Differentiation into Endoderm Lineage: Pancreatic differentiation from Embryonic Stem Cells

Dong Hyeon Lee¹, Hyung Min Chung²

¹Department of Physiology, School of Medicine, CHA University, Seongnam, ²CHA Stem Cell Institute & CHA Bio&Diotech, CHA University, Seoul, Korea

The endoderm gives rise to digestive and respiratory tracts, thyroid, liver, and pancreas. Representative disease of endoderm lineages is type 1 diabetes resulting from destruction of the insulin-producing β cells. Generation of functional β cells from human embryonic stem (ES) cells *in vitro* can be practical, renewable cell source for replacement cell therapy for type 1 diabetes. It has been achieved by progressive instructive differentiation through each of the developmental stages. In this article, important studies of differentiation into pancreatic β cells from ES cells are reviewed through pancreatic developmental stages as definitive endoderm, primitive gut tube/foregut, and pancreatic cells. The investigation of differentiating ES cells from definitive endoderm to pancreas using signaling, arrays, and proteomics is also introduced.

Keywords: Endoderm, Primitive gut tube, Pancreatic β cell, Embryonic stem cell

Introduction

The endoderm gives rise to the epithelial lining of the digestive and respiratory tracts, and organs as the thyroid, liver, gall bladder, and pancreas (1). Type 1 diabetes and chronic hepatitis are representative diseases involved in organs derived from endoderm. Specific cell type losses as pancreatic β cells and limitations of medical treatment lead this disease to be a preferred candidate for cell-based therapy.

Type 1 diabetes results from destruction of the insulin-secreting β cells in the pancreatic islets of Langerhans by T cell-mediated autoimmune (2). Type 1 diabetes has been treated by injection of exogenous insulin, and glucose monitoring combining with insulin injections. The more physiological treatment is replacement of β cells by

whole pancreas or transplantation of islets of Langerhans from human donors, however, it is a great obstacle of transplantation that the donor of pancreatic tissue is rare. Researchers are trying to develop the ways to generate replacement sources of pancreatic β cells for cell therapy and some of the studies involve the directed differentiation of embryonic stem (ES) cells to pancreas development into glucose-responsive β cells (3). It has been achieved by progressive instructive differentiation through each of the developmental stages (4, 5). Generation of functional β cells from human ES cells *in vitro* can be practical, renewable cell source for replacement therapy.

The ES cells that are generated from the inner cell mass of blastocyst-stage embryos represent a promising source of cells for transplantation or cell-based therapy of any damaged cells. They can be maintained in culture, renew for themselves, and proliferate unlimitedly as undifferentiated ES cells (6). The ES cells are capable of differentiating into all cell types of the body as the ectoderm, mesoderm, and endoderm lineage cells or tissues. The major benefit of ES cells is stable self-renewal in culture and the potential to differentiate. These unique intrinsic properties lead the researchers to invent tailored cell therapy

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Correspondence to **Dong Hyeon Lee**

Department of Physiology, School of Medicine, CHA University,
222 Yatap-dong, Bundang-gu, Seongnam 463-836, Korea

Tel: +82-31-725-8307, Fax: +82-31-725-8339

E-mail: leedh@cha.ac.kr

for incurable disease or malignancy. The important task in using ES cells for cell therapy is to discovery protocols that direct their differentiation into specific and functional cells. In type 1 diabetes, resolutions of this issue are a thorough understanding of pancreatic islet development and underlying developmental mechanism, and characterization of each development stage according to the derivation process for promoting cell therapy.

In this article, important studies of differentiation into pancreatic β cells from ES cells are reviewed through pancreatic developmental stages as definitive endoderm, primitive gut tube/foregut, and pancreatic endocrine cells. Considering the difficulty of handling human embryos and the differences between humans and other species, differentiating human ES cells to pancreas would allow for the investigation of the normal developmental processes or congenital diseases. Thus, the investigation of differentiating ES cells from definitive endoderm to pancreas using signaling, arrays, and proteomics are also introduced.

Differentiation to definitive endoderm from ES cells

The definitive endoderm is generated *in vivo* from the inner cell mass by the process of gastrulation of embryogenesis, in which epiblast cells are instructed to form the three germ layers. Definitive endoderm gives rise to diverse cells and tissues that contribute to vital organs as the pancreatic β cells, liver hepatocytes, lung alveolar cells, thyroid, thymus, and the epithelial lining of the alimentary and respiratory tract (1). It is different from the primitive endoderm of extraembryonic tissues, which gives rise to the visceral and parietal endoderm. The definitive endoderm derived from ES cells is theoretically capable of becoming any endoderm derivatives, and directing ES cells into the endoderm lineage is a prerequisite for generating therapeutic endoderm derivatives.

The ability to identify and regulate endoderm precursor populations is a major issue (7, 8). It is possible that the signals regulating endoderm differentiation during normal embryonic development could also instruct ES cells to commit to an endoderm fate. The first study to derive definitive endoderm was introduced by Kubo, which differentiated mouse ES cells into definitive endoderm with activin (7). The formation of endoderm cells from mouse embryoid bodies (EB) was induced by high levels of activin A which mimics the action of Nodal. Nodal is a ligand for transforming growth factor- β (TGF- β) superfamily and activates a series of downstream signaling events and

transcriptional network that regulates definitive endoderm development (9). EB stimulated by activin consisted of more than 50% endoderm, based on the expression of transcription factor forkhead box A2 (Foxa2). Yasunaga reported that mouse ES cells cultured as monolayer were induced into definitive endoderm by treatment of activin A (10). Detection of Goosecoid (Gsc) and SRY (sex determining region Y)-box 17 (Sox17) markers allowed separation of mesendoderm-derived definitive endoderm (Gsc⁺ Sox17⁺) from visceral endoderm (Gsc⁻ Sox17⁺), which can define culture conditions that permit selective differentiation to either definitive or visceral endoderm.

Subsequently human ES cells were also efficiently differentiated into definitive endoderm using high concentration of activin A (11). First of all, mesendoderm differentiation was promoted by culturing ES cells in monolayer at a low concentration of fetal bovine serum (FBS) with Wnt3A and activin A for the first day of differentiation. Then, the differentiation of ES cells to definitive endoderm was achieved by culturing ES cells with activin A for another 2 days. The differentiated human ES cells consisted of 80% definitive endoderm, as measured by co-expression of FOXA2 and SOX17, important definitive endoderm differentiation markers. Additionally other markers of definitive endoderm, such as cerberus 1 (CER1) and chemokine (C-X-C motif) receptor 4 (CXCR4), were also up-regulated. The definitive endoderm further differentiated by grafting under the kidney capsule into mature endoderm derivatives as intestinal epithelial cells and liver hepatocytes. It indicates the importance of activin and nodal signaling in inducing definitive endoderm differentiation and superiority of monolayer to EB in culture.

Aforementioned mesendoderm that are prior to definitive endoderm differentiation are important developmental stage of ES cells. The fate of mesendoderm into mesoderm or endoderm is dependent on population with different surface markers and regulated signaling. Application of either activin or nodal under defined conditions selectively induced ES cells into mesendoderm which is represented as a Gsc⁺, E-cadherin (Ecd)⁺, platelet-derived growth factor receptor- α (PDGFR- α)⁺ population (12). It gives rise to two different intermediates; Gsc⁺, Ecd⁺, PDGFR- α ⁻ population and Gsc⁺, Ecd⁻, PDGFR- α ⁺ population, and they will preferentially differentiate into definitive endoderm and mesodermal lineages, respectively. Also, activation of Wnt pathway using Wnt3A or an inhibitor of glycogen synthase kinase 3 β (GSK-3 β) drove mouse and human ES cells cultured in monolayer condition to differentiate into mesendoderm (8). They are brachyury (T)⁺, Flk-1 (kinase insert domain protein re-

ceptor, Kdr^+ , Gsc^+ , and $Foxa2^+$ population and have potential to differentiate to mesodermal cell lineages and possibly to endodermal cell lineages. Using the tetracycline-regulated expression of Nodal, efficient differentiation method of mouse ES cells was introduced (13). The upregulation of Nodal signaling pathway induced the specification of ES cells into definitive endoderm and mesoderm derivatives. This system induced a mesendodermal progenitor population expressing $Cxcr4$, VEGF receptor 2 (VEGFR2), and PDGFR- α in about 70% of cells, which is superior to the application of exogenous activin.

Moreover, various approaches to establish definitive endoderm were investigated. The constitutive expression of SOX17 established stable definitive endoderm progenitors from human ES cells (14). SOX17 overexpressing ES cells demonstrated the ability to differentiate toward hepatic and pancreatic derivatives in the absence of activin A treatment. Recently, the small molecules, IDE1 and IDE2, were identified as mimics of activin A by screening a chemical library and they can efficiently generate SOX17⁺ definitive endoderm from mouse and human ES cells cultured in monolayer (15). These compounds induced nearly 80% of ES cells to form definitive endoderm, which is a higher efficiency than that achieved by activin A or Nodal. The use of these small molecules would be more adequate for large-scale, reproducible, directed ES cell differentiation into definitive endoderm and functional pancreatic β cells.

The definitive endoderm derived from ES cell is a powerful tool to perform cellular, molecular, and biochemical analyses of differentiation process. The research about definitive endoderm cells is necessary to understand not only its derivation but also key areas such as gene expression, signaling pathways, and transcription factors. Suppression of phosphatidylinositol 3-kinase (PI3K) signaling pathways through insulin/insulin-like growth factor (IGF) were reported to be necessary in activin/nodal signaling-induced definitive endoderm differentiation in human ES cells (16). Retinoic acid induced primitive endoderm differentiation of ES cells, which suppressed cell proliferation (17), and it was achieved by restricting nuclear entry of activated MAPK. Recently, proteomic analysis of definitive endoderm cells derived from human ES cells suggested that definitive endoderm cells up-regulated adenomatous polyposis coli (APC) and cyclin B3 (CCNB3), and repressed the expression of 70-kDa heat-shock protein 9 precursor (HSPA9), chaperonin containing TCP1 (CCT2), and tyrosine 3/tryptophan 5-monooxygenase activation protein (YWHAE) (18). They can be involved in the dif-

ferentiation processes and be differentiation markers. Beside, differentiation of definitive endoderm from human ES cells induced switch of the expressions of each cyclin subtype (18). These studies provided a model system and the tools necessary to investigate early human definitive endoderm development and they can supply endoderm cells to various applications.

Differentiation to primitive gut tube and foregut

Precise patterning of anterior-posterior axis of the definitive endoderm eventually forms the primitive gut tube. The definitive endoderm-derived primitive gut tube induces the pharynx, esophagus, stomach, duodenum, small and large intestine along the anterior-posterior axis as well as associated organs, including pancreas, lung, thyroid, thymus, parathyroid, and liver (3, 19). The anterior portion of the foregut of the primitive gut tube becomes lung, thyroid, esophagus, and stomach. The pancreas, liver, and duodenum originate from the posterior portion of the foregut. The midgut and hindgut of primitive gut tube gives rise to the small and large intestine. The anterior foregut expresses developmental markers, NK2 homeobox 1 (NKX2-1) and SRY (sex determining region Y)-box 2 (SOX2); the posterior foregut expresses hematopoietically expressed homeobox (HHEX), pancreatic and duodenal homeobox 1 (PDX1), one cut homeobox 1 (ONECUT1, known as HNF6), and hepatocyte nuclear factor 4 alpha (HNF4A); and the midgut/hindgut expresses caudal type homeobox 1 (CDX1), caudal type homeobox 2 (CDX2), and motor neuron and pancreas homeobox 1 (MNX1) (3, 19, 20).

D'Amour first suggested the primitive gut tube and posterior foregut stages in the efficient differentiation process that converts definitive endoderm derived from human ES cells into pancreatic lineage endocrine cells expressing pancreatic hormone (4). This process mimics *in vivo* pancreatic organogenesis by instructing cells through each endoderm intermediate stage similar to those that occur during pancreatic development; definitive endoderm, primitive gut tube endoderm, pancreatic endoderm, and endocrine precursor. Differentiation process to primitive gut tube was induced by adding FBS and removing activin A from the culture for 3 days. The removal of activin A induced the progression from definitive endoderm to primitive gut endoderm which expressed primitive gut tube markers hepatocyte nuclear factor 1B (HNF1B) and HNF4A (4). The addition of the growth factor, fibroblast growth factor 10 (FGF10), and hedgehog signaling inhibitor, 3-Keto-N-(amino-ethyl-aminocaproyl)-dihydrocin-

namoyl)-cyclopamine, or keratinocyte growth factor (KGF) significantly promotes primitive gut tube differentiation and further β cell differentiation (4, 5). To induce posterior foregut differentiation *in vitro*, the differentiated primitive gut tube cells treated with retinoic acid, cyclopamine, and FGF10 or Noggin, a TGF inhibitor were cultured for 3 days in serum-free conditions (4, 5). Differentiated posterior foregut cells express significant levels of PDX1, HNF6, and SRY (sex determining region Y)-box 9 (SOX9).

FGFs and retinoic acid regulate differentiation of primitive gut tube from definitive endoderm (20-23). FGF2 specifies human ES cell-derived definitive endoderm into different foregut lineages in a dosage-dependent manner (20). Increasing concentrations of FGF2 inhibits hepatocyte differentiation, whereas intermediate concentration of FGF2 promotes differentiation toward a pancreatic cell fate. High FGF2 concentrations increased specification of midgut endoderm into small intestinal progenitors and also promoted differentiation toward an anterior foregut pulmonary cell fate. Retinoic acid and FGF4 jointly directed differentiation of activin-induced definitive endoderm of human ES cells into PDX1⁺ foregut endoderm cells in an efficient manner (22). This combination generates 32% PDX1⁺ foregut endoderm cells which can be committed to pancreatic, posterior stomach, or duodenal endoderm.

Various studies, including omic, signaling, or characterization, are needed to discover the precise differentiation mechanism and properties of human primitive gut tube and foregut. Recently, proteomic analysis of primitive gut tube cells derived from definitive endoderm of human ES cells indicated that the expression levels of three proteins, ras-responsive element-binding protein 1 (RREB1), rod photoreceptor cGMP-specific phosphodiesterase beta-subunit (PDE6B), and CD209, involved in general signal transduction, were increased in primitive gut tube (24). These proteins could be important signaling candidates in the differentiation of primitive gut tube cells from human ES cells.

Differentiation to functional pancreatic endocrine cells

Of particular therapeutic interest for type 1 diabetes, the various techniques for the differentiation of ES cells to the pancreatic β cells have been developed. In the last decade, several different approaches have been described for differentiation of mouse and human ES cells into insulin-expressing cells. In any cases, the successful differ-

entiation to pancreatic β cells should require that differentiated cells synthesize and secrete physiologically appropriate amounts of insulin. It attracted researcher's attentions that the insulin-secreting pancreatic β cells were directly differentiated from human ES cells by mimicking the developmental stages of pancreatic organogenesis *in vivo* (4, 5).

This area of research was initiated by the reports demonstrating the generation of insulin-expressing cells from mouse ES cells (25,26). Using a cell-trapping system, an insulin-secreting cell clone was selected from undifferentiated ES cells, which contained total insulin of 16.5 ng/ μ g protein, corresponding to about 90% of the insulin content of mouse islets, and secreted hormone in regulated manner (25). Other attempts reported the achievement of insulin-producing cell clusters from mouse ES cells using a method based on selection of nestin⁺ progenitor population after spontaneous differentiation of EB *in vitro* (26-28). This experimental strategy is following 5 stages. They are expanding ES cells (stage 1); generating EB for enriching nestin⁺ cell population (stage 2); plating the EBs into a serum-free medium (ITSFn) for increasing the proportion of nestin⁺ cells (stage 3); expanding these cells in N2 serum-free medium with B27 supplement and basic FGF (bFGF) (stage 4); withdrawal bFGF from N2 medium with B27 supplement and nicotinamide for inducing differentiation and promoting cessation of cell division (stage 5). These cells were transplanted into streptozocin (STZ)-induced diabetic mice but the cells did not secrete enough insulin to rescue hyperglycemia (26, 27). Some modification of the protocol was reported to improve differentiation of insulin-producing cells. Mouse ES cells treated with LY294002, an inhibitor of PI3K, showed improved differentiation and greater level of insulin (27).

Although this method was successful at generating a subpopulation of cells that express the pancreatic hormone insulin, the cells had a high degree of cellular heterogeneity and proliferation, and low insulin levels compared to pancreatic islets (29, 30). The insulin⁺ cells derived from ES cells revealed not to be real pancreatic β cells but to be likely to neuronal cells (30). These insulin-producing cells rarely expressed C-peptide and were unable to rescue STZ-induced hyperglycemia. However, Fujikawa reported that these cells expressed insulin mRNA and C-peptide and released insulin in a glucose-dependent manner (31). Even though the transplanted insulin-expressing cells formed immature teratoma, they reversed the hyperglycemic state for 3 weeks after transplanted into diabetic mice.

Some other protocols to generate pancreatic β cell were

suggested. ES cells were cultured with serum and monothioglycerol and reformed to EB cultured in media with serum replacement, which generated PDX1⁺ and insulin I and II⁺ cells (32). The addition of β cell specification and differentiation factors activin β B, nicotinamide, and extendin-4 to ES-derived EB increased the number of insulin⁺ cells to 2.73% of the total population. By non-selective culture protocol, EB made of mouse ES cells in suspension culture with serum spontaneously differentiated into PDX1⁺ pancreatic progenitor cells and islet hormone-expressing cells (33). Furthermore three-step experimental approach induced mouse ES cells to differentiate into insulin-producing cells in 2 weeks and insulin released from these cells was regulated by the glucose concentration (34). This protocol was based on forming EB, expanding insulin-producing precursors, and maturing the insulin-producing cells with combination of activin A, retinoic acid, bFGF, N2 supplement, B27 supplement, laminin, and nicotinamide. It supported the stepwise differentiation of mouse ES cells through major stages of early pancreatic development of embryogenesis.

Human ES cells were spontaneously differentiated *in vitro* in both adherent and suspension culture conditions, which generated cells with characteristics of insulin-producing β cells (35). The differentiated cells positively stained for insulin were about 1~3%. By passing human ES cells through an EB formation step, they can be differentiated into PDX1⁺ pancreatic progenitors (36). Under non-selective conditions, human ES cells spontaneously differentiated to cells expressing the definitive endoderm and pancreatic progenitor markers FOXA2, SOX17, and PDX1, and some cells expressing islet endocrine hormones. However, the frequency of insulin cells derived from human ES cells is very low.

A stepwise protocol directing human ES cell differentiation were developed, which entails differentiation processes that recapitulates the major stages of normal pancreatic endocrine development (4, 5, 36, 37). The differentiation of ES cells to hormone-expressing pancreatic endocrine cells was conducted by transiting ES cells through major stages of embryonic development; differentiation to mesendoderm and definitive endoderm, establishment of the primitive gut endoderm, patterning of the posterior foregut, and specification and maturation of pancreatic endoderm and endocrine precursors (4, 5). Through these stages, ES cells can obtain pancreatic endocrine phenotype and ability of glucose responsive insulin secretion *in vitro*.

In this stepwise differentiation protocol, differentiation into definitive endoderm, primitive gut tube, and posterior

or foregut are former three steps in generating pancreatic β cells, which have been reviewed previously. Briefly the human ES cells were first differentiated into definitive endoderm with activin A in low FBS media through mesendoderm, then cells were treated with a combination of FGF10 or KGF, and KAAD-cyclopamine and then with retinoic acid, KAAD-cyclopamine, and FGF10 or Noggin. These cultures induced the expression of the posterior foregut markers HNF6, HNF1B, HNF4A, SOX9, and PDX1. The next stages are involved in pancreatic endoderm and endocrine precursors. The posterior foregut cells were cultured in serum-free conditions with extendin 4, IGF1, DAPT, and hepatocyte growth factor (HGF) to potentiate insulin secretion in response to glucose (4) or cultured in serum free with no supplements (5). The differentiated ES cells expressed PDX1, NK6 homeobox 1 (NKX6-1), HNF6, NK2 homeobox 2 (NKX2-2), neurogenin 3 (NEUROG3, NGN3), paired box 4 (PAX4), neurogenic differentiation 1 (NEUROD1), ISL LIM homeobox 1 (ISL1), paired box 6 (PAX6), and/or synaptophysin. They contained secretory granules with high concentration of insulin and secreted insulin in response to secretagogue. These cells thus had functional ATP-sensitive potassium channel and voltage-dependent calcium channels, key components of insulin secretory machineries, however, glucose-responsive insulin secretion was absent. Also insulin⁺ cells made up only about 7% of the differentiated ES cell population and pancreatic endocrine (synaptophysin⁺) cells was 13% (4). Probably they are needed to be more matured and the derivation protocol to be more efficient. The last stage of pancreatic β cell differentiation involves maturation of endocrine progenitors to hormone-producing cells. Engrafting differentiated pancreatic endocrine cells into mice promoted their maturation into β cells (5). These cells can synthesis insulin content similar to that found in adult islet cells and secrete insulin in response to glucose, which work more physiologically than the cells are differentiated *in vitro*. After these prominent studies, several other groups modified differentiation protocols following the main developmental stages of the pancreatic endocrine cells (38-41).

Based on the above studies, monolayer cultures are more efficient for differentiation of ES cells into pancreatic endocrine progenitor cells rather than EB formation. The study of Kroon gives the clues that three-dimensional cultures can be more effective for maturation of pancreatic β cell progenitors into functional ones (5). Therefore a combination of monolayer culture and three-dimensional culture or transplantaion protocol can lead to derive more functional pancreatic β cells *in vitro*. Jiang introduced se-

rum-free protocol generating about 24% PDX1⁺ cells and 4% C-peptide⁺ cells (40), which composed of four stages, definitive endoderm induction, pancreatic endoderm formation, endocrine induction, islet-like cluster maturation. Human ES cell grown in monolayer under feeder-free conditions was exposed to sodium butyrate and activin A to generate definitive endoderm expressing SOX17, FOXA2, and CXCR4. This cell population was converted into cellular aggregates cultured in suspension and further differentiated to PDX1⁺ pancreatic endoderm by the treatment of epidermal growth factor (EGF), bFGF, and Noggin. FGF10 signaling expands pancreatic progenitor cells, notch signaling decides divergence to be an endocrine or exocrine cell, and EGF treatment facilitates the expansion of pancreatic progenitors (40, 42). The aggregates were matured by applying IGF-II and nicotinamide. This population contained numerous secretory granules and was able to secrete C-peptide in response to glucose stimulation as well as produce glucagon and somatostatin.

Other study produced a population of cells expressing C-peptide, insulin, glucagon, and GLUT2 by modified differentiation method which generated over 15% C-peptide⁺ cells of total differentiated cells (41). Human ES cells were induced to differentiate into functional insulin-producing cell in a serum-free system through definitive endoderm, pancreatic commitment, and insulin producing cells with activin A and retinoic acid in chemically defined medium, and bFGF and nicotinamide in DMEM/F12. And then EB was formed and cultured in suspension to achieve islet maturation. The derived cells expressing PDX1, NKX6.1, and PAX6 have secretory vesicles and are glucose responsive, which rescue the STZ-induced hyperglycemia. Other protocol of sequential applying serum, activin, and retinoic acid differentiated EB of human ES cells into FOXA2⁺, SOX17⁺, PDX1⁺, and homeobox HB9 (HLXB9)⁺ cell populations, definitive endoderm-derived pancreatic endodermal cells (43). Transplantation into STZ-treated diabetic mice caused PDX1⁺ cells to differentiate into more matured cell types producing C-peptide, insulin, and glucagon, resulting in amelioration of hyperglycemia. Previously described methods (34, 41) were also modified (37). An efficient stepwise protocol directed pancreatic differentiation into mature insulin-producing cells similar to adult islet β cells from the human ES cell. The application of activin A and wortmannin induced definitive endoderm formation and the treatment with retinoic acid, Noggin, and FGF7 induced pancreatic specialization. EGF expanded pancreatic progenitors and a cocktail of bFGF, nicotinamide, exendin-4, and bone morphogenetic protein 4 (BMP4) induced the progenitors to mature. This

approach obtained nearly 25% insulin⁺ cells which released insulin and C-peptide in response to glucose stimuli in a manner comparable to that of adult human islets.

Another trial is overexpressing transcription factors, which direct the differentiation of ES cells to insulin-expressing cells. The transcription factors used in the previous study are critical for β cell development as PDX1 (28, 44-46), PAX4 (28, 47), NEUROG3 (48, 49), NKX2-2 (50), or NEUROD1 (46), however, overexpressing pancreatic transcription factors in ES cells are not an efficient method to generate pancreatic cells. Next discovery is small molecules that can modulate various stages of β cell differentiation from ES cells. Aforementioned IDE1 and IDE2 can be very useful to derivate pancreatic β cells. Definitive endoderm produced by IDE1 and IDE2 initiated pancreatic differentiation in response to retinoic acid, FGF10, and cyclopamine (15). Indolactam V identified by screen of chemical libraries promoted pancreatic progenitor formation from definitive endoderm (51).

Concluding remarks

The accumulated knowledge of pancreas development in embryogenesis has given anticipation to development of techniques to instruct the differentiation of ES cells into pancreatic endocrine cells. Recent outstanding researches have led the differentiation of ES cells into definitive endoderm, primitive gut tube, and pancreatic β cells by directing them to follow major developmental stages. Even though various materials and approaches have been adopted in previous studies, derivation of ES cells through stepwise developmental stages produce differentiated ES cells highly efficiently and they exert more physiological function and are more similar to target cells. It indicates the usefulness of ES cells in major source for the tailored cell therapy for type 1 diabetes and diseases originated from endoderm and in the study of regenerative medicine and human development. The studies that would be necessary to focus are about better practical derivation protocols as well as discovery of important signaling, genes, and proteins for understanding differentiation mechanism.

Potential conflict of interest

The authors have no conflicting financial interest.

References

1. Lu CC, Brennan J, Robertson EJ. From fertilization to gastrulation: axis formation in the mouse embryo. *Curr Opin Genet Dev* 2001;11:384-392

2. Eiselein L, Schwartz HJ, Rutledge JC. The challenge of type 1 diabetes mellitus. *ILAR J* 2004;45:231-236
3. Guney MA, Gannon M. Pancreas cell fate. *Birth Defects Res C Embryo Today* 2009;87:232-248
4. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* 2006;24:1392-1401
5. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J, Agulnick AD, D'Amour KA, Carpenter MK, Baetge EE. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol* 2008;26:443-452
6. Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. *Nat Biotechnol* 2005;23:699-708
7. Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. Development of definitive endoderm from embryonic stem cells in culture. *Development* 2004;131:1651-1662
8. Bakre MM, Hoi A, Mong JC, Koh YY, Wong KY, Stanton LW. Generation of multipotential mesendodermal progenitors from mouse embryonic stem cells via sustained Wnt pathway activation. *J Biol Chem* 2007;282:31703-31712
9. Champeris Tsaniras S, Jones PM. Generating pancreatic beta-cells from embryonic stem cells by manipulating signaling pathways. *J Endocrinol* 2010;206:13-26
10. Yasunaga M, Tada S, Torikai-Nishikawa S, Nakano Y, Okada M, Jakt LM, Nishikawa S, Chiba T, Era T, Nishikawa S. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. *Nat Biotechnol* 2005;23:1542-1550
11. D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* 2005;23:1534-1541
12. Tada S, Era T, Furusawa C, Sakurai H, Nishikawa S, Kinoshita M, Nakao K, Chiba T, Nishikawa S. Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. *Development* 2005;132:4363-4374
13. Takenaga M, Fukumoto M, Hori Y. Regulated Nodal signaling promotes differentiation of the definitive endoderm and mesoderm from ES cells. *J Cell Sci* 2007;120:2078-2090
14. Séguin CA, Draper JS, Nagy A, Rossant J. Establishment of endoderm progenitors by SOX transcription factor expression in human embryonic stem cells. *Cell Stem Cell* 2008;3:182-195
15. Borowiak M, Maehr R, Chen S, Chen AE, Tang W, Fox JL, Schreiber SL, Melton DA. Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. *Cell Stem Cell* 2009;4:348-358
16. McLean AB, D'Amour KA, Jones KL, Krishnamoorthy M, Kulik MJ, Reynolds DM, Sheppard AM, Liu H, Xu Y, Baetge EE, Dalton S. Activin efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. *Stem Cells* 2007;25:29-38
17. Smith ER, Smedberg JL, Rula ME, Xu XX. Regulation of Ras-MAPK pathway mitogenic activity by restricting nuclear entry of activated MAPK in endoderm differentiation of embryonic carcinoma and stem cells. *J Cell Biol* 2004;164:689-699
18. Lee DH, Ji YG, Chung HM, Ko JJ, Kim SK. Proteomic identification of overexpressed Adenomatous polyposis coli and Cyclin B3 during endoderm differentiation from human embryonic stem cells. *Pancreas* 2011;40:271-280
19. Zorn AM, Wells JM. Vertebrate endoderm development and organ formation. *Annu Rev Cell Dev Biol* 2009;25:221-251
20. Ameri J, Ståhlberg A, Pedersen J, Johansson JK, Johannesson MM, Artner I, Semb H. FGF2 specifies hESC-derived definitive endoderm into foregut/midgut cell lineages in a concentration-dependent manner. *Stem Cells* 2010;28:45-56
21. Bayha E, Jørgensen MC, Serup P, Grapin-Botton A. Retinoic acid signaling organizes endodermal organ specification along the entire antero-posterior axis. *PLoS One* 2009;4:e5845
22. Johannesson M, Ståhlberg A, Ameri J, Sand FW, Norrman K, Semb H. FGF4 and retinoic acid direct differentiation of hESCs into PDX1-expressing foregut endoderm in a time- and concentration-dependent manner. *PLoS One* 2009;4:e4794
23. Dessimoz J, Opoka R, Kordich JJ, Grapin-Botton A, Wells JM. FGF signaling is necessary for establishing gut tube domains along the anterior-posterior axis in vivo. *Mech Dev* 2006;123:42-55
24. Lee DH, Ko JJ, Ji YG, Chung HM, Hwang T. Proteomic identification of RREB1, PDE6B, and CD209 up-regulated in primitive gut tube differentiated from human embryonic stem cells. *Pancreas* 2011 (in press)
25. Soria B, Roche E, Berná G, León-Quinto T, Reig JA, Martín F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* 2000;49:157-162
26. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 2001;292:1389-1394
27. Hori Y, Rulifson IC, Tsai BC, Heit JJ, Cahoy JD, Kim SK. Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *Proc Natl Acad Sci U S A* 2002;99:16105-16110
28. Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L, Wobus AM. Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *Proc Natl Acad Sci U S A* 2003;100:998-1003
29. Rajagopal J, Anderson WJ, Kume S, Martinez OI, Melton DA. Insulin staining of ES cell progeny from insulin uptake. *Science* 2003;299:363

30. Sipione S, Eshpeter A, Lyon JG, Korbitt GS, Bleackley RC. Insulin expressing cells from differentiated embryonic stem cells are not beta cells. *Diabetologia* 2004;47:499-508
31. Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am J Pathol* 2005;166:1781-1791
32. Ku HT, Zhang N, Kubo A, O'Connor R, Mao M, Keller G, Bromberg JS. Committing embryonic stem cells to early endocrine pancreas in vitro. *Stem Cells* 2004;22:1205-1217
33. Kahan BW, Jacobson LM, Hullett DA, Ochoada JM, Oberley TD, Lang KM, Odorico JS. Pancreatic precursors and differentiated islet cell types from murine embryonic stem cells: an in vitro model to study islet differentiation. *Diabetes* 2003;52:2016-2024
34. Shi Y, Hou L, Tang F, Jiang W, Wang P, Ding M, Deng H. Inducing embryonic stem cells to differentiate into pancreatic beta cells by a novel three-step approach with activin A and all-trans retinoic acid. *Stem Cells* 2005;23:656-662
35. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. Insulin production by human embryonic stem cells. *Diabetes* 2001;50:1691-1697
36. Xu X, Kahan B, Forgianni A, Jing P, Jacobson L, Browning V, Treff N, Odorico J. Endoderm and pancreatic islet lineage differentiation from human embryonic stem cells. *Cloning Stem Cells* 2006;8:96-107
37. Zhang D, Jiang W, Liu M, Sui X, Yin X, Chen S, Shi Y, Deng H. Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. *Cell Res* 2009;19:429-438
38. Blyszczuk P, Asbrand C, Rozzo A, Kania G, St-Onge L, Rupnik M, Wobus AM. Embryonic stem cells differentiate into insulin-producing cells without selection of nestin-expressing cells. *Int J Dev Biol* 2004;48:1095-1104
39. Schroeder IS, Rolletschek A, Blyszczuk P, Kania G, Wobus AM. Differentiation of mouse embryonic stem cells to insulin-producing cells. *Nat Protoc* 2006;1:495-507
40. Jiang J, Au M, Lu K, Eshpeter A, Korbitt G, Fisk G, Majumdar AS. Generation of insulin-producing islet-like clusters from human embryonic stem cells. *Stem Cells* 2007;25:1940-1953
41. Jiang W, Shi Y, Zhao D, Chen S, Yong J, Zhang J, Qing T, Sun X, Zhang P, Ding M, Li D, Deng H. In vitro derivation of functional insulin-producing cells from human embryonic stem cells. *Cell Res* 2007;17:333-344
42. Jang HK, Kim BS. Modulation of stem cell differentiation with biomaterials. *Int J Stem Cell* 2010;3:80-84
43. Shim JH, Kim SE, Woo DH, Kim SK, Oh CH, McKay R, Kim JH. Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. *Diabetologia* 2007;50:1228-1238
44. Miyazaki S, Yamato E, Miyazaki J. Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells from embryonic stem cells. *Diabetes* 2004;53:1030-1037
45. Lavon N, Yanuka O, Benvenisty N. The effect of over-expression of Pdx1 and Foxa2 on the differentiation of human embryonic stem cells into pancreatic cells. *Stem Cells* 2006;24:1923-1930
46. Saitoh K, Yamato E, Miyazaki S, Miyazaki J. Both Pdx-1 and NeuroD1 genes are requisite for the maintenance of insulin gene expression in ES-derived differentiated cells. *Diabetes Res Clin Pract* 2007;77:S138-S142
47. Liew CG, Shah NN, Briston SJ, Shepherd RM, Khoo CP, Dunne MJ, Moore HD, Cosgrove KE, Andrews PW. PAX4 enhances beta-cell differentiation of human embryonic stem cells. *PLoS One* 2008;3:e1783
48. Treff NR, Vincent RK, Budde ML, Browning VL, Magliocca JF, Kapur V, Odorico JS. Differentiation of embryonic stem cells conditionally expressing neurogenin 3. *Stem Cells* 2006;24:2529-2537
49. Serafimidis I, Rakatzi I, Episkopou V, Gouti M, Gavalas A. Novel effectors of directed and Ngn3-mediated differentiation of mouse embryonic stem cells into endocrine pancreas progenitors. *Stem Cells* 2008;26:3-16
50. Shiroy A, Ueda S, Ougi Y, Saito K, Moriya K, Sugie Y, Fukui H, Ishizaka S, Yoshikawa M. Differentiation of embryonic stem cells into insulin-producing cells promoted by Nkx2.2 gene transfer. *World J Gastroenterol* 2005;11:4161-4166
51. Chen S, Borowiak M, Fox JL, Maehr R, Osafune K, Davidow L, Lam K, Peng LF, Schreiber SL, Rubin LL, Melton D. A small molecule that directs differentiation of human ESCs into the pancreatic lineage. *Nat Chem Biol* 2009;5:258-265