

Characterization of a Novel Umbilical Cord Lining Cell with CD227 Positivity and Unique Pattern of P63 Expression and Function

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Abstract Umbilical cord tissue is gaining attention as a novel source of multipotent stem cells because it is easily obtainable, ethically acceptable and the cells are immunologically naïve. In this study, we have isolated and characterized a new cell type expressing MUCIN1 (CD227) from human umbilical cord lining which we termed MUCIN-expressing Cord Lining Epithelial Cell (CLEC-muc). We found that CLEC-muc is highly proliferative and had significant clonogenic ability. These cells express embryonic stem cell markers OCT-4, NANOG, SSEA-4, REX1 and SOX2. Despite the abundant expression of epithelial cell marker MUCIN1

and cytokeratins, this population is also positive to the mesenchymal stem cell (MSC) marker CD166. CLEC-muc is unique in p63 expression that shuttles from the cytoplasm to the nucleus over time in culture. To understand p63 regulation and function in CLEC-muc, cells were treated with BMP4, a potent morphogen that plays a role in epidermal differentiation via p63 upregulation in ES cell and subsequent analyses were done. We found that BMP4 does not alter cytoplasmic expression of p63 that promotes cell proliferation. However, it increases nuclear p63 expression together with several other epithelial-associated genes such as *GATA3*, *JAGGED1*, *NOTCH1*, *HES1* and *IKK α* . BMP4 has also been found to weakly induce deltaNp63 expression in CLEC-muc. Our results suggest that CLEC-muc is a novel stem cell-like population that can be further differentiated by BMP4 to generate specific cell-types probably destined to form non-keratinized epithelia.

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Introduction

The umbilical cord blood has been found to be an important source of multipotent and immunologically naïve stem cells that has been used effectively for the treatment of various diseases [1, 2]. However, cord blood is rich in haematopoietic stem cells that form blood cells, and lack sufficient mesenchymal and epithelial stem cells that contribute to regeneration of solid organs in the body. In recent years, studies have reported the isolation of both mesenchymal and epithelial stem cells from human Wharton's jelly [3, 4],

amniotic fluid [5], amniotic membrane [6, 7], and umbilical cord [8–11] using different methods. These studies demonstrate that mesenchymal cells derived from umbilical cord are multipotent as these cells have the ability to differentiate into various cell types. It has been shown that the umbilical cord epithelium (UCE) expresses a cytokeratin pattern, which is similar to human epidermis [12], although the general architecture of the umbilical cord epithelium significantly differs from the mammalian epidermis [13]. Furthermore, epithelial cells derived from UCE are capable of forming a stratified epithelium when seeded on fibroblast populated collagen gels [14, 15]. A very recent study also shows that primary neonatal keratinocytes and umbilical cord lining epithelial cells have a very similar pattern of cytokeratin CK1/CK10 and deltaNp63 expression [16].

It has been shown that *p63*, a *p53*-related gene plays crucial roles in epithelial proliferation and differentiation as *p63*-deficient or transgenic mice exhibited an abnormal epidermis and other stratified epithelia formation [17, 18]. More recent studies have demonstrated that *p63* functions to maintain the proliferative capacity of corneal, epidermal, or thymic epithelial stem cells [19, 21]. Medawar et al. [22] recently showed that deltaNp63, which is activated by BMP4, is necessary for the commitment of embryonic stem (ES) cells into CK5/CK14-positive squamous stratified epithelial cells. BMP4 is a member of the transforming growth factor-beta (TGF- β) superfamily, which has been shown to act as a strong epidermal inducer to convert ectodermal cells to epidermal lineage and inhibit neural differentiation [23, 24]. These primitive epidermal cells express basal cytokeratins CK5/CK14 and further differentiation gives rise to tissue-specific cytokeratin CK3/CK12- or CK1/CK10-positive cells [25, 26]. The former set is expressed in differentiated corneal epithelium while the latter in keratinized epidermis. Although a good number of studies demonstrate the molecular pathways toward epithelial lineage formation from ES cell, little is known about such mechanisms in cells derived from umbilical cord, and the different type of cells from this source have not been well characterized. This issue needs to be resolved essentially to determine their proper downstream application.

In this study, we report the isolation and characterization of a novel cell source from human umbilical cord, CLEC-muc, and evaluated the growth characteristics, proliferative capacity, clonogenicity and gene expression pattern. Based on our experimental results from immunocytochemistry, flow-cytometry and qPCR, we demonstrate that these cells express several known markers of ES cell such as, OCT-4, NANOG, SOX2, REX1 and SSEA-4. In addition, CLECs-muc strongly express both epithelial and MSC markers MUCIN1, CD151, CD166

and VIMENTIN, while E-CADHERIN, CD73 and CD105 are weakly expressed. We found that BMP4 does not alter cytoplasmic *p63* whereas this gene is upregulated when transported to the nucleus. Furthermore, BMP4 in our culture system stimulates the expression of several epithelial-morphogenesis genes *GATA3*, *JAGGED1*, *NOTCH1*, *HES1* and *IKK α* , and differentiate CLECs-muc into CK3/CK12-positive epithelial cell type. These novel stem cells have potential clinical applications for use in regenerative medicine.

Materials and Methods

Isolation of Umbilical Cord and Cell Culture

Umbilical cords were obtained from healthy women undergoing delivery after obtaining proper informed consent according to the statutes of the Institutional Review Board and the Singapore Bioethics Advisory Committee. The umbilical cord tissue was cut into 2 cm segments and transferred into 10 cm diameter cell culture dishes, where further washing and disinfection was performed with 70% ethanol followed by multiple washes using phosphate-buffered saline (PBS) containing an antibiotic mixture (50 IU/ml penicillin, 50 μ g/ml streptomycin, 250 μ g/ml fungizone, 50 μ g/ml gentamicin, all purchased from Invitrogen) until the solution became clear. Surgical dissection of the umbilical cord was first performed to separate the umbilical cord lining membrane from the enclosed Wharton's jelly and other internal structures. The isolated cord lining membrane was then divided into small squares (0.5 cm²) for cell isolation. For CLEC-muc separation/cultivation, explant tissue samples were placed on cell culture plastic surfaces and submerged in 5 ml Medium-171 (Cascade Biologics, OR). Cell outgrowth from tissue explants was monitored under phase contrast microscopy. Outgrowing cells were harvested by trypsinization (0.0125% trypsin/0.05% EDTA) in Medium-171 and cryopreserved. In this study, cells were cultured in PTTe-1 medium supplemented with 2.5% fetal bovine serum (FBS), 50 μ g/ml insulin-like growth factor-1 (IGF-1), 50 μ g/ml platelet-derived growth factor-BB (PDGF-BB), 5 μ g/ml transforming growth factor- β 1 (TGF- β 1) and 5 μ g/ml insulin. Cultures were incubated at 37°C, under 5% CO₂ and 95% air, with medium change carried out every 2 days. Upon reaching 70–80% confluence, the cells were subcultured by enzymatic disaggregation with 0.25% trypsin/0.02% EDTA, and subsequently plated in culture dishes at a seeding density of 2×10^3 cells/cm². CLECs-muc were also cultured with various concentrations of BMP4 (PeproTech Inc. New Jersey, USA).

Cell Viability Assay

Cell count and viability assay were performed using GUAVA PCA-96 (Millipore technologies) according to the manufacturer's protocol. Briefly cultured cells were trypsinized and a homogenous suspension was prepared. 2 µl of 0.5 X Viacount Flex reagent was added to 200 µl cell suspension, mixed well by vortexing and incubated for 5 mins at room temperature. Then the sample was loaded onto GUAVA PCA-96 for assay.

Bromodeoxyuridine (BrdU) ELISA Cell Proliferation Assay

The proliferative capacity of CLECs-muc was determined by a BrdU-ELISA cell proliferation assay (RPN250, Amersham Biosciences). Cells were seeded in 96-well plates at a density of 2,000 cells/well, with 100 µl of culture media. Cultured cells were incubated with 10 µM of BrdU labeling solution (Amersham Biosciences, Freiburg, Germany) for 20 h at 37°C, followed by washing with 200 µl of PBS containing 10% serum. Then the cells were fixed and incubated with 100 µl of monoclonal antibody against BrdU for 2 h, followed by 100 µl of peroxidase substrate per well. The BrdU absorbance in each well was measured directly using a spectrophotometric microplate reader (Tecan spectrophotometer, Grodig, Austria) at a test wavelength of 450 nm. The respective plain basal media was used as negative control. The optical density (OD) reading for the negative control was subtracted from the sample readings. This gave us a measure of the degree of proliferation of cells, which we termed the proliferation index.

Number of Population Doublings

Upon reaching 80% confluence, the cells were subcultured by enzymatic disaggregation using 0.25% trypsin/0.02% EDTA for a period of 10 mins. The single-cell suspensions were plated at a density of $3-4 \times 10^4$ cells/cm². The number of population doublings, x , was calculated as follows: $x = \log_2(N/N_0)$, where N is the total number of cells harvested at subculture, and N_0 is the number of viable cells seeded.

Colony Forming Efficiency (CFE)

Cells were detached from culture plates using trypsin and plated on 6-cm culture plates (BD Biosciences) in PTTe-1 medium at a density of 5 cells/cm². Cells were cultured with medium changes every other day for 3 weeks before being fixed with 4% paraformaldehyde

and stained with 3% crystal-violet for 1 h. After a brief wash, the plates were dried, and colonies were counted in triplicate. Colonies having diameters larger than 2 mm were counted. The colony-forming efficiency was determined as follows:

Colony – forming efficiency(%)

$$= \frac{\text{Colonies formed at end of growth period} \times 100\%}{\text{Total number of viable cells seeded}}$$

Flow Cytometry

Cells were detached from culture plates using trypsin-EDTA, fixed in 4% formaldehyde for 15 min at 37°C and blocked for 30 mins in 0.5% BSA. Cells were then incubated for 90 mins with primary antibodies (mouse anti-CD34, mouse anti-CD45, mouse anti-CD73, rabbit anti-MHC class I, rabbit anti-MHC class II, mouse anti-CD151 from Abcam, Cambridge, UK; mouse anti-CD166 from BD Biosciences, USA; mouse anti-CD31, mouse anti-CD105 from Biolegend, San Diego, CA) in blocking solution followed by 30 mins incubation with appropriate secondary antibodies. Propidium iodide was incubated for 15 mins to counterstain the cells. Control samples were incubated with isotype-specific or no primary antibody. FACS was performed on GUAVA PCA-96 using Cytosoft 5.3 software (Millipore technologies).

Immunocytochemistry

Cells were fixed in cold methanol for 15 min at room temperature before blocking and permeabilizing with 2% BSA in PBS with 0.4% Triton X-100. Primary antibodies were incubated overnight at 4°C in blocking buffer and included mouse anti-p63 (4A4), mouse anti-CK1/10, goat anti-CK5, goat anti-MUCIN1 (Santa Cruz Biotechnology Inc., CA), mouse anti-CK7, mouse anti-CK14, mouse anti-KI67, mouse anti-VIMENTIN (Sigma-Aldrich), mouse anti-OCT-4, rabbit anti-E-CADHERIN (Millipore), mouse anti-CK19, rabbit anti-SOX2, mouse anti-SSEA-4, rabbit anti-REX1, mouse anti-p63 (4A4), mouse anti-NANOG (Abcam, Cambridge, UK), mouse anti-CK3/12 (RDI, NJ, USA), rabbit anti-deltaNp63 (Biolegend, San Diego, CA). Cells were stained with the appropriate fluorophore-conjugated secondary antibody (Invitrogen) for 1 h at room temperature and counterstained with DAPI contained in the mounting media (Vectashield, Vector Laboratories, CA). Immunofluorescence images were observed on a Zeiss Axioplan 2 microscope (Zeiss, Germany). For DAB staining, cells were treated with 0.5% periodic acid to block endogenous peroxidase activity before blocking with 10% serum. After overnight incubation with primary

antibody, cells were incubated with anti-mouse or rabbit horseradish-peroxidase conjugated secondary antibody followed by treatment with DAB solution (Vector Laboratories, CA) for 5 mins.

Semiquantitative and Quantitative Reverse Transcription-Polymerase Chain Reaction

RNAs were obtained from cells using the RNeasy Mini kit (Qiagen), and cDNAs were generated using SuperScript III First Strand Kits (Invitrogen). For semiquantitative analysis, polymerase chain reaction (PCR) was conducted using PCR Master mix (2X) (Fermentas Life Sciences). Quantitative PCR was conducted using LightCycler 480 SYBR Green I Master kit (Roche Applied Science). Negative controls using non-reverse transcribed total RNA as

template strands were performed for all experiments. All assays were run in duplicate for 3–5 individual samples. Relative expression levels were calculated using the ΔCt method, normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcription. ΔCt was calculated by subtracting the Ct of GAPDH from the Ct of the target gene. For non comparison study, ΔCt values are graphed, therefore, higher ΔCt values indicate lower gene expression and vice versa. For comparison, the BMP4-untreated cell sample was chosen as the calibrator to compare the relative abundance of each gene transcript in BMP4-treated samples. The fold change in other samples was determined by the formula $2^{(-\Delta\Delta\text{Ct})}$, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{calibrator}}$. PCR products were verified by melting curve analysis and/or 2% agarose gel electrophoresis. The following oligonucleotide primer sequences were used:

Gene	Sequence	Gene	Sequence
<i>p63</i>	For, 5'-GCC ACC TGG ACG TAT TCC ACT G-3' Rev, 5'-TAA AAT TGG ACG GCG GTT CAT C-3'	<i>OCT-4</i>	For, 5'-TCG AGA ACC GAG TGA GAG GC-3' Rev, 5'-CAC ACT CGG ACC ACA TCC TTC-3'
<i>ΔNp63</i>	For, 5'-CTG GAA AAC AAT GCC CAG AC-3' Rev, 5'-GGG TGA TGG AGA GAG AGC AT-3'	<i>SOX2</i>	For, 5'-GCA CAT GAA CGG CTG GAG AGC AACG-3' Rev, 5'-TGC TGC GAG TAG GAC ATG CTG TAG G-3'
<i>CK19</i>	For, 5'-CTG CGG GAC AAG ATT CTT GGT-3' Rev, 5'-CCA GAC GGG CAT TGT CGA T-3'	<i>REX1</i>	For, 5'-GGC GGA AAT AGA ACC TGT CA-3' Rev, 5'-CTC ACC CCT TAT GAC GCA TT-3'
<i>IKKα</i>	For, 5'-GCCATCCACTATGCTGAGGT -3' Rev, 5'-TCCCTGACGTCTTCCATAGG-3'	<i>NANOG</i>	For, 5'-CCA ACA TCC TGA ACC TCA GCT AC-3' Rev, 5'-GCC TTC TGC GTC ACA CCA TT-3'
<i>NOTCH1</i>	For, 5'-CGG GTC CAC CAG TTT GAA TG-3' Rev, 5'-GTT GTA TTG GTT CGG CAC CAT-3'	<i>TERT</i>	For, 5'-GCC AGC ATC ATC AAA CCC C-3' Rev, 5'-CTG TCA AGG TAG AGA CGT GGC TC-3'
<i>GATA3</i>	For, 5'-CTC ATT AAG CCC AAG CGA AG-3' Rev, 5'-GTC TGA CAG TTC GCA CAG GA-3'	<i>GAPDH</i>	For, 5'-GCC AAG GTC ATC CAT GAC AAC-3' Rev, 5'-GTC CAC CAC CCT GTT GCT GTA-3'
<i>JAGGED1</i>	For, 5'-GAA TGG CAA CAA AAC TTG CAT-3' Rev, 5'-AGC CTT GTC GGC AAA TAG C-3'	<i>Tp63</i>	Cat # PPH01032E (SABiosciences)
<i>HES1</i>	For, 5'-GCG GAC ATT CTG GAA ATG ACA-3' Rev, 5'-AGC GCA GCC GTC ATC TG-3'	<i>CK3</i>	Cat # PPH19500E (SABiosciences)

Results

Morphology and In Vitro Growth Characteristics of CLECs-muc

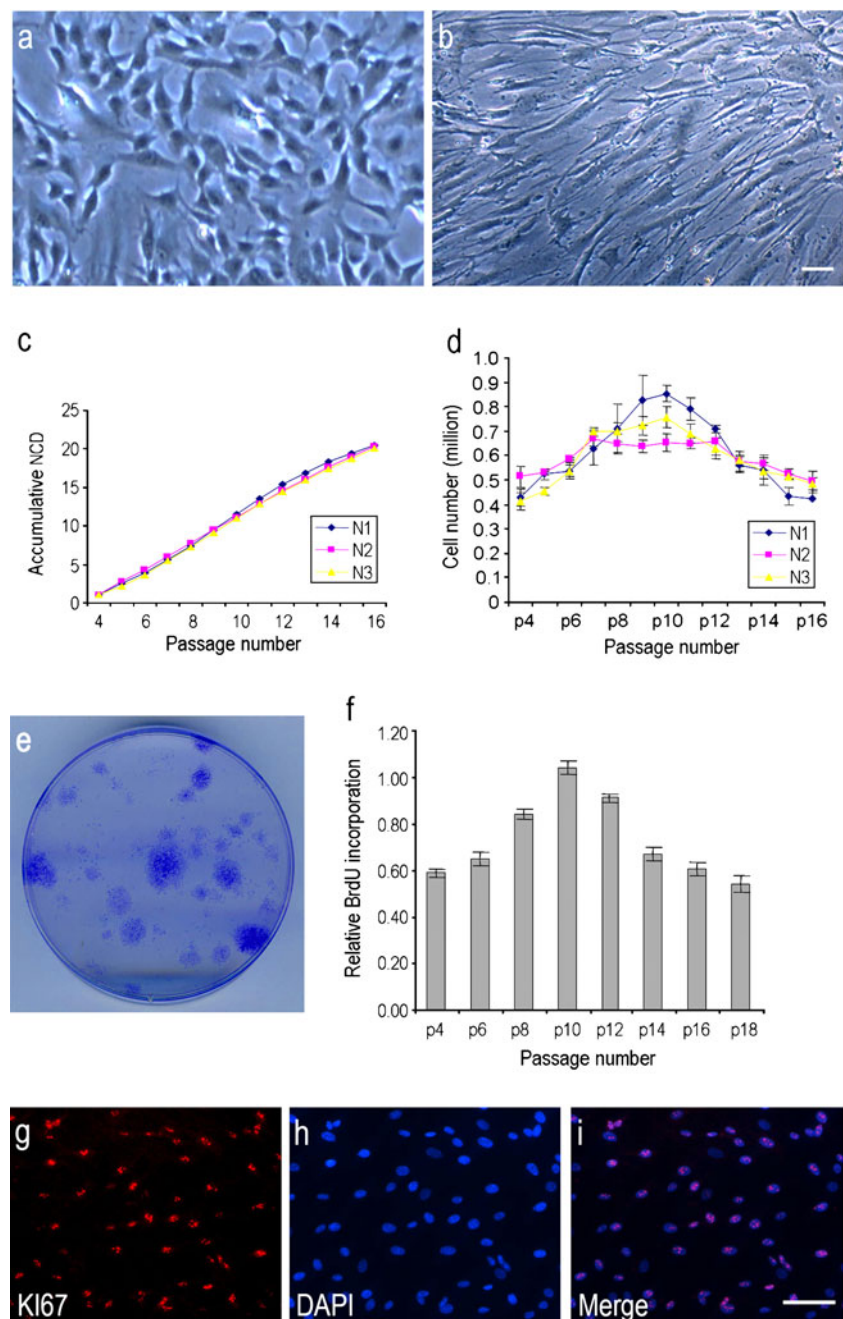
All CLECs-muc used were tested and shown to have no detectable mycoplasma or known viral pathogens. Within 6 h after seeding, most CLECs-muc attached to the plastic plate and started spreading. The cultured cells were serially passaged to examine their expansion potential and morphology. In PTTe-1 medium, CLECs-muc grew as adherent small cells of similar size. In the first 3 days, the cells mostly appeared cuboidal in morphology. However, these cells extended quickly to link with each other and displayed a fibroblast-like

pattern of expansion (Fig. 1a). When these cells reached confluence, they became elongated or oval. The same cell morphology was maintained throughout cultures that were expanded for up to 16 passages. For comparison, MSCs from umbilical cord were also cultured, which appeared different in morphology (Fig. 1b). We counted the cells for each passage and found that passages between 8–12 exhibited a higher number of cells (Fig. 1c).

Number of Population Doublings

We performed population doublings assay from P4 to P16. The results are summarized in Table 1. CLECs-muc showed population doublings of 20.28 ± 0.17 ($n=3$) (Fig. 1d).

Fig. 1 Morphology and growth profile of CLEC-muc in PTTe-1 media. Phase contrast image of CLECs-muc (a) and MSC (b) from umbilical cord tissue. Long-term growth profiles of CLECs-muc from three donors show cell numbers in each passage indicated (c) and total number of cell doublings (d). A representative image shows colony formation of CLEC-muc (e). Graph represents the levels of BrdU incorporation determined for CLECs-muc at different passages (f). Data are represented as mean $\pm$ SD of three independent experiments performed in triplicate. Red fluorescent shows the expression of the proliferation marker KI67 in CLEC-muc (g). Blue DAPI depicts cell nuclei (h) and I shows merged image. Each image is the representative of at least three independent experiments. N, donor. Bar, 100 μ m



Colony Forming Efficiency

We carried out colony forming efficiency assays to assess the growth capacity of CLEC-muc at different passages (p4, p12 and p16) cultured in PTTe-1 medium. CLECs-muc from all passages exhibited colony forming efficiencies between 24% and 28% (Fig. 1e). These results indicate that CLECs-muc were able to retain their clonogenic and proliferative ability over passages, a characteristic of stem cells.

BrdU-ELISA Proliferation Assay

We found that CLECs-muc had a gradual increase in their proliferative capacity and then declined to the baseline at passage 16 when the cells were passaged successively. The highest proliferative capacities were exhibited by cells of passages 8–12 (Fig. 1f). We further stained the cells using mouse monoclonal antibody against KI67, a proliferation marker, and found positive reactivity in a significant number of cells (Fig. 1g–i), which was very similar in all passages.

Table 1 Population doublings of CLEC-muc from P4 to P16

Passage number	Initial cell density (millions)	Incubation time (days)	Mean final cell number (millions)	Number of cell doublings	Accumulative number of cell density	Population doubling time (h)
P4	0.2	4	0.430±0.036	1.104	1.104	86.930
P5	0.2	4	0.523±0.025	1.388	2.492	69.178
P6	0.2	4	0.597±0.056	1.577	3.916	60.878
P7	0.2	4	0.630±0.067	1.655	5.571	57.994
P8	0.2	4	0.710±0.099	1.828	7.399	52.522
P9	0.2	4	0.827±0.100	2.047	9.446	46.891
P10	0.2	4	0.930±0.022	2.217	11.540	43.297
P11	0.2	4	0.790±0.045	1.982	13.522	48.440
P12	0.2	4	0.710±0.014	1.828	15.350	52.522
P13	0.2	4	0.563±0.034	1.494	16.844	64.258
P14	0.2	4	0.542±0.061	1.437	18.281	66.787
P15	0.2	4	0.433±0.038	1.115	19.396	86.062
P16	0.2	4	0.423±0.037	1.082	20.478	88.741

Flow-Cytometric Detection of Cell Surface Antigens

Since cell surface markers represent the easiest cell characterization as flow cytometry-based assays are reproducible, automated and highly objective, we analyzed the expression of different cell surface antigens and the results are shown in Fig. 2a. We found that the specific endothelial cell marker CD31 was not expressed in CLECs-muc, confirming that these cells are not endothelial cells. Analyses of MSC markers CD73, CD105 and CD166 showed that the expression level of CD166 was very high, CD105 was moderately positive, while only weak expression of CD73 was detected in CLECs-muc, indicating that these cells are different from other reported cells isolated from umbilical cord, and may share some mesenchymal characteristics. We also tested the expression of MHC class I and MHC class II antigens; the cells expressed MHC class I, but did not express MHC class II, consistent with the previous report of the cells from the same tissue [5, 11, 27–29]. Expression of hematopoietic markers CD34 and CD45 was relatively low. We showed that MSC marker VIMENTIN was expressed in almost all cells (Fig. 2d, e). We evaluated the expression of epithelial markers E-CADHERIN, CD151 and MUCIN1 (CD227) (Fig. 2a–g) and found that CD151 and MUCIN1 were abundantly expressed while E-CADHERIN expression was found in some cells (Fig. 2a, b, f, respectively).

Detection of Cytokeratins in CLECs-muc

To understand more about epithelial properties, we examined the expression of several cytokeratins including ‘simple’ epithelial cytokeratins CK7, CK19 and ‘stratifica-

tion related’ cytokeratins CK5 and CK14. We were able to observe a weak expression of CK7 (Fig. 2h, i) and CK14 (Fig. 2j, k) in all cells while no reactivity was detected for CK5 (data not shown) by immunostaining. A strong CK19 expression was observed in a limited number of cells (Fig. 2l, m). These data imply that CLECs-muc are not fully undifferentiated and possess epithelial characteristics.

Expression of Embryonic Stem Cell Markers in CLEC-muc

To determine whether CLECs-muc possess any ES cell characteristics, we investigated the expression of several ES cell markers such as NANOG, OCT-4, REX1, SSEA-4, SOX2, and TERT [30–32]. All the cells showed positive reactivity to OCT-4 (Fig. 3a, b) and NANOG (Fig. 3c,d). SSEA-4, a stage specific embryonic antigen, was similarly detected in CLECs-muc (Fig. 3e, f). Although, a previously isolated mesenchymal cell line from umbilical cord was shown to be negative for *SOX2* and *REX1* [11], our CLECs-muc were positive to these markers in more than 70% cells with variable intensity (Fig. 3g–j). To confirm our immunostaining data, conventional and real-time PCR were performed and the results were found to be consistent (Fig. 3k, l). However, no *TERT* expression was observed in CLECs-muc (Fig. 3k,l). These results suggest that our cells derived from umbilical cord are different from other cell lines from the same source, and may have some residual effect of ES cell-specific molecules.

Aberrant Expression of p63

Since cytokeratins are specific to epithelial cell types and p63 functions as a key regulator for stratified epithelial

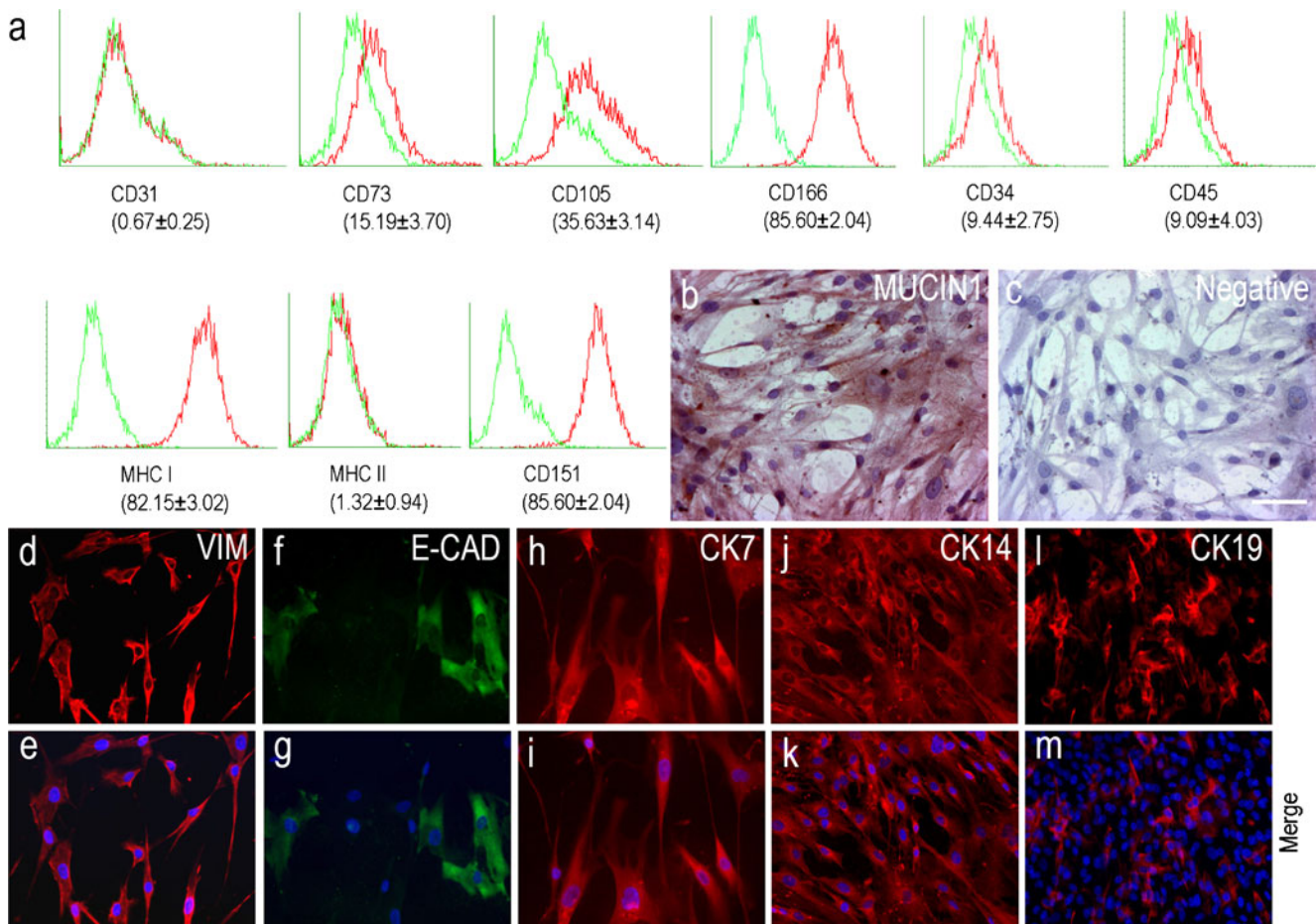


Fig. 2 Characteristic expression of epithelial and MSC markers. Flow cytometric analysis of surface antigen expression including CD31, CD73, CD105, CD151, CD166, CD34, CD45, MHC class I and MHC class II in CLECs-muc (a). Numbers of positive cells among the gated population are shown in parentheses (a). A minimum number of 10,000 events was recorded in all cases. Green line depicts isotype control and red line indicates positive signal for each specific

antibody. DAB-staining shows positive expression of MUCIN1 (CD227) (b) and no expression in negative control (c). Fluorescent immunostaining indicates the expression of VIM (d, e), E-CAD (f, g) CK7 (h, i), CK14 (j, k), CK19 (l, m) in CLECs-muc. Blue DAPI visualizes cell nuclei in merged images (e, g, i, k, m). Each image is representative of at least three independent experiments. Bar, 100 μ m

development [17–22], we evaluated the p63 expression along with its dominant-negative isoform, deltaNp63 in these cells. Immunofluorescent staining of cells in 4-day cultures revealed p63 expression localized in the cytoplasm (Fig. 4a, b), which is different from its regular nuclear expression pattern found in other normal cell types. This finding prompted us to further investigate this issue. We carefully examined its expression in different passages ranging from passage 4–20, however p63 localization was the same in all cases, at all cell densities. We then evaluated its expression at different time intervals of culture from day 2 to day 8 using mouse anti-p63 (4A4) monoclonal antibodies from two manufacturers. Fluorescent immunostaining revealed an interesting pattern of p63 expression. We found that p63 expression was confined to the cytoplasm surrounding the nucleus until day 4, and between days 5–6, the expression largely shifted from cytoplasm to

nucleus (Fig. 4c, d). On day 8, an absolute expression of p63 in the cell nucleus was detected (Fig. 4e, f). We repeated this experiment 11 times using CLECs-muc from different donors and the above phenomena were observed 7 times. A persistent expression of p63 in cytoplasm was found on rest occasions. We confirmed this pattern of protein localization by DAB-immunostaining, which demonstrated exactly the same pattern of expression (data not shown). We analyzed RNA expression by RT-PCR, and found that both conventional and real-time RT-PCR analyses showed positive expression of *p63* (Fig. 4g, h), which was consistent with the immunostaining data. We investigated the expression of deltaNp63 at protein and RNA levels. However this isoform was not found in CLECs-muc by immunostaining and only a very faint expression was detected by qPCR (Fig. 4g, h). These results suggest that CLECs-muc display a unique pattern of

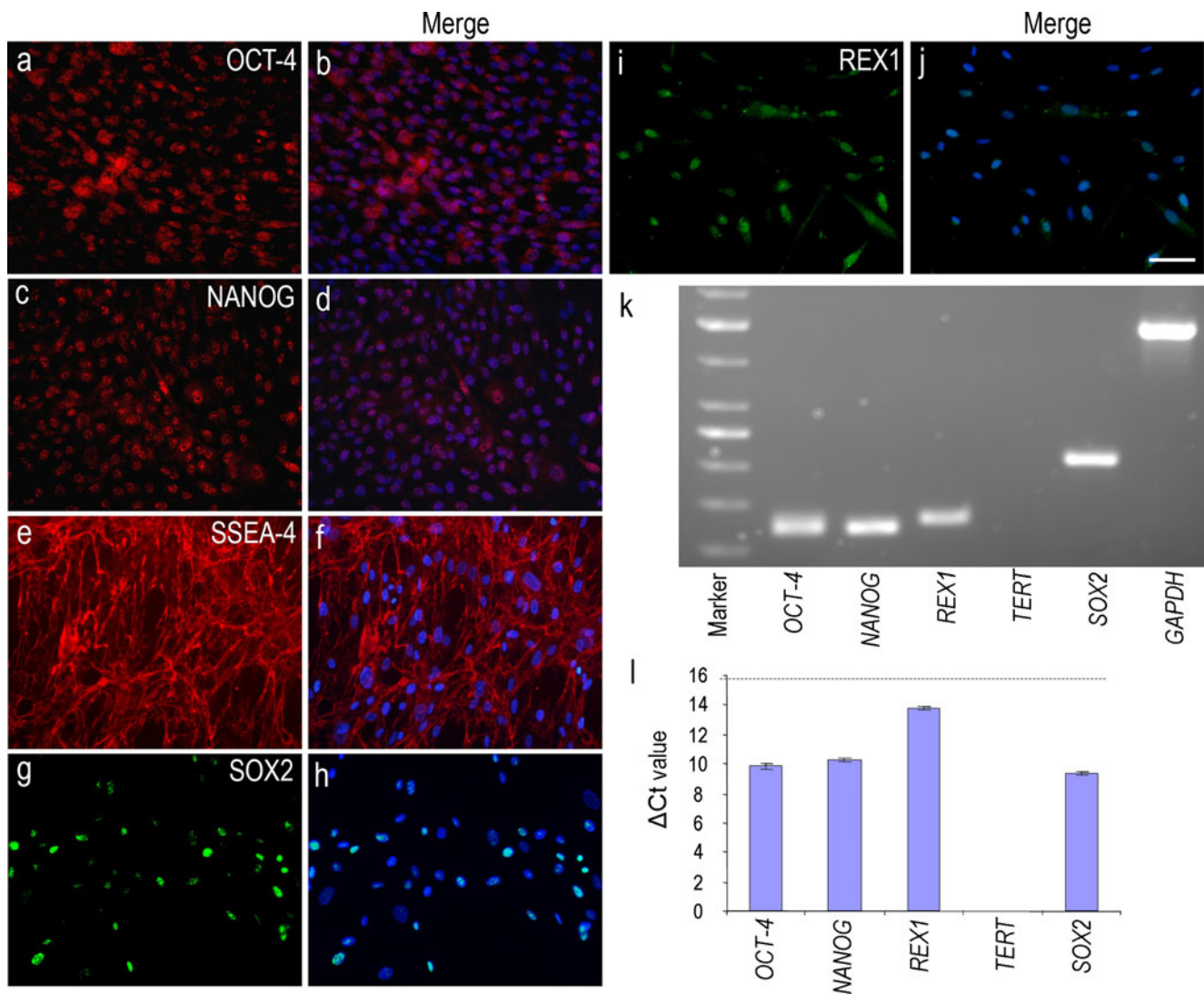


Fig. 3 Expression of embryonic stem cell markers in CLECs-muc. Immunofluorescent staining shows positive expression of OCT-4 (a), NANOG (c), SSEA-4 (e), SOX2 (g) and REX1 (i). Blue DAPI visualizes cell nuclei in the merged images (b, d, f, h, j). Each image is representative of at least three independent experiments. Agarose electrophoresis shows the expression of these genes by conventional

RT-PCR (k). Ct values of real-time RT-PCR from at least three independent experiments reveal different level of gene expression (l). Higher ΔC_t value corresponds to lower gene expression and vice versa. GAPDH was used as endogenous internal control (k). Dot line indicates limit of gene expression (l). Bar, 100 μ m

p63 expression, which slowly shifts from cytoplasm to nucleus during culture.

BMP4 Stimulates CLECs-muc Differentiation

To understand the cellular behaviour of CLEC-muc in response to certain morphogens or signaling cues, we investigated the effect of BMP4, as this morphogen has been shown to play multiple roles during ES cell differentiation and embryonic development. Previous studies have demonstrated that BMP4 blocks neural lineage formation in human and murine ES cells and participates in epithelial cell formation [33–35]. We cultured the CLECs-

muc with BMP4 at different concentration (10 ng, 25 ng, 50 ng and 100 ng) and found that 10 ng/ml of BMP4 concentration had almost no effect on cell death as evidenced by cell viability assay that revealed 92–94% viability in BMP4-added and control cells (Fig. 5a). When CLECs-muc were cultured for 7 days with 10 ng/ml BMP4, a slow morphological change was observed under phase-contrast microscopy showing that the cells typically acquired an epithelial-like phenotype instead of the elongated morphology seen in control (Fig. 5b, c). To understand more about this differentiation, we examined the expression of CK1/10, CK3/12, CK4, CK13 and CK19 by immunostaining, which exhibited negative expression of

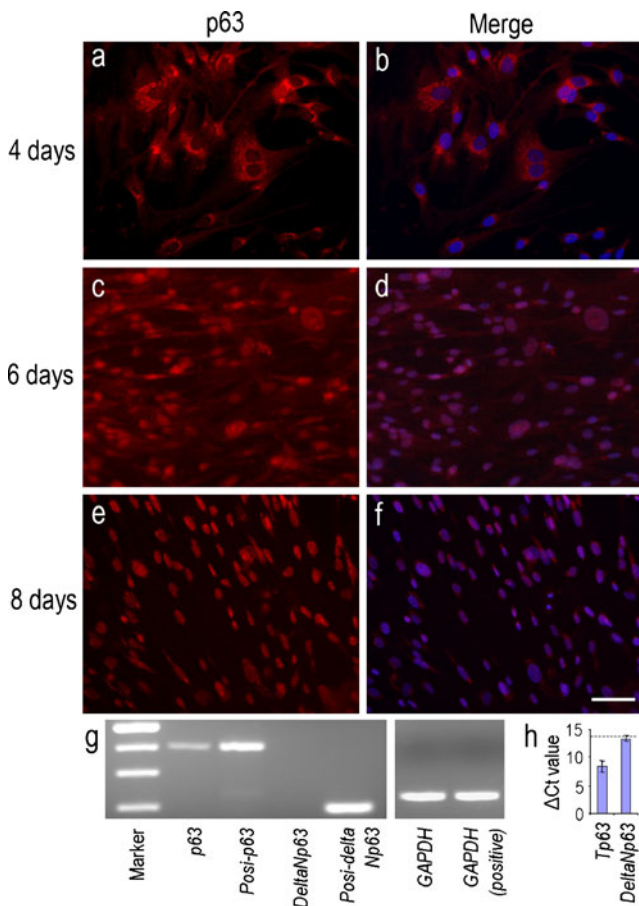


Fig. 4 p63 expression in CLECs-muc. Cells cultured for 4 days show positive expression of p63 in cytoplasm (a). A diffuse expression in 6-day culture (c) and nuclear expression in 8-day culture (e). Immunostaining images are representative of at least three independent experiments. Conventional RT-PCR shows positive expression of *p63* while negative expression of *deltaNp63* (g). Sample from an epithelial cell line was used as a positive control (g). GAPDH was used as endogenous internal control (g). Δ Ct values represent gene expression by real-time RT-PCR from at least three independent experiments performed in duplicate (h). Higher Δ Ct value corresponds to lower gene expression and vice versa. Dot line indicates limit of gene expression (h). Bar, Bar, 100 μ m

CK1/10, CK4, and CK13 in both populations (data not shown). However, CK3/12 expression was enhanced while CK19 expression was decreased in BMP4-added cultures as compared to controls (Fig. 5d–k), suggesting that BMP4 differentiates CLECs-muc into a certain tissue-specific cell type. qPCR was also performed, which revealed consistent results for *CK3* and *CK19* (Fig. 5p). To understand the proliferative status of the cells in this culture condition, ELISA-BrdU assay was performed, which showed a lower level of BrdU incorporation in BMP4-added samples compared to controls (Fig. 5r). Immunostaining for the proliferation marker KI67 revealed that the number of KI67-positive cells decreased significantly in the BMP4-added sample (Fig. 5l–o,q), which was consistent with the

ELISA-BrdU results. Finally, we compared the expression of stem cell markers *SOX2*, *OCT-4* and *NANOG* between controls and BMP4-added cultures by qPCR, which clearly showed a downregulation of these genes in BMP4-added samples (Fig. 5s) suggesting that CLECs-muc have undergone differentiation towards an epithelial cell lineage.

Differential Regulation of p63 by BMP4 in CLEC-muc

It has been shown that BMP4 can upregulate deltaNp63 in mouse ES cell [22]. To determine how p63 expression is regulated by BMP4 in CLEC-muc, BMP4 was supplemented in our culture system and cells were harvested at two different time points, 4 days and 7 days, and the expression of p63 was analyzed in control and BMP4-added samples. Immunostaining revealed no change in fluorescence intensity of p63 in BMP4-added cells as compared to the control in 4-day cultures (Fig. 6a–d). Notably, the p63 protein was localized in the cytoplasm in this case. qPCR consistently exhibited a similar result at the RNA level (Fig. 6m). When we cultured cells for 7 days, the positive reactivity for nuclear p63 expression was visualized in both populations. However, the fluorescence intensity was slightly higher in BMP4-added samples compared to the controls (Fig. 6e–h). Similarly, the higher level of *TP63* transcript was detected by qPCR in BMP4-added cultures (Fig. 6m). We also examined deltaNp63 expression and it was undetected in both populations cultured for 4 days (data not shown). In contrast, deltaNp63 was found to be positively expressed in BMP4-added 7-day cultures, even though it was very weak, while no cells were positive to deltaNp63 in the control sample (Fig. 6j–l). qPCR also revealed a similar induction of *deltaNp63* in BMP4-added cultures (Fig. 6m). Agarose electrophoresis clearly demonstrated the change in gene expression (Fig. 6n). These findings suggest that BMP4 regulates p63 expression in a different way, which is dependent on p63 localization in cells.

p63 Function is Mediated Through Known Epithelial-Morphogenesis Genes

We have observed that BMP4 enhances p63 expression and cell differentiation in 7-day cultures. To gain insight into the mechanism of such differentiation, we examined the classical pathway of p63 in transcribing the downstream genes in epithelial morphogenesis. CLECs-muc were cultured either for 4 days or for 7 days with or without addition of BMP4. At the end of cultures, total RNA was isolated to perform qPCR against several known epidermal morphogenesis-associated downstream genes of *p63* such as *NOTCH1*, *JAGGED1*, *GATA3*, *IKK α* and *HES1* [22]. The data revealed that there was no obvious alteration of

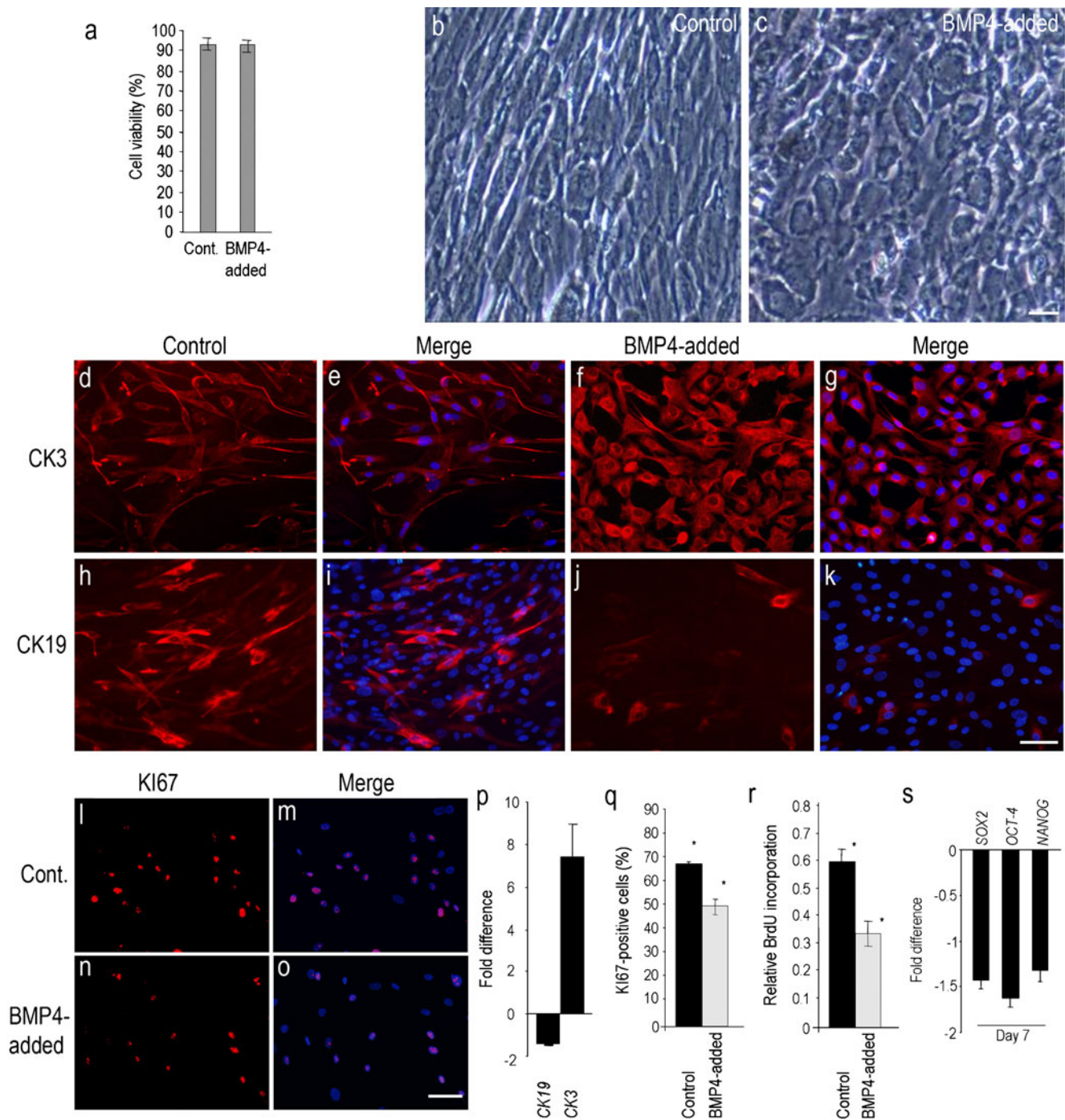


Fig. 5 Addition of BMP4 in culture causes differentiation of CLEC-muc. Cells were cultured for 7 days with BMP4 at a concentration of 10 ng/ml and subjected to viability assay (**a**). Phase contrast images of cells showing morphology in control (**b**) and BMP4-added samples (**c**). Immunofluorescent staining indicates expression of cytokeratins CK3/12 and CK19 in control (**d**, **h**, respectively) and BMP4-added (**f**, **j**, respectively) samples. Also, proliferation marker KI67 was examined in control (**l**) and BMP4-added (**n**) samples and positive cells were counted and expressed as percentage (**q**). Data are

representative of at least three independent experiments. Levels of BrdU incorporation is represented in the graph (**r**). qPCR shows the alteration of *CK3* and *CK19* expressions in RNA level (**p**). Also, qPCR shows downregulation of *SOX2*, *OCT-4* and *NANOG* in BMP4-added cells cultured for 7 days (**s**). The value for each gene was normalized to control CLEC-muc culture, and represents the mean $\pm$ SD of three independent experiments performed in duplicate. Blue DAPI marks cell nuclei in merged images (**e**, **g**, **i**, **k**, **m**, **o**). Asterisks indicate values, $p < 0.05$. Bar, 100 μ m

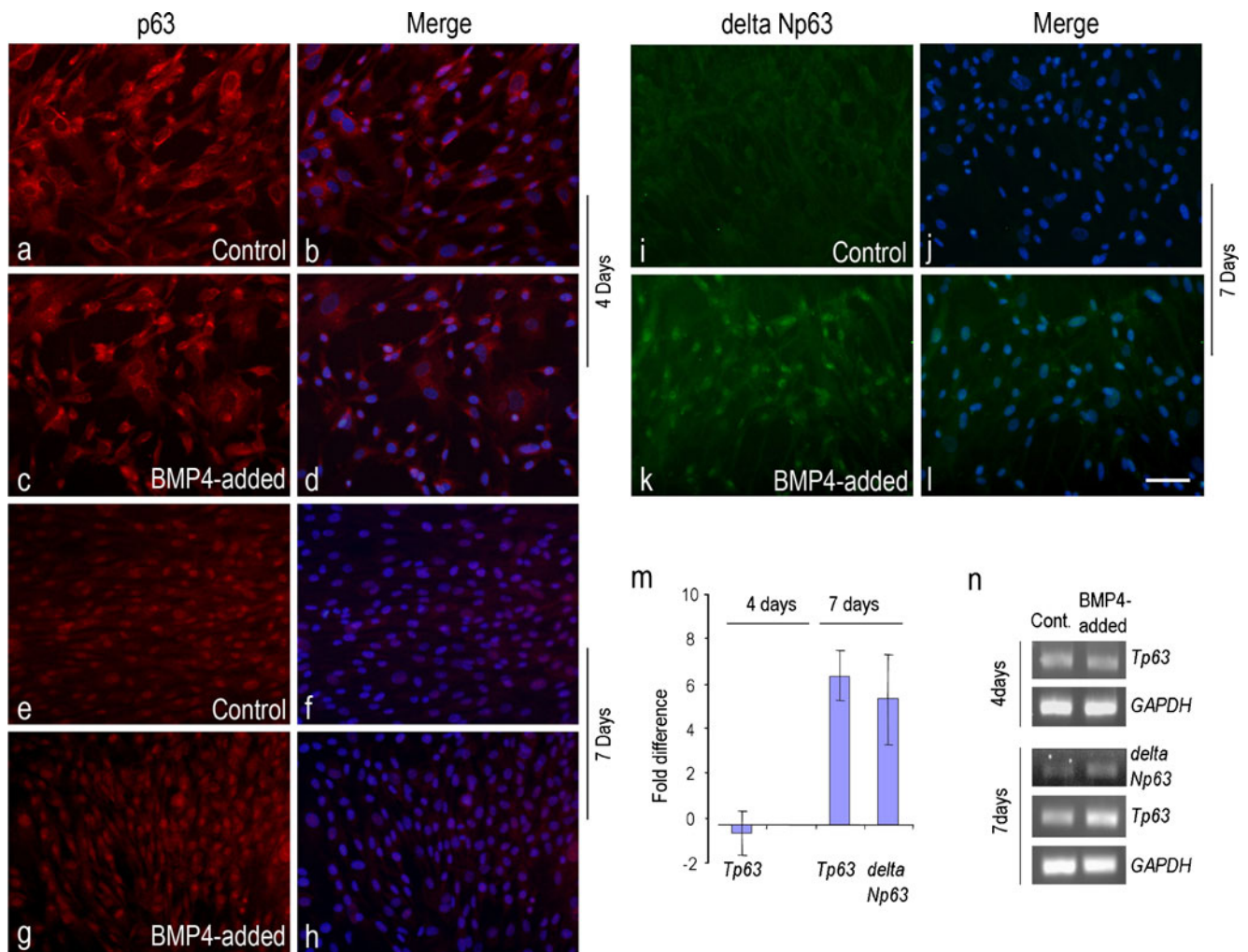


Fig. 6 Effects of BMP4 on p63 expression. Cells cultured for 4 days (a–d) or 7 days (e–g) with or without addition of BMP4 were subjected to immunostaining against p63 antibody. DeltaNp63 expression was also examined in control (i, j) and BMP4-added cultures (k, l). Blue DAPI visualizes cell nuclei in merged images (b, d, f, h, j, l). Immunostaining images are representative of at least three

independent experiments. qPCR was performed on total RNA from these samples using specific primers for *Tp63* and *deltaNp63* (m). The value for each gene was normalized to control CLEC-muc culture, and represents the mean $\pm$ SD of three independent experiments performed in duplicate. Representative qPCR products were analyzed by agarose electrophoresis (n). Bar, 100 μ m

these genes in 4-day culture except *HES1*, which was upregulated presumably by an alternative mechanism, independent of Notch signaling (Fig. 7). In contrast, all other genes were upregulated in the BMP4-added 7-day culture (Fig. 7), indicating that differentiation potential of p63 is restricted to its nuclear localization and that the effect is mediated through its common downstream genes as found in other epithelial differentiation pathways.

Involvement of Cytoplasmic p63 in BMP4-Induced CLEC-muc Proliferation

Our results show that BMP4 neither enhanced p63 nor its target genes in 4-day cultures. This encouraged us to investigate whether cytoplasmic p63 helps in cell proliferation. Accordingly, KI67-expression and BrdU incorpora-

tion assay were performed and the results were compared between control and BMP4-added 4-day samples. We found a higher number of KI67-positive cells in BMP4-added sample than in the control (Fig. 8a, b, g, h, m). BrdU incorporation was slightly decreased, but this was not statistically significant (Fig. 8n). We further checked the expression of SOX2, a universal stem cell marker that has been demonstrated to play role in stem cell self-renewal and cell proliferation [36, 37]. Immunofluorescent staining of cells cultured with BMP4 for 4 days showed an enhanced expression of SOX2 in both number and fluorescence intensity as compared to the control (Fig. 8c, d, i, j, o). Since *SOX2*, *OCT-4* and *NANOG* are mutually regulated, we further examined their expressions by qPCR in control and BMP4-added cells cultured for 4 days. Consistently, we observed an upregulation of all three genes at the RNA

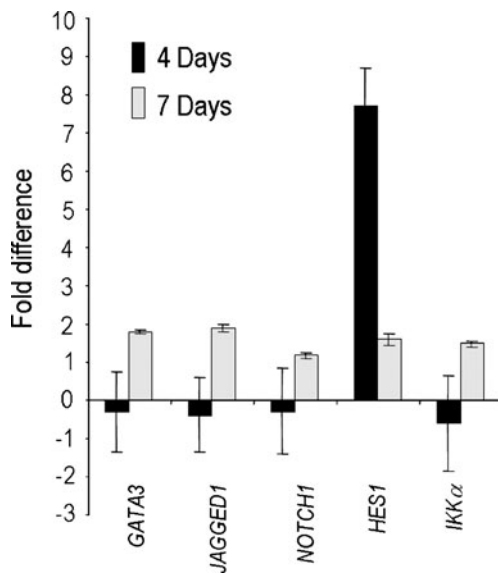


Fig. 7 BMP4 increases epithelial morphogenesis-associated genes through nuclear p63. Total RNAs were isolated from CLECs-muc cultured for 4 days (with or without BMP4) and 7 days (with or without BMP4). qPCR was performed using primers for *GATA3*, *JAGGED1*, *NOTCH1*, *HES1* and *IKKα*. The value for each gene was normalized to control CLEC-muc culture, and represents the mean $\pm$ SD of three independent experiments performed in duplicate. Data show the fold changes

level (8p). However, in 7-day cultures, SOX2 expression was decreased with differentiation (Fig. 8e, f, k, l, q) as would be expected. These results suggest that BMP4 cannot trigger differentiation as long as p63 resides in the cytoplasm. In other words, cytoplasmic p63 possibly favors CLEC-muc proliferation.

Discussion

In this study, we report the successful isolation and characterization of a novel cell-type, CLEC-muc from human umbilical cord. We show that CLEC-muc is unique with regard to p63 expression and this cell is stimulated by BMP4 towards further epithelial differentiation.

CLEC-muc is a Novel Type of Stem Cell Displaying Both Mesenchymal and Epithelial Characteristics

The mesenchymal stem cells obtained from umbilical cord have been previously studied by other groups, however, the morphology and gene expression profiles of their cells are different from ours [11, 38–40]. The culture condition of CLEC-muc is also different as these cells grow well in low serum supplemented (2.5% FBS) PTTe-1 medium containing components that preferentially support epithelial cell growth. Cell screening based on cell surface antigen by flow cytometry revealed that these cells express surface

antigens in patterns that do not mimic any classified cell type. For example, our CLECs-muc strongly express CD166, but are weakly positive to CD105 and CD73 (Fig. 2a), which makes these cells different from earlier reported MSCs derived from umbilical cord (11). The endothelial marker CD31 is totally absent. In contrast, we have detected abundant expression of MUCIN1 (Fig. 2b), which is expressed in epithelial cells. The other cell surface antigen CD151 has also been observed strongly, which may play a role in epithelial cell-to-cell adhesion via interaction with integrin- β 1 found in CLEC-muc (data not shown), as it has been shown previously that mutation of CD151 markedly reduces integrin- α 6 β 1 adhesion strengthening in cultured cells [41]. We have also observed profound expression of MHC class I antigen, indicating that these cells are likely to be immunologically compatible with the recipient. The cells are also positive to CK7, CK14 and CK19 in varying degrees. A moderate number of cells were found to express the epithelial marker E-CADHERIN, which was expected as CLEC-muc is not a fully differentiated epithelial cell, but rather, a unique type of stem cell population. The MSC marker VIMENTIN was moderately expressed in our cells. All these data suggest that CLEC-muc is a novel type of cell having mixed properties of both epithelial and mesenchymal stem cells.

We carried out a thorough screening to determine expression of ES cell markers, including OCT-4, NANOG, REX1, SOX2, TERT and SSEA-4. CLECs-muc exhibited positive expression of all these genes except *TERT* (Fig. 3). Although these cells are not identical to ES cells, expression of these markers suggest that these cells may share some similarities with ES cell. We found that expression of these stem cell markers declined when the cells were induced to differentiate upon BMP4 addition in cultures (Fig. 5s). Substantial expression of SOX2 and REX1 in CLEC-muc further defines this cell as a novel type that had not been previously characterized from umbilical cord. However, we did not observe TERT expression in our cells, which as expected, indicates that CLECs-muc differ from ES cell. Apart from ES cell markers, we have also found positive expression of the adult epithelial stem cell marker p63 (Fig. 4) and basal epithelial markers CK7, CK14, and CK19 in CLECs-muc (Fig. 2d–i), albeit CK19 expression was limited to a small percentage of cells which might represent a certain lineage-specific epithelial stem cell population. Nevertheless, the composite data demonstrate that CLEC-muc is a primitive type of stem cell having both embryonic and adult epithelial stem cell properties.

Atypical p63 Expression and Possible Function in CLECs-muc

p63 is a critical initiator of epithelial stratification and a key regulator of cell adhesion and survival in progenitor cells in

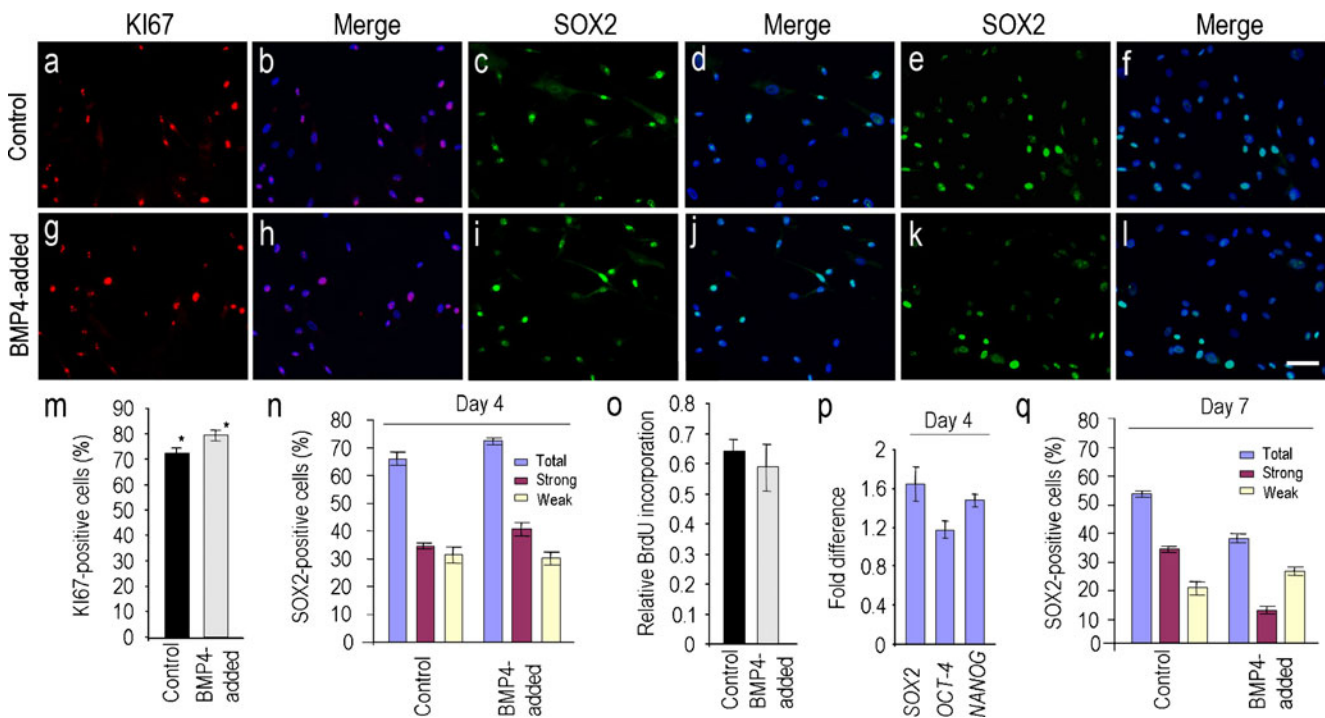


Fig. 8 Cytoplasmic p63 promotes cell proliferation. CLECs-muc cultured for 4 days with or without BMP4 were subjected to immunostaining against KI67 (a, b and g, h, respectively) and SOX2 (c, d and i, j, respectively) antibodies. 7-day culture was also examined for SOX2 expression (e, f, k, l). Number of KI67-positive (m) cells in 4-day culture was counted and levels of BrdU

incorporation were measured (n). SOX2-positive cells in 4-day (o) and 7-day (q) cultures were also counted. qPCR shows upregulation of *SOX2*, *OCT-4* and *NANOG* in BMP4-added cells cultured for 4 days (p). Blue DAPI depicts cell nuclei in merged images (b, d, f, h, j, l). Data represent the mean $\pm$ SD of at least three independent experiments. Asterisks indicate values, $p < 0.05$. Bar, 100 μ m

squamous epithelium. In vivo studies have shown that the BMP-signaling pathway plays a role in ectoderm specification and epithelial development in ES cell cultures [22, 42, 43]. A more recent study shows that in mouse ES cell, exogenous BMP4 induced deltaNp63 and these cells were later differentiated into CK14/CK5-positive keratinocytes upon serum treatment, suggesting that deltaNp63 is essential for epidermal commitment [22]. Our cells are already positive to CK14 expression in their basal state (Fig. 2f, g). Interestingly, in CLECs-muc, p63 expression was detected in the cytoplasm (Fig. 4a, b), which may be due to slow trafficking of this protein from the cytoplasm to the nucleus, as this was later identified in nucleus. A previous study has shown that cells exhibiting secretory changes or secretory carcinomas had cytoplasmic p63 expression [44]. It is possible that the secretory products in CLEC-muc may form transient protein-protein complexes with p63, which slow down the import of p63 protein into the nuclei. We found that cytoplasmic p63 is unresponsive to BMP4. However, later an increase in p63 expression in both protein and RNA levels was detected in 7-day cultures with BMP4 (Fig. 6e–h, m, n), when p63 was present in nucleus. Therefore, we postulate only nuclear p63 is regulated by BMP4 in CLEC-muc.

It has been observed, when cells were cultured for 4 days with BMP4, there was no significant change in the transcription level of epithelial morphogenesis associated *p63*-targets such as *GATA3*, *NOTCH1*, *JAGGED1* and *IKK α* , while only a small increase in the expression of *SOX2*, *OCT-4* and *NANOG* was observed (Fig. 8p). This was probably due to mislocalization of p63 protein, which remained mostly quiescent in the cell cytoplasm. We propose that p63, by retaining its expression in the cytoplasm, may provide some shielding effects against differentiation cues and the concurrent SOX2 expression facilitates cell proliferation. We found an increase of *HES1* expression, although the known upstream regulator *NOTCH1* remained unchanged. This may be due to the selective function of cytoplasmic p63 on *HES1*, which could be cell-specific to maintain their undifferentiated states.

In 7-day cultures, Tp63 upregulation was followed by an enhancement of *GATA3*, *NOTCH1*, *JAGGED1*, *IKK α* and *HES1* expression (Fig. 7). We assume this effect of BMP4 is propelled through nuclear p63 as the protein was detected in the nucleus by day 5 or 6. Hence, it can be conceived that only nuclear Tp63 possesses differentiation potential. Our observation is consistent with a previous report demonstrating that while TAp63 is responsible for initial

steps toward differentiation and stratification during development, the final differentiation requires the involvement of deltaNp63 [45]. In fact, a weak induction of deltaNp63 by BMP4 was detected in 7-day cultures (Fig. 6k, m, n). It is possible that prolonged culture may induce a higher amount of deltaNp63 which might be essential for efficient epithelial differentiation of CLEC-muc. Our findings also reveal that Notch signaling is active during CLEC-muc differentiation, consistent with previous reports demonstrating that Notch signaling plays a crucial role in epidermal stratification and keratinocyte differentiation [46, 47]. Since immunostaining is not a quantitative method, we attempted to perform western blot to measure p63 expression, but this was technically difficult to perform, presumably because of low expression of this protein in cultured cells. We have also observed that SOX2, OCT-4 and NANOG expressions declined but were not abolished in 7-day cultures (Figs. 5s, and 8k, q), suggesting that some cells still possess stem cell properties that may be lost upon complete terminal differentiation. Our study further shows that BMP4 changes cell-fate towards non-keratinized epithelial tissue formation as evidenced by positive expression of CK3/12 (Fig. 5f, g) and negative expression of keratinization markers CK1/10 and involucrin (data not shown).

In summary, to our knowledge this is the first report describing the detailed characterization of a novel cell type, CLEC-muc, from human umbilical cord that possesses both mesenchymal and epithelial cell properties. In addition, CLECs-muc express most, but not all, ES cell markers, suggesting that CLEC-muc is different from ES cell. Another striking feature of this cell is the aberrant expression pattern of p63, which is differentially controlled by BMP4. We have shown that BMP4 stimulates CLEC-muc to differentiate into further epithelial cell types by upregulating *GATA3*, *NOTCH1*, *JAGGED1*, *IKK α* and *HES1* and, interestingly, generates a tissue-specific cell-type positive to CK3/12 but negative to CK1/10. As umbilical cord is easily obtainable and there are no ethical concerns related to its use, CLEC-muc may be an important source of stem cells that may play a significant role in clinical applications and regenerative medicine.

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