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Protocols to differentiate embryonic stem cells into insulin-producing cells

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Abstract

Embryonic stem cells represent an interesting alternative to treat diabetes due to their two main properties: self-renewal by symmetric divisions, and the potential for cell differentiation into insulin-expressing cells under certain culture conditions. Achieving the latter implies the modification of the culture medium adding specific growth factors and/or reprogramming the cells by transfecting specific DNA constructs. Several laboratories have used one or a combination of such strategies in order to obtain insulin-positive cells with different degrees of success. The main obstacles posed in the different protocols concern the amounts of intracellular insulin stored and secreted in response to different stimuli, the correct processing of the hormone, the risk of forming teratomas and the immune rejection once cells are transplanted. Promising results have been obtained from protocols which try to recapitulate the pancreas ontogeny in the culture dish. To this end, it is instrumental to understand the embryonic development process of the human pancreas from definitive endoderm to the adult, as well as its molecular markers and the functional traits of the obtained cells in order to achieve a transplantable product to cure diabetes. This manuscript explores published protocols following this objective, analyzing the main features that could contribute to elaborate a more definitive strategy. The final protocol must be universal, easily transferable to the clinical practice and, most importantly, liberate patients from the insulin injections without the development of secondary complications.

Keywords: diabetes, β-cell, embryonic stem cells, cell bioengineering, insulin.

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Abbreviations:

AFP: α-Fetoprotein; Alb1: Albumin 1; Amn: Amnionless; Bry: Brachyury ; EBs: embryoid bodies; ECD: E-Cadherin; ESCs: embryonic stem cells; GFP: green fluorescent protein; GLP-1: glucagon-like peptide-1; Gsc: Goosecoid; LIF: leukaemia inhibitory factor; NCD: N-Cadherin; RT-PCR: reverse transcriptase-polymerase chain reaction; Shh: Sonic hedgehog.

Introduction

Spontaneous insulin gene expression in embryonic stem cell (ESCs) cultures alerted many scientific groups of their potential as an alternative β-cell source in future cell replacement clinical trials to treat diabetes. To initiate the spontaneous differentiation pathway towards insulin$^+$ cells, ESCs are transferred from monolayer to suspension cultures in the absence of leukaemia inhibitory factor (LIF). Under these non-adherent conditions, cells tend to aggregate forming clusters called embryoid bodies (EBs). Ectoderm, mesendoderm and primitive endoderm markers can be observed at different culture times in these cell aggregates.[1] Two main questions arise from this observation. One concerns how differentiation programmes can be activated when ESCs form EBs. Gradients in nutrients, growth factors and oxygen levels throughout the EB structure, as well as intercellular connections, could contribute to this process. The second question concerns how the expression of specific embryonic layer markers may be sufficient proof that EB formation recapitulates the key steps in embryonic development. In this context, it is important to mention that it is very seldom for one gene to be exclusively expressed in a specific cell lineage. This is the case of, for example, Amnionless (Amn), a good primitive endoderm marker.[2] On the other hand, Brachyury (Bry) is considered a mesendoderm marker; however this gene is also expressed in the primitive endoderm.[3]

In addition, EBs obtained from ESC monolayers are heterogeneous structures, displaying a large variety of morphologies.[4] This chaotic patterning in EBs could be circumvented using coaxial and/or directional strategies, thereby committing EBs to specific cell fates. However, it is difficult to achieve a 100% commitment to a specific cell lineage, since ESC monolayers contain undifferentiated cells and precursors that very often differentiate into ectoderm derivatives.[5] Also, it is difficult in coaxial strategies to facilitate the access of growth factors and compounds present in the culture medium to all cells located in the EB. Therefore, obtaining definitive endo-

derm, the embryonic layer from which the endocrine pancreas derives, via EBs is difficult to achieve. In the best of cases, EB structures can be modestly enriched in specific cell types that are capable of progressing towards endocrine pancreatic fates.[6] On the other hand, transfection with DNA constructs coding for key transcription factors, an approach used in directional strategies, must face the problem of transcription factor specificity, which usually is not exclusively expressed in pancreatic β-cells.[7] Finally, the existence of culture artefacts must be strongly considered, since some genetic programmes could be modulated in response to particular culture conditions rather than specific embryogenesis programmes that could be spontaneously activated *in vitro*.

Altogether, we must be careful when analyzing the phenotype of a final cell product that expresses insulin. First of all, insulin is not the unique distinctive trait of a pancreatic β-cell. In fact, insulin is also expressed in neuroectoderm-derived tissues as well as primitive endoderm,[8] and can even be taken from the culture medium.[9] In the latter, serum is a good source of extracellular insulin that can be incorporated into cells in vesicle-like structures. Besides insulin, many transcription factors that are ascribed exclusively to β-cells, i.e. Pdx-1, are expressed in other cell types.[10] Second, RT-PCR and immunocytochemistry figures seen in publication should be complemented with functional tests that could give more complete data about the real therapeutic potential of the final cell product. Such functional tests should include static and dynamic insulin secretion assays, analysis of intracellular Ca^{2+} patterns in response to different secretagogues, and demonstration of the existence of a processing pathway that converts preproinsulin to mature insulin, as well as a packaging system of the hormone in secretory vesicles that are connected to a sensor-coupling system.

Therefore, it can be concluded that insulin$^+$ cells obtained from ESCs can have different origins. We have observed that spontaneous differentiation goes to favour the neuroectodermal pathway since neuroectoderm does not require complex signals in order to differentiate.[5] Only coaxial strategies can give rise to modest amounts (2-7%) of insulin-producing cells derived from definitive endoderm.[6,11] One key question concerns the possibility of bioengineering neuroectoderm-derived insulin$^+$ cells into a β-cell phenotype. In this context, the main changes to introduce should be: to increase the amount of hormone synthesised, to achieve a correct processing from preproinsulin to mature insulin (neuroectodermal tissues barely express endopeptidase PC2) and to obtain a nutrient-regulated expression of the insulin gene (i.e. glucose). Alternatively, mimicking the process by which definitive endoderm arises during embryonic development could result in cells bearing a phenotype very similar to pancreatic β-cell precursors.[11] In mouse, this process can be followed by controlling the expression of insulin I gene, exclusively expressed in pancreatic β-cells, versus insulin II expression, typical of neuroectoderm and primitive endoderm tissues.[8] In humans, there is only one insulin gene, requiring complementary proofs to be certain that the final cell product is a cell that fulfils the criteria of a definitive endoderm-derived insulin$^+$ cell. Taking into account this rationale, several protocols have been developed. This manuscript analyzes them in order to find the keys towards a more complete protocol.

Derivation of embryonic stem cells to definitive endoderm

Several protocols have been designed to study the possibility of driving ESCs to definitive endoderm (table 1). Definitive endoderm is the parental embryonic layer from which β-cell derives. Unfortunately this lineage shares many markers with primitive endoderm. Therefore, the main obstacle to circumvent in this group of strategies concerns the distinction between definitive and primitive endoderm. The use of a combination of various markers to establish lineage association has been the proper criterion adopted for the identification of definitive endoderm precursors.[13,15] During embryogenesis, different morphological structures are evident, such as the blastocyst, gastrula, primitive streak, etc. On the contrary, in the culture plate these structures are not so evident and cells display an apparent homogeneous morphology. In addition, a precise time schedule cannot be applied and only RT-PCR and protein expression profiles can give some clues concerning the in vitro time course of the differentiation route, which can differ greatly from the in vivo process (figure 1).

During mouse gastrulation, the primitive streak recruits cells from specific regions of the epiblast, where they undergo the epithelial-to-mesenchymal transition, giving rise to mesoderm (posterior streak) and definitive endoderm (anterior streak).[18,19] This common origin within the primitive streak supports the idea that both lineages could arise in vivo from an original progenitor called the mesendoderm. Despite the controversy in mouse embryogenesis, this concept, when applied in vitro, has resulted in human and mouse ESCs to differentiate towards definitive endoderm derivatives (table 1).

The report of Gadue et al.[16] is focused on characterising a cell population isolated by homologous recombination

Table 1. Differentiation strategies to obtain definitive endoderm committed cells from ESCs

Reference	Cell type	Culture conditions	Gene markers
6	Mouse E14.1 (HR: Bry-GFP)	**Undifferentiated:** Feeder layer. DMEM, 1% LIF, 1,5x10^{-4} M MTG and 15% FCS **Differentiation:** A) 1.1- EB (10^3- 8x10^4 cells/ml). IMDM, 2 mM Glutamine, 0.5 mM ascorbic acid, 4.5x10^{-4} M MTG, 5% PFHM-II, 200 μg/ml Transferrin and 15% FCS (2.5 days) 1.2- EB. IMDM , 2 mM Glutamine, 0.5 mM ascorbic acid, 4.5x10^{-4} M MTG and 15% KSR (4 days) B) 1.1- EB (10^3- 8x10^4 cells/ml). IMDM, 2 mM Glutamine, 0.5 mM ascorbic acid, 4.5x10^{-4} M MTG, 5% PFHM-II, 200 μg/ml Transferrin and 15% FCS (2.5 days) 1.2- EB. Stem Pro 34 Medium, 2 mM Glutamine, 0.5 mM ascorbic acid, 4.5x10^{-4} M MTG, 1% Kit ligand and 15% KSR (2 days) 1.3- EB. IMDM, 2mM Glutamine, 0.5 mM ascorbic acid, 4.5x10^{-4} M MTG, 100 ng/ml Activin A and 15% KSR	– Foxa2 (RT-PCR, IC) – Tcf1 (RT-PCR) – Hnf4 (RT-PCR) – Sox17 (RT-PCR) – AFP (RT-PCR) – Alb1 (RT-PCR, IC) – Hhex (RT-PCR) – TTR (RT-PCR) – AAT (RT-PCR) – TAT (RT-PCR) – Cps1 (RT-PCR) – Pdx1 (RT-PCR) – Mixl1 (RT-PCR) – Sftpc (RT-PCR)
12	Mouse 129/SvJ	**Undifferentiated:** DMEM, 0.1 mM βME, 1000 U/ml LIF, 1X NEAAs and 15% FBS **Differentiation:** A) EB. DMEM, 0.1 mM βME, 1X NEAAs and 15% FBS (54 days) B) 1. EB. DMEM, 0.1 mM βME, 1X NEAAs and 15% FBS (14 days) 2. EB disaggregated and culture in gelatin. 100 ng/ml aFGF. 15% FBS(2 days) 3. 20 ng/ml HGF. 15% FBS (3 days) 4. Replated on Matrigel. 20 ng/ml HGF, 10 ng/ml Oncostatin M, 10^{-7} M dexamethasone, ITS (5 mg/ml insulin, 5 mg/ml transferrin and 5 μg/ml selenious acid). 15% FBS (3 days)	– AFP (RT-PCR, IC) – GATA4 (RT-PCR, IC) – Aldolase B (RT-PCR) – Alb (RT-PCR) – Apoa2 (RT-PCR) – G6P (RT-PCR) – PAH (RT-PCR) – TTR (RT-PCR) – TAT (RT-PCR)
13	Human CyT25 CyT49 CyT203 BG01 BG02 BG03 H7 H9	**Undifferentiated:** Feeder layer. DMEM/F12, 1 mM NEAAs, 0.55 mM βME, 4 ng/ml FGF2 and 20% KSR (sometimes 10 ng/ml Activin A is added to maintain undifferentiated state) **Differentiation:** A) Monolayer. RPMI. 100 ng/ml Activin A and different concentrations of FBS (0.5-10%) (5 days) B) Monolayer. RPMI. 100 ng/ml Activin A or 100 ng/ml BMP4, 5 μM SU5402 and FBS (0% 1 day; 0,2% 1 day and 2% 2 days)	– Sox17 (RT-PCR, IC) – Gsc (RT-PCR) – Foxa2 (RT-PCR, IC) – Lhx1 (RT-PCR) – Wnt3 (RT-PCR) – Bry (RT-PCR, IC) – Fgf8 (RT-PCR) – Nodal (RT-PCR)
14	Mouse EB5 (HR: Oct3/4-Blasticidin S / Gsc-GFP	**Undifferentiated:** Gelatin. G-MEM, 0.1 mM NEAAs, 1 mM sodium pyruvate, 0.1 mM βME, 1,000 U/ml LIF, 20 μg/ml Blasticidin S, 1% FCS and 10% KSR **Differentiation:** A) Monolayer. Type IV collagen-coated 10 cm dishes (1x10^5 cells/dish). O3 medium, 0.1% BSA, 50 μM βME and/or 10 ng/ml Activin A, 10 ng/ml BMP4 and 1000 ng/ml Nodal. Serum free (4 days) B) EB (3x10^4 cells/dishes 6 cm). O3 medium, 0.1% BSA, 50 μM βME and/or 10 ng/ml Activin A, 10 ng/ml BMP4 and 1000 ng/ml Nodal. Serum free (4 days) C) Monolayer. Sorting Gsc$^+$ECD$^+$ and Gsc$^+$ECD^{low}. Type IV collagen-coated 10 cm dishes (1x10^5 cells/dish). O3 medium, 0.1% BSA, 50 μM βME and 10 ng/ml Activin A. Serum free (4-6 days)	– Bry (RT-PCR, IC) – Sox17 (RT-PCR) – Hex (RT-PCR) – Mixl1 (RT-PCR) – ECD (RT-PCR, IC) – Gsc (RT-PCR) – CK8 (RT-PCR) – CK18 (RT-PCR, IC) – CK19 (RT-PCR) – GATA4 (RT-PCR) – Claudin 6 (RT-PCR) – Foxa2 (RT-PCR, IC)

Table 1. Differentiation strategies to obtain definitive endoderm committed cells from ESCs *(continuation)*

Reference	Cell type	Culture conditions	Gene markers
15	Mouse EB5 (HR: Gsc-GFP / Sox17-hCD25 / Oct3/4-Blasticidin S)	**Undifferentiated:** Gelatin. G-MEM, 0.1 mM NEAAs, 1 mM sodium pyruvate, 0.1 mM βME, 1,000 U/ml LIF, 20 μg/ml Blasticidin S, 1% FCS and 10% KSR **Differentiation:** A) Definitive endoderm. Monolayer. Type IV collagen-coated 10 cm dishes. O3 medium and 10 ng/ml Activin A Serum free (6 days) B) Visceral endoderm. Monolayer (10^5 cells/ml). Gelatin or human fibronectin coated dishes. O3 medium. Serum free (6 days) C) Monolayer. Sorting $Gsc^+ECD^+Sox17^+$ and $Gsc^-ECD^+Sox17^+$. Type I collagen-coated. O3 medium, 20 ng/ml EGF, 20 ng/ml BMP4, 20 ng/ml aFGF and 5 ng/ml bFGF. Serum free (4-6 days)	– ECD (RT-PCR, IC) – Sox17 (RT-PCR, IC) – Gsc (RT-PCR) – Bry (RT-PCR) – Mixl1 (RT-PCR) – Foxa2 (RT-PCR) – Alb (RT-PCR) – AFP (RT-PCR) – TAT (RT-PCR) – Cyp7a1 (RT-PCR) – Cxcr4 (RT-PCR, IC)
16	Mouse E14.1 (HR: Bry-GFP / Foxa2-hCD4)	**Undifferentiated:** 50% Neurobasal medium, 50% DMEM/F12, 0.5X N2, 0,5X B27, 0.05% BSA, ? LIF, 10 ng/ml BMP4 and $1.5x10^{-4}$ M MTG. Free serum (without feeder) or containing ? serum (with feeder layer) **Differentiation:** 1) EB ($1.5x10^5$ cells/ml). 75% IMDM, 25% Ham´s F12 medium, 0.5X N2, 0.5X B27, 0.05% BSA, 2 mM Glutamine, 0.5 mM ascorbic acid and $4.5x10^{-4}$ M MTG. Free serum or containing ? serum (2 days) 2) EB dissociated and reaggregated. 1 or 25 ng/ml Activin A, 100 ng/ml Wnt3, 150 ng/ml DKK1 or 10 μM SB. Free serum (3-4 days)	– Cer1 (RT-PCR) – Gsc (RT-PCR) – Foxa1 (RT-PCR) – Foxa2 (RT-PCR) – Hnf4 (RT-PCR) – Pdx1 (RT-PCR) – AAT (RT-PCR) – Alb1 (RT-PCR) – Bry (HR: GFP)
17	Mouse E14.1 (HR: Bry-GFP / Foxa2-hCD4)	**Undifferentiated:** 50% Neurobasal medium, 50% DMEM/F12, 0.5X N2, 0,5X B27, 0.05% BSA, ? LIF, 10 ng/ml BMP4 and $1.5x10^{-4}$ M MTG. Free serum **Differentiation:** 1) EB (9,000 cells/ml for step 2.A or 20,000 cells/ml for step 2.B). 75% IMDM, 25% Ham´s F12 medium, 0.5X N2, 0.5X B27, 0.05% BSA, 2 mM Glutamine, 0.5 mM ascorbic acid and $4.5x10^{-4}$ M MTG. Free serum (2 days) 2.A) EB. 50 ng/ml Activin A (2 days) 2.B) EB dissociated and reaggregated. 50 ng/ml Activin A (2 days) 2.B.1 – EB dissociated, populations isolated by cell sorting and reaggregated (250,000 cells/ml). Combination of: 50 ng/ml BMP4, 10 ng/ml bFGF, 50 ng/ml Activin A, 10 ng/ml VEGF (2 days) 2.B.2 – Outgrowth. Presence of adherent colonies and suspended aggregates. Combination of: 50 ng/ml BMP4, 10 ng/ml bFGF, 50 ng/ml Activin A, 10 ng/ml VEGF (6 days)	– Alb1 (RT-PCR, IC) – AFP (RT-PCR, IC) – Sox17 (RT-PCR) – Hhex (RT-PCR) – Cyp7a1 (RT-PCR) – Cyp3a11 (RT-PCR) – Ipf1 (RT-PCR) – Spc (RT-PCR) – TAT (RT-PCR) – Bry (HR:GFP) – Foxa2 (HR: hCD4) – c-Kit (FACS) – Cxcr4 (FACS)

Abbreviations used: aFGF: acidic fibroblast growth factor, bFGF: basic fibroblast growth factor, BMP4: bone morphogenetic protein 4, Bry: brachyury, BSA: bovine serum albumin, βME: beta-mercaptoethanol, DKK1: dickkopf-1 (inhibitor of Wnt signalling), DMEM: dulbecco´s modified Eagle´s medium, EB: embryoid body, EGF: epidermal growth factor, FACS: fluorescence-activated cell sorting, FBS: fetal bovine serum, FCS: fetal calf serum, FGF2: fibroblast growth factor 2, GFP: green fluorescent protein, G-MEM: Glasgow minimum essential medium, Gsc: Goosecoid, HGF: Hepatocyte growth factor, HR: Homologous recombination, IC: Immunocytochemistry, IMDM: Iscove´s modified Dulbecco´s medium, ITS: Insulin-Transferrin-Selenious acid, KSR: Knockout serum replacement, LIF: Leukemia inhibitory factor, MTG: Monothioglycerol, NEAAs: Nonessential amino acids, PFHM: Protein free hybridoma medium, RPMI: Culture medium, RT-PCR: Reverse transcriptase-Polymerase chain reaction, SB: Inhibitor SB-431542, SU5402: Inhibitor of FGFr1 (fibroblast growth factor receptor-1), VEGF: Vascular endothelial growth factor, ?: Unknown concentration.

(Bry-GFP^+/Foxa2-$CD4^{High}$) using anterior primitive streak markers, which are therefore definitive endoderm precursors. The expression of additional markers (table 1) supports this hypothesis. However, these markers are also expressed in the visceral endoderm, suggesting that the presence of a mixed population of definitive and visceral endoderm cannot be excluded. In this report,[16] commitment to definitive endoderm is enhanced by activating Wnt and Nodal pathways using pharmacological agonists. Again the pattern of gene expression observed

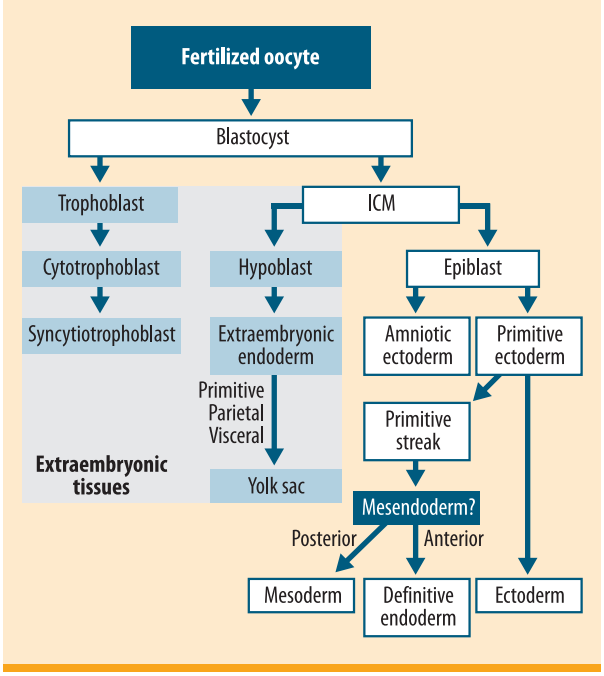

Figure 1. Schematic representation of the mouse and human early development. ICM: Inner cell mass

under these conditions cannot exclude the presence of visceral endoderm in the final cell population obtained.

Differentiation to mesendoderm, as a precursor of definitive endoderm, has been exploited in the report of Kubo et al.[6] using homologous recombined cells in the Bry locus (Bry-GFP). Although Bry is also expressed in visceral endoderm[3], the most convincing results of definitive endoderm commitment were obtained when the sorted Bry$^+$ population was incubated in the presence of dexamethasone. Under these conditions, the resulting cell population expressed TAT and AAT genes, very liver-specific, indicating that a part of the Bry$^+$ population can derive to definitive endoderm. In the same culture conditions, but in the presence of activin A, early hepatic endoderm was obtained according to the expression of Tcf1 and Sftpc.

In the protocol of Choi et al.,[12] EBs cultured for 2 weeks were capable of, through spontaneous differentiation, to give rise to cells expressing aldolase B and ApoA2, which are liver specific markers. We cannot, however, discard that this could be a specific cell line property. A coaxial protocol incubating EBs derived from these ESCs with different growth factors and compounds resulted in obtaining definitive endodermal cells and hepatic precursors (table 1).

Yasunaga et al.[15] reported that it was possible to isolate mouse cells committed to definitive endoderm through the use of specific selection strategies and culture conditions. Based on the expression of Gsc (goosecoid), Sox17/CD25 and E-Cadherin (ECD), they established a mesendoderm precursor line (Gsc$^+$/Sox17$^+$/ECD$^+$) vs a visceral endoderm line (Gsc$^-$/Sox17$^+$/ECD$^+$). However, Gsc is also expressed in visceral endoderm, making it difficult to understand how a Gsc$^-$ population could be considered a visceral endoderm precursor. Nevertheless, the expression of Pem and Amnionless in the Gsc$^-$ population ratifies its commitment to visceral/parietal endoderm. In the same context, the expression of Cxcr4 in the Gsc$^+$ population supports its definitive endoderm precursor fate.

In the protocol of D'Amour et al.,[13] two cell populations were obtained, which differed in the gene expression pattern. One population, expressing Sox7, Bry, Meox1, Sox1 and Zic1, was committed to primitive endoderm. Nevertheless, the presence of neuroectoderm (Sox1$^+$/ Sox7$^+$/ Zic1$^+$) cannot be discarded. The other population committed to definitive endoderm and expressed, mainly in the presence of activin A and low serum, Sox17, Gsc, Mixl1 and Foxa2 which are also primitive endoderm markers. The incubations in the presence of BMP4 and SU5402 were non-conclusive, since the selected markers (Nodal, FGF8, Bry) were expressed both in definitive and visceral endoderm. The same could be extended to the downregulation of ECD vs upregulation of N-Cadherin (NCD), since both markers were very ubiquitous. The most solid argument was the isolation of a Cxcr4$^+$ sorted population, a very specific definitive endoderm marker. In this population, Sox17, Gsc, Mixl1 and Foxa2 were expressed at higher levels than in the Cxcr4$^-$ population.

Tada et al.[14] isolated a cell population Gsc-GFP$^+$ that was committed to definitive endoderm in absence of serum. All markers used to discriminate visceral from definitive endoderm are also expressed in the neuroectoderm and mesoderm. The expression of Cxcr4 (definitive endoderm marker) or Amn (primitive endoderm marker) were not documented. However, at long culture periods they observed the expression of homeotic gene Prox1, suggesting that the Gsc$^+$/ECD$^+$ population bears a definitive endoderm profile. No data concerning Prox1 expression were documented in the line Gsc$^+$/ECD$^-$ (mesoderm) in order to ratify the selection protocol.

The population obtained through cell sorting (Bry-GFP$^+$/ Foxa2-CD4$^+$/c-Kit$^+$) by Gouon-Evans et al.[17] seemed to be definitive endoderm precursors, since α-fetoprotein (AFP) and albumin 1 (Alb1) were not expressed. Nevertheless, the sorting was made very early and Amn expression should have been included to ensure that primitive endoderm precursors were not present. The presence

of activin A favoured the commitment to definitive endoderm of the sorted population, which derived to a population that expressed typical hepatic tissue markers.

From definitive endoderm to insulin-producing cells

Although spontaneous differentiation from ESCs to insulin$^+$ cells has been documented[20, 21], it is usually necessary to apply specific culture conditions to drive the cells from definitive endoderm towards endocrine pancreatic cells (table 2). In this sense, endoderm-derived organs follow specific developmental patterns which can be mimicked in vitro by adding specific factors to the culture medium (table 2). However, the varied results seen in the published reports suggest that the efficiency could be related to different aspects, such as the factor concentration in the culture medium, exposition time and cell type, among others. Nevertheless, the efficiency of these factors to obtain insulin-producing cells increases consistently when the cells are committed to definitive endoderm.

The reference protocol in this group of strategies is described in the report of D'Amour et al.[11]. This protocol mimics in vitro the key differentiation steps from definitive endoderm to endocrine pancreatic islets. This protocol presents interesting aspects that deserve some attention. First of all, some of the markers used in the initial stages of the protocol to assess the progression towards a pancreatic fate are also expressed in the primitive endoderm and neuroectoderm. For instance, in step 3 (posterior foregut) Hnf6 was expressed, however when the cells were committed to neuroectoderm by adding follistatin, Hnf6 (neuroectoderm marker) was not expressed. It is possible that some cells were not properly following the corresponding differentiation pathway. Second, the protocol duration was approximately 15 days, whereas in vivo the process to obtain endocrine pancreatic cells takes around 3 months. Third, the resulting cells possessed high insulin contents, similar to those found in mature β-cells, yet it seems that the hormone is unprocessed. Fourth, cells responded to multiple secretagogues, except glucose, the main inducer of insulin release. Fifth, some cell clusters were co-expressing insulin and glucagon, an observation difficult to assimilate when in the pancreas embryogenesis insulin$^+$ and glucagon$^+$ cells derive from independent lineages. Sixth, MafA, a transcription factor required to obtain mature β-cells,[30] was not detected. Seventh, not all ESC lines that can commit to definitive endoderm can progress in vitro through the endocrine pancreas pathway.

In any case, this excellent report represents a working base for future research, provided that certain changes may be introduced in order to improve the efficiency and function of the resulting insulin-secreting cells. Jiang et al.[27] differentiated H9-human ESCs cells to insulin-secreting cells, whereas in the protocol of

Table 2. Differentiation strategies to obtain insulin-secreting cells from definitive endoderm committed cells derived from ESCs

Reference	Cell type	Culture conditions	Gene markers
22	Mouse EB3 (HR: Oct3/4-Blasticidin S)	**Undifferentiated:** DMEM (4.5 mg/l D-glucose), 0.1 mM 2 βME, 0.1 mM NEAAs, 1 mM sodium pyruvate, 1000 U/ml LIF and 10 % FBS **Differentiation:** 1) Hanging drop (500 cells/20 μl) without LIF (5 days) 2) Outgrowth in gelatin (20 EBs/ dish) 10% FBS (23 days)	– Foxa2 (RT-PCR) – Pdx1 (RT-PCR) – ProINS I (RT-PCR) – ProINS II (RT-PCR) – GCG (RT-PCR) – PP (RT-PCR) – GLUT2 (RT-PCR) – IGRP (RT-PCR) – INS (IC)
23	Mouse R1 E14.1 CCE	**Undifferentiated:** Feeder layer. DMEM, ? LIF, $3x10^{-4}$ M MTG and 15% FCS **Differentiation:** 1) EB ($5\text{-}2x10^3$ cells/ml). IMDM, 50 μg/ml ascorbic acid, $6x10^{-3}$ M MTG and 15% FCS (2 days) 2) EB. IMDM, 50 μg/ml ascorbic acid, $6x10^{-4}$ M MTG and 15% FCS or KSR (4 days) 3.1. EB or Outgrowth in gelatin. DMEM/F12 (1:1) with or without 10 ng/ml FGF2 and 15% KSR (5-11 days) 3.2. EB coming from step 2 with 15% FCS. DMEM/F12 (1:1), 10 mM Nicotinamide, 0.1 nM Exendin-4, 10 ng/ml Activin βB and 15% KSR (13 days)	– Amylase 2 (RT-PCR) – GCG (RT-PCR, IC) – Foxa2 (RT-PCR) – INS I and II (RT-PCR) – INS (IC) – Pdx1 (RT-PCR) – Sox17 (RT-PCR) – C-peptide (IC)

Table 2. Differentiation strategies to obtain insulin-secreting cells from definitive endoderm committed cells derived from ESCs *(continuation)*

Reference	Cell type	Culture conditions	Gene markers
11	Human CyT25 CyT49 CyT203 BG01 BG02 BG03	**Undifferentiated:** Feeder layer. DMEM/F12, 1 mM NEAAs, 0.55 mM βME, 4 ng/ml FGF2 and 20%KSR.(sometimes 10 ng/ml Activin A is added to maintain undifferentiated state) **Differentiation:** 1) Monolayer. RPMI, 100 ng/ml Activin A, 25 ng/ml Wnt3a (first 2 days) and FBS (0% 2 days; 0.2% 2 days) 2) Monolayer. RPMI, 50 ng/ml hFGF10, 0.25 μM KAAD-cyclopamine and 2% FBS (4 days) 3) Monolayer. DMEM, 50 ng/ml hFGF10, 0.25 μM KAAD-cyclopamine, 2 μM RA and 1% B27 (4 days) 4) Monolayer. DMEM, 1 μM DAPT, 50 ng/ml Exendin 4 and 1% B27 (3 days) 5) Monolayer. CMRL, 50 ng/ml Exendin 4, 50 ng/ml IGFI, 50 ng/ml HGF and 1% B27 (>3 days)	– Sox17 (WB, IC) – Foxa2 (WB) – Hnf1βWB, IC – Pdx1 (WB, IC) – Nkx2.2 (WB, IC) – Nkx6.1 (WB, IC) – C-peptide (WB) – Cer (RT-PCR) – Cxcr4 (RT-PCR) – Hnf4a (RT-PCR) – Hnf6 (IC) – Ngn3 (IC) – INS (IC) – GCG (IC) – Ghrelin (IC) – SST (IC) – PP (IC) – Isl1 (IC)
24	Mouse R1	**Undifferentiated:** Feeder layer. DMEM (4.5 g/l glucose), 2 mM L-glutamine, ? NEAAs, 100 μM βME, 1000 U/ml LIF and 15% FCS **Differentiation:** 1) Hanging drop (600 cells/ 20 μl). IMDM, 2 mM L-glutamine, ? NEAAs, 450 μM MTG and 20% FCS (2 days) 2) EB in suspension. IMDM, 2 mM L-glutamine, ? NEAAs, 450 μM MTG and 20% FCS (3 days) 3) Outgrowth with gelatin. IMDM, 2 mM L-glutamine, ? NEAAs, 450 μM MTG and 20% FCS (9 days) 4) Outgrowth with poli-L-ornithine-laminin. DMEM/F12 (4.5 g/l glucose), 20 nM progesterone, 100 μM putrescine, 1μg/ml Laminin, 10 mM nicotinamide, 25 μg/ml insulin, 50 μg/ml transferrin and ? B27. Serum free (6 or 18 days)	– Isl1 (RT-PCR) – Pdx1 (RT-PCR) – INS 1 (RT-PCR) – INS 2 (RT-PCR) – INS (IC) – GCG (RT-PCR) – SST (RT-PCR) – CK19 (IC) – C-peptide (IC)
25	Human Miz-hES4 Miz-hES6	**Unifferentiated:** Feeder layer. DMEM/F12, 0.1 mM βME, 1% NEAAs, 1 mM Glutamine, 4 ng/ml bFGF and 20% KSR **Differentiation**: 1) EB. DMEM/F12, 0.1 mM βME, 1% NEAAs, 1mM Glutamine and 20% FBS (4 days) 2) EB. DMEM/F12, 0.1 mM βME, 1% NEAAs, 1mM Glutamine, 30 ng/ml Activin A. Serum free (4 days) 3) EB. DMEM/F12, 0.1 mM βME, 1% NEAAs, 1mM Glutamine, 10 μmol/l RA. Serum free (2 days) 4) Outgrowth. ITS medium and 5 μg/ml Fibronectin. Serum free (10-13 days)	– Foxa2 (RT-PCR, IC) – Sox17 (RT-PCR, IC) – Mixl1 (RT-PCR) – Bry (RT-PCR, IC) – Pdx1 (RT-PCR, IC) – Hlxb9 (RT-PCR) – Cdx2 (RT-PCR) – Ngn3 (RT-PCR) – NeuroD1 (RT-PCR) – Nkx2.2 (RT-PCR) – Nkx6.1 (RT-PCR) – Isl1 (RT-PCR) – GATA4 (IC) – INS (IC) – GCG (IC)

Table 2. Differentiation strategies to obtain insulin-secreting cells from definitive endoderm committed cells derived from ESCs *(continuation)*

Reference	Cell type	Culture conditions	Gene markers
26	Human H1 H7 H9	**Undifferentiated:** Conditionated medium of MEF cells: 80% KO-DMEM, 1mM Glutamine, 0.1 mM βME, 1% NEAAs, 4 ng/ml hbFGF and 20% KSR **Differentiation:** 1) Monolayer. RPMI 1640, 11.1 mM Glucose, 1X B27, 4 nM activin A, 1 mM NaB (1 day) and then 0.5 mM NaB (6 days) 2) Suspension. RPMI 1640, 11.1 mM Glucose, 1X B27, 20 ng/ml EGF, 2 ng/ml bFGF and 100 ng/ml Noggin (14 days) 3) Suspension. RPMI 1640, 11.1 mM Glucose, 1X B27, 20 ng/ml EGF and 100 ng/ml Noggin (7 days) 4) Suspension. RPMI 1640, 11.1 mM Glucose, 1X B27, 0.5% BSA, 10 mM Nicotinamide and 50 ng/ml IGF-II (5 days) and them without IGF-II (2 days)	– Sox17 (RT-PCR, IC) – Foxa2 (RT-PCR, IC) – Hnf4a (RT-PCR) – Pdx1 (RT-PCR, IC) – Cxcr4 (IC) – C-peptide (IC) – GCG (RT-PCR, IC) – SST (IC) – CK19 (IC) – Ptf1a (RT-PCR) – Ngn3 (RT-PCR) – Isl1 (RT-PCR) – INS (RT-PCR) – Glut2 (RT-PCR) – Amylase (RT-PCR)
27	Human H1 H9	**Undifferentiated:** Feeder layer. DMEM (4.5 mg/l D-glucose), 1 mM Glutamine, 0.1 mM βME, 1% NEAAs and 20% FBS **Differentiation:** 1) Monolayer. CDM (1:1 IMDM:F12 NUT-MIX), ITS-A (1:100), 450 μM MTG, 5 mg/ml Albumin fraction V or X-Vivo10 with 55 μM βME and 0.1% Albumin fraction V (2 days) Free serum 2) Monolayer. CDM and 50 ng/ml Activin A (4 days) 3) Monolayer. CDM and 10^{-6} M RA (4 days) 4) Monolayer. DMEM/F12 1:1, ITS-A (1:100), 2 mg/ml Albumin fraction V and 10 ng/ml bFGF (3 days) 5) Monolayer. DMEM/F12 1:1, ITS-A (1:100), 2 mg/ml Albumin fraction V, 10 ng/ml bFGF and 10 mM Nicotinamide (5 days) 6) Suspension. DMEM/F12 1:1, ITS-A (1:100), 2 mg/ml Albumin fraction V, 10 ng/ml bFGF and 10 mM Nicotinamide (2 days)	– Sox17 (RT-PCR, IF) – Pdx1 (RT-PCR, IF) – Hnf4α (RT-PCR) – INS (RT-PCR,ELISA) – Glut2 (RT-PCR) – Amylase (RT-PCR,IC) – GCG (RT-PCR, IC) – SST (RT-PCR, IC) – GK (RT-PCR) – IAPP (RT-PCR) – C-peptide (IC)
28	Mouse D3	**Undifferentiated:** DMEM (25 mM glucose), 1400 U/ml LIF, 0.1 mM βME and 10% FCS **Differentiation:** 1) EB. Without LIF, DMEM (25 mM glucose), 0.1 mM βME and 10% FCS (1 day) 2) EB. DMEM (25 mM glucose), 0.1 mM βME, 10^{-7} M RA and 10% FCS (4 days) 3) EB. DMEM (25 mM glucose), 0.1 mM βME, 10^{-7} M RA and 2 nM Activin A or 1 nM BTC or 1 mM NaB (expression of INS I) and 10% FCS (4 days)	– PreproINS (RT-PCR) – Insulin (IC) – SST (RT-PCR) – PP (RT-PCR) – GCG (RT-PCR) – Amylase (RT-PCR)

D'Amour et al., these cells were unable to complete the differentiation process. The addition of Activin A increased the expression of Bry and Sox17, both being visceral endoderm and mesendoderm markers. The control population in the absence of activin A is negative for Bry, presents a low expression of Sox17 and high expression levels of AFP, indicating the presence of visceral endoderm. After the subsequent differentiation steps, the obtained population shared phenotypical and functional traits with pancreatic β-cells, such as glucose-dependent insulin secretion.

Nakanishi et al.[29] demonstrated that obtaining a pancreatic endocrine lineage depends of the concentration of activin A and all-trans retinoic acid. The final mouse cell product obtained expressed insulin I, an excellent pancreatic β-cell marker. However, functional studies are required to validate the therapeutic potential of these cells.

Table 2. Differentiation strategies to obtain insulin-secreting cells from definitive endoderm committed cells derived from ESCs *(continuation)*

Reference	Cell type	Culture conditions	Gene markers
29	Mouse E14 CMTI-1	**Undifferentiated:** Feeder layer. DMEM (4.5 mg/l D-glucose), ? NEAAs, 0.001% βME, 1,500 U/ml LIF and 15% FBS **Differentiation:** 1) EB without LIF and 15% KSR (4 days) 2) EBs with 15% KSR (2 days), 10-50 ng/ml Activin A and 0.001-1 μM RA. (Exocrine →10 ng/ml Activin A and 0.1 μM RA; INS II → 25 ng/ml Activin A and 0.1 μM RA) 3) Outgrowth with gelatin and 10% KSR (6-12 days)	– Alb (RT-PCR) – AFP (RT-PCR) – Amylase(RT-PCR,IC) – G6P (RT-PCR) – GCG (RT-PCR, IC) – INS I and II (RT-PCR) – Pdx1 (RT-PCR, IC) – SST (RT-PCR) – PP (RT-PCR) – Sox17 (RT-PCR) – TAT (RT-PCR) – Ptf1a (RT-PCR) – C-peptide (IC)

Abbreviations used (see table 1): BTC: Betacellulin, CMRL: Culture medium, DAPT: γ-secretase inhibitor, GCG: Glucagon, hbFGF: Human basic Fibroblast growth factor, hFGF10: Human fibroblast growth factor 10, IGFI: Insulin-like growth factor I, IGFII: Insulin-like growth factor II, INS: Insulin, NaB: Sodium butyrate, PP: Pancreatic polypeptide, RA: All trans Retinoic acid, SST: Somatostatin, WB: Western blotting.

Altogether, it is necessary to design culture environments that mimic in vivo niches where cells can progress into the differentiation pathway. Proteomic approaches could be applied to discover key factors that can be transferred to an in vitro system. However, this is not an easy task if we consider the dynamics that occur in these niches in terms of concentration, half-life and interactions between the different factors. This interesting idea must be sustained in protocols in which ESCs differentiate to cells that derive from the definitive endoderm and not from visceral endoderm.[31] The detection of insulin I versus insulin II gene expression is an easy starting point for a more complete subsequent characterization.

Conclusions

Taken into account this published information, it is possible to establish some instrumental steps towards a reproducible protocol. We cannot consider the currently published protocols as universal, since they still greatly depend on the assayed cell line (tables 1 and 2). On the other hand, the use of activin A and serum withdrawal seem to be the key determinants in the commitment from undifferentiated ESCs to definitive endoderm in the majority of the cell lines tested. In the subsequent stages from definitive endoderm to insulin-secreting cells, there is no consensus protocol in terms of determinants used (table 2).

One remaining question is to know whether the classical approach via EB formation could offer a higher differentiation capacity than in monolayer. It seems that monolayer culture allows the direct access of extracellular factors to the cells, improving the reproducibility of the protocols. In addition, EBs present a high degree of heterogeneity, in which it is still not established which one is more committed to pancreatic fates.[32] A possible advantage of using EBs versus monolayers during differentiation processes is the presence of different cell types, such as those derived from the ectoderm, mesoderm and primitive endoderm that could release key factors and thereby mimic certain steps of pancreatic development. Nevertheless, this point remains to be fully demonstrated inside EB structures.

The presence of glucagon mRNA in spontaneous differentiation protocols could suggest that EBs may give rise to definitive endoderm derivatives after long periods of culture.[1] However, this observation is in contradiction with the production of Shh by EB structures, most likely repressing any endocrine pancreas differentiation.[33] In this context, Shh inhibition using cyclopamine could improve the efficiency of insulin-producing cells in differentiation protocols.[11] The correct characterization of these glucagon$^+$ cells could give key information in order to design a definitive protocol, not only towards insulin-secreting cells, but to a bioartificial islet.

Note added in proof: A recent article published in Nature Biotechnology from the group of Baetge describes the derivation of glucose-responsive insulin-secreting cells from human stem cells. The reading of this key paper[34] is strongly recommended.

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Key points

- Embryonic stem cells have still the potencial for self-renewal and for cell differentiation to insulin-expressing cells under certain culture conditions.
- Different strategies have been used to obtain cells that expressed insulin. Among others, endoderm cells, which are the origin of adult pancreas, have been used.
- Detection of intracellular insulin in a cultured cell is not enough to consider that this cell should be defined as a pancreatic cell.

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