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Sequential introduction and dosage balance of defined transcription factors affect reprogramming efficiency from pancreatic duct cells into insulin-producing cells

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ABSTRACT

While the exogenous expression of a combination of transcription factors have been shown to induce the conversion of non-β cells into insulin-producing cells, the reprogramming efficiency remains still low. In order to develop an *in vitro* screening system for an optimized reprogramming protocol, we generated the reporter cell line mPac-MIP-RFP in which the reprogramming efficiency can be quantified with red fluorescent protein expressed under the control of the insulin promoter. Analysis with mPac-MIP-RFP cells sequentially infected with adenoviruses expressing Pdx1, Neurog3, and Mafa revealed that expression of Pdx1 prior to Neurog3 or Mafa augments the reprogramming efficiency. Next, infection with a polycistronic adenoviral vector expressing Pdx1, Neurog3 and Mafa significantly increased the expression level of insulin compared with the simultaneous infection of three adenoviruses carrying each transcription factor, although excessive expression of Mafa together with the polycistronic vector dramatically inhibited the reprogramming into insulin-producing cells. Thus, *in vitro* screening with the mPac-MIP-RFP reporter cell line demonstrated that the timing and dosage of gene delivery with defined transcription factors influence the reprogramming efficiency. Further investigation should optimize the reprogramming conditions for the future cell therapy of diabetes.

1. Introduction

To date, insulin-producing β-like cells have been shown to be generated from various differentiated cell types in adult organs, such as acinar cells and α-cells, as well as from embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells) [1–3]. While some humoral factors can induce β-cell neogenesis under some specific conditions [3,4], it has been shown that the ectopic expression of pancreas-specific transcription factors in non-β cells can change their cell fate into insulin-expressing cells [5,6].

Among the genes which play essential roles in β-cell differentiation and function, Pdx1, Neurog3, and Mafa can efficiently induce the reprogramming from acinar cells or liver cells into β-like cells, resulting in the amelioration of hyperglycemia in diabetic model mice [2,7]. However, it remains a challenge to improve the reprogramming efficiency to the level to cure diabetes. One point of consideration is that the expressions of these transcription factors are not simultaneously initiated during endocrine differentiation, and their spatio-temporal expressions are tightly regulated by different factors and at different levels [5,8]. Therefore, we hypothesized that optimal timing of gene transfer and optimal dosage balance of the three transcription factors may improve the reprogramming efficiency for β-cell neogenesis. To investigate this hypothesis, we generated a new reporter cell line to evaluate the reprogramming efficiency, and found that Pdx1 expression prior to that of Neurog3 and Mafa can increase the number of β-like cells, whereas excessive expression of Mafa has a negative effect on this reprogramming.

2. Material and methods

2.1. Construction of the insulin promoter-RFP plasmid

To generate stable cell lines, pcDNA3.1/Hygro (Invitrogen, Carlsbad, CA) was used and the CMV promoter was removed at

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NruI and NheI sites. A 0.7 kb fragment of DsRed-E5 was fused to mouse insulin 1 promoter (MIP) which was excised from MIP-GFP-pGEM-11Zf (+) [9], and then inserted into pcDNA3.1/Hygro.

2.2. Generation of MIP-RFP stable transformants

The pcDNA3.1-MIP-RFP/Hygro plasmid was transfected into the mouse pancreatic duct cell line, mPac cells. After transfection, the cells were allowed to recover for 1 day, and selection was started with medium containing hygromycin as a selection marker. After 14–21 days of selection, 30 hygromycin-positive individual colonies were manually picked and expanded. In order to identify a clone with a high expression level of RFP, the cells were infected with adenoviruses expressing Pdx1, Neurog3, and Mafa, and observed by confocal laser scanning microscopy (FV1000D; Olympus). The RFP expression was confirmed in 18 out of 30 cell lines.

2.3. Preparation of adenoviruses

Recombinant adenoviruses expressing Pdx1, Neurog3, or Mafa were prepared using the AdEasy system (kindly provided by Dr. Vogelstein, Johns Hopkins Cancer Center) [10]. The rat Pdx1, mouse Neurog3 and Mafa genes were cloned into pAdTrack-CMV, and was introduced into *Escherichia coli* BJ5183 cells by electroporation (2500 V, 200 Ω, 25 μF). The resultant plasmids were then linearized with PacI and then transfected into the adenovirus packaging cell line 293. To generate a polycistronic "Ad-Pdx1 (2A) Neurog3 (2A) Mafa" adenovirus, each sequence encoding Pdx1, Neurog3, and Mafa, was fused by "PCR-mediated overlap extension" method [11], and then transfected into the adenovirus packaging cell line 293. The adenovirus titer was roughly 10^8 infectious units per mL (ifu/mL) after treatment with the Virakit virus purification kit (Virapure, San Diego, CA).

2.4. RNA isolation and real-time PCR analysis

Total RNA was isolated using the RNeasy Mini Plus kit (Qiagen, Valencia, CA). One microgram total RNA was reverse-transcribed at 42 °C for 30 min with anchored oligo-dT and random hexamer primers using the Verso cDNA synthesis kit according to manufacturer's protocol (Thermo Fisher, Rockford, IL). Quantitative real-time PCRs were run using the SYBR Green Master Mix Kit (Applied Biosystems, Foster City, CA). The sequences of the primer sets were as follows: mouse insulin 2 (numbering relative to ATG, forward −45 TCCGCTACAATCAAAAACCA, reverse +239 GGTCTGAAGGTCAC CTGCTC, 284 bp), mouse β-actin (+778 GCTCTTTTCCAGCCTTCCTT, +945 CTTCTGCATCCTGTCAGCAA, 168 bp). The signal fluorescence magnitude was detected with an ABI Prism 7900 sequence detection system. The data are normalized to the β-actin signal.

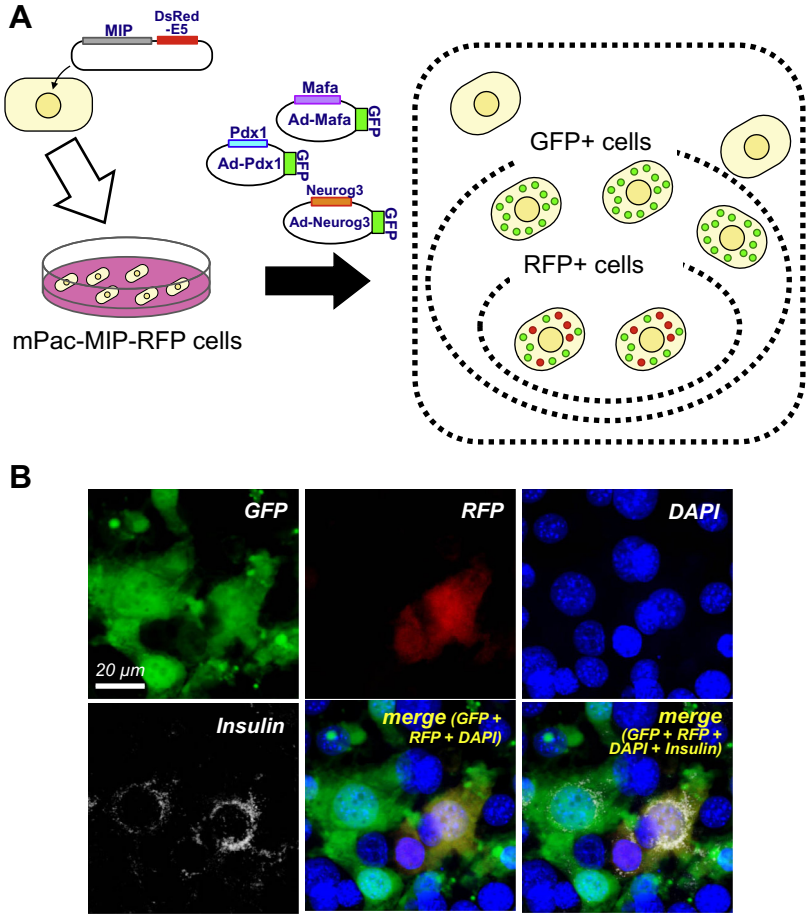

Fig. 1. Generation of pancreatic duct cell line expressing a fluorescent reporter protein. (A) Schematic protocol to examine reprogramming efficiency into β-like cells. A plasmid encoding the mouse insulin1 promoter (MIP) followed by red fluorescent protein (RFP) was transfected into the mPac cells to create mPac-MIP-RFP cells. After the mPac-MIP-RFP cells were infected with adenoviruses encoding Pdx1, Neurog3 and Mafa, together with eGFP, the number of green- and/or red-fluorescent cells was counted. The ratio of red fluorescent cells to green fluorescent cells was calculated to evaluate the reprogramming efficiency. (B) Red fluorescence in mPac-MIP-RFP cells indicates insulin expression. The mPac-MIP-RFP cells were infected with adenoviruses expressing Pdx1, Neurog3, and Mafa, together with eGFP, and immunostained using anti-insulin antibody. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; blue). Some of the eGFP-expressing cells (green) exhibit red fluorescence derived from RFP. Red fluorescent cells (red) were positive for the insulin protein (white).