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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,