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Epithelial to mesenchymal transition (EMT) induced by bleomycin or TFG_{b1}/EGF in murine induced pluripotent stem cell-derived alveolar Type II-like cells

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ABSTRACT

Induced pluripotent stem (iPS) cells are derived from reprogrammed somatic cells and are similar to embryonic stem (ES) cells in morphology, gene/protein expression, and pluripotency. In this study, we explored the potential of iPS cells to differentiate into alveolar Type II (ATII)-like epithelial cells. Analysis using quantitative real time polymerase chain reaction and immunofluorescence staining showed that pulmonary surfactant proteins commonly expressed by ATII cells such as surfactant protein A (SPA), surfactant protein B (SPB), and surfactant protein C (SPC) were upregulated in the differentiated cells. Microphilopodia characteristics and lamellar bodies were observed by transmission electron microscopy and lipid deposits were verified by Nile Red and Periodic Acid Schiff staining. C3 complement protein, a specific feature of ATII cells, was present at high levels in culture supernatants demonstrating functionality of these cells in culture. These data show that the differentiated cells generated from iPS cells using a culture method developed previously (Rippon et al., 2006) are ATII-like cells.

To further characterize these ATII-like cells, we tested whether they could undergo epithelial to mesenchymal transition (EMT) by exposure to drugs that induce lung fibrosis in mice, such as bleomycin, and the combination of transforming growth factor beta1 (TGF_{b1}) and epidermal growth factor (EGF). When the ATII-like cells were exposed to either bleomycin or a TGF_{b1}–EGF cocktail, they underwent phenotypic changes including acquisition of a mesenchymal/fibroblastic morphology, upregulation of mesenchymal markers (Col1, Vim, a-Sma, and S100A4), and downregulation of surfactant proteins and E-cadherin.

We have shown that ATII-like cells can be derived from skin fibroblasts and that they respond to fibrotic stimuli. These cells provide a valuable tool for screening of agents that can potentially ameliorate or prevent diseases involving lung fibrosis.

1. Introduction

Epithelial to mesenchymal transition (EMT) is one of the processes in the body responsible for organogenesis and cellular plasticity giving rise to mesenchymal phenotypes such as fibroblasts and myofibroblasts from other types of mature cells. This transformation is characterized by loss of cell to cell contact marked by a decrease in cell adhesion protein E-cadherin (Kalluri and Neilson, 2003; Rippon et al., 2006) and a switch to N-cadherin (Brabletz et al., 1998), a change of morphology from a flattened epithelial cell type into a spindle-shaped, fibroblast-like appearance, and subsequent acquisition of cytoskeletal markers (S100A4, a-Sma, Vim, b-Catenin), extracellular matrix proteins (Col1, Col3, Fn1, Lama5), and transcription factors (Snai1, Snai2, Zeb1, Twist1, Lef-1, Ets-1, Gsc) (Kalluri and Neilson, 2003; Zeisberg and Neilson, 2009).

Several factors have been shown to induce EMT. Oncogenic pathways such as Ras, Wnt/b-Catenin, and Notch can induce EMT associated with repression of E-cadherin (Boyer et al., 1997). Environmental factors such as hypoxia and oxidative stress also contribute to the EMT phenotype (Manotham et al., 2004). Activation of the canonical Smad signaling pathway via tandem

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effects of transforming growth factor beta1 (TGF_{b1}) and epidermal growth factor (EGF) have been shown to be major mediators of EMT (Piek et al., 1999). Bleomycin, which acts by preventing incorporation of thymidine into the DNA, promotes EMT by inducing DNA strand breaks (Yamamoto et al., 2000). Serious complications of EMT in tissues involve impaired normal organ functions, and may eventually result in fibrotic lung diseases.

Embryonic stem cells (ESCs) are ideal for studying cell differentiation because of their unlimited self renewal property and pluripotency (Andrews, 2002; Bodnar et al., 2004; Ohtsuka and Dalton, 2008). Various laboratories have shown that alveolar epithelial cells can be derived from ESC *in vitro* (Coraux et al., 2005; Rippon et al., 2008; Wang et al., 2007a) but the use of ESC has major drawbacks such as immune rejection and ethical issues due to the utilization of embryonic tissues. In 2006, a method for retrodifferentiation of somatic cells to embryonic-like cells emerged by introduction of defined transcription factors (Takahashi and Yamanaka, 2006). These cells have indistinguishable properties to ESC such as morphology, gene expression profile, and pluripotency. Success in utilizing iPS cells in studying disease states has been presented by various laboratories including our own (Alipio et al., 2010; Hanna et al., 2007; Xu et al., 2009).

Here we demonstrate the ability to induce EMT in an *in vitro* cell culture system of ATII-like cells derived from iPS cells. These cells are useful tools in searching for new biomarkers of EMT or novel drugs that can prevent EMT.

2. Materials and methods

2.1. Generation of induced pluripotent stem (iPS) cells from murine tail-tip fibroblasts

Normal fibroblasts from the tail-tip of green fluorescent protein (GFP) transgenic mice [(C57BL/6-Tg(UBC-GFP) 30Scha/J Stock#004356, Jackson Laboratory, Bar Harbor, ME)] were transduced with four retroviruses encoding transcription factors Oct4, Sox2, Klf4, and C-Myc (purchased from Stemgent, San Diego, CA) and iPS cells were produced and characterized as previously described (Alipio et al., 2010; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Fourteen to twenty days post transduction, 10 iPS subclone colonies were picked and expanded. Two representative clones with similar morphology and intensity of GFP expression were used for alveolar Type II (ATII) epithelial cell differentiation. These clones exhibited all the characteristics of typical iPS cells: expression of transducing transcription factor mRNA as well as additional embryonic stem cell markers and the ability to form teratomas when injected subcutaneously into the flanks of nude mice, properties that we and others have reported previously (Alipio et al., 2010; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007).

2.2. In vitro differentiation of iPS cells into alveolar Type II (ATII-like) epithelial cells

Alveolar Type II (ATII-like) cells were derived from iPS cells by a three-stage differentiation process, following previously described methods for murine ES cells with slight modifications (Rippon et al., 2006). The modifications include the following: (1) enhanced attachment of Embryoid Bodies (EBs) at Stage 2 by culture of EBs in media containing FBS instead of Knock Out Replacement Serum (KOSR), (2) improved viability of ATII-like epithelial cells by passaging the differentiating cells at Stage 2 Day 5 of alveolar cell differentiation instead of continuous culture for 11 days during the Stage 2 process, and (3) improved viability of ATII-like epithelial cells by supplementing the small airway growth medium with 10% FBS in Stage 3.

2.3. Epithelial to mesenchymal (EMT) induction of ATII cells

Stage 3, Day 7 ATII-like cells grown to 80% confluency were treated with bleomycin (APP Pharmaceuticals LLC, Schaumburg, IL) at 1, 2, 4, 8, and 16 μg/ml final concentration. Concurrently, the combined effects of TGF_{b1} and EGF on the Stage 3, Day 7 ATII-like cells were also assessed. We tested three doses of TGF_{b1} and EGF (both from Millipore Billerica, MA): 5 ng/ml TGF_{b1} + 50 ng/ml EGF, 10 ng/ml TGF_{b1} + 100 ng/ml EGF, and 20 ng/ml TGF_{b1} + 200 ng/ml EGF. The cells were exposed to either of the fibrosis-inducing treatments for 2, 4, and 7 days. Culture conditions were repeated at least three times in independent experiments.

2.4. Nile red

To assess lipid deposits, Stage 3 Day 7 ATII-like cells grown to 80% confluency were stained with Nile Red (Sigma, St. Louis, MO) according to the manufacturer's instruction.

2.5. PAS staining

To determine the presence of glycolipids, Stage 3 Day 7 ATII-like cells grown to 80% confluency were stained with Periodic Acid Schiff staining (EMD Chemicals Inc, Gibbstown, NJ) according to manufacturer's instruction.

2.6. Polymerase chain reaction (PCR)

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) and 2 μg of total RNA was reverse transcribed using cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR reactions were ran using Platinum PCR Supermix High Fidelity (Invitrogen) and quantitative real time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Data was acquired using 7500 FAST Real Time PCR system (Applied Biosystems, Foster City, CA) and analyzed using Comparative CT method via SDS FAST System software version 1.4.0. Primer sequences for endogenous/total retroviral transcripts (Boyer et al., 1997; Kalluri and Neilson, 2003), the lung markers (Rippon et al., 2006), and other gene specific oligonucleotides (obtained from Roche's UniversalProbe library) are reported in the Supplementary Table S1.

2.7. Immunofluorescence staining

Immunofluorescence staining was performed on actively dividing cells cultured in 12 well plates as previously described (Alipio et al., 2010; Hanna et al., 2007; Xu et al., 2009). Primary antibodies diluted at 1:100 in 1% BSA were used for immunofluorescence staining: Oct4 and SSEA-1 (Chemicon, Billerica, MA), Sox2, Nanog, SPA, SPB, SPC, ABCA3, CC10, Cytochrome P450 A1A (all from Santa Cruz Biotechnology, Santa Cruz, CA), and Aquaporin 5 (Abcam, Cambridge, MA). The secondary antibodies used were goat anti-mouse PE (Sigma, St Louis, MO), goat anti-rabbit-PE and rabbit anti-goat-PE (both from Southern Biotech, Birmingham, Alabama). Nuclei were counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole (Sigma, St Louis, MO) for 10 min. Images were captured by QImaging camera (QImaging, Surrey, BC) and analyzed by QimagingPro software (QImaging, Surrey, BC).

2.8. Transmission electron microscopy (TEM)

One 100 mm semi-confluent tissue culture dish containing Stage 3 Day 7 ATII-like epithelial cells was trypsinized, the cells were washed with serum free medium containing 0.5% bovine serum albumin and centrifuged for 5 min at 300*g*. The cell pellet