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MafA differentiates rat intestinal cells into insulin-producing cells

Satoshi Nomura [a], Takaaki Nakamura [b], Tetsuya Hashimoto [a], Yoshihiko Nishio [a], Hiroshi Maegawa [a], Motoi Kudo [b], Atsunori Kashiwagi [a,*]

[a] *Division of Endocrinology and Metabolism, Department of Medicine, Shiga University of Medical Science, Japan*
[b] *Department of Anatomy, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan*

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Abstract

Recent studies have suggested that basic leucine zipper transcription factor MafA has a crucial role in pancreatic β-cell-specific insulin gene transcription. Thus, we investigated whether MafA overexpression in the intestine induces insulin production in small-intestinal epithelial cells in vivo. Recombinant adenovirus containing MafA gene (Ad-MafA) was prepared and administered orally to streptozocin-treated diabetic rats. Insulin gene expression was observed in the intestine by RT-PCR analysis, and then insulin protein was detected by immunohistochemical analysis after Ad-MafA administration. Furthermore, MafA overexpression in the intestine increased plasma insulin levels and ameliorated hyperglycemia. These results indicate that MafA overexpression in the intestine induces intestinal epithelial cells newly to produce and release insulin.

Keywords: MafA; Insulin; Adenovirus; Intestine; Streptozocin; Diabetes

Expression of insulin gene in adult mammals is restricted to pancreatic β-cells [1]. MafA transcription factor has a crucial role in tissue-specific expression of insulin [2]. MafA is exclusively expressed in β cells with in a pancreatic content [3] and binds to a cis-regulatory element called RIPE3b in the insulin gene promoter region [4] to regulate insulin transcription in response to serum glucose levels [5,6]. MafA is also required for the maintenance of adult pancreatic architecture and function, since MafA-deficient mice display decrease in β-cell/α-cell ratio because of reduced expression of Pdx1 or Beta2/NeuroD transcription factors [7].

The intestinal tract is a possible target organ for gene delivery with adenovirus [8,9] or in transgenic animals [10]. Cheung et al., demonstrated that transgenic mice with human insulin gene linked to the 5′-regulatory region of the gene encoding glucose-dependent insulinotropic polypeptide (GIP) produce insulin specifically in K cells [11]. This experimental evidence indicates that intestinal epithelial cells can differentiate into insulin-producing cells in vivo.

Our previous reports have presented evidence that undifferentiated rat intestinal epithelial cells (IEC-6 cells) can be differentiated into enteroendocrine cells with overexpression of transcription factor Pdx1 [12] and into insulin-producing cells with combined expression of Pdx1 and transcription factor Isl1 [13] in vitro. Furthermore, we have reported that Isl1 enables IEC-6 cells to express potassium inwardly rectifying channel (Kir6.2) [14]. These results suggest that introduction of the specific transcription factors into rat intestinal epithelial cells enable to newly synthesize insulin in vivo. Thus, to examine this possibility, we firstly constructed a recombinant adenovirus expressing MafA, and then we administered this virus into rat intestine in vivo to see whether newly synthesized insulin was observed in the rat intestine. Furthermore, we also assessed whether this synthesized insulin ameliorated hyperglycemia in streptozocin (STZ)-treated diabetic rats.

* Corresponding author. Present address: Shiga University of Medical Science, Seta-Tsukinowa-cho, Otsu, Shiga 520-2192, Japan. Fax: +81 77 543 3858.
E-mail address: kasiwagi@belle.shiga-med.ac.jp (A. Kashiwagi).

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Materials and methods

Cell culture. Rat pancreatic β-cells (RIN-5F cells) and human kidney epithelial cells (HEK 293 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Recombinant adenovirus. IRES-EGFP-SV40 poly(A) site was amplified from a pIRES2-EGFP vector (Clontech, Mountain View, CA) using a forward primer with linkers for restriction sites for *Nhe*I, *Bam*HI, and *Pme*I, and a reverse primer with linker for *Eco*RI. The fragment was then cloned into a pShuttle2 vector (Clontech) at the *Nhe*I and *Eco*RI sites, creating the pShuttle2-EGFP vector. cDNA encoding for wild-type murine MafA was amplified using a forward primer with linker for *Bam*HI, and a reverse primer with linker for *Eco*RV. This fragment (*Bam*HI/*Eco*RV sites) was cloned into the *Bam*HI/*Pme*I sites of the pShuttle2-EGFP vector to create the pShuttle2-MafA vector. Expression cassette of pShuttle2-MafA vector was cloned into Adeno-X viral DNA (Clontech) to create Ad-MafA. We also constructed LacZ adenovirus (Ad-LacZ) as a control. Viruses were amplified in HEK 293 cells and isolated using the freeze-thaw method. Plaque-forming units (pfu) were assayed using End-Point Dilution Assay according to the manufacturer's protocol (Clontech).

Animals. Six-week-old male Sprague–Dawley rats were purchased from SLC (Shizuoka, Japan). Rats were made diabetic by intravenous injection of STZ (Sigma Chemical Co., St. Louis, MO) (50 mg/kg) freshly dissolved in 0.1 M citrate buffer (pH 4.5). Four days after STZ injection, non-fasting blood glucose levels were measured; rats with blood glucose levels of more than 300 mg/dl were selected for experiments. Blood glucose levels were measured regularly by glucose oxidase method with a portable blood glucose level monitor (GLUTEST PRO; Sanwa Kagaku Kenkyusho, Aichi, Japan) after tail snipping.

Measurement of insulin content. On the fourth and sixth days after STZ injection, 2 ml of Ad-MafA, or Ad-LacZ (1×10^9 pfu/ml) was administered orally to the diabetic rats. On the ninth day after STZ injection, fasting blood glucose levels were measured and fasting plasma insulin levels were determined using rat insulin ELISA Kit (U-type) (Shibayagi, Gunma, Japan), which detects insulin levels in the range from 50 to 3000 pg/ml.

Analysis of tissue mRNA expression. Total RNA was extracted from the intestine using TRIzol Reagent (Invitrogen, Carlsbad, CA). The first-strand cDNA was synthesized with random hexamers and superscript II reverse transcriptase (Invitrogen). Subsequent PCR was performed using KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). The following primer pairs were used to amplify fragments: insulin, 5′-CTT CCT ACC CCT GCT GGC CCT GC-3′ (forward) and 5′-GGG CTT CCT CCC AGC TCC AGT TG-3′ (reverse); MafA, 5′-TCA ACG ACT TCG ACC TGA TG-3′ (forward) and 5′-GGG CAG AGT GAT GAT GGT G-3′ (reverse); Pdx1, 5′-ACA TCT CCC CAT ACG AAG TG-3′ (forward) and 5′-TGA TGT GTC TCT CGG TCA AG-3′ (reverse); β-actin, 5′-ATG GAT GAC GAT ATC GCT G-3′ (forward) and 5′-ATG AGG TAG TCT GTC AGG T-3′ (reverse).

Immunoblotting. HEK 293 cells transduced with adenovirus and RIN-5F cells were homogenized in buffer (50 mM Tris, pH 6.8, 2% SDS, 6% of 2-mercaptoethanol, 10% glycerol). Cellular homogenates including proteins (10 μg) were separated by SDS–PAGE and then transferred to polyvinylidene fluoride membrane. Immunoblot analysis was performed using Western Lightning (Perkin-Elmer, Wellesley, MA). Antibody to MafA (M-153, Santa Cruz Biotechnology, Santa Cruz, CA) was commercially obtained.

Immunohistochemistry. Intestines of rats were excised 5 days after the first adenovirus administration and fixed overnight in 4% paraformaldehyde. Fixed tissues were frozen in powdered dry ice and cut into 20-μm sections in a cryostat. For peroxidase staining, the sections were incubated overnight with antibody against insulin (18-0067, Invitrogen). The avidin–biotinylated enzyme complex (ABC) method was applied using Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. For fluorescence immunohistochemistry, the sections were incubated overnight with DAPI (4′,6-diamino-2-phenylindole dihydrochloride), or antibodies against MafA (M-153, Santa Cruz), insulin (18-0067, Invitrogen), Chromogranin A (AB9254, Chemicon, Temecula, CA), prohormone covertase 1/3 (PC1/3) (AB1260, Chemicon) and Potassium channel Kir6.2 (AB5492, Chemicon). The sections were then incubated with the species-specific secondary antibody mixture of Alexa Fluor 350 and 546 (Invitrogen). Sections were observed under a fluorescence microscope.

Statistical analysis. Results are expressed as means $\Delta \pm$ SE, unless otherwise stated. The unpaired Student's t -test was used to determine the significance of any differences between two groups. A p value of <0.05 was considered significant.

Results

To evaluate the effects of MafA expression on insulin gene expression in the intestine, we constructed MafA-expressing adenovirus (Ad-MafA) and a control adenovirus (Ad-LacZ). Ad-MafA was designed to express enhanced green fluorescent protein (EGFP) in addition to MafA from a single transcript (Fig. 1A). To confirm the expression of MafA, Ad-MafA was transduced into HEK 293 cells (final concentration, 1.0×10^7 pfu/ml). The cells were subjected to immunoblotting. MafA protein was identified in the extracts from both HEK 293 cells with Ad-MafA and RIN-5F cells as a positive control, whereas no signals were seen in HEK 293 cells with Ad-LacZ (Fig. 1B). The molecular weight of MafA is 48 kDa here, and this is consistent with a previous report [4].

To evaluate the expression of MafA in rat intestine, on the fourth and sixth days after STZ injection, Ad-MafA (2×10^9 pfu) was orally administered twice to STZ-treated diabetic rats. On the fifth day after the first adenovirus administration, rats were sacrificed and the intestine was subjected to immunofluorescence analysis. As shown in Fig. 2, MafA was detected in the nuclei of intestinal epithelial cells (Fig. 2A) and EGFP was detected in the cytoplasm

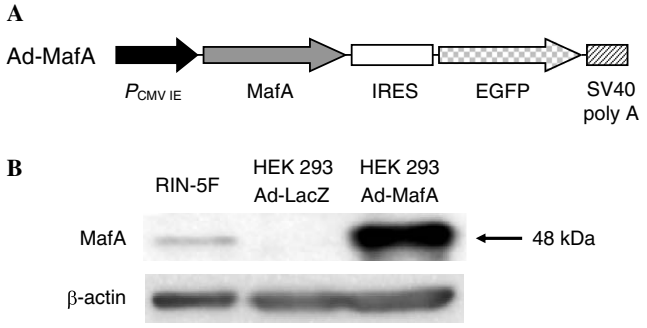

Fig. 1. The structure of the expression cassette integrated into the adenoviral vector and adenovirus-mediated MafA expression in HEK 293 cells. (A) The expression cassette contained the cytomegalovirus immediate early promoter ($P_{CMV\ IE}$), mouse MafA cDNA, followed by an internal ribosome entry site (IRES) and EGFP cDNA, which led to the concomitant expression of EGFP, detected by fluorescence microscopy. (B) Expression of mouse MafA protein (48 kDa) was detected by Western blotting using anti-MafA antibody three days after adenovirus infection. Cell extracts from RIN-5F cells and HEK 293 cells transduced with Ad-LacZ were used as positive and negative controls, respectively.

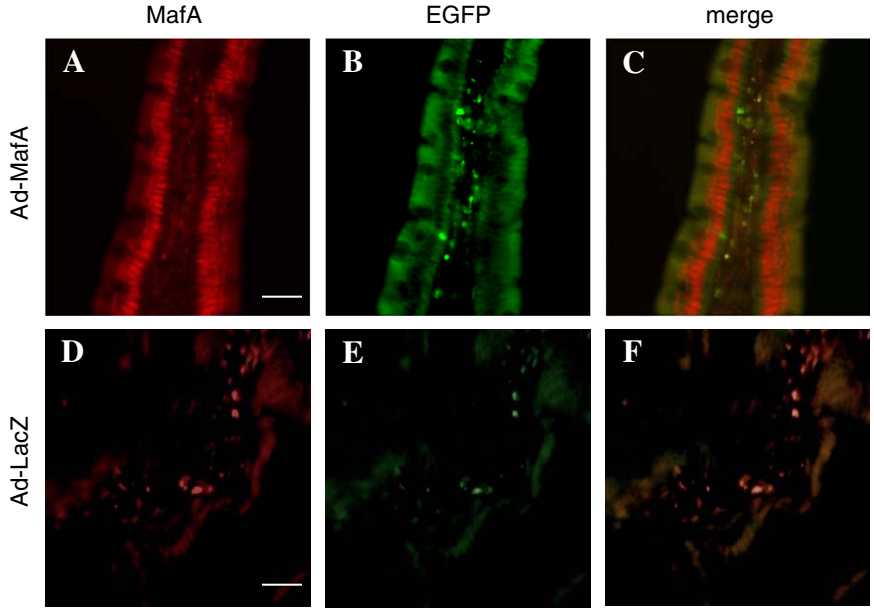

Fig. 2. Adenovirus-mediated expression of MafA and EGFP in rat intestine. Ad-MafA (A–C) or Ad-LacZ (D,E) was given to each rat orally. On the fifth day after the first adenovirus transduction, intestine sections were immunohistochemically stained for MafA (A,D) and EGFP (B,E). Merged images are shown on the right (C,F). The results shown are representative of three independent experiments. Bars, 150 μm.

(Fig. 2B). We also investigate how long MafA and EGFP were co-localized in epithelial cells (Fig. 2C). No staining of these transgenes was found in intestine with Ad-LacZ (Fig. 2D–F). We also confirmed no staining of these genes in the liver with Ad-MafA, suggesting that Ad-MafA did not transfect rat liver cells at all (data not shown). Next, to confirm whether MafA expressed in the nuclei of the cells, the nuclei was stained by DAPI and the positive staining of both DAPI (Fig. 3B) and MafA (Fig. 3A) were observed in the same cells (Fig. 3C) with Ad-MafA, whereas no such stain was identified with Ad-LacZ (Fig. 3D–F).

To study the effect of MafA expression on expression of several pancreas-related genes in the intestine, RT-PCR analysis was performed. As shown in Fig. 4, insulin gene was detected in the intestine after transduction with Ad-MafA but not with Ad-LacZ. Expression of Pdx1 gene was identified with both Ad-MafA and Ad-LacZ. All of these genes were identified in extracts from rat embryonic pancreas.

Because MafA can induce insulin gene expression in the intestine, to reconfirm insulin protein production, we performed peroxidase immunostaining for insulin. Insulin-positive cells were observed in the intestinal epithelial cells with Ad-MafA (Fig. 5A and B), whereas no staining was detected in the intestine with Ad-LacZ (Fig. 5C and D). To study whether Ad-MafA induced the expression of

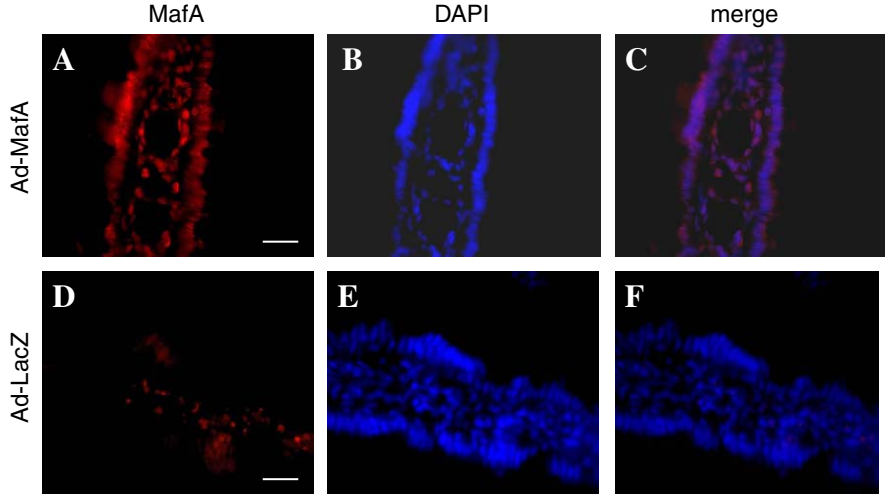

Fig. 3. Adenovirus-mediated expression of MafA and DAPI in rat intestine. Ad-MafA (A–C) or Ad-LacZ (D,E) was given to each rat orally. On the fifth day after the first adenovirus transduction, intestine sections were immunohistochemically stained for MafA (A,D) and DAPI (B,E). Merged images are shown on the right (C,F). The results shown are representative of three independent experiments. Bars. 150 μm.

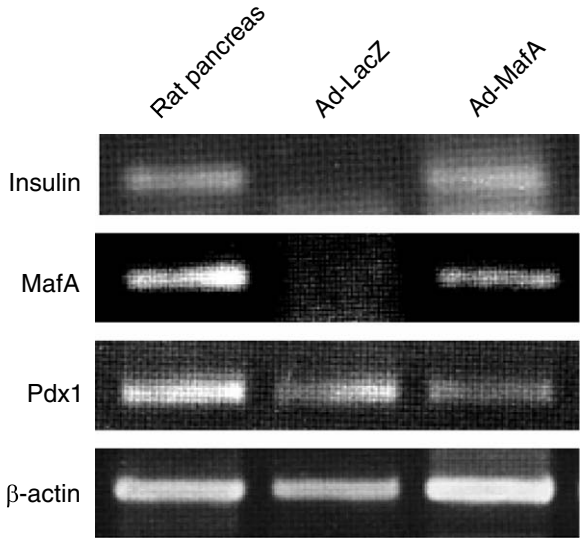

Fig. 4. RT-PCR analysis of gene expression in rat epithelial cells after transduction with adenovirus. On the fifth day after the first adenovirus transduction, total RNA was extracted from the rat intestinal epithelial cells. RT-PCR was used to probe for the presence of insulin, MafA, Pdx1, and β-actin in total RNA isolated from rat pancreas (left lane), and in intestinal epithelial cells transduced with Ad-LacZ (middle lane) and with Ad-MafA (right lane). The expected sizes of amplified cDNA were obtained. The results shown are representative of three independent experiments.

insulin in vivo, double staining for both MafA and insulin was performed by fluorescence immunohistochemistry. As shown in Fig. 6, the stain for MafA was identified in the nuclei of epithelial cells (Fig. 6A) and insulin positive cell was found among those cells (Fig. 6B and C). These results indicated that Ad-MafA induced the expression of insulin. However, neither of MafA nor insulin was identified with Ad-LacZ (data not shown).

To further identify the type of epithelial cells that produce insulin, we performed fluorescence immunohistochemistry. Since we found that the insulin-positive cells assumed the typical shape of enteroendocrine cells as seen in Fig. 5, we performed double staining of insulin and chromogranin A (CgA), which is believed to be an enteroendocrine cell marker [15]. As shown in Fig. 7, CgA-positive cells were identified among the specific epithelial cells with both Ad-MafA (Fig. 7A) and Ad-LacZ (Fig. 7D); those cells were considered enteroendocrine cells. Insulin-positive cell was observed among a certain portion of epithelial cells only with Ad-MafA (Fig. 7B), and not with Ad-LacZ (Fig. 7E). Thus, insulin-positive cells were also stained with CgA in the cytoplasm of the intestinal epithelial cells only with Ad-MafA (Fig. 7C). To evaluate how many enteroendocrine cells could express insulin, we measured the number of both insulin-positive cells and CgA-positive cells. About 50 insulin-positive cells per 100 jejunal villi were found in a section from rat intestine with Ad-MafA, whereas about 500 CgA-positive cells per 100 jejunal villi were found in the same section (data not shown). These results indicate that MafA may induce about 10% of enteroendocrine cells to produce insulin.

It is important that insulin-producing cells should be examined for expression of other islet cell enriched products, such as prohormone convertase and ATP sensitive potassium channel. Thus, we examined co-expression of prohormone convertase PC1/PC3 or potassium channel Kir6.2 in insulin-producing epithelial cells in the presence of Ad-MafA as shown in Fig. 8. Both PC1/PC3 (Fig. 8A) and Kir6.2 (Fig. 8D) were colocalized in insulin-producing cell (Fig. 8B and E). These results indicate that Ad-MafA can differentiate intestinal epithelial cells into insulin-producing cells expressing PC1/PC3 and

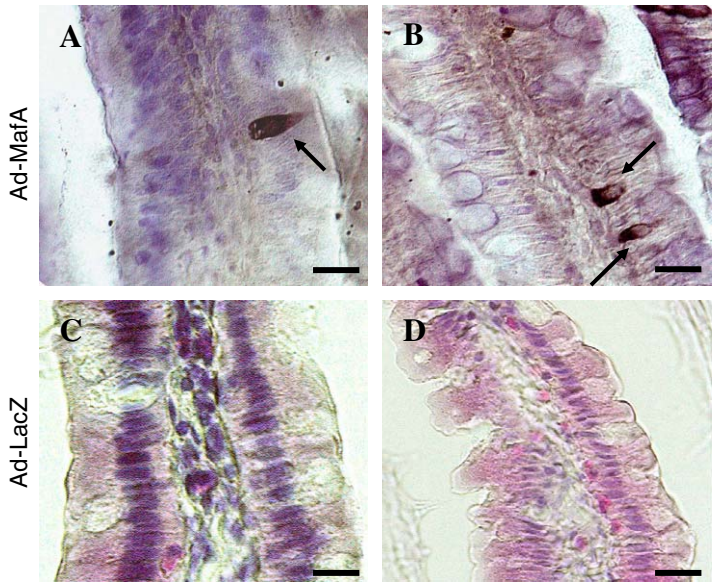

Fig. 5. Peroxidase staining for insulin in the rat intestine after transduction with adenovirus. On the fifth day after the first adenovirus transduction, intestinal sections from the rat transduced with Ad-MafA (A,B) and with Ad-LacZ (C,D) were peroxidase stained with anti insulin antibody. (A,B) Independent studies. Bars, 20 μm.

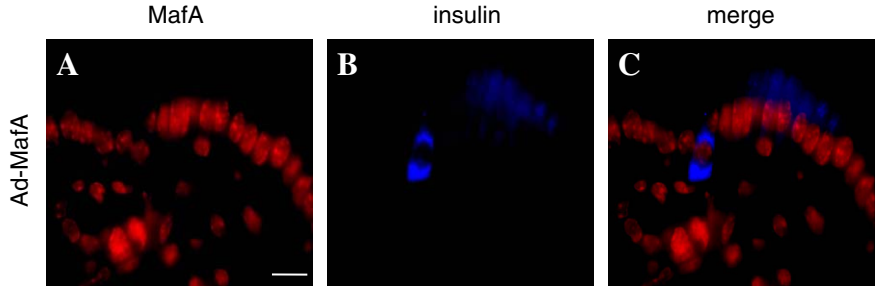

Fig. 6. Double stainining results by immunohistochemistry for insulin and MafA in the rat intestine. On the fifth day after the first adenovirus transduction, intestinal sections from the rat transduced with Ad-MafA (A–C) were double-immunostained with insulin (B) and MafA (A) antibodies, respectively. Merged images are shown on the right (C). Bars, 20 μm.

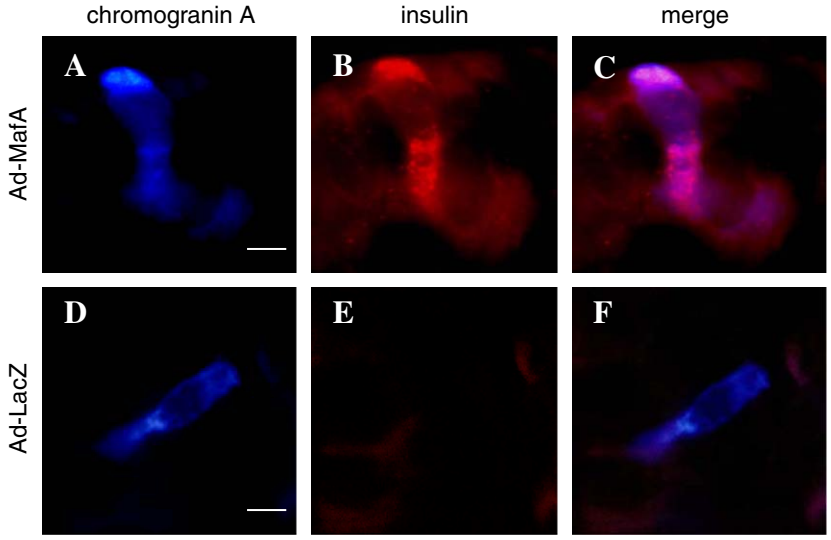

Fig. 7. Double-immunostaining results for insulin and chromogranin A in the rat intestine. On the fifth day after the first adenovirus transduction, intestinal sections from the rat transduced with Ad-MafA (A–C) and with Ad-LacZ (D–F) were double-immunostained with chromogranin A (A,D) and insulin (B,E) antibodies, respectively. Merged images are shown on the right (C,F). Bars, 10 μm.

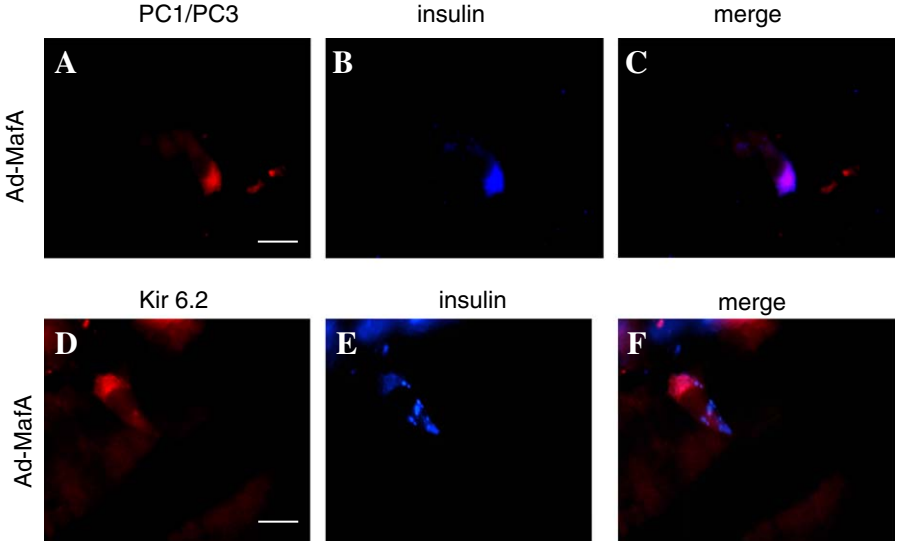

Fig. 8. Double-immunostaining results for insulin, PC1/PC3 and/or Kir6.2 in the rat intestine. On the fifth day after the first adenovirus transduction, intestinal sections from the rat transduced with Ad-MafA were double-immunostained with insulin (B, E), PC1/PC3 (A) and/or Kir6.2 (D) antibodies, respectively. Merged images are shown on the right (C, F). Bars, 20 μm.

Table 1
Blood glucose and insulin levels before and after adenovirus transduction

	Blood glucose levels (mg/dl)		Plasma insulin levels (pg/ml)
	Before transduction (ad lib)	After transduction (overnight fasting)	After transduction (overnight fasting)
Ad-MafA ($n = 10$)	448 ± 16	108 ± 13*	173 ± 20*
Ad-LacZ ($n = 10$)	434 ± 24	337 ± 14	79 ± 42

Data are expressed as mean ± SE. Blood glucose and insulin levels were measured on the fifth day after the first adenovirus transduction.
* $p < 0.01$ vs. Ad-LacZ.

Kir6.2, which are pancreatic β cell specific properties as well as insulin.

We showed that MafA enables enteroendocrine cells to produce insulin in vivo, but it was still unclear whether the blood glucose levels are decreased in response to this insulin. To investigate the effects of newly synthesized insulin on STZ-induced hyperglycemia, we measured fasting blood glucose (Table 1). No differences of blood glucose levels among the rats were identified before adenovirus administration. Fasting blood glucose levels were significantly decreased in Ad-MafA-administered rats, whereas no such effect was observed in Ad-LacZ-administered rats. Furthermore, to confirm insulin secretion from enteroendocrine cells, we measured plasma insulin levels in STZ-treated diabetic rats after the adenovirus administration. As shown in Table 1, fasting plasma insulin levels were increased after Ad-MafA administration. These insulin levels were consistent with previous reports that constitutively active Pdx1 induced efficient insulin production in adult murine liver [16]. We performed oral glucose tolerance text (1g/kg body weight) in Ad-MafA-treated STZ rats, and found that insulin levels did not increase during 60–120 min (167 ± 31.7 pg/ml, $n = 6$), suggesting that Ad-MafA dependent insulin secretion is not glucose regulated.

Discussion

In this study, we showed that oral administration of recombinant adenovirus expressing MafA efficiently induced rat enteroendocrine cells to produce and release insulin. Insulin-positive cells were co-localized with Chromogranin A in enteroendocrine cells. In addition, fasting blood glucose levels were significantly decreased after Ad-MafA administration in STZ-treated diabetic rats, whereas no such effect was observed in Ad-LacZ-administered rats. Furthermore, plasma insulin levels were significantly increased in the rats with Ad-MafA compared to the rat with Ad-LacZ.

A number of studies have addressed the feasibility of gene therapy for production of insulin in diabetic patients. Several tissues are possible targets for regeneration of insulin-producing cells. Target tissues tested include liver [16–18], muscle [19], pituitary [20], hematopoietic stem cells [21], and gastrointestinal cells [11,22]. Among these tissues, we have focused on the intestinal cells because pancreatic endocrine and exocrine cells are known to be originated from the gut endoderm during embryogenesis [23]. We have previously reported that IEC-6 cells that have the characteristics of immature intestinal crypt cells differentiate into insulin-producing cells by co-expression of the transcription factors Pdx1 and Isl1 in vitro [13]. Thus, we investigated whether rat crypt cells differentiated into insulin-producing cells by overexpression of these transcription factors in vivo. Because RT-PCR analyses have revealed normal expression of both Pdx1 [24] and Isl1 [25] in the intestine, we selected MafA transcription factor. MafA has an important role in pancreatic β-cell-specific expression of insulin [2]. Kaneto et al., showed that MafA overexpression in the liver, together with Pdx1 and NeuroD, markedly increase insulin gene expression [18]. According to their report, adenovirus transduction with Pdx1, Beta2/NeuroD, and MafA can induce insulin promoter activity and exert strong synergistic effects on insulin gene expression in the liver, although no such effects were observed with MafA alone. On the contrary, our data suggest that expression of MafA alone induces intestinal epithelial cells to produce insulin in vivo. Because transcription factors, such as Pdx1, Ngn3, Isl1, Beta2/NeuroD, Pax4, and Nkx2.2, that participate in pancreatic β-cell differentiation, are already expressed in the intestine [24–27], expression of MafA alone may enable rat intestinal cells to differentiate into insulin-producing cells. We also showed that MafA might induce about 10% of enteroendocrine cells to produce insulin in this study. Approximately 15 different enteroendocrine cells, such as GLP-1 and GIP producing cells, show characteristic distributions migrating to intestinal epithelial cells and secrete hormones into circulation. The number of each enteroendocrine cell is very few in intestinal villi, however, the mass of each enteroendocrine cell in jejunum are thought to be large as endocrine system. Thus, the cells mass of 10% of enteroendocrine cells which produce insulin can be equal to that of GLP-1 or GIP producing cells in jejunum and the high blood insulin levels in diabetic rats after treatment with Ad-MafA are consistent.

Next, we also investigate that how long insulin expression is maintained within the intestine. Whereas turnover of enteroendocrine cells is longer than enterocytes and it is in 6 days, adenovirus can bind to and be internalized to greater degree in both differentiated and undifferentiated enteroendocrine cells. However, insulin-positive cells were not observed in intestinal cells at two and four weeks post infection. These results indicate that viral MafA expression can induce insulin production in ente-

roendocrine cells within only a week because of the rapid turnover.

Our study suggests that MafA is a useful regimen for inducing islet neogenesis and that the intestinal tract could serve as an important source of tissue for generating functional insulin-producing cells instead of malfunctioning β-cells in patients with type 1 diabetes. However, as a practical problem, we administered gene repeatedly, because of the rapid turnover of intestinal epithelial cells; intestinal epithelial cells migrate up from crypt to villus tip every 3–4 days [28]. Furthermore, we also need a vector with good efficacy and with the best safety profile available as well as a gene delivery tool. Chitosan may be a low-immunogenic and non-toxic carrier for successful oral delivery of plasmid DNA in future [29]. In any case, we have to climb over a significant barrier for clinical application of this gene transfer method in type 1 diabetes.

In conclusion, MafA overexpression induces insulin production in the intestine and ameliorates fasting plasma glucose levels in STZ-treated diabetic rats, suggesting that enteroendocrine cells may play a crucial role in insulin production in future.

Acknowledgments

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