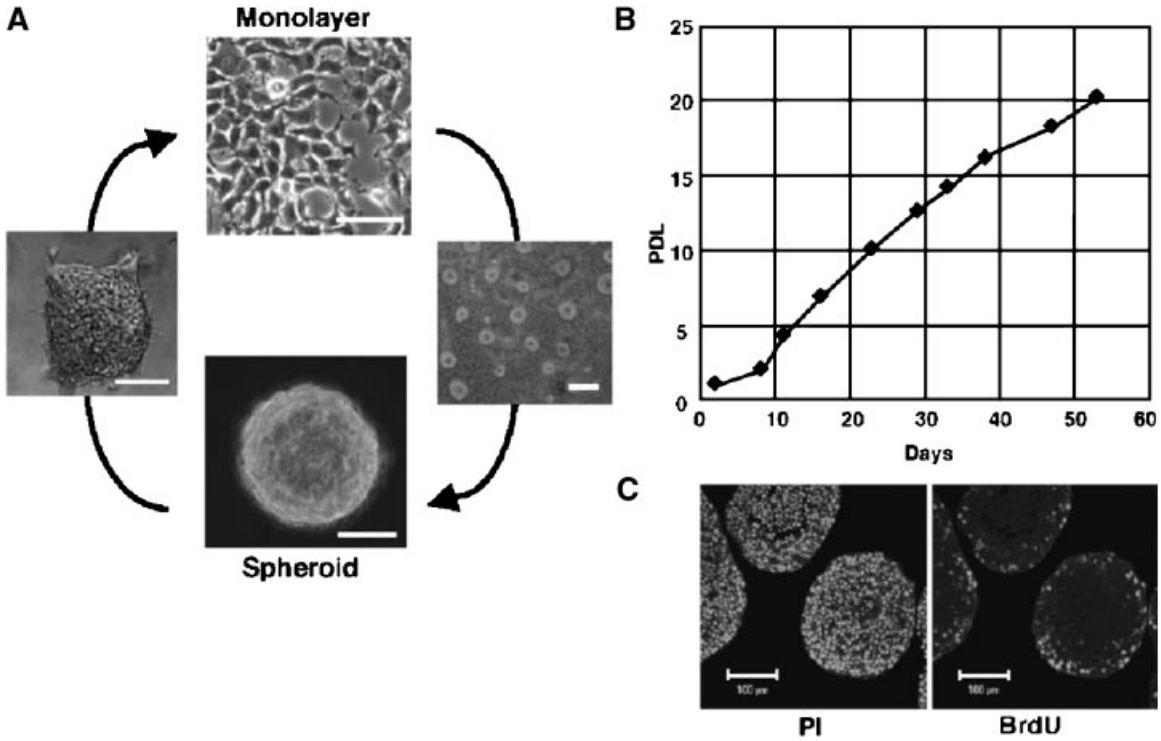


**Fig. 4.** Cultivation of DEEP-1 cells in spheroids. **A**: Convertible culture conditions of DEEP-1 cells. DEEP-1 cells readily formed spheroids either when maintained in high density in monolayer culture or when inoculated into a non-adherence plastic dish. Scale bars: dotted line, 50 μm; thin line, 100 μm; thick line, 200 μm. **B**: A cumulative growth curve of DEEP-1 cells serially passaged in spheroids. **C**: In spheroids, BrdU-positive cells were mostly found in the periphery. PI, propiodium iodide. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DEEP cells that co-expressed aquaporin-1 when injected into the kidney (Fig. 7C) and neuronal markers such as tubulin βIII, GFAP, and nestin when the cells were injected into the brain (Fig. 7D). Although DEEP-1 cells were derived from embryonic dermis, the cells injected into the subcutaneous region of SCID mice did not undergo any appreciable differentiation pathway but remained as an undifferentiated cell mass.

The majority of the injected DEEP-1 cells that did not commit to any differentiation pathways grew to some extent and retained their original properties but remained benign. For example, when injected into the testicle, mouse embryonic stem cells progressively grew, resulting in the formation of a teratoma and destruction of the seminiferous tubules, while DEEP-1 cells grew moderately, leaving the seminiferous tubules intact (Fig. S3).

### Presence of Cytokeratin 8-Positive Cells in Mouse Embryonic Dermis

To gain an insight into the origin of DEEP cells, we stained mouse embryonic skin at 15.5 dpc for CK 18. As shown in Figure 8, we detected CK 18-positive cells scattered in the dermis. Although DEEP cells expressed some epithelial cell markers, no CK 18-positive cells were observed in the epidermis. These results indicate that DEEP cells probably derived from the CK 18-positive cells observed in the dermis at the embryonic stage.

## DISCUSSION

In the present study, we isolated cells possessing some stem/progenitor cell properties using a synthetic thermo-sensitive polymer gel. The present method has the following advantages: (1) it enables cultivation of progenitor/stem cells as a single cell clone from the primary culture, (2) it is simple and easy without a need for any sophisticated apparatus such as a cell sorter, (3) it enables simple recovery of colonies by reducing the temperature, and (4) it can be used for isolation of stem cells other than skin stem cells, for example, neural stem cells and bone marrow mesenchymal stem cells (manuscript

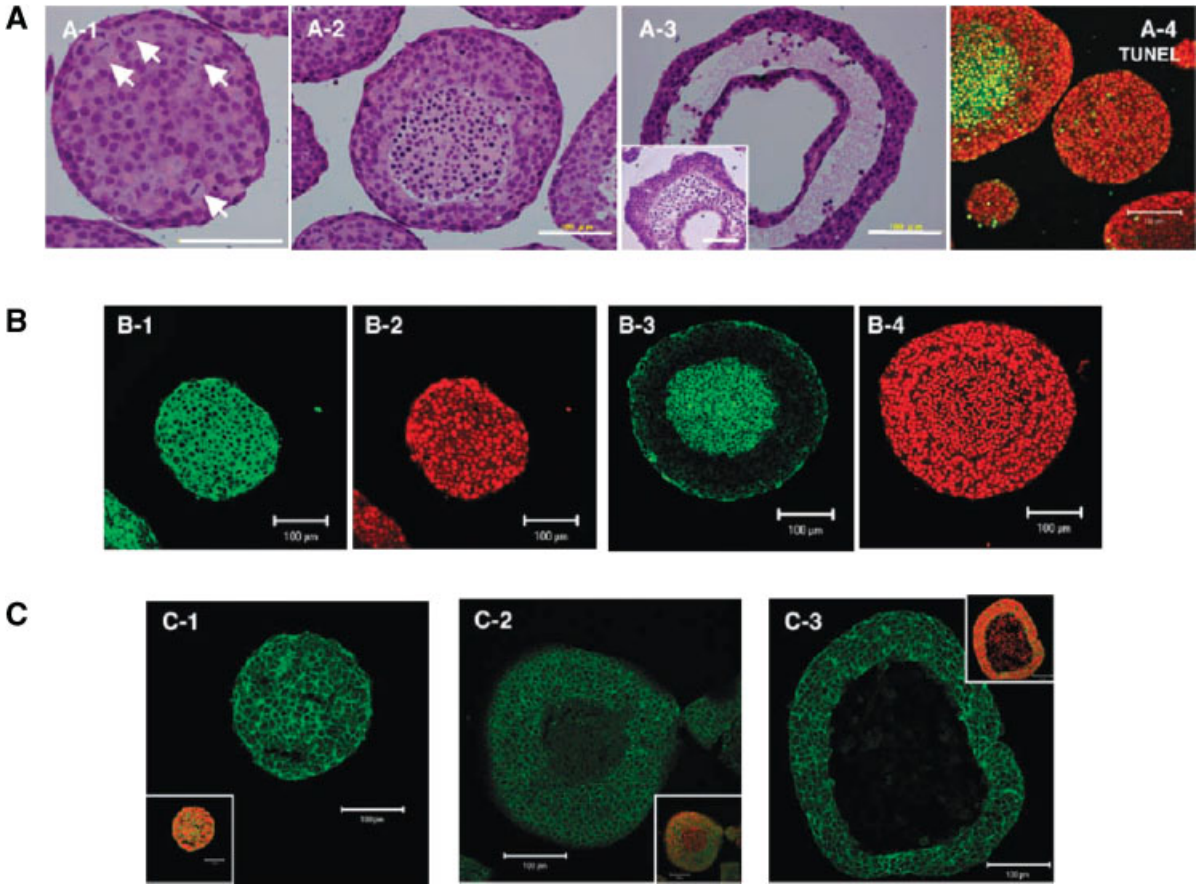


**Fig. 5.** Characteristic natural history of DEEP-1 spheroids. **A1∼3**: Hematoxylin-eosin staining. A-1: DEEP-1 spheroids with diameters less than 150 μm were mostly solid. Mitotic figures were frequently observed in the periphery (white arrows). A-2: As the size of spheroids increased, the central core of the spheroids underwent apoptosis. A-3: At later stages, the cortical area of many spheroids also underwent apoptosis, leaving viable cells only in the inner and outer shells, thus resulting in the formation of a double-layered structure. **A-4**: TUNEL staining of the spheroids shown in green with nuclear staining with propiodium iodide in red. **B**: Expression of cytokeratin 18 in the spheroids at different stages observed by a confocal laser microscope. **B-1** and **B-3**: Immunostaining for CK 18 shown in green; (**B-2** and **B-4**) stained for nuclei with propiodium iodide. **C**: Expression of E-cadherin in the spheroids at the early (**C-1**), middle (**C-2**), and late (**C-3**) stages observed by a confocal laser microscope. Insets, pictures merged with those stained with propiodium iodide.

in preparation). Since the polymer is biologically inert, cells that formed colonies in the gel apparently grew anchorage-independently [Tsukikawa et al., 2003]. This is in accordance with results of previous studies in which stem cells from nervous tissues [Weiss et al., 1996; Uchida et al., 2000] and mammary glands [Dontu et al., 2003] were isolated by cultivating the cells in a suspension.

DEEP-1 cells could be propagated for more than 200 population doublings without any appreciable alteration of phenotype, indicating the possession of self-renewing capacity. DEEP-1 cells expressed marker genes characteristic of stem/progenitor cells and epithelial cells such as S100A6, p63, mRif1, integrin β1, CK 8, CK 18, CK 19, E-cadherin, and ZO-1 (Figs. 2 and 3). In culture, DEEP-1 cells underwent epithelial mesenchymal transition in response to TGFβ1 (Fig. 6). When injected into various adult mouse tissues, some of the injected DEEP-1 cells migrated into the neighboring region and acquired a novel phenotype characteristic of the surrounding cells, such as expression of neural cell markers, aquaporin-1, and albumin (Fig. 7). It is unlikely, though the possibility is not absolutely excluded, that the acquisition of a new phenotype by the injected DEEP-1 cells was solely due to cell fusion because fusional events have been reported to be rare in undamaged organs within rather limited observation periods [Harris et al., 2004; Jang et al., 2004] as was the case in the present study. The apparent self-renewing capacity and lineage-

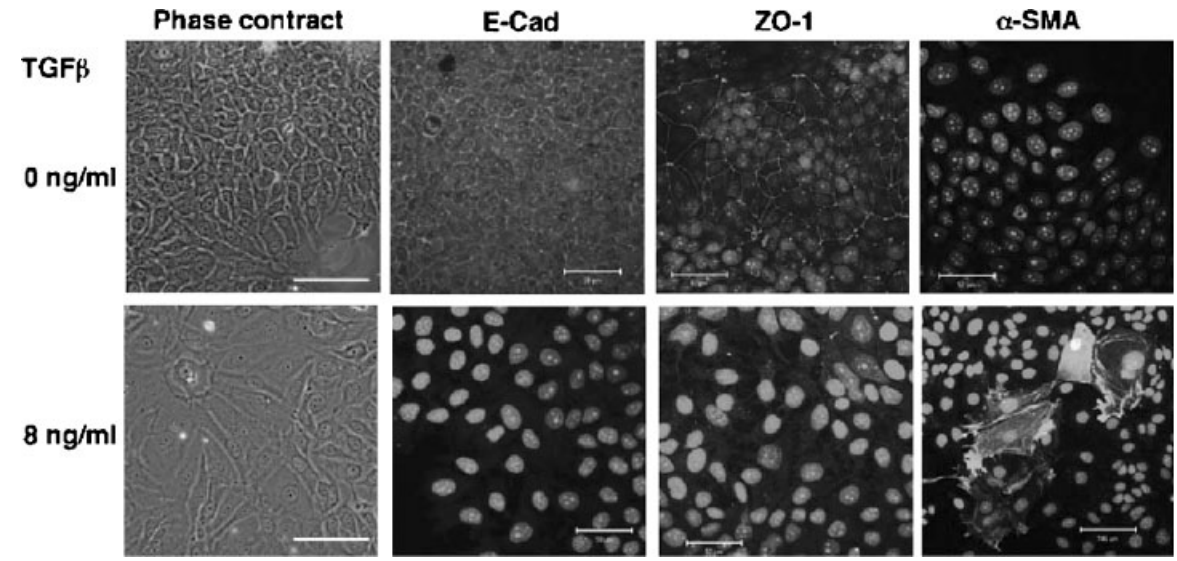


**Fig. 6.** Epithelial mesenchymal transition of DEEP-1 cells by TGFβ1 DEEP-1 cells were cultivated with or without TGFβ1 (8 ng/ml) for 1 week. Morphology of the cells changed from cobble stone-like arrangement to spindle shapes with downregulation of E-cadherin (E-Cad) and ZO-1 and induction of α-smooth muscle actin (α-SMA) at least in some of the cells. The specific signal was shown in green with nuclear staining with propiodium iodide in red. Scale bars in phase contrast pictures, 100 μm; scale bars in immunostained pictures, 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

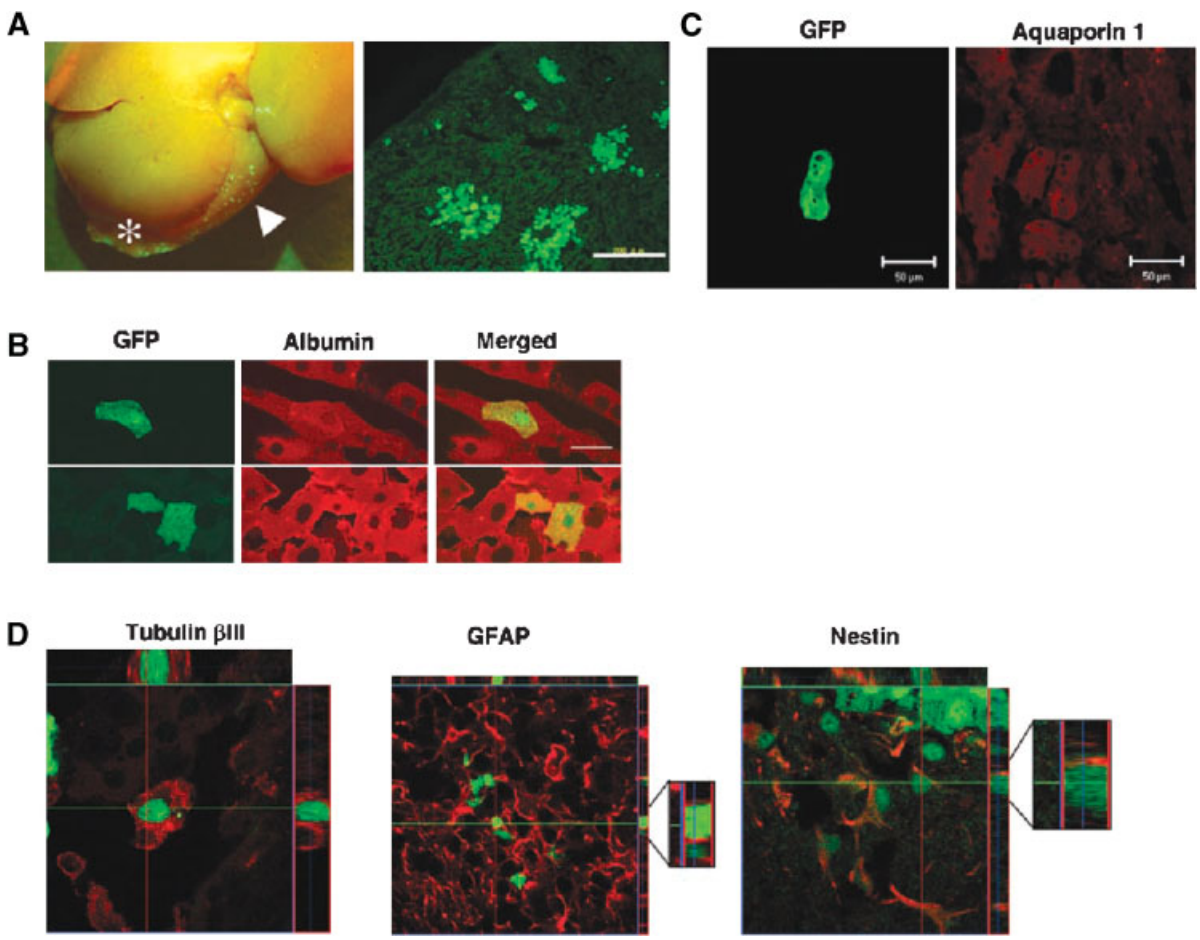


**Fig. 7.**

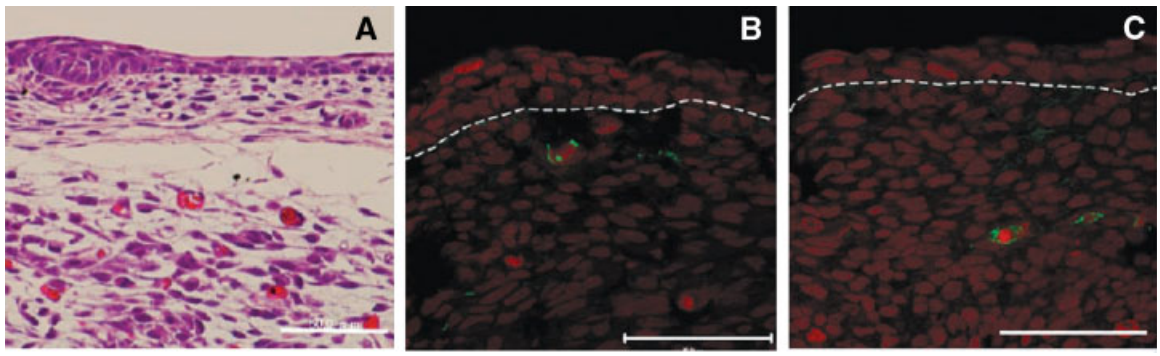


**Fig. 8.** Immunostaining of mouse embryonic skin at 15.5 dpc for CK 18. **A**: Hematoxilin-Eosin staining. **B**, **C**: Immunostaining for CK 18 (green). Nuclei are couterstained with propiodium iodide (red). Dotted white line indicates the boundary between the epidermis and dermis. Scale bars, 50 μm.

unrestricted nature of DEEP-1 cells feature them as a stem/progenitor cell population.

The origin of DEEP-1 cells has not been unequivocally determined. Lack of expression of CK 1, CK 5, CK 14, and CK 6 (Fig. 2) excludes the possibility that DEEP-1 cells are of epidermal keratinocyte origin. Multipotent epidermal stem cells isolated by Liang and Bickenbach [2002] were positive for expression of CK 14. SKPs that were isolated from the mammalian dermis and shown to differentiated into several different cell lineages were consistently positive for expression of nestin [Toma et al., 2001; Fernandes et al., 2004; Amoh et al., 2005] and thus distinct from DEEP-1 cells. Merkel cells are known to express CK 8, CK 18, CK 19, and CK20 but not the cytokeratins featuring more mature keratinocytes such as CK 5, CK 14, and CK 1 [Moll et al., 1984, 1996; Moll and Moll, 1992], this profile being partially shared by DEEP-1 cells. However, CK 20, the most specific marker of Merkel cells [Moll and Moll, 1992], and another marker synaptophysin [Moll et al., 1996] were not detected in DEEP-1 cells (Figs. 2 and S2). In addition, S100A6, p63, and integrin β1, which are frequently expressed in stem/progenitor cells in skin [Jones and Watt, 1993; Ito and Kizawa, 2001; Pellegrini et al., 2001], were detected in DEEP-1 cells (Fig. 2C). It is not known whether these proteins are expressed in Merkel cells. Thus, DEEP-1 cells are distinct from the stem/progenitor cells thus far reported, although they might be related to the precursor of Merkel cells.

DEEP-1 cells showed a peculiar behavior in spheroids (Figs. 4 and 5). In general, cell death is usually observed in the central part of spheroids as their diameter exceeds 200 μm. However, the sharply demarcated boundary between the apoptotic core and cortex area of DEEP-1 spheroids and further apoptotic cell death in the cortex region leaving a double layered-structure (Fig. 5) clearly indicate that the process is not simply a passive one due to the lack of nutrient supply but an active autonomous one reflecting some kind of morphogenic process in vivo. Expression of CK 18, but not that of E-cadherin, was downregulated in the cortical area of DEEP-1 spheroids (Fig. 5B,C).

In conclusion, this is the first trial to apply a synthetic hydrogel for clonal isolation of stem/progenitor cells. Since skin is a relatively easily accessible organ, isolation of stem-like cells possessing a capacity to actively proliferate and to differentiate into multiple cell lineages is of high potential value for future medical applications. The results of the present study using a new simple method contribute to the extension of research activities in such a direction.

**Fig. 7.** Differentiation of DEEP-1 cells in vivo. **A**: Mouse liver injected with DEEP-1 cells. White arrowhead, small nests formed by GFP-labeled DEEP-1 cells that had migrated out of the injected site (*). Scale bar, 200 μm. **B**: Cells positive for both GFP and albumin observed 10 days after intrahepatic injection of GFP-labeled DEEP-1 cells. **C**: Cells positive for both GFP and aquaporin-1 observed 6 weeks after intrarenal injection of GFP-labeled DEEP-1 cells. **D**: GFP-positive cells with co-expression of neuronal markers, tubulin βIII, GFAP, and nestin observed 10 days after intracerebral injection of GFP-labeled DEEP-1 cells. The upper and right protrusions are pictures sectioned along the green and red lines, respectively. The accessory boxes are enlarged pictures.

## REFERENCES


Adams IR, McLaren A. 2004. Identification and characterisation of mRif1: A mouse telomere-associated protein highly expressed in germ cells and embryo-derived pluripotent stem cells. Dev Dyn 229:733–744.

Amoh Y, Li L, Katsuoka K, Penman S, Hoffman RM. 2005. Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. Proc Natl Acad Sci USA 102:5530–5534.

Bhatia M. 2001. AC133 expression in human stem cells. Leukemia 15:1685–1688.

Colter DC, Sekiya I, Prockop DJ. 2001. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 98:7841–7845.

D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. 2004. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J Cell Sci 117:2971–2981.

Dennis JE, Carbillet JP, Caplan AI, Charbord P. 2002. The STRO-1+ marrow cell population is multipotential. Cells Tissues Organs 170:73–82.

Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS. 2003. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17:1253–1270.

Fernandes KJ, McKenzie IA, Mill P, Smith KM, Akhavan M, Barnabe-Heider F, Biernaskie J, Junek A, Kobayashi NR, Toma JG, Kaplan DR, Labosky PA, Rafuse V, Hui CC, Miller FD. 2004. A dermal niche for multipotent adult skin-derived precursor cells. Nat Cell Biol 6:1082–1093.

Harris RG, Herzog EL, Bruscia EM, Grove JE, Van Arnam JS, Krause DS. 2004. Lack of a fusion requirement for development of bone marrow-derived epithelia. Science 305:90–93.

Huelsken J, Vogel R, Erdmann B, Cotsarelis G, Birchmeier W. 2001. beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell 105: 533–545.

Ito M, Kizawa K. 2001. Expression of calcium-binding S100 proteins A4 and A6 in regions of the epithelial sac associated with the onset of hair follicle regeneration. J Invest Dermatol 116:956–963.

Jang YY, Collector MI, Baylin SB, Diehl AM, Sharkis SJ. 2004. Hematopoietic stem cells convert into liver cells within days without fusion. Nat Cell Biol 6:532–539.

Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. 2002a. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol 30:896–904.

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. 2002b. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49.

Jones PH, Watt FM. 1993. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 73: 713–724.

Joshi CV, Enver T. 2002. Plasticity revisited. Curr Opin Cell Biol 14:749–755.

Lendahl U, Zimmerman LB, McKay RD. 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595.

Liang L, Bickenbach JR. 2002. Somatic epidermal stem cells can produce multiple cell lineages during development. Stem Cells 20:21–31.

Miyazaki M, Akiyama I, Sakaguchi M, Nakashima E, Okada M, Kataoka K, Huh NH. 2002. Improved conditions to induce hepatocytes from rat bone marrow cells in culture. Biochem Biophys Res Commun 298:24–30.

Moll I, Moll R. 1992. Early development of human Merkel cells. Exp Dermatol 1:180–184.

Moll R, Moll I, Franke WW. 1984. Identification of Merkel cells in human skin by specific cytokeratin antibodies: Changes of cell density and distribution in fetal and adult plantar epidermis. Differentiation 28:136–154.

Moll I, Paus R, Moll R. 1996. Merkel cells in mouse skin: Intermediate filament pattern, localization, and hair cycle-dependent density. J Invest Dermatol 106:281–286.

Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y. 2001. Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell 104:233–245.

Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F, De Luca M. 2001. p63 identifies keratinocyte stem cells. Proc Natl Acad Sci USA 98:3156–3161.

Randall TD, Weissman IL. 1998. Characterization of a population of cells in the bone marrow that phenotypically mimics hematopoietic stem cells: Resting stem cells or mystery population? Stem Cells 16:38–48.

Rochat A, Kobayashi K, Barrandon Y. 1994. Location of stem cells of human hair follicles by clonal analysis. Cell 76:1063–1073.

Scholer HR, Balling R, Hatzopoulos AK, Suzuki N, Gruss P. 1989. Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. EMBO J 8:2551–2557.

Spangrude GJ, Heimfeld S, Weissman IL. 1988. Purification and characterization of mouse hematopoietic stem cells. Science 241:58–62.

Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM. 2000. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 102:451–461.

Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD. 2001. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 3:778–784.

Tsukikawa S, Matsuoka H, Kurahashi Y, Konno Y, Satoh K, Satoh R, Isogai A, Kimura K, Watanabe Y, Nakano S, Hayashi J, Kubota S. 2003. A new method to prepare multicellular spheroids in cancer cell lines using a thermoreversible gelation polymer. Artif Organs 27:598–604.

Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL. 2000. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci USA 97:14720–14725.

Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA. 1996. Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. J Neurosci 16:7599–7609.

Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. 2001. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7:1028–1034.