

# Construction of tissue engineered skin with human amniotic mesenchymal stem cells and human amniotic epithelial cells

S.-C. YU<sup>1</sup>, Y.-Y. XU<sup>2,3</sup>, Y. LI<sup>1</sup>, B. XU<sup>1</sup>, Q. SUN<sup>2</sup>, F. LI<sup>1,2</sup>, X.-G. ZHANG<sup>1,2,4</sup>

<sup>1</sup>Department of Human Anatomy, Histology and Embryology, School of Biology and Basic Medical Sciences, Soochow University Suzhou, Jiangsu, China

<sup>2</sup>Institute of Medical Biotechnology, Soochow University, Jiangsu Key Laboratory of Stem Cells, Suzhou, Jiangsu, China

<sup>3</sup>Institute of Pediatrics, Children's Hospital Affiliated to Soochow University, Suzhou, Jiangsu, China

<sup>4</sup>Jiangsu Institute of Clinical Immunology, the First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China

*Shuichang Yu and Yunyun Xu contribution equally to this work*

**Abstract. – OBJECTIVE:** To establish a new model for construction of tissue engineered skin with human amniotic mesenchymal stem cells (hAMSCs) and human amniotic epithelial cells (hAECs).

**MATERIALS AND METHODS:** hAMSCs and hAECs were isolated from amniotic membrane. The morphology and phenotype of hAMSCs and hAECs were confirmed by microscope and flow cytometry, respectively. Then, we performed RT-PCR and immunofluorescence staining to assess the expression of stem cells and keratinocyte markers. Moreover, cell co-culture was performed to observe the growth and phenotype characteristics of hAMSCs and hAECs *in vitro*. In addition, tissue engineered skin with hAMSCs and hAECs was constructed and assessed with histological methods.

**RESULTS:** hAMSCs and hAECs were successfully isolated, exhibiting fibroblast-like morphous and cobblestone-shape epithelial morphous, respectively. The surface biomarker analysis showed that hAMSCs and hAECs were both positive for CD73, CD90 and CD105, and negative for CD34 and HLA-DR. The RT-PCR showed that hAMSCs expressed stem cell marker Nanog and c-MYC, and keratinocyte marker K19,  $\beta$ 1 integrin and K8, whereas hAECs expressed stem cell marker KLF4 and c-MYC, and keratinocyte marker K19,  $\beta$ 1 integrin, K5 and K8. The expression of keratinocyte proliferation antigen K14 was also found on hAECs. Furthermore, we found co-culture has no impact on the phenotype of hAMSCs and hAECs, but increased the proliferation activity of hAECs and decreased the proliferation activity of hAMSCs. Finally, the histological analysis showed that the tissue engineered skin exhibited similar structure as normal skin.

**CONCLUSIONS:** Tissue engineered skin with hAMSCs and hAECs was successfully constructed and shown a similar feature as a skin equivalent. The tissue engineered skin might have good application prospects in regenerative medicine.

*Key Words:*

Human amniotic mesenchymal stem cells, Human amniotic epithelial cells, Stem cells, Skin, Tissue engineering.

## Introduction

Skin, which is the largest organ of the human body, plays multiple functions in maintaining the homeostasis of the human body<sup>1</sup>, and skin damage is one of common clinical skin disease. The loss of this barrier requires a closure of the wounded surface and the main curable method is the use of skin graft<sup>2</sup>. However, the demand of the skin graft is far greater than that currently available. Modern tissue engineered living skin replacements provide hope to fill this gap. Therefore, it is necessary to conduct the research to find the ideal skin substitute<sup>3</sup>.

Mesenchymal stem cells (MSCs) derived from bone marrow, adipose tissue, umbilical cord and many other tissues, are multipotent stem cells which can display self-renewal and multi-lineage differentiation ability, including osteoblasts, adipocytes, chondrocytes, myocyte and even neu-

rons and epithelial cells<sup>4-8</sup>. Besides above potentials, MSCs also have a low immunogenicity and immunoregulatory action by affecting the function of the immune cells. Therefore, they have been used for skin defects repair and have exhibited satisfactory effects of repairing<sup>9,10</sup>. However, limited number of cells hindered the progress.

Since rapid migration and proliferation of the epithelial cells located in the basal layer of epidermis were involved in the healing process, the human amniotic epithelial cells (hAECs) have attracted the attentions from skin reconstruction<sup>11</sup>. The advantages of hAECs such as easy to obtain, high cell yield and immune tolerance<sup>12</sup>, make them good candidates for skin regeneration medicine.

Here, we isolated the human MSCs (hMSCs) and hAECs from term amnion membrane, and studied their characteristics *in vitro*. In addition, we constructed a tissue engineered skin with hMSCs and hAECs to evaluate the possible feasibility of skin defect repair.

## Materials and Methods

### **Ethical Approval of the Study Protocol**

The study protocol was approved by the institutional Broad Committee of the First Affiliated Hospital of Soochow University and was in full compliance with the Declaration of Helsinki.

### **Isolation and Culture of hAMSCs**

Amniotic membrane was peel off from the human placenta that was harvested within 2 h delivery after cesarean sections, and washed several times in phosphate buffered saline (PBS) to remove the blood and cellular debris. The amniotic membrane was minced into about 1 mm<sup>3</sup> small pieces and digested with type IV collagenase (0.25 mg/ml; Sigma, St. Louis, MO, USA) in the medium with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) at 37°C for 2 h. The cell suspensions were filtered through 100-mm strainers, centrifuged, and cultured in low glucose Dulbecco's modified Eagle's medium (LG-DMEM) supplemented with 10% FBS, 20 ng/ml bovine basic fibroblast growth factor at 37°C, and 5% CO<sub>2</sub>. The medium was, then, changed every 3-4 days. When the cells reached 80% confluence, they were digested with 0.25% trypsin containing 0.02% ethylene diaminetetraacetic acid (Life Technology, Carlsbad, CA, USA) and split with a ratio of 1:5.

### **Isolation and Culture of hAECs**

The isolation of hAECs was modified from the method described by Miki et al<sup>13</sup>. Briefly, the amniotic membrane was incubated at 37°C with 0.05% trypsin/0.02% EDTA. The cells from the first 10-min digestion were discarded to exclude debris. The cells from the second and third 30-min digestion were filtered through 100-mm strainers, centrifuged, and then cultured DMEM containing 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, 55 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% penicillin-streptomycin, 10 ng/ml epidermal growth factor (EGF; R&D, Minneapolis, MN, USA) at 37°C, and 5% CO<sub>2</sub>. The medium was, then, changed every 3-4 days. After grown for about 12 weeks, they were digested with 0.25% trypsin containing 0.02% ethylene diaminetetraacetic acid (Life Technology, Carlsbad, CA, USA) and split with a ratio of 1:3.

### **The Phenotype Characterization**

To identify the cell phenotype, hAMSCs and hAECs were characterized using phycoerythrin- or fluorescein isothiocyanate-conjugated monoclonal antibodies against human CD34, CD73, CD90, CD105 and HLA-DR (BD Pharmingen, San Diego, CA, USA). Briefly, about 2 × 10<sup>5</sup> cells were suspended in 5 mL PBS/2% FBS and incubated with above antibodies or isotype control IgGs according to the manufacturer's instructions. Cells were washed with phosphate buffered saline (PBS) twice and resuspended in PBS/1% paraformaldehyde. Samples were stored in 4°C until checked on FACSaria (Becton-Dickinson, Mountainview, CA, USA).

### **Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

Total RNA was extracted from the culture cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 1 μg of total RNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, CA, USA) according to the manufacturer's instruction. The PCR reaction were performed using specific primers of Wnt signaling pathway related genes and TaKaRa Ex Taq® reagent (Takara, Dalian, China). The PCR products were analyzed by electrophoresis on 2% agarose gel. The electrophoretic products were photographed using a gel imaging system. The primer sequences used in here were listed in Table I. As a control cells, human embryonic stem cells were purchased from Beijing Biocytogen Co.Ltd, China.

**Table 1.** The primer sequences annealing temperature and product length specific for each target gene.

Gene name	Primer sequence 5'-3'	Annealing temperature (T <sub>m</sub> ; °C)	Product length (bp)
OCT-4	Forward: CGAGCAATTTGCCAAGCTCCTGAA Reverse: TTCGGCACTGCAGGAACAAATTC	60	396
NANOG	Forward: CTGCTTGGCTTTGAAGCATCCGA Reverse: CTGCAGAAGTGGGTTGTTTGCCTT	60	212
KLF-4	Forward: AATTACCCATCCTTCCTGCCCGAT Reverse: TGTGTTTACGGTAGTGCCTGGTCA	60	313
c-MYC	Forward: TCAAGAGGCGAACACACAACGTCT Reverse: GTTCTCGTCGTTTCCGCAACAAGT	60	216
KRT-19	Forward: AGGTGGATTCCGCTCCGGGC Reverse: ATCTTCCGTCCTCGAGCA	57	461
β1-integrin	Forward: AATGTTTCAGTGCAGAGCC Reverse: TTGGGATGATGTCGGGAC	56	260
KRT-5	Forward: TGCCCTGATGGACGAGATCAACTT Reverse: GTTGGCACACTGCTTCTTGACGTT	58	346
KRT-8	Forward: AGGCATCACCGCAGTTACG Reverse: TTGCTTCGAGCCCGTCTTCT	59	222
GAPDH	Forward: GGTGGTCTCCTCTGACTTCAACA Reverse: GTTGCTGTAGCCAAATTCGTT	60	412

### Immunofluorescence

hAECs ( $1 \times 10^6$  cells) were plated onto slides and incubated overnight. After paraformaldehyde fixation (4%) was performed for 30 min at 4°C, blocking was carried out with goat serum for 1 hr to minimize non-specific binding of the antibody. The K14 primary antibodies (Abcam, Cambridge, UK) were applied at a 1:100 dilution for 1 hr at room temperature. As a negative control, PBS was used instead of the primary antibodies to exclude non-specific binding of the secondary antibody. After rinsing with PBS, slides were incubated with the corresponding immunofluorescence labeled secondary antibodies (Vector Laboratories, Burlingame, CA, USA) for 15 min at 37°C. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and mounting medium was added before confocal microscopy examination.

### Coculture of hAMSCs/hAECs

hAECs and hMSCs were cocultured and plated in 6-well plates or 24-well plates (Nunc, Roskilde, Denmark) at a ratio of 3:1. For both cell types, under passage 4 were used. The cell loading density was  $1.5 \times 10^4$ /mL hAECs and  $0.5 \times 10^4$ /mL hMSCs in LG-DMEM medium supplemented with 10%FBS, 20 ng/mL bFGF and 10 ng/mL EGF. As control groups, hAECs and hMSCs were seeded onto 6-well plates in the same

medium. The culture medium of both groups was changed twice a week.

Due to the different adherent properties of hAECs and hMSCs, hMSCs can be separated by two-step trypsinization. For growth curve, hMSCs in the coculture group were removed by treatment with 0.125% trypsin/0.02%EDTA (Life Technology, Carlsbad, CA, USA), for 3 min at 37°C. Detached hMSCs were removed with PBS wash; hAEC and hMSCs controls were treated similarly. Finally, the hAECs from both groups were detached with 0.25% trypsin/0.02% EDTA for 5 min at 37°C. Following a 5 min centrifugation at 800 g, hAEC and hMSCs were collected from coculture and control groups. The cell pellets from 24-well plate were counted by trypan blue exclusion. The cell pellets from 6-well plate were used for flow cytometry analysis.

### Reconstruction of the Skin Equivalent

Construction of the tissue engineered (TE) skin with hAECs and hMSCs was processed by using the method modified from previous description<sup>14</sup>. At first, a collagen solution was made of by mixing 10%v/v 10 × LG-DMEM, 10% v/v FBS, 50% v/v type I collagen (final concentration 0.8%) and hMSCs ( $1 \times 10^6$  cells/mL) in 30% v/v 1 × LG-DMEM. The collagen solution with cells was then added into an upper chamber of a 75-mm

Transwell plate, incubated at 37°C for 30 min in the CO<sub>2</sub> incubator and allowed to gelation. The collagen gel with the hMSCs were, then, incubated for 4 days in the CO<sub>2</sub> incubator with the lower chamber filled with LG-DMEM supplemented with 10% FBS, 20 ng/mL bFGF and 10 ng/mL EGF. Then primary cultured hAECs were harvested and dispensed into a single cell suspension at a density of  $1 \times 10^6$  cells/mL, and seeded onto the collagen gel mentioned above. The collagen gel containing the cells was incubated for 3 days. It was, then, raised onto the liquid-air surface for 2 weeks until ready for histology studies.

### Histological Analysis

Normal human skin, TE skin with human fibroblasts and keratinocytes were used as control group. Normal human skin, TE skin with human fibroblasts and keratinocytes, and TE skin with hAECs and hMSCs were fixed in 10% formalin and embedded in paraffin, and 5  $\mu$ m sections

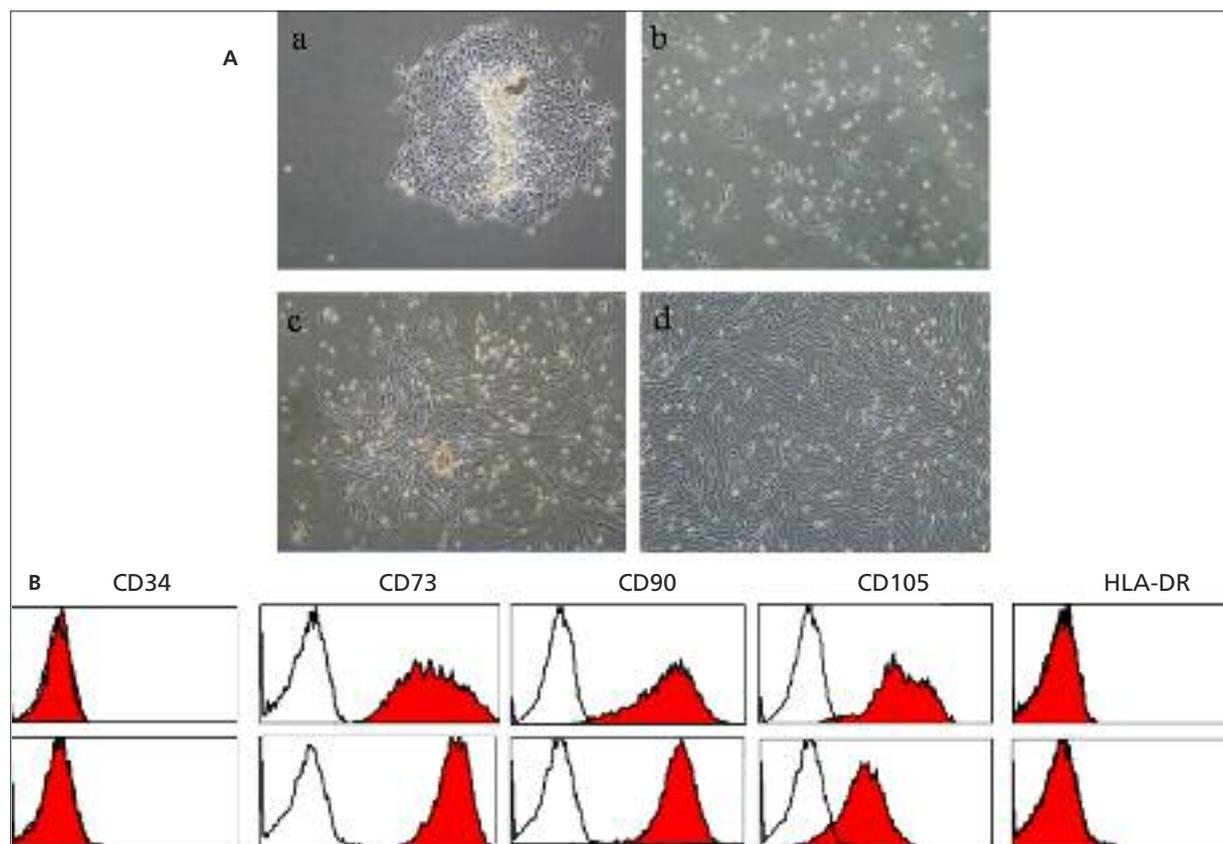
were prepared. Sections were stained with H&E and observed by microscope.

## Results

### Isolation and Characterization of hAMSCs and hAECs

**Morphology:** As shown in Figure 1A, primary cells of hAMSCs and hAECs were heterogeneous, which displayed different cell shapes, including round shape, irregular shape and spindle-shaped cells. The cultured cells from passage 3 were in good condition and showed typical morphology: the hAMSCs showed fibroblast-like and fusiform shape and the hAECs formed a cobblestone-shaped epithelial cells (Figure 1A).

**Phenotype:** The flow cytometry analysis showed that hMSCs showed the characteristic pattern of mesenchymal surface markers



**Figure 1.** Isolation and characterization of human amniotic mesenchymal stem cells (hAMSCs) and human amniotic epithelial cells (hAECs). **A**, The hAECs colony was formed after cultured for 4 days (**a**; 100 $\times$ ); the passage 3 hAECs showed cobblestone shaped epithelial cells (**b**; 100 $\times$ ); The hAMSCs colony was formed after cultured for 4 days (**c**; 100 $\times$ ); the passage 3 hAMSCs showed fibroblast like cells (**d**; 100 $\times$ ). **B**, Flow cytometry analysis of the expression of hAMCs and hAECs surface markers related to CD34, CD73, CD90, CD105 or HLA-DR.

including CD73, CD90, and CD105, and negatively expressed hematopoietic marker CD34 and major histocompatibility complex antigen HLA-DR. hAECs expressed a high percentage of CD73 and CD90, whereas CD105 was positive to some extent. No expression of CD34 and HLA-DR was found in hAECs (Figure 1B).

#### **Comparison of the Expression of Stem Cells Markers on hAMSCs and hAECs**

RT-PCR was, then, employed to assess the expression of stem cell markers on hAMSCs and hAECs. The results showed that hAMSCs expressed stem cell marker Nanog and c-MYC, and keratinocyte marker K19,  $\beta$ 1 integrin and K8, whereas hAECs expressed stem cell marker KLF4 and c-MYC and keratinocyte marker K19,  $\beta$ 1 integrin, K5 and K8. The expression of keratinocyte proliferation antigen K14 was also found on hAECs (Figure 2A). In addition, we found the expression of keratinocyte proliferation related protein K14 by immunofluorescence staining (Figure 2B).

#### **Coculture Changes the Growth Characteristics of hAMSCs and hAECs**

As shown in Figure 3A, the hAECs were surrounded by hAMSCs during the culture. By comparing with control group with only hAECs, we found the number of the hAECs in coculture system increased (Figure 3A, a,b). Moreover, the expression of surface marker was also evaluated by flow cytometry analysis, the results showed that coculture had no impact on the surface marker of both cell types (Figure 3B). According to the growth curve, we found increased proliferation on hAECs and decreased proliferation on hAMSCs were found on coculture system after 4 days' coculture (Figure 3C).

#### **Histological Results of Skin Equivalents**

TE skin with hAECs and hMSCs developed into epithelium composed of several layers of amniotic epithelial cells presented in the epidermis (Figure 4C, F) whereas TE skin with human fibroblasts and keratinocytes developed epithelium consisted of several layers of keratinocytes and cornified layers (Figure 4B, E). Normal human skin section showed a similar feature as the TE skin with hAECs and hMSCs (Figure 4A, C). Moreover, cutin analogue was evident in TE skin with human fibroblasts and keratinocytes (Figure 4E) and only few cutin analogue was found in

TE skin with hAECs and hMSCs (Figure 4F). In addition, stratum granulosum could be found in all the three kinds of skins (Figure 4D-F).

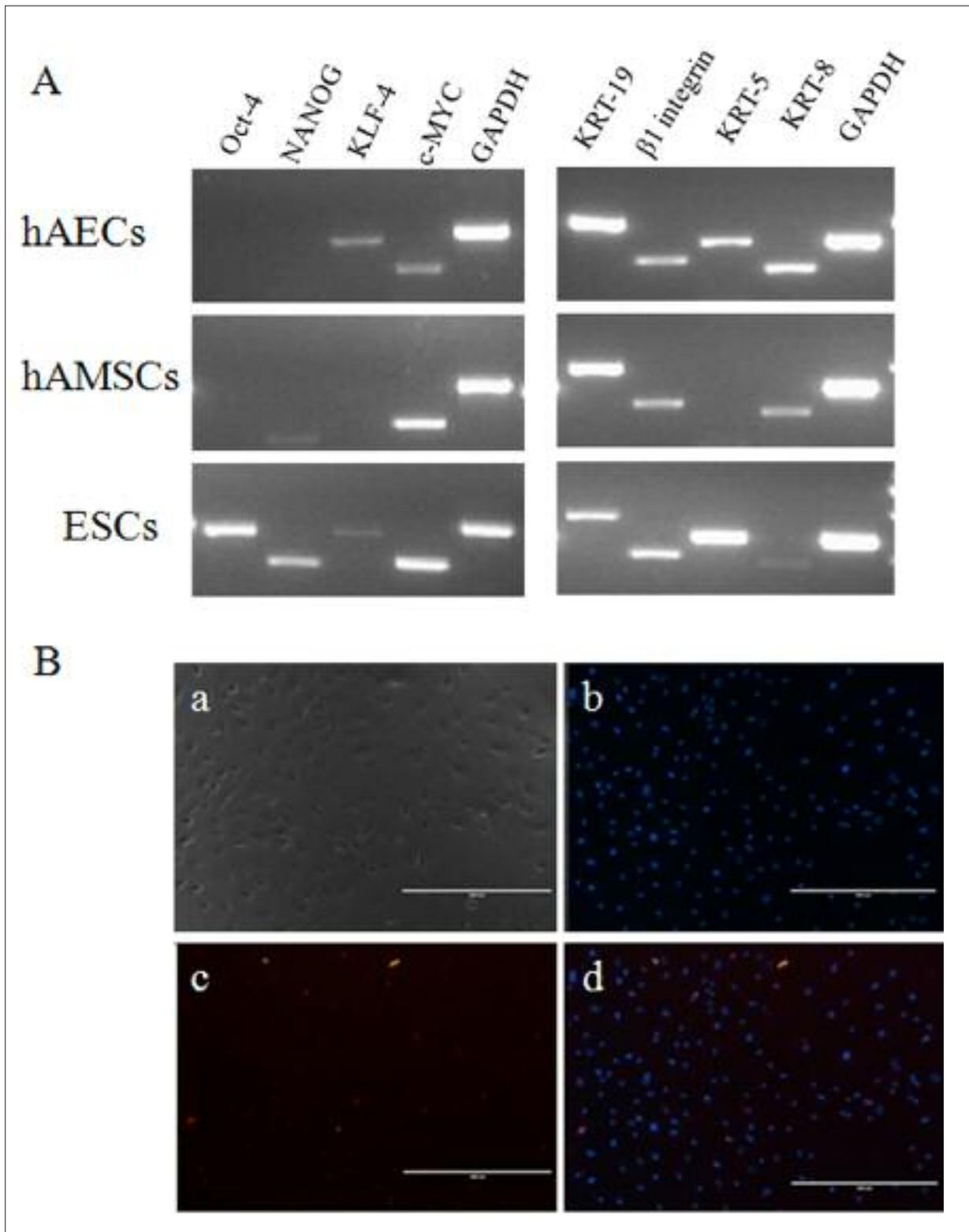
## **Discussion**

In present study, hAMSCs and hAECs were successfully isolated from human term amniotic membrane and characterized by expression of mesenchymal markers (CD73, CD90 and CD105) but not hematopoietic cell markers (CD34 and HLA-DR). Moreover, we found the expression of certain embryonic stem cells and epithelial stem markers on hAMSCs with hAECs. *In vitro* coculture system was also employed and coculture hAMSCs with hAECs has no impact on the cell phenotype but increased and decreased the proliferation of hAECs and hMSCs, respectively. In addition, we also constructed the TE skin with hAECs and hMSCs and found this type of skin showed similar feature as the normal skin.

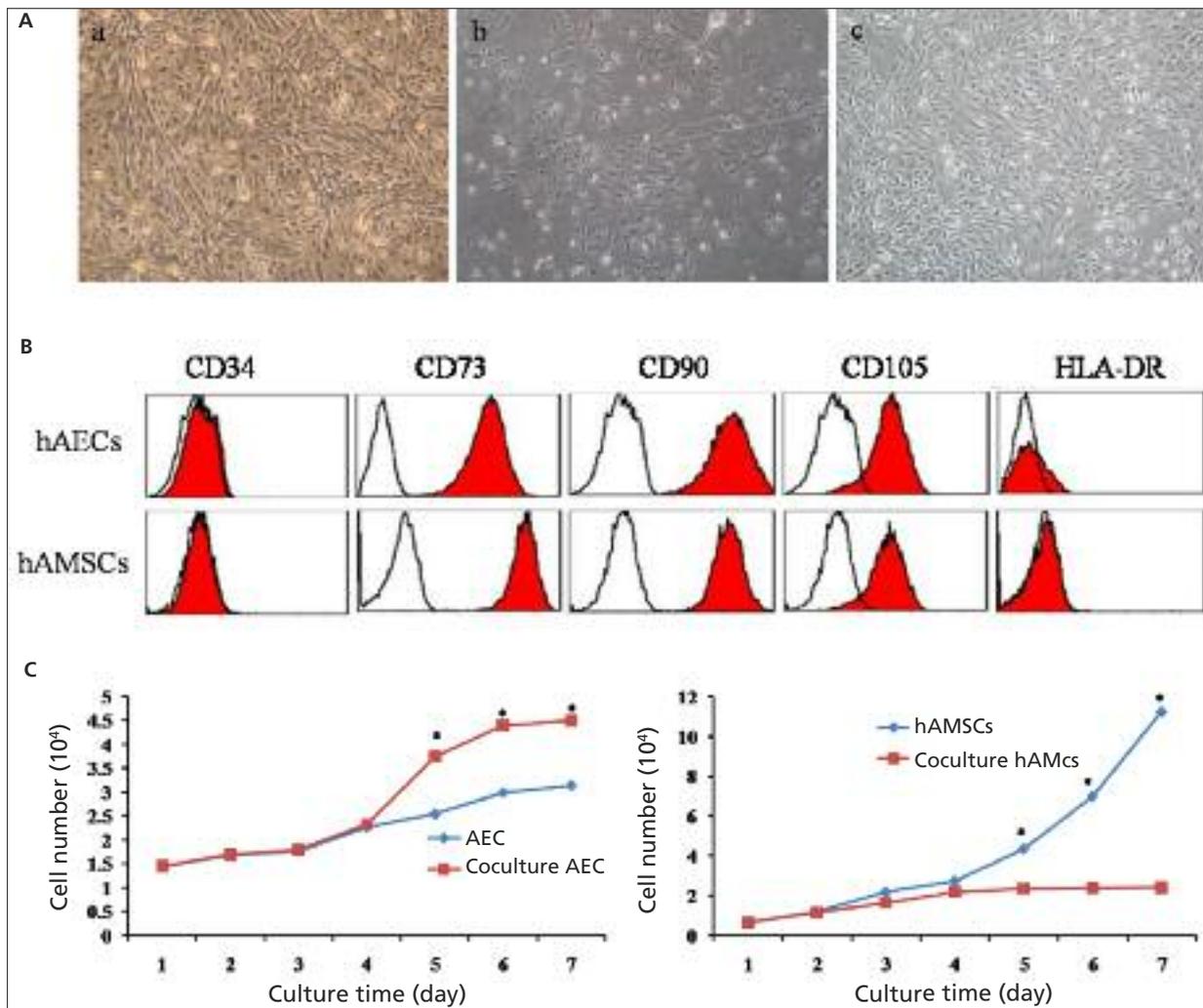
Studies<sup>15</sup> have shown that stem cells can exert satisfactory repair effects. hAECs and hAMCs express stem cell markers and have the ability to differentiate toward all three germ layers<sup>16</sup>. Miki et al<sup>17</sup> proved that cells isolated from amniotic and chorionic membranes do not elicit an allogeneic or xenogeneic immune response but effectively suppress lymphocyte proliferation. Besides, the advantages of the amniotic cells include nearly unlimited availability, easy procurement, and low processing costs for therapeutic application.

After isolation and characterization of hAMSCs and hAECs, we used the *in vitro* coculture system to explore the interaction between hAMSCs and hAECs, and found that the growth of the hAECs was along with hAMSCs and surrounded by hAMSCs. Moreover, we found increased proliferation of hAECs and decreased proliferation of hAMSCs in the coculture system. The reason resulted in this phenomenon was that coculture system could mimic the situation of cell growth *in vivo*. *In vivo*, MSCs cells were in quiescent state and located in certain position of the tissues<sup>18</sup>. The effect of MSCs *in vivo* was believed exerted through cytokine paracrine mechanisms<sup>19</sup> and the paracrine cytokines could result in the proliferation increasing on the hAECs.

As an essential way to understand the homeostasis of adult tissues and their regeneration as well as to elucidate the mechanisms of embryogenesis, studies about cell-cell interactions are critical<sup>20</sup>. Interactions between mesenchymal and



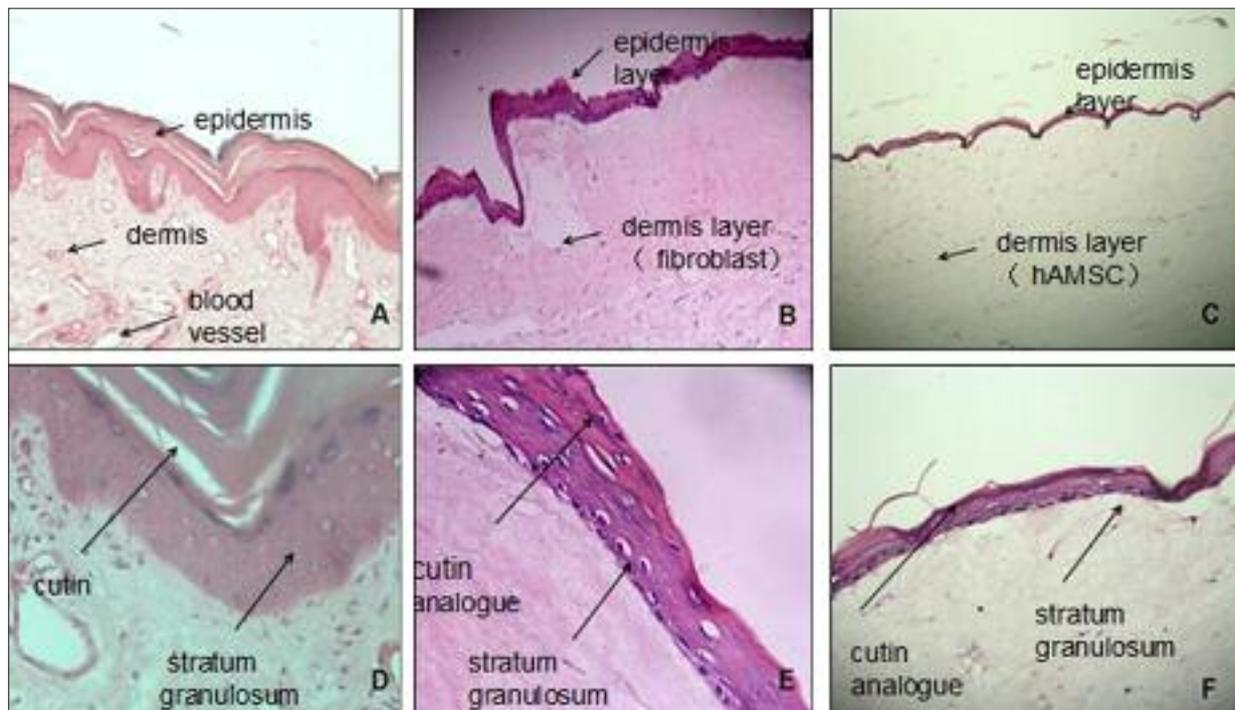
**Figure 2.** The expression of stem cell markers on human amniotic mesenchymal stem cells (hAMSCs) and human amniotic epithelial cells (hAECs). **A**, RT-PCR analysis of the expression of embryonic stem cell markers related to OCT-4, NANOG, KLF4 or c-MYC and epithelial stem cell markers related to KRT-19,  $\beta$ 1 integrin, KRT-5 or KRT-8. **B**, Immunofluorescence staining of epithelial proliferation marker K14: hAECs under bright field (**a**; 40 $\times$ ), nuclei staining of DAPI (**b**; 40 $\times$ ), PE staining of K14 (**c**; 40 $\times$ ) and Merge image of DAPI and PE staining (**d**; 40 $\times$ ).



**Figure 3.** Coculture of human amniotic mesenchymal stem cells (hAMSCs) and human amniotic epithelial cells (hAECs). **A**, Representative images of cocultured hAECs and hAMSCs (**a**; 100 $\times$ ); hAEC cultured without hAMSCs (**b**; 100 $\times$ ); hAECs isolated from coculture system (**c**; 100 $\times$ ). **B**, Flow cytometry analysis of the expression of hAMCs and hAECs surface markers related to CD34, CD73, CD90, CD105 or HLA-DR in the coculture system. **C**, The growth curve analysis of cocultured hAMCs and hAECs. \*represents  $p < 0.05$ .

epithelial cells are involved in the homeostasis and organogenesis of the skin and the effect of interaction could be exerted through cell surface proteins and soluble factors produced by resident cells<sup>21</sup>. In the present study, using the cell-cell interaction mechanism in a tissue engineering context, we established a new bilayered TE skin with hAMSCs and hAECs which presented stem cell characteristics (such as stem cell markers) *in vitro*. After 14 days of culture at the air-liquid interface, a stratified epidermis is formed. This is reminiscent of traditional TE skin. Our work described the *in vitro* production of the novel human skin substitutes reconstructed with hAMCs and hAECs.

Not exactly like traditional TE-skin with fibroblasts and keratinocytes, keratinosis of TE skin with hAMSCs and hAECs is not found in our system; the reason may due to that hAECs are simple epithelia and keratinocytes are stratified epithelia. Multilayered (“stratified”) epithelia differ from one-layered (“simple”) polar epithelia by various architectural and functional properties<sup>22</sup>. Differentiation of hAECs may require certain environments: some reports have found that hAECs could differentiate into stratified squamous epithelium resembling adult skin when it was implanted into subcutaneous sites, wrapped into omentum, under the kidney capsule<sup>23</sup>. In this study, we also observed the forma-



**Figure 4.** Histological analysis of the tissue engineered skin with human amniotic mesenchymal stem cells (hAMSCs) and human amniotic epithelial cells (hAECs). **A**, Normal skin section (40 $\times$ ), **B**, Tissue engineered skin with fibroblasts and keratinocytes (40 $\times$ ). **C**, Tissue engineered skin with hAECs with hAMSCs (40 $\times$ ). **D**, Normal skin section (100 $\times$ ). **E**, Tissue engineered skin with fibroblasts and keratinocytes (100 $\times$ ). **F**, Tissue engineered skin with hAECs with hAMSCs (100 $\times$ ).

tion of a stratum corneum *in vitro*. We suppose that the environments of culture medium and hMSCs may contribute to the differentiation of AC-TE skin.

### Conclusions

We here successfully reconstructed skin equivalents with hAMSCs and hAECs *in vitro*. We will also apply our TE skin into an animal model to explore the *in vivo* effect and confirm the efficacy of the TE skin. Our skin model may contribute to improve performance and clinical outcome in the treatment of injuries/defects in the near future.

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### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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