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Enrichment and characterization of mouse putative epidermal stem cells

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Abstract

Epidermis, a continuously renewing tissue, is maintained by stem cells that proliferate and replenish worn out or damaged cells in the tissue during life. Cultured epidermal stem cells have great potential in scientific research and clinical application. However, isolating a pure and viable population of epidermal stem cells and culturing them has been challenging. In this study, putative epidermal stem cells of mouse were isolated by combining Hoechst 33342 and propidium iodide staining with fluorescence-activated cell sorting. Molecular markers expression pattern analysis showed that cytokeratin 14, integrin β_1 and p63 are expressed in the sorted putative stem cells, but not active β-catenin, nestin and involucrin. Our results provide further supporting data that mouse putative epidermal stem cells could be successfully isolated by combining Hoechst dye staining with fluorescence-activated cell sorting and cultured in vitro. The cultured mouse putative epidermal stem cells could be used as a potent tool for studying stem cell biology and testing stem cell therapy.

Keywords: Epidermal stem cell; FACS; Hoechst 33342; Molecular marker

1. Introduction

Mammalian epidermis, a stratified squamous epithelium, is made up of multiple layers of keratinocytes. A subpopulation of those keratinocytes in the basal layer, known as epidermal stem cells, gives rise to transit amplifying (TA) cells by symmetric or asymmetric division (Watt and Hogan, 2000). As located in a continually self-renewing tissue, it was supposed that a single epidermal stem cell has sufficient proliferative capacity to produce enough new epidermis to cover the body surface (Fuchs and Raghavan, 2002). Epidermal stem cells play a central role in homeostasis and wound repair and also represent a major target of tumor initiation and gene therapy (Watt, 2000; Perez-Losada and Balmain, 2003). Because epidermis is an excellent example of self-renewing tissue containing stem cells, it is of interest to examine the current status of this field (Lavker and Sun, 2000).

Earlier, studies of mouse epidermal stem cells revolved around the concept of label-retaining cell (LRC). In mouse epidermis, a small population of keratinocytes that slowly cycle and retain a nucleotide label has been identified as LRCs and believed to represent epidermal stem cells for decades (Potten, 1974). But these LRCs could not be used for biological studies that require living cell because they have to be fixed to detect the existing of nucleotide label (Watt, 2001; Terunuma et al., 2003). Clearly, appropriate cell surface markers will facilitate the isolation and characterization of mouse epidermal stem cells. A population of cells with an SP phenotype after a Hoechst 33342 staining has been found in many tissues including hematopoietic system, muscle, brain and pancreas. Most of those cells

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with SP phenotype probably represent primitive stem cells (Goodwell et al., 1996; Gussoni et al., 1999; Terunuma et al., 2003). In mouse epidermis, although it was not called SP population, a population of basal cells with low Hoechst 33342 staining has been isolated with similar method and defined as genuine epidermal stem cells (Dunwald et al., 2001). But molecular markers expressed in this population are unknown yet. The same cell population has not been found in human epidermis when we tried to isolate human epidermal stem cells from aborted fetuses with Hoechst staining (Zhou et al., 2004). Furthermore, recently it has been reported that side population keratinocytes resembling bone marrow side population stem cells are distinct from traditional LRCs (Terunuma et al., 2003). So we hypothesize that the reliability of using Hoechst staining to isolate epidermal stem cells needs to be confirmed in further study.

The p63 transcription factor, a p53 homologue, has been shown to be a specific marker for keratinocyte stem cells. It helps distinguish human keratinocyte stem cells from their TA progeny (Mills et al., 1999; Pellegrini et al., 2001). Our previous study also showed that it appeared in the basal layer of human fetal epidermis, where epidermal stem cells are supposed to be located (Zhou et al., 2004). But its expression in the mouse epidermal SP cells is not unclear. The phosphorylated β-catenin, reflecting Wnt signaling activities, appears to be expressed at a higher level in cultured epidermal stem cells than TA cells (Zhu and Watt, 1999). Its expression in the mouse epidermal SP cells is not also unknown yet.

Our goal here has been to determine the reliability of using Hoechst staining to isolate mouse epidermal stem cells by exploring the molecular marker expression pattern in the putative mouse epidermal SP cells.

2. Materials and methods

2.1. Animal

Kunming white strain mice (Experimental Animal Center, the Genetic Institute of Chinese Academy of Sciences) were housed in the animal facility of the State Key Laboratory of Reproductive Biology. Each pregnant mouse was bred in a separate cage. Neonatal mouse was used for collecting skin sample at first or second day after its birth.

2.2. Medium and antibodies

S-MEM medium and fetal calf serum were purchased from Invitrogen. The EGF growth factor (EGF), Hoechst 33342, hydrocortisone, minoguanidine nitrate and cholera toxin were purchased from Sigma. The anti-integrin β_1 mouse monoclonal antibody (sc-9970, Santa Cruz), anti-p63 antibody (sc-8431, Santa Cruz), anti-involucrin antibody (sc-15223, Santa Cruz), anti-nestin antibody (MAB353, Chemicon) and anti-active-β-catenin antibody (05-665, Upstate) were used in the immunofluorescent staining at dilutions suggested by the suppliers.

2.3. Cell preparation for FACS

Neonatal mouse was humanely sacrificed when necessary and skin sample collected. The skin sample was then placed in 70% ethanol for 1 min. After an extensive wash with PBS without Ca^{++} and Mg^{++}, the skin was incubated in 0.25% trypsin for overnight at 4 °C. Next day, the epidermis was removed from the dermis and a single cell suspension was acquired by gently shaking the epidermis. After being washed twice in 1% BSA, the cell population was incubated in 1% BSA solution containing 5 μg/ml Hoechst 33342 for 2 h at 37 °C. The cells were washed with 1% BSA and suspended in S-MEM medium containing 1 μg/ml PI. Stained cells were kept on ice until the flow cytometric analysis.

2.4. FACS

Cells' sorting was performed on a FACSDiva cell sorter (Becton–Dickinson). Gates were set using FSC and SSC. At least 50,000 events were acquired in list mode for each sample. Forward and orthogonal scatter signals were generated using 100 mW at 488 nm. PI and Hoechst 33342 were excited with 100 mW UV radiation (351–364 nm). Hoechst fluorescence was detected through a 440/60 nm band pass filter and PI was detected through a 670/14 nm band pass filter. Debris and cells positive for PI were gated out. The selected pressure is 20 PSI and pressure difference is 0.8.

2.5. Immunocytochemistry

For the immunocytochemistry analysis of sorted cells, cells were air dried onto glass slides, fixed with a mixture of methanol and acetone (1:1) for 10 min at room temperature, rinsed in PBS for three times, and incubated with a primary antibody at 4 °C for overnight. Next day, after three washes with PBS, the appropriate FITC-labeled secondary antibody was added and incubated for 45 min at 37 °C. After many washes in PBS, PI (1 μg/ml) was added and incubated for 2 min. The slides were washed again with PBS and examined under a confocal microscope (Leica). To confirm the negative expression of β-catenin, nestin and involucrin in the sorted putative epidermal stem cells, putative human epidermal stem cells, $nestin^+$ mouse ES cells (kindly gifted from Dr. Shiyan) and suspension-induced putative human epidermal stem cells (Zhou et al., 2004) were used as positive controls.

2.6. Cultivation of sorted mouse putative epidermal stem cells

Sorted putative epidermal stem cells were cultured on a collagen type IV-coated (100 μg/ml) plate. After sorting, the putative epidermal stem cells were planted into a 24-well plate at a density of 10^5cell per well. The medium was the same as previously described (Dunwald et al., 2001) and changed every 2 days. Cultures were passaged when they reached 80% confluence.

3. Results

3.1. Putative epidermal stem cells isolation by flow cytometry

Fig. 1a shows cells with a lower forward light-scatter (FSC) and side light-scatter (SSC) that gated in region P1. Label-retaining in vivo showed that ~10% of basal cells were genuine epidermal stem cells. So we first chose ~10% of the total basal cells for further analysis (Fig. 1c). To sort stem cells from this small cell population, cells with the lowest Hoechst fluorescence were further gated in region P3. The cells here were our putative epidermal stem cells (Fig. 1c). This cell population was ~4.9% of the total basal cells. The transit amplifying (TA) cells were collected as cells in region P2 with higher FSC and SSC without regard to the Hoechst fluorescence (Fig. 1a). Under a phase microscope, the sorted putative stem cell population was small, round and homogenous cells (Fig. 2a), but the TA population were inhomogeneous cells with big or small volume (Fig. 2b).

3.2. Positive markers in the putative epidermal stem cells

To identify the putative stem cell population, we examined the expression of some previously reported markers using immunofluorescence. Under a fluorescent microscope, integrin β_1 (Fig. 3a) and p63 (Fig. 3b) were highly expressed on all of the sorted putative stem cells. p63 Protein was only expressed in the nucleus of sorted putative stem cells (Fig. 3b). Cytokeratin 14, a previously reported marker of undifferentiated mouse basal

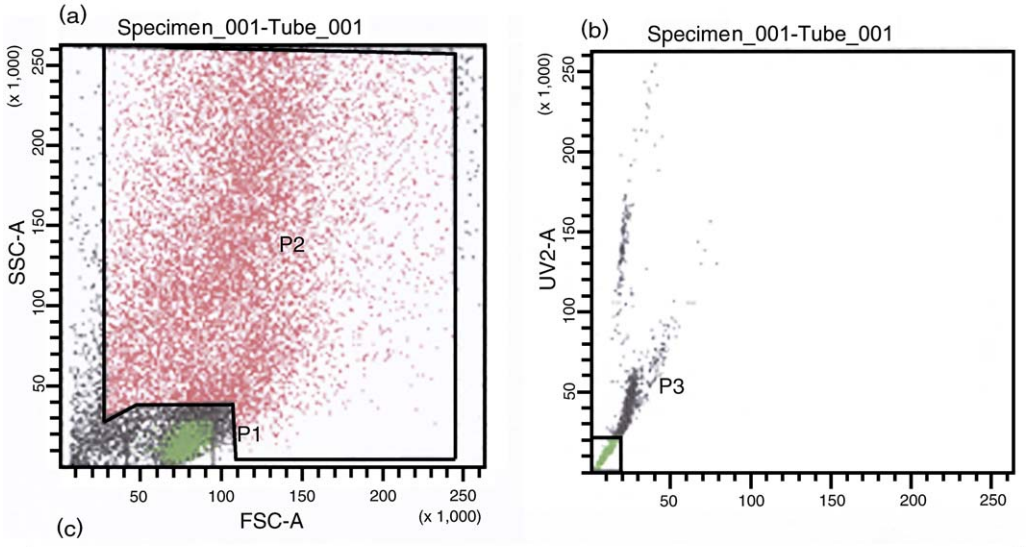

Fig. 1. Fluorescence-activated cell sorting of mouse putative epidermal stem cells and transit amplifying cells. (a) Dot plot shows the FSC and SSC of a suspension of mouse basal keratinocytes. Cells with lower or higher FSC and SSC are gated in region P1 and P2, respectively. The TA cells were collected without regard to the Hoechst fluorescence. (b) Cells with the lowest Hoechst fluorescence were gated in region P3 and collected as putative epidermal stem cells. (c) Percentage of each gated cell population. Cells in region P3 were about 4.9% of total basal cells.

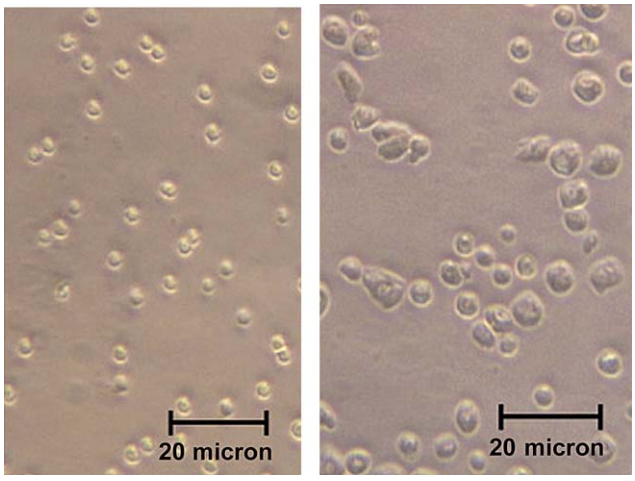

Fig. 2. Morphology of sorted putative stem cells and TA cells. (a) Stem cells. (b) TA cells. Scale bar = 20 μm.

keratinocyte, also appeared in the membrane of sorted putative stem cells (Fig. 3c), consistent with these other reports. The results showed that this sorted population probably represented a genuine epidermal stem cell population.

3.3. Negative markers in the putative epidermal stem cells

Some other markers were also detected in the sorted putative epidermal stem cells. Surprisingly, we found that active β-catenin, a molecule tightly related with self-renewal potential of human epidermal stem cells, was not expressed in the sorted putative epidermal stem cells (Fig. 3g). In addition, we have found that nestin, a putative marker widely appeared in neural stem cells and other adult stem cells, did not appear in the sorted putative epidermal stem cells (Fig. 3h). Involucrin, a previously reported marker of differentiated mouse basal keratinocyte, did not appear in the sorted putative stem cells (Fig. 3i).

3.4. Cultivation of sorted mouse putative epidermal stem cells

We found that the putative stem cells required to be inoculated at a higher than normal concentration to grow in culture after the physical stress of sorting (about 10^5 cells per well of a 24-well plate). After planting in a collagen type IV-coated plate, the putative stem cells grew very slowly at first and after ~6 days they began to grow more quickly (Fig. 4), taking about 10 days to reach confluence (data not shown). The average doubling-time of stem cells was 10–12 days.

4. Discussion

In our study, the putative mouse epidermal stem cells have been isolated from the neonatal epidermis using Hoechst staining and FACS. The sorted putative stem cells express cytokeratin 14, integrin β_1 and p63, but not active β-catenin, nestin and involucrin. There are at least two factors important for isolating epidermal stem cells. One is choosing specific surface molecular markers, and the other is separating basal keratinocyte population containing epidermal stem cells from the rest of the epidermis. One of the reported methods used to enrich mouse epidermal stem cells is with integrin α_6 and transferring receptor CD71 as stem cell markers (Tani et al., 2000). But these could not be used widely because they remain relatively poorly defined. In fact, lack of specific molecular markers has been a major obstacle in epidermal stem cells research (Lavker and Sun, 2000). Our results further support the notion that Hoechst 33343 staining combined with FACS may be a reliable method for isolating mouse epidermal stem cells.

We report that the sorted putative mouse epidermal stem cells by Hoechst staining express integrin β_1 and p63, but not active β-catenin and nestin. This raises a very interesting question: is there anything in common between human and mouse epidermal stem cells, even with other adult stem cells? There are many reports of inconsistency between human and mouse epidermal stem cells. Our previous study and others have shown that integrin β_1 can function as a reliable marker for isolating human epidermal stem cells (Jones and Watt, 1993; Zhou et al., 2004), but it could not be used for isolating much mouse epidermal stem cells.

It was shown that the active β-catenin was expressed at a higher level in cultured human epidermal stem cells than TA cells (Zhu and Watt, 1999). There are also data showing that β-catenin is essential in deciding the fate of skin stem cells (Joerg et al., 2001). Other recently reported data further imply that β-catenin signaling plays an important role in maintaining self-renewal potential or regulating lineage selection in human ES cells or other adult stem cells (Niemann et al., 2003; Sato et al., 2004). But in this study we found it did not appear in the mouse epidermal stem cells. We suppose that the putative stem cells in vitro have been down-regulated with regard to active β-catenin protein. But to consolidate this data with our results, further investigations are needed. Nestin is a widely distributed marker in adult stem cells except for neural stem cells (Lendahl et al., 1990; Lechner et al., 2002). We have boldly assumed that neural stem cells had some commonality with epidermal stem cells because these two tissues both originate from ectoderm. But here we found that, it did not appear in the mouse putative epidermal stem cells. Furthermore, we have found that our culture system—conducive for human epidermal stem cells—did not

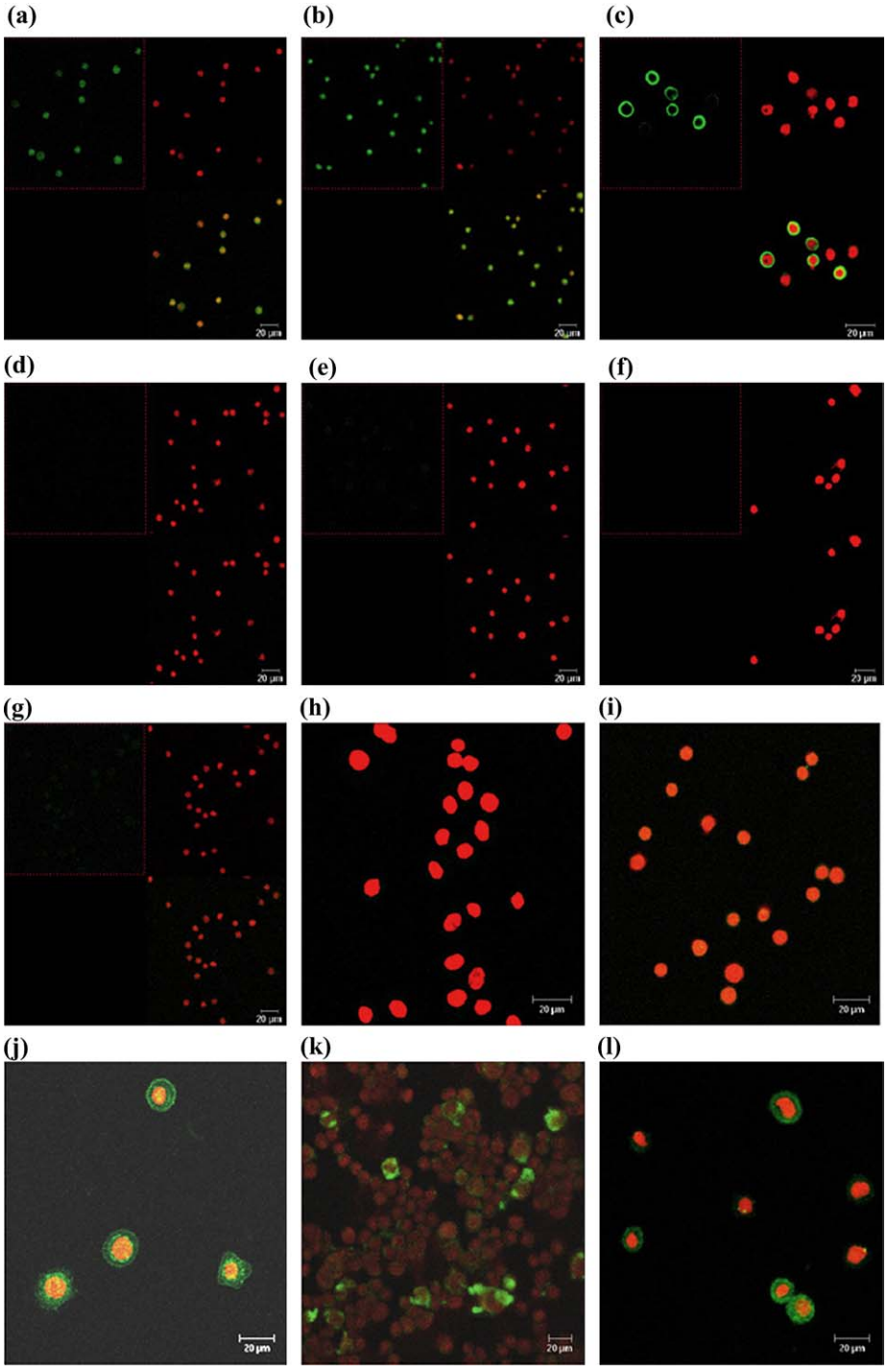

Fig. 3. Molecular markers expressed in the sorted putative epidermal stem cells. (a) Expression of integrin β_1 in the sorted putative epidermal stem cells. (b) Expression of p63 in the sorted putative epidermal stem cells. It presents in the nucleus of the cells. (c) Expression of cytokeratin 14 in the sorted putative epidermal stem cells. It mainly locates in the membrane of the cells. (d–f) Negative controls of (a–c), respectively. (g) Negative expression of β-catenin in the sorted putative stem cells. (h) Negative expression of nestin in the sorted putative stem cells. (i) Negative expression of involucrin in the sorted putative stem cells. (j–l) Positive controls of (g–i), respectively. (j) Expression of β-catenin in the sorted putative human epidermal stem cells. (k) Expression of nestin in mouse ES cells. (l) Expression of involucrin in suspension-induced putative human epidermal stem cells. The green colour represents positive staining of primary antibody and the red colour indicates nucleus staining. The yellow colour represents convergence of green and red. Scale bar = 20 μm.

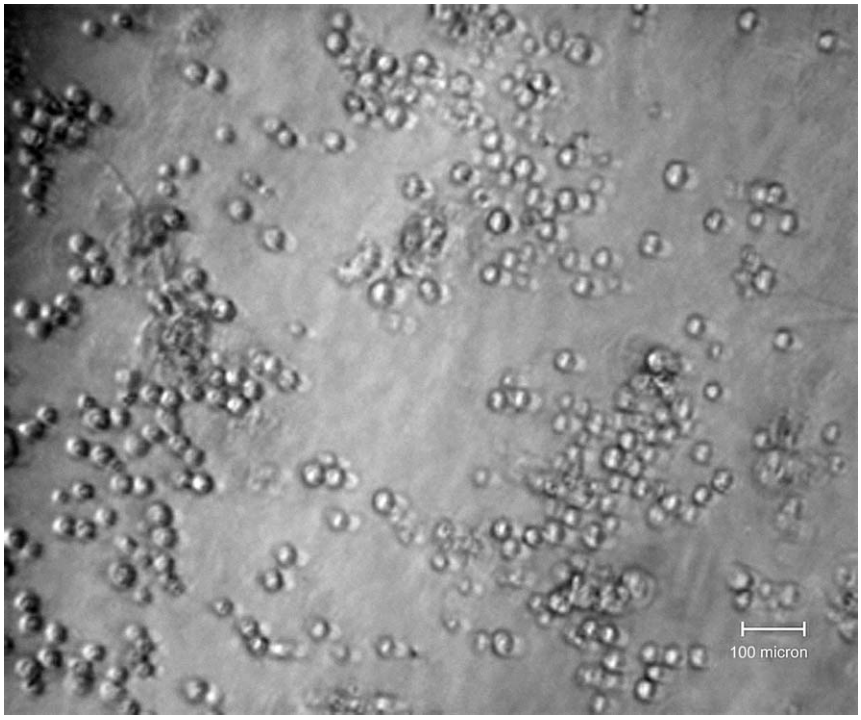

Fig. 4. Morphology of cultured mouse putative epidermal stem cells. Scale bar = 100 μm.

support the growth of mouse epidermal stem cells (unpublished data), and little is known about these differences.

In conclusion, mouse putative epidermal stem cells have been successfully isolated from neonatal epidermis using FACS and cultured in vitro. Our results confirmed previous observations that mouse putative epidermal stem cells could be isolated by Hoechst staining and FACS. The isolated epidermal putative stem cells population will be extremely useful in future for studying stem cell biology and the potential of generating functional differentiated cells other than epidermal cells.

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