Published in final edited form as:

Curr Opin Organ Transplant. 2009 February ; 14(1): 56–63. doi:10.1097/MOT.0b013e3283186fc1.

Development of Insulin Producing Cells From Primitive Biologic Precursors

Carmella Evans-Molina^{1,2}, George L. Vestermark¹, and Raghavendra G. Mirmira^{1,2,3,*}

¹Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202

²Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202

³Department of Pediatrics, Indiana University, Indianapolis, IN 46202

Abstract

Purpose of review—The differentiation of pluripotent and multipotent stem cells into insulin producing cells has the potential to create a renewable supply of replacement β cells with tremendous utility in the treatment of diabetes. The purpose of this review is to summarize recent advancements in the field, with emphasis on the limitations of this technology as it relates to the β cell.

Recent findings—Multiple groups have developed successful *in vitro* protocols to differentiate human embryonic stem cells and selected tissue specific stem cells into progenitors capable of insulin production and glucose-stimulated insulin secretion. The resulting cells are immature β like cells that co-express multiple islet hormones and lack the full complement of genes necessary for normal function. Protocols that include *in vivo* maturation in immune compromised mice produce cells with a more mature phenotype.

Summary—Although tremendous progress has been made in differentiating stem cells into insulin producing cells, there is still more research needed to produce a fully functional adult β cell.

Keywords

human embryonic stem cells; insulin producing cells; differentiation protocols; β cells

INTRODUCTION

Diabetes mellitus results from either a complete or relative deficiency of insulin, a protein whose production is restricted almost entirely to the pancreatic β cells (1). Worldwide, diabetes is estimated to affect 246 million individuals, and the prevalence of this disease is increasing to epidemic proportions (2–4). Currently, pancreatic or islet transplantation are the only treatments that offer patients the possibility of a cure. Both methods of transplantation, however, are limited by organ availability. Moreover, enthusiasm for islet transplantation has been tempered by disappointing results of long term outcome studies, which suggest that only 10–15% of patients demonstrate continued insulin independence 5 years post transplantation (5). Thus, recent research efforts have focused on exploiting the principles that guide β cell development with the hope of creating a renewable and limitless source of insulin producing cells from primitive biologic precursors (6–10). In the current

*Corresponding Author: Raghavendra G. Mirmira, Indiana University School of Medicine, 635 Barnhill Drive, MS 2031B, Indianapolis, IN 46202. rmirmira@iupui.edu, Telephone: (317) 274-4140, Fax (317) 274-4107.

review, we will summarize the state of this field, with emphasis on the development of insulin producing cells from human embryonic stem cells (hESCs). An accompanying review describes the status of current research efforts focused on the production of functional β cells from resident pancreatic precursor cells.

An overview of β cell development

The adult pancreas has dual exocrine and endocrine functions. The exocrine tissue comprises about 80% of the pancreas and consists of acinar and ductal cells (11). The endocrine pancreas is composed of clusters of hormone producing cells known as the islets of Langerhan. Islets are made up of α , β , γ , ϵ , and F cells, which synthesize and secrete glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, respectively (12). A common progenitor cell is responsible for the formation of acinar, ductal, and islet tissue. A variety of gene deletion studies have been performed to define the pathways of differentiation for each cell type. These studies have provided a framework for pancreas formation, which is characterized by a spatial and temporal signaling cascade that includes both cell-intrinsic (e.g. transcription factors) and non-intrinsic (e.g. intercellular signaling) mechanisms. The subject of pancreatic organogenesis has been extensively reviewed elsewhere (13, 14). Because the fundamentals of embryogenesis form basis of strategies to convert primitive precursors to β cells, we will consider briefly the embryonic pathway from a common progenitor to a functional insulin producing β cell.

The pancreas develops from definitive endoderm, which forms the primitive gut tube. Initiation of the pancreatic program requires extrinsic signals that lead to inhibition of hedgehog signaling and increased retinoid signaling (15, 16) within the discrete region destined to become pancreas. Subsequently, a series of cell-intrinsic signals from transcription factors direct the gene expression program that guides the developmental tree. The master transcription factors that sit atop this developmental tree are the homeobox transcription factor, Pdx-1, and the basic helix loop helix protein, Ptf1a, which together mark cells of endodermal origin destined to form the mature pancreas (17–20). The transcription factor responsible for driving Pdx-1/Ptf1a-positive progenitor cells to an endocrine rather than an exocrine fate is the basic helix-loop-helix (bHLH) protein Neurog3. Notch signaling appears to be instrumental in maintaining a balance between endocrine and exocrine pathways via a “lateral inhibition” pathway of intercellular communication. Cells expressing Neurog3 signal through Notch ligands to neighboring cells, which subsequently express Hes-1, a potent suppressor of Neurog3 expression (21). Thus, this pathway allows for the spatial separation between endocrine and exocrine components of the pancreas as seen in the mature organ. A subset of Neurog3-positive cells that are destined to become β cells then initiates a cascade of transcription factors that share responsibility in guiding cells towards this predetermined lineage. This list includes Insm1, NeuroD1/Beta2, Pax4, Nkx2.2, Nkx6.1, Hlxb9, and MafA, and again Pdx-1, which plays an additional role to maintain mature β cell function. Table 1 summarizes our current understanding of the transcriptional regulators of pancreatic development.

Human embryonic stem cells as precursors

There are at least two major types of stem cells: tissue- or organ-specific stem cells and embryonic stem cells. Tissue specific stem cells are also referred to as adult stem cells. These cells are capable of self-renewal and are considered to multipotent, which means that they possess the ability to produce a limited number of cell types. This is in contrast to embryonic stem (ES) cells, which are derived from the inner cell mass of the developing blastocyst. ES cells are pluripotent, or capable of forming virtually any cell type. (47). Given these unique characteristics, there is obvious enthusiasm for the use of ES cells to generate a limitless supply of insulin producing cells

The first successful strategies to develop β cells from ES cells utilized embryoid body (EB) formation as the initial step (48, 49). When ES cells are cultured in the absence of leukemia inhibitory factor or mouse embryonic fibroblasts, they differentiate spontaneously into three dimensional aggregates known as EBs. EB formation is followed by the differentiation of all three germ cell layers, a process known as gastrulation (50). In the early years of experimentation, a relatively low percentage of insulin-positive cells were recovered using this approach. As a result, a variety of enhancement and selection techniques were developed (51–53). These methods were often combined with the ectopic overexpression of transcription factors that are known to drive β cell formation during development (see Table I) (54, and others). Subsequent careful analysis refuted the results of a many of these early differentiation protocols and suggested that insulin-positive staining in these cultures may have actually resulted from insulin uptake from the culture medium. Thus, current standards for analysis of cultures differentiated *in vitro* include measurement of C-peptide, insulin gene expression, electron microscopy for identification of insulin granules, and ultimately demonstration that such cells reverse hyperglycemia for sustained periods in animal models of diabetes (55).

As the field has developed, more sophisticated methods have been used to generate insulin producing β cells. Whereas some protocols still utilize EB formation, others have adopted approaches to avoid this initial step. In these protocols, ES cells have been first driven to form definitive endoderm (DE). This strategy purportedly offers the advantage that it increases the yield of insulin positive cells, as the maintenance of a two dimensional culture system would allow for the uniform exposure of all cells to differentiation factors (8, 9). However, in the absence of all factors that promote differentiation, those groups that continue to favor the initial formation of EBs argue that the three-dimensional structure of EBs promotes necessary inter-cellular interactions that more closely recapitulate embryonic development (56).

Under normal conditions, DE forms in the presence of high levels of TGF β /Nodal signaling (57). Activin, a TGF β signaling ligand, has been used in combination with a variety of factors including Wnt3a, sodium butyrate, Bmp4, and retinoic acid, to induce the formation of DE (8, 9, 56). In addition to the activation of Nodal signaling, the specification of DE also appears to require the release of inhibitory signaling from insulin and insulin-like growth factor 1 (IGF1) via the phosphatidylinositol 3-kinase (PI3K) pathway. Thus, specification of DE has been accomplished through the use of serum-free conditions or chemical inhibitors of PI3K, such as LY294002 and AKT1-II (58). Expression of SOX17, CER, FOXA2, CXCR4, and HNF α have been used as markers to confirm the presence of endodermal tissue. (6, 9, 59, 60).

Several groups have published recent accounts of hESC differentiation into insulin producing cells by utilizing many of the above advancements. The protocols developed by Phillips et al. and Jiang et al. consider 4 main steps in the conversion of hESCs to β cells: (1) formation of DE, (2) formation of pancreatic endoderm, (3) formation of endocrine tissue, and (4) β cell maturation (Fig. 1a). Phillips and colleagues allowed EBs to form and subsequently treated with activin and Bmp4 to enhance formation of definitive endoderm. FGF18 was used alone in Step 2, and this was followed by treatment with a number of additional growth factors in Step 3 for endocrine induction, which included EFG, TGF α , IGF1, IGFII, and VEGF. In the final maturation step, cells were exposed to HGF, pancreatic polypeptide, and forskolin. In this optimized protocol, about 85% of progenitors expressed Pdx-1 at day 12, and 65% of cells developed into endocrine cell types. Approximately 30% of EBs expressed insulin by *in situ* hybridization by day 34. Consistent with an immature endocrine cell-type, glucagon and somatostatin were present as well. These cells had a modest C-peptide response to glucose challenge (about 1.5 times baseline). Following

transplantation into STZ-treated SCID mice, they retained the ability to express Pdx-1 and insulin but lacked the ability to consistently lower blood glucose in the diabetic mice (56).

Jiang et al. developed a 36 day protocol involving the use of feeder cell-free suspension cultures and avoided the spontaneous formation of embryoid bodies (Fig.1b). DE was specified by treatment with activin and sodium butyrate, a histone deacetylase inhibitor. In the second step; noggin, bFGF, and EGF were used to form pancreatic endoderm. Noggin is a protein known to inhibit TGF β signaling. Following the withdrawal of bFGF to avoid hepatic differentiation, cells were treated with nicotinamide and IGFII for continued maturation to a β cell lineage. Resulting cells expressed insulin, glucagon, and somatostatin, similar to results seen by Phillips et al. The islet like clusters were shown to have morphology similar to β cells using electron microscopy, and these cells were able to release C-peptide in response to glucose exposure for prolonged periods (8).

D'Amour and colleagues published a detailed differentiation protocol in 2006. The protocol differed in those described previously in that several additional steps of development were considered (Fig. 2a). They also avoided the formation of embryoid bodies but used mouse fibroblast feeder layers. In Stage 1, DE was specified by actin and Wnt3a. This was followed by treatment with FGF10 and the hedgehog signaling inhibitor, KAAD-cyclopamine to induce formation of the primitive gut tube, and posterior foregut was specified by the addition of retinoic acid. Stage 4 involved the differentiation of pancreatic endoderm with DAPT, which was used to inhibit Notch signaling, and Exendin 4. Exendin 4 acts as an agonist of GLP-1 and has been shown to have proliferative and anti-apoptotic effects on islet tissue (61, 62). In the final stage, an endocrine phenotype was specified by the addition of IGF1 and HGF. Resulting cells were found to express insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin. These cells release c-peptide in response to glucose. The same group published a follow-up paper in 2008, in which their protocol was modified slightly (Fig. 2b). At day 12 in this new protocol, at a point when the cells exhibited a phenotype of committed pancreatic endoderm, they were transplanted into SCID mice. After 3 months, the grafts displayed architecture consistent with a mature islet, and co-staining of islet hormones was not noted within the same cells. During glucose tolerance testing, insulin was secreted in a regulated, predictable manner. Streptozotocin (STZ) was then used to preferentially ablate the native β cells of recipient mice; hyperglycemia was successfully prevented by the grafts in these animals (6, 9, 57).

In a similar experiment, Shim et al. cultured EBs in the presence of serum for four days, followed by serum withdrawal and the addition of activin and retinoic acid. The resulting cells were cultured for several more days and then transplanted into STZ-treated mice. Within four days of transplantation, blood glucose in the recipient mice improved and they remained normoglycemic until the grafts were removed. The grafts contained structures that resembled islets but many cells stained positive for insulin and glucagon, indicating an immature phenotype.

Multipotent stem cells as precursors

Given the ethical, political, and safety concerns surrounding research with human ES cells, the identification and differentiation of adult tissue-specific stem cells into insulin producing tissue offers an attractive alternative. Cai-Xia and colleagues recently published a protocol using immortal liver epithelial progenitor cells (LEPCs), which is a stem-like cell line derived from regenerative liver. Pdx-1 was stably expressed in LEPCs, under high glucose/low serum conditions, followed by treatment with a variety of growth factors. The resulting cells stained positive for insulin and C-peptide and robustly secreted insulin in response to high glucose. Further, diabetes was ameliorated after transplantation into STZ-treated SCID mice (63).

Similarly, Hori et al. used neural progenitor cells and subjected them to a differentiation protocol consisting of glucose deprivation and retinoic acid. The resulting cells expressed low levels of insulin and secreted insulin in response to a glucose challenge. Following transplantation into immunocompromised mice, the engrafted cells responded to glucose challenge, but absolute insulin release remained quite low (64).

Given the ease of isolation and ready availability, bone marrow derived stem cells represent a particularly attractive source of progenitors for differentiation protocols. To date, there has been ample controversy in this field. Many believe there is no definite evidence to support the idea that bone marrow derived cells and progenitors have the ability to differentiate into insulin-producing cells. They do, however, acknowledge that cells of this type may play a role in supporting β cell regeneration and in abrogating immune injury in Type 1 DM (65). Notwithstanding this debate, several interesting *in vitro* observations have been made recently. Moriscot, et al. isolated multipotent stem cells from human bone marrow aspirates. Following overexpression of Pdx-1, HLXB9, and FOXA2 using an adenovirus system, insulin expression could be detected (66). Ai et al published a recent account where mesodermal progenitor cells (MPCs) were isolated from human fetal bone marrow aspirates. Previous studies suggested that MPCs may have pluripotent properties (67). These cells were labeled with green fluorescent protein and engrafted with human fetal pancreatic tissue into SCID mice for *in vivo* maturation. The resulting islet-like clusters were glucose responsive and able to reverse hyperglycemia in NOD-SCID mice. Importantly, the islet clusters remained GFP positive pointing to the differentiation of MPCs rather than a pancreatic stem cell (68).

Limitations of current technologies

Despite the success of the protocols described in this review, there is significant work to be done before these ideas can be applied clinically. Paramount to consider are the safety implications of transplanting grafts that may be contaminated with undifferentiated pluripotent cells, as the potential for teratoma formation exists. At the completion of their study, Koon et al. removed transplanted grafts from recipient SCID mice. Pathological examination of grafts revealed a teratoma in 1/46 grafts and teratomatous tissue in six others (6). Additionally, karyotypic instability has been described in hECS cultured long term, and future studies should be designed to address these issues (69).

Notwithstanding these safety concerns, there are additional technical considerations. Most importantly, none of the protocols described have recapitulated precisely the exquisite machinery that enables the normal β cell to sense glucose and release insulin in a truly normal manner. In this regard, *in vivo* maturation of partially differentiated cells by factors yet to be defined has offered the best results, but only highlights the fact that there is still much to learn about pancreatic development and islet biology. Although not specifically discussed here, transplantation of any cell type brings with it concerns regarding immune tolerance, and there are numerous studies under way to investigate strategies aimed at inducing tolerance prior to stem cell transplantation (70). In addition to these concerns, there are issues of scale as even the most successful protocols produce relatively low numbers of insulin-positive cells. One could envision revisiting the idea of forced overexpression of selected transcriptional regulators like Pdx-1 or Neurog3 at certain steps to increase the fidelity of this process. Dominquez-Bendala et al. published a report describing fusion of Neurog3 to the HIV/TAT protein transduction domain. This is a technology that ensures efficient uptake of a protein into a cell of interest, allows for transient overexpression, and could have utility in differentiation protocols as a means to increase the yield of insulin-positive cells (71). Finally, no discussion of the future of stem cell therapy would be complete without the acknowledgement that political and ethical considerations may impact the pace of future discovery in this field (72).

Conclusions

Even in the context of the limitations outlined above, it is important to acknowledge that there has been incredible progress in the field of stem cell research within the past several years. While it should be emphasized that none of the protocols discussed here have been successful in reproducing a fully functional mature β cell, the generation of insulin producing cells that demonstrate glucose responsivity is impressive and encouraging. Although significant research is still required, a future where diabetes is treated with β cell replacement seems an attainable goal.

Acknowledgments

We gratefully acknowledge Shari Upchurch for her assistance in preparing this manuscript.

This work was supported by grants R01 DK60581 and a Juvenile Diabetes Research Foundation Award (both to R.G.M.), and grant K08 DK080225 (to C.E.M.)

REFERENCES

Papers of particular interest, published within the annual period of review, have been highlighted as follows:

* Of special interest

* * Of outstanding interest

1. Prentki M, Nolan CJ. Islet beta cell failure in type 2 diabetes. *J Clin Invest*. 2006; 116:1802–1812. [PubMed: 16823478]
2. International Diabetes Federation. <http://www.idf.org> (September 3, 2008).
3. Narayan KM, Boyle JP, Geiss LS, et al. Impact of recent increase in incidence on future diabetes burden: U.S., 2005–2050. *Diabetes Care*. 2006; 29:2114–2116.
4. Narayan KM, Boyle JP, Thompson TJ, et al. Lifetime risk for diabetes mellitus in the United States. *JAMA*. 2003; 290:1884–1890. [PubMed: 14532317]
5. Berney T, Toso C. Monitoring of the islet graft. *Diabetes Metab*. 2006; 32:503–512. [PubMed: 17130809]
6. Kroon E, Martinson LA, Kadoya K, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol*. 2008; 26:443–452. [PubMed: 18288110] One of the recent hallmark articles outlining a successful protocol for differentiating human embryonic stem cells into insulin producing cells. This protocol included a final maturation step in immune compromised mice that resulted in a more mature final phenotype.
7. Shim JH, Kim SE, Woo DH, et al. Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. *Diabetologia*. 2007; 50:1228–1238. One of the recent hallmark articles outlining a successful protocol for differentiating human embryonic stem cells into insulin producing cells.
8. Jiang W, Shi Y, Zhao D, et al. In vitro derivation of functional insulin-producing cells from human embryonic stem cells. *Cell Res*. 2007; 17:333–344. [PubMed: 17426693] One of the recent hallmark articles outlining a successful protocol for differentiating human embryonic stem cells into insulin producing cells.
9. D'Amour KA, Bang AG, Eliazar S, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol*. 2006; 24:1392–1401. [PubMed: 17053790] One of the first accounts outlining a successful protocol for differentiating human embryonic stem cells into insulin producing cells. The authors considered a multi-step differentiation protocol that recapitulated the signaling cascade of normal development.

10. Hori Y, Rulifson IC, Tsai BC, et al. Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *Proc Natl Acad Sci U S A*. 2002; 99:16105–16110. [PubMed: 12441403]
11. Murtaugh LC. Pancreas and beta-cell development: from the actual to the possible. *Development*. 2007; 134:427–438. [PubMed: 17185316] A comprehensive review of pancreatic and islet development.
12. Edlund H. Pancreatic organogenesis--developmental mechanisms and implications for therapy. *Nat Rev Genet*. 2002; 3:524–532. [PubMed: 12094230]
13. Oliver-Krasinski JM, Stoffers DA. On the origin of the beta cell. *Genes Dev*. 2008; 22:1998–2021. [PubMed: 18676806] A very recent and extremely comprehensive review of cell development.
14. Babu DA, Deering TG, Mirmira RG. A feat of metabolic proportions: Pdx1 orchestrates islet development and function in the maintenance of glucose homeostasis. *Mol Genet Metab*. 2007; 92:43–55. [PubMed: 17659992] A comprehensive review of the role of Pdx-1 in pancreatic development and cell function.
15. Kawahira H, Ma NH, Tzanakakis ES, et al. Combined activities of hedgehog signaling inhibitors regulate pancreas development. *Development*. 2003; 130:4871–4879. [PubMed: 12917290]
16. Stafford D, Hornbruch A, Mueller PR, Prince VE. A conserved role for retinoid signaling in vertebrate pancreas development. *Dev Genes Evol*. 2004; 214:432–441. [PubMed: 15322880]
17. Ahlgren U, Jonsson J, Edlund H. The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. *Development*. 1996; 122:1409–1416. [PubMed: 8625829]
18. Hale MA, Kagami H, Shi L, et al. The homeodomain protein PDX1 is required at mid-pancreatic development for the formation of the exocrine pancreas. *Dev Biol*. 2005; 286:225–237. [PubMed: 16126192]
19. Kawaguchi Y, Cooper B, Gannon M, et al. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat Genet*. 2002; 19:19.
20. Wiebe PO, Kormish JD, Roper VT, et al. Ptf1a binds to and activates area III, a highly conserved region of the Pdx1 promoter that mediates early pancreas-wide Pdx1 expression. *Mol Cell Biol*. 2007; 27:4093–4104. [PubMed: 17403901]
21. Jensen J, Pedersen EE, Galante P, et al. Control of endodermal endocrine development by Hes-1. *Nat Genet*. 2000; 24:36–44. [PubMed: 10615124]
22. Li H, Arber S, Jessell TM, Edlund H. Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9. *Nat Genet*. 1999; 23:67–70. [PubMed: 10471501]
23. Harrison KA, Thaler J, Pfaff SL, et al. Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice. *Nat Genet*. 1999; 23:71–75. [PubMed: 10471502]
24. Jacquemin P, Durviaux SM, Jensen J, et al. Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. *Mol Cell Biol*. 2000; 20:4445–4454. [PubMed: 10825208]
25. Jacquemin P, Lemaigre FP, Rousseau GG. The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade. *Dev Biol*. 2003; 258:105–116. [PubMed: 12781686]
26. Ahlgren U, Jonsson J, Jonsson L, et al. beta-cell-specific inactivation of the mouse *Ip1/Pdx1* gene results in loss of the beta-cell phenotype and maturity onset diabetes. *Genes Dev*. 1998; 12:1763–1768. [PubMed: 9637677]
27. Stoffers DA, Zinkin NT, Stanojevic V, et al. Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. *Nat Genet*. 1997; 15:106–110. [PubMed: 8988180]
28. Krapp A, Knofler M, Ledermann B, et al. The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. *Genes Dev*. 1998; 12:3752–3763. [PubMed: 9851981]
29. Seymour PA, Freude KK, Tran MN, et al. SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc Natl Acad Sci U S A*. 2007; 104:1865–1870. [PubMed: 17267606]

30. Wilson ME, Yang KY, Kalousova A, et al. The HMG Box Transcription Factor Sox4 Contributes to the Development of the Endocrine Pancreas. *Diabetes*. 2005; 54:3402–3409. [PubMed: 16306355]
31. Kim SK, Selleri L, Lee JS, et al. Pbx1 inactivation disrupts pancreas development and in *Ipfl*-deficient mice promotes diabetes mellitus. *Nat Genet*. 2002; 30:430–435. [PubMed: 11912494]
32. Dutta S, Gannon M, Peers B, et al. PDX:PBX complexes are required for normal proliferation of pancreatic cells during development. *Proc Natl Acad Sci U S A*. 2001; 98:1065–1070. [PubMed: 11158595]
33. Haumaitre C, Barbacci E, Jenny M, et al. Lack of TCF2/vHNF1 in mice leads to pancreas agenesis. *Proc Natl Acad Sci U S A*. 2005; 102:1490–1495. [PubMed: 15668393]
34. Poll AV, Pierreux CE, Lokmane L, et al. A vHNF1/TCF2-HNF6 cascade regulates the transcription factor network that controls generation of pancreatic precursor cells. *Diabetes*. 2006; 55:61–69. [PubMed: 16380477]
35. Gradwohl G, Dierich A, LeMeur M, Guillemot F. *neurogenin3* is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A*. 2000; 97:1607–1611. [PubMed: 10677506]
36. Ahlgren U, Pfaff SL, Jessell TM, et al. Independent requirement for *ISL1* in formation of pancreatic mesenchyme and islet cells. *Nature*. 1997; 385:257–260. [PubMed: 9000074]
37. Naya FJ, Huang HP, Qiu Y, et al. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in *BETA2/neuroD*-deficient mice. *Genes Dev*. 1997; 11:2323–2334. [PubMed: 9308961]
38. Gierl MS, Karoulias N, Wende H, et al. The zinc-finger factor *Insm1* (*IA-1*) is essential for the development of pancreatic beta cells and intestinal endocrine cells. *Genes Dev*. 2006; 20:2465–2478. [PubMed: 16951258]
39. St-Onge L, Sosa-Pineda B, Chowdhury K, et al. *Pax6* is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature*. 1997; 387:406–409. [PubMed: 9163426]
40. Smith SB, Ee HC, Connors JR, German MS. Paired-homeodomain transcription factor *PAX4* acts as a transcriptional repressor in early pancreatic development. *Mol Cell Biol*. 1999; 19:8272–8280. [PubMed: 10567552]
41. Artner I, Bianchi B, Raum JC, et al. *MafB* is required for islet beta cell maturation. *Proc Natl Acad Sci U S A*. 2007; 104:3853–3858. [PubMed: 17360442]
42. Nishimura W, Kondo T, Salameh T, et al. A switch from *MafB* to *MafA* expression accompanies differentiation to pancreatic beta-cells. *Dev Biol*. 2006; 293:526–539. [PubMed: 16580660]
43. Zhang C, Moriguchi T, Kajihara M, et al. *MafA* is a key regulator of glucose-stimulated insulin secretion. *Mol Cell Biol*. 2005; 25:4969–4976. [PubMed: 15923615]
44. Sussel L, Kalamaras J, Hartigan-O'Connor DJ, et al. Mice lacking the homeodomain transcription factor *Nkx2.2* have diabetes due to arrested differentiation of pancreatic beta cells. *Development*. 1998; 125:2213–2221. [PubMed: 9584121]
45. Doyle MJ, Loomis ZL, Sussel L. *Nkx2.2*-repressor activity is sufficient to specify {alpha}-cells and a small number of {beta}-cells in the pancreatic islet. *Development*. 2007; 134:515–523. [PubMed: 17202186]
46. Sander M, Sussel L, Connors J, et al. Homeobox gene *Nkx6.1* lies downstream of *Nkx2.2* in the major pathway of beta-cell formation in the pancreas. *Development*. 2000; 127:5533–5540. [PubMed: 11076772]
47. Scott CT, Reijo Pera RA. The road to pluripotency: the research response to the embryonic stem cell debate. *Hum Mol Genet*. 2008; 17:R3–R9. [PubMed: 18632694] This article includes practical definitions of a stem cell and outlines some of the controversies associated with stem cell research.
48. Soria B. In-vitro differentiation of pancreatic beta-cells. *Differentiation*. 2001; 68:205–219. [PubMed: 11776473]
49. Soria B, Roche E, Berna G, et al. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes*. 2000; 49:157–162. [PubMed: 10868930]

50. Kurosawa H. Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. *J Biosci Bioeng.* 2007; 103:389–398. [PubMed: 17609152] Informative review of the concept of an embryoid body.
51. Assady S, Maor G, Amit M, et al. Insulin production by human embryonic stem cells. *Diabetes.* 2001; 50:1691–1697. [PubMed: 11473026]
52. Fukazawa T, Matsuoka J, Naomoto Y, et al. Development of a novel beta-cell specific promoter system for the identification of insulin-producing cells in in vitro cell cultures. *Exp Cell Res.* 2006; 312:3404–3412. [PubMed: 16934249]
53. Leon-Quinto T, Jones J, Skoudy A, Burcin M, Soria B. In vitro directed differentiation of mouse embryonic stem cells into insulin-producing cells. *Diabetologia.* 2004; 47:1442–1451. [PubMed: 15309294]
54. Lavon N, Yanuka O, Benvenisty N. The effect of overexpression of Pdx1 and Foxa2 on the differentiation of human embryonic stem cells into pancreatic cells. *Stem Cells.* 2006; 24:1923–1930. [PubMed: 16675598]
55. Rajagopal J, Anderson WJ, Kume S, et al. Insulin staining of ES cell progeny from insulin uptake. *Science.* 2003; 299:363. [PubMed: 12532008]
56. Phillips BW, Hentze H, Rust WL, et al. Directed differentiation of human embryonic stem cells into the pancreatic endocrine lineage. *Stem Cells Dev.* 2007; 16:561–578. [PubMed: 17784830] One of the recent hallmark articles outlining a successful protocol for differentiating human embryonic stem cells into insulin producing cells.
57. D'Amour KA, Agulnick AD, Eliazar S, et al. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol.* 2005; 23:1534–1541. [PubMed: 16258519]
58. McLean AB, D'Amour KA, Jones KL, et al. Activin efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. *Stem Cells.* 2007; 25:29–38. [PubMed: 17204604] The authors show that in addition to activin signaling, PI3K inhibition is necessary to specify definitive endoderm.
59. Pearsall RS, Canalis E, Cornwall-Brady M, et al. A soluble activin type IIA receptor induces bone formation and improves skeletal integrity. *Proc Natl Acad Sci U S A.* 2008; 105:7082–7087. [PubMed: 18460605]
60. Lavon N, Narwani K, Golan-Lev T, Buehler N, Hill D, Benvenisty N, et al. Derivation of euploid human embryonic stem cells from aneuploid embryos. *Stem Cells.* 2008; 26:1874–1882. [PubMed: 18450823]
61. Ferdaoussi M, Abdelli S, Yang JY, et al. Exendin-4 protects beta-cells from interleukin-1 beta-induced apoptosis by interfering with the c-Jun NH2-terminal kinase pathway. *Diabetes.* 2008; 57:1205–1215. [PubMed: 18252896]
62. Gedulin BR, Nikoulina SE, Smith PA, et al. Exenatide (exendin-4) improves insulin sensitivity and {beta}-cell mass in insulin-resistant obese fa/fa Zucker rats independent of glycemia and body weight. *Endocrinology.* 2005; 146:2069–2076. [PubMed: 15618356]
63. Jin CX, Li WL, Xu F, et al. Conversion of immortal liver progenitor cells into pancreatic endocrine progenitor cells by persistent expression of Pdx-1. *J Cell Biochem.* 2008; 104:224–236. [PubMed: 17979180] Account of a protocol to convert LEPCs into insulin producing cells.
64. Hori Y, Gu X, Xie X, Kim SK. Differentiation of insulin-producing cells from human neural progenitor cells. *PLoS Med.* 2005; 2:e103. [PubMed: 15839736]
65. Liu M, Han ZC. Mesenchymal stem cells: Biology and clinical potential in Type 1 Diabetes Therapy. *J Cell Mol Med.* 2008 Comprehensive review of the status of research efforts to convert mesenchymal stem cells into insulin producing cells.
66. Moriscot C, de Fraipont F, Richard MJ, et al. Human bone marrow mesenchymal stem cells can express insulin and key transcription factors of the endocrine pancreas developmental pathway upon genetic and/or microenvironmental manipulation in vitro. *Stem Cells.* 2005; 23:594–603. [PubMed: 15790780]
67. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature.* 2002; 418:41–49. [PubMed: 12077603]
68. Ai C, Todorov I, Slovak ML, et al. Human marrow-derived mesodermal progenitor cells generate insulin-secreting islet-like clusters in vivo. *Stem Cells Dev.* 2007; 16:757–770. [PubMed: 17204604]

17999597] The authors outline a protocol used to differentiate human mesodermal progenitor cells into insulin producing cells.

69. Mantel C, Guo Y, Lee MR, et al. Checkpoint-apoptosis uncoupling in human and mouse embryonic stem cells: a source of karyotypic instability. *Blood*. 2007; 109:4518–4527. [PubMed: 17289813]
70. Chidgey AP, Layton D, Trounson A, Boyd RL. Tolerance strategies for stem-cell-based therapies. *Nature*. 2008; 453:330–337. [PubMed: 18480814]
71. Dominguez-Bendala J, Klein D, Ribeiro M, et al. TAT-mediated neurogenin 3 protein transduction stimulates pancreatic endocrine differentiation in vitro. *Diabetes*. 2005; 54:720–726. [PubMed: 15734848]
72. Denker HW. Human embryonic stem cells: the real challenge for research as well as for bioethics is still ahead of us. *Cells Tissues Organs*. 2008; 187:250–256. [PubMed: 18268403] Discussion of some of the ethical concerns associated with stem cell research.

Figure 1. Four-step protocol for the differentiation of human embryonic stem cells into insulin producing cells

Depicted are the protocols used by Phillips et al (A) and Jiang et al (B). The factors used for differentiation are denoted above and below the arrows, and the relative stages of development are shown in boxes. The relevant genes expressed at each stage are shown above and below the boxes. Abbreviations: Bmp4, Bone morphogenetic protein 4; FGF18, Fibroblast growth factor 18; bFGF, basic Fibroblast growth factor; EGF, Epidermal growth factor; TGF α , Transforming growth factor α ; IGF1, Insulin-like growth factor I; IGFII, Insulin-like growth factor II; VEGF, Vascular endothelial growth factor; HGF, Hepatocyte growth factor; PP, Pancreatic polypeptide; Gck, Glucokinase; Sur1, Sulfonylurea Receptor-1; Gcg, glucagon; Sst, somatostatin. Adapted with permission from (A) Phillips et al. 2007. *Stem Cells Dev*; 16: 561–578 and (B) Jiang et al. 2007. *Cell Res*. 2007; 17: 333–344.

Figure 2. Expanded five-step protocol for the differentiation of human embryonic stem cells into insulin producing cells

Depicted are the protocols used by D'Amour et al (A) and Kroon et al (B). The factors used for differentiation are denoted above and below the arrows, and the relative stages of development are shown in boxes. The relevant genes expressed at each stage are shown within parentheses. Abbreviations: FGF10, Fibroblast growth factor 10; CYC, KAAD-cyclopamine; KGF, Keratinocyte growth factor; DAPT, γ -secretase inhibitor; IGFI, insulin-like growth factor I; HGF, Hepatocyte growth factor; Iapp, islet amyloid polypeptide; Gcg, glucagon; Sst, somatostatin; Ppy, pancreatic polypeptide; Ghrl, Ghrelin. Adapted with permission from (A) D'Amour et al. 2006. *Nat Biotechnol*; 24: 1392–1401 and (B) Kroon et al. 2008. *Nat Biotechnol*; 26: 443–452.

Table 1Transcriptional Regulation of β Cell Development

Development Stage	Transcription Factor (Reference[s])	Role in Pancreatic Development	Knock-Out Phenotype
Pancreatic Endoderm	Hlxb9 (22, 23)	Expression required for timely activation of Pdx-1 in dorsal pancreas; also required later for β cell development	Dorsal lobe of the pancreas fails to form; abnormal architecture in remaining islets with reduced size and number of β cells
	HNF6 (24, 25)	Expression required for timely activation of Pdx-1 early in development; regulates expression of Neurog3 later	Hypoplastic pancreas and abnormal islet architecture
	Pdx-1 (17, 26, 27)	Initiates onset of pancreogenesis; expressed in all pancreatic progenitors	Pancreatic agenesis
	Ptf1a (28)	Expressed in all pancreatic progenitors; regulates differentiation of exocrine cells	Hypoplastic pancreas and loss of exocrine cells
	Sox9 (29)	Promotes proliferation and maintenance of pancreatic progenitor cells	Pancreatic hypoplasia and reduction of pancreatic progenitors
	Sox4 (30)	Expressed in pancreatic buds during early development	Reduced number of endocrine cells
	Pbx1 (31, 32)	Forms a complex with Pdx-1 that is necessary for pancreatic progenitor pool expansion	Pancreatic hypoplasia and defects in endocrine and exocrine cell differentiation
	Tcf2 (33, 34)	Controls expression of Hnf6	Pancreatic agenesis
	Neurog3 (35)	Expression required for the specification all four endocrine cell types	Complete absence of endocrine cells
Endocrine Precursors	Isl1 (36)	Required for the development of dorsal pancreatic mesenchyme	Absence of islet cells
	NeuroD1 (37)	Expressed in endocrine progenitors	Marked reduction in β cells and altered islet architecture
	Insm1 (38)	Necessary for endocrine precursor differentiation	Endocrine precursors form but lack hormone expression
	Pax6 (39)	Required for endocrine cell differentiation	Altered islet morphology and decrease in all endocrine cell types

Development Stage	Transcription Factor (Reference[s])	Role in Pancreatic Development	Knock-Out Phenotype
β Cell Specification	Pax4 (40)	Regulates development of endocrine cells	Reduced numbers of β and δ cells
	MafB (41)	Expressed in immature α and β cells during development	Reduced α and β cells.
	MafA (42, 43)	Switch from MafB to MafA observed in differentiation of β cells; required for insulin gene transcription	No defects in development but impaired insulin secretion
	Nkx2.2 (44, 45)	Has dual roles as a transcriptional activator and repressor during development	Reduced α , β , and PP cells but increased numbers of ϵ cells.
	Nkx6.1 (46)	Plays a role in β cell formation and maturation postnatally.	Reduced numbers of mature β cells