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Title
MafA regulates expression of genes important to islet β cell function

Short title
Different response to MafA overexpression between various endodermal cells

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Abstract

Insulin transcription factor MafA is unique in being exclusively expressed at the secondary and principal phase of insulin-expressing cell production during pancreas organogenesis, and is the only transcriptional activator present exclusively in islet β cells. Here we show that ectopic expression of MafA is sufficient to induce a small amount of endogenous insulin expression in a variety of non-β cell lines. Insulin mRNA and protein expression was induced to a much higher level when MafA was provided with two other key insulin activators, PDX-1 and BETA2. Potentiation by PDX-1 and BETA2 was entirely dependent upon MafA, and MafA binding to the insulin enhancer region was increased by PDX-1 and BETA2. Treatment with activin A and HGF induced even larger amounts of insulin in AR42J pancreatic acinar cells, compared with other non-β endodermal cells. The combination of PDX-1, BETA2, and MafA also induced the expression of other important regulators of islet β cell activity. These results support a critical role of MafA in islet β cell function.
Introduction

Pancreatic islet β-cell-specific expression of the *insulin* gene is principally regulated by the actions of distinct islet-enriched transcription factors on the conserved A3 (-201/-196 bp), C1 (-118/-107 bp), and E1 (-100/-91 bp) elements of the enhancer region, which is found between -340 to -91 base pairs relative to the transcription start site. Thus, the islet β- and δ-cell enriched PDX-1 homeodomain protein (formerly known as IPF-1, STF-1, and IDX-1 (1-3)) controls A3 element activation (3-6), while a heterodimer composed of islet cell enriched BETA2 (7) and the generally distributed HEB (8) or E2A (9-12) basic helix-loop-helix protein stimulates through an E1 element. These transcription factors also control expression of other gene products associated with β cell identity, including glucokinase (13, 14), islet amyloid polypeptide (15-18), and glucose transporter type 2 (19). Significantly, dysfunctional mutations in PDX-1 (20) and BETA2 (21) contribute to the development of diabetes in humans, presumably due in part to reduced expression of target genes required for glucose sensing. In addition, each of these factors plays a critical role in islet cell development during embryogenesis (22). Collectively, the data strongly suggest that PDX-1 and BETA2 are necessary for the formation and maintenance of physiologically functional islet β cells.

In contrast to PDX-1 and BETA2, β cell development does not appear to be affected in mice deficient for the basic leucine-zipper-containing MafA, an activator of *insulin* C1, although adult mutant animals are glucose intolerant due to diminished *insulin* transcription and impaired glucose-stimulated insulin secretion (23). The unimportance of MafA in β cell development is surprising, since this factor is only produced in insulin-producing cells of the second and principal phase of β cell production during embryogenesis in mice (24). Significantly, the expression pattern of MafA is unique, as no other transcription factor is expressed this late in islet cell development or in such a restricted fashion. For example, closely related MafB is detected in both first and second wave insulin+ and glucagon+ (i.e. islet α cell) cells during pancreas organogenesis (25), while PDX-1 (26) and BETA2 (26) are found widely in pancreatic acinar/islet and islet progenitors, respectively. Because both MafA and MafB are co-expressed in developing β cells and capable of activating insulin expression (25, 27), MafB may be compensating for the loss of MafA in null mice. Collectively, these findings indicate that MafA and/or MafB is normally critical to the assembly of the *insulin* transcription unit in developing β cells, a proposal consistent with insulin expression being induced by MafA alone in a stably expressing islet α cell line (i.e. αTC-6 (24))
and suppressed insulin expression accompanied by reduction of endogenous MafA expression under hyperglycemic (28, 29), or reduced insulin signaling (30) conditions.

In this study, we show that adenoviral MafA overexpression is independently capable of activating endogenous insulin mRNA synthesis in several distinct non-β cell lines (αTC6, AR42J, IEC-6). In addition, co-expression of PDX-1 and BETA2 was found to greatly enhance MafA-mediated activation, especially in AR42J cells. The combination of MafA, PDX-1 and BETA2 in AR42J and IEC-6 cells also stimulated the expression of other important β cell regulators (e.g. GLUT2 and PC2), with activation in AR42J cells entirely dependent upon the presence of growth factors critical to islet cell differentiation. Our studies show that a combination of islet enriched transcription factors and signaling mediators is capable of producing high level insulin expressing β-like cells from non-insulin-producing cells in vitro.

Results

MafA induces insulin and other β cell-enriched factors in many non-β cell lines

Stable expression of MafA selectively induced low-level insulin expression in αTC-6 cells (27). To more broadly evaluate the ability of this factor to initiate the insulin transcriptional program, adenoviruses expressing MafA, PDX-1 and BETA2 (Ad-MafA, Ad-PDX-1, and Ad-BETA2, respectively) were generated. Western blotting of nuclear extracts prepared from each adenovirus-infected cells confirmed that the appropriate sized product was produced by each adenovirus, which was approximately 2- to 7-fold higher than that in the MIN6 β cell line (Fig. 1A). The expression level was almost same between the cell lines used (data not shown).

Total RNA was isolated and RT-PCR was performed to first determine if rat insulin 1 and/or 2 mRNA expression is affected by infection with MafA versus control GFP expressing adenovirus (Ad-GFP) (Rodents have two non-allelic insulin genes, which are regulated by similar, but not identical, enhancer control sequences (31-34)). Insulin 2 gene expression was induced by MafA in αTC6 (i.e. islet α like), AR42J (pancreatic acinar), and IEC-6 (intestinal epithelial) cells, but not in HepG2 (hepatic) cells (Fig. 1B). In contrast, insulin 1 expression was not detected clearly under these conditions (Fig. 1D), which may reflect the relatively weak MafA/C1 binding site within this gene (35). The insulin 2 RNA product was also correctly spliced in αTC6, AR42J, and IEC-6 cells, as judged by the size of the PCR product generated with the intron spanning primers and by direct DNA sequencing. These results suggest that MafA is able to induce insulin 2 mRNA
expression in many, but not all, endoderm-derived cell lines, although the produced amount varied considerably among cell lines infected with Ad-MafA.

Ad-MafA, -PDX-1 and -BETA2 were next used in IEC-6 and AR42J cells to examine their effects on the endogenous expression of other gene products associated with β cell identity, specifically glucose transporter 2 (GLUT2), β cell type glucokinase (GK), ATP-sensitive inward rectifier potassium channel (Kir6.2), sulfonylurea receptor 1 (SUR1), prohormone convertase 2 (PC2). An insulin 2, GLUT2, and PC2 mRNA signal was clearly detected upon infection with Ad-MafA alone in IEC-6 cells, but only insulin 2 was activated clearly in AR42J cells (Fig. 1C & D). Significantly, GLUT2 and PC2 expression was induced in AR42J cells when MafA was added in combination with BETA2 and/or PDX-1 (Fig. 1C & D). However, these β cell-enriched products were only induced in AR42J cells in the presence of both activin A and HGF (Fig. 1D), growth factors involved in β cell differentiation (36). Interestingly, insulin 1 expression was both detected in IEC-6 and AR42J cells when MafA was co-expressed with PDX-1 and BETA2 (Fig. 1C & D). Real-time PCR was performed to precisely quantify the induced amount of each β cell product in AR42J and IEC6 cells (Fig. 2). It was found that most of evaluated factors, with the exception of Kir6.2, were increased by the combination of MafA, PDX-1, and BETA2, and that the amount of insulin produced in AR42J cells was comparable to rat insulinoma cells, RINm5F. These results demonstrate that MafA, PDX-1 and BETA2 act together to activate endogenous transcription of a subset of key β cell genes like insulin, GLUT2 and PC2 in a variety of distinct endodermally derived cell lines.

GLUT2 appears to be regulated by MafA in islet β cells

To determine if MafA regulates GLUT2 and PC2 transcription, MIN6 β cells were infected with adenoviruses producing small interference RNAs to MafA (i.e. +66/+84 and +82/+102 bp (37)). As expected, the MafA level was profoundly reduced upon infection with both the +66/+84 and +82/+102 viruses (i.e. 30.4% of control Ad-GFP), and it was lesser effected with either +66/+84 (65.9%) or +82/+102 (37.8%) alone (Fig. 3A). GLUT2 mRNA was reduced upon reduction of MafA (Fig. 3B), while PC2 expression was unaffected (Fig. 3C). As expected, insulin 1 and 2 were also significantly suppressed by the MafA +66/+84 and +82/+102 siRNAs (37) (Fig. 3D and E). These results indicate that MafA is a bona fide regulator of insulin and GLUT2 transcription in β cells.

PDX-1 and BETA2 potentiate MafA binding to the insulin enhancer region

In IEC6 and AR42J cells, the presence of PDX-1 and BETA2 enhanced MafA-mediated activation of -238 Luc, a
rat insulin 2-luciferase reporter activity driven by enhancer/promoter sequences spanning -238 to +2bp (Fig. 4A & B). However, activation of transiently transfected -238 LUC was independent of activin A and HGF treatment (Fig. 4B), in contrast to transcription of the chromosomal insulin genes (Fig. 1D & see below). Chromatin immunoprecipitation analysis was next performed over the enhancer region with MafA and PDX-1 antibodies in AR42J cells, to examine factor binding within the insulin control region. The level of precipitated DNA products was determined by real-time PCR analysis; western blotting showed that a similar amount of PDX-1 and MafA protein was observed between samples (data not shown). The insulin enhancer region was selectively pulled down in Ad-MafA-infected cells (Fig. 4C), although the amount of α-MafA precipitation was enhanced by PDX-1 and BETA2, with PDX-1 being most effective. In contrast, the insulin enhancer signal detected upon α-PDX-1 precipitation was insensitive to MafA and/or BETA2 (Fig. 4D). These results suggest that PDX-1 and BETA2 potentiate MafA binding to the insulin enhancer region. Strikingly, PDX-1 and MafA binding occurs in the absence (or the presence of low level) of insulin transcription, as concluded from the very similar binding pattern observed with and without activin A and HGF treatment (Fig. 1D & 4C). Collectively, these results imply that activin A and HGF impact insulin transcription by recruiting factors important to MafA, PDX-1, and/or BETA2 activation on the chromatin.

MafA, PDX-1, and BETA2 act together to induce insulin expression in AR42J and IEC-6 cells.

The level of insulin protein produced by the combined actions of MafA, PDX-1, and BETA2 paralleled insulin mRNA expression in IEC-6 and AR42J cells. Hence, the highest insulin mRNA levels were observed in the presence of all three activators in HGF and activin A-treated AR42J cells (Fig. 2A & B, supplemental Fig. 1A & B). Insulin protein levels analyzed by ELISA were also most effectively produced in AR42J cells (149.4 mg/g protein), with roughly 15-fold increase over untreated cells (Table 1). Insulin was clearly detected in the cytoplasm of Ad-MafA, Ad-PDX-1 and Ad-BETA2-treated cells (Fig. 5), although insulin secretion was not enhanced by glucose (AR42J, 49.4/52.6 pg/ml/10^5 cells in 18/1.5 mM glucose medium; MIN6, 2571.3/662.7).

Discussion

PDX-1 and BETA2 play a principal role in both islet cell development and adult islet function in vivo. The importance of PDX-1 (20) and BETA2 (21) in humans was also established upon demonstrating their mutational association with type 2 diabetes. In contrast, the recently
isolated MafA transcription factor appears to principally be involved in adult islet β cell activity (23). Cell-line based experiments performed with insulin-driven reporter constructs strongly suggest that high level transcription in β cells requires the coordinated actions of MafA, BETA2, and PDX-1 (37, 38). Here we show that MafA, together with BETA2 and PDX-1, provided β-like insulin expression properties to IEC-6 and AR42J cell lines.

The capability of adenoviral MafA expression alone to induce endogenous insulin expression was initially compared among a variety of endoderm-derived cell lines: IEC-6, AR42J, αTC6, and HepG2. Low-level insulin 2 expression was observed in all but HepG2 cells (Fig. 1B, supplemental Fig. 2). MafA induces insulin independently, as overexpression did not induce detectable level of PDX-1 or BETA2 by western blotting (data not shown). The ability of MafA to activate only insulin 2, but not insulin 1, was also observed in stably MafA-expressing αTC-6 cells (24). Combination of MafA, PDX-1 and BETA2 caused notably higher insulin expression than either individual factor in IEC-6 and AR42J cells, with mRNA and protein expressed at a significant fraction of rat islets and RINm5F β cells (Fig. 2 & 5). Only MafA and PDX-1 or BETA2 were necessary in AR42J cells to detect significant amount of insulin (Fig. 1D), while all were necessary in IEC6 (Fig. 1C). Although it is unclear why activation differed between such closely-related cell lines, we assume that this is due to difference in expression of various transcriptional regulators which positively or negatively modulate insulin gene transcription.

Activation of endogenous insulin by MafA was entirely dependent upon the presence of two key β-cell differentiation factors, activin A and HGF (Fig. 1D). These agents had been appreciated for their ability to induce low-level insulin production in AR42J cells (39, 40), as does the betacellulin differentiation factor in IEC6 cells (41, 42). (Betacellulin does not influence MafA-mediated activation of insulin in IEC6 cells (data not shown)). In striking contrast to the effect of activin A and HGF on endogenous insulin, activation of the transfected insulin-driven -238 LUC reporter by MafA, PDX-1, and BETA2 was not impacted by such factors (Fig. 3B). Similarly, binding to the endogenous insulin enhancer region in chromatin immunoprecipitation assays was also insensitive to the presence of these differentiation agents in AR42J cells. In addition, the expression level of p300/CBP, a co-activator for assembly of MafA, PDX-1 and BETA2, was also unchanged by activin A and HGF (data not shown). Thus, it remains unclear why activin A and HGF only impact expression of the endogenous insulin gene, but we assume that activin A and HGF are involved in promoting the assembly of the chromatin remodeling machinery by recruiting other activating factor(s).

MafA appears to mediate
expression of other key islet β cell genes, like GLUT2 and PC2 (Fig. 1C & D). In addition, knock-down experiments also suggested that MafA directly regulates GLUT2 gene expression, although this may not be the case for the PC2 gene (Fig. 3). The importance of MafA in GLUT2 expression was also indicated in MafA−/− mice (23). Taken together, our findings clearly illustrate the central role that MafA plays in promoting insulin expression and the significance of functional interactions between other key islet regulators in regulating gene expression in β cells.

**Materials and Methods**

**Cell culture and preparation of nuclear extract**

The IEC-6 (Riken Gene Bank (Tsukuba, Japan)) and HeLa cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM), and HepG2 cells in minimum essential medium (MEM) both of which were supplemented with 10% (vol/vol) heat-inactivated fetal calf serum and 1% penicillin-streptomycin. Monolayer pancreatic cell line cultures (β: RINm5F (43), MIN6 (44), α: aTC6 (45), and acinar: AR42J B-13 (39)) were grown as described previously. In some experiments, 2 nM betacellulin (a gift from Dr. Reiko Sasada, Takeda Pharmaceutical, Tsukuba, Japan) or 2 nM human activin A (R&D systems, Minneapolis, MN) plus 100 pM hepatocyte growth factor (HGF; R&D systems) were added to the medium. Human islets were provided by the Juvenile Diabetes Research Foundation Distribution Program at Washington University and were cultured in CMRL medium (GIBCO BRL, Gaithersburg, MD) with 10% heat-inactivated fetal calf serum. Cell line nuclear extracts were prepared by the procedure described by Schreiber et al (46), except that 1 mM phenylmethylsulfonyl fluoride was included in the high salt nuclear resuspension buffer.

**Preparation of expression plasmids and reporter analysis**

The -238 insulin firefly luciferase (Luc) expression plasmids contain rat insulin 2 gene sequence from -238 to +2 bp (47). The construction of the cytomegalovirus (CMV) enhancer-driven MafA expression vector (MafA/pcDNA3.1) was described previously (27), while PDX-1/pcDNA3.1 and BETA2/pcDNA3.1 were constructed by subcloning the coding sequences from mouse PDX-1 and hamster BETA2 into pcDNA3.1 (Invitrogen, San Diego, CA). Insulin -238 LUC (0.25 µg) was transfected into cells with MafA/pcDNA3.1 (0.25 µg), PDX-1/pcDNA3.1 (0.25 µg), and/or BETA2/pcDNA3.1 (0.25 µg) using the lipofectamine procedure (Invitrogen, San Diego, CA), with the cotransfected tyrosine kinase promoter-driven renilla luciferase expression plasmid (phRL-TK, 20 ng; Promega Madison, WI) serving as an internal control.
Preparation of adenoviruses

Recombinant adenoviruses expressing MafA, PDX-1, or BETA2 were prepared using the AdEasy system (kindly provided by Dr. Bert Vogelstein, Johns Hopkins Oncology Center) (48), with the pAdTrack-CMV shuttle vector used in cloning. The adenoviruses expressing small interfering RNAs (siRNA) were driven by the RNA polymerase III H1 gene promoter, and constructed using the following oligonucleotides: Ad-siMafA +66/+84 (37) (mafA sequences are underlined), 5'-CGCGTGCAGACTCCGAATTTGAGAGGAATTCGCTTCAAGGGTCGAAGTCGTTTTTTGGAAA-3' and 5'-AGCTTTTCAAAAAACGACTTCGACCTGATGAAGGGAATTCGCTTCA AGGGTCGAAGTCGTTTTTTGGAAA-3'; Ad-siMafA +82/+102 5'-CGCGTCCAAGTTCGAGGTGAAGAACGAGTTCTCTTCACCTCGAACTT TTTTTTTGGAAA-3' and 5'-AGCTTTTCAAAAAACGACTTCGACCTGATGAAGGGAATTCGCTTCA AGGGTCGAAGTCGTTTTTTGGAAA-3'; Ad-siScramble 5'-CGCGTGCGCGCTTTGTAGGATTCGGGAATTCGCGAACAAAGCGCGCTTTTTTTGGAAA-3' and 5'-AGCTTTTCAAAAAACGACTTCGACCTGATGAAGGGAATTCGCTTCA AGGGTCGAAGTCGTTTTTTGGAAA-3'. These oligonucleotides were inserted in the MluI/HindIII sites of the pRNAT-H1.1/Adeno shuttle plasmid (GenScript, Piscataway, NJ). Adenovirus titer was roughly 10^10 plaque forming units (PFU)/ml after treatment with the Adeno-X™ Virus Purification kit (Clontech), as estimated using the Adeno-X™ Titer kit (Clontech).

RNA isolation and RT-PCR analysis

Total cellular RNA was isolated using the TRIZOL Reagent (Invitrogen, San Diego, CA). Two micrograms of total RNA were reverse-transcribed at 42 °C for 60 min with 0.5 µg of oligo(dT)15 primer using the SuperScript™II system (Invitrogen, San Diego, CA). The PCR reactions were run for 30 cycles with 25 pmol of primer under the following conditions: 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The sequences of the primer sets were as follows: mouse insulin 2 (Numbering relative to ATG, Forward -57 AGCCCTAAGTGATCCGCTACAA, Reverse +331 AGTTGCACTAGTTCTCCAGCTG, 388 bp product), rat insulin 2 (-57 AGCCCTAAGTGACCAGCTACAG, +198 CAGTTGTGCCACTTGTTGGGT, 255 bp), human insulin (-7 TTCTGCCATGCCCCTGTTGGAT, +331 AGTTGCACTAGTTCTCCAGCTG, 338 bp), rat insulin 1 (-50 AGTGACCAGCTAATCATAG, +252 AACTCTCAGTGGCAAGGTCT, 302 bp), rat GLUT2 (+56 TGGGTTCCTTCAGTTGGG, +238 AGCGTCTGGTGGTGGTAT, 183 bp), rat
glucokinase (+9
TGACAGAGCGAGTGGAG, +307
TCTTCACGCTCCACTGCC, 299 bp), rat
Kir6.2 (+717
CATGGGAAGGCTGTTGGG, +915
CAGATAGGAGGTGCGGGC, 199 bp), rat
SUR1 (+3904
CCAGACCAAGGGAAGATCCA, +4170
GTCCTGTAGGATGATAGCA, 267 bp), rat
PC2 (+1629
CTCCAAGGGGGCTTGTGAC, +2053
GCAAGCAAAGCTTCAGACCA, 425 bp),
β-actin (+53
AGGCCGGCTTCGCGGGCA, +302
CTTCCTCAGGGCCACACG, 250 bp).
The products were resolved on a 1.5% agarose gel run in TAE buffer, and visualized by ethidium bromide staining. The correctness of the amplified products was determined by diagnostic restriction-enzyme digestion and DNA sequencing.

Real-time PCR analysis

One microliter of the reverse-transcribed products obtained as described above was used in a 25-µl reaction mixture including 1x Applied Biosystems SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 4 µl of 2.5 µM primer for mouse insulin 1 (Numbering relative to ATG, Forward -47
GACCGCTATAATCCAGAGCC, Reverse +331
AGTTGCAGTAGTTCTCCAGCTG, 378 bp), mouse GLUT2 (+557
CCCTGGGTACTCTTCACCAA, +666
GCAAGTAGGATGTGCAAT, 110 bp), mouse PC2 (+1646
ACAAGTGCCCCATTCAGACC, +1773
GTAAGCATGGGTTCTTCATTTm 128 bp), mouse β-actin (+778
GCTCTTTTCCAGCTTCTCCTT, +945
CTTCTGCATCCTGTGCAGCAA; 168 bp), or human β-actin (+813
CTGTGGCATCCAGAAACTA, +1012
AGTACTTGCGCTCAGGAGGA, 200 bp). Other primer sets for real-time PCR analysis were described in other part. The initial cycling conditions involved a hold at 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, then 60°C for 60 s. The signal fluorescence magnitude was detected with an ABI PRISM 7700 Sequence Detector. The data are normalized to the β-actin signal and presented as mean amount ± standard deviation (SD) relative to the control adenovirus (Ad-GFP) infection.

Chromatin immunoprecipitation analysis

Control and HGF + activin A-treated AR42J cells (~10^8 cells/point) were formaldehyde cross-linked three days after adenovirus infection and the sonicated chromatin-DNA complexes isolated as described previously (49). αMafA (10 µg; Bethyl Laboratories) or αPDX-1 antibodies were added to the sonicated chromatin and the antibody-protein-DNA complexes isolated with A/G-agarose (Santa Cruz Biotechnology, Inc.). PCR was performed
on one-twentieth of the purified immunoprecipitated DNA using 25 pmol of each primer, with the rat insulin 2 primers detecting binding to both the rat 1 and 2 genes (-263 GAGACAATGTCCCCTGCTGT-3', -53 CCCCTGGGACTTTGCTGTTTG-3'). PCR cycling parameters were 1 cycle of 95°C (3 min) and 30 cycles of 95°C (20 sec), 60°C (20 sec), and 72°C (20 sec). Amplified products were electrophoresed through a 1.5% agarose gel in TAE buffer and visualized by ethidium bromide staining. The same primer sets were used to quantify by RT-PCR the amount of DNA precipitated by αMafA.

Northern blotting analysis

Total RNA (10 µg) was isolated from RINm5F and adenovirus-infected IEC-6 and AR42J cells and separated under denaturing conditions on 1% agarose gels. The transferred RNA was hybridized with a 32P-labeled mouse insulin cDNA probe isolated from the pMIN2C plasmid (50), which was a kind gift of Drs. J.M. Chirgwin (University of Texas Health Science Center, San Antonio, TX) and M.A. Permutt (Washington University School of Medicine, St. Louis, MO).

Western immunoblot analysis

MIN6 and IEC-6 nuclear protein was fractionated on 10% SDS-PAGE, transferred to nitrocellulose, and probed with αMafA (1:2,500 dilution), αPDX-1 (1:5,000) (51), and αBETA2 (1:2,000) (#N-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antiserum. Antibody binding was detected using horseradish peroxidase coupled to goat α-rabbit or rabbit α-goat IgG (1:5,000 dilution), with the complex visualized by incubation with the Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA)

ELISA for insulin

Whole cell extracts were obtained from adenovirus-infected AR42J, IEC6 or HepG2 cells by treating for 24 h at 4°C in acid-ethanol. The insulin content of the extract was determined with the Insulin ELISA Kit/Rat Ultra Sensitive using a rodent insulin standard (Morinaga Biochemicals, Yokohama, Japan), and the rat insulinoma RINm5F cell line was used as a positive control. Insulin concentration was normalized with total cellular protein, as measured using the Bio-Rad protein assay kit (Bio Rad Laboratories, Richmond, CA).

Immunocytochemistry analysis

AR42J cells were fixed on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) with 4% paraformaldehyde. Insulin staining was
performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The slides were incubated with ABC reagent for 30 min, and positive guinea-pig anti-insulin antibody (1:2,000; DAKO, Glostrup, Denmark). Its reactivity was visualized with the 3,3’-diaminobenzidine tetrahydrochloride substrate (Zymed Laboratories, San Francisco, CA).

**Statistical analysis**

Data are expressed as means ± SD. Statistical analysis was performed using the one-way ANOVA followed by Scheffe’s test. A value of \( p < 0.05 \) was considered to be statistically significant.

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Figure 1. Adenoviral MafA expression induces β cell products in a variety of non-β cell lines

A) IEC6 cells were infected with Ad-MafA, Ad-PDX-1, Ad-BETA2, or control Ad-GFP for 48 hours. Nuclear protein was then isolated and western blotting performed with α-MafA, α-PDX-1 or α-BETA2 antiserum. The same amount of MIN6 nuclear extract was used as a positive control. The relative amount of each factor to MIN6 cells is represented below the panel. B) Total RNA was isolated three days after Ad-MafA (MafA) or Ad-GFP (-) infection. Activin A and HGF were added in the medium for AR42J. RT-PCR was performed using mouse insulin 2 (lane 1 to 3), rat insulin 2 (lane 4 to 8) and human insulin specific primer sets (lane 9 to 11). The signals from mouse islet (i.e. for αTC-6), rat islet (AR42J, IEC), and human islet (HepG2) serve as species positive controls. Total RNA from C) IEC6 or D) AR42J cells was isolated three days after infection of Ad-MafA, PDX-1 and/or BETA2, and RT-PCR analysis performed to examine expression of the specified genes. Note that an RT-PCR signal was not detected without the addition of reverse transcription or RNA. The RINm5F signal served as the positive control. The number of PCR cycle was 30 unless indicated as 25 cycle which was decreased cycle to semi-quantitate the amount of mRNA.

Figure 2. Quantification of induced β cell products in non-β cell lines by MafA, PDX-1 and BETA2 overexpression

Using the prepared RNA in the same way as Figure 1 with or without activin A and HGF, real-time PCR analysis was performed to precisely quantify and compare the amount of mRNA of A) insulin 1, B) insulin 2, C) GLUT2, D) glucokinase, E) Kir6.2, F) SUR1, G) PC2 and control β-actin in AR42J, IEC6 and positive control cells such as rat islet and RINm5F cells. Each mRNA level was normalized with β-actin level and are presented as relative amounts ± SD after Ad-GFP-infected control IEC6 being arbitrarily set at 1.0 (n = 4).

Figure 3. Suppression of MafA reduces GLUT2 and insulin expression

A) MIN6 nuclear extracts were prepared after Ad-siMafA, Ad-GFP or Ad-scramble infection and western blotted with MafA antibody. Relative amount of MafA protein is indicated as means ± SD, with the amount after treatment with Ad-GFP being arbitrarily set at 100% (n = 4). Real-time RT-PCR analysis was performed to detect B) GLUT2, C) PC2, D) insulin 1, E) insulin 2, and control β-actin levels using total RNA. GLUT2, PC2, insulin 1 and insulin 2 mRNA level were normalized with β-actin level and are presented as relative amounts ± SD with the ratio of each mRNA level after Ad-GFP treatment being arbitrarily set at 1.0 (n = 4). *, p < 0.05, **, p < 0.01
versus control Ad-GFP.

**Figure 4. PDX-1 and BETA2 enhance MafA activity through their binding to the insulin enhancer region in AR42J cells.**

The rat insulin 2 (-238 to +2 bp)-driven firefly luciferase expression plasmid (-238 Insulin Luc) was transiently co-transfected with MafA, PDX-1 and/or BETA2 expression plasmids into A) IEC6 or B) AR42J cells. The firefly luciferase activity from -238 Insulin Luc was normalized with the co-transfected phRL-TK renilla luciferase signal. The relative -238 Insulin Luc activity was calculated as the ratio of Luc activity after MafA, PDX-1 and/or BETA2/pcDNA3.1 transfection to that after control pcDNA3.1 transfection. Experiments were performed at least four times and the relative values are expressed as means ± standard deviation (SD).

The cross-linked DNA from Ad-GFP, Ad-MafA, Ad-PDX-1, and/or Ad-BETA2 infected AR42J cells was sonicated and the DNA-protein complexes were immunoprecipitated with (C) α-MafA or (D) α-PDX. Top panel: The total (lane 1) and precipitated DNA (lanes 2 to 11) was analyzed by PCR using rat insulin (-263/-53 bp) enhancer region primers. The no DNA template control is also shown (lane 12). Middle panel: real-time PCR was performed to determine the relative amount of MafA binding to the insulin enhancer region under each condition. The results are presented as relative values to that after Ad-GFP treatment without activin A nor HGF. Data are presented as means ± SD of four independent experiments. Lower panel: The similar size of the sonicated DNA products from each sample is illustrated. The immunoprecipitations were preformed with the same amount of sonicated DNA.

**Figure 5. Combination of MafA, PDX-1 and BETA2 induces large amounts of insulin protein in IEC6 and AR42J cells**

Insulin immunostaining was performed with AR42J cells infected with Ad-GFP, Ad-MafA, Ad-PDX-1 or/and Ad-BETA2. RINm5F cells were used as a positive control.
AR42J, IEC6 or HepG2 cells were infected with Ad-MafA, Ad-PDX-1, and/or Ad-BETA2 with or without 2 nM activin A and 100 pM HGF. Insulin protein level was measured after 72 hr incubation, with rat islets and RINm5F cells serving as a positive control. The normalized values ± SD were calculated from four independent experiments. N.D., not detectable.

Table 1. MafA, PDX-1 and BETA2 induce substantial amounts of insulin protein in AR42J cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Growth Factor</th>
<th>Insulin Content (mg/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat islet</td>
<td>-</td>
<td>27153.11 ± 445.71</td>
</tr>
<tr>
<td>RINm5F</td>
<td>-</td>
<td>3645.20 ± 0.51</td>
</tr>
<tr>
<td>AR42J</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>AR42J (MafA)</td>
<td>Activin A + HGF</td>
<td>N.D.</td>
</tr>
<tr>
<td>AR42J (PDX-1)</td>
<td>Activin A + HGF</td>
<td>N.D.</td>
</tr>
<tr>
<td>AR42J (BETA2)</td>
<td>Activin A + HGF</td>
<td>N.D.</td>
</tr>
<tr>
<td>AR42J (MafA + PDX-1)</td>
<td>Activin A + HGF</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>AR42J (MafA + BETA2)</td>
<td>Activin A + HGF</td>
<td>126.07 ± 4.21</td>
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<tr>
<td>AR42J (PDX-1 + BETA2)</td>
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<td>N.D.</td>
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<td>AR42J (MafA + PDX-1 + BETA2)</td>
<td>Activin A + HGF</td>
<td>143.39 ± 4.05</td>
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<tr>
<td>AR42J (MafA + PDX-1 + BETA2)</td>
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<td>10.23 ± 0.52</td>
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<td>IEC6 (MafA + PDX-1 + BETA2)</td>
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<td>0.20 ± 0.03</td>
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<tr>
<td>HepG2 (MafA + PDX-1 + BETA2)</td>
<td>Activin A + HGF</td>
<td>N.D.</td>
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</table>
Fig. 1B

- mouse islet
- αTC6
- rat islet
- AR42J
- IEC6

(-) MafA

Insulin 2
1 2 3 4 5 6 7 8

- human Islet
- HepG2

(-) MafA

Insulin
9 10 11
Fig. 1C

<table>
<thead>
<tr>
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<tr>
<td>MafA</td>
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<tr>
<td>RINm5F</td>
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<td>dH2O</td>
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- Insulin 1
- Insulin 2
- Insulin 2 (25 cycle)
- GLUT2
- Glucokinase
- Kir 6.2
- SUR1
- PC2
- β-actin
Fig. 1D

<table>
<thead>
<tr>
<th>Gene</th>
<th>AR42J (+Activin &amp; HGF)</th>
<th>AR42J (-)</th>
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<tbody>
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<td>MafA</td>
<td>- + - - + + - +</td>
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<td>PDX-1</td>
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<td>Insulin 1</td>
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<tr>
<td>Insulin 1 (25 cycle)</td>
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<td>Insulin 2</td>
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<td>Insulin 2 (25 cycle)</td>
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<td>Kir 6.2</td>
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<tr>
<td>β-actin</td>
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</table>
Fig. 2A

Rat insulin 1

- - - - + + + + - - + + + +
- - - - - - + + - - - - - + +
- - - - - - + + - - - - - + +
- - - - + - + - + - + - + - +

MafA
PDX-1
BETA2
Activin A + HGF

RINm5F rat islet AR42J IEC6
Fig. 2B

Rat insulin 2

MafA
PDX-1
BETA2
Activin A + HGF

RINm5F
rat islet
AR42J
IEC6
Fig. 2C

Rat GLUT2

<table>
<thead>
<tr>
<th>Condition</th>
<th>RINm5F</th>
<th>rat islet</th>
<th>AR42j</th>
<th>IEC6</th>
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<tr>
<td>MafA</td>
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<tr>
<td>PDX-1</td>
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<td>BETA2</td>
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<tr>
<td>Activin A + HGF</td>
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</table>
Fig. 2D

Rat glucokinase

MafA:
- - - - + + + + - - + + + +

PDX-1:
- - - - - - + + - - - - + +

BETA2:
- - - - - - - + - - - - + +

Activin A + HGF:
- - - - + - + + - + - + +

RINm5F:
rat islet

AR42J

IEC6
Fig. 2E

Rat Kir6.2

- - - - - + + + + - - + + + +

MafA

- - - - - - - + + + + - - - - + +

PDX-1

- - - - - - + + + - - - - - + +

BETA2

- - - - - - - + + - - - - - + +

Activin A + HGF

- - - + - + - + - + - + - + +

RINm5F

rat islet

AR42J

IEC6
Fig. 2F

Rat Sur1

MafA
- - - - + + + + - - + + + + +
PDX-1
- - - - - - + + - - - - + + + +
BETA2
- - - - - - + + - - - - + + + +
Activin A + HGF
- - - + - + - + - + - + - + +

RINm5F  Rat islet  AR42J  IEC6
Fig. 2G

Rat PC2

MafA: - - - - + + + + - - + + + +
PDX-1: - - - - - - + + + - - - + +
BETA2: - - - - - - + + - - - - + +
Activin A + HGF: - - - + - + - + - + - + - +

RINm5F rat islet AR42J IEC6
Fig. 3

A) MafA

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<tr>
<th>Treatment</th>
<th>Relative Amount</th>
<th>Standard Deviation</th>
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<tr>
<td>Ad-GFP</td>
<td>100</td>
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<tr>
<td>Ad-scramble</td>
<td>95.2 ± 7.0</td>
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<tr>
<td>Ad-siMafA +66/+84</td>
<td>65.9 ± 7.9</td>
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<td>Ad-siMafA +82/+102</td>
<td>37.8 ± 5.0</td>
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<tr>
<td>Ad-siMafA +66/+84 &amp; +82/+102</td>
<td>30.4 ± 3.1</td>
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</table>

B) GLUT2

- Ad-GFP: 1.0 ± 0.1
- Ad-scramble: 1.1 ± 0.2
- Ad-siMafA +66/+84: 0.7 ± 0.1
- Ad-siMafA +82/+102: 0.6 ± 0.1
- Ad-siMafA +66/+84 & +82/+102: 0.8 ± 0.1

C) PC2

- Ad-GFP: 1.0 ± 0.1
- Ad-scramble: 1.1 ± 0.2
- Ad-siMafA +66/+84: 0.9 ± 0.1
- Ad-siMafA +82/+102: 0.8 ± 0.1
- Ad-siMafA +66/+84 & +82/+102: 0.7 ± 0.1

* p < 0.05
** p < 0.01
Fig. 3

D) Insulin 1

E) Insulin 2
Fig. 4

A) Relative Activity

- MafA
- PDX-1
- BETA2

B) Relative Activity

- Activin & HGF (+)
- Activin & HGF (-)
**Fig. 4C**

**α-MafA**

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</table>

**Insulin enhancer region**

**Activin & HGF (-)**

**Activin & HGF (+)**

**Real-time PCR**

**Sonicated DNA**

(before precipitation)

**Marker**
Fig. 4D

**α-PDX-1**

<table>
<thead>
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<th>PDX-1</th>
<th>PDX-1 + MaA</th>
<th>PDX-1 + MaA + BETA2</th>
<th>GFP</th>
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Insulin enhancer region

Real-time PCR

Fold Association

Sonicated DNA (before precipitation)

Marker
Fig. 5

AR42J

Ad-GFP  Ad-MafA  Ad-PDX-1  Ad-BETA2

Ad-MafA  Ad-PDX-1  Ad-BETA2

RINm5F