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METHODS AND REAGENTS

Efficient definitive endoderm induction from mouse embryonic stem cell adherent cultures: A rapid screening model for differentiation studies

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Abstract Definitive endoderm (DE) differentiation from mouse embryonic stem cell (mESC) monolayer cultures has been limited by poor cell survival or low efficiency. Recently, a combination of TGFβ and Wnt activation with BMP inhibition improved DE induction in embryoid bodies cultured in suspension. Based on these observations we developed a protocol to efficiently induce DE cells in monolayer cultures of mESCs. We obtained a good cell yield with 54.92% DE induction as shown by Foxa2, Sox17, Cxcr4 and E-Cadherin expression. These DE-cells could be further differentiated into posterior foregut and pancreatic phenotypes using a culture protocol initially developed for human embryonic stem cell (hESC) differentiation. In addition, this mESC-derived DE gave rise to hepatocyte-like cells after exposure to BMP and FGF ligands. Our data therefore indicate a substantial improvement of monolayer DE induction from mESCs and support the concept that differentiation conditions for mESC-derived DE are similar to those for hESCs. As mESCs are easier to maintain and manipulate in culture compared to hESCs, and considering the shorter duration of embryonic development in the mouse, this method of efficient DE induction on monolayer will promote the development of new differentiation protocols to obtain DE-derivatives, like pancreatic beta-cells, for future use in cell replacement therapies.

Abbreviations: ActA, activin A; AKP, 1-azakenpaullone; BIO, 6-bromo indirubin-3-oxine; CDM, chemically defined medium; CHIR, CHIR99021; DE, definitive endoderm; EBs, embryoid bodies; hESC, human embryonic stem cells; KOSR, knockout serum replacement; mESC, mouse embryonic stem cells; NG, noggin.

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Introduction

Embryonic stem cells have a great potential in regenerative medicine because they generate somatic cell types from all three germ layers. For example, insulin-producing pancreatic beta-cells derived from human embryonic stem cells (hESCs) can be applied in type-1 diabetes. One of the problems to overcome is that it has proven very difficult, if not impossible, to obtain fully differentiated and functional beta-cells in vitro. Currently, it is possible to generate pancreatic progenitors from hESCs, and they were shown to differentiate into functional beta-cells after a prolonged period of engraftment in mice (Kroon et al., 2008; Mfopou et al., 2010a; Nostro et al., 2011; Rezania et al., 2012). Further optimization is needed to establish suitable conditions required for beta-cell differentiation in vitro. To this end, the study of mouse embryonic stem cells (mESCs) can be useful for several reasons. First, in vitro differentiation protocols are intended to mimic the conditions in the developing embryo and most knowledge on embryonic development of the pancreas was accumulated from mouse studies. Furthermore, the timeline for the specification and maturation of a particular cell is theoretically shorter in mice than humans (respectively 3 weeks and 40 weeks). Second, mESCs are easier to maintain in culture as they grow faster, are more resistant to enzymatic dissociation during passaging, and have less tendency to spontaneously differentiate. mESCs can thus be used as a model to rapidly tweak protocols that thereafter could be implemented on hESCs, for example to obtain beta-cells. However, despite the similarities in their general properties, embryonic stem cells from mouse and human differ in their manipulation in vitro, which is related to the developmental origin of these cells. Indeed, in contrast to mESCs, pluripotent cells derived from mouse epiblast stage embryos (epiblast stem cells, EpiSCs) can be considered as the true developmental counterparts of hESCs. Interestingly, both EpiSCs and hESCs were shown to require the same culture conditions (Brons et al., 2007; Tesar et al., 2007). Human and mouse ESCs also differ in the conditions needed for definitive endoderm (DE) induction, the first step towards commitment into pancreatic and other gastrointestinal fates (Mfopou et al., 2010b). Whereas hESCs cultured as monolayers and stimulated with Activin A and Wnt3a in a basic medium efficiently generate DE progenitors, mESCs cultured under similar conditions usually fail to survive or they generate DE cells with low efficiency (<25%) (D'Amour et al., 2005; Hansson et al., 2009; Morrison et al., 2008; Sulzbacher et al., 2009; Tada et al., 2005; Yasunaga et al., 2005). On the contrary, mESCs cultured as embryoid bodies generate DE cells in the presence of ActA with an efficiency that can reach 85% if Noggin is also supplemented (Gadue et al., 2006; Kubo et al., 2004; Li et al., 2011). We have previously shown that embryoid bodies do not constitute an optimal environment for efficient differentiation into pancreatic phenotypes (Mfopou et al., 2005, 2007). Interestingly, monolayer cultures are also technically more practical and simple to rapidly analyze microscopically; e.g. they don't need to be embedded and sectioned. They are thus optimal for high throughput screening of growth factor and small molecule combinations.

DE cells were recently generated from mouse embryoid bodies using a combination of ActA (TGFβ activator), Noggin (BMP antagonist), and lithium chloride (Wnt pathway activator) (Li et al., 2011). Whereas ActA and Wnt activators are commonly used for DE induction, Noggin supplementation is justified by the requirement for low BMP signaling to direct the mesendoderm towards anterior primitive streak derivatives (D'Amour et al., 2005; Sumi et al., 2008; Wang et al., 2012). In the present study, we implemented this protocol in mESC monolayer cultures using different GSK3β inhibitors to activate the Wnt pathway. We show an efficient DE-derivation from these cultures and the generation of Pdx1 + Nkx6.1 + pancreatic progenitors following the strategies that we previously developed with hESCs (Mfopou et al., 2010a; Sui et al., 2012). Furthermore, we present data indicating that the mESC-derived DE cells also give rise to hepatocyte-like cells. Therefore, these findings constitute an optimal and rapid model for further screening growth factor and small molecule combinations in view of the differentiation of endoderm progenies such as the pancreatic beta cells.

Materials and methods

Cell culture and differentiation

Mouse ESCs were maintained undifferentiated on inactivated mouse embryonic fibroblasts (feeders) in knockout DMEM (Life Technologies, Paisley, UK) supplemented with 15% knockout serum replacement (KOSR; Life Technologies), 1× non-essential amino-acid (Sigma, Saint-Louis, USA), 1× Glutamax (Life Technologies), 1× Penicillin–Streptomycin solution (Sigma), 0.55 mM beta-mercaptoethanol (Life Technologies) and 1000 U/ml leukemia inhibitory factor (LIF; Sigma). Cells were passaged on new feeder layers every 3 days by dissociation with recombinant trypsin (TrypZean; Sigma). For differentiation studies, dissociated mESCs (about 10^5 cells/cm^2) were seeded with the carry-over feeders on gelatin-coated plates. Initially, definitive endoderm was induced in RMPI or DMEM (Life Technologies) two days after seeding mESCs and using ActA (10 to 50 ng/ml; RnD Systems, Minneapolis, USA), Wnt3a (25 ng/ml; RnD Systems), FGF2 (10 ng/ml; Life Technologies), EGF (50 ng/ml; Sigma), BMP4 (10 ng/ml; RnD Systems), LY294002 (10 μM; Sigma), FBS (0.2 to 5%; PAA, Pasching, Austria), KOSR (2 to 5%; Life Technologies) and B27 supplement (1%, Life Technologies). Later on, DE was induced with a combination of ActA (50 ng/ml), Noggin (200 ng/ml; RnD Systems) and a GSK3β inhibitor (1 μM of 6-bromo indirubin-3-oxine (BIO; Merck KGaA, Darmstadt, Germany); 2.5 μM of 1-azakenpaullone (AKP; Sigma) or 5 μM of CHIR99021 (CHIR; Stemgent, San Diego, USA) (Li et al., 2011)). The GSK3β inhibitor was supplemented for 2 days (2+ and 2 + 2−) or for the entire 4 days (4+) and the chemically defined medium (CDM) used was made of 50% DMEM, 50% DMEM-F12, 0.2% bovine serum albumin (Life Technologies), 2 mM Glutamax, 0.5 mM ascorbic acid (Sigma) and 0.55 mM beta-mercaptoethanol. An overview of the conditions tested is given in Supplementary Table 1.

For the differentiation towards pancreatic lineages (Mfopou et al., 2010a; Nostro et al., 2011; Sui et al., 2012, 2013), DE cells (stage 1) were cultured in DMEM + 1% B27 and exposed to Noggin (100 ng/ml; RnD Systems), KAAD-cyclopamine (250 nM; Merck KGaA), retinoic acid (2 μM, Sigma) and FGF10 (25 ng/ml; RnD Systems) for 6 to 8 days, then to FGF10 (50 ng/ml) for 4 days (stage 2) and finally to Noggin (100 ng/ml) and the Notch