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SHORT REPORT

Generation of organized anterior foregut epithelia from pluripotent stem cells using small molecules☆

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Abstract Anterior foregut endoderm (AFE) gives rise to therapeutically relevant cell types in tissues such as the esophagus, salivary glands, lung, thymus, parathyroid and thyroid. Despite its importance, reports describing the generation of AFE from pluripotent stem cells (PSCs) by directed differentiation have mainly focused on the Nkx2.1$^+$ lung and thyroid lineages. Here, we describe a novel protocol to derive a subdomain of AFE, identified by expression of Pax9, from PSCs using small molecules and defined media conditions. We generated a reporter PSC line for isolation and characterization of Pax9$^+$ AFE cells, which when transplanted *in vivo*, can form several distinct complex AFE-derived epithelia, including mucosal glands and stratified squamous epithelium. Finally, we show that the directed differentiation protocol can be used to generate AFE from human PSCs. Thus, this work both broadens the range of PSC-derived AFE tissues and creates a platform enabling the study of AFE disorders.

1. Introduction

The ability to generate specific tissues from pluripotent stem cells (PSCs) is an important goal for developing human cell replacement therapies and to enable the generation of disease-specific models. While much attention has focused on the derivation of organs involved in metabolic diseases, such as the pancreas and liver, fewer studies have addressed organs derived from anterior foregut endoderm (AFE). Arising from a regional domain of definitive endoderm (DE), the AFE contributes to many organs including the salivary glands, esophagus, stomach, trachea, and lungs. It

Abbreviations: AFE, anterior foregut endoderm; BMPi, bone morphogenic protein inhibitor; DE, definitive endoderm; ESCs, embryonic stem cells; MEF, mouse embryonic fibroblasts; PSCs, pluripotent stem cells; TGFβi, transforming growth factor inhibitor

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also gives rise to the pharyngeal endoderm, which in turn forms the thymus, parathyroid glands, thyroid gland and ultimobranchial bodies (Zorn & Wells, 2009).

One approach to generate specific cell populations is to direct PSCs toward a desired cell type by sequential progression through intermediate cell populations, mimicking the stages of embryonic development. Monolayer differentiation approaches allow homogeneous accessibility of cells to exogenous signals required to guide cell fate decisions. Indeed, monolayer differentiation strategies have been employed to produce definitive endoderm (DE), the precursor of AFE, from PSCs through activation of the wingless (WNT) and TGFβ signaling pathways (Sherwood et al., 2011; D'Amour et al., 2005). More recently, AFE has been generated from PSC-derived definitive endoderm (DE) *in vitro* and upon further differentiation can be directed to express the lung- and thyroid-specific transcription factor Nkx2.1 (Mou et al., 2012; Longmire et al., 2012; Wong et al., 2012; Green et al., 2011). The generation of organized AFE epithelia has also been reported, however these are currently limited to respiratory epithelial lineages (Mou et al., 2012; Wong et al., 2012), necessitating further work to produce additional organized AFE epithelia from PSCs. In order to develop a protocol to produce AFE capable of forming a broader spectrum of AFE-derived epithelia, we selected the transcription factor Pax9 for use as an AFE marker based on its broad expression domain (Fig. 1A). Pax9 is expressed from e9.0 in the developing murine pharyngeal foregut endoderm and is required for the development of the thymus, parathyroid and ultimobranchial bodies (Peters et al., 1998). Pax9 expression is retained in several AFE-derived mature cell types including the oral epithelium, salivary glands, parathyroid gland, thymus, esophagus and fore-stomach (Peters et al., 1998). Although the generation of PAX9+ cell containing AFE cultures has been reported from human PSCs (Green et al., 2011), upon transplantation, obtained organized epithelial structures expressed genes indicative of lung epithelial cells rather than other PAX9$^+$ AFE derived epithelia. Thus, there is a need to explore alternative approaches to generate PAX9$^+$ cells from PSCs in order to produce the spectrum of PAX9$^+$ AFE epithelia.

We report a monolayer differentiation approach using small molecules and chemically defined media to generate PAX9$^+$ AFE cells from both mouse and human PSCs, thereby minimizing the contribution of uncharacterized signals during directed differentiation. Through the use of a novel mouse reporter PSC line, we isolated Pax9$^+$ AFE cells for molecular and functional characterization. Generated Pax9$^+$ AFE cells not only exhibit molecular signatures of anterior and pharyngeal foregut *in vitro*, but also are competent to form a variety of organized AFE epithelial structures upon transplantation *in vivo*. Thus, these results advance the AFE differentiation field by generating a transcriptional signature for Pax9$^+$ AFE, broadening the spectrum of AFE epithelia currently generated from PSCs, and creating a platform to study AFE developmental disorders.

2. Materials and Methods

2.1. Mouse pluripotent stem cell culture

All mouse PSC lines; AV3, p2lox40, Sox17^{GFP}, and Pax9^{Venus} were maintained on mouse embryonic fibroblasts (MEF) in KO DMEM (GIBCO, 10829) supplemented with 15% Hyclone FBS (Thermo Scientific, SH30070.03), 1% Glutamax (GIBCO, 35050079), 1% NEAA (Cellgro, 25-025-CI), 100 μM β-mercaptoethanol (Invitrogen, 21985023), 1 × 10^3 units/ml ESGRO LIF (Millipore, ESG1107). Cells were fed daily and split every 2–3 days with 0.25% trypsin (GIBCO, 25200114).

2.2. Mouse pluripotent stem cell differentiation

Maintenance cultures were split and MEF depleted for 30 min in maintenance media. PSCs were collected and resuspended in differentiation media 1 [A-DMEM (GIBCO, 12491) supplemented with 1% N2 (GIBCO, 17502-048), 2% B27 minus vitamin A (GIBCO, 12587-010), 1% Glutamax (GIBCO, 35050079) 1% P/S (Cellgro, 30-001-CI)] and 10 μM Y-27632 (Selleck Chemicals, S1049). Cells were plated at 5 × 10^3 cells/cm^2 onto 0.1% gelatin-coated 6 well plates. On day 2 cells were fed with differentiation media 1 with 5nM GSK-3 inhibitor XV (Calbiochem, 361558) and 5 μM IDE-1 (Tocris, 4015). On days 3, 4, and 5, cells were fed with differentiation media 2 [A-DMEM (GIBCO, 12491) supplemented with 2% B27 minus vitamin A, 1% Glutamax, 1% P/S] plus 5 μM IDE-1. On day 6 cultures were split with 0.25% trypsin and plated at 3 × 10^5 cells/cm^2 onto 804G conditioned medium-coated plates with 10 μM Y-27632 in differentiation media 2. Cells were treated with 500 nM A83-01 (Tocris, 2939) and 100 nM LDN193189 (Selleck Chemicals, S2618).

2.3. Human pluripotent stem cell culture

HUES64 were maintained on mouse embryonic fibroblasts in KO DMEM (GIBCO, 10829) supplemented with 20% KOSR (Invitrogen, 10828-028), 1% Glutamax (GIBCO, 35050079), 1% NEAA (Cellgro, 25-025-CI), 55 μM β-mercaptoethanol (Invitrogen, 21985023), 10 ng/ml bFGF (GIBCO, PH0023). Cells were fed daily and split every 4–5 days with TrypLE Express (GIBCO, 12604).

2.4. Human pluripotent stem cell differentiation

Maintenance cultures were split with TrypLE Express and cells plated at 1–1.5 × 10^5 cells/cm^2 onto growth factor reduced matrigel (BD Bioscience, 356231)-coated 6 well plates in mTESR (Stem Cell Technologies, 05850). On day 3, cells were fed with differentiation media [RPMI 1640 (GIBCO, 21870-092) 2% B27 minus vitamin A (GIBCO, 12587-010), 1% Glutamax (GIBCO, 35050079) 1% P/S (Cellgro, 30-001-CI)] supplemented with 5 nM GSK-3 inhibitor XV (Calbiochem, 361558) and 5 μM IDE-1 (Tocris, 4015). On days 4–6, cells were fed with differentiation media supplemented with 5 μM IDE-1. On days 7–13, cells were fed with differentiation media supplemented with 500 nM A83-01 (Tocris, 2939) and 100 nM LDN193189 (Selleck Chemicals, S2618).

2.5. Quantitative real time PCR

RNA was isolated using Trizol (Invitrogen, 15596-018) according to the manufacturer's instructions and 2 μg of RNA was reverse transcribed to cDNA using SuperScript III (Invitrogen,