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**Advances and challenges in the differentiation of pluripotent stem cells into pancreatic β Cells**

Abdelalim EM *et al.* Pluripotent stem cell-derived pancreatic β Cells

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**Abstract**

Pluripotent stem cells (PSCs) are able to differentiate into several cell types, including pancreatic β cells. Differentiation of pancreatic β cells depends on certain transcription factors, which function in a coordinated way during pancreas development. The existing protocols for *in vitro* differentiation produce pancreatic β cells, which are not highly responsive to glucose stimulation except after their transplantation into immune-compromised mice and allowing several weeks for further differentiation to ensure the maturation of these cells *in vivo.* Thus, although the substantial improvement that has been made for the differentiation of induced PSCs and embryonic stem cells toward pancreatic β cells, several challenges still hindering their full generation.Here, we summarize recent advances in the differentiation of PSCs into pancreatic β cells and discuss the challenges facing their differentiation as well as the different applications of these potential PSC-derived β cells.

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**Key words:** Embryonic stem cells; Induced pluripotent stem cells; Insulin-secreting cells; *In vitro*; *In vivo;* Differentiation; Maturation

**Core tip:** Pluripotent stem cells (PSCs) including induced PSCs and embryonic stem cells are valuable sources for cell replacement therapies and disease modeling of diabetes. Although several studies reported the differentiation of PSCs into pancreatic β cells *in vivo*, still their response to glucose is very limited in *vitro* due to their immature nature. In this review, we summarize the current knowledge about the differentiation of PSCs into pancreatic β cells and discuss the challenges facing the differentiation and application of PSC-derived β cells.

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**INTRODUCTION**

Diabetes mellitus (DM) is a common disease, characterized by hyperglycemia caused by insufficient insulin production and/or resistance to insulin. Two different kinds of DM are well characterized, type 1 (T1D) and type 2 (T2D). T1D is an autoimmune disease in which insulin-secreting β cells in pancreatic islets are permanently damaged by autoimmune attack, resulting in a lack of insulin production[[1](#_ENREF_1)]. T2D occurs when the pancreas produces insufficient amounts of insulin and/or the tissues of the body become resistant to normal or even high levels of insulin[[2](#_ENREF_2)]. Thus, T1D and T2D patients suffer from insulin insufficiency and damage of insulin-secreting β cells by distinct mechanisms[[3](#_ENREF_3),[4](#_ENREF_4)].

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced PSC (iPSCs) have the ability to differentiate into all cell types of the body[[5](#_ENREF_5),[6](#_ENREF_6)], which make them valuable sources for cell replacement therapies and other applications. Several reports have showed the generation of iPSCs from patients with different forms of diabetes[[7-12](#_ENREF_7)]. Also, the generation of patient-specific ESCs has been recently generated from somatic cells of diabetic patients[[13](#_ENREF_13)]. Thus, generation of functional β cells from human PSCs represents the most promising approach to treat diabetes. In addition to their high potential in cell therapy, β cells derived from patient-specific PSCs can be used as a disease model, where they provide a new approach to culture cells with a disease genotype and re-establishing pathogenesis *in vitro.* Disease modeling with such cells has the potential to give insights into the molecular mechanisms underlying diabetes and enable new cell-based drug discovery as well as autologous transplantation[[13](#_ENREF_13),[14](#_ENREF_14)].

Several studies reported the generation of pancreatic β cells from ESCs[[15-18](#_ENREF_15)] and iPSCs[[7](#_ENREF_7),[8](#_ENREF_8),[12](#_ENREF_12),[15](#_ENREF_15),[19](#_ENREF_19)]. However, a number of obstacles have arisen that render the generation of fully functional pancreatic β cells from PSCs[[14](#_ENREF_14)]. Therefore, in this review we summarize the current knowledge about the differentiation of ESCs and iPSCs into pancreatic β cells and discuss the challenges facing the differentiation and application of PSC-derived β cells.

**DIFFERENTIATION OF PLURIPOTENT STEM CELLS INTO INSULIN-SECRETING β CELLS**

It has been well documented that both hESCs and hiPSCs share the same characteristics and properties and are able to differentiate into various types of cells under certain cultural conditions[[6](#_ENREF_6),[20](#_ENREF_20),[21](#_ENREF_21)]. Among these cells, insulin-secreting β cells differentiation from both hESCs and hiPSCs has been studied using similar differentiation protocols (Figure 1)[[8](#_ENREF_8),[15-17](#_ENREF_15),[19](#_ENREF_19)]. However, variation in the efficiencies of differentiation has been reported between different hPSC lines[[22](#_ENREF_22),[23](#_ENREF_23)]. These variations might occur due to the use of different protocols and different cell lines. The process of β cells differentiation is controlled by a complex network that depends on transcriptional regulation of genes involved in the development of the pancreas, the type and number of the differentiation factors, and the type and conditions of stem cell culture. Although many factors are important for successful generation of pancreatic insulin-secreting β cells from PSCs, successful manipulation of culture conditions stands out as one of the more critical factors. Several initial studies have used the step-wise approach to differentiate insulin-secreting cells from hESCs. Under these cultural conditions, hESCs generate up to 12% pancreatic β cells; however, the ability of these differentiated cells to produce insulin in response to glucose was very limited[[16](#_ENREF_16),[18](#_ENREF_18),[24](#_ENREF_24)]. Therefore, a number of modifications in culture conditions have been done to improve the efficiency of β cell differentiation[[16](#_ENREF_16),[18](#_ENREF_18),[25](#_ENREF_25),[26](#_ENREF_26)]. These modifications include, but are not limited to, monolayer cultures[[16-18](#_ENREF_16),[25](#_ENREF_25),[27](#_ENREF_27),[28](#_ENREF_28)] or embryoid body (EB) technique[[29](#_ENREF_29),[30](#_ENREF_30)]. Also, several studies have used xeno-free culture system to generate pancreatic β cells from PSCs *in vitro*[[31-34](#_ENREF_31)]*.* Furthermore, *in vitro* modifications of cultural conditions to regulate signaling pathways demonstrated the vital role of specific pathways in the enhancement of β cell differentiation (Nostro *et al*[29], 2011). In hESCs, theregulation of transforming growth factor β (TGFβ) signaling produces high yields of insulin-secreting cells that reaches up to 25%[[29](#_ENREF_29)]. Importantly, it has been proven that such critical refining in pancreatic differentiation protocols efficiently enhances the differentiation and maturation of hESC-insulin-secreting cells *in vivo*. These generated insulin-secreting cells showed several properties of functional β cells and were capable to respond efficiently to glucose stimulation in engrafted mice[[25](#_ENREF_25)].

***Differentiation of pluripotent stem cells into definitive endoderm***

Similar to embryonic pancreatic development, PSCs differentiation into pancreatic lineage is excised in several steps that start with the differentiation into definitive endoderm (DE) (Figure 1), which is recognized by the expression of specific markers. Previous studies showed a high percentage (60%-80%) of hESC-differentiated cells express a panel of specific DE endodermal markers such as SOX17, FOXA2, CXCR4, and GSC, but not the visceral endodermal marker, SOX7[[15](#_ENREF_15),[16](#_ENREF_16),[25](#_ENREF_25),[35-37](#_ENREF_35)]. The initiation of DE differentiation is properly induced in hESCs and hiPSCs by NODAL and WNT signals[[15](#_ENREF_15),[18](#_ENREF_18),[35](#_ENREF_35),[38](#_ENREF_38)]. NODAL signals have been previously reported to be the main inducer of endogenous endoderm[[39](#_ENREF_39)] and is activated by one of the members of TGFβ family, activin A. Notably, the dose of activin A appears to be crucial for Nodal signaling activation and in-turn DE differentiation. A previous study showed that the use of activin A in a concentration of (50-100 ng/mL) leads to an efficient DE differentiation as compared to lower concentrations[[30](#_ENREF_30),[40](#_ENREF_40)]. In association with activin A, other factors have been shown to play an important role in DE differentiation. A recent study showed that treatment of hESCs with a combination of activin A, wortmannin (PI3K inhibitor), and CHIR99021 improves the percentage (90%) of the generated SOX17-positive cells[[41](#_ENREF_41)]. Furthermore, it has been previously shown that the combination of activin A with sodium butyrate[[16](#_ENREF_16)], PI3K pathway antagonists[[15](#_ENREF_15),[38](#_ENREF_38)], or Wnt signaling activators (WNT3A or CHIR9902) enhances the efficiency of DE differentiation in PSCs. It is worth to note that CHIR99021 has been found to be more potent in promoting SOX17-and FOXA2-positive endodermal cells than Wnt3A[[28](#_ENREF_28)]. Like wise, treating hESCs with GSK3β inhibitor instead of WNT3A increases DE generation[[42](#_ENREF_42)]. Also, another TGFβ family member, GDF8 (myostatin), has been found to be effective for stimulating DE[[43](#_ENREF_43)]. However, the efficiency of DE differentiation not only depends on GDF8, or activin A and its associated factors but also is enhanced by small molecules such as IDE1 and IDE2, which has been found to significantly induce the differentiation of approximately 80% of ESCs into SOX17-expressing DE cells[[44](#_ENREF_44)].

It is well known that DE eventually generates both pancreatic and hepatic tissues. To direct DE cells towards pancreatic differentiation *in vitro*, the alternative hepatic lineage differentiation is inhibited by treating the cells with different types of growth factors inhibitors, such as SU5402 (FGF receptor antagonist) and Noggin (BMP antagonist)[[36](#_ENREF_36)]. It has been shown that BMP signaling exerts two opposite effects during pancreatic differentiation [[45](#_ENREF_45)]. In the dorsal endoderm, BMP signaling inhibition has been found to be required for specific differentiation into pancreatic lineage, whereas it is presence after the formation of pancreatic cells is essential to maintain pancreatic and duodenal homeobox 1 gene (PDX1) expression[[45](#_ENREF_45)].

***Differentiation into pancreatic progenitors and endocrine cells***

In the dorsal endoderm, BMP signaling inhibition has been found to be required for specific differentiation into pancreatic lineage, whereas its presence after the formation of pancreatic cells is essential to maintain pancreatic and PDX1 expression[[45](#_ENREF_45)]. PDX1 is a transcription factor that is expressed on all pancreatic precursor cells and has been shown to be essential for early pancreatic development[[46](#_ENREF_46)]. It has been found that the expression of PDX1 is correlated with the pancreas developmental stages. During the early stages of endocrine specification, PDX1 expression becomes restricted, whereas at later stages during β cells development its expression is upregulated as the protein enhances β cell function and is involved in insulin secretion[[46](#_ENREF_46)]. The differentiation of PDX1-expressing cells *in vitro* is regulated by several factors that range from signaling pathways inhibitors to protein kinase activators. For example, in hESCs, the differentiation of pancreatic progenitors expressing PDX1 is induced by a small molecule, Indolactam V, that activates protein kinase C[[17](#_ENREF_17)], and enhanced by retinoic acid (RA) and dorsomorphin (a BMP type 1 receptor inhibitor) treatments [[28](#_ENREF_28)], whereas its proliferation is increased by inducing epidermal growth factor (EGF) signaling[[15](#_ENREF_15)]. Two other signaling pathways, NOTCH and HEDGEHOG, have been shown to be involved in the DE differentiation into pancreatic endocrine cells[[16](#_ENREF_16)] (Figure 1). The inhibition of HEDGEHOG-signaling by Cyclopamine or KAAD cyclopamine induces the generation of PDX1-expressing cells[[17](#_ENREF_17),[18](#_ENREF_18),[25](#_ENREF_25),[27](#_ENREF_27),[29](#_ENREF_29)], whereas Fibroblast growth factor 10 (FGF10) activates NOTCH signaling, which is involved in the proliferation of PDX1-expressing pancreatic progenitors.

Another transcription factor to consider as a marker for late pancreatic cell development is neurogenin 3 (NGN3). NGN3 expression peaks during endocrine differentiation stage, which is subsequent to the generation of PDX1 pancreatic progenitors. It has been previously shown that the capacity of PSCs to differentiate into pancreatic endocrine cells is highly affected by the seeding density of initial cultures. For example, Gage *et al*[[47](#_ENREF_47)] demonstrated that high density hESCs led to remarkable increase in that rate of cell differentiation into PDX1- and NGN3-positive cells as compared to low density cultured cells. In contrast to PDX1, NGN3 expression is stimulated by a reduction in NOTCH signaling. It has been previously shown that the blockage of NOTCH signaling at this stage may be driven by DAPT (the gamma secretase inhibitor) treatment and thus drive NGN3-expressing cells formation[[17](#_ENREF_17),[18](#_ENREF_18),[27](#_ENREF_27)]. Interestingly, these cells have been found to be differentiated from PDX1-expressing pancreatic progenitors after specific treatments. A recent study used a xeno-free culture system showed that a high NOGGIN concentration is crucial for inducing the differentiation of iPSCs into pancreatic progenitors (PDX1-positive) and then pancreatic endocrine progenitors (NGN3-posisitve cells)[[34](#_ENREF_34)]. A previous study showed that TGFβ type 1 receptor inhibitor (SB431542) stimulates the differentiation of hESC-derived PDX1-positive pancreatic cells into NGN3-positive pancreatic endocrine progenitors[[28](#_ENREF_28),[48](#_ENREF_48)]. Another recent study reported the generation of NGN3-positive endocrine precursors after the inhibition of vesicular monoamine transporter 2 (VMAT2) by reserpine and tetrabenazine (TBZ) in PDX1-positive cells[[49](#_ENREF_49)].

***Differentiation into pancreatic β cells***

The generation of mature pancreatic β cells from hESCs and hiPSCs is characterized by the expression of a panel of different factors, such as PDX1, MAFA, NKX6.1, NEUROD, ISL-1, and GLUT2, C-peptide, and INS (insulin)[[15](#_ENREF_15)]. Of these factors, NKX6.1 expression has been shown to be essential for the production of functional mature β cells. This significant role of NKX6.1 was remarkably clear in the *in vivo* studies done by Rezania and colleagues[[50](#_ENREF_50)], where they showed that hyperglycemia significantly reduced in diabetic mice engrafted with cells expressing substantial amounts of NKX6.1. On the other hand, diabetic mice transplanted with cells expressing low levels of NKX6.1 remains hyperglycemic. In another study, it has been demonstrated that mice lacking Nkx6.1 does not develop pancreatic β cells. However, re-expression of Nkx6.1 in pancreatic progenitors (Pdx1-positive) restores the development of pancreatic β cells[[51](#_ENREF_51)]. It has been also reported that overexpression of some pancreatic development-transcription factors enhance pancreatic β cell differentiation[[52-54](#_ENREF_52)]. In human ESCs, enforced expression of the transcription factor PAX4 significantly promotes cell differentiation into pancreatic β cells[[55](#_ENREF_55)]. Also, overexpression of human PAX4 in hESC-derived pancreatic progenitors increases the number of insulin-secreting β cells, which produce only one hormone (insulin) by suppressing the expression of ARX and glucagon[[56](#_ENREF_56)]. In contrast, overexpression of PDX1 induces pancreatic endocrine cell differentiation, but not in vitro β-cell formation during the differentiation of hESCs into EBs[[57](#_ENREF_57)].

Several treatments, forskolin (an adenylate cyclase activator) and dexamethasone (a synthetic adrenocortical steroid)[[28](#_ENREF_28)], hepatocyte growth factor (HGF), insulin growth factor 1(IGF-1) and glucogon-like peptide 1 (GLP-1)[[9](#_ENREF_9)], have been used to enhance pancreatic β cell maturation *in vitro*. Several studies have previously reported the successful production of insulin-secreting cells *in vitro* from either hESCs or hiPSCs[[15](#_ENREF_15),[16](#_ENREF_16),[18](#_ENREF_18),[19](#_ENREF_19),[24](#_ENREF_24),[25](#_ENREF_25),[29](#_ENREF_29),[30](#_ENREF_30),[36](#_ENREF_36),[58-60](#_ENREF_58)]. However these differentiated β cells showed little ability to respond to glucose, which is considered one of the well-known and important parameters that recognize functional and mature β cells. These differentiated cells also lack the expression of specific mature pancreatic β cell markers such as NKX6.1 and MAFA[[19](#_ENREF_19)], that categorize them as immature non-functional β cells. Moreover, hPSC-derived β cells co-express multiple hormones such as INS, GCG (glucagon), and C-peptide instead of secreting only insulin hormone, which is secreted only by mature β cells. Therefore, controversy exists as to the ability of PSCs to differentiate into fully functional pancreatic β cells[[61](#_ENREF_61)]. A previous study showed that endocrine precursors could be differentiated into glucose responsive β cells[[49](#_ENREF_49)], which is considered one of the major challenges during β cell differentiation process.

***In vivo maturation of pluripotent stem cell-derived pancreatic β cells***

Despite these contentious *in vitro* evidences, mentioned above, *in vivo* microenvironment is likely to be important for pancreatic β cells maturation. Several transplantation studies have demonstrated the differentiation of immature pancreatic β cells or pancreatic progenitors into mature β cells *in vivo*. In either healthy[[25](#_ENREF_25),[30](#_ENREF_30)] or streptozotocin (STZ)-induced diabetes mice[[62](#_ENREF_62)], functional and mature pancreatic β cells are successfully developed from hESC-derived pancreatic progenitors transplanted either under the mouse kidney capsule or fat pad. Interestingly, another study showed that pancreatic progenitor cells are still capable to differentiate into mature pancreatic β cells that efficiently secrete insulin within macroencapsulation devices that have been transplanted into diabetic mice[[63](#_ENREF_63)]. Like wise, in two different mouse models that mimic human T1D and T2D[[64](#_ENREF_64),[65](#_ENREF_65)] *in vivo*, transplanted iPSC-derived β cells efficiently differentiate into glucose-responsive pancreatic β cells. In these studies as well as another study that has used monkey iPSC-derived β cells [[48](#_ENREF_48)], the generated functional pancreatic β cells were found to improve hyperglycemia in the transplanted mouse models. Other studies reported that transplantation of pancreatic progenitors derived from PSCs into mice induces the differentiation of polyhormonal cells into glucose responsive pancreatic β cells (Figure 1). Interestingly, it has been found that such transition into mature β cells is associated with dynamic chromatin remodeling[[58](#_ENREF_58),[66-68](#_ENREF_66)].

Moreover, it has been found that urocortin 3 (Ucn3), which is a corticotropin-releasing factor, is highly expressed in pancreatic β cells and regulates glucose-stimulated insulin secretion[[69](#_ENREF_69)]. Although Ucn3 expression is detected in pancreatic β cells after *in vivo* maturation, it has not been detected after *in vitro* differentiation, suggesting the difference between *in vitro* and *in vivo* differentiation[[70](#_ENREF_70)]. Interestingly, the level of Ucn3 expression increases more than sevenfold between immature and mature pancreatic β cells[[70](#_ENREF_70)]. Thus *in vivo* maturation is critical for both the functionality and maturity of PSC-derived pancreatic β cells, indicating that there may be specific signals at the transplantation sites that induce and enhance β cell differentiation and maturation.

**CHALLENGES AND FUTURE PERSPECTIVES**

Several challenges need to be addressed before using PSCs in clinical applications and disease modeling. Differentiation of PSCs into mature, glucose responsive β cellsis required for proper cellular therapies as well as for re-establishing the disease phenotypes *in vitro* to understand the mechanisms underlying different forms of diabetes. Pancreatic β cell maturation is defined based on glucose-stimulated insulin secretion (GSIS). To date, although several reports showed an improvement in generating glucose-responsive insulin-secreting β cells *in vivo*, still their response to glucose is very limited in *vitro* due to their immature nature[[8](#_ENREF_8),[15-17](#_ENREF_15),[19](#_ENREF_19)]. A recent study has reported that insulin-secreting β cells differentiated from hPSCs are highly similar to human fetal pancreatic β cells than adult β cells[[71](#_ENREF_71)]. Also, it has been suggested that the inability of pancreatic β cells to produce adequate insulin is due to the gradual loss of a specific group of transcription factors, which is important for insulin secretion and glucose responsiveness. Previous studies reported that dysfunctional pancreatic β cells derived from hESCs fail to secrete PDX1, NKX6.1, and MAFA[[18](#_ENREF_18),[25](#_ENREF_25),[72](#_ENREF_72)], indicating that restoring the transcription factors is essential to recover β cell functions. Another study found that some genes related to early embryonic development (DPPA4, LIN28A, and LIN28B) continue to be expressed in the cells differentiated from PSCs, suggesting that the cells differentiated from PSCs are similar to the cells found in the very early stage of human development[[73](#_ENREF_73)]. Notwithstanding the above findings, transplantation of pancreatic progenitors derived from PSCs into immune-compromised mice induces their differentiation into glucose responsive mature β cells[[7](#_ENREF_7),[50](#_ENREF_50),[58](#_ENREF_58),[66-68](#_ENREF_66)]. Thus, future studies should focus on the signaling pathways regulating the maturation of pancreatic β cells *in vitro.*

Another problem hindering cell therapies of PSCs in diabetes is the low efficiency in producing insulin-secreting β cells. The current protocols are still lacking critical signals required for efficient generation of insulin-secreting β cells. Therefore, future studies should focus on studying the molecular mechanisms, guiding normal pancreatic development and to develop highly efficient differentiation protocols to obtain large number of insulin-secreting β cells.

Furthermore, one of the problems facing the stem cell therapies is the potential for unwanted cell growth. Undifferentiated PSCs are characterized by their ability to produce teratoma *in vivo* due to their high pluripotential capabilities. Previous studies suggested that encapsulation of the transplanted cells prevents overgrowth[[74](#_ENREF_74),[75](#_ENREF_75)]. In addition, encapsulation was found to be crucial to differentiate PSCs into pure culture of insulin-secreting β cells to avoid their contamination with undifferentiated cells. To use the functional pancreatic β cells to cure T1D, the repair of self-tolerance or eliminate the influences of autoimmunity must be considered, thus the immune system cannot destroy the newly transplanted insulin-secreting β cells. Therefore, several studies reported the use of encapsulated devices to protect pancreatic β cells from immunological attack. Glucose responsive insulin-secreting grafts have been generated from encapsulated CyT49-luc hESC-derived pancreatic epithelium (hESC-PE) and found that maturation occurs without an increase in cell biomass[[75](#_ENREF_75)]. Thus, encapsulation is considered as a possible tool to overcome some of the grand challenges in diabetes treatment by stem cell therapy.

Another challenge is related to the use of ESCs in cell therapy due to ethical concerns and immune rejection problem. The use of patient-specific ESCs, which are immunologically compatible to the patient, would overcome the immune rejection issue since ESCs can be generated from the somatic cells of the patients using SCNT[[13](#_ENREF_13),[76](#_ENREF_76)].

In conclusion, there is a lot of work need to be done to obtain fully functional pancreatic β cells *in vitro*. For example, a genome-wide transcriptional analysis should be performed in each stage during pancreatic β celldifferentiation to recognize the defects in the transcription factors. Also, comparing *in vivo* pancreatic development with *in vitro* pancreatic differentiation is important to identify the difference in gene expression, which may account for the functional differences. Finally, different differentiation protocols should be applied to understand the signaling pathways controlling the differentiation process *in vitro*.

In conclusion, there is a lot of work need to be done to obtain fully functional pancreatic β cells *in vitro*. For example, a genome-wide transcriptional analysis should be performed in each stage during pancreatic β celldifferentiation to recognize the defects in the transcription factors. Also, comparing *in vivo* pancreatic development with *in vitro* pancreatic differentiation is important to identify the difference in gene expression, which may account for the functional differences. Different differentiation protocols should be applied to understand the signaling pathways controlling the differentiation process *in vitro*.

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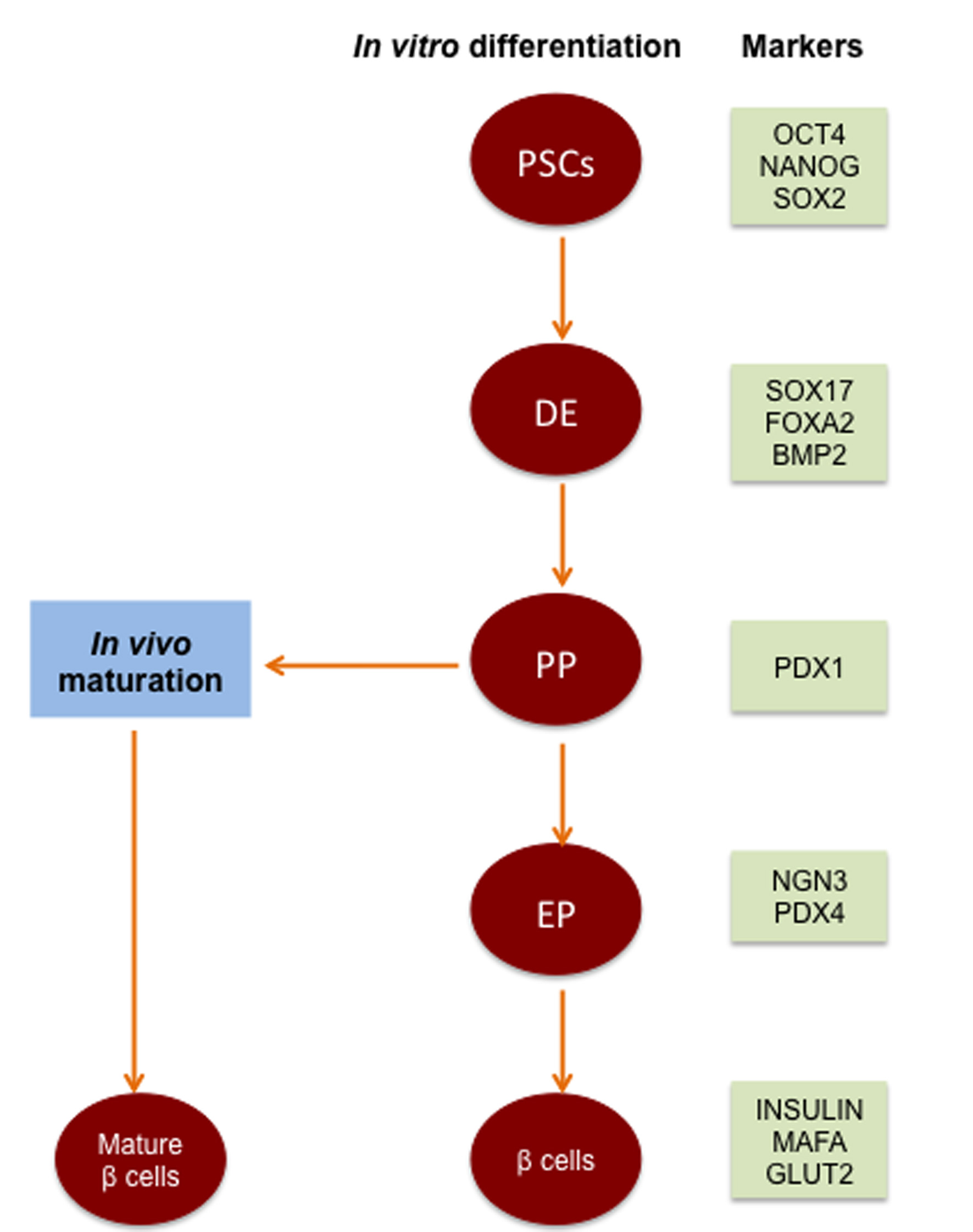
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**Figure 1 Schematic representation of the differentiation of pluripotent stem cells into pancreatic β cells.** Pluripotent stem cells (PSCs) can be differentiated *in vitro* into pancreatic ß cells after a step-wise differentiation protocol into definitive endoderm (DE), pancreatic progenitors (PP), pancreatic endocrine (PE) progenitors, and ß cells. Transplantation of pancreatic progenitors derived from PSCs into immune-compromised mice induces their differentiation into glucose responsive pancreatic β cells (mature β cells). During pancreatic β cell differentiation, specific transcription factors are expressed at different stages.