Biochemistry and Biophysics Reports 10 (2017) 192–197

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Adenovirus vector-mediated macrophage erythroblast attacher (MAEA) overexpression in primary mouse hepatocytes attenuates hepatic gluconeogenesis

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ARTICLE INFO

Keywords:
Adenovirus vector
Diabetes mellitus
Glucose metabolism
GWAS
MAEA

ABSTRACT

Japanese patients with type 2 diabetes mellitus present a different responsiveness in terms of insulin secretion to glucose and body mass index (BMI) from other populations. The genetic background that predisposes Japanese individuals to type 2 diabetes mellitus is under study. Recent genetic studies demonstrated that the locus mapped in macrophage erythroblast attacher (MAEA) increases the susceptibility to type 2 diabetes mellitus in East Asians, including Japanese individuals. MAEA encodes a protein that plays a role in erythroblast enucleation and in the normal differentiation of erythroid cells and macrophages. However, the contribution of MAEA to type 2 diabetes mellitus remains unknown. In this study, to overexpress MAEA in the mouse liver and primary mouse hepatocytes, we generated a MAEA-expressing adenovirus (Ad) vector using a novel Ad vector exhibiting significantly lower hepatotoxicity (Ad-MAEA). Blood glucose and insulin levels in Ad-MAEA-treated mice were comparable to those in control Ad-treated mice. Primary mouse hepatocytes transduced with Ad-MAEA showed lower levels of expression of gluconeogenesis genes than those transduced with the control Ad vector. Hepatocyte nuclear factor-4α (HNF-4α) mRNA expression in primary mouse hepatocytes was also suppressed by MAEA overexpression. These results suggest that MAEA overexpression attenuates hepatic gluconeogenesis, which could potentially lead to improvement of type 2 diabetes mellitus.

1. Introduction

Type 2 diabetes mellitus results from the interaction of genetic, aging, and environmental factors. Relatively few diabetic individuals in Japan are obese, and impairment of insulin secretion frequently results in the onset of type 2 diabetes mellitus [1,2]. Furthermore, patients with type 2 diabetes mellitus in Japan present lower insulin resistance than Europeans patients [1]. Therefore, the etiology of type 2 diabetes mellitus possibly differs between the Japanese and European populations.

A recent genome-wide association study (GWAS) identified susceptibility loci for type 2 diabetes mellitus in East Asians [3]. Macrophage erythroblast attacher (MAEA), alias erythroblast macrophage protein (Emp), is one of the type 2 diabetes mellitus-susceptibility genes of East Asians. Other GWAS also identified MAEA as a locus associated with type 2 diabetes mellitus in the Japanese population [4]. MAEA encodes a protein involved in erythroblast-macrophage cell attachment, terminal maturation and enucleation of erythroid cells, and inhibition of erythroblast apoptosis [5–7]. MAEA, originally detected in erythroblasts and macrophages, is expressed in other tissues [8], suggesting additional functions besides its role in hematopoiesis. However, the precise biological functions of MAEA, including that in type 2 diabetes mellitus, remain unexplained.

The liver is mainly responsible for the regulation of glucose metabolism, and impaired hepatic glucose metabolism contributes to the development of type 2 diabetes mellitus [9]. In order to explore the

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http://dx.doi.org/10.1016/j.bbrep.2017.04.010
Received 21 July 2016; Received in revised form 27 December 2016; Accepted 18 April 2017
Available online 20 April 2017

association between MAEA and type 2 diabetes mellitus, we overexpressed MAEA in the mouse liver using Ad vector (Ad-MAEA). Systemic administration of Ad vectors results in the liver-specific expression of exogenous genes [10], while the main adverse effect of Ad vector is hepatotoxicity [11,12]. To reduce Ad vector-induced hepatotoxicity, we utilized a novel Ad vector with miR-122a-targeted sequences in the 3′-untranslated region of the E4 gene in the Ad genome, named Ad-E4-122aT [13]. Ad-E4-122aT exhibits lower liver injury than conventional Ad vector. Therefore, it is suitable not only for gene therapy, but also for gene function analysis in the liver. Furthermore, we examined the effects of MAEA overexpression in primary mouse hepatocytes in details.

2. Materials and methods

2.1. Mice

Male C57BL/6 mice aged 6–7 weeks were obtained from Nippon SLC (Hamamatsu, Japan). Male KKAy mice aged 6 weeks were obtained from CLEA Japan (Tokyo, Japan). All experimental procedures used in this study were performed in accordance with the institutional guidelines for animal experiments at Osaka Ohtani University.

2.2. Plasmids and Ad vectors

Ad vectors were constructed by using an improved *in vitro* ligation method [14,15]. MAEA complementary DNA (cDNA) was cloned from liver cDNA from C57BL/6 mouse. The CA promoter (a fusion promoter composed of chicken β-actin promoter and cytomegalovirus (CMV) enhancer)-driven MAEA-expression plasmid, pHMCA-MAEA, was constructed using pHMCA6 [16]. pHMCA-MAEA and pHMCA6L, which is a firefly luciferase-expressing plasmid, were digested with I-CeuI/PI-SceI. The fragments of pHMCA-MAEA and pHMCA6L were ligated into I-CeuI/PI-SceI-digested Ad vector plasmid pAdHM4-E4-122aT [13], producing pAd-MAEA and pAd-Luc, respectively. All Ad vectors (Ad-MAEA and Ad-Luc) were purified as described previously [13]. The virus particles (VPs) were determined using a spectrophotometric method [17] and biological titers were measured using an Adeno-X-rapid titer kit (Clontech, Mountain View, CA, USA).

2.3. MAEA mRNA expression in mouse tissues

Total RNA was extracted from mouse tissues and primary mouse hepatocytes using TRIzol (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The total pancreatic RNA was isolated as previously described [18]. The reticulocytes were prepared from mouse peripheral blood using Optiprep (Nycomed Amersham, Oslo, Norway) following the manufacturer's protocol. Relative levels of MAEA mRNA in the mouse organs were assayed by real-time RT-PCR using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and normalized to β-actin mRNA levels. The protocol for thermal cycling consisted of 60 s at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 63 °C. The sequences of the primers used in this study were as follows: mouse MAEA forward, 5′-CAC TGA ACA AAC GCT TCC GAG-3′; mouse MAEA reverse, 5′-GGC AAC TAC TCA AGG TCT TCT C-3′; mouse β-actin forward, 5′-GGC TGT ATT CCC CTC CAT CG-3′; mouse β-actin reverse, 5′-CCA GTT GGT AAC AAT GCC ATG T-3′.

2.4. MAEA protein expression in primary mouse hepatocytes

For immunocytochemistry, primary mouse hepatocytes were isolated from a mouse using the hepatic portal perfusion technique as described previously [19]. One day after isolation, the primary mouse hepatocytes were transduced with Ad-MAEA or Ad-Luc at an MOI of 100. The medium containing Ad vectors was replaced with fresh medium after 24 h of incubation. At 48 h after transduction, the primary mouse hepatocytes were fixed in 4% paraformaldehyde phosphate solution (Nacalai, Kyoto, Japan) for 20 min at 4 °C. After washing in phosphate-buffered saline (PBS), the cells were solubilized with 0.1% Triton X-100 in PBS for 1 h at room temperature (RT). After washing in PBS, the cells were treated with 2% goat serum in PBS for 1 h at RT. The cells were then incubated with rabbit anti-MAEA antibody (OriGene Technologies, Rockville, MD, USA) for 4 h at RT. After washing in PBS, the cells were incubated with Alexa Fluor 488-conjugated rabbit anti-goat IgG (Thermo Fisher Scientific, Waltham, MA, USA) and DAPI (Dojindo, Kumamoto, Japan) for 1 h at RT.

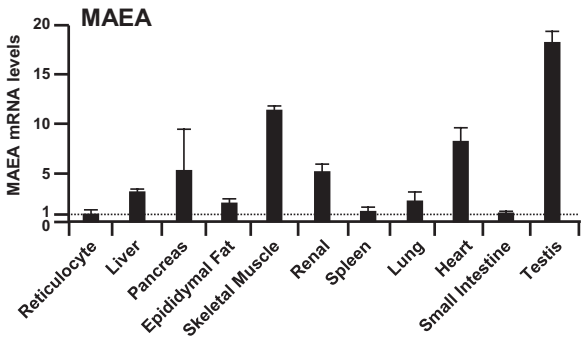

Fig. 1. Distribution of MAEA in mouse tissues. MAEA mRNA levels in mouse tissues were determined by real-time RT-PCR. Normalized data are expressed relative to the corresponding value for reticulocytes. The data are expressed as mean ± SD (n = 4–5).

2.5. Serum glucose and insulin analyses

Ad vectors were intravenously administered into C57BL/6 mice at a dose of 5×10^9 infectious units (IFU)/mouse *via* the tail vein. The blood samples were obtained from fasted (13–15 h) mice 2 weeks after administration of Ad vectors. The serum levels of glucose were determined by Glutest Sensor Neo (Sanwa Kagaku Kenkyusho, Nagoya, Japan). An ELISA kit was used to measure the serum insulin levels (Morinaga, Tokyo, Japan).

2.6. Glucose tolerance tests

Ad vectors were intravenously administered into C57BL/6 mice at a dose of 5×10^9 infectious units (IFU)/mouse *via* the tail vein. Glucose tolerance tests were performed on 16 h-fasted mice injected intraperitoneally with glucose (2 g/kg) 7 and 14 days after Ad vector administration. Blood glucose levels were determined immediately before and 30, 60, and 120 min after injection as determined by the Glutest Sensor Neo.

2.7. Analysis of gene expression in primary mouse hepatocytes

Primary mouse hepatocytes were seeded into a 12-well collagen-coated plate at 1×10^5 cells/well. One day after isolation, primary mouse hepatocytes were transduced with Ad-MAEA or Ad-Luc at an MOI of 100. The medium containing Ad vectors was replaced with fresh medium after 24 h of incubation. Primary mouse hepatocytes were harvested at 48 h after transduction. Total RNA were extracted from primary hepatocytes. Hepatocyte nuclear factor-4α (HNF-4α) and gluconeogenesis mRNA levels were determined by real-time RT-PCR. The sequences of the primers used in this study were as follows: mouse glucose-6-phosphatase (G6Pase) forward, 5′-CTG TTT GGA CAA CGC CCG TAT-3′; mouse G6Pase reverse, 5′-AGG TGA CAG GGA ACT GCT TTA-3′; mouse phosphoenolpyruvate carboxykinase (PEPCK) forward, 5′-CTG CAT AAC GGT CTG GAC TTC-3′; mouse PEPCK reverse, 5′-GCC TTC CAC GAA CTT CCT CAC-3′; mouse fructose-1, 6-bisphosphatase (FBPase) forward, 5′-CAC CGC GAT CAA AGC CAT CT-3′; mouse FBPase reverse, 5′-CCA GTC ACA TTG GTT GAG CCA-3′; mouse glucokinase

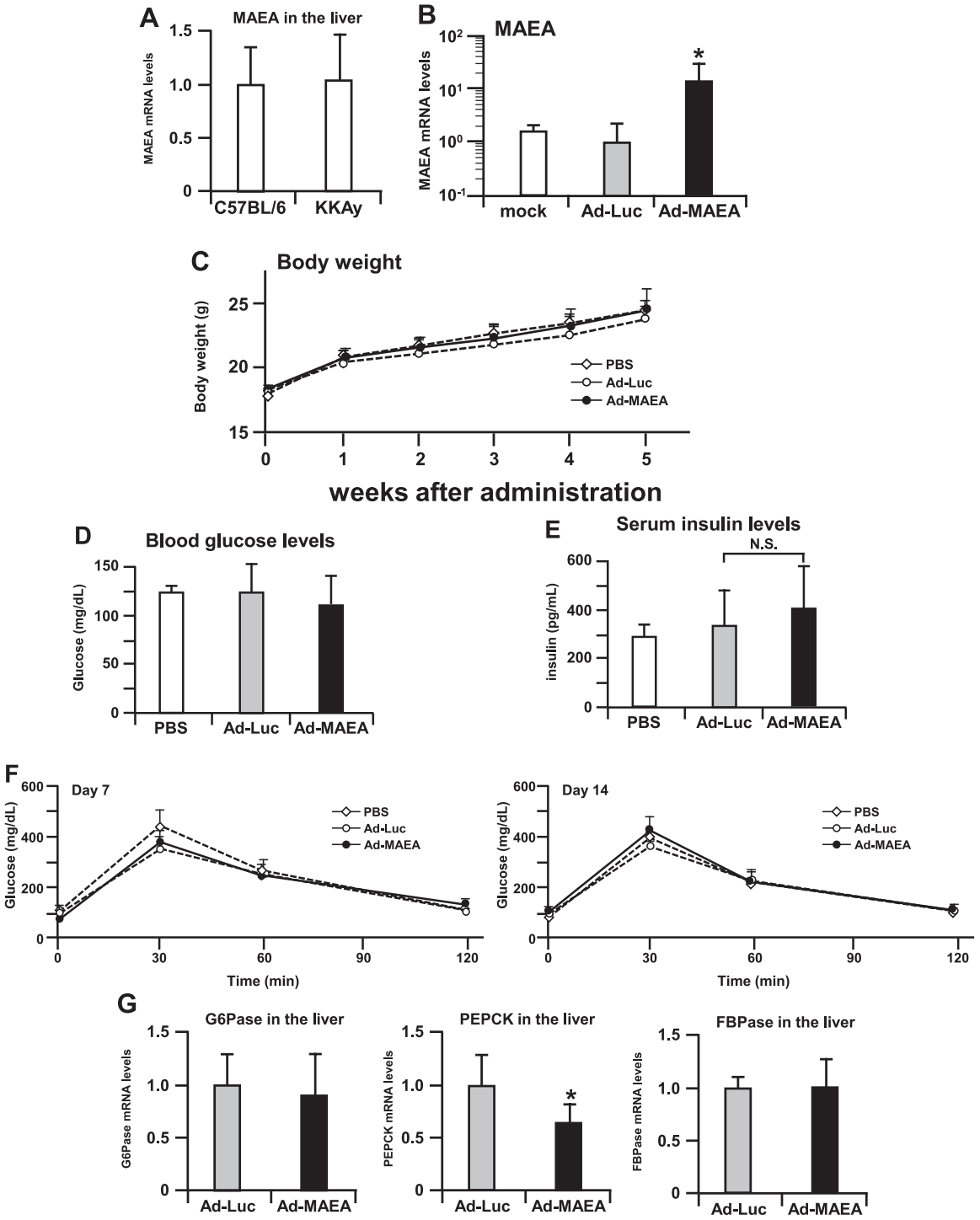

Fig. 2. Metabolic phenotypes of the mice. (A) MAEA mRNA levels in the liver of C57BL/6 and KKAy mice. (B) Hepatic MAEA mRNA levels in C57BL/6 mice transduced with Ad-Luc or Ad-MAEA at week 2. (C) Body weights of C57BL/6 mice transduced with Ad-Luc or Ad-MAEA. Serum glucose (D) and insulin (E) levels in fasted (overnight) mice 2 weeks following Ad-Luc or Ad-MAEA administration. (F) Glucose tolerance test of mice transduced with Ad vectors at day 7 and 14. (G) Hepatic mRNA levels of gluconeogenesis genes in C57BL/6 mice transduced with Ad-Luc or Ad-MAEA at week 2. The data are expressed as mean ± SD (A, F: n = 4; B-E, G: n = 6). *, $P < 0.05$ in comparison with Ad-Luc. N.S., not significant.

forward, 5′-AGG AGG CCA GTG TAA AGA TGT-3′; mouse glucokinase reverse, 5′-CTC CCA GGT CTA AGG AGA GAA A-3′; mouse pyruvate kinase forward, 5′-CCG CAT CTA CAT TGA CGA CG-3′; mouse pyruvate kinase reverse, 5′-CCG TGT TCC ACT TCG GTC AC-3′; mouse HNF-4α forward, 5′-CAC GCG GAG GTC AAG CTA C-3′; mouse HNF-4α reverse, 5′-CCC AGA GAT GGG AGA GGT GAT-3′.

2.8. Statistical analysis

Statistical significance ($p < 0.05$) was determined using Student's *t*-test. Data are presented as means ± SD.

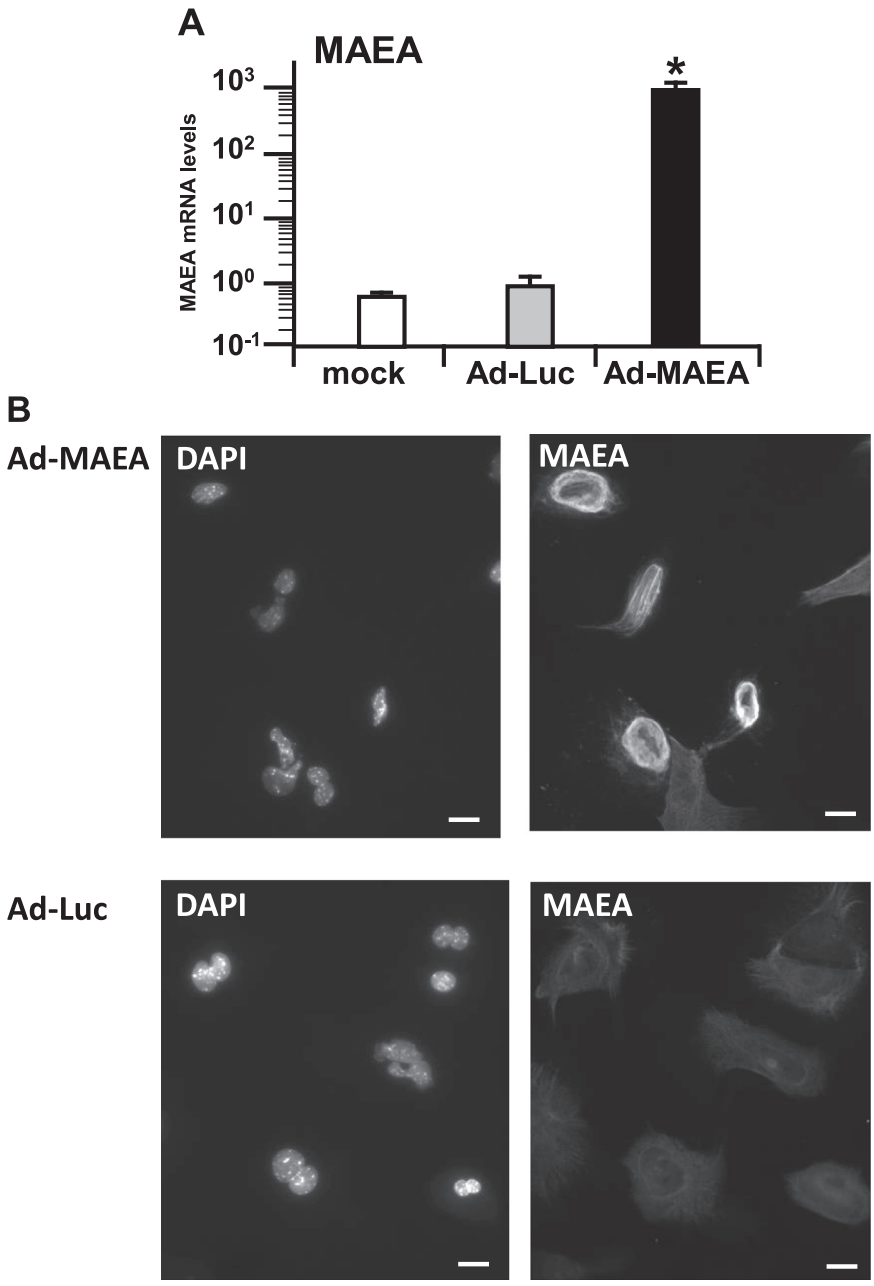

Fig. 3. Overexpression of MAEA in primary mouse hepatocytes. Primary mouse hepatocytes were transduced with Ad-Luc or Ad-MAEA at an MOI of 100. (A) MAEA mRNA levels in primary mouse hepatocytes were determined by real-time RT-PCR. Normalized data are expressed relative to the corresponding value of the primary mouse hepatocytes transduced with Ad-Luc. The data are expressed as mean ± SD (n = 4). *, $P < 0.05$ in comparison with Ad-Luc. (B) MAEA protein in primary mouse hepatocytes determined by immunocytochemistry. Bar = 10 μm.

3. Results

3.1. Distribution of MAEA mRNA in mouse tissues

In order to determine the distribution of MAEA in tissues, MAEA mRNA levels were determined in C57BL/6 mice. MAEA mRNA expression was observed in all tissues examined in this study. Higher expression of MAEA mRNA was observed in the liver, pancreas, epididymal fat, skeletal muscle, renal, lung, heart, and testis than that in reticulocytes (Fig. 1). Especially, MAEA mRNA was abundantly expressed in the skeletal muscle and testis.

3.2. Glucose metabolism and insulin secretion in mice overexpressing MAEA

The liver is an essential metabolic organ. Therefore, we determined the MAEA mRNA expression levels in the liver of KKAy, type 2 diabetic, and C57BL/6 mice. The MAEA expression levels in the liver of KKAy mice were comparable to that of C57BL/6 mice (Fig. 2A). MAEA has been identified as containing a type 2 diabetes mellitus-associated SNP by some GWASs [3,4]. It is unknown whether the MAEA SNP affects MAEA function. Therefore, we tried to overexpress MAEA in the mouse liver.

To explore the effect of MAEA on the metabolic profile *in vivo*, an MAEA-expressing Ad vector (Ad-MAEA) or a control luciferase-expressing Ad vector (Ad-Luc) was administered intravenously to C57BL/6 mice. The injection of Ad-MAEA into C57BL/6 mice increased hepatic MAEA expression 14-fold compared with that in Ad-Luc-treated mice (Fig. 2B). The increase in body weight was similar in Ad-MAEA- and Ad-Luc-treated mice (Fig. 2C). Next, we monitored blood glucose and insulin levels under fasting conditions 2 weeks after administration of

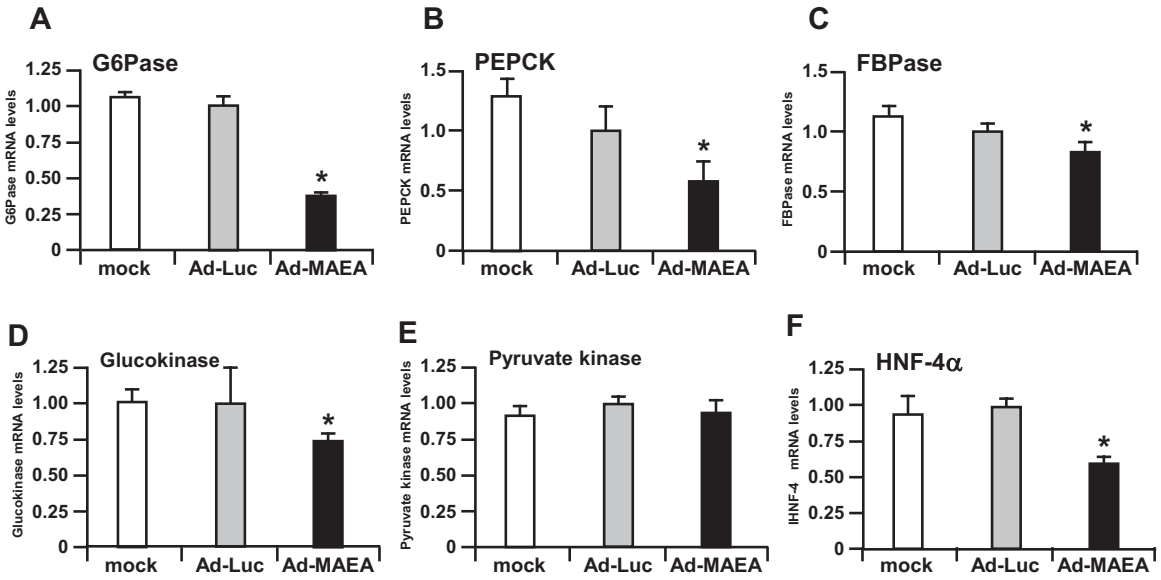

Fig. 4. Expression of gluconeogenesis-related genes in primary mouse hepatocytes. Primary mouse hepatocytes were transduced with Ad-Luc or Ad-MAEA at an MOI of 100. mRNA levels in primary mouse hepatocytes were determined by real-time RT-PCR. Normalized data are expressed relative to the corresponding value for the primary mouse hepatocytes transduced with Ad-Luc. The data are expressed as mean ± SD (n = 4). *P < 0.05 in comparison with Ad-Luc.

Ad-MAEA or Ad-Luc (Fig. 2D and E). No significant difference in blood glucose and insulin levels was observed between Ad-MAEA- and Ad-Luc-treated mice. Furthermore, we examined glucose tolerance 7 and 14 days after Ad vector administration. Glucose tolerance was similar between Ad-MAEA-treated mice and control Ad-treated mice at both day 7 and day 14 (Fig. 2F). However, the gene expression of PEPCK, a key gluconeogenic gene, was significantly suppressed in Ad-MAEA-treated mice (Fig. 2G). These results indicated that MAEA overexpression in the liver did not influence fasting glucose and insulin levels *in vivo*.

3.3. Gene expression profile in primary mouse hepatocytes

To explore the impact of MAEA on hepatic gluconeogenesis and glucose metabolism in details, primary mouse hepatocytes isolated from C57BL/6 mice were transduced with Ad-MAEA or control Ad-Luc. MAEA mRNA expression in primary mouse hepatocytes transduced with Ad-MAEA was 1000-fold higher than that in Ad-Luc-transduced primary mouse hepatocytes (Fig. 3A). We also determined MAEA protein levels in the primary mouse hepatocytes by immunocytochemistry. MAEA protein levels were considerably higher in primary mouse hepatocytes transduced with Ad-MAEA than in control Ad-transduced hepatocytes (Fig. 3B).

Next, we investigated the expression profiles of glucose metabolism-related genes. The gene expression level of G6Pase, one of the key gluconeogenic enzyme, was significantly suppressed by 2.7-fold in primary mouse hepatocytes transduced with Ad-MAEA, compared with Ad-Luc-transduced primary mouse hepatocytes (Fig. 4A). Expression levels of other gluconeogenic genes such as PEPCK and FBPase, were significantly decreased by 1.5- and 1.2-fold, respectively, in the primary mouse hepatocytes treated with Ad-MAEA (Fig. 4B and C). We also examined the mRNA expression levels of glucokinase and pyruvate kinase, two glycolysis-related genes. Although the expression level of glucokinase was slightly reduced by 1.4-fold in Ad-MAEA-transduced primary mouse hepatocytes compared with that in Ad-Luc-transduced hepatocytes (Fig. 4D), pyruvate kinase mRNA expression was not affected by Ad-Luc treatment (Fig. 4E).

To elucidate the molecular mechanisms of gluconeogenesis suppression in Ad-MAEA-treated primary hepatocytes, we focused on HNF-4α, a liver-specific transcriptional factor and a central regulator of gene expression in the liver [20]. Expression of HNF-4α induces the transcription of key gluconeogenic enzymes such as G6Pase and FBPase [21]. Therefore, we examined the expression levels of the HNF-4α in the primary mouse hepatocytes. HNF-4α expression in Ad-MAEA-treated primary hepatocytes significantly decreased by 1.7-fold compared to that in Ad-Luc-treated primary hepatocytes (Fig. 4F). These results suggested that MAEA overexpression suppressed HNF-4α mRNA levels in primary mouse hepatocytes, leading to the attenuation of hepatic gluconeogenesis.

4. Discussion

GWAS allowed the identification of susceptibility loci for type 2 diabetes. However, the biological mechanisms underlying their contribution to the susceptibility to type 2 diabetes mellitus remain unknown. In this study, we investigated the mechanism underlying the association between MAEA and type 2 diabetes mellitus. C57BL/6 mice were transduced with Ad-MAEA to determine the effect of MAEA on type 2 diabetes mellitus. As shown in Fig. 2, MAEA overexpression in the mouse liver had no effect on body weight, blood glucose, and insulin levels, although PEPCK mRNA levels were suppressed in the liver of Ad-MAEA-treated mice. In addition, MAEA overexpression in primary mouse hepatocytes suppressed the expression of gluconeogenesis genes such as G6Pase, PEPCK, and FBPase.

MAEA overexpression might be responsible for the differences in the effects observed *in vivo* and *in vitro*. MAEA mRNA expression in hepatocytes transduced with Ad-MAEA was 1000-fold higher than that in the hepatocytes transduced with the control Ad vector (Fig. 3A). In contrast, MAEA mRNA expression in the liver of Ad-MAEA-treated mice was 10-fold higher than that of mice treated with the control Ad vector (Fig. 2B). Therefore, the relatively low expression of the MAEA gene could not influence blood glucose *in vivo*.

MAEA is homologous to *Saccharomyces cerevisiae* glucose induced degradation deficient 9 (GID9), which is one of the proteins constituting the glucose induced degradation deficient (GID) complex [22]. GID complex mediates polyubiquitination of FBPase. In this study, MAEA suppressed FBPase expression in primary mouse hepatocytes at the transcriptional level. Mammalian MAEA and its orthologs might be conserved and involved in the suppression of FBPase expression.

In type 2 diabetes mellitus, gluconeogenesis is increased and mainly attributed to excessive hepatic glucose production. Increased gluconeogenesis is responsible for fasting hyperglycemia. The activation of

gluconeogenesis is directly regulated by the transcriptional expression levels of gluconeogenesis related genes such as G6Pase and PEPCK. Attenuation of gluconeogenesis (*e.g.*, suppression of the expression of gluconeogenesis-related genes) has therapeutic effects on type 2 diabetes mellitus. In this study, MAEA overexpression in hepatocytes resulted in the reduction of the expression levels of gluconeogenesis genes, glucokinase and HNF-4α. HNF-4α mediates glucose metabolism in the liver by controlling the transcription of both G6Pase and glucokinase, which catalyze the last and first rate-limiting step in gluconeogenesis and glycolysis, respectively [23]. Thus, the suppression of G6Pase and glucokinase mRNA levels by MAEA overexpression may be attributed to HNF-4α attenuation. The expression of other gluconeogenic enzymes such as PEPCK and FBPase was also suppressed by MAEA overexpression. Therefore, MAEA may be a candidate gene for the suppression of gluconeogenesis. However, this study presents some limitations. One limitation is that we did not directly demonstrate the suppression of gluconeogenesis genes and glucokinase mRNA levels *via* attenuation of HNF-4α by MAEA overexpression. Further studies are needed to confirm these findings.

Among antidiabetic drugs, biguanide acts on the liver and attenuates gluconeogenesis [24,25]. The effect of MAEA on the liver is similar to that of biguanide. The mechanisms of biguanide on diabetes mellitus have not been fully explained. Thus, MAEA might help explain how biguanide suppresses gluconeogenesis. Furthermore, studies on MAEA may lead to the identification of new mechanisms and new potential drugs for the treatment for type 2 diabetes mellitus.

In summary, MAEA, one of the type 2 diabetes mellitus-susceptibility genes in the Japanese population, is ubiquitously expressed in mouse tissues. MAEA overexpression in hepatocytes suppressed the expression of gluconeogenesis and HNF-4α genes. MAEA may be a new therapeutic target for the treatment of diabetes mellitus.

Conflicts of interest

None.

Acknowledgements

We thank Dr. J Miyazaki for kindly providing the CA promoter. This study was supported by JSPS KAKENHI Grant Number JP15K18939 (K. S.), and Osaka Ohtani University Research Foundation (K. S.).

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