

Activin A, exendin-4, and glucose stimulate differentiation of human pancreatic ductal cells

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Abstract

Islet transplantation is one treatment option for diabetes mellitus. However, novel sources of pancreatic islets or insulin-producing cells are required because the amount of donor tissue available is severely limited. Pancreatic ductal cells are an alternative source of β -cells because they have the potential to differentiate into insulin-producing cells. We investigated whether treatment of human pancreatic ductal cells with activin A (ActA) and exendin-4 (EX-4) stimulated transdifferentiation of the cells, both *in vitro* and *in vivo*. We treated human pancreatic ductal cells with ActA and EX-4 in high-glucose media to induce differentiation into insulin-producing cells and transplanted the cells into streptozotocin-induced diabetic nude mice. Co-treatment of mice with ActA and EX-4 promoted cell proliferation, induced expression of pancreatic β -cell-specific markers, and caused glucose-induced insulin secretion compared with the ActA or EX-4 mono-treatment groups respectively. When pancreatic ductal cells treated with ActA and EX-4 in high-glucose media were transplanted into diabetic nude mice, their blood glucose levels normalized and insulin was detected in the graft. These findings suggest that pancreatic ductal cells have a potential to replace pancreatic islets for the treatment of diabetes mellitus when the ductal cells are co-treated with ActA, EX-4, and glucose to promote their differentiation into functional insulin-producing cells.

Key Words

- ▶ Activin A
- ▶ Exendin-4
- ▶ Glucose
- ▶ Human pancreatic ductal cells
- ▶ Differentiation

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Introduction

One of the major determinants of both type 1 and type 2 diabetes mellitus is loss of β -cell mass or dysfunction in the pancreas. Transplantation of an entire pancreas or pancreatic islets is an effective method to treat diabetes. However, there is a severe shortage of available donor tissue. Most studies have demonstrated that the growth capacity of islets *in vitro* is limited (Brelje *et al.* 1993).

Moreover, culture of human adult islets requires preparation of an extracellular matrix and several growth factors. However, most of the growth protocols for islets result in a decrease in the insulin-producing capacity of the islets (Yuan *et al.* 1996, Lefebvre *et al.* 1998, Beattie *et al.* 1999). Other sources of β -cells, such as embryonic stem cells, bone marrow stem cells, pancreatic acinar cells,

pancreatic duct epithelium cells (ductal cells), and pancreatic stem or progenitor cells, have been shown to be able to differentiate into insulin-producing cells (Bonner-Weir *et al.* 2004, Bonner-Weir & Weir 2005, Yue *et al.* 2006). Among these, human pancreatic ductal cells have the advantage that they can be harvested from pancreatic remnants after islet isolation.

Activin A (ActA) is a member of the transforming growth factor β superfamily, and it regulates the proliferation and differentiation of many types of cells (Hemmati-Brivanlou & Melton 1994) as well as embryonic axial patterning and the functions of foregut-derived organs (Moriya *et al.* 2000). Moreover, ActA can promote pancreatic bud formation, facilitate the differentiation of endocrine and exocrine cells in the pancreas, and induce the formation of pancreatic islets (Yamaoka *et al.* 1998). ActA has been reported to induce amylase-secreting pancreatic exocrine cell lines to differentiate into pancreatic polypeptide-producing endocrine cells (Mashima *et al.* 1996). Furthermore, injection of ActA with beta-cellulin reduced hyperglycemia in neonatal streptozotocin (STZ)-induced diabetic rats (Li *et al.* 2004). ActA is also needed at relatively early stages of the differentiation of embryonic stem cells, and it promotes induced pluripotent stem cells to change into endocrine cells (Tateishi *et al.* 2008, Maehr *et al.* 2009, Zhang *et al.* 2009). We previously reported that ActA could potentiate the differentiation of rat pancreatic ductal cells (Park *et al.* 2007). Therefore, we hypothesized that ActA might have the potential to stimulate both the growth and differentiation of pancreatic ductal cells.

Glucagon-like peptide-1 (GLP1) is an intestinal insulinotropic hormone that is secreted from L-cells of the distal ileum and colon (Drucker 1998). GLP1 administration in patients with type 2 diabetes mellitus can decrease their glucose levels (Gutniak *et al.* 1992, Nauck *et al.* 1993, Thorens *et al.* 1993). Moreover, GLP1 increases the β -cell mass by inducing the differentiation and neogenesis of ductal cells into insulin-producing cells through regulation of PDX1 expression (Mojsov *et al.* 1987, Knudsen & Pridal 1996, Todd *et al.* 1997, Drucker 2002, Sudre *et al.* 2002). The GLP1 analog, exendin-4 (EX-4), is a potent insulinotropic molecule with a much longer half-life (Goke *et al.* 1993, Thorens *et al.* 1993, Chen & Drucker 1997). Injection of EX-4 was shown to have long-term beneficial effects on blood glucose levels in diabetic mice and rat (Greig *et al.* 1999) and induced the expression of the GLP1 receptor in pancreatic ducts and β -cell replication (Xu *et al.* 1999, Tourrel *et al.* 2001). These results suggest that EX-4 has the potential to induce the

differentiation of ductal cells into insulin-producing cells. In this study, we investigated for the first time the possibility that human pancreatic ductal cells may be a useful β -cell source in a diabetic animal model, and we determined the effects of ActA and EX-4 treatment on the differentiation of human pancreatic ductal cells.

Materials and methods

Isolation of human islets

Human pancreatic islets were isolated by organ distension using intraductal collagenase (Boehringer Mannheim) and digested using a modified automated method (Ricordi *et al.* 1988). Islet purification was performed with a COBE 2991 processor (COBE, Lakewood, CA, USA) and a large-scale continuous density gradient (Robertson *et al.* 1993) prepared from Ficoll-diatrizoate media using the pancreatic digests from cadavers ($n=5$). Islet purity was determined subjectively by visual assessment using two 100 μ l sampling strips. Islet yields were expressed as islet equivalents (of islets 150 μ m in diameter) (Ricordi *et al.* 1990). After islet isolation, purified islets were incubated for at least 24–48 h at 37 °C in culture medium.

Culture of human pancreatic ductal cells

After human pancreatic islet isolation for transplantation, residual cells were collected and plated in M199 media (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco) in a T-175 culture flask at 37 °C in an incubator. Geneticin (50 μ M) (G418; Cellgro, Manassas, VA, USA) was added to inhibit fibroblast proliferation. After 2 days, cells were dissociated with cell dissociation kit (Sigma) and plated on appropriate plates. To remove residual β -cells, 5 mM STZ (Sigma) in growth media were added to the cells followed by a 5-day incubation. The 5 mM STZ containing M199 media were changed every day. Then, the human ductal cells were treated with 2 nM ActA (R&D Systems, Minneapolis, MN, USA) and 10 nM EX-4 (Sigma) in 11 mM glucose containing M199 media. The use of human pancreatic islets and ductal cells was approved by Samsung Medical Center Institutional Review Board (IRB number; SMC 2005-11-013-007).

Glucose-induced insulin secretion

Differentiated human ductal cells (1×10^5) and 20 islets per well in a 24-well plate were washed with Krebs–Ringer bicarbonate HEPES (KRBH) buffer (pH 7.37) and incubated

for an additional 60 min in 1 ml KRBH buffer that contained 5.6 or 16.7 mM glucose. Then, the media were stored at -20°C until required for insulin determination. The concentration of secreted insulin was determined using a human insulin ELISA kit (Mercodia, Uppsala, Sweden).

Analysis of gene expressions

Total RNA from human ductal cells and islets was obtained using Trizol reagent (Invitrogen) according to the manufacturer's recommendation. First-strand cDNA was synthesized from 1 μg total RNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). This cDNA (5% of first RT reaction volume) was amplified directly by a quantitative PCR (q-PCR) system. q-PCR was performed with SYBR green PCR master mix (Applied Biosystems) and analyzed with the 7500 real-time PCR system (Applied Biosystems). Primer annealing temperatures were suggested by the manufacturer (Bioneer, Daejeon, Korea) and the primer sequences were as follows: cytokeratin-19 (*CK19*) (*KRT19*): CTTTTCGCGCGCCAGCATT and GATCTTCTGTCCCTCGAGC; *NGN3* (*NEUROG3*): AGACGACGCGAAGCTCACC and AAGCCAGACTGCCTGGGCT; *PDX1*: CTGCTTTCCCATGGATGAA and CGCTTCTTGTCTCCTCCTTT; *NEUROD* (*NEUROD1*): ATCCCAACCACCACCAACC and CAGCGGTGCCTGAGAAGATT; PREPROINSULIN: CATCAAGCACATTGTCC and CTGTTCAAGGGCTTTATTC; INSULIN: GCAGCCTTTGTGAACCAACA and TCTGCGGTCATCAAATGAGG; *NKX6.1* (*NKX6-1*): TCTTCTGGCCGGGGTGATG and AGCCGCGTGCTTCTTCTCC; *GLUT2* (*SLC2A2*): CAAATCTGACTCCAAAGCTC and CTGTCTCTTGCAAGATCAGC; *PAX4*: AGGAGGACCAGGGACTACCGT and TTAGGTGGGTGTCACTCAG; *PAX6*: CAAAAGTCCAAGTGCTGACA and CCCATCTGTTGCTTTTCGCT; *GAPDH*: AGAGCTATGAGCTGCCTGAC and CTGATCCACATCTGCTGGAA.

Experimental animals and transplantation

All procedures were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Samsung Biomedical Research Institute (SBRI), Sungkyunkwan University School of Medicine (Permit Number: H-B0-043). For transplantation, male Balb/C-nude mice (Orientbio, Seongnam, Gyeonggi-do, Korea) were housed at a

constant temperature ($23.5 \pm 2.0^{\circ}\text{C}$) and humidity ($50 \pm 5\%$) under a 12 h light:12 h darkness cycle. At 6 weeks of age, diabetes was induced by a single i.p. administration of 200 mg/kg body weight (BW) of STZ dissolved in 0.05 mmol/l citrate buffer (pH 4.5). After verification of hyperglycemia for 1 week, the left kidney of the mouse was exposed through a small incision in the flank under inhalational isoflurane anesthesia. A small channel was made under the renal subcapsular space of the kidney with a 26 gauge needle. Human ductal cells (1×10^6) infected with 1×10^8 pfu/ml adenovirus-GFP were transplanted into the subcapsular space of the kidney. ActA was injected i.p. at 100 ng/g BW and EX-4 was injected s.c. at 3 ng/g BW. Blood glucose levels were determined using the Accu-Check Active System (Roche) every 2 days for 60 days. At 60 days after transplantation, transplant grafts were removed from mice and then blood glucose was measured for 8 days.

Immunocytochemistry and immunohistochemistry

The samples were washed three times in PBS and then fixed with 4% paraformaldehyde for 10 min at room temperature. After permeabilization with 0.5% Triton X-100 for 15 min at room temperature, primary antibodies were added overnight at 4°C . After washing with PBS, secondary antibodies were added for 1 h at room temperature and DAPI (Dako, Glostrup, Denmark) was used for nuclear staining. The cells were then examined by fluorescence microscopy (Olympus). The primary antibodies we used were as follows: monoclonal mouse anti-CK19, polyclonal guinea pig anti-insulin (Dako), monoclonal mouse anti-carbohydrate antigen 19-9 (CA19-9) (Leica Biosystems, Nussloch, Germany), monoclonal mouse anti-BrdU, platelet/endothelial cell adhesion molecule (PECAM; Sigma), polyclonal rabbit anti-PDX1 (Abcam, Cambridge, MA, USA), and polyclonal anti-goat GLUT2 (Santa Cruz Biotechnology). Secondary antibodies were conjugated with FITC or Texas red (Vector, Burlingame, CA, USA).

Apoptosis assay

To monitor apoptosis in STZ-treated human ductal cells and the sectioned transplanted grafts and pancreas tissues, we performed the TUNEL assay with an *in situ* cell death detection kit (Roche) according to the manufacturer's recommendation. DNase I (3 U/ml) (Roche) was added as a positive control and propidium iodide (Dako) was used to stain nuclei.

Statistical analysis

The data are presented as the means \pm s.d. Mann–Whitney *U* tests were performed to compare the differences between two independent groups. One-way ANOVA with *post hoc* analyses was used to compare the differences between several groups. *P* values <0.05 were considered statistically significant (PRISM; Graphpad Software Corp., San Diego, CA, USA).

Results

Isolation and cultivation of human pancreatic ductal cells

After human islet isolation for transplantation, residual cells were cultured in growth media containing 50 μ M geneticin for 2 days. Suspended cells were discarded, and attached cells were re-seeded in appropriate culture dishes. In this step, isolated cells were immunostained with CK19; only a

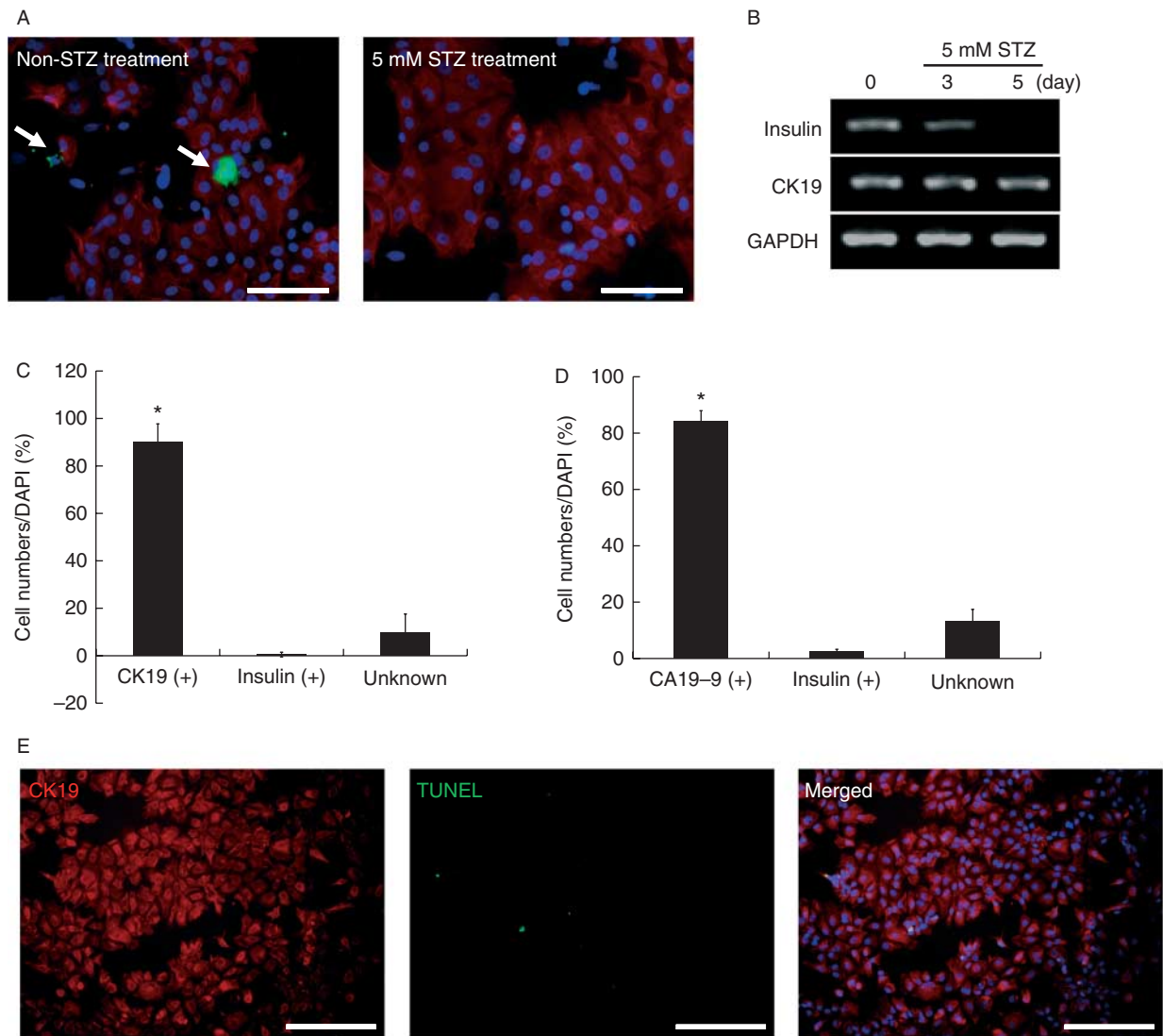


Figure 1

Characteristics of pure isolated human ductal cells. (A) During the initial stages of human ductal cell culture, CK19 (red)- and insulin (green)-positive cells were detected by immunostaining. Insulin-positive cells (arrow) were detected in the non-STZ treatment group (left). Treatment with 5 mM STZ removed residual β -cells (right). $\times 400$, scale bar = 100 μ m. (B) Insulin mRNA levels were decreased by STZ treatment. However, CK19 mRNA levels were not altered. (C and D) After completing geneticin and STZ treatment, we evaluated the purity of human ductal cells by CK19 and CA19-9

immunostaining. After immunostaining, the number of CK19-, CA19-9-, and insulin/DAPI-positive cells was counted (CK19: $89.9 \pm 7.84\%$, insulin: $0.49 \pm 0.99\%$, unknown: $9.6 \pm 7.97\%$; $n=4$, $*P < 0.001$ vs insulin (+), unknown) (CA19-9: $84.31 \pm 3.62\%$, insulin: $2.48 \pm 0.79\%$, unknown: $13.2 \pm 4.21\%$; $n=4$, $*P < 0.001$ vs insulin (+), unknown). (E) A small number of TUNEL-positive cells were observed after STZ treatment. $\times 200$, scale bar = 200 μ m.

few β -cells were detected among the human ductal cells (Fig. 1A). To remove residual β -cells and to further purify the human ductal cells, 5 mM STZ were added to the culture media for 5 days. After STZ treatment, insulin mRNA expression was decreased. However, *CK19* mRNA expression was not altered (Fig. 1B). We monitored the expression of CK19 and CA19-9 as ductal cell-specific markers and found that the total proportion of CK19- and CA19-9-positive human ductal cells was more than 85% (Fig. 1C and D). We performed an apoptosis assay to determine whether STZ induced cell death in human ductal cells. Small numbers of TUNEL-positive cells were detected (Fig. 1E).

Effects of ActA and EX-4 on β -cell marker expression in human pancreatic ductal cells

We measured the mRNA levels of pancreatic β -cell-specific markers over time. As shown in Fig. 2, β -cell-specific

markers were not fully expressed at 10 days. Interestingly, co-treatment of cells with ActA and EX-4 induced the expression of β -cell-specific markers earlier than treatment of cells with 11 mM high glucose (HG), ActA, or EX-4. At 10 days after co-treatment with ActA, EX-4, and HG, expression of β -cell-specific markers were increased, except *GLUT2*. Moreover, the ActA- and EX-4-co-treated ductal cells showed similar expression levels of these β -cell-specific markers compared with normal islets at 30 days of differentiation.

Effects of ActA and EX-4 on the proliferation of human pancreatic ductal cells

At 30 days of differentiation, co-treatment of cells with ActA and EX-4 induced more cell proliferation than was seen in the other treatment groups (Fig. 3A). Replication of human ductal cells was monitored by the BrdU uptake

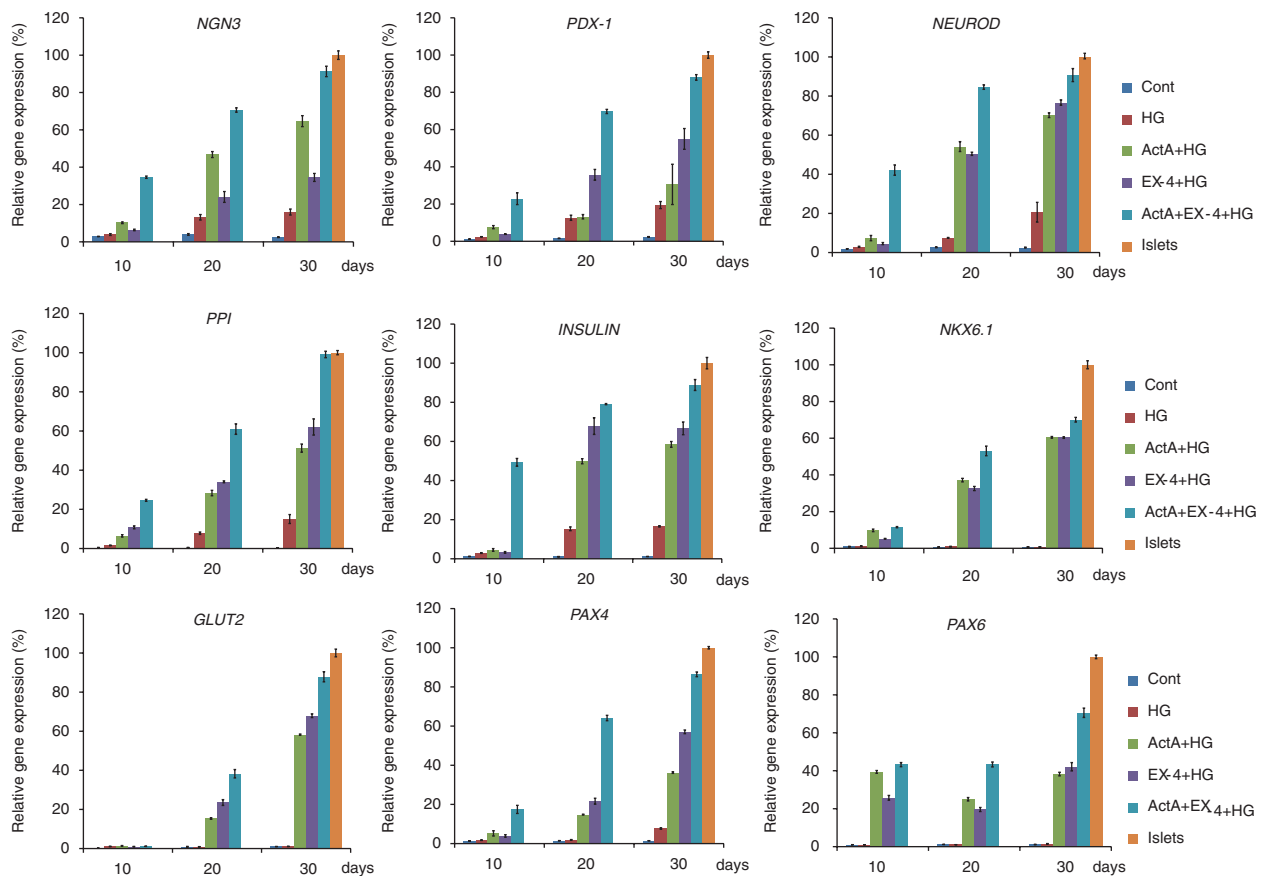
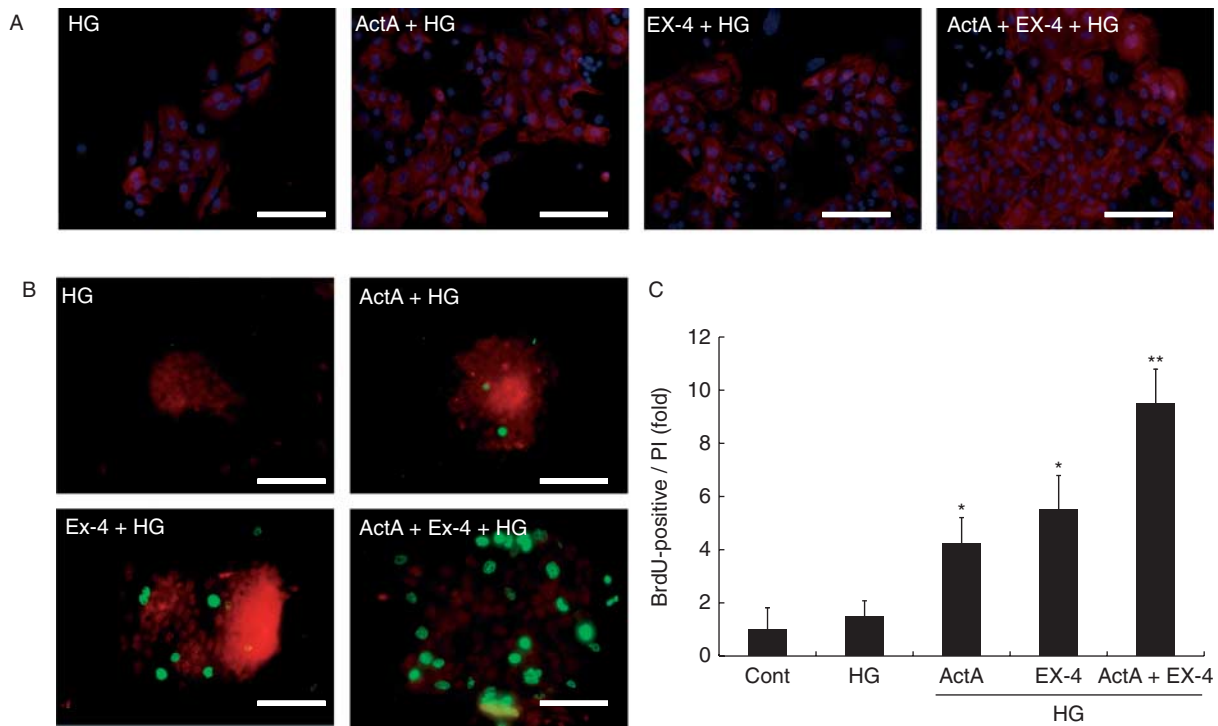


Figure 2

Effects of ActA, EX-4, and glucose on the differentiation of human ductal cells. ActA and EX-4 treatment with 11 mM glucose (HG) stimulated the expression of β -cell-specific markers in a time-dependent manner. At 30 days of differentiation, co-treatment of cells with ActA, EX-4, and HG

induced differentiation of human ductal cells (*NGN3*: neurogenin 3, *PPI*: preproinsulin). The β -cell-specific marker expression of human islets is also presented for comparison with human ductal cells.

**Figure 3**

Effects of ActA and EX-4 on the proliferation of human ductal cells. (A) ActA and EX-4 treatment increased the number of human ductal cells at 30 days of differentiation. $\times 400$, scale bar = 100 μm . (B) Co-treatment of

cells with ActA and EX-4 further increased cell proliferation. (C) Treatment of cells with ActA and EX-4 also increased BrdU-positive human ductal cells ($n=4$, * $P<0.05$ vs Cont, HG; ** $P<0.001$ vs Cont, HG, ActA and EX-4).

assay. ActA (4.25 ± 0.95) or EX-4 (5.5 ± 1.29) treatment promoted cell proliferation compared with the control group (1 ± 0.81) and the high-glucose treatment group (1.5 ± 0.57). Moreover, co-treatment with ActA and EX-4 (9.5 ± 1.29) promoted greater cell proliferation than treatment with ActA or EX-4 alone (Fig. 3B and C).

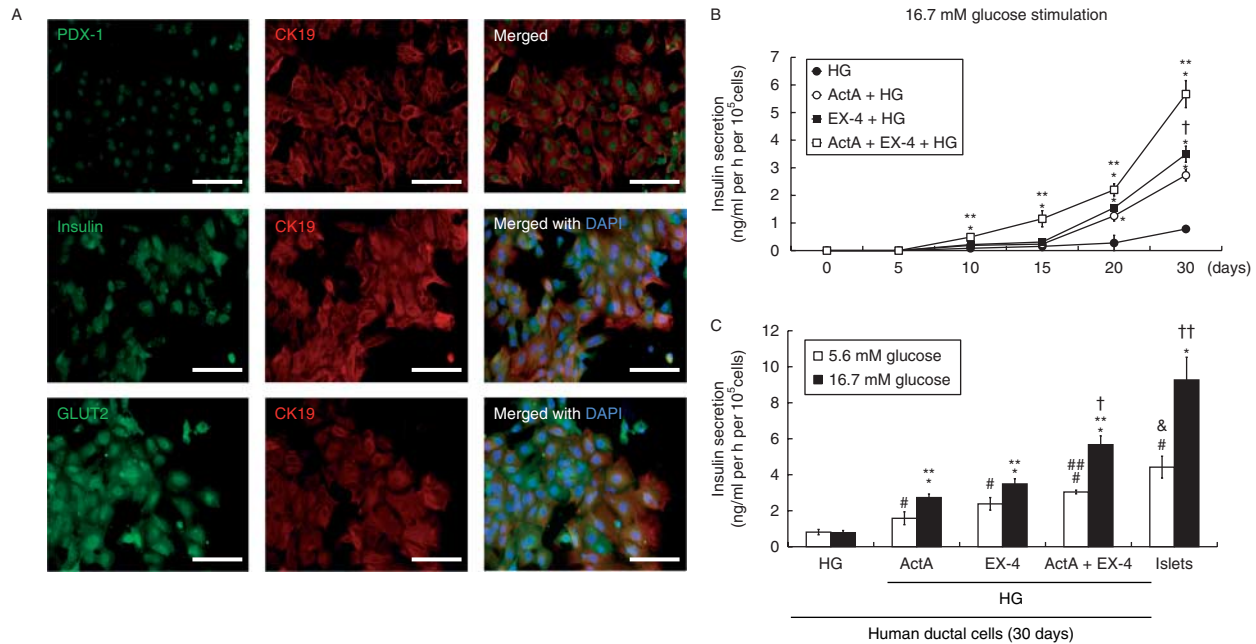
Effects of ActA and EX-4 on the differentiation of human pancreatic ductal cells

Next, we observed PDX1, insulin, and GLUT2 protein expression by immunostaining at 30 days of differentiation. Co-treatment with ActA and EX-4 resulted in upregulation of PDX1, insulin, and GLUT2 expression (Fig. 4A). To determine whether differentiated ductal cells could functionally secrete insulin in response to glucose stimulation, we performed a glucose-induced insulin secretion (GIIS) assay depending on differentiation time (5, 10, 15, 20, and 30 days). ActA and EX-4 treatment significantly stimulated insulin secretion from 20 days of differentiation. Compared with the ActA treatment group, the EX-4-treated ductal cells showed significantly increased

insulin secretion. Furthermore, co-treatment of cells with ActA and EX-4 resulted in increased insulin secretion from 10 days compared with treatment of cells with HG, ActA, or EX-4 (Fig. 4B). The GIIS ability of differentiated human ductal cells was less than normal islets (Fig. 4C).

Transplantation of human pancreatic ductal cells into STZ-induced diabetic nude mice

After differentiation (for 30 days), we transplanted human ductal cells into diabetic mice to determine whether these cells could normalize hyperglycemia. After STZ injection, we verified β -cell destruction by insulin immunostaining and blood glucose measurements (data not shown). Differentiated ductal cells (1×10^6) infected with 1×10^8 pfu adenovirus-GFP were transplanted into the subcapsular spaces of the left kidneys of each group of mice. To determine whether hypoxia was induced in the graft, we performed TUNEL assays on the transplanted grafts 3 days after transplantation. A small number of TUNEL-positive cells were observed in the transplanted sites (Fig. 5A). Blood glucose levels gradually normalized

**Figure 4**

Effects of ActA and EX-4 on insulin, PDX1, and GLUT2 protein expression and the GIIS of human ductal cells. (A) At 30 days of differentiation, ActA and EX-4 treatment upregulated the protein expression of PDX1, insulin, and GLUT2. $\times 400$, scale bar = 100 μm . (B) The insulin secretion at 16.7 mM glucose stimulation was detected by ELISA. Co-treatment of cells with ActA, EX-4, and HG promoted insulin secretion of human ductal cells ($n=4$, $*P<0.05$ vs HG; $**P<0.05$ vs EX-4 + HG; $^\dagger P<0.001$ vs ActA + EX-4 + HG).

(C) GIIS ability of differentiated human ductal cells was compared with that of human islets ($n=4$, $*P<0.05$ vs 5.6 mM glucose; $**P<0.01$ vs 16.7 mM glucose of HG; $^\# P<0.05$ vs 5.6 mM glucose of HG; $^\dagger P<0.01$ vs 5.6 mM glucose of ActA + HG; $^\ddagger P<0.01$ vs 16.7 mM glucose of ActA + HG, EX-4 + HG; $^\dagger^\dagger P<0.01$ vs 16.7 mM glucose of ActA + EX-4 + HG; and $^\& P<0.01$ vs 5.6 mM glucose of ActA + EX-4 + HG).

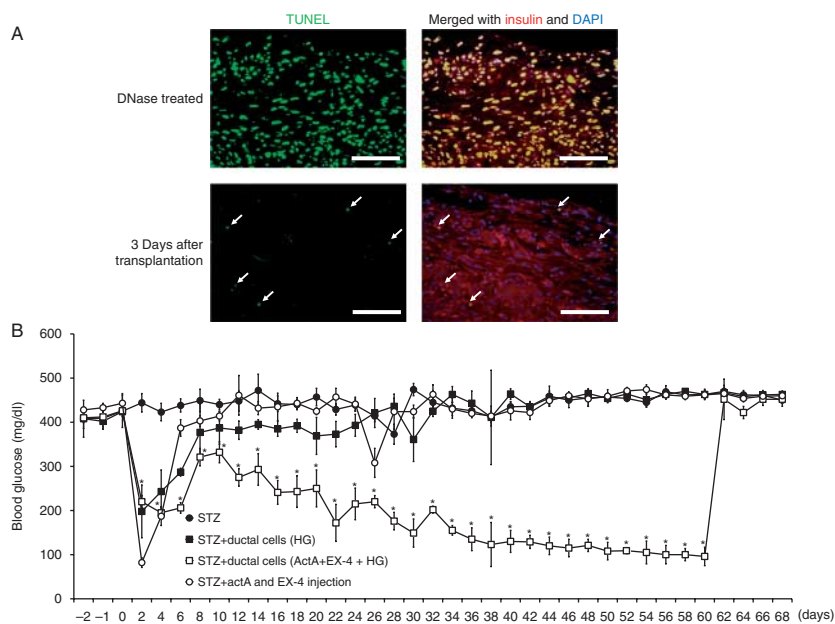
in the mice that had received differentiated ductal cell transplants. We injected ActA and EX-4 into diabetic mice at the same time as a control transplantation group. A single injection of ActA and EX-4 transiently decreased blood glucose levels; however, this decrease in blood glucose levels was not maintained. After removal of transplant graft in mice transplanted with differentiated human ductal cells, blood glucose rapidly increased the level before transplantation (Fig. 5B). And then, we examined insulin expression in the graft by immunostaining. Strong expression of insulin was found in GFP-positive cells at the transplant site (Fig. 6A). In addition, angiogenesis was observed by PECAM immunostaining. PECAM-positive blood vessels were located on the insulin-expressing area (Fig. 6B).

Discussion

In this study, we isolated human pancreatic ductal cells and induced them to differentiate into insulin-producing cells by treating them with ActA and EX-4. In particular, we enriched pure CK19- and CA-19-9-positive human

ductal cells for differentiation. We treated cells with 5 mM STZ to obtain pure human ductal cells. By treating cells with STZ, we ruled out the possibility that residual β -cells influenced the differentiation of the human ductal cells. It has been reported that human β -cells are resistant to STZ. Yang & Wright (2002) transplanted adult rat, mouse, fish, and human pancreatic islets into nude mice and then injected STZ at different doses. As a result, STZ was not toxic to the adult human islets. However, this study only examined the effects of a single injection of STZ at a dose of up to 450 mg/kg. We used a relatively high concentration of STZ (5 mM) and treated the human ductal cells for 5 days. In addition, Tuch *et al.* reported that only fetal human islets were resistant to STZ-induced toxicity (Tuch *et al.* 1989, Tuch & Chen 1993).

In addition, we removed fibroblast-like cells in human pancreatic tissues by geneticin treatment during the initial stages of culture. Overgrowth of fibroblast-like cells inhibits human ductal cell attachment and growth because these cells have a higher growth rate and they attach earlier than ductal cells. Recent studies reported the switch of cell phenotype in epithelial cell culture, and this

**Figure 5**

Changes in fasting blood glucose levels and immunohistochemical staining results in STZ-treated nude mice. (A) To identify potential hypoxia-induced apoptosis, a TUNEL assay was performed on the sectioned transplant graft tissue at 3 days after transplantation. A small number of TUNEL-positive cells (green, arrows) were detected at the transplant site compared with the positive control (DNase treated). Tissue sections were counterstained with DAPI. $\times 400$, scale bar = 100 μm . (B) After transplantation of differentiated human ductal cells (1×10^6 cells), blood glucose levels

gradually normalized ($*P < 0.05$ vs STZ). Closed circle, STZ ($n = 5$): no transplantation group; open circle, STZ + ActA, and EX-4 injection ($n = 5$): ActA and EX-4 injection group; closed square, STZ + ductal cells (HG) group ($n = 5$): ductal cells treated with 11 mM glucose for 30-day transplantation group; open square, STZ + ductal cells (ActA + EX-4 + HG) ($n = 5$): ductal cells co-treated with ActA and EX-4 with HG for 30 day-transplantation group. The transplant graft was removed from all the animals in this group (open square) after 60 days of transplantation.

change was due to epithelial–mesenchymal transition (EMT). EMT plays an important role in dedifferentiation, proliferation, and redifferentiation of pancreatic epithelial cells (Saika *et al.* 2004, Ulianich *et al.* 2008) and may be associated with tissue regeneration in liver and pancreas and cultured human β -cells (Gershengorn *et al.* 2004, Ouziel-Yahalom *et al.* 2006, Russ *et al.* 2009). EMT is rapidly undergone and most cultured pancreatic epithelial cells changed into fibroblast-like cells. We were trying to rule out this change to verify the differentiation potency of human ductal cells only, so 50 μM geneticin were used for elimination of fibroblast-like cells from initial stage of culture. Treatment of geneticin inhibited the morphological change of human ductal cells into fibroblast-like cells and outgrowth. Growth media containing geneticin were used until treatment of ActA, EX-4, and HG, any other fibroblast-like cells were not observed during culture. Human ductal cells can also be isolated by automated magnetic cell sorting (auto-MACS). Gmyr *et al.* (2001, 2004) performed human ductal cell auto-MACS using a CA19-9 antibody. In Gmyr's study, geneticin was applied to inhibit the proliferation of

fibroblast-like cells in the pancreas. This isolation method can potentially be used for the large-scale and rapid isolation of human ductal cells for clinical trials.

Our q-PCR and GIIS results indicate that normal islets have more potent insulin secretory capacity than differentiated human ductal cells. However, treatment with ActA and EX-4 resulted in human ductal cell differentiation.

ActA was known as a differentiation factor for endocrine cell differentiation. Treatment of ActA induces the expression of *NKX1.1* and *PAX* genes in embryonic stem cell-induced embryoid bodies (Yamada *et al.* 1994) and *NGN3* expression in pancreatic acinar AR42-B13 cells (Ogihara *et al.* 2003). Moreover, ActA promoted *PAX4* gene expression in AR42J cells and pancreatic β -cell line, NIT1, and INS-1 cells (Ueda 2000, Kanno *et al.* 2006). In addition, it has been reported that EX-4 induced the expressions of *Pdx1* and *Ngn3* in pancreas of STZ-induced diabetic mice (Kodama *et al.* 2005). These genes have an important role in pancreatic development and endocrine cell differentiation. In this study, treatment of ActA and EX-4 induced these gene expressions in human ductal cells. However, differentiation of human

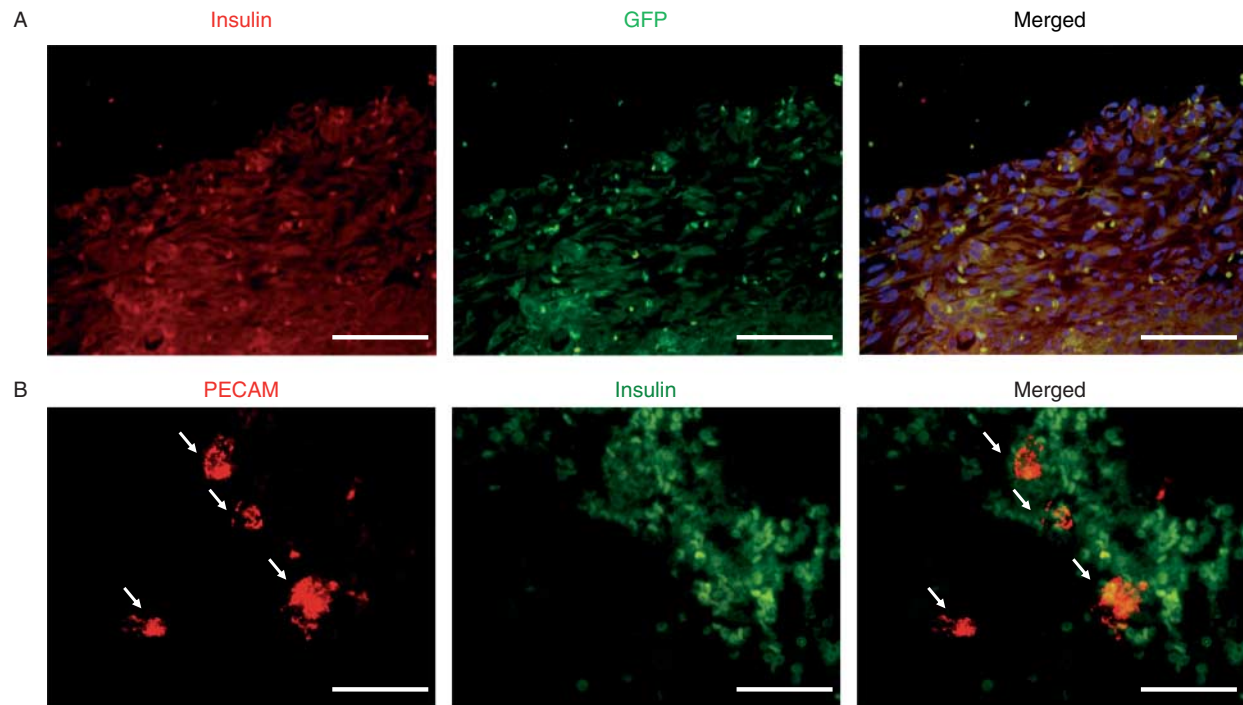


Figure 6

Histochemical analysis of the transplant graft. (A) At 60 days after transplantation, the transplant grafts were removed and immunostained with insulin antibodies. Differentiated human ductal cells were insulin positive (red) and GFP positive (green). $\times 400$, scale bar = 100 μm .

(B) PECAM (red) and insulin (green) double staining in the transplant graft was assessed to monitor angiogenesis. PECAM-positive signals (red) were located nearby the insulin-positive site (arrows). $\times 1000$, scale bar = 40 μm .

ductal cells required a relatively long time. At 10 days of differentiation, expression of insulin gene was detected, but these cells were not functional because *GLUT2*, which is necessary for insulin secretion in response to glucose stimulation, was not expressed. *GLUT2* was expressed only after 20 days of differentiation. In Fig. 4A, β -cell-specific markers, PDX1, insulin, and GLUT2, were detected; however, CK19 was still expressed. This result suggests that these ductal cells are still in differentiation progress and are not fully differentiated. In the results of the β -cell-specific gene expression analysis and GIS assay with differentiated human ductal cells, functions of differentiated human ductal cells were less than normal islets (Figs 2 and 4C). Complete full differentiation of human ductal cells and a reduction in the period required for differentiation would make human ductal cells an attractive alternative to islet transplantation for treating diabetes.

This is the first study to report the transplant of differentiated human ductal cells into diabetic animals and the induction of normal glycemic control as a result. Interestingly, the human ductal cells treated with HG (11 mM) that were only transplanted into the diabetic animals induced a transient reduction in blood glucose

levels (Fig. 5B). We applied HG *in vitro* for differentiation. In a study using a human pancreatic duct cell line, PANC 1 cells, serum-free media with a high-glucose concentration induced low levels of islet hormone mRNA and protein expression (Hardikar *et al.* 2003). In this study, high-glucose treatment also induced the expression of β -cell-specific markers (Fig. 2). However, the expression levels of these genes were lower than the expression levels observed in the other treatment groups.

To compare the glucose-lowering effects of differentiated human ductal cells, we co-injected ActA and EX-4 into diabetic mice, which resulted in a transient decrease in their blood glucose levels. It has been reported that injection of ActA and betacellulin induced persistently improved glucose homeostasis in a STZ-treated newborn mouse because ActA and betacellulin upregulated the proliferation and differentiation of β -cells, δ -cells, and ductal cells (Li *et al.* 2004). In addition, EX-4 treatment has been reported to stimulate β -cell neogenesis and improve glucose homeostasis (Xu *et al.* 1999, Tourrel *et al.* 2001). However, these results were achieved by the repeated injection of ActA, betacellulin, and EX-4. Consistent with the previous studies, we found that a single injection of

ActA and EX-4 did not induce stable glucose homeostasis. The transient blood glucose-lowering effect may be due to the effects of EX-4 rather than the effects of ActA. We plan to further study the effects of ActA and EX-4 combination therapy in diabetic animals.

According to Zhang *et al.* (1996) and Ichikawa *et al.* (2001), ActA reduces DNA synthesis and growth of hepatocytes. However, ActA had no effects on cell proliferation and apoptosis of human ductal cells in our study. By contrast, ActA treatment upregulated cell proliferation (Fig. 3). These results suggest that ActA has different effects in different cell types. We collected pancreatic tissues from diabetic mice single injected with ActA and EX-4 and then performed the TUNEL assay to detect apoptosis. We found no significant difference in apoptosis between the control group and the ActA- and EX-4-injected groups.

At 3 days after transplantation, the grafts were isolated for TUNEL assay to determine whether the injection of a large amount of ductal cells (1×10^6 cells) caused hypoxia at the transplantation site. However, a small number of apoptotic cells were detected in the grafts. In addition, we performed insulin and PECAM double immunostaining to identify angiogenesis at 60 days after transplantation. As shown in Fig. 6B, PECAM-positive signals overlapped with insulin-positive cells. This result suggests that angiogenesis was induced at the transplant sites and that insulin secretion in response to glucose was achieved after human ductal cell transplantation.

Cellular replacement therapy is a promising treatment option for long-term blood glucose control in diabetes mellitus. However, islet transplantation is limited by the amount of available tissue. To overcome the limited donor supply, diverse therapeutic approaches such as *in vitro* islet mass expansion, xenotransplantation, and stem cell technology have been widely investigated. In this study, we have shown that it is possible to replace islet cells with human ductal cells obtained from the residual pancreatic tissue after islet isolation. Furthermore, we evaluated the normoglycemic effects of differentiated human ductal cells stimulated with ActA, EX-4, and glucose in a diabetic animal model for the first time. Our results suggest that human pancreatic ductal cells induced to differentiate by ActA, EX-4, and glucose treatment are a promising therapeutic option for diabetes treatment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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