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Betacellulin and nicotinamide sustain *PDX1* expression and induce pancreatic β-cell differentiation in human embryonic stem cells

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Abstract

The major obstacle in cell therapy of diabetes mellitus is the limited source of insulin-producing β cells. Very recently, it was shown that a five-stage protocol recapitulating *in vivo* pancreatic organogenesis induced pancreatic β cells *in vitro*; however, this protocol is specific to certain cell lines and shows much line-to-line variation in differentiation efficacy. Here, we modified the five-stage protocol for the human embryonic stem cell line SNUhES3 by the addition of betacellulin and nicotinamide. We reproduced *in vivo* pancreatic islet differentiation by directing the cells through stages that resembled *in vivo* pancreatic organogenesis. The addition of betacellulin and nicotinamide sustained *PDX1* expression and induced β-cell differentiation. C-peptide—a genuine marker of *de novo* insulin production—was identified in the differentiated cells, although the insulin mRNA content was very low. Further studies are necessary to develop more efficient and universal protocols for β-cell differentiation.

Keywords: Embryonic stem cell; Betacellulin; Nicotinamide; Insulin; Pancreatic β cell

Since defective insulin secretion by pancreatic β cells is the essential pathophysiologic mechanism underlying diabetes mellitus, cell therapy that augments the insulin secretory function of these cells in diabetic patients will undoubtedly be effective. Recent remarkable technical improvement in pancreatic islet transplantation has resulted in insulin independence with good metabolic control in type 1 diabetic patients [1]. However, a long-term follow-up study after pancreatic islet transplantation revealed that prolonged insulin independence was maintained in only a small number of patients [2]. This suggests that repeated transplantation to boost the insulin secretory function is mandatory for maintaining insulin independence with the current protocol. At this time, the major obstacle in the cure of diabetes is the shortage of transplantable pancreatic islets or equivalent β cells. As human embryonic stem (ES) cells have practically unlimited replication capacity, they have been extensively studied for renewable sources of insulin-producing cells. Aside from mouse ES cell studies, many studies have reported the differentiation of insulin-producing cells from human ES cells [3–5].

Since the first report regarding β-cell differentiation from ES cells via lineage selection of nestin-positive cells [6], there has been a great deal of controversy regarding whether the insulin-containing cells that differentiated from nestin-positive progenitor cells are genuine β cells or merely the results of passive uptake of exogenous insulin in the medium from the cells undergoing apoptosis [7–9]. It was suggested that lineage selection of nestin-positive cells should be avoided because these cells are already

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committed to a neural fate before pancreatic differentiation is induced; therefore, the procedure for inducing pancreatic β-cell differentiation would eventually activate apoptotic pathways [8].

A more rational approach to pancreatic β-cell differentiation may be to direct ES cells through a process that recapitulates *in vivo* pancreatic development. Very recently, it has been reported that a five-stage protocol comprising the sequential phases of inducing a definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, and cells that express endocrine hormones mimics *in vivo* pancreatic organogenesis and successfully produced pancreatic β cells from human ES cells *in vitro* [10]. However, this protocol was primarily optimized for the cell line used in that study, and therefore, it requires modification in order to be applicable to other cell lines.

In this study, we modified the previous five-stage protocol by the addition of betacellulin and nicotinamide to the differentiation media. Betacellulin—a 9.5-kDa glycoprotein highly expressed in the pancreas—belongs to the epidermal growth factor family [11] and promotes the differentiation, regeneration, and proliferation of pancreatic β cells under various *in vivo* and *in vitro* conditions [12–15]. Nicotinamide—a poly(-ADP-ribose) synthetase inhibitor—is known to increase the mitotic indices of β cells after pancreatectomy [16] and is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells [17]. By the addition of betacellulin and nicotinamide, we found here that the modified protocol sustained *PDX1* expression and induced pancreatic β-cell differentiation in the human ES cell line SNUhES3.

Materials and methods

Culture of human ES cells

The human ES cell line SNUhES3 was used in this study. The derivation process and characteristics of SNUhES3 have been previously described [18]. The Institutional Review Board at Seoul National University Hospital approved the study protocol, and the Korean Ministry of Health and Welfare also approved the study protocol according to the Life Ethics Law.

SNUhES3 was maintained on STO (mouse fibroblast) feeder layers in knockout Dulbecco's modified Eagle's medium (KO-DMEM) or DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 20% knockout serum replacement (Invitrogen), 0.4 ng/ml basic fibroblast growth factor (FGF) (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% nonessential amino acid (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO), 50 U/ml penicillin, and 50 μg/ml streptomycin (PEST; Invitrogen). The culture medium was preincubated in an incubator at 37 °C, 1 day prior to use. The culture medium for expansion of the STO cells comprised DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), and PEST. The expanded STO cells were mitotically inactivated by mitomycin-C treatment and stored in liquid nitrogen. After thawing, these cells were seeded on 0.1% gelatin-coated culture dishes 1 day before the SNUhES3 cells were plated. The SNUhES3 cultures were manually passaged at a 1:4–1:6 split ratio every 6–7 days.

Differentiation protocol

For the most part, differentiation experiments were carried out according to the previously described five-stage protocol [10]. A brief description of our modified five-stage protocol follows.

Stage 1 (definitive endoderm induction). To induce the mesendoderm, SNUhES3 cells were cultured in RPMI 1640 medium (Invitrogen) containing 100 ng/ml activin A (R&D systems, Minneapolis, MN) and 25 ng/ml Wnt3a (R&D systems) for 1 day. At this stage, FBS was not added to the culture media. After 24 h, the medium was replaced with fresh RPMI 1640 supplemented with 100 ng/ml activin A and 0.2% FBS for 2 days to further differentiate the cells into the definitive endoderm.

Stage 2 (primitive gut tube induction). To induce the primitive gut tube, the culture medium was replaced with RPMI 1640 supplemented with 50 ng/ml FGF10 (R&D systems) and 0.25 μM KAAD-cyclopamine (Toronto Research Chemicals, Downsview, Ont., Canada) for 3 days.

Stage 3 (posterior foregut induction). We replaced the culture medium with DMEM supplemented with 1% B27 (Invitrogen), 2 μM all-*trans* retinoic acid (Sigma), 50 ng/ml FGF10, and 0.25 μM KAAD-cyclopamine.

From stage 4 onward, we used 2 protocols either without (*protocol 1*) or with (*protocol 2*) betacellulin and nicotinamide supplementation.

Stage 4 (pancreatic endoderm induction). In this stage, the cells were cultured with DMEM containing 1% B27, 1 μM γ-secretase inhibitor DAPT (Sigma), and 50 ng/ml exendin-4 (Sigma) for 2 days (protocol 1). In protocol 2, 10 nM betacellulin (R&D systems) and 10 mM nicotinamide (Sigma) were added to the same culture medium.

Stage 5 (pancreatic β-cell induction). In the final stage, the culture medium was replaced with CMRL (Invitrogen) containing 1% B27, 50 ng/ml exendin-4, 50 ng/ml insulin-like growth factor (IGF)-1 (Sigma), and 50 ng/ml hepatocyte growth factor (HGF) (PeproTech, Rocky Hill, NJ) for 6 days (protocol 1). In protocol 2, 10 nM betacellulin and 10 mM nicotinamide were added to the same culture medium.

Immunofluorescence

Cells from each differentiation stage were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature (RT) for 15 min, permeabilized in 0.1% Triton X-100/PBS at RT for 1 min, and blocked with 5% normal goat serum or 3% BSA/PBS at RT for 1 h. The cells were then incubated overnight at 4 °C with the primary antibodies listed below. After three washes with PBS, the cells were incubated with the secondary antibodies at RT for 1 h. The cells were incubated with 10 nM DAPI (Invitrogen) at RT for 15 min, followed by three washes with PBS, and then mounted with DakoCytomation Fluorescent Mounting Medium (Dako, Carpinteria, CA). The resulting fluorescent signal was imaged with a Radiance 2000 confocal microscope (Bio-Rad, Hercules, CA).

The following primary antibodies and dilutions were used: rabbit anti-brachyury (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:100; goat anti-*SOX17* (Santa Cruz), 1:100; goat anti-*FOXA2* (Santa Cruz), 1:100; rabbit anti-*HNF4A* (Santa Cruz), 1:100; mouse anti-*PDX1* (R&D), 1:100; mouse anti-C-peptide (Chemicon, Temecula, CA), 1:100; and rabbit anti-glucagon (Dako), 1:100. The following secondary antibodies (all from Invitrogen) and dilutions were used: goat anti-rabbit immunoglobulin (Ig)G Alexa 488, 1:100–1:200; donkey anti-goat IgG Alexa 555, 1:100–1:200; goat anti-mouse IgG Alexa 488, 1:100; donkey anti-rabbit IgG Alexa 555, 1:100; and goat anti-mouse Alexa594, 1:100.

Reverse transcriptase polymerase chain reaction

To monitor the expression levels of marker genes during differentiation, total RNA from the cells was prepared using the RNeasy mini kit (Qiagen, Valencia, CA). To compare the insulin gene expression levels of differentiated cells, total RNA from isolated human pancreatic islet cells was used. cDNA was obtained using 1 μg RNA with oligo-dT20 (Invitrogen) and avian myeloblastosis virus reverse transcriptase (Invitrogen). Polymerase chain reactions (PCRs) were carried out with 1 μl cDNA template, 10 pM of each primer, and *HiPi* PCR premix (ELPIS-Biotech., Inc., Taejeon, Korea) in 20 μl total reaction volume. The sequences of the primers and conditions of PCR were identical to those used in the previous studies: those for brachyury and *SOX17* were obtained from Ref. [19];

those for *HNF4A* and *FOXA2* (*HNF3B*), from Ref. [20]; and those for *PDX1*, glucagon, insulin, and *GAPDH*, from Ref. [5].

Results

We repeated the five-stage protocol for inducing insulin-producing cells from human ES cells, which was recently published [10]. In stage 1 (definitive endoderm induction), human ES cells differentiated via mesendoderm positive for brachyury (Fig. 1A), which appeared rather specifically during the mesendoderm stage (Fig. 2), to the definitive endoderm in differentiation media supplemented with a high concentration of activin A (100 ng/ml) and low concentration of FBS. These cells expressed the definitive endoderm marker *SOX17* (Fig. 2), which appeared in the early mesendoderm stage, and its expression level peaked in the late mesendoderm and definitive endoderm stages. Thereafter, its expression level decreased with further differentiation into the pancreatic endoderm. The cells at the end of stage 1 also expressed the anterior definitive endoderm marker *FOXA2* (Fig. 1B). Most *FOXA2*-positive cells also expressed the primitive gut tube marker *HNF4A* with co-localization to the nucleus (Fig. 1C). In the reverse transcription (RT)-PCR analysis, *FOXA2* (also known as *HNF3B*) appeared just before the appearance of *HNF4A*.

In stage 2 (primitive gut tube induction), activin A was removed, and KAAD-cyclopamine, a hedgehog inhibitor, and FGF10 were added. Numerous *HNF4A*-positive cells were observed, but none of the cells expressed *PDX1* at this stage (Fig. 1D). In stage 3 (posterior foregut induction), the gut tube endoderm was treated with all-*trans* retinoic acid, KAAD-cyclopamine, and FGF10. At this stage, the cells rapidly began to express *PDX1* (Fig. 3). Some *PDX1*-posi-

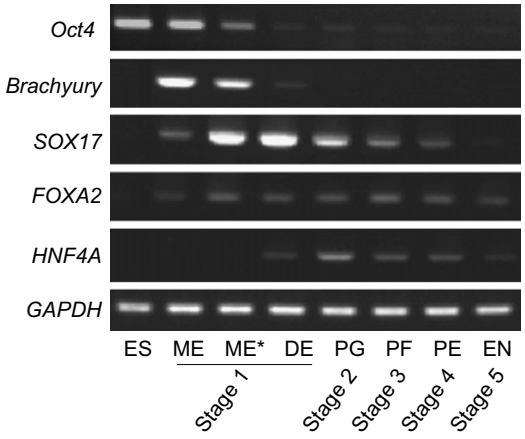

Fig. 2. RT-PCR analyses for several key markers of endoderm differentiation. Abbreviations: ES, undifferentiated ES cells; ME, mesendoderm; ME*, mesendoderm on day 2 of stage 1; DE, definitive endoderm; PG, primitive gut tube; PF, posterior foregut; PE, pancreatic endoderm; and EN, endocrine cells.

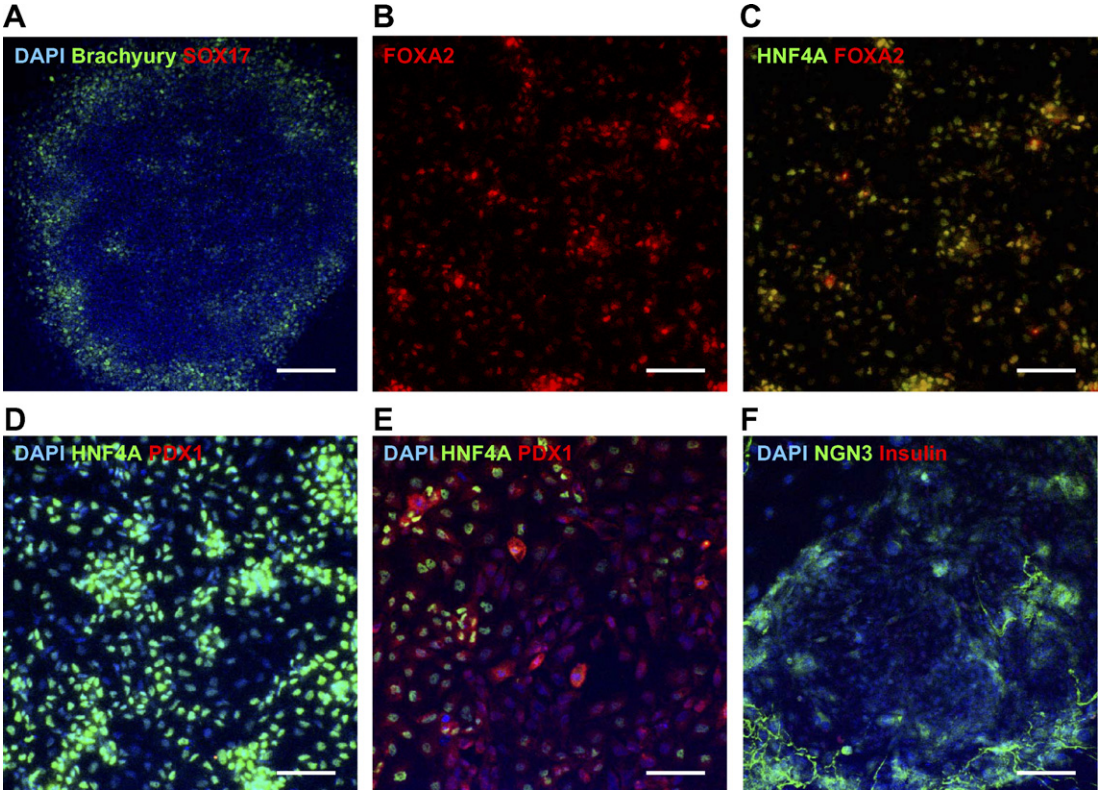

Fig. 1. Immunofluorescence analysis of differentiation of undifferentiated human ES cells to pancreatic endoderm. (A) In the mesendoderm stage (day 1 of stage 1), cells at the periphery of the cell cluster express brachyury (green). (B) In the definitive endoderm stage (day 3 of stage 1), cells express *FOXA2* (red). (C) In the same stage as in (B), *HNF4A* (green) is co-expressed with *FOXA2*. (D) In the primitive gut tube stage (stage 2), numerous cells express *HNF4A* (green). (E) In the posterior foregut stage (stage 3), cells are stained for *PDX1* (red) and *HNF4A* (green). (F) In the pancreatic endoderm stage (stage 4), *NGN3*-positive cells (green) are observed. Scale bars, 200 μm (A,F) and 100 μm (B, C, D, and E).

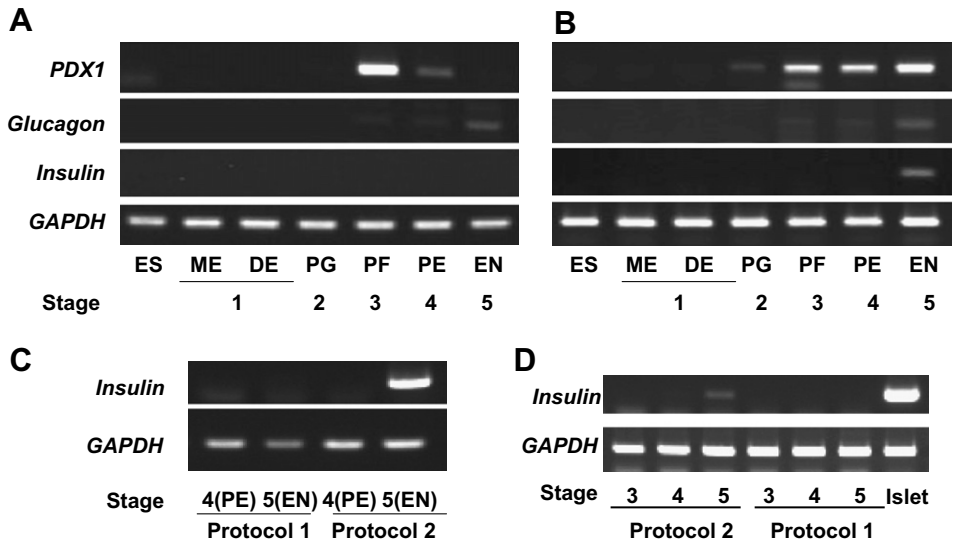

Fig. 3. RT-PCR analyses for pancreatic endocrine cells. (A) Protocol 1: no betacellulin and nicotinamide supplementation. (B) Protocol 2: betacellulin and nicotinamide supplementation. (C) Here, on increasing the number of PCR amplification cycles to as high as 40, insulin gene expression is clearly observed in stage 5 of protocol 2. (D) Insulin gene expression in stage 5 in protocol 2 was compared to that by isolated human islet cells. Abbreviations: ES, undifferentiated ES cells; ME, mesendoderm; DE, definitive endoderm; PG, primitive gut tube; PF, posterior foregut; PE, pancreatic endoderm; and EN, endocrine cells.

tive cells also showed positive staining for *HNF4A* in the nuclei (Fig. 1E). In stage 4 (pancreatic endoderm induction), the γ-secretase inhibitor DAPT and exendin-4 were added. We observed the expression of *NGN3*, particularly around the peripheral portion of cell clusters; however, we could not observe any insulin staining (Fig. 1F). *PDX1* expression was abruptly decreased in stage 4, and virtually disappeared in stage 5 in protocol 1, i.e., without betacellulin and nicotinamide supplementation (Fig. 3A), whereas it was sustained in stage 4 and slightly increased in stage 5 in protocol 2, i.e., with nicotinamide and betacellulin supplementation (Fig. 3B).

In stage 5 (pancreatic β-cell induction) in protocol 1, endocrine cells expressing C-peptide—a genuine marker of *de novo* insulin production—were not observed, although some cells expressing glucagon were noted (data not shown). In contrast, in protocol 2, clusters of cells expressing C-peptide and glucagon were present (Fig. 4B and C). Immunofluorescence study findings were confirmed by RT-PCR. The cells at the end of stage 5 expressed only glucagon in protocol 1 (Fig. 3A), whereas they expressed both insulin and glucagon in protocol 2 (Fig. 3B). For differentiation of insulin-producing cells, both betacellulin and nicotinamide were necessary and either of them alone was not sufficient enough (data not shown). Insulin gene expression was clearly observed after 40 cycles of PCR amplification in protocol 2, but it was not detected at all in protocol 1 (Fig. 3C). At the end of stage 5, most of the cells were non-endocrine cells that did not express either C-peptide or glucagon; however, approximately 15–20% of the total cells in a monolayer expressed C-peptide in the cytoplasm (Fig. 4C). Considerable number of cells at this stage co-expressed glucagon and C-peptide, while a certain proportion of cells showed predominant C-peptide expression (Fig. 4D). In accordance with this observation, the expression level of insulin in the total cell culture level at the end of stage 5 in protocol 2 was much lower than that of human pancreatic islet cells (Fig. 3D, 30 cycles of PCR amplification using the same amount of cDNA).

Discussion

In this study, we modified the previous five-stage protocol recapitulating *in vivo* pancreatic organogenesis [10] by the addition of betacellulin and nicotinamide to differentiation media and found that the modified protocol sustained *PDX1* expression and induced insulin-producing cells from SNUhES3 human ES cells.

The insulin-producing cells observed in this study were induced through a sequence of stages that resembled the induction of a definitive endoderm (*SOX17* and *FOXA2* positive), gut tube endoderm (*HNF4A* positive), posterior foregut (*PDX1* positive), pancreatic endoderm, and endocrine precursor (*NGN3* positive). The differentiation process reported in this study was consistent with that of a previous study [10]. During RT-PCR analysis, *FOXA2* (also known as *HNF3B*) appeared just before the appearance of *HNF4A*, which was consistent with the fact that *HNF4A* expression is known to be regulated by *FOXA2* (*HNF3B*) [21]. *FOXA2* is also known to play an essential role in the cell type-specific transcription of the *PDX1* gene in the pancreas [22].

The most important finding in this study is that the addition of betacellulin and nicotinamide sustained *PDX1* expression up to the later differentiation stage and induced

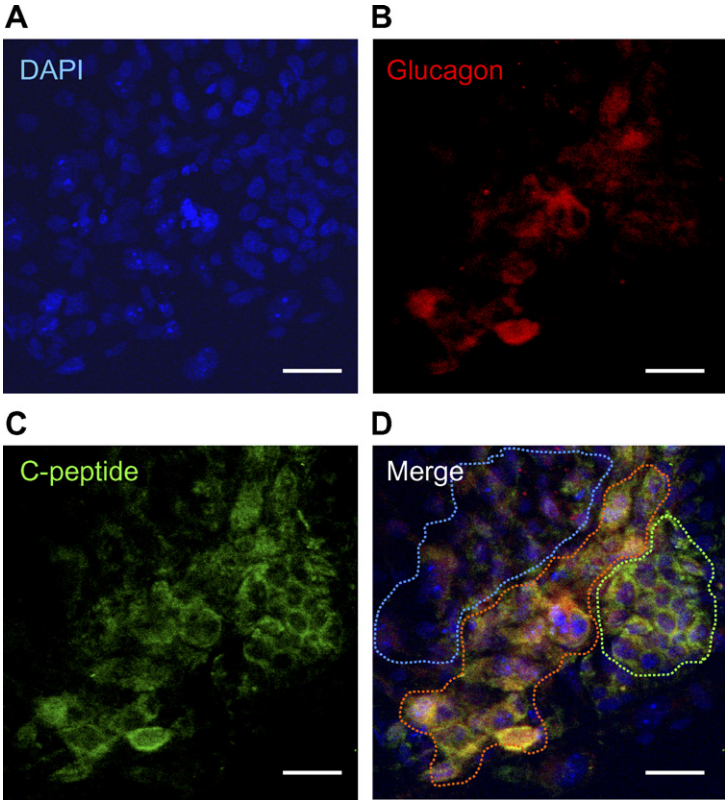

Fig. 4. Immunofluorescence analysis of stage 5 cells supplemented with betacellulin and nicotinamide (protocol 2). Note the DAPI staining in (A) shows that this field of cells is a monolayer, which is adequate for the assessment of co-expression of insulin and glucagon. (B) Glucagon-expressing cells are shown in red. (C) C-peptide-expressing cells are shown in green. (D) Merged image showing the areas of non-endocrine cells (blue dotted line), multi-hormone positive α/β cells (red dotted line), and putative β cells (green dotted line). Scale bars, 50 μm.

β-cell differentiation. Betacellulin is known to promote the differentiation, regeneration, and proliferation of pancreatic β cells under various *in vivo* and *in vitro* conditions. Betacellulin, together with activin A, can convert amylase-secreting exocrine AR42J rat pancreatic tumor cells into insulin-secreting cells [14]. Intraperitoneal injection of betacellulin promotes β-cell regeneration in 90% pancreatectomized Wistar rats [13]. It induces insulin and glucokinase gene expression in *PDX1*-expressing glucagonoma cells [15] and promotes the proliferation of the rat insulinoma INS-1 cell line [12]. Interestingly, betacellulin was reported to increase *PDX1* expression and insulin production [23], which is consistent with the findings of protocol 2 in our study. Since *PDX1* induces the expression of the insulin gene [15,24], sustained expression of *PDX1* in the later stage of β-cell development is crucial.

Nicotinamide—a poly(ADP-ribose) synthetase inhibitor—is also known to increase the mitotic indices of β cells after pancreatectomy [16] and is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells [17]. Therefore, nicotinamide might play an important role in terminal endocrine differentiation observed in this study.

The putative β cells in this study are believed to produce insulin *de novo* since insulin gene expression was detected by RT-PCR and the cells stained positive for C-peptide—a genuine marker of *de novo* insulin synthesis [7,9]. However, a considerable portion of the cells expressing C-peptide also positively stained for glucagon, and the efficiency of β-cell differentiation was relatively low. Although there is a controversy, particularly regarding rodents [25], multi-hormonal cells have been reported during early fetal development in both rodents [26] and humans [27,28]. In addition, the increase of insulin concentration in culture media after stimulation of various secretagogues was very modest and statistically insignificant (data not shown). Taken together, the cells expressing both C-peptide and glucagon observed in this study may be immature β cells, which may also explain the low insulin mRNA expression as compared to normal human islet.

It was previously reported that culture in low glucose (5 mmol/l) medium with nicotinamide supplementation increased insulin content approximately 20-fold [29]. In the current study, we also tested the low glucose condition in protocol 2. However, this condition did not result in the differentiation of insulin-producing cells (data not shown).

In summary, the addition of betacellulin and nicotinamide to the previous five-stage protocol [10] sustained *PDX1* expression and induced insulin-producing cell differentiation from SNUhES3 human ES cells. Further studies are necessary to develop more efficient and universal protocols for β-cell differentiation.

Acknowledgments

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