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Reprogramming of enteroendocrine K cells to pancreatic β-cells through the combined expression of Nkx6.1 and Neurogenin3, and reaggregation in suspension culture

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ABSTRACT

Recent studies have demonstrated that adult cells such as pancreatic exocrine cells can be converted to pancreatic β-cells in a process called cell reprogramming. Enteroendocrine cells and β-cells share similar pathways of differentiation during embryonic development. Notably, enteroendocrine K cells express many of the key proteins found in β-cells. Thus, K cells could be reprogrammed to β-cells under certain conditions. However, there is no clear evidence on whether these cells convert to β-cells. K cells were selected from STC-1 cells, an enteroendocrine cell line expressing multiple hormones. K cells were found to express many genes of transcription factors crucial for islet development and differentiation except for *Nkx6.1* and *Neurogenin3*. A K cell clone stably expressing *Nkx*6.1 (Nkx6.1$^+$-K cells) was established. Induction of *Neurogenin3* expression in Nkx6.1$^+$-K cells, by either treatment with a γ-secretase inhibitor or infection with a recombinant adenovirus expressing *Neurogenin3*, led to a significant increase in *Insulin1* mRNA expression. After infection with the adenovirus expressing *Neurogenin3* and reaggregation in suspension culture, about 50% of Nkx6.1$^+$-K cells expressed insulin as determined by immunostaining. The intracellular insulin content was increased markedly. Electron microscopy revealed the presence of insulin granules. However, glucose-stimulated insulin secretion was defective, and there was no glucose lowering effect after transplantation of these cells in diabetic mice. In conclusion, we demonstrated that K cells could be reprogrammed partially to β-cells through the combined expression of *Nkx6.1* and *Neurogenin3*, and reaggregation in suspension culture.

1. Introduction

Islet transplantation is the only curative treatment in patients with type 1 diabetes mellitus [1]. However, the limitation of pancreas donors is still a major obstacle. This problem has led to development of regenerative medicine, the goal of which is to induce expansion of existing β-cells and to generate β-cells from embryonic stem cells or other adult cells [2].

Switching cell fate in adult cells, which is called cell reprogramming, involves two processes [3,4]. One is pluripotent reprogramming, in which one type of adult cells is converted into pluripotent stem cells and then re-differentiated to another cell type [5,6]. The other process is lineage reprogramming, in which one type of adult cells is directly converted into another cell type or into progenitor cells. Lineage reprogramming from non-β-cells to β-cells has been reported in pancreatic exocrine cells [7], liver cells [8], bone marrow cells [9], and even pancreatic alpha cells [10]. However, the "induced" β-cells are generated only infrequently and are functionally immature in most cases [2].

Enteroendocrine cells (endocrine cells within the gut epithelium) secrete various hormones, including glucagon-like peptide-1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP) [11]. These cells are derived from the endoderm and, after birth, arise continually from stem cells in the crypts of the intestine. Interestingly, enteroendocrine cells and β-cells share the expression of specific transcription factors during the course of differentiation [12]. Notch signaling plays a critical role in determining endocrine cell fate in both the gut and pancreas. Neurogenin 3 (NGN3), one of transcription factors regulated by Notch signaling, plays key roles in the early stages of enteroendocrine cell differentiation.

Abbreviations: Adv, adenovirus; DAPI, 4′,6′-diamidino-2-phenylindole; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; DMEM, Dulbecco's Modified Eagle's Medium; GFP, green fluorescent protein; GIP, glucose-dependent insulinotropic polypeptide; GIPP, glucose-dependent insulinotropic polypeptide promoter; GLP-1, glucagon-like peptide-1; GLUT2, glucose transporter 2; ITS, insulin/transferring/sodium selenite; KRB, Krebs Ringer Buffer; MOI, multiplicity of infection; NGN3, Neurogenin 3; PC, prohormone convertase; PDX1, pancreatic and duodenal homeobox-1.

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Because of similar pathways of differentiation between enteroendocrine cells and β-cells, stem cells of the intestinal crypts might be converted to β-cells under certain conditions. There were earlier reports that an intestinal crypt cell line, IEC-6, could be induced to express insulin after induction of pancreatic and duodenal homeobox-1 (PDX1) expression [13,14]. Recently, Talchai et al. [15] reported that ablation of *Foxo1* in Ngn3-positive enteroendocrine progenitor cells gave rise to insulin-positive cells. However, there is no direct evidence on whether differentiated, mature enteroendocrine cells are also able to convert to β-cells. One study showed that oral administration of a recombinant adenovirus containing *MafA* to rats led to detection of insulin-positive cells in the intestine [16]. Other studies showed that intra-intestinal injection of a recombinant adenovirus expressing GLP-1 [17] or treatment with GLP-1 [18] produced insulin-positive cells in the intestine of mice. Nevertheless, it cannot be excluded that stem cells in the crypts of the intestine could have differentiated into β-cells in these studies.

One type of enteroendocrine cells is K cells that secrete GIP. Very interestingly, K cells express many of the key proteins found in β-cells, including PDX1 [19], glucokinase, prohormone convertases PC1/3 and PC2 [12] and even KIR6.2/SUR1 [20,21]. This feature has made K cells an attractive target for a new therapeutic approach to type 1 diabetes mellitus. As one approach, genetic engineering of K cells to secrete insulin in a diabetic state has been attempted [22,23]. However, the major problem of this approach is inappropriate secretion of insulin in response to glucose leading to hypoglycemia. This is because K cells express the sodium-dependent glucose transporter 1 with a low *K*m for glucose [21], not the glucose transporter 2 (GLUT2) with a high *K*m for glucose, which is the primary isoform of GLUTs expressed by β-cells. The other approach is to reprogram K cells to β-cells. In our preliminary experiments [24], K cells were found to express many genes of transcription factors crucial for islet development and differentiation except for *Nkx6.1* and *Ngn3*. In addition, a K-cell clone expressing *Nkx6.1* started to express *Insulin1* mRNA after exendin-4 treatment and serum deprivation. However, there was only a little increase in intracellular insulin content and extensive cell death occurred during culture. In this study, we aimed to reprogram K cells to β-cells more efficiently through the induction of both *Nkx6.1* and *Ngn3* expression and use of optimal culture conditions.

2. Materials and methods

2.1. Selection of K cells from STC-1 cells

STC-1 cells, an enteroendocrine cell line expressing multiple hormones including GIP [25], were kindly supplied by Dr. Hanahan (University of California, USA). For selecting K cells from STC-1 cells, a *GIP* promoter-expressing vector was transfected into STC-1 cells [24]. Briefly, nucleotides −1153 to +7 of the *GIP* promoter (GIPP) region were amplified from rat genomic DNA by PCR. The PCR product was subcloned into an Epstein-Barr virus-based plasmid (pCEP4). The green fluorescent protein (GFP) cDNA was subcloned into a pEGFP-C2 plasmid (Clontech, Mountain View, CA). Then, the GIPP/GFP cDNA construct was subcloned to generate the pGIPP/GFP/CEP4 plasmid (Supplementary Fig. 1). To obtain GIP-expressing cell lines, STC-1 cells were transfected with the pGIPP/GFP/CEP4 plasmid. The cell lines produced were designated as K cells. In addition, rat *Nkx6.1* cDNA construct, kindly supplied by Dr. Serup (Hagedorn Research Institute, Denmark), was subcloned into a pcDNA3.1 plasmid (Invitrogen Life Technologies, Carlsbad, CA). K cells were transfected with the pcDNA3.1 plasmid expressing *Nkx6.1*. After selecting transfected cells, a K-cell clone expressing *Nkx6.1* stably was established and designated as Nkx6.1^{+}-K cells.

2.2. Culture conditions

The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose and 10% FBS. To induce reprogramming to β-cells, the cells were cultured in serum-free DMEM containing 10 mM nicotinamide (Sigma–Aldrich, St. Louis, MO) and an insulin/transferring/sodium selenite mixture (ITS; 5 mg/L insulin, 5 mg/L transferrin and 5 mg/L sodium selenite; Roche Diagnostics, Mannheim, Germany).

The expression of NGN3 was induced either by treatment with 10 μM *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT; Sigma–Aldrich) or by infection with a recombinant adenovirus expressing *Ngn3* (Adv-Ngn3), kindly provided by Dr. Kaneto (Osaka University, Japan) [26]. Cells were infected at a determined multiplicity of infection (MOI) with Adv-Ngn3 for 2 h.

In some experiments, cells were reaggregated and incubated in suspension culture. Briefly, cells at 70–80% confluence were harvested with 0.25% trypsin–EDTA and, after washing, were placed in a dish with Ultra-Low Attachment surface (Corning Incorporated, Corning, NY).

2.3. RT-PCR

Total RNA isolation, first-strand cDNA synthesis, and PCR were performed with standard procedures. The primers and cycle numbers used for PCR are shown in Supplementary Table 1. The expression of cyclophilin was used as an internal control.

2.4. Western blot analysis

Western blot analysis was performed with standard procedures. The primary antibodies were anti-NGN3 antibody (1:1000; BCBC Antibody Core, Nashville, TN), anti-β-actin antibody (1:5000; Abcam, Cambridge, MA).

2.5. Immunostaining

The cells were grown on cover glasses, and fixed in 4% paraformaldehyde for 10 min. Immunostaining was performed using the following primary antibodies: monoclonal anti-NKX6.1 IgG (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA), guinea pig polyclonal anti-insulin IgG (1:200; Zymed Laboratories, San Francisco, CA), and mouse anti-C-peptide IgG (1:100; Cell Signaling Technology Inc., Beverly, MA). After overnight incubation with primary antibodies at 4 °C, slides were incubated with rhodamine-conjugated goat anti-guinea pig IgG (1:100) as the secondary antibody. For NKX6.1 immunostaining, slides were developed with 3,3′-diaminobenzidine (Sigma–Aldrich), and counterstained with hematoxylin. The nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI).

2.6. Intracellular insulin content

Cell pellets were sonicated three times, and then incubated overnight in acid-ethanol solution (1.5% HCl in 70% ethanol) at 4 °C. After centrifugation, the supernatant was collected and neutralized 1:1 with 1 M Tris, pH 7.5. Insulin was measured using a RIA kit (Millipore Corporation, Billerica, MA), and normalized to protein content. Protein content was measured using the Bradford method.

2.7. Glucose-stimulated insulin secretion

After removal of the culture medium, cells were washed with Krebs Ringer Buffer (KRB) containing 25 mM HEPES, 115 mM NaCl,

24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% BSA, and 2.7 mM glucose. The cells were then pre-incubated for 1 h in KRB containing 2.7 mM glucose. Then aliquots of the cells were incubated in KRB containing 2.7 mM or 25 mM glucose for 2 h. Insulin concentration was measured using a RIA kit (Millipore Corporation).

2.8. Electron microscopy

For electron microscopic analysis, the specimens were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde for overnight. After washing with PBS, the specimens were post-fixed with 1% osmium tetroxide for 1 h. And after dehydration and embedding in Epon 812, areas of interest were excised and glued onto resin blocks. Ultrathin sections (70–90 nm thick) were examined in a transmission electron microscope.

2.9. Transplantation

Diabetes was induced in nude mice by administering streptozotocin intraperitoneally at a dose of 150 mg/kg body weight. Mice were considered diabetic if their blood glucose level reached a stable value over 400 mg/dL for 3 consecutive days. $Nkx6.1^+$-K cells were reaggregated after Adv-Ngn3 infection and cultured in suspension for 3 days. Then, 10,000 clusters of these cells were transplanted under the kidney capsule of the diabetic mice.

2.10. Statistical analysis

Data are expressed as the means ± SEM. ANOVA was used to analyze quantitative variables between groups, followed by post hoc testing with Fisher's least significant difference test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Selection of K cells from STC-1 cells

K cells were identified visually by GFP expression. Fig. 1A demonstrated GFP expression in K cells, not in STC-1 cells (Fig. 1A). RT-PCR showed that K cells had more expression of *GIP* mRNA than did the STC-1 cells. *Glucokinase* mRNA was also expressed in K cells as well as isolated mouse islets and STC-1 cells (Fig. 1B).

3.2. Gene expression of transcription factors and insulin secretion/exocytosis-related proteins

RT-PCR revealed that *Pdx1, NeuroD, MafA, Isl1, Pax6, and Nkx2.2* mRNAs were all detected in K cells as in islets. However, *Nkx6.1 and Ngn3* mRNAs were not expressed in K cells. *Pax4* mRNA was present in K cells, but not in islets. As expected, *Insulin1* and *Insulin2* mRNAs were absent in K cells (Fig. 1C). And most genes of insulin secretion/exocytosis-related proteins were expressed in K cells

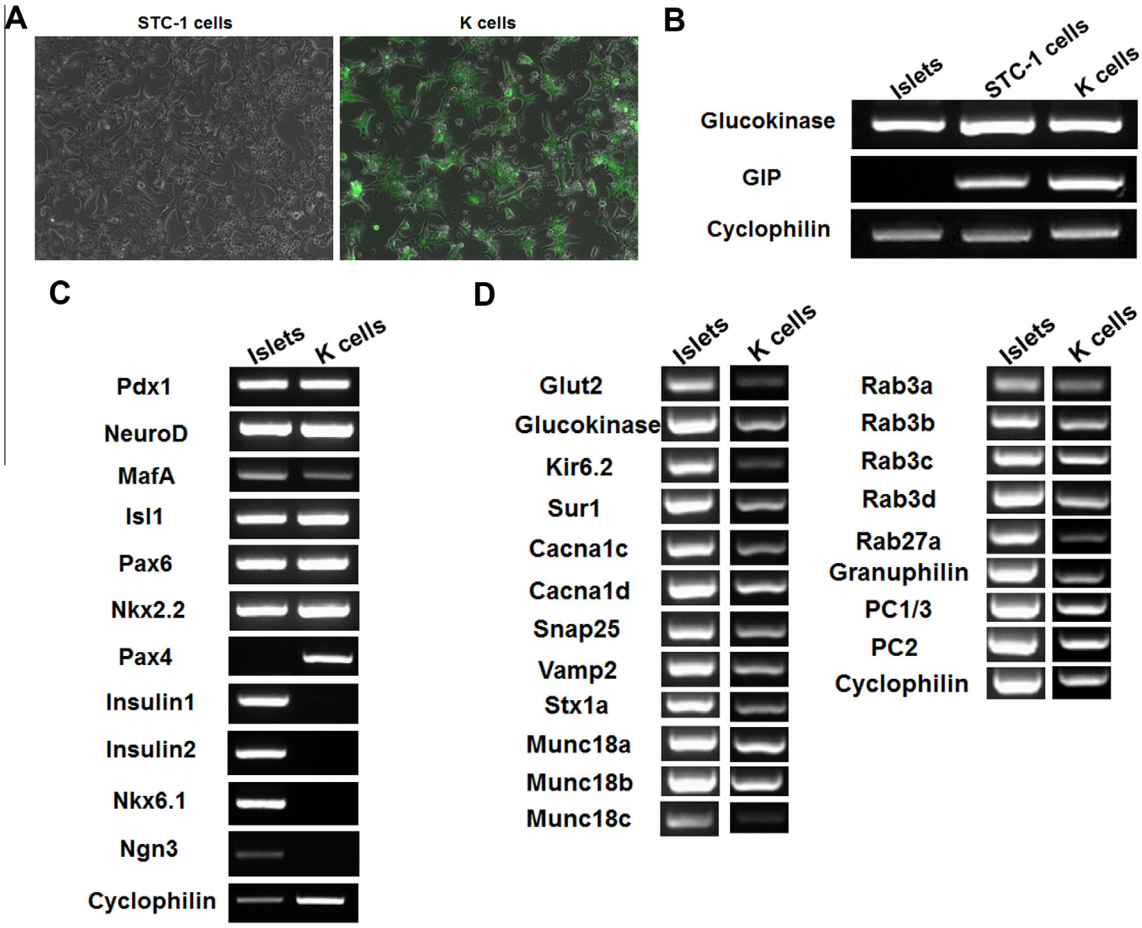

Fig. 1. Selection of K cells from STC-1 cells. Fluorescence microscopy showed the expression of GFP in K cells (A). Magnification 100×. RT-PCR compared mRNA expression of glucokinase, GIP, transcription factors, and insulin secretion/exocytosis-related proteins (B–D).

although some glucose-sensing components such as *Glut2* and *Kir6.2* were expressed much less than in islets (Fig. 1D).

3.3. Reprogramming of K cells to β-cells

First, K cells were incubated in serum-free DMEM (containing nicotinamide and ITS) for 1 week. However, there was little detection of *insulin1* mRNA by RT-PCR.

Next, we induced *Nkx6.1* expression in K cells because *Nkx6.1* was absent in these cells. We established a K cell clone expressing *Nkx6.1* (Nkx6.1$^+$-K cells) by stable transfection of rat *Nkx6.1* into K cells. RT-PCR showed the expression of rat *Nkx6.1* mRNA in Nkx6.1$^+$-K cells, but not in K cells (Fig. 2A). Immunostaining confirmed the presence of NKX6.1 in the nuclei of Nkx6.1$^+$-K cells, but not in those of K cells (Fig. 2B). When Nkx6.1$^+$-K cells were incubated in the same culture conditions described above, *Insulin1* mRNA expression was increased a little (Fig. 2C). We then induced the expression of *Ngn3*, another transcription factor not expressed in K cells, in Nkx6.1$^+$-K cells to enhance the reprogramming process. RT-PCR showed that the Notch receptors (*Notch1* and *Notch2*) and the Notch ligands (*Jag1, Jag2*, and *Dll1*) were expressed in K cells as well as in islets, suggesting the presence of Notch pathway components in K cells (Fig. 2D). The expression of *Ngn3* was induced by either treatment with DAPT, a γ-secretase inhibitor, or infection with Adv-Ngn3. Western blot analysis showed that NGN3 expression paralleled increases in the MOI of Adv-Ngn3 in Nkx6.1$^+$-K cells (Fig. 2E). The optimum MOI for NGN3 expression and cell viability was 50 and this dose was used in the following experiments. Treatment of Nkx6.1$^+$-K cells with DAPT or Adv-Ngn3 infection in the same culture condition described above for 1 week induced the clear expression of *Insulin1* mRNA, but not *Insulin2* mRNA (Fig. 2F). Confocal microscopy showed that insulin immunostaining was positive in about 20% of Nkx6.1$^+$-K cells at 1 week after Adv-Ngn3 infection (Fig. 2G).

Finally, we reaggregated Nkx6.1$^+$-K cells after Adv-Ngn3 infection and then cultured them in suspension in serum-free DMEM to reduce cell death occurring during monolayer culture and promote the reprogramming process. During suspension culture, the cells formed aggregates and these became smooth spheroids within 24 h. After 6 days of culture, the spheroids were composed mostly of living cells as determined by DAPI staining (Fig. 3A). Confocal microscopy showed that some cells in the spheroids became insulin-positive by 3 days and these insulin-positive cells had increased in number by 6 days (Fig. 3B). Quantification data showed that the percentage of insulin-positive cells increased significantly after treatment for 6 days, up to about 50% (Fig. 3C). C-peptide was also found to be present, similar to insulin (Fig. 3D). RT-PCR showed that *Glucagon*, *Somatostatin*, or *Pancreatic polypeptide* mRNA expressions were not increased although these mRNAs were present in K cells (Fig. 3E).

Electron microscopy revealed the presence of secretory granules having a crystalline core surrounded by a halo and a clearly defined limiting membrane, a characteristic of insulin granules in mouse β-cells (Fig. 4A), in some Nkx6.1$^+$-K cells at 6 days after Adv-Ngn3 infection and reaggregation in suspension culture (Fig. 4B). The intracellular insulin content was markedly increased

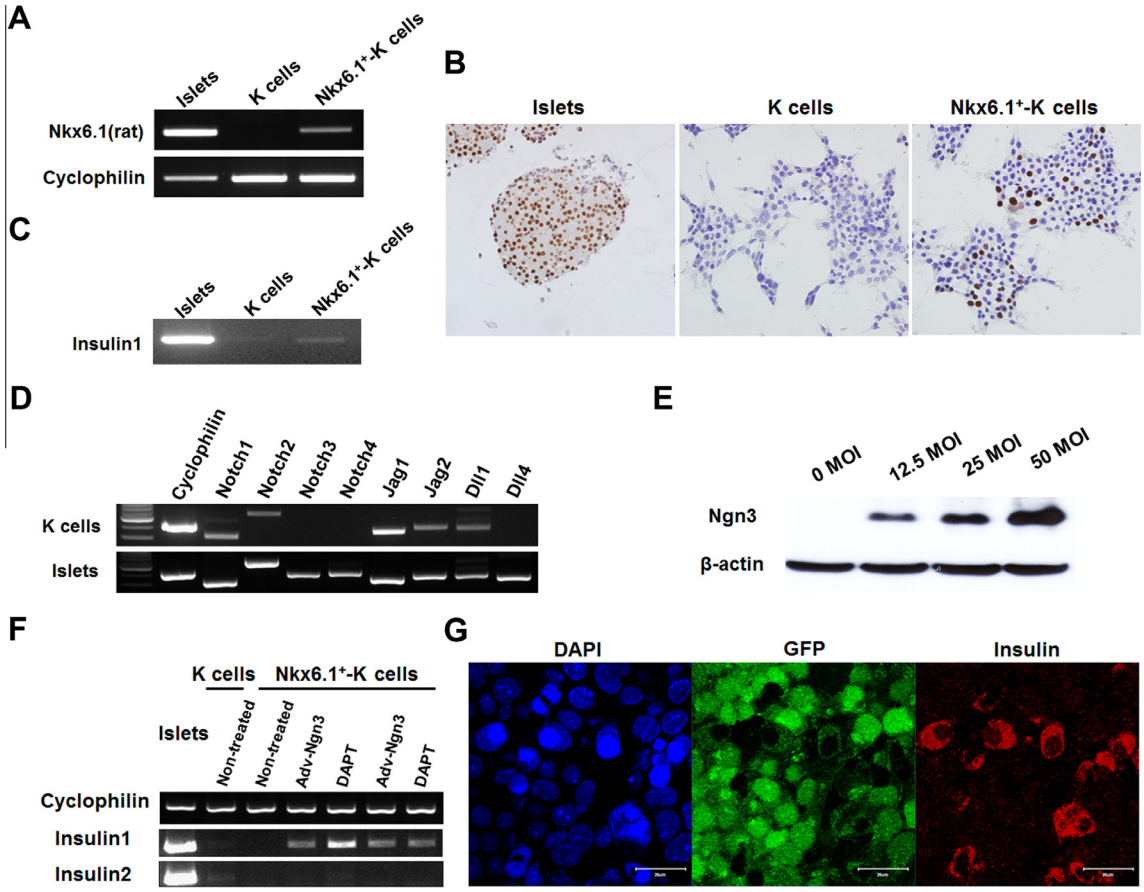

Fig. 2. Reprogramming of Nkx6.1$^+$-K cells to β-cells. RT-PCR showed mRNA expression of NKX6.1, insulin, and Notch receptors and ligands (A, C, D, F). Immunostaining showed the presence of NKX6.1 (brown) in the nuclei (B). Magnification 100×. Western blot analysis showed NGN3 expression after Adv-Ngn3 infection (E). Confocal microscopy showed Nkx6.1$^+$-K cells stained for DAPI, GFP and insulin (G). Bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

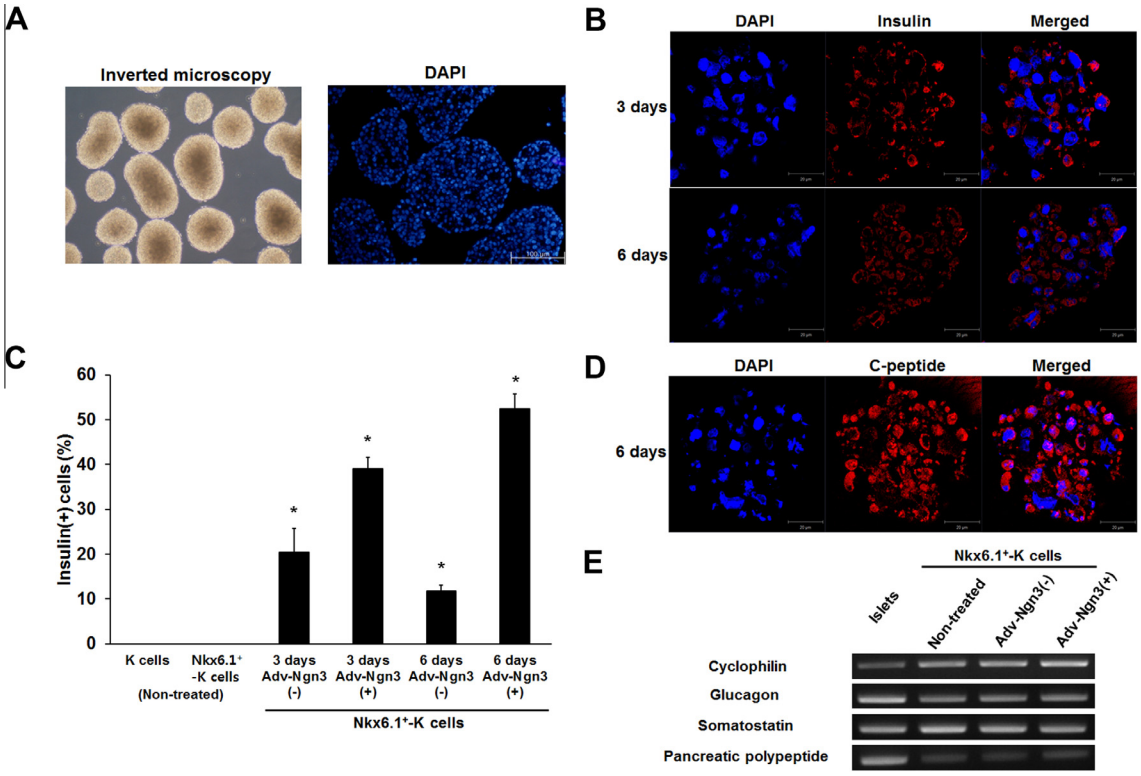

Fig. 3. Reprogramming of Nkx6.1$^+$-K cells to β-cells after Adv-Ngn3 infection and reaggregation in suspension culture. Inverted microscopy showed cell aggregates (spheroids) (A). Bar, 100 μm. Confocal microscopy showed insulin- or C-peptide-positive cells (B, D). Bar, 20 μm. The percentage of insulin-positive cells was calculated (C). Data are means ± SEM ($n = 3$). $^*P < 0.05$ vs. K cells or Nkx6.1$^+$-K cells before treatment. RT-PCR showed mRNA expression of glucagon, somatostatin, and pancreatic polypeptide (E).

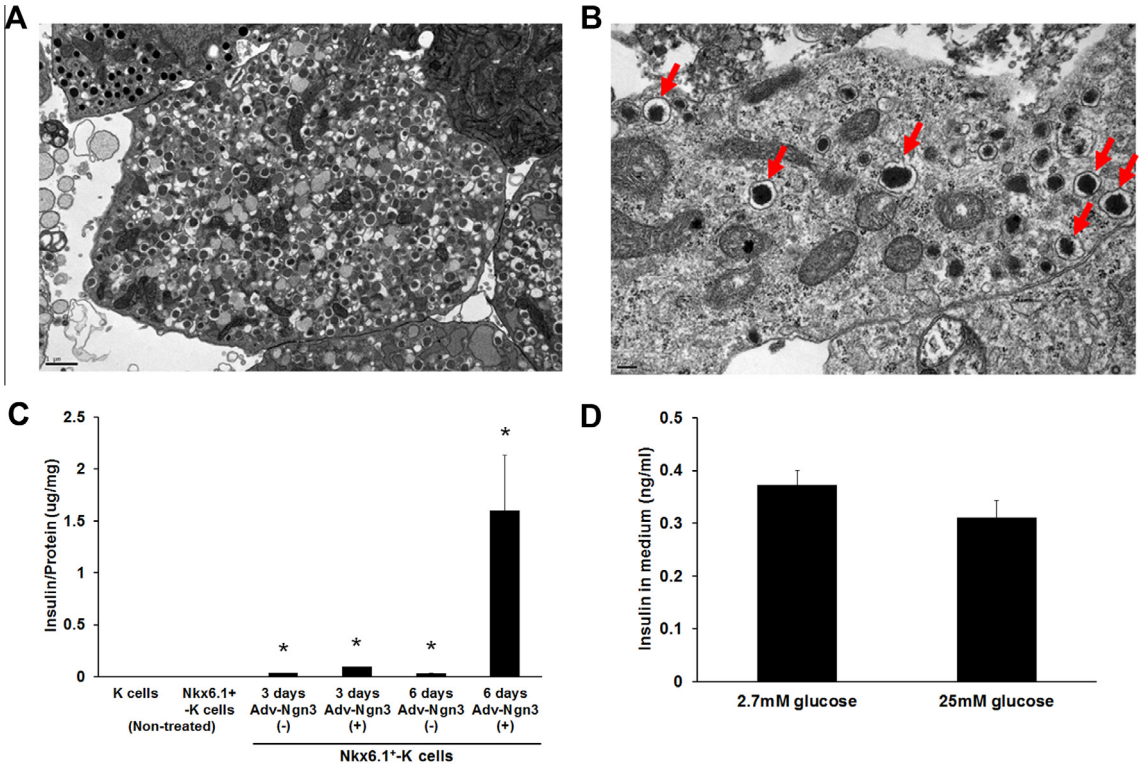

Fig. 4. Ultrastructural analysis, intracellular insulin content and glucose-stimulated insulin secretion. Electron microscopy showed insulin granules in mouse β-cells (A) and Nkx6.1$^+$-K cells after Adv-Ngn3 infection, reaggregation, and suspension culture in serum-free DMEM for 6 days (B). Arrows indicate typical insulin granules in Nkx6.1$^+$-K cells. Bar, 1 μm (A) or 0.2 μm (B). Intracellular insulin content was measured (C). Glucose-stimulated insulin secretion was measured by incubation with 2.7 or 25 mM glucose in KRB for 2 h (D). Data are means ± SEM ($n = 3$). $^*P < 0.05$ vs. K cells or Nkx6.1$^+$-K cells before treatment.

in Nkx6.1$^+$-K cells after Adv-Ngn3 infection and reaggregation in suspension culture (Fig. 4C). In response to glucose stimulation, the cells did secrete insulin, but in a defective manner, showing little effect of glucose (Fig. 4D).

To determine whether maturation of reprogrammed K cells could occur *in vivo*, we did the transplantation experiment. However, there was no decrease in blood glucose levels after transplantation and only a few insulin-positive cells were found in the grafts (data not shown).

4. Discussion

In this study, we demonstrated that K cells could convert to β-cells *in vitro* with an increase in insulin expression and intracellular insulin content, and the presence of insulin granules.

To obtain K cells, we established GIP-expressing cell lines by transfection of a GIPP-expressing vector into STC-1 cells. *Pdx1*, *NeuroD*, *MafA*, *Isl1*, *Pax4*, *Pax6*, and *Nkx2.2* mRNAs were all detected in K cells; however, *Nkx6.1* and *Ngn3* mRNAs were not expressed. A recent study [27] using microarray analysis in primary mouse K cells revealed that *Pdx1*, *NeuroD*, *Isl1*, *Pax6*, *Nkx2.2*, and *Ngn3* mRNAs were expressed with mean Robust Multi-chip Average (RMA) values of >100. However, *MafA*, *Pax4*, and *Nkx6.1* mRNAs were expressed at low levels with mean RMA values of <100. These discrepancies regarding the gene expression profiles of *MafA*, *Ngn3*, and *Pax4* could be because our K cells originated from a multiple hormone-positive STC-1 cells. As for the expression profiles of insulin secretion/exocytosis-related genes, K cells were also similar to islets.

Nkx6.1 is found almost exclusively in β-cells and some enteroendocrine cells after birth [28,29]. Its role in the differentiation and functions of these cells is not yet defined. Recently, Gefen-Halevi et al. [30] reported that *Nkx6.1* promoted the reprogramming of hepatocytes to β-cells. *Ngn3* is expressed in both the developing pancreas and immature cells of the intestinal crypts, but shows little expression in differentiated islets or mature enteroendocrine cells [12]. Knockout of *Ngn3* resulted in the loss of islet and enteroendocrine cells [31,32]. The ectopic expression of *Ngn3* in the gut endoderm of chick embryos caused endodermal cells to transform into endocrine cells expressing glucagon and somatostatin [33]. Moreover, the ectopic expression of *Ngn3* in human pancreatic duct cells induced the expression of insulin [34].

In the present study, little *Insulin1* mRNA expression was detected in K cells after culture in serum-free DMEM supplemented with nicotinamide and ITS. As *Nkx6.1* expression was defective in K cells, a stable K cell clone expressing *Nkx6.1* (Nkx6.1$^+$-K cells) was established. When Nkx6.1$^+$-K cells were incubated in the aforementioned culture medium, *Insulin1* mRNA expression became evident, but it was still much less than islets. Next, we induced the expression of NGN3 in Nkx6.1$^+$-K cells by DAPT treatment after observing the presence of Notch pathway components in K cells, or by Adv-Ngn3 infection. As a result, the expression of *Insulin1* mRNA was significantly increased with the appearance of insulin-positive cells. Finally, reaggregation in suspension culture after Adv-Ngn3 infection have reduced cell death and promoted the cell reprogramming process. The percentage of insulin-positive cells increased up to about 50%. The presence of C-peptide and insulin granules was demonstrated by immunostaining and electron microscopy, respectively. Taken together, the ectopic expression of *Nkx6.1* and *Ngn3*, and reaggregation in suspension culture facilitated the reprogramming of K cells to β-cells.

However, reprogramming of K cells to β-cells was not complete in the present study. Intracellular insulin content was markedly increased, up to about 1.5 μg/mg protein. However, it did not reach the amount of insulin content in mouse islets, about 250 μg/mg protein [35]. And insulin secretion was defective in response to glucose stimulation. Furthermore, the transplantation experiment in diabetic mice did not show *in vivo* maturation or any glucose lowering effect of the reprogrammed K cells.

In conclusion, K cells could be partially reprogrammed to β-cells through the combined expression of *Nkx6.1* and *Ngn3*, and reaggregation in suspension culture. However, more studies on molecular mechanism of β-cell reprogramming will be needed to generate more mature and functional β-cells from K cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.093.

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