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ORIGINAL ARTICLE

Stem cell-like properties of human umbilical cord lining epithelial cells and the potential for epidermal reconstitution

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Abstract

Background aims. Stem cells are particularly attractive for many cell-based therapeutic interventions because of their ability to self-renew and their capacity to differentiate into site-specific differentiating cells. Restoration of the integrity of epithelial continuity is an essential aspect of wound repair and tissue regeneration. We are currently looking at the potential of human umbilical cord lining cells as a source of epithelial stem cells with appropriate differentiation capacity for potential epidermal reconstitution. *Methods*. We isolated human umbilical cord lining epithelial cells (CLEC) and characterized their phenotype from the perspective of proliferative potential, telomere length, expression of epidermal differentiation markers, as well as stem cell-specific markers, and clonogenicity. Their potential for epidermal reconstitution was investigated in an organotypic culture model. *Results*. The results demonstrated that CLEC present a long telomere length and have a relatively high proliferative potential and passaging ability in culture. CLEC display some of the stem cell-specific markers for epithelial as well as pluripotent stem cells, including CK19, p63, OCT-4, SSEA-4, TRA-1–60, SOX2 and Nanog. CLEC are capable of generating a fully stratified epithelium in organotypic culture. *Conclusions.* The potential of CLEC to be used in clinical applications for specialized epithelial reconstruction is still unexplored. The demonstration that CLEC have stem cell-like properties and are capable of generating fully stratified epithelium provides support for their potential clinical application in epidermal reconstitution.

Key Words: *epidermal reconstitution, human umbilical cord, stem cells*

Introduction

The umbilical cord connects the fetus to the placenta. In addition to its essential role in prenatal development, the human umbilical cord has now been recognized as an ethically acceptable, safe and sustainable source of stem cells (1–3). The stem cell potency of mesenchymal cells isolated from the matrix of cord has been reported in the literature (2,4). However, there has been little investigation of the nature of the covering epithelium, the cord lining. The cord lining is generally thought to be derived from amniotic epithelium and is contiguous with the fetal skin. It is typically a single layer of epithelial cells. Previous observations have demonstrated a structural resemblance between umbilical cord epithelium and early fetal epidermis (5,6). Recent studies have demonstrated further common features of umbilical cord epithelial (UCE) cells and epidermal keratinocytes in terms of the expression patterns of cytokeratins, cell-surface markers and their response to epidermal differentiation stimuli (7,8). However, few, if any, studies have explored the stem cell properties of these cells.

Stem cells are the best choice for most cell-based therapeutic interventions because of their ability to self-renew and capability of differentiating into site-specific differentiating cells. When looking at the reconstitution of epithelial constructs, it is preferable to find a potential source of epithelial stem cells (ESC). The skin itself contains a variety of stem cells (9,10). The interfollicular epidermis and sebaceous

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glands contain unipotent progenitors that can maintain homeostasis of their respective tissue by regenerating their respective cell lineage (11). On the other hand, multipotent ESC reside predominantly in the bulge area of the hair follicle, which can give rise to all lineages of skin epithelium by contributing to hair follicles and sebaceous glands in normal homeostasis and to epidermis after injury (12,13). With the introduction of the laboratory culture of keratinocytes in 1975, there has been widespread application of autologous keratinocytes in treating cutaneous wounds following burns and other forms of trauma (14,15). However, this approach is very much influenced by the time for cellular proliferation, availability of donor tissue and biocompatibility of both the cellular and connective tissue elements of repair. Of particular relevance are the immunologic barriers when using differentiated allogeneic cells. We are currently looking for an alternative source of ESC with appropriate differentiation capacity. Our attention is focused on the human umbilical cord lining.

We have isolated human umbilical cord lining epithelial cells (CLEC) and characterized their phenotype from the perspective of proliferative potential, telomere length, expression of epidermal differentiation markers, as well as stem cell-specific markers, and clonogenicity *in vitro*. Their potential for epithelial reconstitution was investigated in an organotypic culture model.

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Methods

Tissue samples and CLEC isolation

Human umbilical cords were collected after Cesarean section from full-term births with written consent of the mothers from the Department of Obstetrics and Gynaecology, the Chinese University of Hong Kong (CUHK, Hong Kong, China). Surgical discarded foreskin tissues were collected from the Department of Paediatrics, Alice Ho Miu Miu Ling Nethersole Hospital (Hong Kong, China), with informed consent of the patients. All these procedures were approved by the Clinical Research Ethics Committee of CUHK (reference numbers CRE-2004.433 and CRE-2006.434).

Primary culture of keratinocytes from foreskin specimens was performed as described previously (16). Our protocol for isolating the lining epithelial cells from umbilical cord was developed by modifying methods described previously (17,18). Briefly, the cords were cut into 2–3-cm lengths; the blood was removed and the cords flushed with sterile saline. The cords were then opened with a lengthwise incision and the vessels, together with surrounding matrix, were removed by sharp dissection with a scalpel blade. The cord lining pieces were washed again with saline to remove the blood and other debris and then immersed in an enzyme cocktail (dispase 4 mg/mL, collagenase 1 mg/mL and hyaluronidase 1 U/mL) in 100-mm culture plates with the lining surface facing upwards. After digestion at 37°C for 2 h, the lining samples were collected, washed and digested again in 0.05% trypsin/ethylene diamine tetra acetic acid (EDTA) at 37°C for 5–8 min with agitation. The resulting cell suspension was filtered through a cell strainer (70 μm) and pelleted by centrifugation. The cells were then resuspended in fresh keratinocyte serum-free growth medium (K-SFM) and seeded at a density of 5000 cells/cm^2 onto culture dishes. Cultures were passaged regularly upon cells reached 80% confluence. Cells at passage 3–4 were used for the following experiments unless otherwise specified.

Growth curve and population doubling time

CLEC at passage (P) 2 were seeded at a density of 3×10^4/well into 96-well plates and cultured in K-SFM for 11 consecutive days. Cell proliferation was determined by the MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]) assay using standard methodology (n = 4). A growth curve was generated using the optical density (OD) values (y-axis) versus growth time (days) (x-axis). For calculation of the population doubling time (PDT), cells were counted separately in an increased logarithmic phase ($n = 4$) and PDT was calculated according to the formula $PDT = (t - t_0)\ \lg 2/(\lg n_t - \lg n_0)$, where t_0 and t represent the start time and end time of cell culture, respectively, and n_0 and n_t represent the cell number of the start culture and end culture, respectively.

Telomere length assay

Genomic DNA was extracted from CLEC at different passages (P2, P6 and P8) using a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). The length of the telomere regions of genomic DNA from cells was assessed using a Telo TAGGG kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Control DNA with long telomere regions was provided with the kit.

Fluorescence-activated cell sorter analysis

Cell-surface CD markers, including CD44, CD90, CD166, CD105, CD71, CD34, CD45 and HLA-DR, and integrin-α6 and integrin-β1 were examined for their expression in CLEC by fluorescence-activated cell sorter (FACS) analysis using standard

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methods. All antibodies and associated working conditions used in the present study are listed in supplementary Table I.

Colony-forming efficiency

To test for self-renewal capability, single-cell suspensions were prepared and dispensed into 60-mm Petri dishes at a clonal density of 50 cells/cm^2 (19). Cultures were maintained for 2–3 weeks to monitor the development of colonies. The cultures were then fixed and stained for rhodamine B. A cluster of more than 50 cell numbers was considered to be a clonal colony. The colony-forming efficiency was calculated as the percentage of (number of colonies/number of cells seeded).

Immunohistochemical and immunocytochemical analyses

For immunohistochemistry, paraffin-embedded tissue sections (5 μm) were dewaxed, rehydrated and treated in dual endogenous enzyme blocker solution to inactivate the endogenous peroxidase. The sections were then boiled in 10 mM citrate buffer (pH 6.0) for 10 min to expose the antigen location (sections for p63 did not require this pre-treatment), cooled down and blocked with 5% goat serum in 1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) for 30 min prior to incubation with primary antibodies (30 min at room temperature or 4°C overnight). Detection was performed using an EnVision$^+$ system/HRP, rabbit/mouse (DAB$^+$) kit (Dako, Glostrup, Denmark) and counterstained with hematoxylin.

For immunocytochemical studies, CLEC were plated in 8-well chamber slides (BD Biosciences, California, USA) and cultured to reach 80% confluence. Cells were then washed with PBS, fixed in 4% paraformaldehyde and permeabilized with 0.1% TritonX-100/PBS. After exposure to endogenous enzyme blocker solution, the slides were blocked in 1% fetal bovine serum (FBS) in PBS and incubated with primary antibodies. Detection was performed using an EnVision$^+$ system/HRP, rabbit/mouse (DAB$^+$) kit (Dako) and counterstained with hematoxylin. The primary antibodies and associated working conditions used are listed in supplementary Table I.

Reverse transcription–polymerase chain reaction

Cell or tissue total RNA was extracted by Trizol Reagent™ (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentration was measured spectrophotometrically at 260 nm wavelength using a Pharmacia GeneQuant machine (Scientific Support, Inc, CA, USA). Two micrograms of total RNA was reverse transcribed (RT) to cDNA by Superscript II reverse transcriptase (Invitrogen) in 20 μL reaction mixture containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 500 μM dNTPs. cDNA, 0.5 μL each, was used as a template in the polymerase chain reaction (PCR) using gene-specific primer pairs and conditions as listed in supplementary Table II. The GAPDH gene was used as an internal control to normalize the template quantity. The PCR products were identified in 1.5% agarose gel stained with ethidium bromide.

Organotypic culture of CLEC on a fibroblast-populated collagen lattice

Organotypic culture of CLEC was performed using the same conditions as organotypic culture of epidermal keratinocytes on a fibroblast-populated collagen lattice (FPCL), as described previously (20), with a few modifications. Briefly, collagen solution was prepared by mixing rat tail type I collagen at 5 mg/mL with 3 × Dulbecco's modified Eagle medium (DMEM) and neutralized with 5 N NaOH to make a final collagen concentration of 3 mg/mL. The collagen solution was then mixed with human dermal fibroblasts to make a fibroblast cell density of 1×10^5/mL; 600 μl of this FPCL solution were put into each Transwell insert within a 12-well plate (10.5 mm in diameter, 8.0 μm pore size; BD Biosciences). Collagen gel polymerization was usually achieved within 2–3 h at 37°C, and the FPCL were then cultured overnight in DMEM supplemented with 10% FBS. The next day, 2×10^5 CLEC suspended in keratinocyte growth medium (KGM) were seeded onto each FPCL with the help of a 1-cm diameter cylinder. After the cells attached overnight, the cylinder was removed carefully and the Transwell insert was lifted to the air–liquid interface and cultured for an additional 7, 14, 21 and 28 days. For comparison, foreskin keratinocytes were plated individually on FPCL and cultured in the same conditions. KGM was prepared with a 1:3 mixture of Ham's F12 and DMEM, supplemented with 10% FBS, 5 μg/mL insulin, 1.8×10^{-4} M adenine sulfate, 10^{-10} M cholera toxin, 0.4 μg/mL hydrocortisone, 1 ng/mL epidermal growth factor (EGF) and 0.1% BSA. The medium was changed every 2 days. The cultured equivalents were harvested at the end of experiment and processed for histologic and immunohistochemical analysis.

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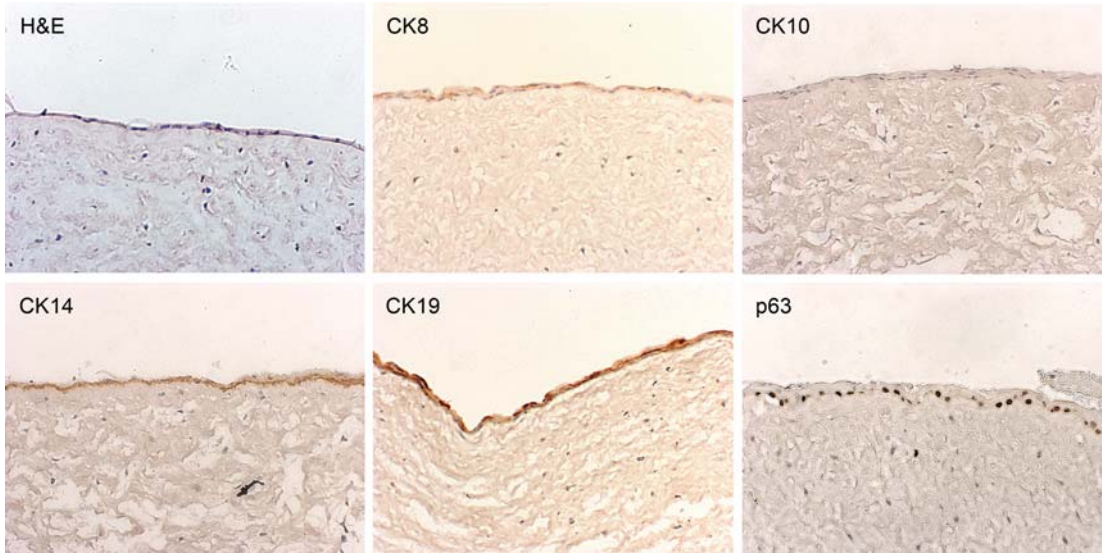

Figure 1. The histology (H&E) and immunohistochemical staining of epidermal differentiation cytokeratin markers and p63 in human umbilical cord lining tissue specimens. CK8, CK14, CK19 and p63 were positively expressed, but CK10 was undetectable. Original magnification was 200× for all panels.

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Results

The expression pattern of cytokeratins and p63 in human umbilical cord lining

Histologic examination demonstrated that the cord lining was generally a monolayer of flat epithelial cells (Figure 1, H&E panel). To look at the epithelial differentiation stage of the umbilical cord lining, we examined the expression pattern of cytokeratin markers in the cord lining tissue specimens by immunohistochemical staining. The cord lining presented CK8, the cytokeratin of simple epithelium type (Figure 1). It was also positive for CK14, a cytokeratin that is usually expressed in basal keratinocytes of stratified epithelium, but negative for CK10, the cytokeratin characteristic of epidermal differentiation. It positively expressed CK19, which it has been suggested is a biochemical marker for skin stem cells (21). Other cytokeratin components examined included CK15 and CK17, both negatively expressed (data not shown). p63, the marker for progenitor cells of skin epidermis and appendages (22,23), was strongly expressed in the cord lining cells.

The phenotypic characterization of isolated CLEC

The primary cultured CLEC showed a cobblestone appearance characteristic of typical epithelial cells in K-SFM culture medium (Figure 2A). These cells grew relatively slowly at the beginning but after initiation of clonal colonies they began to expand faster and formed large, tightly packed colonies of 10^3–10^4 cells (Figure 2B). It took 14–18 days on average for the primary culture to reach a flourish and need to be passaged. After passaging, these cells became rapidly proliferative. The average total PDT from P2 to P3 cells was 65 ± 18 h, which was much quicker compared with the average PDT (102 ± 20 h) of neonatal keratinocytes cultured in the same conditions. The neonatal keratinocyte culture could not be maintained after P4–P5. In contrast, CLEC could be propagated to P10 (Figure 2C), which corresponded to about 40 population doublings, although the cells started to develop the morphologic features of senescence after P7 in most cases. With the telomere length assay, it was found that CLEC contained long telomeres, indicating a relative long replicative lifespan of these cells. It was, however, noted that the mean telomere length of CLEC shortened with cell passaging in culture (Figure 2D), which may be a reflection of cell aging undergoing in the current culture conditions. It was also interesting that, regarding clonogenicity, assessed by single-cell colony assay, CLEC appeared to have a very low colony-forming efficiency, generating only one clonal colony/1000 cells seeded (Figure 2E).

FACS analysis (Figure 3) demonstrated that CLEC were negative for most of the mesenchymal and hemopoietic stem cell markers, including CD90, CD105, CD45, HLA-DR and CD71; however, these cells positively expressed CD166 and CD44. They were also positive for CD34 in a small cell portion. CLEC highly positively expressed the cell adhesion molecules integrin-α6 and integrin-β1, which is a pattern commonly seen in the basal layer of undifferentiated keratinocytes.

We further examined the expression of pluripotent stem cell-specific markers in CLEC. With

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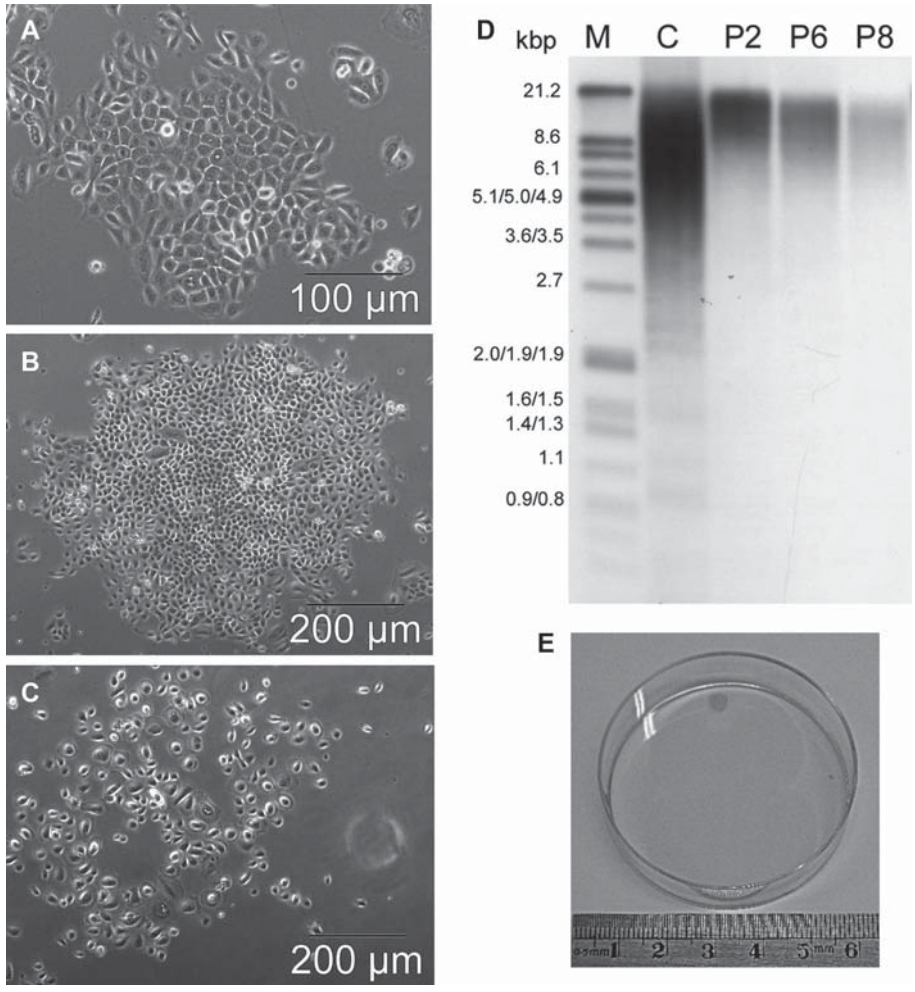

Figure 2. The cell morphology, telomere length and colony-forming efficiency in CLEC. (A) CLEC in primary culture on day 7; (B) CLEC in primary culture on day 12; (C) CLEC at P10. (D) The mean length of telomeric ends in genomic DNA extracted from CLEC at different culture passages. M, molecular weight marker; C, control DNA with long telomeres from the assay kit. (E) The colony-forming efficiency assay.

immunofluorescence staining (Figure 4A–G), OCT-4, SSEA-4 and TRA-1–60 were found to be present randomly in a small proportion of CLEC; in contrast, p63, CK8 and CK14 were distributed more generally in the majority of the cells. With RT-PCR analysis, the expression of OCT-4 mRNA was confirmed in CLEC. Moreover, SOX2 and Nanog, two transcription factors characteristic of pluripotency, were found to be expressed positively by CLEC (Figure 4H). However, it was noted that in late passages of CLEC expression of SOX2 was lost, which was accompanied with a decreased expression of p63 in these cells. The presence of pluripotent stem cell markers in CLEC may indicate that these cells are more primitive cells, remaining closer to the embryo in terms of stage of differentiation; on the other hand, loss of the expression of these markers during serial propagation may indicate the progression of cell differentiation as a result of long-term culture.

Reconstitution of stratified epithelium by CLEC organotypic culture

The CLEC-constructed skin equivalent was made by seeding CLEC onto a fibroblast-populated collagen matrix and culturing using the air–liquid interface method. After 2 weeks of organotypic culture, CLEC formed a well-stratified multilayered epithelium with five to six layers of cells (Figure 5B). The histology was very similar to the one constructed using foreskin keratinocytes (Figure 5A); the only difference was that the keratinocyte-constructed skin equivalent formed an apparent cornified strata layer at 2 weeks of air–liquid interface culture but this was not present in CLEC-derived constructs cultured even for 28 days.

In addition, as shown in Figure 5 and supplementary Table III, the immunohistochemical assessment of the expression of epidermal differentiation markers in these constructed epithelia showed that CK14

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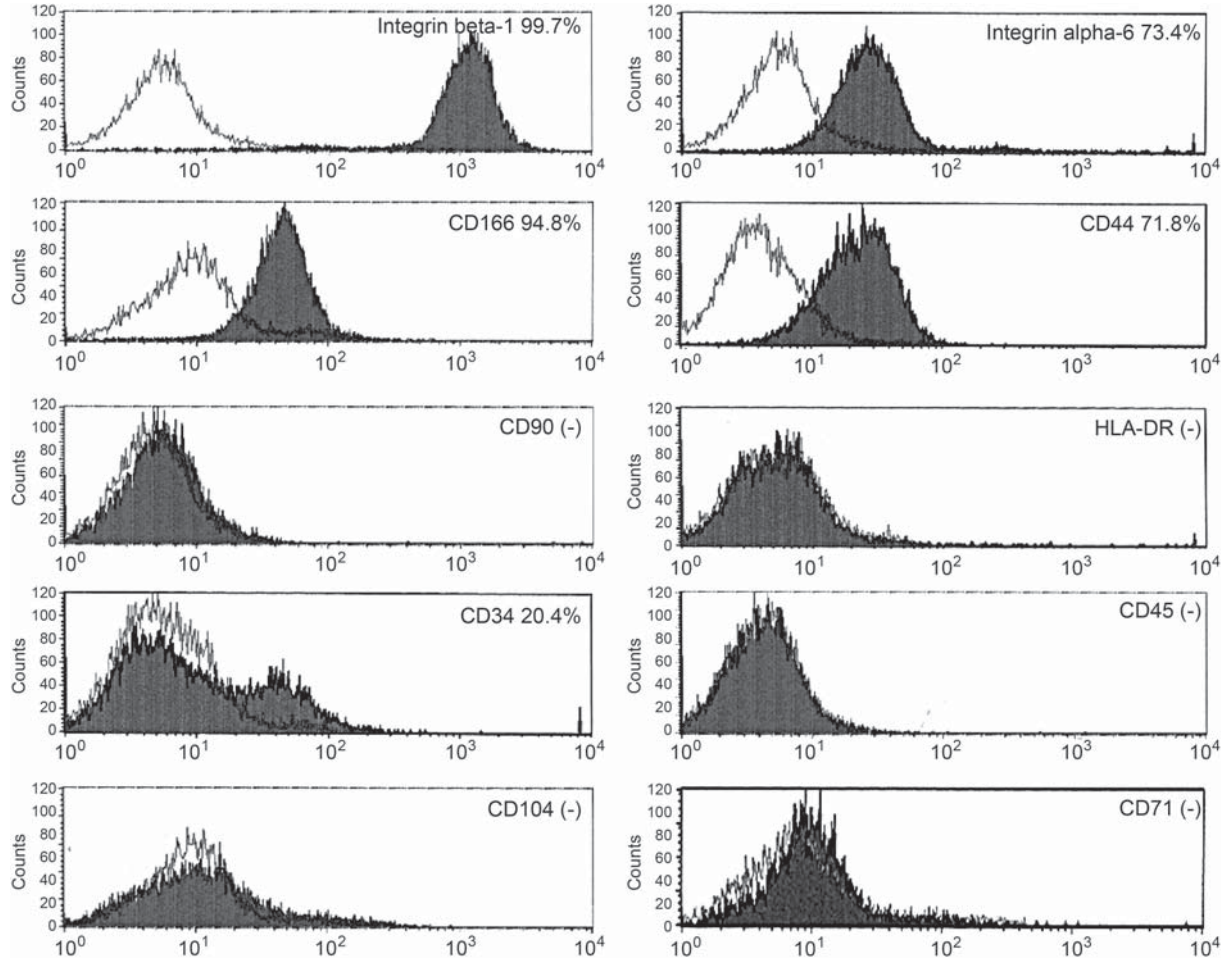

Figure 3. The expression profile of cell-surface markers in CLEC by flow cytometric analysis.

was present throughout the whole epithelium in both types of constructs. CK8 was only detectable in the basal layer of CLEC-derived epithelium cultured for 2 weeks and lost its expression at 4 weeks. Similar results were found for CK4, CK10, CK15 and CK16 and involucrin in both types of constructed epithelium: CK4, CK10 and CK15 were undetectable, but CK16 was present in the upper suprabasal layer and involucrin was expressed throughout the entire epithelium. However, CK19 stained intensively in the basal layer as well as upper suprabasal layer of the CLEC-constructed epithelium but there were very few cells stained positively for CK19 in the keratinocyte-constructed epithelium. More p63-positive cells presented in the basal and suprabasal layers of the CLEC-constructed epithelium compared with keratinocyte-constructed ones. Immunohistochemical assessment of the expression of basement components in these reconstructed epithelia showed similar results, as laminin-332 appeared as a continuous deposit and collagen IV as an intermittent deposit at the junctional zone between the lowermost layer of reconstructed epithelium and the fibroblast-populated collagen matrix; collagen VII had not appeared even at 28 days of culture.

Discussion

There has been considerable progress in the research of stem cell applications to modulate the course of skin wound healing (24). The challenge is to have a safe, reliable, sustainable and ethical source of such cells. Bone marrow (BM)-derived mesenchymal stromal cells (MSC) have been shown to be capable of giving rise to functional skin cells, including keratinocytes and dermal fibroblasts, thus promoting skin tissue regeneration; they also have an impact on wound healing through the release of paracrine factors (25,26). Incorporating BM MSC into skin substitutes has been demonstrated recently to improve wound repairing, with better keratinization, less wound contraction and more vascularization in a burns pig model compared with non-stem cell-containing skin substitutes (27). In addition, there have been reports exploring the capacity of umbilical cord blood-derived MSC to differentiate

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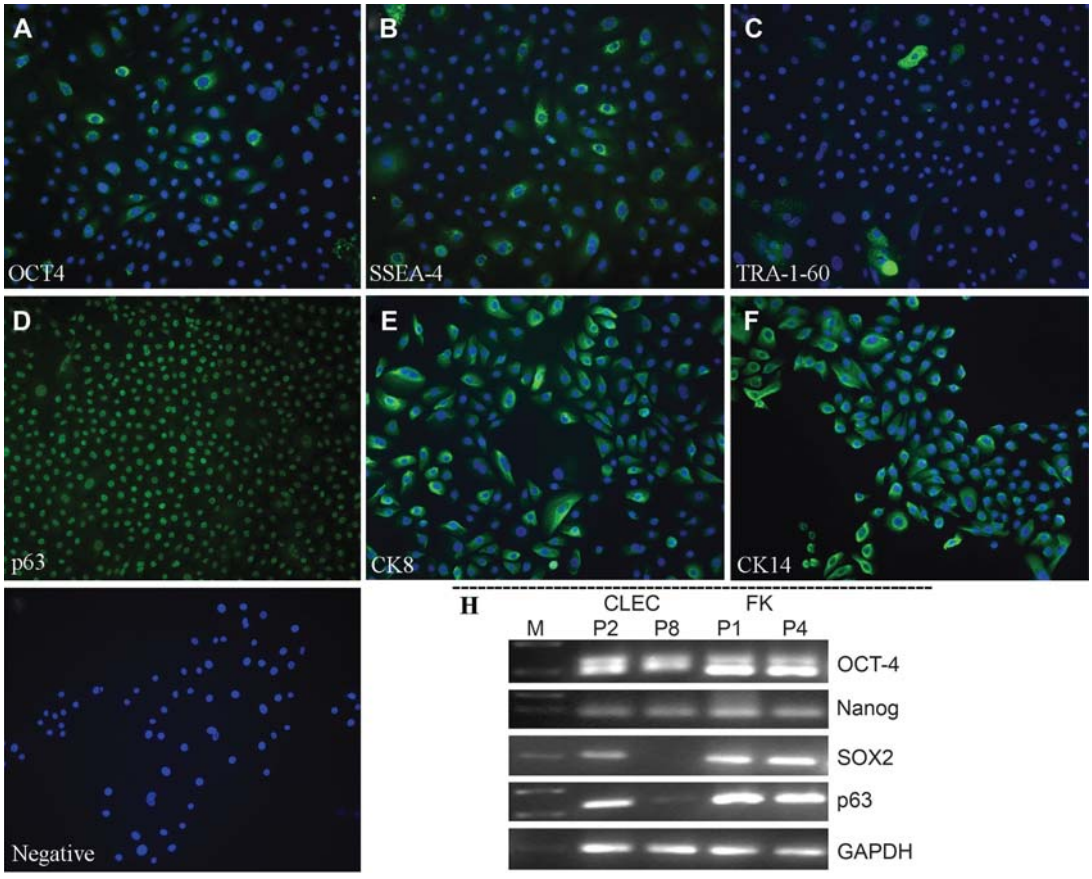

Figure 4. (A–G) The immunofluorescence staining of stem cell-specific markers OCT-4, SSEA-4, TRA-1–60 and p63, and CK8 and CK14, in cultured CLEC cells. The section that was not incubated with primary antibody but only secondary antibody (FITC-conjugated anti-mouse IgG) served as a negative control (G). DAPI was used as the nuclear counterstain. Original magnification was 200× for all panels. (H) The expression of OCT-4, Nanog, SOX2 and p63 in early and late passages of CLEC (P2 and P8) by RT-PCR analysis. The GAPDH gene was used as an internal control in RT-PCR to normalize the template quantity. FK P1 and P4 refer to the fetal keratinocytes at passages 1 and 4.

into keratinocytes *in vitro* (28) and adipose tissue-derived MSC to promote dermal repair and accelerate the re-epithelialization of cutaneous wounds (29). The potential benefits of stem cell application in cutaneous repair and regeneration are obvious; however, risks still remain, including inappropriate stem cell migration, immune rejection in the case of allogeneic sources and even tumor formation in the case of embryonic stem cells. Where allogeneic stem cell sources are concerned, the immune properties of the cells have fundamental implications for potential clinical applications. However, allogeneic skin substitutes, such as Apligraf®, which is a bi-layered living skin equivalent consisting of allogeneic keratinocytes and fibroblasts extracted from neonatal foreskin, have been used satisfactorily to promote the healing of deep partial and full thickness burns (30,31). Skin substitutes containing allogeneic stem cells of low immunogenicity may be considered a good temporary skin cover for wounds, that will not only prevent the loss of body fluids and prevent infection, but also aid with skin regeneration (32).

In the present study we have demonstrated the nature of human umbilical cord lining to be a simple epithelium containing putative epithelial stem cells. CK8, the simple epithelial cytokeratin, and CK14, characteristic of a differentiating epithelium, are both present in cord lining cells, while CK10, whose expression strictly parallels the extent of epidermal differentiation, is absent. It is well known that CK14 is usually expressed in the basal keratinocytes of stratified epithelium, representing the differentiated stratified state. However, CK14 has also been demonstrated in various other non-stratified epithelia and is suggested to be characteristic for epithelial cell types that have the potential to produce metaplasias and other forms of stratified epithelium (33,34). Thus the presence of CK14 in cord lining cells may indicate their differential potential towards a stratified epithelium. This has been demonstrated further in the organotypic CLEC culture. By seeding CLEC onto a FPCL and culturing at the air–liquid interface, CLEC formed a remarkably well-stratified epithelium consisting of basal, spinous and granular

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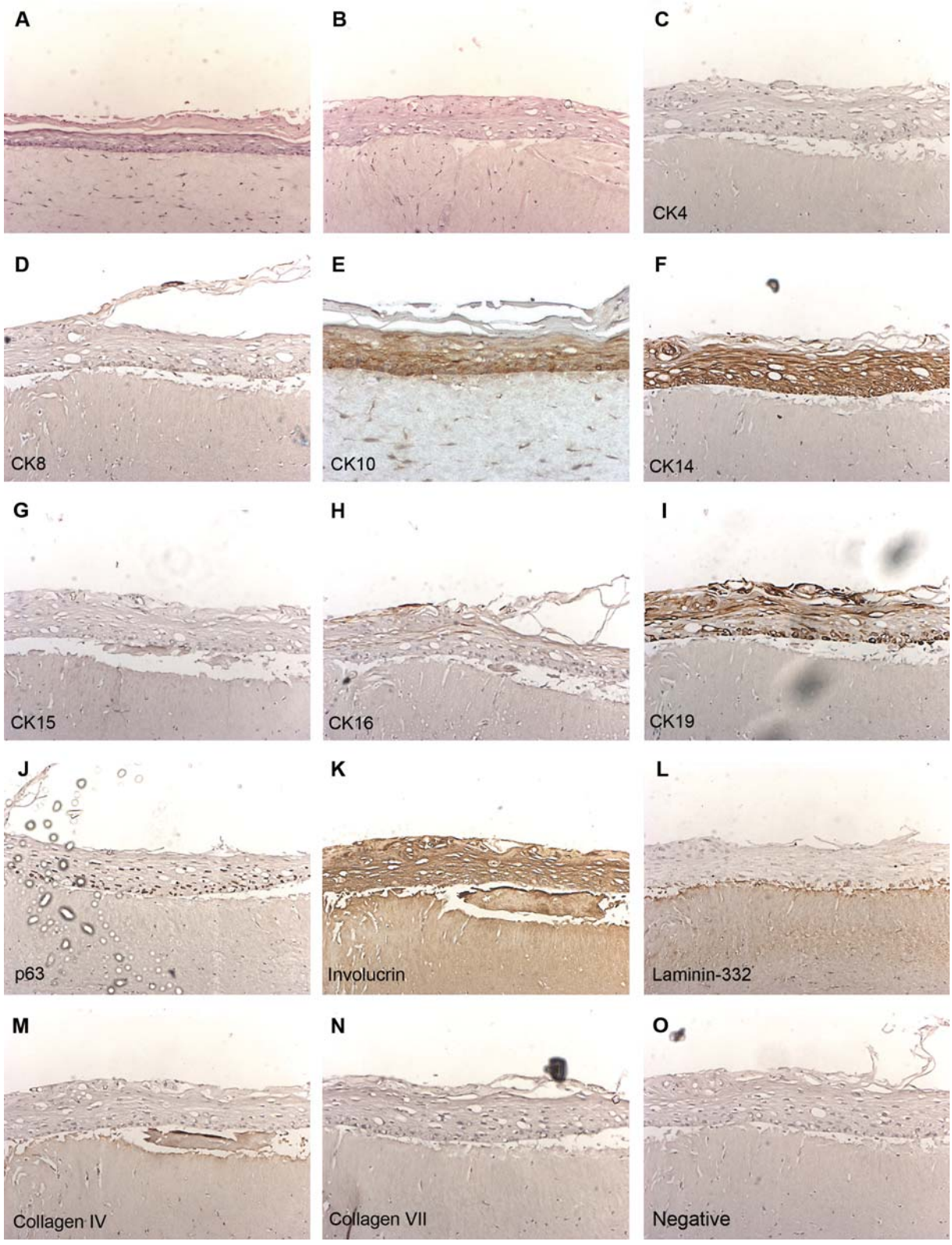

Figure 5. The histology and immunohistochemical staining of CLEC-constructed skin equivalents after 2 weeks of culture. (A, B) H&E staining of foreskin keratinocyte-constructed skin equivalent and CLEC-constructed skin equivalent, respectively; (C–N) immunohistochemistry of cytokeratins CK4, CK8, CK10, CK14, CK15, CK16 and CK19, p63, involucrin, basement membrane components laminin-332, collagen types IV and VII, respectively. The section that was not incubated with primary antibody served as the negative control (O). Original magnification was 200× for all panels.

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layers but without cornified strata. The formation of a typical four-layered epidermis is a continuous process of terminal differentiation of epidermal keratinocytes, which was observed in our control cultures using foreskin keratinocytes. This may reflect the fact that, in contrast to foreskin keratinocyte-constructed stratified epithelium, the CLEC-constructed one is not yet in a final stage of epidermal differentiation in our model. The 'reconstituted' CLEC are still highly proliferative, which critically will be beneficial for clinical applications where a highly proliferative cultured skin equivalent is needed. This is particularly suitable for the coverage of large wounds, where the concept of mesh or stamp grafting can be used. The highly proliferative skin equivalent will produce a more rapid re-epithelialization than the fully differentiated graft.

Of note, a previous study has demonstrated that a fully stratified and keratinized epithelial layer can be generated from organotypic culture of UCE cells (35). The culture conditions used in their study were very similar to ours except that a serum-free medium was used and the calcium concentration was adjusted to a very high level, at 1.8 mM. When looking at the expression of epidermal differentiation markers in their UCE-constructed skin equivalents, the data also differed from ours. It was reported in their paper that stratified organotypic UCE continued to express its original tissue-specific keratins, including CK4, CK13 and CK8, which are typical of simple or mucous epithelium *in vivo*. However, in our stratified organotypic CLEC culture, these typical simple or mucous epithelium keratins were disappearing; in the meantime, a pattern of stratified epidermal cytokeratins, including CK14 and CK16 together with the epidermal differentiation marker involucrin, was present. It is well documented that CK16 is not expressed by normal interfollicular epidermal cells; however, its expression can be induced in hyperproliferative conditions such as psoriasis and wound healing, and in organotypic cultures (36,37). The expression of CK16 in our CLEC constructs may indicate an activated hyperproliferative state of the reconstructed epithelium. On the other hand, the data on basement membrane formation are more consistent between the two studies done by us and the Japanese group. Both studies indicate the formation of an incomplete basement membrane, with a continuous deposition of laminin-332 but an intermittent deposition of collagen type IV at the junctional zone. Collagen type VII was undetectable in our model until 4 weeks of culture, but was reported to appear as a very low signal by the Japanese group (35). We postulate that these discrepancies may be a reflection of epigenetic factors caused by varying culture conditions between us and the Japanese group.

Although there is still controversy regarding the specific markers for epithelial stem/progenitor cells, a number of characteristics have been used preferentially. High levels of integrin-α6 and -β1 expression coupled with low levels of the transferring receptor CD71 have been used as markers for enriching epidermal stem cells *in vitro* by cell-sorting techniques (38,39). p63 is among the best markers of epithelium progenitor cells (22,23). CK19 has also been reported as an epidermal stem cell marker (21) and the proportion of CK19-positive cells has been found to correlate with keratinocyte culture lifespan (40). CK19 is positively present in CLEC. This, together with the observation that p63 is positive in CLEC in the nucleus, may suggest the epithelial stem/progenitor cell capacity of CLEC. The cell-surface antigens and transcription factors characteristic of embryonic/pluripotent stem cells, including OCT-4 , SSEA-4, TRA-1–60, SOX2 and Nanog (41–45), are also reported here for the first time as being expressed in CLEC, using immunofluorescence staining and/or RT-PCR analysis. This may indicate that these cells remain closer to the embryo in terms of stage of differentiation; they are more primitive cells. Notably, however, the cell portion that displays the characteristics of pluripotent stem cells is very small and the cells appear to lose some of the markers during serial propagation in the current culture conditions; in addition, the colony-forming efficiency of the current cell isolates is limited. It would be interesting to optimize the culture conditions in future studies to favor the maintenance of stem cell-like characters, and to enrich the stem cell fraction by cell sorting and compare the self-renewal and tissue regenerative capacities of the sorted, unsorted and leftover cells. Whether CLEC are multipotent, capable of contributing to hair follicle and sebaceous gland regeneration or capable of generating other types of epithelia where the situation mandates, is an intriguing question for future studies.

Last but not least, the immune properties of the CLEC and how these might change with serial propagation have fundamental implications for potential clinical applications. We are undertaking a series of *in vitro* and *in vivo* studies to characterize the immunogenic profile of the cord lining cells. Ongoing studies have demonstrated a suppression of the lymphocyte proliferation response in mixed-lymphocyte culture experiments by CLEC (unpublished observations, L. Huang *et al.*). We have also demonstrated that xenotransplantation of human umbilical cord lining into the kidney capsule of immunocompetent mice does not cause an acute immune rejection, and they survived for 8 weeks (unpublished observations and limited study period, L. Huang *et al.*). These data suggest a low immunologic profile of human umbili-

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cal CLEC. However, further work is needed to determine whether CLEC or associated constructs can be engrafted long-term *in vivo* and display self-renewal and multi-differentiation potency, as such, to demonstrate whether CLEC are a true stem cell population with an immunosuppressive function.

In summary, the present study has demonstrated that CLEC share some of the characteristics of epithelial as well as pluripotent stem cells. The demonstration that these cells have stem cell-like properties and are capable of generating fully stratified epithelium provides support for their potential clinical application in epidermal reconstitution.

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Declaration of interest: The authors declare no potential conflicts of interest.

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Table I. List of antibodies and associated working conditions used in the present study.
Table II. PCR reaction conditions and primer pairs.
Table III. The expression of cytokeratins, basement membrane components and other differentiation markers in CLEC- or keratinocyte (K)-constructed skin equivalents.

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