Rapid enrichment of stem cell population by filter screening and biomarker-immunoassay from human epidermis

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Abstract

Background. It is known that there are some biomarkers for the epidermal stem cells (ESC), such as p63, k19 and β1 integrin. *Objective.* This study was to explore rapid isolation of the epidermal stem cell population (ESCP) from human epidermis and to identify their biological characteristics. *Methods.* The ESCP were enriched from the digested single keratinocytes (KC) solution through screening filters with different pore sizes (mu) and through ELISA plate and Sepharose 4B mini-column immunoassays. The biological characteristics of the enriched putative ESCP were identified by Wright's stain in cell morphology, p63/k19 biomarker in immunostaining and their colony formation rate and the confluence time in culture. *Results.* Based on identification of the putative ESCP with Wright's stain and p63/k19 immunostaining, the ESCP mainly included the smaller ESC (< 11 μm in diameter) and the larger transient-amplifying cells (TA) (11 μm – 20 μm in diameter). About 50% p63immunoreactivity (IR) ESCP could be enriched through screening filter of 300 mu/350 mu, and about 90% p63-IR/k19-IR ESCP could be isolated by ELISA-plate or Sepharose 4Bmini-column immunoassays. In comparison of ESCP with regular KC in culture, the former with less seeded cell number showed higher colony formation rate and earlier confluence time; and the confluent epidermis of ESCP showed stratified and p63-IR in some basal cells. *Conclusion.* In enrichment of the ESCP, the immunoassay technique is more efficient than the filter screening alone; but the latter method was much simple. The viable ESCP with higher efficiency in colony formation and confluence may be attributable to their higher biological potential. [Life Science Journal. 2008; 5(4): 33 – 37] (ISSN: 1097 – 8135).

Keywords: epidermal stem cell population; enrichment; filter screening; biomarker immunoassay; human ESCP culture

1 Introduction

The epidermis is mainly composed of the keratinocytes (KC), occupying 95% of total epidermal cells. The rest 5% includes the melanocyte, Langerhans' cell and Merkel cell. The KC can be divided into epidermal stem cells (ESCs), transient-amplifying cells (TA) and post-mitotic differentiating cells (PMD). The PMD without proliferation ability are terminally desquamated and replaced by the underlying epidermal progenitor cells. The ESC are able to differentiate into TA which possess restricted proliferating capacity through 3 – 5 generations to become PMD and committed differentiating capacity[1]. The ESC located at the human basal layer of epidermis only occupy less than 10% of the basal cells, and they self-renew slowly and can differentiate throughout their life to replace the terminal differentiated epidermal cells to maintain epidermis homeostasis. Since the ESC exhibit biological potential for proliferation, they play a central role in both research fields and clinical application to diseases[2].

Isolation of ESC and TA from human interfollicular epidermis is crucial in study of the biological experiment and clinical application for their regeneration potential[3]. Several techniques have been used for isolating ESC/TA, mainly including collagen IV/β1 integrin adhesion, fluorescence-activated cell sorting (FACS), magnetic force

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stirring and etc[1,4,5]. Park *et al* applied physical force using magnetic stirrer to isolate ESC in addition to separating single epidermal cells with trypsin and EDTA[5]. Besides the physical force, the molecules, such as integrin family members expressed on the cell surface of ESC/TA could be used to isolate them by adhesion and FACS techniques. Zhou et al. could enrich ESC from aborted human fetuses by FACS technique based on their high expression of β1 integrin[6]. Kim *et al* successfully isolated the ESC from TA and non-adherent cells; in this case, the rapid adhesion of ESC could be completed in 10 – 20 minutes, while the TA with lower adhesion capacity needed more than 60 minutes to 24 hours[7]. Besides, he demonstrated that the putative stem cell population could be isolated by the double labeled technique, when α6 integrin positive and CD71 negative were used as biomarkers. By using of the FACS technique, Li and Kaur isolated the viable ESC, TA and early differentiating KC stained with α6 integrin and CD71 immuno-fluorescence from human or murine skin[4]. In the adhesive technique with collagen, FACS and application of physical force technique, the enriched stem cells could be identified by β1 integrin as a biomarker of ESC[7,4,5].

The p63, a homologue of p53 tumor suppressor gene, is involved in morphogenesis of stratified epidermis. The p63 molecule expressed by the epidermal progenitor cells could be used to identify ESC/TA[8]. Larouche *et al* demonstrated that the ESC marker, k19, could be used to identify label-retaining stem cells with ^{3}H-thymidine due to their slow cycling[9].

We consider that the ESCP isolated by the immunoassay with p63 or k19 biomarker, not by the adhesive technique, maybe more contribute to further ESC gene transfer experiment. In this study we evaluated filter screening and biomarker-immunoassay techniques for enrichment of the ESCP, including putative ESC and TA. Meanwhile, their cell morphology, immuno-phenotype marker and biological characteristics in culture were identified.

2 Materials and Methods

2.1 Preparation of epidermal single keratinocyte solution

From 4 healthy adults aged 25-30 years, the surgically resected foreskin samples were isolated from the underlying dermis by digestion with 0.5 mg/ml thermolysin (Sigma, US) at 37 ºC for 2 hours. After the epidermis was cut into pieces at size of 1 mm × 1 mm, the epidermal pieces were treated with 0.25% trypsin plus 0.02% EDTA at 37 ºC for 10 minutes and the digestion process was terminated by adding equal volume of the complete keratinocyte culture medium. The keratinocytes were separated into single cells through 240 mu filter.

2.2 Enrichment of ESCP by screening filters

The digested single keratinocytes in solution were passed through 240 mu, 300 mu and 350 mu nylon screening filters respectively and seeded onto a six-well plate precoated with 10 μg/ml mitomycin C (Kyowa, Japan) treated NIH-3T3 cells. The keratinocytes were cultivated with a 3 : 1 DMEM-F12 (Gibco, US) complete medium, containing 10 μg/L EGF, 5 mg/L insulin, 0.4 mg/L hydrocortison, 0.1 nM cholera toxin, 4 mM glutamine, 0.1 mM calcium chloride (after KC confluence the concentration was promoted up to 1.3 mM) and 100 mg/L penicillin and streptomycin (all reagents purchased from Sigma, US) and supplemented with 10% fetal bovine serum (TBD, China).

2.3 Enrichment of ESCP using ELISA-plate immunoassay

The anti-p63 IgG from 50 μl p63 monoclonal antiserum (SantaCruz, US) was extracted by $(NH_4)_2SO_4$ precipitation method and quantified with Comassie brilliant blue G250. The anti-p63 IgG was dialyzed against 0.04 M $NaHCO_3$ buffer (pH 8.5). 50 μl of the final concentration of dialyzed solution (diluted to 1 : 50) was plated onto each well of a six-well plate and dried at 4 ºC. Each well was added (blocked) with 50 μl of 0.01 M Tris-Tween-20-buffer saline (TTBS, pH 7.4) at room temperature for 20 minutes and rinsed once with TBS without Tween-20. The single keratinocytes in 25 μl D-Hanks (pH 7.2) were added onto each well at 4 ºC incubation for 2 hours. After the incubation the keratinocytes were eluted with D-Hanks (pH 6.2) twice. The eluates were collected and the smear slides were prepared separately.

2.4 Enrichment of ESCP using Sepharose 4B mini-column

The swollen sephrose colloid was prepared by 11.4 mg Sepharose 4B powder (Pharmacia, US) dissolved in 2.2 ml 1 mM HCl and transferred into a mini-column at size of 5 cm in length × 0.5 cm in diameter made by our research center. After the mini-column was thoroughly washed with 1 mM HCl for 15 minutes, 60 μl of p63 IgG in coupling buffer ($NaHCO_3$ plus NaCl, pH 8.3) was added into the mini-column at 4 ºC, overnight. The recessive ligand was removed in 3 cycles using the coupling buffer. In each cycle the mini-column was washed with 5 volume buffer vs. the colloid, containing 0.1 M acetate buffer (pH 4.0) plus 0.5 M NaCl, and subsequently washed by Tris-

buffer (pH 8.0) plus 0.5 M NaCl. The single keratinocytes in 100 μl D-Hanks (pH 7.2) was added into the mini-column at 4 ºC incubation for 30 minutes. The keratinocytes were eluted twice with 0.1 M $NaHCO_3$ buffer (pH 6.0) plus 0.5 M NaCl, and the smear slides with twice eluates and that prior to elution were prepared separately.

2.5 Identification of collected ESCP

2.5.1 Wright's staining. After passing through the filter of 240 mu, 300 mu or 350 mu, the single keratinocytes on the smear slides were stained with Wright's dye routinely. The percentages of putative ESCP were counted under an oil-microscope.

2.5.2 Immuno-staining. The confluent epidermis section and the smear slides treated with the immunoassay and filter screening via different mus were immunostained respectively with p63 or k19 (Zymed, US) monoclonal antibody, and the SP kit (Zhongshan, China) according to manufactory's instruction. The slides were lightly counterstained with Eosin. The PBS substituted for the specific primary antibody was performed as the negative control.

2.5.3 Biological characteristics of the enriched ESCP culture. The eluted viable single keratinocytes via ELISA-plate immunoassay were cultured in DMEM-F12 complete medium. The number of colony with more than 50 KC, the colony formation rate (the number of colony / the number of seeded KC) and the confluent time were recorded. After three weeks, the confluent epidermis was fixed with 4% formaldehyde and prepared into paraffin-embedded sections which were p63-immunostained and counterstained with Eosin.

2.6 Statistical analysis

ANOV and *r* (correlation coefficient) were analyzed for the data with SPSS 10 software, and $\alpha = 0.05$ was considered as significant level.

3 Results

3.1 ESCP screened by passing through filters

In Wright's staining, the basal cells of KC appeared small round in shape, less than 11 μm in diameter, the nucleus in deep violet-color surrounded with less cytoplasm in a high nuclear-cytoplasm ratio. The epidermal superficial layer KC in polygonal shape without nuclei were much larger, more than 20 μm in diameter, and stained in reddish color. The shape and size of the middle layer KC were located between the epidermal basal and superficial layers. The keratinocytes with 10 μm – 20 μm in diameter were counted as putative ESCP. In p63 immunostained slides, both the ESC less than 11 μm in diameter, and TA with 11 – 20 μm in diameter displayed p63-immunoreactivity (IR) and were counted as ESCP. Other KC than ESC and TA didn't show p63-IR (Table 1 and Figure 1 a, b).

3.2 ESCP enriched with immunoassays

The p63 monoclonal antibody was coated to the ELISA-plate or coupled into the Sepharose 4B mini-column. The KC in the first and second eluates along with those prior to elution were stained with p63/k19 immunocytochemistry. On the slide, about 90% ESCP, including ESC (less than 11 μm in diameter) and TA (11 μm – 20 μm in diameter) showed p63-IR or k19-IR; other KC than KSC and TA didn't display p63-IR or k19-IR. The p63-IR% and k19-IR% in the first or second eluate were higher than those prior to elution; and there was significantly positive correlation between p63-IR% and k19-IR%, $r = 0.985$, $P < 0.05$ and between the ELISA-plate and Sepharose 4B mini-column immunoassays, $r = 0.959$, $P < 0.05$ (Table 2 and Figure 2 a & b).

3.3 The seeding cell number, colony formation rate and confluence time in enriched ESCP culture

The seeding cell number, colony formation rate and confluence time of enriched ESCP measured in ELISA-plate immunoassay were compared with control that was only treated with 240 mu screening filter alone. The former had higher colony formation efficiency and appeared confluence 5 days earlier than the latter. Furthermore, no correlation was found between the seeding cell number and colony formation rate or between the colony formation rate and the confluence time (Table 3, Figure 3 a & b).

3.4 The biological character of the enriched ESCP culture through ELISA plate immunoassay

When the colony appeared confluence 7 days after seeding, the calcium was supplemented into the medium to facilitate its differentiation. Three weeks after confluence the epidermis developed stratified from the confluent mono-layer; and p63-IR resided in some basal cells by immunostaining (Figure 4 a & b).

4 Discussion

In comparison with adhesion technique, the magnetic stirring could isolate ESC by physical force. The filter mesh screening, that allowed KC passing through restricted pore size exerted physical force to enrich ESC/TA, was studied in this experiment. The results showed

Table 1. The putative ESCP% in Wright's stain and ESCP% identified by p63-IR after screening through 240 mu, 300 mu and 350 mu filters respectively

Staining/filters	240 mu	300 mu	350 mu
P63-IR%	40.3 ± 1.71[1]	52.3 ± 0.96[2]	53.0 ± 1.41[3]
Wright's stain%	24.5 ± 1.29[4]	50.3 ± 1.71[5]	51.5 ± 1.29[6]

[1] *vs.* [2] or [3], $P < 0.05$; [4] *vs.* [5] or [6], $P < 0.05$; [2] *vs.* [3] or [5], $P > 0.05$; [6] *vs.* [3] or [5], $P > 0.05$.

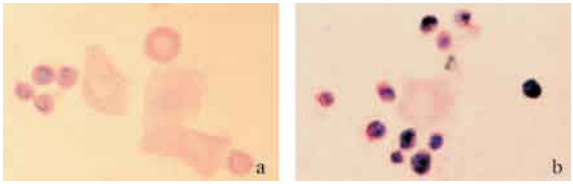

Figure 1. a: Filter screened KC via 240 mu, more cells in larger size, Wright's staining; b: Filter screened putative ESCP via 350 mu, more cells in smaller size (Wright's staining, × 400).

Table 2. The p63-IR% and k19-IR% in the first and second eluates compared with those prior to elution by ELISA plate and Sepharose 4B mini-column immunoassays

Eluate/Assays	ELISA-plate immunoassay		Mini-column immunoassay	
	P63-IR %	K19-IR %	P63-IR %	K19-IR %
Prior to elution	67.3 ± 2.6[1]	64.7 ± 1.26[4]	74 ± 1.63[1']	75.5 ± 2.08[4']
1st eluate	90.0 ± 0.82[2]	89.5 ± 2.52[5]	90.25 ± 2.22[2']	93.25 ± 3.77[5']
2nd eluate	91.3 ± 2.5[3]	90.2 ± 1.71[6]	92.25 ± 1.26[3']	93.5 ± 0.58[6']

[1] *vs.* [2] or [3], $P < 0.05$; [2] *vs.* [3], $P > 0.05$; [4] *vs.* [5] or [6], $P < 0.05$; [5] *vs.* [6], $P > 0.05$; [1'] *vs.* [2'] or [3'], $P < 0.05$; [2'] *vs.* [3'], $P > 0.05$; [4'] *vs.* [5'] or [6'], $P < 0.05$; [5'] *vs.* [6'], $P > 0.05$.

that about 50% ESCP could be obtained after screening with 300 mu or 350 mu filter; there was no significant difference in ESCP% between 300 mu and 350 mu filter screening which was significantly higher than that with 240 mu filter. The filter screening method despite of lower screening efficacy was much simple and less expensive, if the collected numbers of ESCP would be enough for corresponding research requirement.

Pellegrini provided evidence that the p63 transcription factor could distinguish human keratinocyte stem cells from the transient amplifying cells and could be used as an epidermal stem cell marker[8]. The k19 was a potential biochemical marker for skin stem cells both *in vivo* and *in vitro*[9,10]. However, Janes *et al* indicated that no molecular marker could be used to distinguish ESC from TA[1]. Based on difference in expression levels of the surface molecular marker between ESC and TA, the adhesive technique and FACS were able to distinguish ESC from TA to certain extent. Park *et al* could identify full range of basal epidermal cells, including stem cells, transiently amplifying cells and differentiating cells by the levels of surface β1 integrin expression[5]. Similarly, the p63 expression is not limited to "stem" cells which in fact express the highest levels of p63, one marker of the epithelial progenitor cells[11].

Our results showed that the biomarker of p63 or k19, one kind of molecules, could be expressed by ESC/TA and was located in the nucleus/cytoplasm and partial periphery of cells on the smear slides. The ESCP could be enriched through transiently immuno-coupling by p63 or k19 antibody with the corresponding ligand followed by eluation easily through modified pH and ionic strength in the immunoassays of both ELISA plate and Sepharose 4B immunoassays performed in this study. Radu *et al* reported that 65% putative epidermal stem cells identified by p63 expression could be enriched rapidly[12]. In this experiment about 60% – 70% ESCP prior to eluation were already immuno- coupling onto the ELISA plate or onto the Sepharose 4B pretreated with p63/k19 antibody. In the first or second eluate more ESCP% could be obtained approaching up to about 90%. Our results showed that there was no significant difference in immuno-staining between p63-IR% and k19-IR% and in ESCP enrichment efficacy between ELISA plate and Sepharose 4B mini-column immunoassays. Furthermore, the ELISA-plate immunoassay expend less time than the latter; besides, the viable ESCP could be gained ralatively not easily to be contaminated by the immunoassay or filter screening technique, and they need not to be passed through special equipments, such as FACS and magnetic stirrer.

The p63 with more complicate function than p53 is the molecular switch for initiation of an epithelial stratification program. Koster *et al* displayed that the murine ectoderm lacking p63 never stacked up and failed to form a stratified epithelium[13]. Yang *et al* reported that the mice with $p63^{-/-}$ pattern did not exhibit stratified epidermis[14]. Our results showed that the enriched viable ESCP through ELISA-plate could possess higher colony formation efficiency and confluent ability, and the confluent epidermis displayed stratified character with p63-IR in some basal

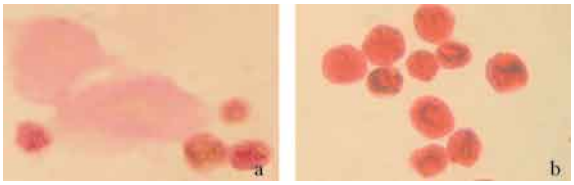

Figure 2. a: Filter screened KC via 240 mu, p63-IR in dark brownish color; b: Filter screened ESCP via 350 mu, p63-IR in dark brownish color (p63 immunostaining, Eosin counterstaining × 1000).

Table 3. The seeding cell number, colony formation rate and confluence time of the enriched ESCP via ELISA-plate immunoassay compared with those treated with 240 mu filter alone as the control

Enrichment/ assay	Seeding number	Colony formation rate	Confluence time
240 mu filter	10500 ± 577.35[1]	7.3 ± 0.96[2]	12.8 th ± 0.5d[3]
240 mu filter + ELISA-plate immunoassay	207.5 ± 9.57[4]	26.3 ± 0.96[5]	6.8 th ± 0.50d[6]

Correlation between [1] and [2], $r = 0.302$, $P > 0.05$; [2] and [3], $r = 0.174$, $P > 0.05$; [4] and [5], $r = 0.455$, $P > 0.05$; [5] and [6], $r = 0.174$, $P > 0.05$.

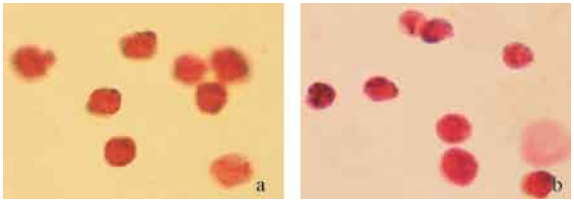

Figure 3. a: The ELISA plate -eluted ESCP, p63-IR in dark brownish color; b: The Sepharose 4B minicolumn-eluted ESCP, k19-IR in dark brownish color (k19 immunostaining, Eosin counterstaining × 1000).

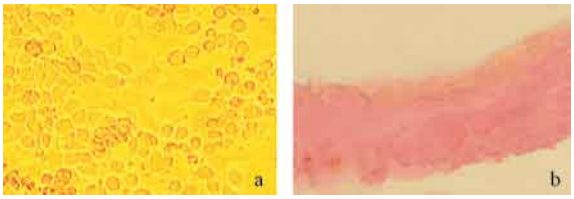

Figure 4. a: The cultured eluted ESCP appeared confluence from the colony (under inverted microscope, × 400); b: A section of formed stratified epidermis from the eluted viable ESCP, some basal cells appeared p63-IR in dark brownish color (p63 immunostaining, counterstained with Eosin, × 400).

cells. It suggests that in further gene transfer experiment the immunoassay with p63/k19 and filter screening to enrich relatively naked ESCP may be advantageous over that with collagen IV/β1 integrin adhesion technique.

5 Conclusion

Any unique characteristic can be used to isolate a pure population of stem cell is still lacking[15]. There is few specific biomarker found for ESC alone, but ESC and TA are able share some biomarkers. If a research requires different numbers of enriched ESCP, rapid enrichment with filter screening or ELISA plate immunoassay may be optimal.

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