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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.

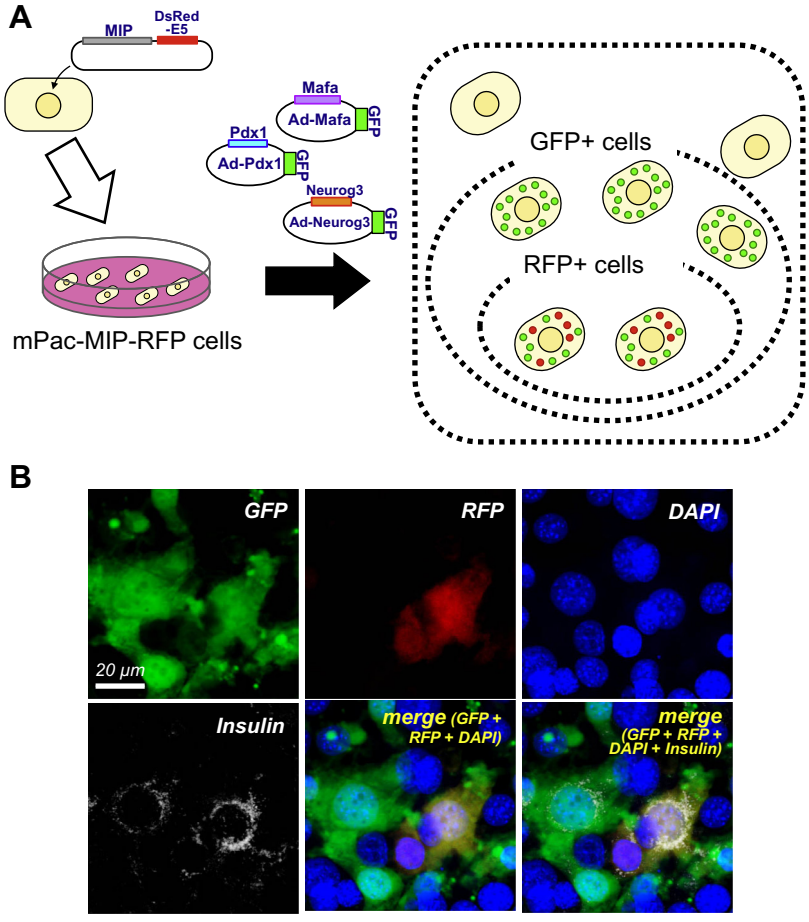

Fig. 1. Generation of pancreatic duct cell line expressing a fluorescent reporter protein. (A) Schematic protocol to examine reprogramming efficiency into β-like cells. A plasmid encoding the mouse insulin1 promoter (MIP) followed by red fluorescent protein (RFP) was transfected into the mPac cells to 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).

2.5. Immunocytochemical analysis

The mPac-MIP-RFP cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature (RT) and washed three times in PBS. After the samples were pretreated with 0.25% Triton X-100 for 10 min and 5% normal horse serum for 30 min at RT, they were incubated with guinea pig anti-insulin antibody (Dako, Carpinteria, CA, diluted 1:1000) in 5% normal horse serum, overnight at 4 °C. Then the samples were washed in PBS and incubated with biotinylated goat anti-guinea pig IgG antibody (Vector Laboratories, Burlingame, CA, diluted 1:200) for 30 min, and were treated with Alexa Fluor 647-conjugated streptavidin at a dilution of 1:200 (Invitrogen, Eugene, OR). After three washes in PBS, sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). The immunostained cells were observed by confocal laser scanning microscopy (FV1000D; Olympus).

2.6. Statistical analysis

Data are expressed as means ± SE. Statistical analyses were carried out using the student's unpaired *t*-test or one-way ANOVA followed by the Tukey's test. The values in Fig. 4B were analyzed with a Wilcoxon rank-sum test due to non-normality of the data. A p value <0.05 was considered to indicate a statistically significant difference among groups.

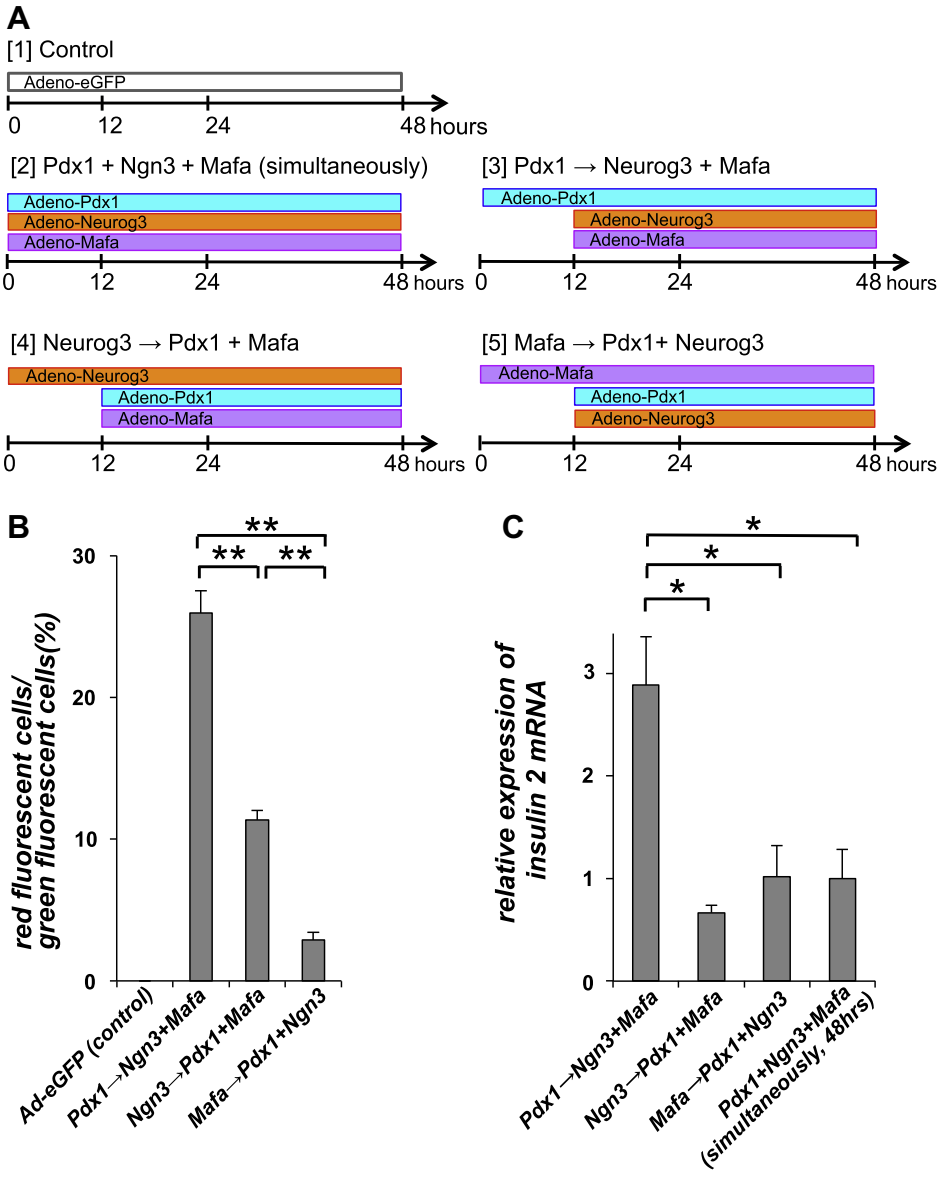

Fig. 2. Expression of Pdx1 prior to Neurog3 and Mafa improves reprogramming efficiency from mPac-MIP-RFP cells into insulin-producing cells. (A) Schematic illustration of the overall experimental design. For simultaneous infection, adenoviruses expressing Pdx1, Neurog3 and Mafa were introduced into cells simultaneously at 0 h or 12 h. For sequential infection, a viral vector expressing one transcription factor was infected first, and those expressing the two other factors were introduced 12 h after the first infection. The number of green and red fluorescent cells was counted at hours 48. (B) The ratio of red to green fluorescent cells was quantified in four sequential combinations of adenoviral infection. The first infection of Mafa significantly lowered the ratio of green to red cells, compared to the other combinations of infection. (C) Total RNAs were isolated 48 h after the first adenovirus infection. Real-time PCR analysis was performed to precisely quantify mRNAs for insulin 2 and β-actin. The expression levels for insulin 2 mRNAs were normalized with β-actin mRNA, and presented as relative expression levels ±standard error (SE), compared to that of simultaneous infection. Data are shown as means ± SE of at least three independent experiments. One-way ANOVA was performed for the statistical analysis. $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Generation and validation of an insulin reporter cell line

To evaluate the efficiency of reprogramming into insulin-producing cells, we aimed to generate a pancreatic duct cell line expressing a fluorescent reporter protein under the control of the insulin promoter (Fig. 1A). The sequence of DsRed-E5, a functional red fluorescent protein (RFP) was placed downstream of the mouse insulin 1 promoter (MIP), then transfected into the mouse pancreatic duct cell line mPac [12], which has ductal characteristics (expression of cytokeratins) and progenitor-like properties, expressing endocrine-specific genes induced by Neurog3 [13].

When the reporter "mPac-MIP-RFP" cells were infected with adenoviruses expressing Pdx1, Neurog3, and Mafa and observed under a confocal microscope, red fluorescence was detected only in the green fluorescent cells labeled with an anti-insulin antibody (Fig. 1B), showing that the mPac-MIP-RFP cells can be used to visualize β-cell neogenesis without using anti-insulin antibody. Since adenoviruses used in this study express enhanced green fluorescent protein (eGFP) together with each transcription factor, the reprogramming efficiency can be evaluated by counting the percentage of red fluorescent cells in the green fluorescent cells (Fig. 1A).

3.2. Expression of Pdx1 prior to Neurog3 and Mafa enhances reprogramming efficiency into insulin-producing cells

Since the pancreas-specific transcription factors Pdx1, Neurog3, and Mafa are expressed in different developmental stages from common progenitors to mature β cells, we hypothesized that the sequential introduction of these genes in mPac-MIP-RFP cells would affect the reprogramming efficiency. In order to verify this hypothesis, Pdx1, Neurog3, and Mafa were sequentially introduced so that one transcription factor was infected first, and then the two other factors were added 12 h after the first infection (Fig. 2A). Forty-eight hours after adenovirus infection, green/red double positive cells were detected among the mPac-MIP-RFP cells expressing all three transcription factors (Fig. 2B), whereas no red fluorescent cells were observed in the cells infected with a control adenovirus expressing only GFP (Fig. 2B), or an adenovirus expressing only one transcription factor (data not shown). When Neurog3 or Mafa expression were introduced before the other transcription factors, 11.4% and 2.9% of the green fluorescent cells were also red fluorescent. On the other hand, when Pdx1 was introduced earlier than Neurog3 and Mafa, over one-fourth of the green fluorescent cells were red fluorescent (Fig. 2B), implying that the introduction of Pdx1 before the other factors improves reprogramming efficiency.

To further investigate the effect of sequential adenovirus infections on reprogramming efficiency, quantitative real-time PCR was performed for insulin 2 mRNA in mPac-MIP-RFP cells harvested 48 h after the first adenovirus infection. Consistent with the data of cell counting in Fig. 2B, the earlier infection of Pdx1 significantly elevated the level of insulin mRNA, compared with other patterns of infection (Fig. 2C). These results indicate that the expression of Pdx1 prior to Neurog3 and Mafa increase the reprograming efficiency from pancreatic duct cells to insulin-producing cells.

3.3. Polycistronic expression of defined transcription factors enhances reprogramming efficiency into insulin-producing cells

In order to improve the introduction efficiency of all three genes into a cell, a single adenoviral vector carrying Pdx1, Neurog3, and Mafa (Ad-PNM) was generated using a polycistronic strategy by 2A peptide-mediated cleavage [14,15] (Fig. 3A). As shown in Fig. 3B, a significantly higher number of red fluorescent cells were observed in the mPac-MIP-RFP cells infected with Ad-PNM. In addition, the expression level of insulin 2 mRNA was significantly increased using Ad-PNM, compared to simultaneous infection with three adenoviruses carrying each transcription factor (Fig. 3B).

3.4. Excessive expression of Mafa together with Pdx1 and Neurog3 inhibits reprogramming into insulin-producing cells

It has been shown that an excessive dose of a transcription factor changes the cell fate during pancreas differentiation, as we previously reported that overexpression of Pdx1 induces acinar-to-ductal transdifferentiation [16,17]. To examine whether an excessive dose of a transcription factor affects reprogramming efficiency, we treated mPac-MIP-RFP cells with the polycistronic adenovirus Ad-PNM (Fig. 3A), together with an adenovirus expressing one of the three transcription factors (Fig. 4A). As shown in Fig. 4B, the number of green and red fluorescent cells was significantly

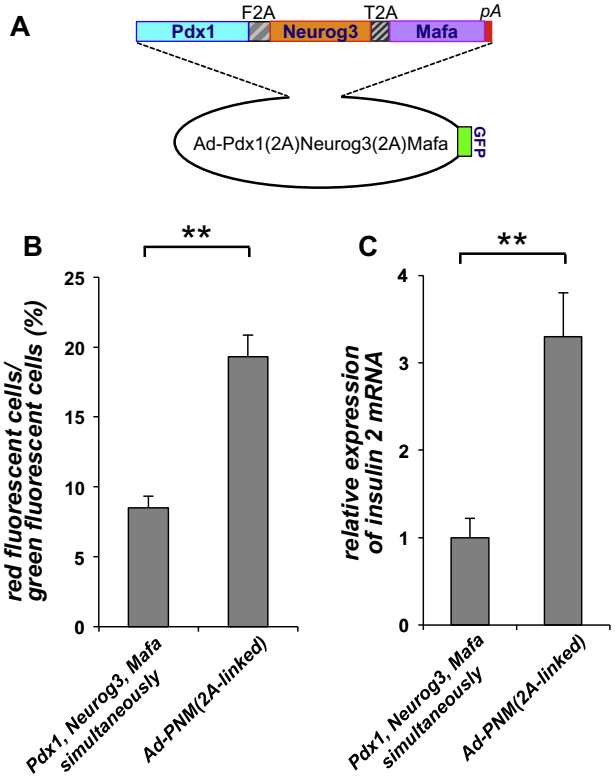

Fig. 3. Polycistronic expression of Pdx1, Neurog3 and Mafa improves reprogramming efficiency from mPAC-MIP-RFP cells into insulin-producing cells. (A) Schematic of the polycistronic vector used to induce the simultaneous exogenous expression of Pdx1, Neurog3, and Mafa. The Pdx1, Neurog3 and Mafa genes were fused with 2A peptide sequences (F2A and T2A), which cause autocleavage of the extending polypeptide, and were cloned into the shuttle vector pAdtrack-CMV, generating a single adenovirus vector, Ad-Pdx1 (2A) Neurog3 (2A) Mafa. F2A and T2A are 'self-cleaving' 2A peptide sequences from foot-and-mouth disease virus and *Thosea asigna* virus, respectively. (B) Pdx1, Neurog3 and Mafa were introduced by a single polycistronic adenovirus or three different adenoviruses carrying each transcription factor. The polycistronic adenoviral infection significantly elevated the ratio of red to green cells, compared to the infection with monocistronic adenoviruses. (C) Total RNAs were isolated 72 h after the first adenovirus infection. Real-time PCR analysis was performed to precisely quantify mRNAs for insulin 2 and β-actin. The expression levels for insulin 2 mRNAs were normalized with β-actin mRNA, and presented as relative expression levels ±standard error (SE), compared to the infection with monocistronic adenoviruses. Data are shown as means ± SE of at least three independent experiments. Student's unpaired *t*-test was used for statistical analysis. $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

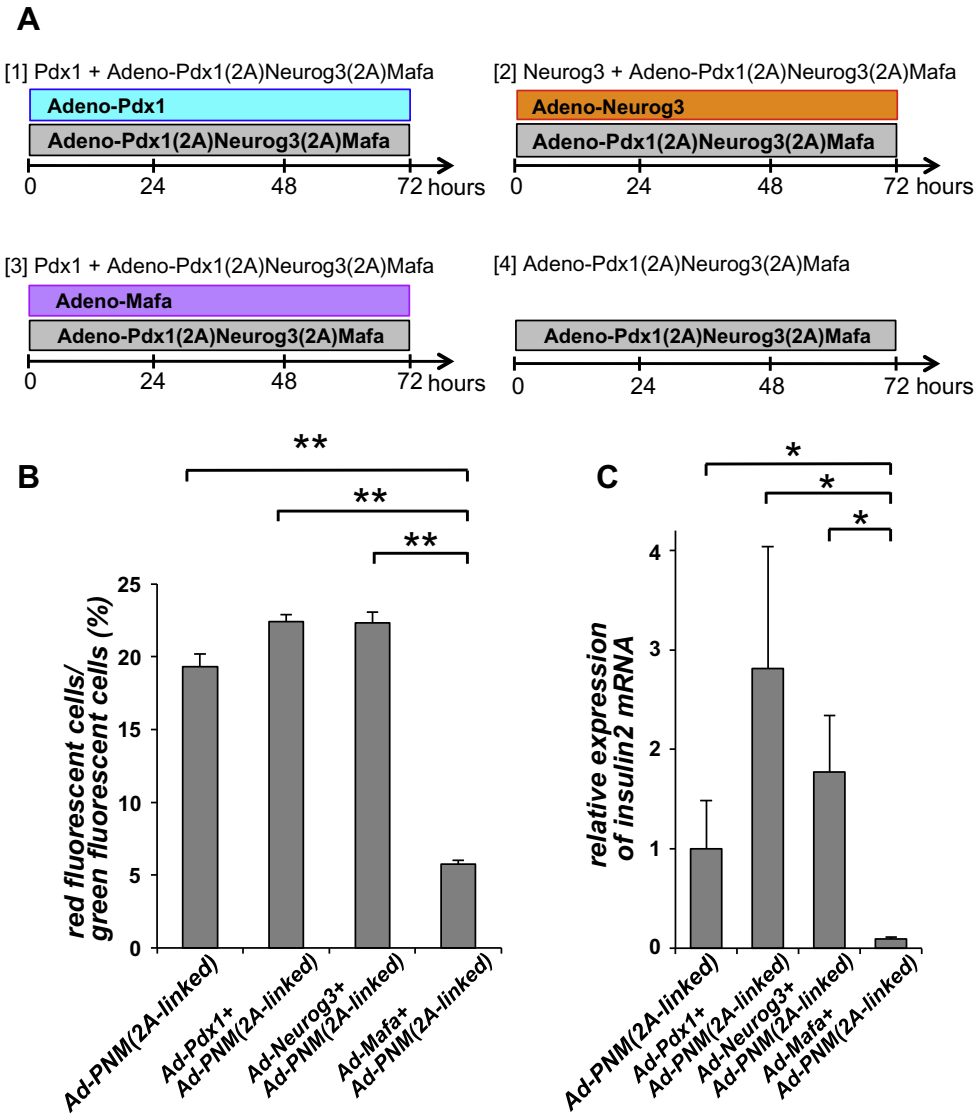

Fig. 4. Excessive expression of Mafa lowers reprogramming efficiency from mPac-MIP-RFP cells into insulin-producing cells. (A) Schematic protocol to examine reprogramming efficiency into β-like cells. The mPac-MIP-RFP cells were infected with the polycistronic adenovirus, together with an adenovirus expressing one of the three transcription factors at 0 h. The number of green and red fluorescent cells was counted at 72 h. (B) Ratio of red to green fluorescent cells upon infection with the four adenovirus combinations. Infection of the polycistronic adenovirus in addition to Ad-Mafa significantly lowered the ratio of red to green fluorescent cells, compared to the other combinations of infections. (C) Total insulin 2 mRNAs in cells infected with the four adenovirus combinations. Real-time PCR analysis was performed to precisely quantify mRNAs for insulin 2 and β-actin. The expression levels of insulin 2 mRNA were normalized with β-actin mRNA, and presented as relative expression levels ± standard error (SE), compared to that of the single infection of the polycistronic adenovirus. Data are shown as means ± SE of at least three independent experiments. The student's unpaired *t*-test was performed for the statistical analysis. One-way ANOVA or Wilcoxon rank-sum test was performed for the statistical analysis. $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lower in mPac-MIP-RFP cells treated with Ad-Mafa in addition to Ad-PNM, compared to the other patterns of infections (Fig. 4B). The data of the real-time PCR analysis were consistent to those of the cell counting (Fig. 4C), suggesting that excessive expression of Mafa together with Pdx1 and Neurog3 inhibits the reprogramming of pancreatic duct cells into insulin-producing cells.

4. Discussion

In this study, we generated a new cell line for evaluating the efficiency of reprogramming of pancreatic duct cells into insulin-producing β-like cells. Although real-time RT-PCR for insulin mRNA expression has been a useful tool to prove the existence of insulin-expressing cells induced from non-β cells, it provides no spatio-temporal information on the insulin-positive cells. While histological approaches, such as immunostaining or in situ hybridization, can be used to quantify the number of insulin-producing cells, less time-consuming methods were anticipated to perform high-throughput screening for evaluating the reprogramming efficiency. The mPac-MIP-RFP cells presented here reflect β-cell neogenesis through red fluorescence (Fig. 1A), and provide us with information for optimizing the reprogramming conditions.

Zhou et al. demonstrated that the simultaneous introduction of adenoviruses expressing Pdx1, Neurog3, and Mafa induced the *in vivo* reprogramming of adult acinar cells into insulin-producing cells, and ameliorated hyperglycemia in a diabetic mouse model [2]. During pancreas development in mice, there is no developmental stage when all three of these transcription factors are highly co-expressed in a cell. In fact, Pdx1 is expressed first around embryonic day 8.5 (E8.5) in the gut when the foregut becomes committed to common pancreatic precursors. Neurog3 is

transiently expressed in endocrine precursors, and Mafa is expressed exclusively in β cells [5]. Therefore we hypothesized that the sequential introduction of these transcription factors, according to the expression profiles in embryos, may change the reprogramming efficiency of mPac-MIP-RFP cells, and found that the preceding of Pdx1 prior to Neurog3 and Mafa enhances the expression of the insulin gene, compared with the simultaneous induction of these three transcription factors (Fig. 2C). Further investigation is required to clarify the mechanisms by which a 12-h time interval between the two adenoviral infections leads to different outcomes.

We then found that the simultaneous expression of Pdx1, Neurog3, and Mafa using the polycistronic adenoviral vector Ad-PNM increases the reprogramming efficiency of mPac-MIP-RFP cells. When three individual adenoviruses carrying each transcription factor are used for gene transfer, only a portion of the adenovirus-infected cells express all these transcription factors, in contrast to the Ad-PNM-infected cells which express all three transcription factors in all infected cells, resulting in a high reprogramming efficiency. This result is consistent with a previous report showing that a similar polycistronic Ad-PNM vector efficiently induces *in vivo* reprogramming from liver cells to insulin-producing cells [7].

Since the polycistronic Ad-PNM vector expresses the same amount of each transcription factor, we further investigated whether excessive expression of one of the three transcription factors together with Ad-PNM could improve the reprogramming efficiency. However, no favorable effect was observed by the excessive expression of a transcription factor; on the contrary, excessive expression of Mafa drastically decreased the cellular reprogramming by Ad-PNM (Fig. 4B and C). It is noted that the expression of Mafa before the other transcription factors resulted in lower efficiency, of the neogenesis of insulin-producing cells, as shown in Fig. 2. These findings are also supported by a previous *in vivo* report that the early expression of Mafa in pancreatic progenitors suppresses normal endocrine differentiation [18]. Although Mafa is a potent transcriptional activator for insulin gene expression [19], these results suggest that optimizing the timing and dose of exogenous Mafa expression is essential to yield a better reprogramming efficiency.

The mPac-MIP-RFP cells that we generated in this study led us to discover better reprogramming conditions for β-cell neogenesis. This system can be used for further optimization of the timing and dosage of expression of defined transcription factors required for β-cell neogenesis. Moreover, the sequential induction of these genes should be confirmed in human ES cells and/or iPS cells for generating surrogate β cells to cure diabetes.

Acknowledgments

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