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Differentiation of umbilical cord blood-derived multilineage progenitor cells into respiratory epithelial cells

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Background

Umbilical cord blood (UCB) has been examined for the presence of stem cells capable of differentiating into cell types of all three embryonic layers (i.e. endo-, ecto- and mesoderm). The few groups reporting success have typically confirmed endodermal potential using hepatic differentiation. We report differentiation of human UCB-derived multipotent stem cells, termed multilineage progenitor cells (MLPC), into respiratory epithelial cells (i.e. type II alveolar cells).

Methods

Using a cell separation medium (PrepaCyte-MLPC; BioE Inc.) and plastic adherence, MLPC were isolated from four of 16 UCB units (American Red Cross) and expanded. Cultures were grown to 80% confluence in mesenchymal stromal cell growth medium (MSCGM; Cambrex BioScience) prior to addition of small airway growth medium (SAGM; Cambrex BioScience), an airway maintenance medium. Following a 3–8-day culture, cells were characterized by light microscopy, transmission electron microscopy, immunofluorescence and reverse transcriptase (RT)-PCR.

Results

MLPC were successfully differentiated into type II alveolar cells (four of four mixed lines; two of two clonal lines). Differentiated cells were characterized by epithelioid morphology with lamellar bodies. Both immunofluorescence and RT-PCR confirmed the presence of surfactant protein C, a protein highly specific for type II cells.

Discussion

MLPC were isolated, expanded and then differentiated into respiratory epithelial cells using an off-the-shelf medium designed for maintenance of fully differentiated respiratory epithelial cells. To the best of our knowledge, this is the first time human non-embryonic multipotent stem cells have been differentiated into type II alveolar cells. Further studies to evaluate the possibilities for both research and therapeutic applications are necessary.

Keywords

endodermal differentiation, respiratory epithelium, stem cells, umbilical cord blood.

Introduction

Conflicting data in the literature initially led to debate over the presence of mesenchymal stromal cells (MSC) in umbilical cord blood (UCB) [1–3]. UCB has since become an accepted source of MSC, stem cells capable of differentiating into cells of connective tissue lineage [4]. More recently, UCB has been examined for the presence of cells capable of differentiating into cell types of all three embryonic layers (i.e. endo-, ecto- and mesoderm). The few groups reporting success with UCB have used hepatic differentiation for confirmation of endodermal potential [5,6].

Investigators working with stem cells derived from sources other than UCB have been successful with differentiation to an alternative cell type of endodermal origin, respiratory epithelium [7,8]. Ali *et al*. [7] first demonstrated derivation of type II alveolar cells from murine embryonic stem cells (ESC) using an off-the-shelf medium designed for maintenance of fully differentiated respiratory epithelial cells. Panoskaltsis-Mortari *et al*. [8]

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later used similar techniques to differentiate murine BM-derived multipotent adult progenitor cells (MAPC) into 'type II pneumocyte-like cells'. Following similar methods based upon their institutional experience with murine ESC [7], Samadikuchaksaraei *et al*. [9] recently were able to derive type II cells from human ESC.

Human multipotent stem cells from sources other than the embryo, however, have yet to be shown to have the *in vitro* capacity to yield respiratory epithelial cells. We report for the first time differentiation of human UCB-derived multipotent stem cells, termed multilineage progenitor cells (MLPC; BioE Inc., Saint Paul, MN, USA), into respiratory epithelial cells (i.e. type II alveolar cells). These efforts demonstrate further endodermal potential of human UCB-derived stem cells, and offer potential for research and clinical applications.

Methods

Cell culture

PrepaCyte-MLPC (BioE Inc.), an Ab-based cell separation medium, was added to a UCB unit (American Red Cross Cord Blood Program, Saint Paul, MN, USA). Following homo- and heterophilic aggregation of undesired cell populations and subsequent sedimentation to gravity, the supernatant containing stem cells was expressed. After overnight incubation (5% CO_2/37°C) in a T-flask in MSC growth medium (MSCGM; Cambrex BioScience, Walkersville, MD, USA), non-adherent cells were washed, leaving adherent cells to expand in culture. As MLPC colonies were observed, cells were further enriched by detachment (PBS/0.1% EGTA), generally at 60–70% confluence, and transfer to a new T-flask. Cloning was achieved by a standard limited-dilution technique.

For differentiation assays, cultures were grown to approximately 80% confluence prior to addition of special differentiation medium. In the case of respiratory epithelial cell differentiation, stem cells were placed into a T-flask with small airway growth medium (SAGM; Cambrex BioScience). SAGM consists of basal medium plus the following factors: bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B, retinoic acid and BSA-fatty acid free. SAGM was changed on days 3–4; cells were harvested (PBS/0.1% EGTA) on day 8 and analyzed by transmission electron microscopy (TEM) and reverse transcriptase (RT)-PCR. For immunofluorescence (IF) staining, MLPC were initially plated at 2×10^4/well in a non-coated four-well chamber slide (Lab-Tek II; Nalge Nunc International, Rochester, NY, USA) then cultured as described above. Clonal cell lines were differentiated as above; however, they were harvested on day 3 for analysis by TEM and RT-PCR.

Light and transmission electron microscopy

Cells were visualized by light microscopy (Eclipse TS100; Nikon Inc., Melville, NY, USA) throughout culture. Upon harvest, a cell pellet was made and prepared for analysis by TEM. Briefly, the pellet was rinsed in PBS and fixed in 2.5% glutaraldehyde in 0.1 m PBS buffer for 30 min. The sample was then post-fixed in 1% osmium tetroxide in 0.1 m PBS (30 min) and rinsed in PBS (three washes, 10 min each). The cells were enrobed and pelleted in 2% molten agarose, chilled at 4°C for 30 min, diced into 1-mm cubes for dehydration through graded ethanol, and embedded in EMbed812 epoxy resin (EMS, Hatfield, PA, USA). Ultra-thin sections of silver–gold interference color were stained in 3% aqueous uranyl acetate (20 min) then in Sato triple lead stain (3 min) prior to examination using an FEI CM12 Electron Microscope (FEI Co., Hillsboro, OR, USA).

RT-PCR

Total RNA was isolated from MLPC in culture by employing the method of Chomczynski [10]. Briefly, TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) was added directly to the cells in culture flasks, causing simultaneous cell lysis and RNA solubilization. RT was accomplished in a reaction containing 2.5 μg RNA, 1.0 μL random hexamers (5 μm final), 1 μL reverse transcriptase (Superscript™ Life Technologies, Rockville, MD), 8 μL dNTP (2.5 mm each), 4 μL MgCl (1.5 mm) and 2 μL 10 × buffer in a final volume of 20 μL, with incubation at 42°C for 45 min followed by 15 min at 70°C.

We employed a two-step nested RT-PCR strategy to amplify the surfactant protein C cDNA. Table 1 contains the primer sequences and expected RT-PCR product sizes for surfactant protein C (SPC) [11,12]. One microliter from the cDNA pool was used in the first PCR amplification with the following conditions: 5 min hot start at 95°C, 30 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 30 s and a final extension for 5 min at 72°C. Two microliters of each primer diluted to 20 pmol/μL were used in each reaction. One microliter of the product from the first reaction was used as a template for the nested reaction, with the same

Table 1. Primer sequences and expected RT-PCR product sizes for SPC

		Primer sequence	Product size (bp)
SPC	First primer	F 5′AAAGAGGTCCTGATGGAGAGC3′ R 5′TAGATGTAGTAGAGCGGCACCT3′	456
	Nested	F 5′AACGCCTTCTTATCGTGGTG3′ R 5′GTGAGAGCCTCAAGACTGG3′	313

cycling conditions and primer concentrations outlined above. RT-PCR amplification of a housekeeping gene, beta-globin, was used to monitor the quality of the mRNA and control for the efficiency of the RT step. PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Product sizes were compared to a 100-bp ladder (Invitrogen, Carlsbad, CA, USA).

IF staining

The immunostaining procedure was similar to Ali *et al*. [7] with minor modifications. Cells were washed with HBSS + 1% BSA twice, treated with 4% paraformaldehyde and incubated at room temperature for 20 min. They were then washed twice with HBSS + 1% BSA. The primary pro-SPC Ab (Chemicon, Temecula, CA, USA) was added to the cells at a 1:125 dilution and incubated overnight at 4°C. After the primary Ab incubation, slides were washed twice with HBSS + 1% BSA. The secondary Alexa Fluor 594 goat anti-rabbit Ab (Invitrogen) was then added at a 1:50 dilution and incubated with the cells for 20 min. Final HBSS + 1% BSA washes were performed, and cover slips were placed onto the glass slides. Cells were viewed under a fluorescence microscope (Eclipse E200; Nikon Inc., Melville, NY, USA).

Results

MLPC were isolated from four of 16 (25%) UCB units and then expanded. Expanded cells ($CD9^+$ $CD13^+$ $CD29^+$ $CD44^+$ $CD73^+$ $CD90^+$ $CD105^+$ $CD34^-$ $CD45^-$ $CD106^-$) were successfully differentiated into cell types representative of each embryonic layer (e.g. osteocytes, hepatocytes, neuronal cells; data not shown).

Light and transmission electron microscopy

By day 8 of culture (day 3 for clonal lines), cells in SAGM possessed a more epithelioid morphology; controls held in MSCGM maintained a fibroblast-like morphology (Figure 1). Ultrastructure consistent with the type II alveolar cell was confirmed with a moderate number of cells from all mixed stem cell lines ($n = 2$) and all clonal stem cell lines ($n = 2$) tested by TEM. Differentiated cells showed lamellar bodies, multivesicular bodies and apparent lipid-laden vacuoles. Cells appeared metabolically active, with abundant mitochondria and distended rough endoplasmic reticulum (RER). Multiple small vesicles near the cell surface (appearing as endocytic vesicles originating from what resembled clathrin-coated pits) and throughout the cytoplasm were observed, suggestive of cellular product transport/trafficking (Figure 2). The ultrastructural findings of the control cells were substantially different from that of the test cells. RER was present, although not nearly as distended, and endocytic-type vesicles as well as multivesicular bodies were much less common. Rare organelles consistent with lamellar bodies were noted, however.

RT-PCR

SPC mRNA was evident in RNA samples from mixed ($n = 4$) and clonal ($n = 2$) stem cells differentiated in SAGM. However, SPC mRNA was not identified in RNA samples from MLPC controls (Figure 3).

IF staining

The presence of pro-SPC protein was confirmed by IF staining of both mixed ($n = 3$) and clonal ($n = 2$) stem cell lines. A substantial number of cells of each culture induced in SAGM were successfully differentiated. MLPC controls (maintained in MSCGM) were clearly negative (Figure 4). Table 2 summarizes all the results.

Discussion

Investigators have previously reported success in deriving distal airway epithelium from murine ESC [7], murine marrow stem cells [8] and, more recently, human ESC [9]. Using culture methods similar to these investigators [7–9], we were able to demonstrate successful differentiation of

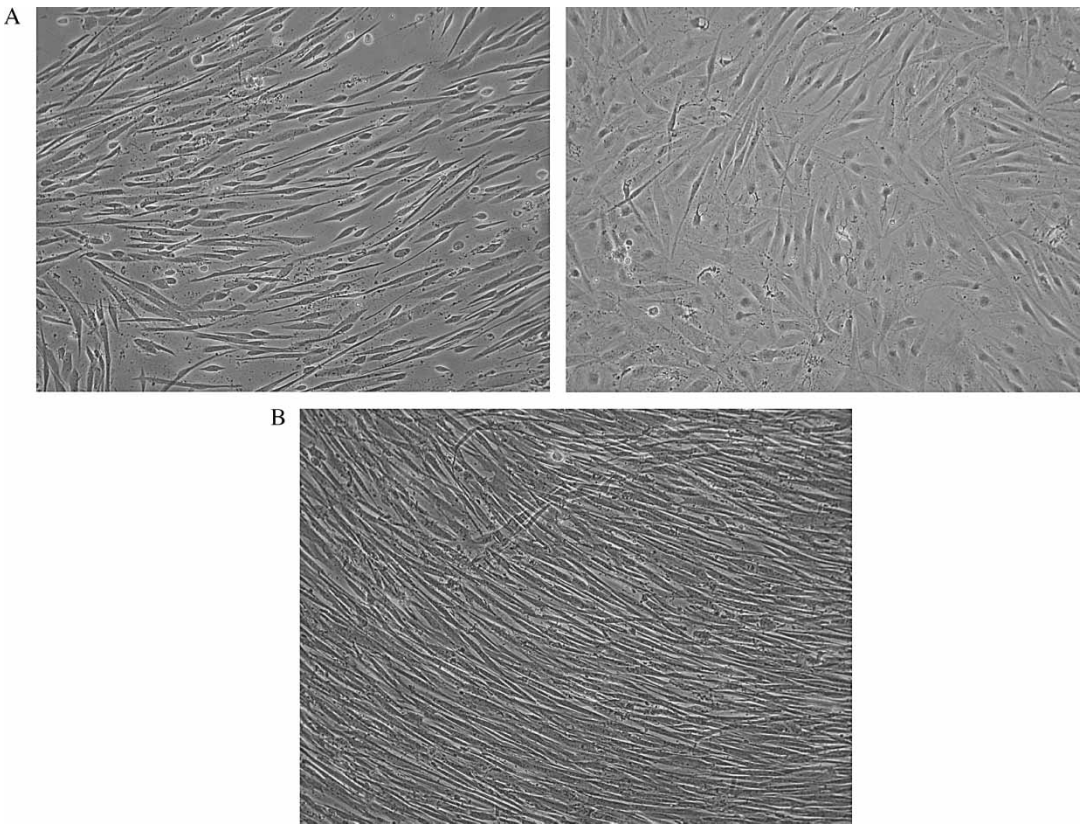

Figure 1. *Morphology by light microscopy. (A) Stem cells induced in SAGM show a spectrum of epithelioid morphology by day 8 (mixed stem cell lines) or day 3 (clonal stem cell lines). (B) Control stem cells (i.e. cells held in MSCGM) exhibit a more fibroblast-like morphology.*

UCB-derived stem cells (MLPC) to type II alveolar cells. To the best of our knowledge this is the first time that human non-embryonic multipotent stem cells have been differentiated into type II alveolar cells.

Type II alveolar cells are the cells responsible for surfactant production/secretion and are essential for the terminal airway repair process following injury [13]. In response to injury, type II cells are able to proliferate and give rise to type I alveolar cells, which serve as the epithelial component of gas exchange in the lung. Type II cells appear relatively late in fetal development, in the late second trimester/early third trimester. Alveolarization, the transformation of immature, saccular lung to mature lung with developed alveoli able to facilitate gas exchange, begins at approximately 36 weeks of gestation and continues through the first few postnatal years. Interestingly, it is estimated that only 15% of alveoli are formed at birth [14]. Considering this relatively late emergence of type II cells in development, it would seem logical to consider UCB an optimal source of stem/progenitor cells of the distal airway; this, however, has not been proven.

MLPC differentiation to respiratory epithelium was, in part, based upon light and transmission electron microscopy findings. An epithelioid morphology by light microscopy coupled with ultrastructural findings consistent with type II alveolar cells (e.g. lamellar bodies, multivesicular bodies) lends strong support to successful differentiation. Lamellar bodies have been identified in a variety of cell types under both normal and pathologic conditions. Lamellar bodies are secretory lysosomes, a subclass of lysosome-related organelles; rather than serving a function of degradation, like typical lysosomes, they are specialized for storage and secretion [15]. Lamellar bodies of type II alveolar cells, which are considered the most extensively studied, serve as the storage form of lung surfactant [16]. Surfactant protein production and packaging occurs through the RER, Golgi apparatus and multivesicular bodies. The final step in the delivery of newly synthesized surfactant proteins to the lamellar body occurs by fusion of the multivesicular body with the lamellar body. Recycling of surfactant proteins from the alveolar surface occurs via the endocytic pathway and

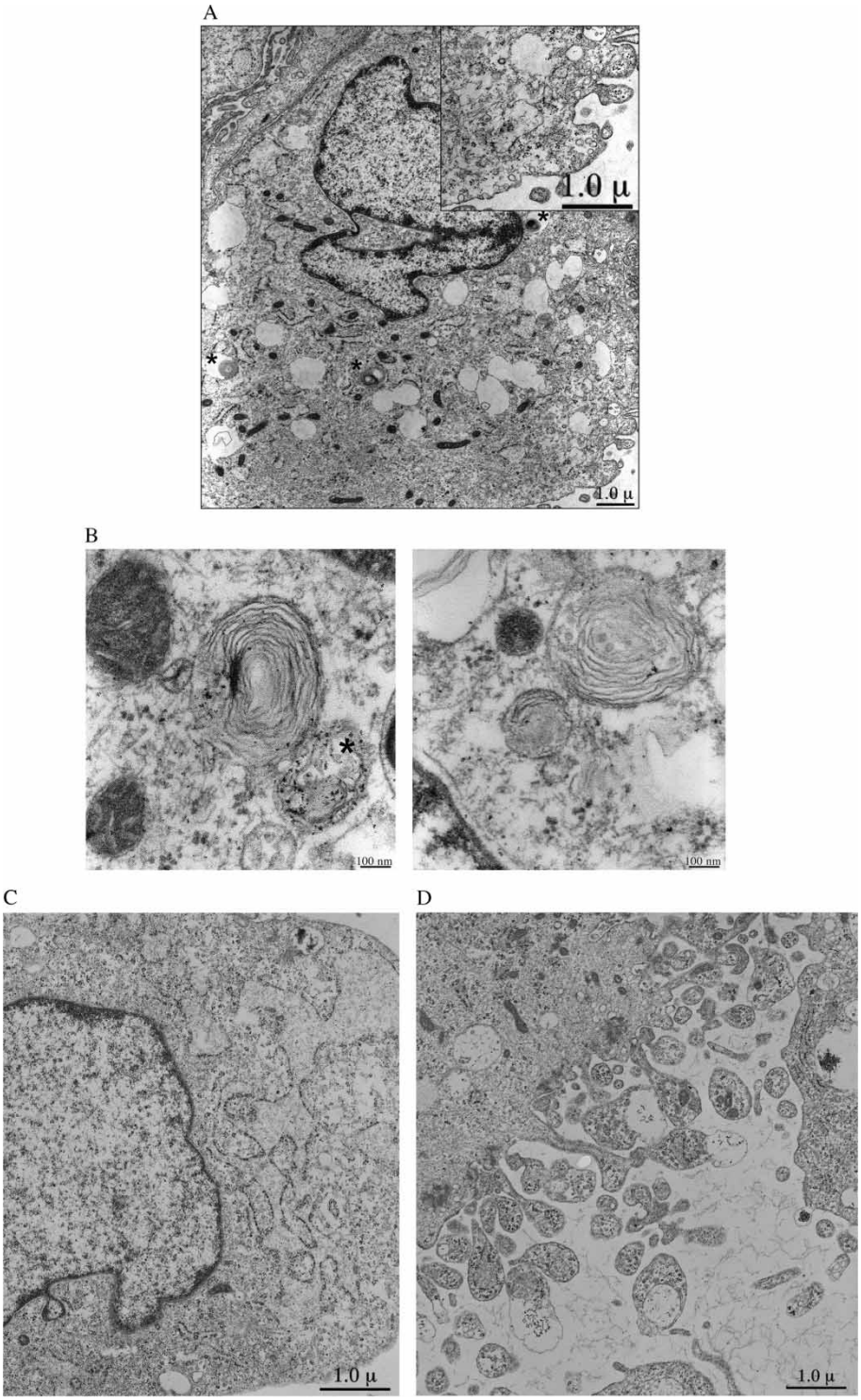

Figure 2. *Transmission electron microscopy of UCB-derived stem cells (MLPC) differentiated to type II alveolar cells. (A) Low power (6300 ×) micrograph of a differentiated cell. Three lamellar bodies (indicated by asterisks), numerous vacuoles, some multivesicular bodies, endocytic-type vesicles (enlarged inset) and abundant RER and mitochondria are noted. (B) Lamellar bodies (75 000 ×), one with multivesicular body fusing (indicated by asterisk). (C) Distended RER (10 000 ×). (D) Microvilli-like structures (10 000 ×).*

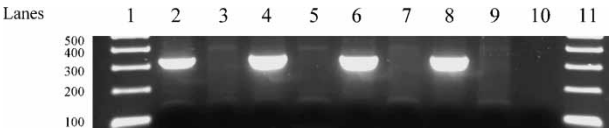

***Figure 3**. RT-PCR. Lane 1, 100-bp ladder; lane 2, clonal stem cell line (CC) 1; lane 3, control (Ct)l CC1; lane 4, CC2; lane 5, Ctl CC2; lane 6, mixed stem cell line (MC) 1; lane 7, Ctl MC1; lane 8, MC2; lane 9, Ctl MC2; lane 10, water control; lane 11, 100-bp ladder. To test for the presence of SPC mRNA, two sequential RT-PCR reactions were performed. The primers for the first reaction exhibited complementarity to exonic sequences within the SPC gene. The presence of a substantial intervening intronic sequence between the forward and reverse primers prevented non-specific amplification of contaminating genomic DNA. The primers for the second reaction were nested within the boundaries of the first RT-PCR product.*

involves the multivesicular body as well [15]. Both the biosynthetic and recycling pathways of surfactant have been studied extensively by various methods, including electron microscopy [17,18].

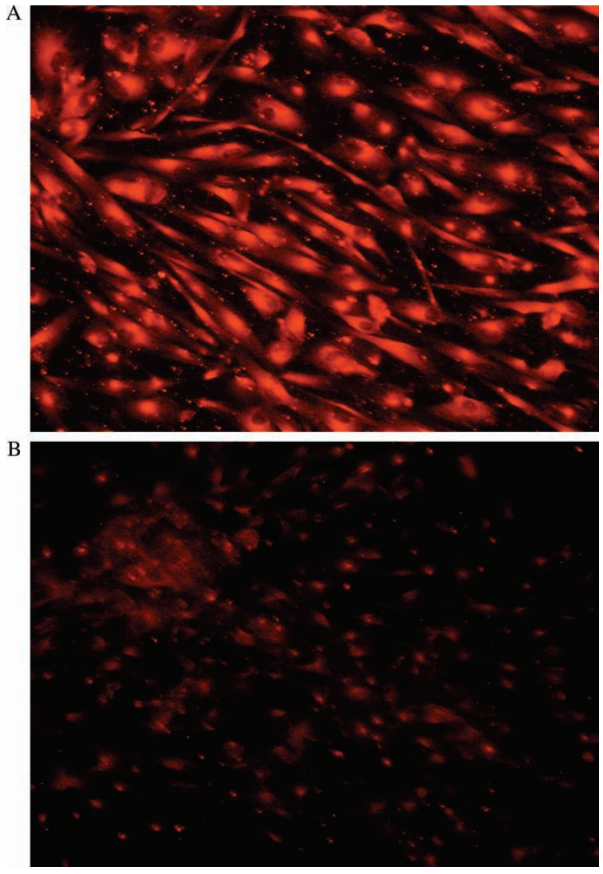

***Figure 4**. Immunofluorescence. (A) Representative micrograph of MLPC cultured in SAGM. (B) Corresponding negative control.*

Table 2. Summary of results

Cell ID	LM	TEM	IF	RT–PCR
MC1	+	NA	+	+
Ctl MC1	–	NA	–	–
MC2	+	+	+	+
Ctl MC2	–	*	–	–
MC3	+	NA	†	+
Ctl MC3	–	NA	–	–
MC4	+	+	+	+
Ctl MC4	–	NA	–	–
CC1	+	+	+	+
Ctl CC1	–	NA	–	–
CC2	+	+	+	+
Ctl CC2	–	NA	–	–

*MC, mixed stem cell line; CC, clonal stem cell line; Ctl, control; LM, light microscopy changes; TEM, transmission electron microscopy findings; IF, IF (pro-SPC+); RT-PCR, reverse transcriptase-polymerase chain reaction (SPC mRNA+); NA, not applicable (i.e. not performed); *rare lamellar-like bodies present; †inconclusive.*

Several of our additional ultrastructural findings (i.e. plentiful mitochondria, distended RER) provide further support for a functional, metabolically active type II alveolar cell. The copious vesicles, most notably near the plasma membrane, are very suggestive of endocytic vesicles involved in the recycling and trafficking of surfactant. TEM with immunogold labeling could verify this supposition and we are planning these studies. Additionally, although the orientation of the differentiated cells was lost in processing (as was the case for Samadikuchaksaraei *et al*. [9]), microvilli-like structures were noted at the cell surface.

Further, more specific evidence of differentiation was based upon amplification of SPC mRNA by RT-PCR and the presence of pro-SPC protein shown by IF. SPC is an integral membrane protein that is only expressed in type II alveolar cells [19]. SPC is transferred from the luminal vesicles of the multivesicular body to the lamellar body at the time of multivesicular body/lamellar body fusion. Surfactant protein B (SPB) then mediates incorporation of the vesicles into the internal membranes of the lamellar body. Processing of the pro-protein to the mature protein occurs in the multivesicular body/lamellar body. While SPB is heavily involved in the intracellular packaging of surfactant, the role of SPC is limited to the formation and maintenance of the surfactant film [15]. As the presence of SPC is absolutely specific for type II alveolar cells, it

serves as a logical means to confirm the identity of type II cells. We, like others using murine and human ESC [7,9], were able to verify the presence of both SPC mRNA and protein in cells cultured in SAGM. Panoskaltsis-Mortari *et al*. [8] reported positive IF staining for pro-SPC with their murine marrow-derived stem cells. Although they did not carry out RT-PCR studies for SPC mRNA, they did indicate detection of FGFR2-IIIb mRNA, consistent with type II alveolar cells [8].

Samadikuchaksaraei *et al*. [9] found that only a very small percentage (2%) of human ESC in culture with SAGM actually appeared to be differentiated to type II cells, as determined by IF. Ali *et al*. [7] reported immunoreactive cells in murine cultures grown in both ESC medium and SAGM; they found that the quality of IF staining and clumping of cells disallowed quantitative analysis. A comment appearing in a later publication by the same group described this differentiation protocol [7,9] as yielding 'a barely detectable level of type II-like cells in the differentiated ESC population' [20]. Unidirectional differentiation, and, hence purity, of differentiated ESC in culture has been challenging. However, our experience indicates that essentially all UCB-derived stem cells in a given culture can be induced successfully to type II alveolar cells.

Uniform differentiation of cells may have advantages with certain applications. For instance, as a research tool (e.g. terminal airway model) consistency of cell type may be critical to experimentation. While control of differentiation will certainly be important in the clinical setting as well, the ideal components of targeted stem cell therapies, in general, remain to be determined. The optimal therapy undoubtedly will depend upon the clinical use and desired outcome. Multipotent stem cells, respiratory progenitor cells and terminally differentiated airway cells may all have a role to play. Therefore approaches to promote minimal or early differentiation may prove useful for clinical applications. Rippon *et al*. have recently described a method to promote differentiation of murine ESC to distal lung epithelial progenitor cells [20]. Our group will be investigating ways to culture UCB-derived respiratory progenitor cells as well.

In conclusion, we have demonstrated that respiratory epithelial cells (i.e. type II alveolar cells) can be differentiated from UCB-derived stem cells (MLPC). These cells and the differentiation platform may have utility as a research tool (e.g. in studying lung pathophysiology, development). Ultimately, it is our hope that MLPC, and possibly more committed cell populations derived from MLPC, will provide therapeutic benefits to patients suffering from lung disease.

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