Embryonic stem cells form glandular structures and express surfactant protein C following culture with dissociated fetal respiratory tissue

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The directed differentiation of embryonic stem cells into desired lineages can be achieved by a number of generic protocols. For example, HESCs are reported to differentiate into cardiomyocyte-like cells after being cocultured with spontaneously differentiating visceral endoderm-like derivatives of the P19 embryonal carcinoma cell line (22). Similarly, γ-irradiated S17 murine bone marrow stromal and C166 yolk sac endothelial cell lines are reported to induce HESC differentiation into hematopoietic colonies (12). An ability to similarly generate protocols relevant to the directed differentiation of HESCs into respiratory lineages would have great implications for developing treatments to a wide range of respiratory-specific diseases.

The importance of epithelial/mesenchymal interactions for normal respiratory epithelial morphogenesis and differentiation during branching morphogenesis and organogenesis is well documented (19, 31). In the absence of such interactions, epithelia fail to form complex architectural structures and fail to undergo cellular maturation characteristics of differentiated adult tissue. Recombination experiments ectopically transplanting mesenchymal cell populations from various sites within the fetal lung anlagen have demonstrated that the mesenchyme effects site-specific regulation of epithelial morphogenesis (1, 9, 31). For example, mesenchyme associated with bronchioles ectopically transplanted to the presumptive tracheal epithelium stimulates bud formation and respiratory cytodifferentiation. Similarly, mesenchyme associated with presumptive trachea ectopically transplanted to bronchiolar epithelium inhibits budding and induces cytodifferentiation characteristic of the developing trachea. Heterologous epithelial/mesenchymal recombinations have further demonstrated that mouse lung mesenchyme induces a mouse-like branching pattern when grafted to either mouse salivary gland epithelium or the normally nonbranching chick air sac epithelium (4). This body of evidence demonstrates that the mesenchyme 1) supports epithelial viability, 2) determines geometrical patterning of the associated epithelium, and 3) possesses an instructive effect on functional cytodifferentiation of heterotypicallyrafted epithelia.

After dissociation of the fetal lung explant into single cells, organotypic morphogenetic interactions reconstitute the branching configuration of the fetal lung (8). This report describes the directed differentiation of β-galactosidase Zin40 and enhanced green fluorescent protein (EGFP) constitutively expressing HESCs into surfactant protein C (Sftpc)-positive, diploid respiratory-like derivatives by their incorporation into respiratory differentiation; coculture; morphogenetic cues

MOUSE EMBRYONIC STEM CELLS (MESCs) are derived in vitro from the inner cell mass or epiblast of developing blastocysts (18). MESCs can be maintained indefinitely in an undifferentiated pluripotent state in vitro, with their capacity to contribute to functional derivatives of all cells of the body being demonstrated by mouse tetraploid embryo complementation studies (24, 25). MESC represent the most extensively characterized embryonic stem cell lines, and in addition to their pluripotent properties described in vivo, are reported to differentiate at a high frequency into desired cell lineages in vitro (6). With the demonstrated capability to isolate and maintain pluripotent human embryonic stem cells (HESCs) in vitro, a rapid increase in methods to direct HESCs into specific lineages relevant to the application of drug discovery and human cell and gene therapies has occurred.

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fetal lung tissue undergoing organotypic regeneration. MESC s are induced to organize into tubular structures, with ~24% of their cellular derivatives displaying immunoreactivity to the respiratory-specific marker Sftp c and 40% displaying immunoreactivity to an epithelia-specific pan-keratin antibody. Combined immunofluorescence and whole mount in situ hybridization demonstrate that MESC derivatives in this system express Sftp c transcripts. Fluorescence in situ hybridization (FISH) demonstrates that MESC derivatives do not require tetraploidization (fusion) for expression or maintenance of Sftp c expression. Ontogenic studies further demonstrate that ubiquitous Sftp c immunolocalization throughout the cytoplasm of the MESC derivatives observed at 8 days of coculture changes to a predominant apical immunolocalization within the cell membrane by 12 days, thus suggesting a maturation program consistent with differentiation into functional respiratory derivatives.

EXPERIMENTAL PROCEDURES

Mice and cell culture. All animal procedures were approved by the Monash University Animal Ethics Committee. Embryonic day 11.5 E11.5 fetal lung buds were collected from F1(C57B/6J WEHI female × CBA/CaH WEHI male) × F1 natural matings, dissociated in 0.05% trypsin/1 mM EDTA in PBS and cultured as previously described (20). Zin40 MESC s and mouse EGFP MESC s were propagated according to previously described methods (23). MESC s and dissociated lung cells were combined in 2 μl of lung bud culture medium and placed on a Millipore filter (GIBCO BRL).

Immunohistochemical staining. Serial sections of MESC/lung aggregates were incubated with either rabbit anti-Sftp c (Research Diagnostic, 1:300) or mouse anti-pan-keratin (Biogenesis, 1:100) primary antibodies according to standard procedures. Binding of primary antibodies was detected using either rabbit anti-mouse IgG1-conjugated (Zymed Laboratories, 1:300) or goat anti-rabbit IgG-conjugated (Molecular Probes, 2 μg/ml) secondary antibodies and horseradish peroxidase-conjugated streptavidin. Sections were viewed using a Leica DMR immunofluorescent microscope Leica MPS60 image capture system.

Combined whole mount in situ hybridization and immunofluorescence and FISH. Whole mount in situ hybridization and RNA riboprobe synthesis were performed using a 190-bp Stu I-Bam HI DNA fragment that had been subcloned from a previously described 683-bp mouse Sftp c cDNA (20). After whole mount in situ hybridization, aggregates were frozen in optimum cutting temperature compound sectioned at 8 μm for EGFP detection using a rabbit anti-EGFP antibody (Molecular Probes, 1:500) and Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes, 2 μg/ml). Slides were incubated with a biotinylated chromosome 11 mouse-specific paint probe according to the manufacturer’s instructions (Cambio). Sections were counterstained with propidium iodide and viewed under a Zeiss Axioplans 2 fluorescent imaging microscope using the triple green/orange/4′,6-diamidino-2-phenylindole dihydrochloride bypass filter.
Statistical analysis. One-way ANOVA followed by Tukey’s post-tests were used to determine significant differences between the percentages of Zin40 MESCs at various time points for each antibody used.

RESULTS AND DISCUSSION

Respiratory-specific transcripts have been amplified from MESCs after culture as embryoid bodies in the presence of growth factors such as hepatocyte growth factor (2). Furthermore, culture of MESCs on collagen type I induces their differentiation into Clara cells capable of generating a tracheobronchial epithelium, and Sftpc-positive cells have been identified following fetal mesenchymal coculture experiments (7, 33). MESCs can thus be induced to display characteristics of differentiated respiratory epithelia in culture. We hypothesized that the highly inductive morphogenetic interactions inherent to organotypic regeneration would provide a highly efficient instructive environment for the differentiation of MESCs into an Sftpc-positive respiratory lineage. E11.5, E12.5, and E13.5 fetal lungs dissociated into single-cell reformed pseudoglandular structures (Fig. 1A and data not shown). Aggregates comprising either 8 × E11.5, 4 × E12.5, or 2 × E13.5 dissociated fetal mouse lung buds in combination with either 1 × 10⁴, 2 × 10⁴, or 5 × 10⁴ dissociated Zin40 MESC were, therefore, empirically tested for organotypic regeneration and Zin40 MESC incorporation during a 12-day culture period. Zin40 MESCs constitutively express nuclear localizing β-galactosi-

Fig. 2. Surfactant protein C (Sftpc) immunohistochemistry of E11.5 mouse fetal lung aggregates. A and B: 8-μm cryostat sections of E11.5 lung aggregate at day 6 showing MESC derivatives forming pseudoglandular structures and displaying immunoreactivity to Sftpc (arrows). C and D: E11.5 lung aggregate at day 12 showing MESC derivatives with apical staining for Sftpc. E: Sftpc immunoreactivity of MESC derivatives when cultured with E11.5, E12.5, and E13.5 mouse fetal lung tissue for 4–12 days of culture (E11.5 = blue, E12.5 = red, E13.5 = yellow; **P < 0.001).
dase activity (21). In each case, the addition of Zin40 MESC did not inhibit dissociated fetal lung from undergoing organotypic regeneration (Fig. 1B). Furthermore, combined β-galactosidase staining and pan-keratin immunoreactivity demonstrated ~40% of all Zin40 MESC derivatives incorporated into and formed pseudoglandular epithelial-like structures with a striking efficiency, most notable after coculture with dissociated E11.5 lung buds (Fig. 1, C and D; data not shown). These pseudoglandular structures were morphologically similar to Sftpc-expressing cells seen within the developing mouse lung (Fig. 1E).

Sftpc expression is restricted to primitive respiratory epithelia and differentiated type II pneumonocytes (27). Sftpc-positive progenitors give rise to nearly all intrapulmonary epithelial cells. To determine whether Zin40 MESC had been induced to differentiate into respiratory lineages, 4- to 12-day cultured E11.5, E12.5, and E13.5 lung bud/Zin40 MESC aggregates were serially sectioned and examined for β-galactosidase activity and Sftpc immunoreactivity. All β-galactosidase-positive cells in addition to all cells displaying colocalization of β-galactosidase activity and Sftpc immunoreactivity were quantified (n > 600 cells/time point from n = 3 aggregates/time point). Dissociated E11.5 lung buds were found to induce Sftpc immunoreactivity at an incidence of 10.6 and 23.7% of all Zin40 MESC derivatives at 4 and 6 days of culture, respectively (Fig. 2, A, B, and E). Although Zin40 MESC incorporated into the E12.5 and E13.5 lungs undergoing organotypic regeneration, a significantly reduced level of β-galactosidase and Sftpc colocalization was observed at all corresponding time points examined (Fig. 2E). Sftpc-immunopositive Zin40 MESC derivatives organized into pseudoglandular-like structures, displaying similar morphology to endogenous Sftpc respiratory epithelium. Sftpc-immunopositive pseudoglandular-like tubules composed of both mixed populations of endogenous respiratory endothelium and induced Zin40 MESC derivatives in addition to Sftpc-immunopositive pseudoglandular-like tubules composed only of Zin40 MESC derivatives were observed. Sftpc immunoreactivity displayed a ubiquitous pattern throughout the cytoplasm, indicating a lack of preferential trafficking of the Sftpc protein to the cell surface. After 6 days of culture, the percentage of Sftpc-immunoreactive Zin40 MESC derivatives per aggregate decreased steadily to 2.4% by day 12 of culture (Fig. 2, C–E). At this time, Sftpc immunoreactivity was localized specifically in association with the cell membrane, thus according with a functional maturation program of the Sftpc-immunopositive cells. E12.5 aggregates displayed similar Sftpc immunoreactivity induction kinetics, yet with a relatively reduced incidence. At 4 and 6 days of culture, 2.2 and 6.0%, respectively, of all Zin40 MESC derivatives displayed Sftpc immunoreactivity. The incidence of Sftpc immunoreactivity per aggregate then again decreased, reaching 1.2% by 12 days of culture (Fig. 2E). Dissociated E13.5 lung buds were found to be highly inefficient at inducing Zin40 MESC derivative Sftpc immunoreactivity. Only 0.02% of all Zin40 MESC derivatives detected in E13.5 lung bud combination aggregates at 6 days of culture displayed detectable Sftpc immunoreactivity. No Zin40 MESC derivative Sftpc immunoreactivity was detected at any other time point examined. These results demonstrate that fetal respiratory tissue efficiently induces cultured mouse Zin40 MESC to display immunoreactivity to the primitive respiratory epithelial and differentiated type II pneumonocyte-specific marker Sftpc. Furthermore, the ability of dissociated fetal lung buds to induce Zin40 MESC Sftpc immunoreactivity demonstrates a temporal dependence, with E11.5 lung buds possessing a greater induction capacity than both E12.5 and E13.5 lung buds (P < 0.001). Last, induced Sftpc-immunopositive Zin40 MESC derivatives undergo a maturation process in culture in respect to the transportation kinetics of the Sftpc protein to the cell membrane. Whether the decreased incidence

Fig. 3. Whole mount in situ of E11.5 day 8 mouse lung aggregate showing Sftpc mRNA expression and chromosome 11 analysis of day 6 aggregates by fluorescence in situ hybridization. A: bright-field image of 8-μm cryostat section of an aggregate with Sftpc mRNA staining (purple; see arrow). B: negative image of bright field with Sftpc mRNA staining (white) and enhanced green fluorescent protein (EGFP; see arrow) MESCs identified by immunoreactivity to EGFP (red). C: 3-μm paraffin sections of day 6 aggregates showing pseudoglandular structure with 1 or 2 chromosome 11 signals (arrow) for each nuclei. No tetraploid signals were detected.
of Sftpc-immunoreactive Zin40 MESC derivatives in aggregates after 6 days of culture results from a relative and disproportionate increase in Sftpc-immunonegative cells, differentiation of Sftpc-immunopositive progenitor-like cells into Sftpc-immunonegative respiratory phenotypes, or a combination of these two possibilities, is not known.

These data strongly suggest that in vitro cultivated fetal mouse lung provides a unique cellular milieu in which instructed differentiation of Zin40 MESC into respiratory lin- eages can be achieved. Insulin immunoreactivity of embryonic stem cell derivatives under culture conditions designed to induce formation of pancreatic islet-like cells in vitro may be explained by uptake of insulin from the culture media rather than by production of the embryonic stem cell derivatives themselves (28). The possibility that MESC derivative Sftpc immunoreactivity in coculture could be explained solely by uptake of Sftpc present in the culture media, provided by the cocultured endogenous fetal lung epithelium, was therefore investigated. EGFP MESC were combined with dissociated E11.5 lung buds for 6 days in coculture and examined for Sftpc expression using whole-mount Sftpc mRNA in situ hybridization and EGFP immunohistochemistry. EGFP MESC used in this study ubiquitously express EGFP under the control of the β-actin promoter in all tissues, including the lung, of transgenic mice (26). After induction with E11.5 dissociated lung buds, EGFP MESC derivatives displayed a strong Sftpc hybridization signal (Fig. 3, A and B). No Sftpc mRNA transcripts were detected when EGFP MESC were grown in the absence of dissociated fetal lung buds (data not shown). Thus in addition to demonstrating that pseudoglandular respiratory induction is not unique to one particular MESC line, these data demonstrate that E11.5 lung tissue induces MESC derivatives to transcribe Sftpc mRNA.

Stem cell derivatives can display apparent properties of differentiated cell types in vitro and in vivo as a result of fusion with already differentiated cell types rather than as a result of instructed differentiation of diploid cells (3, 32, 34, 35). To determine whether Zin40 MESC derivative Sftpc immunoreactivity resulted from cell fusion with Sftpc-immunopositive fetal lung cells, 6-day cultured aggregates were subjected to FISH using a mouse chromosome 11-specific paint probe. Examination of all cells forming luminal structures demonstrated the presence of only one or two hybridizing signals for the chromosome 11-specific paint probe (Fig. 3C). These data thus strongly suggest that Zin40 MESC derivatives do not require cell fusion to display Sftpc immunoreactivity.

Instructive signals inherent to epithelial-mesenchymal interactions during a defined period of early lung morphogenesis induce MESC 32C-7 lineage to undergo a pseudoglandular respiratory-like developmental program with high efficiency in culture. Defining the active signaling molecules directing differentiation in this system using microarray analysis may facilitate scale-up procedures relevant to transplantation technologies. MESC derivative transplantations have been reported to improve heart function after infarction, rescue genetic blood disease, normalize weight, longevity, and (temporarily) insulin levels in diabetic mice, reduce symptoms of Parkinson’s disease, and partially repair damaged spinal cords (5, 10, 11, 14–17, 30). Several studies have demonstrated the potential of Clara cells and bronchoalveolar stem cells to regenerate the respiratory epithelium after induced alveolar and bronchial damage (13, 29). Determining whether the Sftpc-immunopositive cells described in this study may possess similar properties or serve as precursors to these regenerating populations will have important implications for transplantation therapies aimed at treating a range of diseases affecting the peripheral respiratory airways such as cystic fibrosis and idiopathic pulmonary disease.

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