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In vivo suppression of mafA mRNA with siRNA and analysis of the resulting alteration of the gene expression profile in mouse pancreas by the microarray method

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Abstract

Maf is a family of transcription factor proteins that is characterized by a typical bZip structure, and one of the large mafs, mafA is a strong transactivator of insulin. To explore the role of mafA in the pancreas, we modified the mafA mRNA level *in vivo* in mice by the RNA interference (siRNA) technique and analyzed the resulting alteration of the expressed gene profile with a microarray system. The mafA expression level in siRNA-treated mice was reduced approximately 60% compared with control-siRNA-treated animals. Microarray analysis revealed changes in the expression level of several genes in the siRNA-treated mice, with prominent down-regulated expression of the genes encoding insulin, glucagon, and adipocytokines, suggesting possible role of mafA in the pathophysiological states of impaired metabolic responses or inflammatory reactions.

Keywords: Adipocytokine; β Cell; Insulin; mafA; Microarray; siRNA

Maf is a family of transcription factors characterized by a typical bZip structure, which is a motif for protein dimerization and DNA binding, that has been reported to regulate several distinct developmental processes, cell differentiation, and the establishment of cell functions [1–3]. In a previous study, we elucidated the expression profiles of the large maf family in developing human and porcine pancreas [4], and induction and alteration of large maf expression was observed in mature rat pancreas under acute oxidative stress conditions, suggesting several potential biological roles of mafs in the development and regulation of pancreatic cell function. One of the large mafs, mafA, is a distinct molecule that has been attracting the attention of researchers, because it is a strong transactivator of insulin in pancreatic β cells [5,6], and there have been many reports that the mafA transcription factor contributes to β cell function and differentiation [7–10]. A recent study has demonstrated that loss of mafA protein causes a decrease in insulin gene expression in glucotoxic β cells [11], and another study has shown that insulin transcription is markedly reduced in mafA-deficient mice, even though the insulin content of the β cells is not significantly diminished [12].

To explore the role of mafA in the pancreas, we modified the mafA mRNA level by the RNA interference technique *in vivo* in mice. The hydrodynamic method was used and the resulting alteration of the gene profile expressed in the pancreas of siRNA treated mice was analyzed with a microarray system.

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Materials and methods

Animal preparation. Eight-week-old male mice were maintained under stable conditions. All procedures were performed in accordance with guidelines set by the National Institute of Health and the Institutional Animal Care and Use Committee of Tokyo Women's Medical University.

MafA siRNA. A designed siRNA oligomer constructed in a plasmid was purchased from Takara Bio (Takara Bio Co., Japan). The target sequence and designed siRNA sequence are shown in Table 1.

siRNA injection. Anesthetized mice were intravenously injected with the siRNA constructed in the plasmid by hydrodynamic method according to the procedure described in a Hamar's report [13]. Briefly, immediately after immersing the tail in a 55 °C warm water bath for 5 s to the dilate tail veins, siRNA dissolved in TransIT^{R}-QR Hydrodynamic Delivery Solution (Mirus Bio Corporation, Madison, WI) according to the manufacture's instruction manual was rapidly injected into the tail vein within 5 s.

Reverse-transcription (RT) and real-time PCR. RNA isolation and real-time PCR were performed as previously described [14]. Briefly, total RNA was isolated from the pancreas with TRIzol Reagent (GIBCO/BRL). Relative quantitation by the real-time PCR method was performed by using SYBR Green PCR Reagents and an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reactions were performed using 1.0 μl RNA at a concentration of 50 ng/μl in a reaction volume of 25 μl. RT was performed at 48 °C for 30 min, followed by PCR consisting of AmpliTaq activation for 10 min at 95 °C, then 40 cycles of heating to 95 °C for 15 s and cooling to 60 °C for 1 min, and the mRNA levels were normalized to the levels of GAPDH mRNA. Specific primers for real-time PCR were designed and are summarized in Table 2.

Western blotting. Frozen pancreata were immersed in a lysis buffer (20 mM Tris buffer, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin from bovine lung (Wako, Tokyo), 2 mM DL-dithiothreitol, 1% polyoxyethylene sorbitan monolaurate, 1 mM ethylenediamine tetraacetate) and then homogenized on ice. After centrifugation at 5000 rpm for 10 min, 30 μg of protein from each sample was suspended in a loading buffer, separated on a 12.5% polyacrylamide gel (Readygels J, Bio-Rad, Tokyo, Japan), and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked with 3% skim milk for 1 h at room temperature. A primary antibody against mafA (BL1069 Bethyl laboratories Inc.) was used at a 1:3000 dilution and applied overnight at 4 °C. After two 10 min washing steps with washing buffer (0.3% Tween 20 in PBS), the membrane was incubated with horseradish-peroxidase-conjugated immunoglobulins (DAKO, Tokyo, Japan) for 1 h at room temperature, then with an ECL Western blotting system (Amersham Biosciences, Tokyo), and, finally, X-ray film was exposed to the membrane.

DNA microarray analysis. The DNA microarray analysis was performed as described previously [15]. Briefly, Affymetrix Gene Chip technology was used as follows. cDNA was synthesized from total RNA with a Superscript Choice kit (GIBCO/BRL) and T7 polymerase (Mega Script T7 kit; Ambion, Austin, TX). The total RNA (8 μg) was annealed to T7-(dt)24 primer (100 pmol/ml) at 70 °C for 10 min, and reverse transcription was carried out. The reaction mixture was incubated at 16 °C for 2 h, 2 μl of T4 DNA polymerase at 5 U/μl was added, and incubation at 16 °C was continued for 5 min. After termination, biotin-labeled cRNA was synthesized with an In Vitro Transcription Kit (Ambion Inc., Austin, TX). The reaction was allowed to proceed at 37 °C for 5 h in a mixture with 7.5 mM ATP, 7.5 mM GTP, 5.6 mM UTP, 1.9 mM biotinylated UTP, 5.6 mM CTP, 1.9 mM biotinylated CTP, 1× T7-Transcription Buffer, and 1× T7-Enzyme Mix (Enzo Diagnostics, Farmingale, NY). A 20 μg sample of the fragmented cRNA was hybridized to the GeneChip U74A Array Set (Affymetrix, Santa Clara, CA) at 45 °C in a rotisserie hybridization oven at 60 rpm for 16 h. The probe arrays were exposed to antibody solution [1× MES solution, 0.005% antiform, 2 mg/ml acetylated BSA, 0.1 μg/μl normal goat IgG (Sigma, St. Louis, MO), 3 μg/μl goat-anti-streptavidin, biotinylated antibody (Vector Laboratories, Burlingame, CA)] at 25 °C for 10 min, and after washing and staining, the probe array was scanned twice at 3 μm resolution with a GeneChip System confocal scanner (Hewlett–Packard, Santa Clara, CA) controlled by GeneChip 3.1 software (Affymetrix, Santa Clara, CA).

Statistical analysis. All results are expressed as means ± SEM. Differences in expression levels were analyzed by the paired Student's *t* test with Bonferroni correction, and differences were considered significant when the *p* value was <0.05. The experiments were repeated five times.

Table 2
Primers for real-time PCR

Gene	Forward	Reverse
MafA	ccagctggtatccatgtcc	ttctgtttcagtcggatgacc
MafB	aacgctgcaactctcaagtg	gggacttgaacaccaccatt
c-maf	ctggagtcggagaagaacca	ctccttgtaggcgtcccttt
InsulinI	tggtccccacctggtaaag	cacttgtgggtcctccactt
Preproglucagon	gaagacaaacgccactcaca	cggttcctcttggtgttcat
PDX-1	cctttcccgtggatgaaat	acgggtcctcttgttttcct
Nkx6.1	aaacacaccagacccacgtt	tctctctggtcctgccaagt
Adipsin	tcaatcatgaaccggacaac	aatggtgactaccccgtcat
Acrp30/Adiponectin	ccaatgtacccattcgcttt	tgctgccgtcataatgattc

Results

Suppression of mafA mRNA in mouse pancreas by siRNA

An almost 60% reduction in mafA expression in the pancreas was achieved *in vivo* by the intravenous hydrodynamic method of administering siRNA. The suppressive effect of siRNA was assessed by comparison with pancreata from mice injected with control siRNA. The relative expression level of mRNA was determined by using the real-time PCR method to compare the sample of siRNA and control RNA. The maximum suppressive effect of siRNA on the mafA expression level was obtained at the 200 μg concentration (Fig. 1A), and the reduction in expression continued for 72 h. mRNA extracted from the pancreas of mice injected with 200 μg siRNA and removed at 24 h was therefore adopted for analysis of the mRNA

Table 1
Target sequence and designed siRNA sequence

MafA (NM_194350) siRNA	
Target sequence	AGCGGGACCCTGTACAAGGA
Sense oligo	gtttAGTGGGACTTGTACAGGGAACGTGTGCTGTCCGTTCCTTGTACAGGTCCCGCTTTTTT
Antisense oligo	atgcAAAAAAGCGGGACCTGTACCAGGAACGGACAGCACACGTTCCCTGTACAAGTCCCACT
Vector	pcPURmU6icassette
Control siRNA	
Sense oligo	GTTTTTTTTTT
Antisense oligo	ATGCAAAAAAA

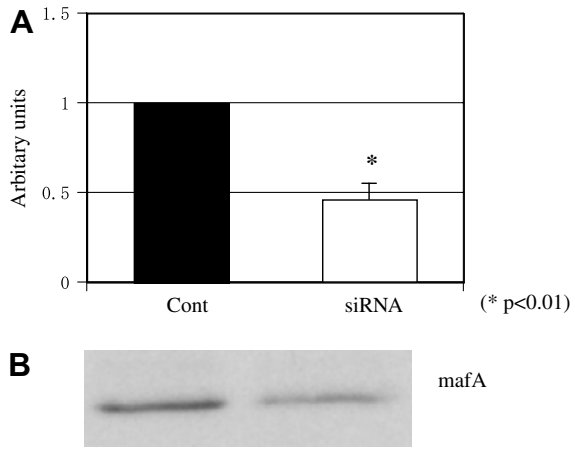

Fig. 1. Maximal effect of siRNA on mafA expression. (A) Real-time PCR and (B) Western blotting demonstrate *in vivo* siRNA function in the pancreas and show decreased mafA mRNA and protein levels.

expression profile by microarray. Western blot analysis was performed to confirm the results of real-time PCR. Fig. 1B shows a representative blot and data demonstrating that the protein expression of mafA was significantly down-regulated by siRNA.

Microarray analysis

The gene expression profiles of the pancreata mafA-siRNA-treated mice were analyzed by the microarray method and compared with those of control-siRNA-treated mice. mRNAs were extracted from the pancreas of both and subjected to microarray analysis (Fig. 2). The genes whose expression level changed by more than 2-fold are listed in Table 3. Nineteen genes were picked up in the list. Expression of 11 genes was up-regulated, and expression of 8 genes was down-regulated. Some genes are unknown origin and sequenced tag, however, of the altered genes, some genes are closely related to the glucose and lipid metabolism.

Confirmation of gene expression by real-time PCR

We performed real-time PCR with specific primers to confirm and observe relative changes in the expression level of the genes listed in Table 3. We also investigated the expression of two members of the large maf family, mafB and c-maf, and transcriptional factors that are closely related to glucose metabolism, including PDX-1 and NKx6.1. As shown in Fig. 3A and B, insulin gene expres-

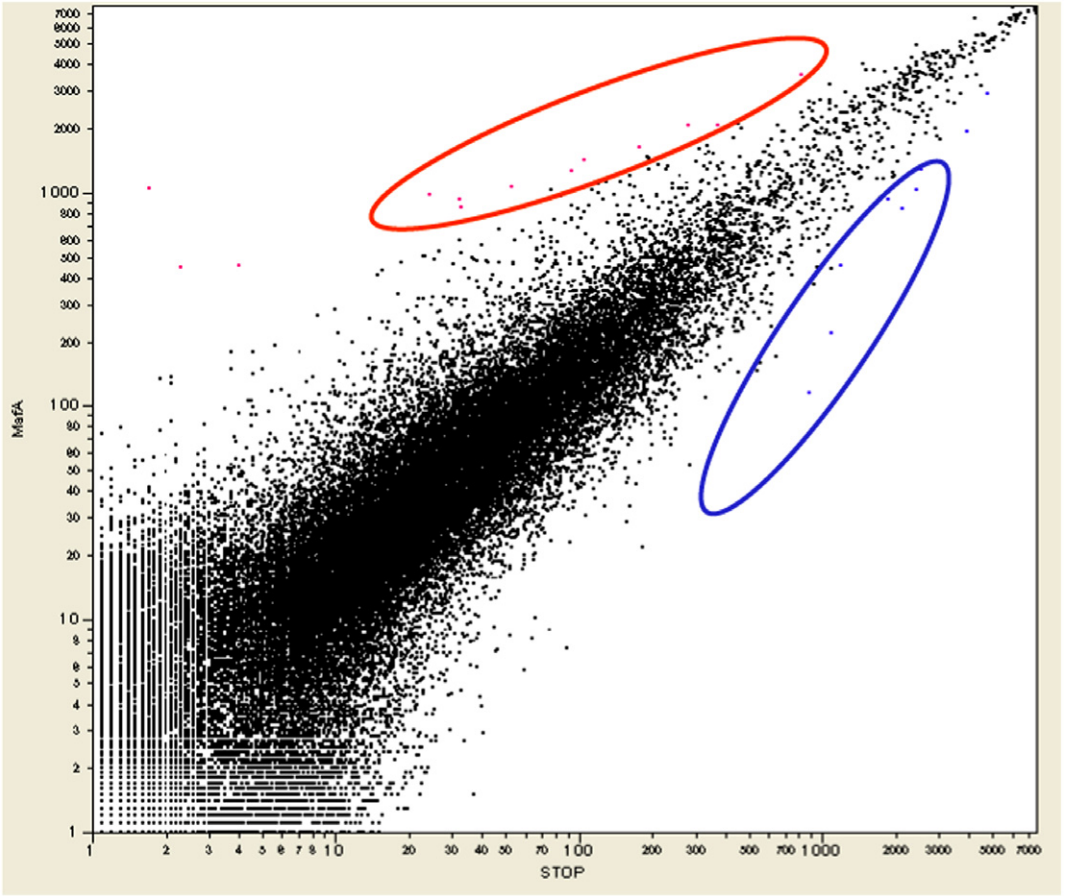

Fig. 2. Comparison between the gene expression profiles of the pancreas of siRNA-treated mice analyzed by microarray, and of the pancreas of control mice. Red circle indicates up-regulated genes and blue circle indicates down-regulated genes.

Table 3
Genes whose expression was altered in the pancreas of siRNA treated mice

Accession No.	Gene name	Expression difference (time folds)
Genes whose expression were increased in the pancreas of siRNA treated mice		
BC003438.1	Iron-regulated transporter	3.2
NM_007655.1	Immunoglobulin-associated alpha (Iga)	2.8
NM_134051.1	AI893585[a]	2.8
BE686578	Membrane-spanning 4-domains	2.8
BB731671	Coagulation factor C	4.2
BM241008	AI447904[b]	3
BB533076	cDNA[c]	2.2
NM_011693.1	Vcam1	3
NM_010442.1	Hmox1	3.8
NM_133245.1	Eraf	4
AK008551.1	cDNA[d]	2.6
Genes whose expression were decreased in the pancreas of siRNA teated mice		
NM_013459.1	Adipsin	0.25
NM_008386.1	Insulin I	0.24
NM_009605.1	Acrp30/Adiponectin	0.24
AF276754.1	Preproglucagon	0.28
NM_017370.1	Haptoglobin	0.32
BI661339	AW493766[e]	0.2
AK007420.1	Phosphatidylserine decarboxylase	0.42
BM505868	cDNA[f]	0.22

[a] A gene similar to IG alpha-1 chain C region (human).
[b] Mouse hematopoietic stem cell cDNA library clone.
[c] Zero day neonate lung cDNA clone.
[d] Adult male small intestine cDNA clone.
[e] NCI_CGAP_Mam4 cDNA clone.
[f] Melton mouse E16 5 pancreas library cDNA clone.

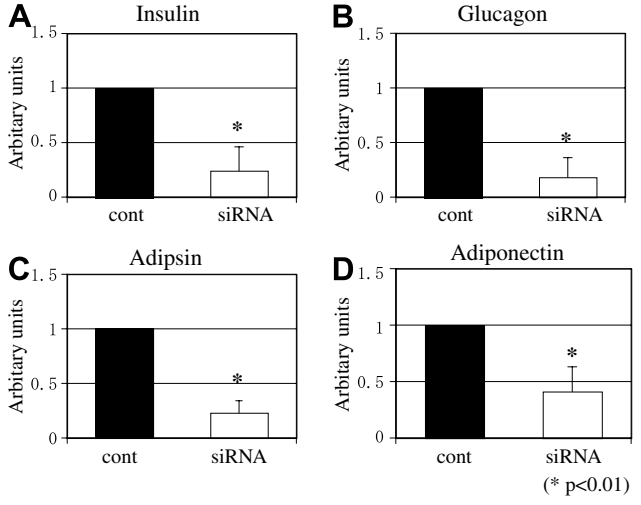

Fig. 3. Real-time PCR of genes in the pancreas of siRNA-treated mice, showing significant decreases in pancreatic mRNA. (A) Insulin mRNA, (B) glucagon mRNA compared with the controls, (C) adipsin mRNA, and (D) adiponectin mRNA compared with the controls.

sion and glucagon gene expression decreased to 30% and 22%, respectively, of control level. Genes derived from adipose tissue were down-regulated in addition to the genes encoding pancreatic endocrine hormones. Expression of adipcin decreased to less than 20% of the control level, and expression of adiponectin decreased to about 40% of the control level (Fig. 3C and D).

To investigate associations between mafA and other related genes, expression of genes for the maf family and transcriptional factors related to the glucose metabolism that were not based on the information in the array system was estimated by real-time PCR. MafB mRNA expression was slightly but not significantly decreased, and c-maf expression tended to increase, but the changes were not

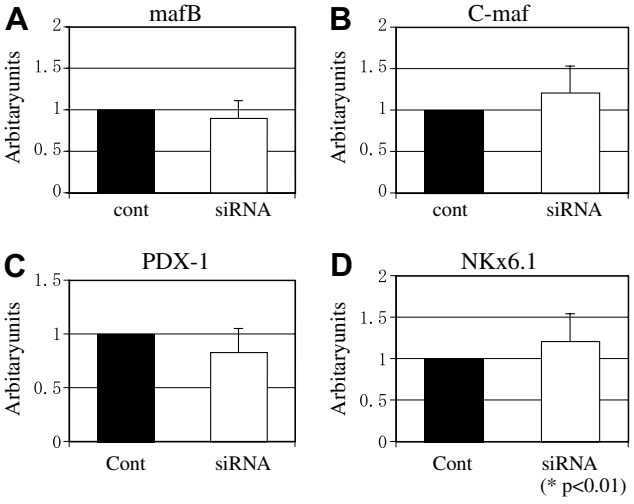

Fig. 4. Real-time PCR of genes in the pancreas of mafA siRNA-treated mice showing no significant change in other mafs, PDX-1 and NKx6.1 mRNA in the pancreas of siRNA-treated mice.

statistically significant. By contrast, expression of representative transcriptional factors, such as PDX-1 or NKx6.1, regulate the differentiations of pancreatic endocrine cells was not significantly affected by the modulation of mafA mRNA expression (Fig. 4).

Alteration of the expression level of several genes was detected in this study, especially the up-regulation of 11 genes. We investigated changes in their expression level by real-time PCR with specific primers and described their Accession No. in Tables 2 and 3. Since these genes seem to be closely related to glucose metabolism and endocrine function, we have not yet investigated their significance, and it remains unclear.

Discussion

This study revealed transcriptional factor, mafA-related gene expression *in vivo* in mouse pancreas by using RNA interference and the hydrodynamic method. We used the method of *in vivo* siRNA injection described in Hamar's report [13]. Although it is unclear how hydrodynamic injection method is effective in producing RNA interference, they hypothesized that parenchymal cells are transduced oligonucleotide by a sudden increase in intravascular volume. There have been several reports of successful suppression of targeted RNA in several organs and cells in *vivo* [16,17]. We used an siRNA-expressing plasmid DNA (pDNA) instead of the oligonucleotide form of siRNA with the aim of achieving longer lasting suppression of mRNA. Kobayashi et al., reported the efficacy of vector-based *in vivo* RNA interference [18] and observed targeted gene suppression in the liver, kidney, lung, and muscle. The reduction in transgene expression became apparent 1 day after the injection.

In our preliminary study siRNA suppressed target mRNA expression in the liver and pancreas. In the pancreas, Bradley et al. reported that their target gene, insulin2, was significantly suppressed by a 100 μg dose of synthetic siRNA *in vivo,* and they observed a reduction in mRNA level and the presence of siRNA in isolated islet cells [19]. In our study the maximal effect of mafA mRNA suppression in the pancreas was observed 24 h after intravenous injection of the 200 μg dose of siRNA. mRNA suppression was confirmed at the protein level by Western blotting (Fig. 1B).

The gene expression profiles of the pancreas of siRNA-treated mice in which mafA mRNA was almost 60% reduced were analyzed with microarrays and compared to those of control mice. mRNAs were extracted from the pancreas of both and subjected to microarray analysis. The genes whose expression level changed more than 2-fold increase or less than one half decrease are listed in Table 3. Interestingly, genes for pancreatic endocrine hormones and adipocyte-related genes were detected among the altered genes.

It is well known that mafA is closely related to pancreatic β cell differentiation and normal function, and several recent reports have revealed a role of mafA in the final differentiation or replication of β cells [11,12]. The observation in this study that mafA interference down regulated insulin gene expression is consistent with these reports. Expression of the mafB and c-maf genes, on the other hand, was not significantly affected by mafA interference. Nishimura et al. suggested that mafB is located upstream of pancreatic endocrine cell differentiation [20]. There may be relationship between mafA and mafB or between mafA and c-maf, however, transient suppression of mafA expression was not accompanied by significant changes in the expression levels of other mafs. Significant changes in the expression levels of other mafs were not observed in this study, but cross talk between mafs, especially mafB and c-maf, may occur. Aziz et al. recently, reported that $mafB^{-/-}$ macrophages expressed increased level of multiple genes, especially c-maf expression in $mafB^{-/-}$ macrophages was significantly increased and $mafB^{-/-}$ macrophages showed intact basic macrophage function. They suggested that mafB's function in macrophage differentiation may be compensated for by other, closely related members of the maf family [21]. MafB redundancy for c-maf and expression compensation may result in no significant changes in the expression levels of each factor.

On the other hand, expression of both the glucagon gene and insulin gene was down regulated despite the absence of any change in mafB expression. A recent study demonstrated that α cells, which secrete glucagon, only express mafB in adult mouse pancreas, whereas mafB has been shown to be expressed in developing α and β cells as well as in proliferating hormone-negative cells during pancreatogenesis [22]. Since the down-regulation of the glucagon gene was not explained by mafB expression in the pancreas of this study, how mafA interference down-regulated glucagon gene expression remains unclear. C-maf or mafB expression may indirectly mediate the down-regulation of glucagon expression or other unknown factors to play a role in the modulation of energy balance and glucose homeostasis, regulating the expression of endocrine genes.

In addition to its effects on β cells, mafA may be involved in adipocyte differentiation and regulation of lipid metabolism through the adipokine network in the pancreas. In the present study mafA interference induced down-regulation of adiponectin and adipsine. Although expression of the adiponectin or adipsin gene has not been reported in the pancreas, adiponectin receptor is expressed in pancreatic islets. There is a possibility that the expression of the adiponectin gene detected in the mRNA from the pancreas originated in intra-pancreatic adipose tissue or other cells. It has recently been reported that adiponectin has insulin-sensitizing, anti-atherogenic, and anti-inflammatory actions but dose not affect insulin secretory function [23]. Adipsin, an adipocytokine, is secreted by adipocytes, and the secretion of adipsin from the cell surface is stimulated by insulin. Adipsin is a transcriptional factor for adipocyte differentiation and also contributes to host defense by functioning as factor D in the alternative path-

way of complement activation. Adipsin and adiponectin are thought to cooperate in promoting adipocyte development. Moreover, although it is unclear how adipocytokines act at the local level, they may be involved in metabolic mechanisms allowing cross talk with distant organs and the coordination of a variety of biological process, including energy metabolism, the inflammatory cascade, and insulin-stimulated secretion. Although the precise mechanism and cross-link have not been clarified in this study, maf-regulated pancreatic endocrine function appears to affect the adipokine network [24,25].

In summary, gene silencing with siRNA administered by the hydrodynamic method was found to be an effective tool for tracing altered gene expression *in vivo* in this study. The gene silencing with siRNA technique used in this paper is an effective tool for physiologically chasing cross talk between cells, tissues or organs, when the cross talk can not otherwise be observed in knockout mice because of the gene defects and the resulting compensation mechanisms at birth. Suppression of mafA mRNA expression in the pancreas *in vivo* induced a decrease in the expression of genes related to endocrine function and it was concluded that mafA may modify the adipokine network in the pancreas.

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