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Distinct gene expression signatures in human embryonic stem cells differentiated towards definitive endoderm at single-cell level

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ABSTRACT

Characterization of directed differentiation of pluripotent stem cells towards therapeutically relevant cell types, including pancreatic beta-cells and hepatocytes, depends on molecular markers and assays that resolve the signature of individual cells. Pancreas and liver both have a common origin of anterior definitive endoderm (DE). Here, we differentiated human embryonic stem cells towards DE using three different activin A based treatments. Differentiation efficiencies were evaluated by gene expression profiling over time at cell population level. A panel of key markers was used to study DE formation. Final DE differentiation was also analyzed with immunocytochemistry and single-cell gene expression profiling. We found that cells treated with activin A in combination with sodium butyrate and B27 serum-free supplement medium generated the most mature DE cells. Cell population studies were useful to monitor the temporal expression of genes involved in primitive streak formation and endoderm formation, while single-cell analysis allowed us to study cell culture heterogeneity and fingerprint individual cells. In addition, single-cell analysis revealed distinct gene expression patterns for the three activin A based protocols applied. Our data provide novel insights in DE gene expression at the cellular level of in vitro differentiated human embryonic stem cells, and illustrate the power of using single-cell gene expression profiling to study differentiation heterogeneity and to characterize cell types and subpopulations.

1. Introduction

In the efforts of developing new sources of insulin producing cells for treatments of Type I diabetes, human embryonic stem cells (hESCs) hold a great promise as an unlimited source of insulin supply. Protocols for efficient generation of definitive endoderm (DE) and its subsequent differentiation to pancreatic progenitors and pancreatic β-cells (beta-cells) have been extensively reported [1–12]. Although, the cells generated with these protocols do not express the same combination of markers as their in vivo counterparts, glucose responsive beta-like cells can be generated when maturated in vivo [8]. This data indicate that hESCs have the potential to develop into functional insulin producing cells, but the instructive signals for in vitro maturation of pancreatic progenitors are missing. To overcome this challenge, it is generally believed that the most straightforward strategy of differentiating pluripotent stem cells towards beta-cells is to mimic the signaling pathways of pancreas development, during normal mammalian embryonic development, and to translate this knowledge to human in vitro systems.

The progressive developmental steps behind beta-cell differentiation are first initiated with formation of DE. In vertebrates, this process starts during gastrulation with the appearance of the primitive streak (PS) (reviewed in [13,14]). Pluripotent epiblast cells undergo epithelial to mesenchymal transitions and migrate through the PS and become either mesoderm or endoderm. The cells that first exit PS form the most anterior part of the embryo, while cells exiting later form the posterior part of the embryo. After migration through the PS, DE invades and replaces the extraembryonic endoderm layers of visceral endoderm (VE) that forms the supportive tissues of the embryo. During vertebrate gastrulation the Nodal signaling pathway regulates endoderm and mesoderm formation [15,16]. In the pregastrula embryo, high levels of Nodal induce endoderm and anterior mesendoderm, whereas low levels of Nodal

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promote transcription of mesoderm and posterior endoderm. A key regulatory gene for PS formation is Brachyuru (*T*) that is induced by signals from extraembryonic endoderm [17,18]. Another transcription factor, Mix homeobox-like 1 (*Mixl1*), is expressed during gastrulation, when the early endoderm migrates out from the PS and plays an important role in cell commitment towards the endodermal linage and suppresses mesodermal differentiation [19]. *Mixl1* is also required for cell movement that during gastrulation is associated with anterior expansion of DE and gut tube morphogenesis [19,20]. Cerberus 1 (*Cer1*), SRY (sex determining region Y)-box 17 (*Sox17*) and forkhead box A2 (*Foxa2*) are expressed in DE and are in many aspects key regulatory genes for endoderm development and specification of foregut endoderm, the part of the endoderm that later on gives rise to organs, such as pancreas and liver [21,22]. Absence of *Sox17* in mice results in depletion of DE in the foregut endoderm [23]. In the pre/early-streak embryo, *Sox17* is expressed in the entire extraembryonic/VE endoderm. At mid-streak stage *Sox17* is expressed in endoderm at the anterior end of the PS, but not in VE. At the time of DE movement to the anterior gastrula, *Sox17* expression expands more anterior and is therefore specifically expressed in DE of the gastrula in contrast to other endoderm markers such as *Cer1*, *Foxa2* and hematopoietically-expressed homeobox protein (*Hhex*) that are also expressed in anterior VE [21,22]. The C-X-C chemochine receptor type 4 (*Cxcr4*) is expressed in DE and in mesoderm but not in VE [24–26]. After gastrulation, the endoderm is regionalized along the anterior–posterior axis into foregut, midgut and hindgut, where foregut later on is specified into thyroid, lung, hepatic and pancreatic endoderm. *Hhex* is one of the earliest markers that regulates anterior–posterior identity and is expressed in the first anterior DE cells, emerging from the PS [27], and plays an essential role in maintaining anterior identity [28,29]. Moreover, at late gastrulation, LIM homeobox 1 (*Lhx1*), orthodenticle homeobox 2 (*Otx2*), *Cer1* and *Foxa2* have been shown to be required for establishment of the anterior–posterior axis body plan [30–32]. *Sox17*, *FoxA2*, *Hhex* and *Cer1* are all expressed both in DE and VE, while SRY (sex determining region Y)-box 7 (*Sox7*) is exclusively expressed in mouse VE [23]. Markers that are expressed during regionalization of the gut endoderm include alpha fetoprotein (*Afp*) [33], caudal type homeobox 2 (*Cdx2*) [34] and HNF1 homeobox B (*Hnf1b*) [35]. To summarize, genetic studies in vertebrates have shown that there is no exclusive markers that can be used to define anterior DE, which is the origin of both beta-cells and hepatocytes. Instead, cell characterization relies on using a combination of markers that collectively identifies anterior DE and excludes a VE phenotype.

In the attempts of translating developmental biology into strategies for in vitro differentiation of pluripotent stem cells, research tools that define the molecular events within heterogeneous cell populations are needed. Individual cells in a seemingly homogenous cultures or tissues are in many aspects unique in their expression of transcripts and proteins [36]. This implies that cell population data cannot be used to accurately describe individual cells. Immunocytochemistry provides an opportunity to specifically target cells expressing anterior DE markers at the protein level. However, there are few or no published data using these antibodies for immunolocalization in hESCs. Furthermore, only a few proteins may be analyzed simultaneously using immunocytochemistry. This highlights the need for novel assays and tools that, at cellular level, monitor differentiation of hESCs towards DE. The use of single-cell gene expression analysis to understand stem cell heterogeneity and the dynamic transition between cell fates has been recognized for a long time, but lack of analytical techniques sensitive enough to measure few transcripts has limited such experiments. However, recent advances allow robust and reproducible single-cell gene expression measurements to be performed [37–39].

Here, we differentiated hESCs towards DE using three different activin A based protocols. The dynamics of DE markers were analyzed over time using gene expression profiling at cell population level. The temporal analysis of DE associated markers identified an optimal endpoint stage of differentiation that was further characterized by single-cell gene expression profiling. Immunocytochemistry was used to confirm expression of DE markers at protein level. Analysis of multiple markers associated with differentiation at the single-cell level allowed us to determine characteristic transcript signatures for cells generated by the three different activin A treatments. Our data illustrate the value of analyzing multiple markers at the cellular level and how single-cell analysis can be implemented as a research tool to understand hESC differentiation.

2. Material and methods

2.1. Cell cultures and differentiation of human embryonic stem cells

The hESC line SA121 [40] was maintained as undifferentiated cells in DEF™-CS (Cellartis AB), according to Swedish ethics guidelines. For differentiation, hESCs were passaged into differentiation media, containing RPMI1640 (Gibco, Invitrogen) supplemented with 0.1% Penicillin/Streptomycin (Gibco) and activin A 100 ng/ml (Peprotech), up to 7 days according to Fig. 1. For AAFBS, differentiation medium supplemented with 0.2% fetal bovine serum (Sigma–Aldrich) was used day 0–2 and 1% fetal bovine serum was used day 3–7. For the AAB27 and AANaB protocols, differentiation medium was supplemented with 2% B27 serum-free supplement medium (Gibco) all days. In AANaB, 1 mM sodium butyrate (NaB, Sigma–Aldrich) was added day 0–1 and 0.5 mM NaB day 2–7 [12]. Full medium change was performed every day. Bright field images of cells were taken on an inverted microscope (Eclipse TE2000-U, Nikon).

2.2. Immunocytochemistry

Cells were washed once in phosphate buffered saline and fixed in 4% paraformaldehyde for 15 min, washed three times in PBS, permeabilized in 0.5% TritonX-100 for 15 min (all Sigma–Aldrich). Primary antibodies: goat anti-SOX17 (1:500) (R&D Systems), mouse anti-POU5F1 3/4 (1:200, Santa Cruz), mouse anti-AFP (1:500, Sigma–Aldrich), goat anti-FOXA2 (1:500, Santa Cruz Bio-

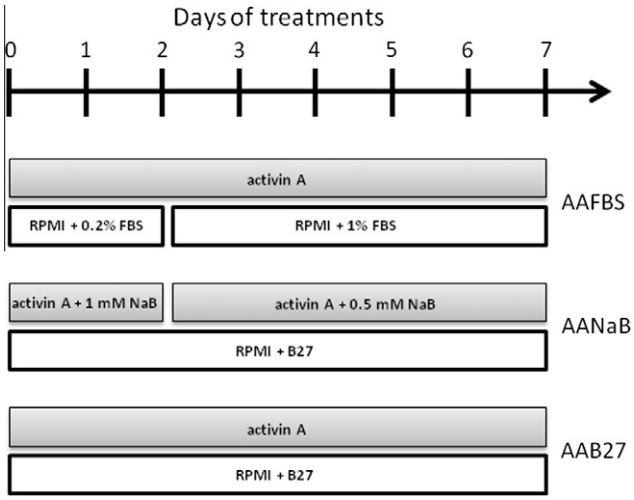

Fig. 1. Definitive endoderm differentiation. Human embryonic stem cells were treated with activin A using three different protocols: AAFBS, AAB27 and AANaB to induce differentiation towards definitive endoderm. Detailed differentiation protocols are described in Section 2.