

Embryonic stem cells to beta-cells by understanding pancreas development

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ABSTRACT

Insulin injections treat but do not cure Type 1 diabetes (T1DM). The success of islet transplantation suggests cell replacement therapies may offer a curative strategy. However, cadaver islets are of insufficient number for this to become a widespread treatment. To address this deficiency, the production of beta-cells from pluripotent stem cells offers an ambitious far-sighted opportunity. Recent progress in generating insulin-producing cells from embryonic stem cells has shown promise, highlighting the potential of trying to mimic normal developmental pathways. Here, we provide an overview of the current methodology that has been used to differentiate stem cells toward a beta-cell fate. Parallels are drawn with what is known about normal development, especially regarding the human pancreas.

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1. Introduction

The incidence of both Type 1 (T1DM) and Type 2 (T2DM) diabetes is increasing worldwide. Both disorders are characterized by high concentrations of blood glucose (hyperglycaemia), which is avoided by appropriate secretion of insulin by the pancreatic beta-cell. The beta-cells are the major constituent of the islets of Langerhans: a composition of several endocrine cell-types that make up ~1–2% of the adult pancreas amongst the more prevalent exocrine and ductal components. The shortfall in insulin reflects an absolute loss of beta-cells in T1DM, a deficit that is increasingly recognized as a relative contributor in T2DM. The major goal in treating diabetes is to regain physiological regulation of circulating glucose levels. Currently, this is achieved in T2DM through dietary control and a range of oral medications, such as insulin secretagogues and sensitizers. Where this is inadequate, or in T1DM where the loss of endogenous insulin secretion is absolute, exogenous insulin is injected to replace pancreatic beta-cell function. Although this regime of frequent daily injections and blood glucose monitoring

has permitted a relatively normal lifestyle for many individuals, it falls a long way short of the perfect treatment—it is not a cure. It also carries a major psychological impact on patients and their relatives. Thus, the search persists for effective cell replacement therapy to restore normal physiological insulin secretion without the need for repeated injections and invasive monitoring.

To date, the most promising form of beta-cell replacement has been islet transplantation (Shapiro et al., 2000). Although encouraging proof-of-principle, the protocol remains far from ideal, since it requires a large supply of cadaveric material, from which whole islets are isolated, and immunosuppression of the recipient. In tune with all organ transplantation programmes, there is not enough material to cater for the millions of individuals with diabetes currently treated by insulin injection. This imbalance has fostered excitement for alternative ‘stem cell therapy’—whereby precursor cells, amenable to expansion, might be directed wholesale to a beta-cell fate, thus providing an unlimited source of material for transplantation. Arguably the most scalable cell-type with a clear potential for beta-cell differentiation is the embryonic stem cell (ESC). Using human ESCs as a starting point also offers the theoretical future of somatic nuclear transfer (SNT; replacing the ESC nucleus with that of a patient’s own cell), or inducible pluripotent stem (iPS) cells (reprogramming achieved via the expression of selected transcription factors). Expansion and differentiation of these latter sources would approximate to an autologous cell product.

To achieve effective cell therapy from ESCs, aside from SNT or iPS cells, a number of desirable characteristics can be assembled: first and foremost, for transplantation in patients, the optimal cell

Abbreviations: T1DM, Type 1 diabetes; T2DM, Type 2 diabetes; ESC, embryonic stem cell; SNT, somatic nuclear transfer; iPS, induced pluripotent stem; ICM, inner cell mass; EB, embryoid body; wpc, weeks post-conception; MODY, maturity onset diabetes of the young; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-(*S*)-phenylglycine *t*-butyl ester; MHC, major histocompatibility complex.

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source would be human; second, the cell product needs to sense blood glucose and secrete insulin exactly like a normal healthy pancreatic beta-cell; finally, given the quality and longevity of life that can be achieved by insulin injection, cell replacement therapy must be safe. It is appropriate that these criteria make for a long-term research goal. Fully functional beta-cells derived from stem cells have yet to be proven *ex vivo*. In this review, we focus on current progress placed into the context of understanding normal beta-cell differentiation during gestation, the process whereby beta-cells first arise.

2. A brief history of embryonic stem cells

A stem cell is defined by the ability to self-renew indefinitely, characteristically by asymmetric cell division, coupled to a propensity for differentiation to one or more specialised cell-types (Weissman, 2000). ESCs are notable for pluripotency, the ability to generate all of the body's cell-types that arise from the earliest lineage fates of the inner cell mass (ICM) of the blastocyst (see Section 4.1). This combination offers scalability and the potential for a wide array of therapeutic cell products.

Historically, ESCs have been derived by taking the ICM of pre-implantation blastocysts into *in vitro* culture (Evans and Kaufman, 1981; Thomson et al., 1998), although it seems that cells from the epiblast offer equivalent potential (Brons et al., 2007). It even appears that pluripotency, akin to that of ESCs, can be imparted by restoring the expression of relatively few transcription factors (Meissner et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Yu et al., 2007). Like the ICM, ESCs retain an ability to differentiate to lineages that are equivalent of gastrulation: the establishment of three germ layers, from which arise all of the body's somatic tissues and organs. Mammalian ESCs were generated first from mouse embryos (Evans and Kaufman, 1981). Seventeen years later, in 1998, human ESCs were reported (Thomson et al., 1998). As a result of these landmarks, many human ESC lines have now been derived and maintained, facilitating investigations of directed differentiation to specific endpoints that include the pancreatic beta-cell.

The optimal strategy for forming beta-cells from ESCs has been keenly debated. In primitive organisms insulin, present in neural cells, acts as a neurotransmitter (Levine, 1981); in mammals, representing millennia of changing evolutionary selection pressures, insulin is restricted to endoderm-derived cells in the pancreas as an endocrine regulator of glucose homeostasis (Murtaugh, 2007). Thus, it seems plausible that arriving at the former cell-type might require fewer, less complex differentiation steps for an ESC compared to the latter navigation of a more circuitous route (Burns et al., 2004). In the laboratory, this raises the idea that human insulin-secreting cells might be generated from ESCs by 'shortcutting' mammalian development and following a relatively direct path. Alternatively, one could attempt mimicry of the normal developmental pathway, more complex, but potentially more reassuring as it builds upon a wealth of developmental biology knowledge and might allow co-differentiation of the other islet cell-types. The following sections describe experiments that can be considered at least partially within these two categories.

3. ESC to insulin-positive cell-types: shortcuts and genetic engineering

Evidence that insulin-secreting cells could be derived from stem cells *in vitro* initially came from studies using mouse ESCs and embryoid body (EB) formation (Soria et al., 2000). Similar results for human ESCs soon followed (Assady et al., 2001). In the latter study,

undifferentiated ESCs were allowed to aggregate inducing spontaneous differentiation within the EB—in effect, a haphazard mimic of gastrulation. Not surprisingly, using this approach, the percentage of insulin-positive cells in EBs was low (~1%). In contrast, Soria et al used transfection and cell selection methodology with an insulin-hygromyocin transgene to obtain an insulin-secreting clone. Transplantation of these cells into diabetic mice restored normoglycaemia (Soria et al., 2000). Recognizing the problems inherent in such a low rate of return from EBs, subsequent studies focused on increasing insulin positive cell numbers by other means. In 2001, Lumelsky et al. devised a five step protocol to enrich for insulin-positive cells from mouse ESCs, by selecting cells after EB formation that expressed the intermediate filament protein Nestin (Lumelsky et al., 2001). Nestin, however, is broadly expressed and commonly interpreted as a marker of neuroprogenitors or neural stem cells (Cattaneo and McKay, 1990; Lendahl et al., 1990). Based on the recognition that many transcription factors regulating beta-cell development and insulin gene expression are also required for neuronal differentiation (Rolletschek et al., 2006), Nestin was considered as a potential marker of pancreatic progenitor cells (Cattaneo and McKay, 1990). Lumelsky and colleagues showed structures at the end of their differentiation protocol, strikingly similar to mouse islets. Others have built on this protocol, such as the inclusion of PI3 kinase inhibitors, to enhance the generation and function of the insulin-positive cell-types (Hori et al., 2002). In the latter study, the effectiveness of transplanted cells was proven by restoration of euglycaemia in diabetic mice and subsequent deterioration following transplant removal (Hori et al., 2002). However, the proximity of these cells to true beta-cells remains contentious, especially as a significant proportion of the intracellular insulin is not *de novo* synthesis but uptake from the culture media by dying cells (Rajagopal et al., 2003; Hansson et al., 2004). Reports of Nestin expression during mouse pancreas development differ (Selander and Edlund, 2002; Bernardo et al., 2006), however, Nestin was not detected during human pancreas development (Piper et al., 2002b). Collectively, these findings have led to suggestions that Nestin expression en route to an insulin-positive cell-type is perhaps more indicative of neuronal differentiation. Either independently or based around Nestin-dependent differentiation protocols, others investigated whether a pancreatic programme could be enforced more rigidly by constitutive (over-) expression of pancreatic transcription factors, such as Pdx1, Ngn3 or Pax4 (Blyszczuk et al., 2003; Miyazaki et al., 2004; Lavon et al., 2006; Treff et al., 2006). Not surprisingly, this strategy induces expression of a number of target genes of these transcription factors. Whereas these experiments have helped our understanding of the pathway from ESC to differentiated beta-cell, it is difficult to assess accurately whether these interventions cause permanent programming of differentiated cell-fate.

In summary, by either accepting random differentiation via EBs or short-cutting normal development by the over-expression of pancreatic progenitor transcription factors, it is possible to generate insulin-positive cells that are capable of restoring euglycaemia in mice. However, these cells are not physiologically normal and whilst they may serve as models for laboratory experiments or, potentially, drug-discovery programmes, it seems unlikely that cell-types for clinical therapy will arise. Thus, we turn to consider the normal developmental pathway from ICM mass to beta-cell and the studies that aimed to mimic it.

4. ESC to beta-cell: the complex pathway of normal development

Given the low frequency of differentiation to desired cell-types by random EB formation, to many researchers, it seemed logical

to try and produce beta-cells by following the normal differentiation that unfolds in utero. The following sections provide sufficient detail on this pathway to allow assessment of a range of ESC studies striving to follow it.

4.1. Gastrulation, the specification of foregut and the formation of pancreatic buds

The ICM is notable for its expression of a number of transcription factors, such as OCT4, SOX2 and NANOG, each of which is required for its establishment and maintenance (Niwa et al., 2000; Nichols et al., 2001; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003). These transcription factors also mark the starting ESC phenotype from which differentiation commences. The ICM gives rise to the primitive endoderm and the epiblast. The latter forms the primitive ectoderm, which progresses through gastrulation, a key event not amenable to direct study in human embryos as it occurs during the third week of development when embryos are inaccessible. Thus, studies using animal models have proved invaluable in guiding our understanding. Gastrulation defines a major cellular reorganization into the three primary germ layers of ectoderm, mesoderm and endoderm, and during which the germ cell lineage is also generated. From endoderm arises the primitive gut tube, which goes on to comprise the foregut, midgut and hindgut. A range of organs develop from the foregut. In addition to lung and thyroid, the liver and pancreas arise from a common set of precursor cells at its distal end where the gut tube forms the proximal part of the duodenum (Deutsch et al., 2001). Gene knockout models in mice have identified several critical transcription factors for formation of foregut endoderm. Mice lacking the *Sex-determining region Y box (Sox) gene 17 (Sox17)* are deficient in gut endoderm (Kanai-Azuma et al., 2002). Similar inactivation studies provide evidence for a requirement of Gata4 and Foxa2 (previously known as Hepatocyte nuclear factor (Hnf) 3 β) (Ang and Rossant, 1994; Weinstein et al., 1994; Molkentin et al., 1997); transcription factors which appear to cooperate in regulating differentiation (Bossard and Zaret, 1998).

Members of the transforming growth factor signalling- β (Tgf- β) protein family appear to be key regulators of endoderm formation and pancreas specification (e.g. Nodal and Activin). For example, formation of definitive endoderm occurs as a result of Nodal signalling, which regulates several genes central to normal endoderm development such as *Mixl1*, *Foxa2*, *Sox17* and *Gata4* (Stainier, 2002). Initially, both Sonic and Indian hedgehog (Shh and Ihh) are expressed throughout the entire gut endoderm (Hebrok et al., 2000). However, in the region destined to become dorsal pancreas contact with the notochord represses their expression; a step thought to be mediated by Activin- β B and Fibroblast growth factor 2 (Fgf2) signalling (Hebrok et al., 1998). Further studies have shown the importance of inductive signals, such as vascular endothelial growth factor (Vegf) emanating from vessels, in early pancreas specification (Lammert et al., 2001). A more global mediator of embryonic patterning is retinoic acid (RA). In RA-deficient zebrafish, pancreas and liver do not form (Stafford and Prince, 2002). RA signalling appears essential for dorsal pancreas formation in the mouse and *Xenopus* (Chen et al., 2004; Martin et al., 2005; Molotkov et al., 2005). At the ventral site of pancreas development there is no notochord contact. Instead, there is a balance between liver or pancreas formation according to the influence of Fgf signalling (Deutsch et al., 2001). More recently, low level Fgf4 signalling has been implicated in pancreas formation (Dessimoz et al., 2006), whereas higher levels promote an intestinal cell fate—collectively raising an important issue about factors not just acting like switches but also signalling dose responsively along gradients.

4.2. Pancreatic growth, endocrine specification and islet cell differentiation

The endodermal cells of the pancreas, distinct from interwoven stromal tissue and blood vessels, give rise to three differentiated components: the exocrine (or acinar) tissue, which comprises the vast majority of the adult organ; the ducts, which drain the digestive secretions from the acinar cells into the duodenum; and the endocrine cells, structured as islets of Langerhans. Cell fate mapping experiments carried out in transgenic mice suggest that these cell populations arise somewhat independently of each other (Gu et al., 2002, 2003).

Knowledge of inter-cellular signalling during development can give clues for exogenous factors that might elicit differentiation of pancreatic progenitors and endocrine cells from ESCs. Mesenchymal Fgf7 and Fgf10 stimulate pancreatic progenitor cell proliferation mediated by the receptor Fgfr2IIIb on their cell surface (Bhushan et al., 2001; Elghazi et al., 2002; Norgaard et al., 2003; Ye et al., 2005). Follistatin also promotes progenitor cell proliferation and, ultimately, exocrine cell differentiation (Miralles et al., 1998). Such pro-proliferative influences act to inhibit endocrine differentiation, which is associated with an exit from the cell cycle (Gittes et al., 1996; Piper et al., 2004; Duvillie et al., 2006). Signalling via the Epidermal growth factor (Egf) receptor (Egfr) is important for islet assembly and beta-cell proliferation (Miettinen et al., 2000). Hepatocyte growth factor (Hgf) also appears important for growth and differentiation of the pancreas (Garcia-Ocana et al., 2001) and plays an important role in postnatal insulin secretion via the Hgf receptor (c-met) (Roccisana et al., 2005).

In humans, the pancreas is first identified at 26 days post-conception (dpc) as ventral and dorsal thickenings of epithelial cells in the distal foregut (Piper et al., 2002a, 2004). Over the following weeks gut rotation brings the two expanding buds together resulting in fusion of a single pancreatic organ by the end of embryonic development (56 dpc). The first insulin-positive cells are also identified at this time and outnumber those positive for the other islet hormones: glucagon, somatostatin (SS) and pancreatic polypeptide (PP) (Clark and Grant, 1983; Stefan et al., 1983; Polak et al., 2000; Piper et al., 2004) (Fig. 1). Primitive islet-like assemblies containing cells independently positive for all four hormones can be detected from 12 weeks post-conception (wpc). A more classical islet distribution is found a week later with a central core of beta-cells surrounded by alpha-cells, delta-cells and gamma-cells expressing glucagon, SS and PP, respectively. In contrast, in mice, glucagon is the first hormone detected at embryonic day (E) 9.5 and islets are only assembled towards the end of gestation. However, as with knowledge of foregut formation, genetic manipulation in mice has been exceedingly valuable in demonstrating a wide range of transcription factors that are important for the differentiation of beta-cells. These are the subject of many comprehensive reviews elsewhere (Edlund, 2002; Kim and MacDonald, 2002; Wilson et al., 2003; Murtaugh, 2007) and are summarized in Table 1. Here, we discuss a small selection for which data are available during human endocrine differentiation.

The earliest human pancreatic progenitors are marked by the transcription factors pancreas duodenal homeobox factor 1 (PDX1) and SOX9 (Piper et al., 2002a, 2004). As its name indicates, PDX1 is not specifically a pancreatic marker. Similarly, SOX9 is also expressed more widely including the basal progenitor cell region of the fetal duodenum and biliary tract (Piper Hanley et al., 2008). Whereas PDX1 is expressed in human fetal and adult beta-cells (where it transactivates the *INSULIN* gene), SOX9 is down-regulated with endocrine differentiation. In the adult pancreas, it persists in the epithelial cells of the pancreatic ducts. Both PDX1 (Jonsson et al., 1994; Offield et al., 1996; Gu et al., 2002) and SOX9 (Piper et al.,

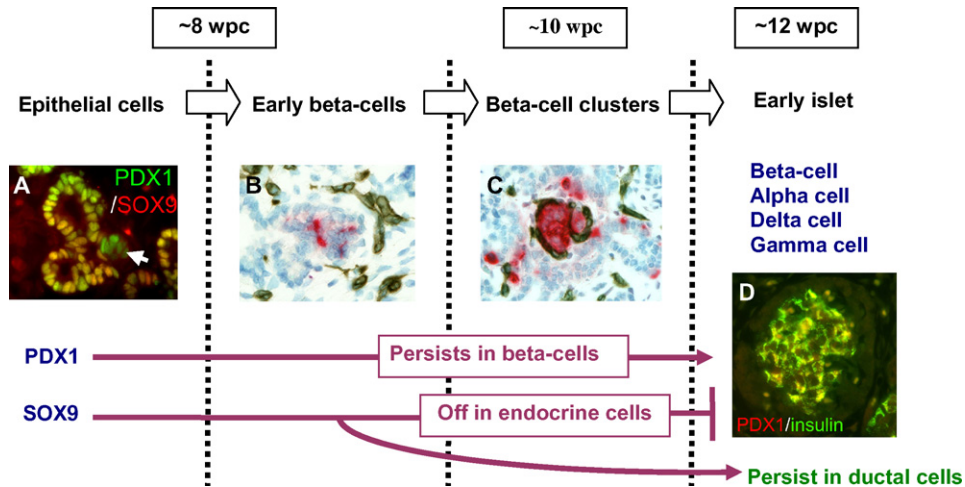


Fig. 1. Summary of human pancreas development (Piper et al., 2004). (A) The majority of early epithelial progenitor cells co-express PDX1 (green) and SOX9 (red) [note beta-cell cluster positive for PDX1 (white arrow) and negative for SOX9]. (B and C) Primitive beta-cells detected at 8 and 10 wpc in close proximity to developing vasculature: insulin (red) and CD34 (vascular marker; brown). (D) Classical islet distribution found at ~12 wpc; beta-cells co-express insulin (green) and PDX1 (red).

Table 1

Summary of transcriptional regulators involved in formation of the early gut tube through to the specification of islet cell types

Cell type	Transcription factor	Function	Reference
Gut tube	SOX17	Formation of gut endoderm	Kanai-Azuma et al. (2002)
	GATA4 ^{a,b}	Foregut morphogenesis; differentiation of both exocrine and endocrine lineage; transactivates glucagon gene	Bossard and Zaret (2000), Ketola et al. (2004) and Ritz-Laser et al. (2005)
	MIXL1	Mesendoderm development and differentiation	Hart et al. (2002)
	FOXA2 ^a	Homozygous knockout lethal in mice due to lack of endoderm and notochord; required for alpha-cell lineage	Monaghan et al. (1993) and Lee et al. (2005)
Pancreas specification	PDX1 ^a	Essential for pancreatic development; differentiation of alpha- and beta-cells; transactivator of insulin gene	Jonsson et al. (1994), Offfield et al. (1996) and Hui and Perfetti (2002)
	SOX9 ^b	Expressed in all pancreatic progenitor cells; islet organisation; restricted to duct cells later in development	Piper et al. (2002a), Akiyama Ddagger et al. (2005) and Seymour et al. (2007)
	SOX4 ^a	Broadly expressed in pancreatic buds with subsequent restriction to islets	Wilson et al. (2005)
	HLXB9 ^a	Expressed in all pancreatic progenitors with restriction to beta-cells during differentiation	Harrison et al. (1999) and Li et al. (1999)
	PBX1 ^{a,b}	Expressed in pre-pancreatic epithelium; required for both exocrine and endocrine differentiation	Dutta et al. (2001) and Kim et al. (2002)
	PTF1 ^{a,b}	Expressed in all pancreatic progenitor cells; essential for exocrine development	Krapp et al. (1996) and Kawaguchi et al. (2002)
	TCF2 (HNF1β)	Expressed in early endoderm, regulator of HNF6 expression	Barbacci et al. (1999) and Poll et al. (2006)
	HNF6 ^{a,b}	Pancreatic precursor cell specification; regulates PDX1 and NGN3 expression; islet and duct cell formation	Jacquemin et al. (2000), Jacquemin et al. (2003) and Poll et al. (2006)
Endocrine specification	NGN3	Differentiation of endocrine lineage	Apelqvist et al. (1999), Jensen et al. (2000), Schwitzgebel et al. (2000) and Sugiyama et al. (2007)
	ISL1 ^a	Differentiation of all islet cell types	Ahlgren et al. (1997)
	NEUROD1 ^a	Differentiation of all islet cell types; islet organisation; transactivates insulin gene	Naya et al. (1997)
	IA1	Differentiation of alpha-, beta- and delta-cells	Gierl et al. (2006)
Islet cell sub-types	HNF4α	Transactivation of HNF1α and insulin gene	Gragnoletti et al. (1997) and Bartoov-Shifman et al. (2002)
	HNF1α	Transactivation of PDX1 and insulin gene	Emens et al. (1992) and Gerrish et al. (2001)
	PAX6	Differentiation of all islet cell types; transactivates glucagon gene	Sander et al. (1997) and St-Onge et al. (1997)
	PAX4	Formation of alpha- and beta-cells	Sosa-Pineda et al. (1997) and Smith et al. (1999)
	NKX2.2	Transactivation of NKX6.1 and insulin genes in beta-cell precursors	Sussel et al. (1998)
	NKX6.1	Differentiation of beta-cells	Sander et al. (2000)
	BRN4	Expressed in alpha-cells; transactivates glucagon gene	Hussain et al. (1997) and Heller et al. (2004)
	ARX	Co-expressed with PAX4; formation of alpha- and beta-cells	Collombat et al. (2005)
	MAFA	Transactivates insulin gene	Kataoka et al. (2002)
	MAFB	Formation of alpha- and beta-cells; controls glucagon expression	Artner et al. (2006)
	GATA6	Differentiation of endocrine lineage; expressed in beta-cells	Ketola et al. (2004)

^a Factors with role in specification of islet sub-types.

^b Factors also involved in specification of acinar and/or duct cells.

2002a; Akiyama Ddagger et al., 2005; Seymour et al., 2007) are critical for survival and proliferation of the pancreatic progenitors and, thus, by supplying the starting cell population, underpin the subsequent differentiation of beta-cells. Disruption of *PDX1* in mouse and human results in pancreatic agenesis (Jonsson et al., 1994; Stoffers et al., 1997b). Heterozygous mutations in the human gene cause Type 4 maturity onset diabetes of the young (MODY 4) (Stoffers et al., 1997a). Mutations in *SOX9* cause campomelic dysplasia with pancreatic hypoplasia and islet abnormalities (Piper et al., 2002a).

Differentiation of all endocrine cell types relies upon appropriate expression of Neurogenin 3 (NGN3) (Apelqvist et al., 1999; Gradwohl et al., 2000; Jensen et al., 2000; Schwitzgebel et al., 2000). In mouse, Ngn3 commits progenitor cells to an endocrine cell fate and is expressed transiently prior to terminal beta-cell differentiation (Schwitzgebel et al., 2000; Heremans et al., 2002; Gasa et al., 2004). Expression in humans appears similar (Sugiyama et al., 2007) (our unpublished data).

In addition to *PDX1*, clues to the roles of other genes come from their identification as causes of MODY. *HNF-1 α* (Yamagata et al., 1996b; Vaxillaire et al., 1997), *HNF-1 β* (Horikawa et al., 1997) and *HNF-4 α* (Bell et al., 1991; Yamagata et al., 1996a) cause MODY 3, 5 and 1, respectively. More recently, *NEUROD1*, encoded by the sixth MODY gene and essential for endocrine cell differentiation and insulin gene transactivation, has been implicated in T2DM when mutated (Malecki et al., 1999).

5. Functional characteristics of normal beta-cells that are required for cell therapy

Acquiring insulin expression from differentiated ESCs raised initial hopes that this important achievement would vouch for a true beta-cell phenotype. This has transpired to be a significant over-simplification. Successful cell therapy demands a far more comprehensive beta-cell phenotype. Insulin production by the beta-cell requires its synthesis, packaging and release, all of which needs to occur proportionate to the prevailing concentration of glucose sensed by the beta-cell. Commonly, the proteins that undertake these functions are expressed from genes under the regulation of specific beta-cell transcription factors, such as *PDX1* and *NEUROD1*. Glucose sensing by the beta-cell is mediated in part by transport of glucose across the beta-cell membrane via the glucose transporter GLUT2 and, particularly in humans, its subsequent ‘trapping’ within the cell by phosphorylation by glucokinase (GCK). This rise in intracellular glucose stimulates ATP generation, which in turn closes ATP-sensitive potassium channels altering the potential of the cell membrane. This causes depolarization and a rise in

intracellular calcium levels, and stimulates the coordinated exocytosis of insulin granules. Within the granules, insulin requires processing from preproinsulin to active insulin and C-peptide by the enzyme prohormone convertase 1/3 (PC1/3) (Steiner, 1998; Goodge and Hutton, 2000). Thus, insulin is co-secreted with C-peptide in equimolar concentrations. However, beta-cell secretion also includes islet amyloid precursor protein (IAPP). All of these considerations create a multitude of markers and physiological characteristics by which to judge the phenotype of differentiated ESCs.

6. ESC differentiation to phenotypically normal beta-cells: a work in progress

In short, ESCs have not been differentiated yet into normal beta-cells. However, progress has been made by trying to follow the normal differentiation pathways that operate during intra-uterine development. As outlined earlier, many of the inductive signals and transcriptional regulators necessary for beta-cell differentiation are reassuringly common to both mouse and human. By initially differentiating mouse ESCs to EBs, the Keller group has described protocols taking Brachyury-positive cells with the potential for both mesoderm and endoderm differentiation towards definitive endoderm via stimulation with Activin-A (Kubo et al., 2004). Mimicking later development with the inclusion of Activin- β B, mouse ESCs gave rise to progeny expressing islet hormones including C-peptide (Ku et al., 2004). However, the majority of hormone-positive cells were positive for both insulin and glucagon. Lineage tracing in mouse has clearly demonstrated that true beta- and alpha-cells arise independently making it difficult to regard dual stained cells as representative of normal islets (Herrera, 2000). Several protocols have now emerged for the differentiation of human ESCs by similar means. Two reports by D’Amour and colleagues have demonstrated the early induction of ESCs, without EB formation, to a foregut-like phenotype via the addition of relatively high amounts of Activin-A followed by later induction towards a more pancreatic phenotype (Fig. 2). The early Activin-A mediated differentiation also requires the inhibition of PI3 kinase, either by withdrawal of serum or via chemical inhibitors (D’Amour et al., 2005; McLean et al., 2007). Cells were subsequently characterized for expression of endodermal markers (e.g. *SOX17* and *GATA4*). Endoderm-like cells were then differentiated toward the pancreas lineage in a step-wise manner that involved inhibition of Hedgehog and Notch signalling using cyclopamine and DAPT, respectively, and the addition of pro-proliferative FGF10 and removal of Activin A to promote the appearance of *HNF1- β* and *HNF4- α* . RA-signalling

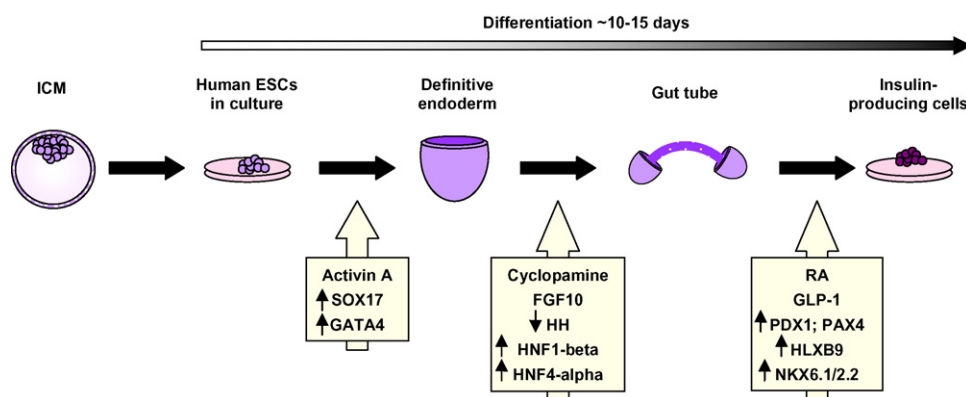


Fig. 2. One step-wise protocol to differentiate human ESCs to insulin-producing cells built upon knowledge of normal development (D’Amour et al., 2006).

was then reported to induce the early pancreatic lineage, characterized by the expression of transcription factors expressed in pancreatic progenitor cells, such as PDX1 and HLXB9 (D'Amour et al., 2006). IGF1 and HGF were then added as growth factors known to support beta-cell proliferation. Despite this progress, the resultant yield of insulin-positive cells was rather low (12% using FACS) and cells were also dually positive for insulin and glucagon. Other groups have described similar differentiation protocols (Jiang et al., 2007; Phillips et al., 2007), including a brief transplantation analysis (Phillips et al., 2007). These results demonstrated graft survival and phenotypic stability over 15 days with some C-peptide production although diabetes was not reversed in mice. However, Kroon and colleagues have demonstrated more successful transplantation of differentiated cells with the restoration of euglycaemia in diabetic mice (Kroon et al., 2008). Proof that the transplanted cells were responsible for the blood glucose control came from the reappearance of diabetes when the transplant was removed. A common addition during the later stages of several attempted beta-cell differentiation protocols is glucagon-like peptide-1 (GLP-1) or analogues thereof. GLP-1 is an alternative cleavage product to glucagon from the same prohormone. GLP-1 analogues or inhibitors of endogenous GLP-1 degradation act as major potentiators of insulin secretion ('incretins') and are new therapeutic agents in T2DM (Baggio and Drucker, 2007). GLP-1 has recently been shown to promote beta-cell differentiation, proliferation and inhibit apoptosis. Mice lacking the GLP-1 receptor develop with relatively normal islets (Scrocchi et al., 1996). However, it remains to be seen whether it is an important mediator of human beta-cell differentiation. Such evidence would support its use in human ESC differentiation protocols.

7. Unsurmounted difficulties for clinical application of ESC derived beta-cells

It remains early days for cell therapy from ESCs. In brief, potential difficulties and obstacles can be summarized in several categories.

7.1. Potential problems inherent to the starting ESCs

One of the major clinical concerns for ESC-derived cell therapy is the risk of tumour formation from any persisting pluripotent stem cells within the transplant. Although fully differentiated ESCs pose no obvious risk, some investigations provide evidence for the persistence of pluripotent cells (Ensenat-Waser et al., 2006). Studies have also recognized karyotypic instability of long term hESC cultures with recurrent chromosomal gain characteristic of some germ cell tumours (Draper et al., 2004). These concerns are not alleviated by standard laboratory transplantation experiments; the length of time used to assess cell replacement in diabetic mice is too short for adequate appreciation of tumour risk in patients (Vaca et al., 2006).

7.2. Immune rejection

As with clinical islet transplantation there are two issues regarding immune rejection. In T1DM, the host autoimmunity that led to initial beta-cell destruction needs suppression. Progress has been made in this area; however, its discussion is beyond the scope of this article. It is worth considering allogeneity that requires immunosuppression to prevent graft rejection as this affects all potential therapies generated from ESCs. Improvements in immunosuppression in recent years are exemplified by the relative success of cadaver islet transplantation with glucocorticoid-free regimens (Shapiro et al., 2000). The induction of immune tolerance may also be possible, for example, thymus induced tolerance from mixed

hematopoietic chimeras (Sykes, 2007) or by a negative vaccination programme prior to transplantation. In both instances, the goal is to nullify potential activation of host antigen presenting cells by transplant antigens. The most ambitious 'designer' strategy to avoid allogeneic rejection would rely on patient-specific ESCs, potentially from somatic cell nuclear transfer, ESCs with manipulated MHC expression (Bradley et al., 2002), or reprogramming of patient somatic cells with pluripotency-inducing transcription factors (iPS cells) (Meissner et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Yu et al., 2007).

7.3. The complexity required for fully functional beta-cells

The majority of this review and the vast proportion of the literature have focused on generating beta-cells, rather than islets from ESCs. To date, this has almost entirely been based on monolayer culture. In addition to complex inter-cellular interactions during normal development of the pancreas, for fully functional beta-cells, three-dimensional assembly is required within the complexity of the multi-cell-type islet (Hauge-Evans et al., 1999). These structures are highly vascular with complex interactions with the surrounding extracellular matrix.

7.4. Scale-up, scale-out and acceptance

Finally, if stem cell therapies are to be readily available for transplantation there is a need to scale up the provision of standardized, phenotypically stable, clinical grade differentiated cell products to treat diabetes. This is likely to be costly, time-consuming and restricted to few specialized centres. The market for monoclonal antibodies as therapeutic agents has only recently become widely recognized, however, the basic research was conducted over 20 years ago. There is a pressing need for advances in the research laboratory to be matched by parallel awareness and development in the pharmaceutical arena so that translation from laboratory bench to patient is timely.

One last slightly concerning consideration is the withdrawal of inhaled insulin by a major pharmaceutical company on account of poor acceptance by patients and physicians, despite the product being regarded as clinically effective. Although treatment rather than cure, the success of injectable insulin, both commercially and therapeutically, presents a formidable benchmark for proposed cell therapy.

8. The future: regenerate or transplant?

Recent advances in ESC research provide the first steps to suggest beta-cell replacement may be attainable to treat diabetes. However, formidable difficulties remain. The potential of endogenous beta-cell regeneration should also be considered (addressed by others in this issue of *Molecular & Cellular Endocrinology*). The two are linked: in vitro ESC differentiation may be inadequate for cell therapy but serve as a platform for the discovery of drugs that recruit or reprogramme adult pancreatic cells as endogenous stem or progenitor cells capable of beta-cell differentiation. To advance therapy in T1DM, success of either approach is desirable.

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