Demethylation of the MafB promoter in a compromised β-cell model

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Abstract

Recent studies suggest that dedifferentiation of pancreatic β-cells is involved in compromised β-cell function in diabetes mellitus. We have previously shown that the promoter activity of MafB, which is expressed in α-cells of adult islets and immature β-cells in embryonic pancreas but not in mature β-cells in mice, is increased in compromised β-cells of diabetic model mice. Here, we investigated a rat β-cell line of INS1 cells with late-passage numbers, which showed extremely low expression of MafA and insulin, as an in vitro model of compromised β-cells. In these INS1 cells, the mRNA expression and the promoter activity of MafB were upregulated compared with the early-passage (‘conventional’) INS1 cells. Analysis of the MafB promoter in these late-passage INS1 cells revealed that specific CpG sites in the MafB promoter were partially demethylated. The reporter assay revealed that the unmethylated promoter activity of the 373 bp region containing these CpG sites was higher than the in vitro methylated promoter activity. These results suggest that the chronic culture of the rat β-cell line resulted in partial DNA demethylation of the MafB promoter, which may have a role in MafB promoter activation and possible dedifferentiation in our compromised β-cell model.

Key Words
- MafB
- β-cells
- dedifferentiation
- diabetes mellitus
- demethylation

Introduction

Diabetes mellitus results from dysfunction of pancreatic β-cells, although the mechanism of impaired secretion of insulin from β-cells is not fully understood. Recent studies suggest that a subpopulation of β-cells in diabetic humans and mice undergo dedifferentiation rather than apoptosis. Upregulation of genes that are normally repressed in mature β-cells, including the transcription factors transiently expressed in endocrine progenitors of the embryonic pancreas, was identified in these compromised β-cells as a hallmark of dedifferentiation (Thorrez et al. 2011, Nishimura et al. 2015) and may result in the impaired insulin release in diabetes mellitus (Thorrez et al. 2011, Pullen & Rutter 2013, Nishimura et al. 2015). However, the mechanism of the β-cell dedifferentiation remains unknown.

MafB, a b-Zip family transcription factor, is expressed in pancreatic α-cells. MafB is also expressed in immature β-cells in embryonic pancreas, but not in mature β-cells in mice (Nishimura et al. 2006, Artner et al. 2010). Recent studies revealed that MafB is upregulated in the islets of MafA knockout (KO) mice, β-cell-specific FoxO1 KO mice with metabolic stress and conditional Pdx1 KO mice as a
hallmark of dedifferentiation (Talchai et al. 2012, Gao et al. 2014, Nishimura et al. 2015). We also reported that the promoter activity of MafB is increased in β-cells of diabetes model mice such as db/db mice and mice that have received low-dose streptozotocin (Nishimura et al. 2015). Because MafB induces genome-wide changes in DNA methylation (Vicente-Dueñas et al. 2012), we speculated the MafB may be a key molecule for the upregulation of β-cell disallowed genes in dedifferentiated β-cells. Thus, we investigated the activity and methylation status of the MafB promoter using an in vitro model of compromised β-cells.

Materials and methods

Cell culture

The rat β-cell line of INS1 cells (Asfari et al. 1992) of the indicated passage (p) numbers were cultured in RPMI-1640 medium (Sigma) supplemented with 10% (w/v) fetal bovine serum (FBS; Thermo Scientific, Rockford, IL, USA), 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, and 55 μM β-mercaptoethanol (Life Technologies) as described previously (Nishimura et al. 2013). HeLa cells (Scherer et al. 1953) were cultured in the DMEM (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. All cell lines were cultured at 37°C under 5% CO2.

Construction of reporter luciferase vectors

The MafB luciferase reporter constructs K6500/LUC were kindly provided by Dr Masaharu Sakai (Huang et al. 2000). The pCpGfree vector (InvivoGen, San Diego, CA, USA) was digested with SpeI and HindIII at the multi-cloning site (MCS), and the promoters for rat MafB (UR6 region), rat Ins2, rat Glut2, and rat Srp2 were cloned using the PCR products generated with the primers listed in Table 1 and the In-Fusion System (Clontech). To clone the human EF1α core promoter, pCpGrich-mcs (InvivoGen) was digested with SpeI and HindIII. The fragment was cloned into the pCpGfree vector digested with SpeI and HindIII using a Quick Ligation Kit (New England BioLabs, Ipswich, MA, USA). The sequences of the inserted were confirmed by sequencing with the universal RVprimer3 (5’-CTAG-CAAAATAGGCTGTCCC-3’).

Quantitative RT-PCR analysis of cultured cells

Quantitative RT-PCR (qRT-PCR) analysis was performed as described previously (Eto et al. 2014). Briefly, total RNA was extracted from the cultured cells using the QIAshredder and RNEasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The concentration of purified RNA was measured by a NanoDrop ND1000 Spectrophotometer (Thermo Scientific). RT was performed using High-Capacity cDNA RT Kits (Applied Biosystems). QPCR amplification was performed using the TaqMan Universal PCR Master Mix Core Reagent Kit (Applied Biosystems) with the probes listed in Table 2 and was analyzed using ABI Prism 7900 HT and StepOnePlus (Applied Biosystems); Ct values were measured in duplicates. The mRNA was quantified with normalization to β-actin expression using the 2-KDΔCt method. The data are presented as the means±S.E.M., and statistical significance was determined using a two-tailed unpaired Student’s t-test. P<0.05 was considered to be significant.

In vitro methylation of plasmid DNA

The pCpG-free vectors were methylated using SssI methylase (New England BioLabs) according to the manufacturer’s instructions. Briefly, 10 μg of plasmid DNA was

Table 1 Primers used for cloning the indicated promoters

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
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<tr>
<td>MafB UR6 promoter</td>
<td>F: CCAACATGTAAGATGTTCTGTGCCCCGTCTTCTTCTAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GTACATTTCTAAGGTGCTGCCGACAGACTCCCTACCC</td>
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<tr>
<td>Ins2 promoter</td>
<td>F: CCAACATGTAAGATGTTCTGTGCCCCGTCTTCTTCTAA</td>
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<tr>
<td></td>
<td>R: GTACATTTCTAAGGTGCTGCCGACAGACTCCCTACCC</td>
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<td>Glut2 promoter</td>
<td>F: CCAACATGTAAGATGTTCTGTGCCCCGTCTTCTTCTAA</td>
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<tr>
<td></td>
<td>R: GTACATTTCTAAGGTGCTGCCGACAGACTCCCTACCC</td>
<td></td>
</tr>
<tr>
<td>Srp2 promoter</td>
<td>F: CCAACATGTAAGATGTTCTGTGCCCCGTCTTCTTCTAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GTACATTTCTAAGGTGCTGCCGACAGACTCCCTACCC</td>
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<tr>
<td>Bisulfited pCpGfree</td>
<td>F: GGAATTTTTGTAGGGTTATTAGGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AAAACAAACACACCCCTATTTCCA</td>
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incubated with or without SssI (2.5 U/µg) in the presence of 160 µM of S-adenosylmethionine (New England BioLabs) for 2 h at 37 °C, and the methylase was then inactivated for 30 min at 65 °C. After purification of plasmid vectors with a NucleoSpin Plasmid QuickPure Kit (Macherey Nagel, Düren, Germany), the plasmids were used for the reporter assay. In parallel, the degree of methylation was examined via bisulfite sequencing. The vectors were linearized with PacI, which was heat-inactivated for 30 min at 65 °C. The linearized vectors were purified again, followed by the bisulfite conversion with an EpiTect Bisulfite Kit (Qiagen). The bisulfited promoters cloned into the MCS of pCPGfree were PCR-amplified with the ‘bisulfited CpGfree’ primers listed in Table 1. The PCR products were cloned into pCR4-TOPO, sequenced and analyzed as described below.

**Reporter assay**

Early-passage (conventional) or late-passage (compromised) INS1 cells and HeLa cells were transfected with 1 µg of 6.5 kb mouse MafB promoter/luciferase vector (−6500/LUC) (Huang et al. 2000) and pRL-TK vector (Promega) as an internal control using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol. The medium was changed 24 h after transfection. At 48 h after transfection, the firefly and renilla luciferase activities of the cells were analyzed using the Renilla-Glo and the Firefly Luciferase Assay System (Promega) as described previously (Eto et al. 2014). For the methylated reporter assay, the late-passage INS1 cells were transfected with 1 µg of in vitro-methylated pCPGfree-reporter vector and 1 µg of pGL4.23 vector (Promega) as an internal control. The medium was changed 24 h after transfection. At 48 h after transfection, 50 µl of the medium was analyzed for luciferase activity using a luminescence microplate reader (LB962 CentroPRO; Berthold Technologies, Bad Wildbad, Germany) for 5 s in each well after automatic shaking of the plates for 10 s. The data are presented as the mean ± S.E.M., and statistical significance was determined using a two-tailed unpaired Student’s t-test. P<0.05 was considered to be significant.

**Prediction of CpG islands and methylation-specific PCR**

For CpG island prediction, the rat MafB promoter (−3000 to +36) was analyzed using MethPrimer (Li & Dahiya 2002). The predicted CpG islands were as follows: UR1, from −564 to −19 (546 bp); UR2, from −921 to −576 (346 bp); UR3, from −1118 to −1011 (108 bp); UR4, from −1450 to −1286 (165 bp); UR5, from −1776 to −1670 (107 bp); UR6, from −2058 to −1835 (224 bp); UR7, from −2648 to −2535 (114 bp); and UR8, from −2883 to −2764 (120 bp). Among these regions, PCR primers for GC-rich UR3, UR5, and UR7 were unable to be set.

A total of 1 µg of genomic DNA was extracted from INS1 cells using a NucleoSpin Tissue Kit (Macherey Nagel). The bisulfite conversion of DNA and methylation-specific PCR (MSP) were performed using an EpiTect Bisulfite Kit (Qiagen) and an EpiScope MSP Kit (Takara Bio, Shiga, Japan) respectively. The primers used for MSP are listed in Table 3. PCR products were analyzed and quantified by MultiNA (Shimadzu Corporation, Kyoto, Japan).

**Bisulfite sequencing**

Bisulfite sequencing was used to analyze the following regions: −237 to +61 for UR1A (299 bp), −321 to −106 for UR1B (216 bp), and −2125 to −1787 (339 bp) for UR6. PCR was performed with the bisulfited DNA using primers listed in Table 4. Nested PCR amplified the regions UR1A and UR1B. The PCR products were cloned into pCR4-TOPO (Life Technologies), which was used to transform DH5α or TOP10 competent cells for bisulfited DNA from INS1 cells, or Mach1 (Life Technologies) for the linearized
plasmid vector, followed by plating. Single colonies were selected, and the bisulfited DNA cloned into pCR4-TOPO was extracted using a NucleoSpin Plasmid QuickPure Kit (Macherey Nagel), followed by sequencing using the universal sequencing primer RVprimer3 or M13 reverse (5'-CAGGAAACAGCTATGAC-3'). The data were analyzed using quantification tool for methylation analysis (QUMA; Kumaki et al. 2008). The data are presented as the mean ± S.E.M., and statistical significance was determined using a two-tailed unpaired Student's t-test. P < 0.05 was considered to be significant.

**Results**

**Upregulation of MafB in the late-passage INS1 cells, a compromised β-cell model**

To analyze the expression of MafB in the compromised β-cells without the increased population of other endocrine cells, the INS1 β-cell line was used, because the change in composition of β- and α-cells would affect these mRNA expression analyses of the islets isolated from diabetic model mice. Late-passage β-cell lines chronically exposed to high glucose have been utilized as models for

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<th>Table 3</th>
<th>Primers used for methylation-specific PCR</th>
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<td><strong>Mice</strong></td>
<td><strong>Primer sequences</strong></td>
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<tr>
<td>Rat MafB UR1A Methylated</td>
<td>F: TTTTAGGTTTTTATTTTTGTTT</td>
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<tr>
<td>Rat MafB UR1A Unmethylated</td>
<td>R: CAGGAAACAGCTATGAC</td>
</tr>
<tr>
<td>Rat MafB UR2 Methylated</td>
<td>F: GGGGGGTATTTTTATTTTTGTTT</td>
</tr>
<tr>
<td>Rat MafB UR4 Methylated</td>
<td>R: CATAAAAACCTTTATCTCATTCAAT</td>
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<th>Table 4</th>
<th>Primers used to amplify and sequence the bisulfite-treated genomic region of the MafB promoter</th>
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</thead>
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<td><strong>CpG island</strong></td>
<td><strong>Primer sequences</strong></td>
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<tr>
<td>UR1A (nested PCR) First PCR</td>
<td>F: GTTAGTTTTTTATTTTTGTTT</td>
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<tr>
<td>Second PCR</td>
<td>R: AAAAAACCTCTACCCTCAAT</td>
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<tr>
<td>UR1B (nested PCR) First PCR</td>
<td>F: TTTTAGGATATAGGAAG</td>
</tr>
<tr>
<td>Second PCR</td>
<td>R: ACCAAACCTTATCTAAAAA</td>
</tr>
<tr>
<td>UR6</td>
<td>F: TTTAGGATATAGGAAG</td>
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glucotoxic β-cells (Harmon et al. 2005). The mRNA expression of molecules critical for β-cell function in INS1 cells with a late passage number (Nishimura et al. 2013) was compared with those with an early passage number. QRT-PCR revealed that the expression of Ins1, Ins2, MafA, and Pdx1 was significantly decreased in late-passage INS1 cells (Fig. 1A). Interestingly, MafB mRNA was significantly increased, in these late-passage INS1 cells with p185 and p239 (Fig. 1B), similar to compromised β-cells in diabetic models, but not in INS1 cells with p42. These results suggest that we could consider late-passage INS1 cells as an in vitro model of compromised β-cells, and these results prompted us to examine the MafB promoter activity in this population of β-cells. A reporter assay using the 6.5 kb MafB promoter in a luciferase vector showed that MafB promoter activity was indeed increased in late-passage INS1 cells compared with those in early-passage INS1 cells or HeLa cells (Fig. 1C). The increased transcriptional activity of MafB is also similar to the β-cells in diabetic model mice (Nishimura et al. 2015).

Lower methylation levels of the MafB promoter in late-passage INS1 cells

Demethylation of a particular region of the Arx promoter is involved in the increased expression of Arx, another α-cell transcription factor, during the conversion of β- to α-cells (Dhawan et al. 2011), suggesting that epigenetic factors can be involved in the mechanism of increased expression of transcription factors during the conversion of cell types. Thus, methylation status of the MafB promoter in early- and late-passage INS1 cells was analyzed. Based on the previous reports on CpG islands in the Arx promoter (Dhawan et al. 2011), in silico analysis was performed in the rat MafB promoter from the transcription start site to the upstream −3000 bp, and this analysis detected eight CpG islands, which were termed UR1–UR8 (Fig. 2A). MSP analyses uncovered various levels of methylation in these regions and showed lower methylation levels of two CpG islands, UR1 (−564 to −19) and UR6 (−2058 to −1835), in late-passage compared to early-passage INS1 cells (Fig. 2B). Bisulfite sequencing of each region in late-passage INS1 cells demonstrated that seven CpG sites in UR6 were under-methylated compared with early-passage INS1 cells (Fig. 3A, B and C; Supplemental Figure 1A, see section on supplementary data given at the end of this article), while no change in UR1 was seen (data not shown). These seven CpG sites were conserved in human, mouse and rat MafB promoters (data not shown). A QUMA revealed that 82.0 ± 7.6%, 85.7 ± 9.3 and 79.5 ± 9.1% of these seven CpG sites in the UR6 region were methylated in early-passage INS1 cells with p29, p30, and p34, respectively, and 57.2 ± 14.4% of these CpG sites were methylated in late-passage INS1 cells with p183. Meanwhile, only 39.3 ± 15.2 and 39.3 ± 11.1% of these CpG sites were methylated in late-passage INS1 cells with p237 and p239-1 (Fig. 3D), which were significantly lower than p29, p30, and p34 INS1 cells respectively (Fig. 3D). Similar results were obtained with

Figure 1

Upregulation of MafB in late-passage INS1 cells. (A) The mRNA expression of the indicated molecules in the INS1 cells with late passage number (p149–158, p176–186, and p227–238) relative to early-passage INS1 cells (p24–38) was analyzed by qRT-PCR; n = 3 for Ins1, Ins2, and MafA; n = 6 for Pdx1. (B) The mRNA expression of the indicated molecules in the INS1 cells with p42, p185, and p239 relative to early-passage INS1 cells (p32) was analyzed by qRT-PCR; n = 4 for MafA and MafB. (C) The reporter assay with the 6.5 kb MafB/luciferase vector and pRL-TK vector as an internal control, transfected into early-passage INS1 (p26–34; n = 6), late-passage INS1 (p234–242; n = 6), and HeLa cells (n = 3). The mean ± S.E.M. are shown. **p<0.01 and *p<0.05.

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Figure 2
Detection of changes in methylation of the MafB promoter in late-passage INS1 cells. (A) Eight CpG islands in the rat MafB promoter are termed UR1–UR8. Regions underlined in blue were analyzed by bisulfite sequencing. (B) MSP revealed demethylation of two CpG islands, UR1 (−564 to −19) and UR6 (−2058 to −1835) in the MafB promoter in the late-passage INS1 cells (p234). The mean ± S.E.M. are shown. **P < 0.01.

Methylation of the UR6 region is involved in the MafB promoter activity in vitro
Although DNA methylation plays an important role in regulating gene transcription in general, the influence of methylation of individual CpG on the promoter activity of a specific gene remains largely unknown (Hashimoto et al. 2013). The relative contribution of DNA methylation of the UR6 region to the MafB promoter activity was investigated in vitro in late-passage INS1 cells. The effects of methylation on promoter activity can be analyzed by reporter assays after the in vitro methylation of CpGfree luciferase reporter vectors. This pCpGfree vector lacks CpG dinucleotides; therefore, the luciferase activity after the in vitro methylation of this vector is only affected by methylated CpG residues in the promoter region cloned into the MCS, suggesting its usefulness for studying the effects of CpG methylation on promoters (Fig. 4A; Klug & Rehli 2006).

The rat MafB promoter UR6 region (21 CpG in 373 bp, GC 57.64%, Fig. 4B), rat Ins2 promoter (two CpG in 333 bp, GC 53.45%, Fig. 4C), rat Glut2 promoter (three CpG in 339 bp, GC 50.44%, Fig. 4D), rat Srpx2 promoter as a negative control (two CpG in 304 bp, GC 45.72%, Fig. 4E; Øster et al. 2013), and human EF1α core promoter as a positive control (16 CpG in 221 bp, GC 58.37%, Fig. 4F; Klug & Rehli 2006) were cloned into MCS of the pCpGfree reporter vector. In vitro treatment of these vectors with or without DNA methylase SssI was performed, followed by the reporter assay using the late-passage INS1 cells. In parallel, linearized vector was analyzed by bisulfite sequencing to examine DNA methylation of the CpG sites in the cloned regions. The luciferase activities driven by unmethylated and methylated Srpx2 promoter are similar (Fig. 4E), and significantly increased activity of unmethylated EF1α promoter was observed (Fig. 4F), both of which were as expected. Results of the methylated reporter assay showed that the promoter activity of unmethylated MafB promoter UR6 was twice as strong as the promoter activity of methylated UR6 (Fig. 4B), while the methylation of the Ins2 or Glut2 promoter, which contain few CpG sites, revealed no changes in promoter activity (Fig. 4C and D). These results revealed that the methylation status of a unique CpG island in the MafB promoter has an effect on promoter activity in late-passage INS1 cells. The Ins2 promoter in either methylated or unmethylated form did not exhibit change in the promoter activity, consistent with the previous report that reduced expression of ins2 in the compromised β-cells (Harmon et al. 2005).

Discussion
In this study, we investigated the mRNA expression of MafB, activity and DNA methylation of the MafB promoter in late-passage INS1 cells, which we considered as a model of compromised β-cells because the expression of Ins1, Ins2, MafA, and Pdx1 is decreased. In these cells, the mRNA expression and the promoter activity of MafB were increased. Concomitant with these results, the particular region in the MafB promoter UR6 was undermethylated. During development, MafB is expressed in the β-cells of embryos and early neonates but downregulated in the
Figure 3

CpG islands in the MafB promoter are partially demethylated in late-passage INS1 cells. (A) CpG sites in the UR6 region of the MafB promoter were analyzed by bisulfite sequencing. CpG islands are highlighted in yellow. The CpG sites indicated by red numbers were demethylated, and the sites with green numbers were not changed in late-passage INS1 cells compared with early-passage INS1 cells. (B) Analysis of DNA methylation by bisulfite sequencing. CpG sites demarcated by red squares corresponding with the red numbers in (A) were demethylated; n = 23 for INS1 p29, n = 20 for INS1 p30, n = 23 for INS1 p34, n = 22 for INS1 p183, n = 20 for INS1 p237, and n = 20 for INS1 p239-1. (C) Quantification of the results shown in (B). (D) The methylated proportion of CpG sites corresponding with the red numbers shown in (C) and Supplementary Figure 1C. The mean ± s.e.m. are shown. **P < 0.01 and *P < 0.05.
Methylation of the MafB promoter UR6 is involved in promoter activity \textit{in vitro}. The experimental protocol (A) explains that the indicated promoters cloned into the pCpGfree vector lacking CpG dinucleotides were \textit{in vitro} methylated with or without DNA methylase SssI, followed by the reporter assay using late-passage INS1 and bisulfite sequencing of those promoter regions, (B) rat MafB (UR6 region), (C) rat Ins2, (D) rat Glut2, (E) rat Srpx2 (negative control), and (F) human \textit{EF1alpha} core promoter (positive control). The luciferase activities of unmethylated promoters, treated without SssI, are shown relative to those of methylated promoters treated with SssI. The results show the activity of unmethylated MafB promoter UR6 was higher than that of methylated UR6, \( n=3 \). The mean ± S.E.M. are shown. ** \( P < 0.01 \).
adult mature β-cells at least in mice (Nishimura et al. 2006, Artner et al. 2010). Using MafB reporter mice (Moriguchi et al. 2006), we previously reported that the MafB promoter was upregulated in β-cells of the diabetic model mice (Nishimura et al. 2015). The present study suggests the possibility that MafB upregulation in compromised β-cells in vivo may also be at least in part due to lower methylation status of the MafB promoter.

The β-cell line is useful for analyzing DNA methylation, as previously reported in a study describing the demethylation of a particular region of the Arx promoter, which is involved in the upregulation of Arx during the β- to γ-cell conversion (Dhawan et al. 2011). This is the first study to provide evidence that the partial DNA demethylation of the MafB promoter can occur in the β-cell line cultured in a high glucose condition. These results, analyzing the promoters of β-cell disallowed genes normally repressed in mature β-cells but upregulated in the compromised β-cells, suggest that DNA demethylation is a possible mechanism for upregulation of these genes. The larger-scale analysis of β-cells in the native islets sorted from normal and diabetes model mice by flow-cytometry, which would be physiologically more relevant, may detect other demethylated promoter regions of β-cell disallowed genes, and MafB may be upstream of these changes in DNA methylation (Vicente-Dueñas et al. 2012) in dedifferentiated β-cells.

Although changes in the expression level of transcription factors affect gene expression in dedifferentiated β-cells, our study suggests that the DNA methylation status of gene promoters critical for β-cell function may play an imperative role. Systematic investigation of epigenetics in β-cells obtained from normal and diabetes model mice may reveal new mechanisms underlying diabetes development.

In conclusion, increased mRNA expression and promoter activity of MafB were observed in the compromised β-cell model of late-passage INS1 cells with reduced expression of Ins1, Ins2, MafA, and Pdx1. The partial demethylation of a specific CpG island in the MafB promoter was detected in these cells, and the methylation status of this region affected the promoter activity in the reconstituted system. These results suggest that epigenetic regulation may be involved in the molecular mechanism of dysfunction and possible dedifferentiation of pancreatic β-cells in diabetes mellitus.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
W N contributed to conception and design of the study, the acquisition, analysis and interpretation of the data and drafting the article. N I, K E, N F, H U, and K Y contributed to interpretation of the data and critical revision of the manuscript. H M, S O, and Y N contributed to critical revision of the manuscript. All authors have approved the final version, and W N is responsible for the integrity of the work as a whole.

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