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# Production of β-cells from human embryonic stem cells

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The making of functional pancreatic islets in renewable numbers has been a goal of stem cell biologists since early 2000. Since that time, many studies have reported successful creation of glucose-responsive pancreatic β-cells. Not until the more recent systematic application of developmental principles to stem cell biology systems were breakthroughs achieved on directed specification of the required early developmental intermediates. The most important first step is the formation of the definitive endoderm (DE) lineage which is compulsory for production of the epithelium of the pancreas and the other important endoderm-derived organs such as the liver, intestine and lung. The formation of DE from embryonic stem cells made possible additional experimentation aimed at directing the endoderm to further specified foregut and pancreatic endoderm lineages. With these discoveries came the first production of immature pancreatic endocrine cells. Most recently, the production *in vivo* of glucose-responsive insulin-producing cells with the capacity to correct Steptozotocin-induced hyperglycaemia in mice has been achieved. The work leading up to this achievement, in relation to the other principle human stem cell studies conducted in this area, will be briefly described. The necessary steps and ideal characteristics of embryonic stem cell-based differentiation to pancreatic β-cells capable of glucose stimulated insulin secretion will be underscored.

Keywords: beta cell (β-cell), definitive endoderm (DE), foregut endoderm, glucose stimulated insulin secretion (GSIS), human embryonic stem cell (hESCs), pancreatic endoderm, C-peptide

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## Introduction

Creation of a renewable supply of human islet cells from an expandable stem cell source can address a major deficit facing the successful development of a broad-based cell therapy for insulin deficient diabetics. A logical starting cell source is human embryonic stem cells (hESCs). The two most important reasons for this are their disproportionately high and stable capacity for self-renewal in defined culture conditions, and their exquisite potential for directed differentiation to the somatic lineages.

Devotion to, and application of, developmental principles is critical to achievement of authentic and efficient differentiation of hESCs to defined terminally differentiated cell types, especially the complex and circuitous path to functional β-cells. In order to achieve the functional β-cell, a critical first step must be achieved with great efficiency, specifically the formation of the definitive endoderm (DE) lineage. As first formed with mouse [1], and later with hESCs [2], the DE lineage is required to produce the principal differentiated epithelium of the fore-, mid- and hind-gut organs including the thyroid, thymus, lung, liver, pancreas, stomach, intestine and bladder. It is unfortunate that many stem cell-based β-cell differentiation procedures have overlooked or ignored this principle tenet of development. When working with pluripotent stem cells, a concerted effort must be dedicated to the development of assays and procedures necessary for efficiently and reproducibly making DE. Without this vital first step, few foregut

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endoderm, pancreatic endoderm or pancreatic endocrine cells will be generated [3,4]. The principle of first forming DE is not only essential to successful differentiation of hESCs to the pancreatic lineage, but also for the differentiation of other pluripotent or multipotent stem cell sources demonstrated or claimed to be capable of making pancreatic endocrine cells. These include but are not limited to human embryonic germ cells [5,6], human spermatogonial stem cells [7–9], human multipotent adult progenitor cells [10] or human placental stem cells [11]. One cannot entirely rule out an unusual trans-lineage differentiation to an upstream endoderm intermediate or the use of direct genetic modification (reprogramming) using a series of critical pancreas transcription factor genes, but in general, these events or procedures are less likely to result in large-scale production of β-cells or stable maintenance of differentiated islet cell function.

## The First Step, Gastrulation and the Formation of DE

A simple model of the principle fate choices that could be taken by hESCs in culture is depicted in figure 1. We have arrived at this model by quantitatively monitoring global gene expression status after manipulation of the culture environment using a combination of signalling factors empirically tested or published by others. A critical tenet in our ability to rapidly and efficiently differentiate hESCs, especially to the mesendoderm and DE lineage, is based on effectively interrupting the stem cell self-renewal cycle. To achieve this it is important to remove from the culture the principal effectors [insulin, IGF (insulin like growth factor), FGF2 (basic fibroblast growth factor)] required for maintaining hESCs in their self-renewal state. Our method employs a washing step to remove hESC medium components (exogenous insulin and FGF2) or factors with strong phosphatidylinositol 3-kinase (PI3K) signalling activity [2,3,12]. We have further demonstrated that inhibition of PI3K signalling while simultaneously stimulating Activin/Nodal signalling, results in efficient and rapid differentiation of hESC cultures to DE [13]. A further useful methodology for determining the final purity of the resultant DE population is via FACS (fluorescence activated cell sorting) analysis (FLOW) using the antibody to the chemokine receptor 4 (CXCR4) which is expressed on nascent DE and mesoderm but not on visceral endoderm or ectoderm [14]. When CXCR4 FLOW analysis is conducted with a second endoderm marker such as FOXA2 or SOX17, a determination of hESC to DE conversion can be made at the earliest postgastrulation stages of differentiation [4,13,15].

## The Second Step, Foregut Endoderm

After determination of efficient DE production our methods require elimination of Transforming Growth Factor Beta (TGFβ) signalling in order to effectively trigger the DE-to-foregut patterning step that is further influenced by FGF

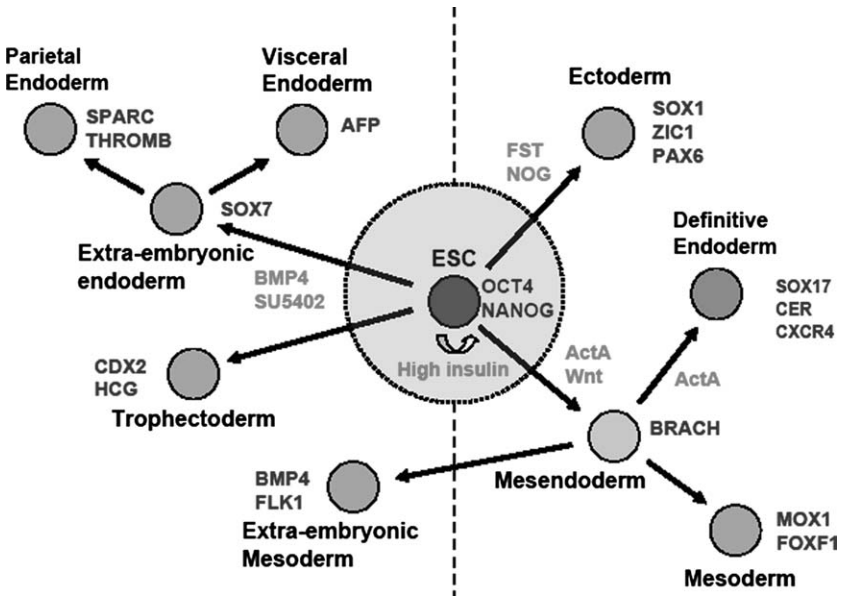


**Fig. 1** Fate choices in hESC cultures.

signalling. FGF10 and FGF7 both signal through FGFR2IIIb which has been shown to be an important signalling pathway involved in specifying many foregut endoderm cell lineages including lung, liver pancreas and intestine [16–22]. In our case, the use of FGF10 or FGF7 [23] results in an optimal specification of DE to foregut endoderm expressing FOXA2, Hepatocyte Nucleur Factor 4 alpha (HNFα) and HNF1β. These markers are not considered specific to foregut but in general are abundantly expressed in the developing gut tube. It should be noted that PDX1, HNF6 or SOX9 are not expressed at anytime during specification of the early foregut endoderm or for that matter DE in our culture system [3,12]. It is important to emphasize here that none of the published stem cell differentiation studies designed to produce pancreatic endocrine cells from human pluripotent stem cells define or incorporate a foregut endoderm stage of differentiation before moving on to pre-pancreatic or pancreatic epithelium (see table 1).

## The Third Step, Pancreatic Epithelium and Endocrine Precursors

A critical regulator of pancreatic endoderm specification and commitment to the endocrine lineage is retinoic acid (RA) signalling. RA signalling has been extensively examined for its effects on pancreas specification in chick [24], frog [25], fish [26–28] and mouse [29–31], and demonstrated to be an important early specifying agent in these contexts. In studies designed to eliminate endogenous RA production during embryonic foregut endoderm differentiation, the enzyme responsible for foregut RA synthesis (RALDH2) was knocked out. These animals have deficits in both lung and dorsal pancreas specification [32,33]. Further studies in frogs [25] and mice [29–31] have demonstrated that application of RA can bias the potential of pancreatic epithelium to produce primarily endocrine as opposed to mature acinar and duct tissues. We and others have therefore explored the stage-specific application of RA, in conjunction with Sonic Hedgehog modulation [34,35], to effect efficient commitment to the pancreatic lineage and to skew differentiation of the pancreatic endoderm to endocrine as opposed to acinar cells, as has been previously shown in mice and frogs.

As demonstrated in our recent work [3,12], after foregut endoderm cultures are exposed to RA, there is a rapid induction of many important transcription factors which have been shown to be essential for correct specification of the pancreatic anlagen and to be important in pancreatic foregut development [36–40], including PDX1, NKX6.1, PTF1α, PROX1, HNF6, HLXB9 and SOX9. The coordinate expression of these genes, first during the RA-inductive phase PDX1, PROX1, HNF6, HLXB9 and SOX9, and then post-RA removal NKX6.1 and PTF1α, appears to closely imitate the timed expression of these factors during this decisive phase of pancreatic development. With the exception of PDX1, few groups have demonstrated the robust temporal expression of these genes at both the message and protein levels in large proportions of the cultured cells (see table 1).

After and during foregut endoderm commitment to the pancreatic epithelium there is a dramatic upregulation of NGN3 mRNA which appears coincidently with NKX2.2. As NGN3 protein is expressed in the cultures, the number of hormone-producing cells rapidly increases. As NGN3 protein expression is labile with a rapid turnover, only a few percent of the cultures will express the protein at any given time. As we observed previously, the NGN3/NKX2.2 positive cells are not dividing in culture and rapidly turn off NGN3 as they become endocrine, hormone-expressing cells [3].

## The Fourth and Fifth Steps, Endocrine Cells and Function

The majority of these early hormone-expressing cells mature in culture to become predominantly multihormonal [3,4]. Most importantly, the insulin-producing cells do not maintain high-level PDX1 protein expression in extended culture and do not express NKX6.1 or MAFA but do express MAFB [3]. Although these endocrine cells produce considerable amounts of insulin and release C-peptide via the majority of classical secretagogues, they do not release insulin or C-peptide upon glucose stimulation [3]. Furthermore, examination of granule morphology and proinsulin processing additionally confirms the immature nature of these cells. Therefore by definition, lack of NKX6.1, MAFA and high-level PDX1 expression results in the formation of immature multiple-hormone-expressing cells that cannot be classified as functional glucose-responsive β-cells [41–43]. In studies similar to ours, demonstration of the production of extensive numbers of multihormonal cells in addition to lack of NKX6.1 or MAFA co-localization in insulin-expressing cells was noted [4,44]. Interestingly, in these studies both groups reported either robust *in vitro* glucose-stimulated C-peptide secretion [4], or partial correction of hyperglycaemia in diabetic animals [44]. In the study by Shim *et al.* no human C-peptide levels were reported from the serum of corrected animals, therefore it is not possible to verify if the transplanted cells were indeed responding to Steptozotocin-induced hyperglycaemia or to glucose infusion by secreting human C-peptide. At the

**Table 1** Human pluripotent stem cell differentiation to pancreatic islet cells

| | Differentiation steps (protein expression) | | | | β-Cell markers (protein expression) | | | | | | β-Cell functional characteristics | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Publication | DE (SOX17, CXCR4) | Foregut endoderm (hNF4a, HNF 1b) | Pancreatic endoderm (NKX6.1, PDX1) | Endocrine precursors (NGN3, NKX2.2) | C-peptide, PDX1 | C-peptide, PDX1, NKX6.1 | C-peptide, MAFA | PC1/3, C-peptide | PC2, C-peptide | Endocrine hormone co-expression | Percentage proinsulin processing | Distinct granule morphology (EM) | GSIS *in vitro* (C-peptide) | GSIS *in vivo* (C-peptide) | Correct STZ hypergly caemia | C-peptide levels* |
| Human islets [12] | NA | NA | NA | NA | Yes | Yes | Yes | Yes | Yes | No | 98% | Yes (single) | Yes | Yes | Yes | 1.0 × 10–15 mol/beta cell (C-peptide content), 2.83 × 10–18 mol/cell (C-peptide GSIS/β-cell *in vivo*)† |
| Kroon *et al.* [12] | Yes | Yes | Yes | ND | Yes | Yes | Yes | Yes | Yes | No | 96–98% | Yes (single) | ND | Yes | Yes | 1.25 × 10–18 mol/beta cell (C-peptide GSIS/β-cell, *in vivo*)† |
| Cho *et al.* [62] | No | No | No | Yes | No | No | No | No | No | Yes | ND | ND | ND | ND | ND | NA |
| Phillips *et al.* [61] | No | No | No | No | Yes | No | No | No | No | No | ND | No | Yes | Yes | No | 3.3 × 10–19 mol/cell (C-peptide content *in vivo*) |
| Jiang *et al.* [46] | No | No | No | Yes | Yes | No | No | No | No | No | ND | No | No | No | Partial | 2.97 × 10–20 mol/cell (C-peptide content *in vitro*); 5.0 × 10–19 mol/cell (C-peptide GSIS, *in vivo*) |
| Jiang *et al.* [4] | Yes | No | No | No | Yes | No | No | No | No | Yes | ND | Yes (mixed morphology) | Yes | No | ND | 9.9 × 10–18 mol/cell (C-peptide GSIS *in vitro*)† |
| Clark *et al.* [6] | No | No | No | No | ND | ND | ND | ND | ND | No | ND | ND | Yes | Yes | ND | 9.0 × 10–20 mol/cell (C-peptide release, *in vivo*)† |
| Shim *et al.* [44] | No | No | No | No | Yes | ND | ND | ND | ND | Yes | ND | ND | ND | ND | Partial | ND |
| Lavon and Benvenisty [60] | No | No | No | No | No | No | No | No | No | ND | ND | ND | ND | ND | ND | NA |
| Baharvand *et al.* [59] | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | Yes (mixed morphology) | Yes (INS) | ND | ND | 4.89 × 10–18 mol/cell (insulin GSIS *in vitro*)‡ |

(*Continued*)

**Table 1** *(Continued)*

| Publication | Differentiation steps (protein expression) | | | | β-Cell markers (protein expression) | | | | | | β-Cell functional characteristics | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | DE (SOX17, CXCR4) | Foregut endoderm (hNF4a, HNF 1b) | Pancreatic endoderm (NKX6.1, PDX1) | Endocrine precursors (NGN3, NKX2.2) | C-peptide, PDX1 | C-peptide, PDX1, NKX6.1 | C-peptide, MAFA | PC1/3, C-peptide | PC2, C-peptide | Endocrine hormone co-expression | Percentage proinsulin processing | Distinct granule morphology (EM) | GSIS *in vitro* (C-peptide) | GSIS *in vivo* (C-peptide) | Correct STZ hyperglycaemia | C-peptide levels* |
| D'Amour *et al.* [3] | Yes | Yes | Yes | Yes | Yes | No | No | ND | ND | Yes | ~50% | Yes (mixed morphology) | No | ND | ND | 3.12 × 10–16 mol/cell (C-peptide content *in vitro*, DTZ enriched) |
| Xu *et al.* [58] | No | No | No | No | No | No | No | No | No | Yes | ND | ND | ND | ND | ND | NA |
| Brolen *et al.* [57] | ND | ND | ND | ND | Yes | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Segev *et al.* [56] | No | No | No | No | No | No | No | No | No | Yes | ND | ND | Yes (INS) | ND | ND | 1.2 × 10–18 mol/cell (insulin GSIS *in vitro*)‡ |
| Assady *et al.* [55] | No | No | No | No | No | No | No | No | No | No | No | No | No | No | No | 10.9 ng insulin/h‡ |

*300 pg protein/cell; 6 pg DNA/cell.
†Mercodia ultrasensitive C-peptide enzyme-linked immunosorbent assay has the following cross-reactivities: Proinsulin Des (64–65) 74%; Proinsulin Split (65–66) 10%; Proinsulin Des (31–32) 3%.
‡Insulin secretion assays are difficult interpret do to extensive insulin in the culture media that may be non-specifically adsorbed and released during glucose stimulated insulin secretion (GSIS) assay.

conclusion of this study, the grafted cells were shown to be multihormonal, and for all the accepted reasons listed above, by definition, cannot be considered glucose responsive [44].

## Putting it all Together

An example of the importance of adherence to rigorous experimental design and thorough quantitative examination of differentiated cells in these complex protocols is exemplified by assessment of another recently published work claiming derivation of functional insulin-producing cells from hESCs [46]. In the first and most important step of the differentiation method the authors fail to clearly delineate the percentage of the culture claimed to be DE by employing previously published methods that employ FACS or immunohistochemistry analysis for CXCR4-SOX17 or CXCR4-FOXA2. Because extraembryonic endoderm (EE) expresses many of the traditional markers expressed in DE, it is essential to use combinations of markers that more specifically rule out EE and confirm DE. As described previously for both mouse and hESC differentiation to DE [2,15], CXCR4 is not expressed on visceral endoderm or ectoderm but is robustly expressed on DE and mesoderm lineage cells immediately postgastrulation [14]. Therefore, the combination of CXCR4, FoxA2 and SOX17 should rule out mesoderm, ectoderm and visceral endoderm, more clearly defining the differentiation status of the DE culture. Further examination of additional EE, neural ectoderm and trophectoderm markers such as SOX7 and thrombomodulin (EE), SOX1, PAX6 and ZIC1 (neural) and CDX2 and HCG (trophectoderm), would also complement this description and better define the purity of the cultures [2]. Even though the expression of BRACHYURY represents an important marker of the mesendoderm state that ideally the entire culture should pass through, it is also vital to demonstrate that BRACHYURY expression is rapidly extinguished when the culture transitions to DE as opposed to the mesoderm lineage. At the end of the DE-inductive phase, ideally greater than 80% of the cell culture should be composed of SOX17-FOXA2-CXCR4 triple-positive cells. Finally, the loss of pluripotent stem cell markers such as OCT4, NANOG and SOX2 in these early endoderm cultures should be demonstrated using quantitative measures such as FACS and QPCR (quantitative polymerase chain reaction). It must be emphasized that the purity and efficiency of hESC-to-DE conversion is essential for productive conversion to foregut followed by pancreatic endoderm and eventually to the endocrine, hormone-expressing cells desired [3].

In the second phase of the differentiation, RA is applied to the DE culture. RA is an extremely potent and pleotrophic signalling molecule and must be utilized in a precisely timed manner to ensure appropriate differentiation of hESC cell cultures. It is important to appreciate that RA signalling appears to be required during the later stages of pancreatic foregut specification into pancreatic epithelium competent to produce endocrine tissue, and has been demonstrated to bias pancreatic endoderm to form pancreatic endocrine as opposed to acinar phenotypes [25,29–31].

In the work by Jiang *et al.* [46] as well as many others (see table 1) protein expression of the critical pancreatic endoderm specifying genes including NKX6.1, PTF1α and PDX1 were not demonstrated. These markers must be localized by double and triple antibody staining within the same cell to specifically define pancreatic epithelium as opposed to other PDX1 expressing intestinal or gastric foregut endoderm cells. Other genes such as HLXB9 [47], PDX1 [48,49] and HNF4α [50] are endodermal markers but by themselves they do not uniquely define pancreatic epithelium. HLXB9 and HNF4α are expressed in dorsal and ventral endoderm cells outside the pancreatic domain, and PDX1-expressing endoderm comprises intestinal and gastric epithelium in addition to pancreatic endoderm [51]. Not only is it important to describe the production of considerable numbers of pancreatic endoderm cells expressing minimally NKX6.1 and PDX1, but it is also required to demonstrate high-level expression of these two critical factors in mature glucose-responsive β-cells [12]. Jiang *et al.* [46] also indicate that NGN3 is co-expressed with C-peptide, which developmentally does not occur [52,53], suggesting improper specification of endocrine cells in these cultures. In the islet-like clusters described in the terminally differentiated cultures in this work [46], the PDX1-C-peptide co-positive cells were not examined simultaneously for co-expression of glucagon or somatostatin to rule out immature multihormonal expression, as previously reported [3,4,44]. Furthermore, there was no attempt to measure protein expression of NKX6.1 or MAFA in combination with PDX1 and C-peptide in the same cell to unambiguously define a glucose-responsive cell. The *in vitro* glucose-stimulated insulin secretion experiments solely examined insulin release as opposed to the well-accepted standard of C-peptide release to rule out non-specific insulin adsorption and re-release in high glucose [54]. This is perplexing because the authors demonstrate immunohistochemical C-peptide expression in their differentiated cells. As presented in table 1, the calculated C-peptide content of the terminally differentiated cells *in vitro* was $2.97–5.95 \times 10^{-20}$ mol/cell. Compared to human adult islets, these levels are nearly 17 000-fold lower. It seems difficult therefore to adequately explain how 30% of the animals engrafted with as few as $10^6$ of these cells achieve improvement of the diabetic state.

Indeed, the majority of the other published studies using human pluripotent embryonic stem or germ cells [3,4,6,44,46,55–62] have not demonstrated a mature β-cell phenotype including full processing of proinsulin to insulin and C-peptide, single hormone expression together, with co-expression at the protein level of the mature pattern of transcription factors PDX1, NKX6.1 and MAFA which are required for glucose sensing (table 1). As described in our previous work [3], β-cells lacking the full complement of these properties are by definition not glucose-responsive, as would also be expected of those with similar characteristics reported by Jiang *et al.* [4]. Finally, conversion of reported C-peptide release levels either *in vitro* or *in vivo* into standard common comparison values (moles C-peptide/cell) indicates that only the study by Kroon *et al.* [12] closely matches human adult islet levels *in vivo*. Not only do these glucose-responsive cells fully correct the diabetic state for >100 days, the C-peptide levels have been validated with respect to mature processing (PC1/3, PC2 protein expression) of proinsulin to insulin and C-peptide.

In summary, the differentiation of human pluripotent stem cells to glucose-responsive β-cells requires meticulous application of developmental principles to the stem cell culture system. More importantly, it necessitates fastidious utilization of multiple quantitative gene (QPCR) and protein (FACS) expression assay systems in concert with high quality immunohistochemical methods for co-localizing two to four fluorophors to the same cell. This is obligatory for determining the homogeneity and efficiency of the multiple differentiation steps and indispensable for verifying the intermediate and terminal differentiated phenotypes at the cellular level. Demonstration of mature β-cell phenotype necessitates that insulin/C-peptide producing cells express all essential transcription factors, processing enzymes and mutually exclusive hormone-containing storage granules. The appropriate transcription factor profile establishes the final differentiated state of a β-cell, which also includes expression of the glucose transporter Glut1 together with glucokinase and the appropriate $K^+_{ATP}$ and calcium channels for proper glucose sensing and insulin release. If the β-cell is specified and differentiated with regard to all of the above, it should be glucose-responsive by definition. Lastly, the demonstration of glucose stimulated insulin secretion (GSIS) (in comparison to human adult

islet controls) should reveal robust levels of human C-peptide (2–10 ng/ml) after IPGTT (intraperitoneal glucose tolerance test) in grafted animals [45]. This is the ultimate readout of mature β-cells. In the end, stable long-term maintenance of normal blood glucose levels (>100 days), in the absence of endogenous β-cells, makes reaching the β-cell proof of principle a very difficult feat to accomplish with any pluripotent or multipotent stem cell line.

For the future, replication of the signals important in the final phase of pancreatic endoderm specification towards acinar and endocrine cells will need to be replicated *in vitro* to achieve proper specification of terminally differentiated endocrine cells which are glucose responsive. This may require simulation of the signalling pathways effected by interaction with mesenchymal elements especially vascularizing endothelial cells shown to be especially important for endocrine and acinar cell specification [63,64]. Proper specification of β-cells from hESCs *in vitro* may also require simulation of appropriate 3-D configurations favourable to formation of appropriate cellular connections and matrix interactions necessary for functional maturation [65,66].

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