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**Original article**

# Transcription factors regulate Forkhead box O1 gene promoter activity in pancreatic β-cells

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***Background:*** Transcription factors of the Forkhead box O (Fox O) family have important roles in cellular proliferation, apoptosis, differentiation, and stress resistance. In pancreatic β-cells, FoxO1 protein plays an important role in β-cells development. The molecular mechanism of transcriptional regulation of basal FoxO1 gene expression in pancreatic β-cells is not fully understood.
***Objectives:*** Explore the potential transcription factors regulating FoxO1 promoter activity using pancreatic β-cell line (RINm5F cells)
***Methods:*** Promoter screening method, luciferase reporter gene analysis, transient expression assay system, and deletion analysis of a -974/-18 bp 5' upstream region of the mouse FoxO1 gene were used in this study.
***Results:*** An inhibition domain (-974/-321) and an activation domain (-321/-18) was identified through deletion analysis of a -974/-18 bp 5' upstream region of the mouse FoxO1 gene. Using the promoter screening method, several transcription factors were selected. Luciferase reporter studies showed that these factors could regulate FoxO1 promoter activity in RINm5F cells. Among these factors, cAMP response-element binding protein (CREB) could positively regulate FoxO1 promoter activity. Signal transducer and activator of transcription 1 (STAT1) played a negative role on FoxO1 promoter. In addition, ETS oncogene family member Elk-1 did not affect the FoxO1 promoter activity.
***Conclusion:*** Two transcription factors (CREB and STAT1) could effectively regulate the mouse FoxO1 gene promoter activity.

***Keywords:*** CREB, FoxO1, promoter activity, STAT1

Transcription factors of the Forkhead box O (Fox O) family have important roles in cellular proliferation, apoptosis, differentiation, and stress resistance. FoxO proteins also play important roles in metabolism of complex organisms. FoxO1 is the most abundant isoform in liver, adipose tissue, and β-cells, and is conventionally viewed as a regulator in glucose and lipid production in liver, food intake in hypothalamus, and cell differentiation in preadipocytes, myoblasts and vascular endothelium [1]. In pancreatic β-cells, the expression pattern of FoxO1 is similar to that of the pancreatic duodenal homeobox-1 (Pdx1), NK-related homeobox 2.2 (Nkx2.2), and paired box gene 4 (Pax4), transcription factors known to be critical for β-cells development [2-4]. Previous studies indicated that FoxO1 inhibited β-cells growth (proliferation) through suppression of Pdx1 [1, 5, 6]. In addition, recent studies revealed that FoxO1 may be involved in the insulin/insulin like growth factor-1 (IGF-1)/insulin receptor substrate (IRS)-2 pathway to regulate β-cells function, especially the glucose-stimulated insulin secretion (GSIS) [7-10]. While majority of FoxO1-related research focuses on the FoxO1 function, the underlying mechanisms that govern FoxO1 gene transcription are largely unknown. Especially, although FoxO1 plays an important role in β-cells function and insulin secretion, no study of FoxO1 expression at the transcriptional level has been performed in pancreatic β-cells.

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In this study, we investigated the transcriptional regulation of FoxO1 promoter in pancreatic $\beta$-cells, using a 1-kilobase mouse FoxO1 gene 5'-flanking region (-974/-18 bp) linked to luciferase reporter gene in pancreatic $\beta$-cell line (RINm5F cells).

## Materials and methods

### *Reagents*

PrimeSTARTM HS DNA Polymerase, Restriction Enzymes (*Xho*I and *Hin*dIII) and T4 DNA Ligase were purchased from TaKaRa (Otsu, Japan). Luciferase reporter plasmids (pGL3-basic) and Luciferase reporter assay system were purchased from Promega (Madison, USA). LipofectAMINE 2000 reagent was purchased from Invitrogen Life Technologies (Grand Island, USA). Plasmid extraction kit and DNA gel extraction kit were purchased from Qiagen (Hilden, Germany). RPMI 1640 and fetal bovine serum (FBS) were purchased from GIBCO (Burlington, USA).

### *Cell line and cell culture*

Rat insulinoma cell line RINm5F was obtained from ATCC and grown in RPMI 1640 medium supplemented with 10% FBS, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100U/mL penicillin and 100 μg/mL streptomycin. The cells were incubated at 37°C in humidified atmosphere containing 95% air and 5% $CO_2$.

### *Searching for transcription factors*

The potential transcription factor binging sites in mouse FoxO1 gene promoter were obtained by screening promoter region (937bp) with two computer programs called Matrix Search for Transcription Factor Binding Sites (MATCH) and Pattern Search for Transcription Factor Binding Sites (PATCH) (provided by Transfac® Professional 7.0, MA, USA). Three transcription factors (cAMP response-element binding protein (CREB), Signal transducer and activator of transcription 1 (STAT1) and ETS oncogene family member (Elk-1) were selected in the search results. These factors were all expressed in pancreatic $\beta$-cells with high matching scores.

### *Plasmid construction*

Luciferase reporter constructs containing the mouse FoxO1 promoter (-974/-18), (-751/-18), (-567/-18), (-321/-18), (-193/-18) and (-131/-18) were prepared using pGL3-basic vector that contained firefly luciferase cDNA. The mouse FoxO1 promoter region was PCR-amplified from the mouse genomic DNA with appropriate primers and using standard polymerase chain reaction (PCR) conditions. The upstream and downstream PCR primers contained *Xho*I and *Hin*dIII restriction sites respectively. The following primers were used: upstream primers, from -974, 5'-AATGCTCGAGCTTATCTCCTTTTGCGACTTC-3'; from -751, 5'-AATGCTCGAGAGGTGCTTGTGCAACAGTGT-3'; from -567, 5'-AATGCTCGAGCCAGGGCAGATGCCAGCTT-3'; from -321, 5'-AATGCTCGAGTTTGCCTCCTAGCAATCC-3'; from -193, 5'-AATGCTCGAGCCCCACCAACCTACTGCC-3'; from -131, 5'-AATGCTCGAGCTGCTTCGGCGGAGACTT-3'; and downstream primer from -18, 5'-AATCAAGCTTCCCGTCTTACGGGATCTGC-3'. The PCR amplifications were performed using a thermocycler (Stratagene, California, USA) under the following conditions: 94°C for five minutes (one cycle); 98°C for 10 seconds, 58°C for 15 seconds, 72°C for one minute (30 cycles); and final extension of 10 minutes at 72°C. Deletion constructs containing various promoter regions of FoxO1 were named pFoxO1 (-974/-18), pFoxO1 (-751/-18), pFoxO1 (-567/-18), pFoxO1 (-321/-18), pFoxO1 (-193/-18), and pFoxO1 (-131/-18), relative to the transcription start site of the FoxO1 gene. Restriction enzyme digestions and direct DNA sequencing (Invitrogen Life Technologies, Shanghai, China) were performed to confirm the proper sequence of all constructs. Plasmids expressing CREB, STAT1, and Elk-1 were described previously [11].

### *Cell transient transfection and luciferase assay*

FoxO1 promoter activity was assessed in RINm5F cells using the FoxO1 promoter luciferase reporter construct. We used a plasmid containing the β-galactosidase gene driven by the cytomegalovirus promoter (Clontech Laboratories, Palo Alto, USA) as an internal control. RINm5F cells were transiently co-transfected with two plasmids (1.6 μg Luciferase reporter plasmid and 0.1 μg β-galactosidase plasmid) or three plasmids (0.8 μg Luciferase reporter plasmid, 0.8 μg expression plasmid and 0.1 μg β-galactosidase

plasmid) using the LipofectAMINE 2000 regent. The day before transfection, RINm5F cells were plated into 12-well cell culture plates at a density of $7.5x10^5$ cells/well. Transfections were performed following the manufacturer's protocol. Four hours after transfection, the medium was removed by aspiration and replaced with normal culture media containing 10% FBS and antibiotics. Thirty-six hours after transfection, cells were washed with PBS and lysed using 1 x passive lysis buffer. The cell extracts were prepared for luciferase determination according to the protocol of the luciferase reporter assay system. Luciferase activity was measured with a luminometer (TD-20/20, Turner Designs, CA, USA) using the luciferase assay system. β-galactosidase activity was detected to normalize any variations in the transfection efficiency. Each experiment was performed in triplicate and repeated at least three times.

### *Statistical analysis*

Comparisons were performed using Student's *t* test between two groups, or ANOVA in multiple groups. Results are presented as means standard deviation (SD). One-way ANOVA plus Tukey's post hoc comparison was used to calculate the significant difference between experimental and control groups. A p-value of less than 0.05 was considered to be statistically significant.

## Results

### *Basal FoxO1 promoter activity*

Deletion mutants of mouse FoxO1 promoter-driven luciferase reporter gene constructs and control pGL3-basic vector were transiently transfected into RINm5F cells for 36 hours, and relative luciferase activity (ratio of firefly luciferase to β-galactosidase) of each reporter gene construct was measured as shown in **Figure 1**.

The -321/-18 bp region gave the highest level of FoxO1 promoter activity. The serial promoter deletions from -974bp to -321bp showed a six-fold increase in promoter activity, consistent with the notion that this region contains inhibitory domains. The deletions from -321 to -18bp caused about 60% loss of luciferase activity. This indicated that the region between -321 and -18bp contains activation domains for basal FoxO1 expression in *β*-cells.

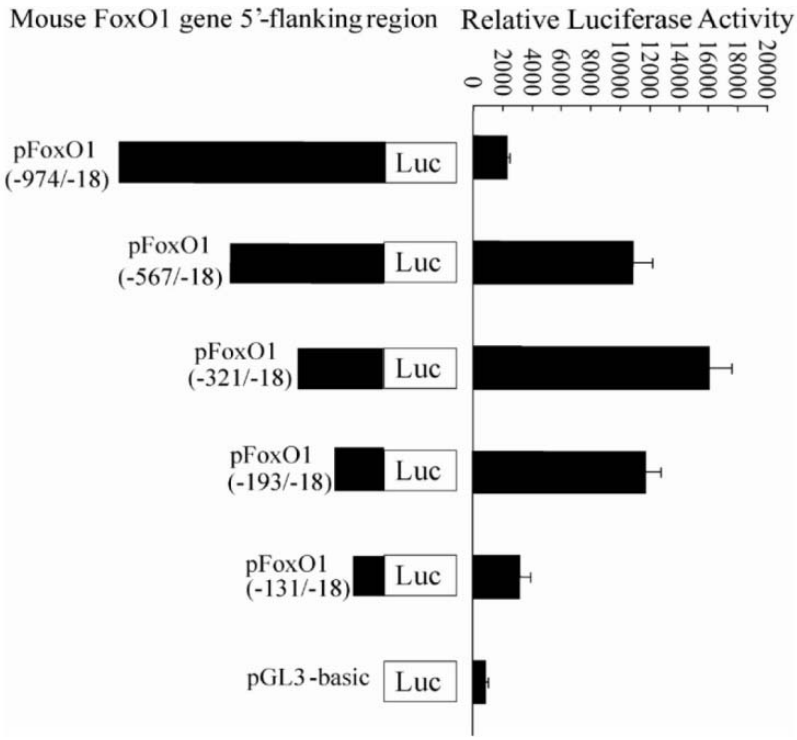


**Figure 1.** Deletion analysis of the mouse FoxO1 promoter in pancreatic *β*-cells. Relative luciferase activities were expressed as mean SD. The experiments were performed in triplicate and repeated three times independently. Serial deletion mutants demonstrated the significance of the -321-bp flanking region for basal promoter activity. Luc=luciferase gene; +1=transcription start site

***Up-regulation by CREB***

The CREB can bind to a few thousand gene promoters [12]. By transiently co-transfecting luciferase reporter construct containing FoxO1 promoter and CREB expression plasmid into RINm5F cells for 36 hours, we measured the relative luciferase activity of each reporter gene construct. **Figure 2** shows relative luciferase activities (ratio of firefly luciferase to β-galactosidase). Interestingly, CREB increased reporter gene activity to 1.69 fold compared with the control vector PCMV3.0b.

***Inhibition by STAT1***

The Janus kinase (JAK)-STAT signaling pathway can be activated by various families of cytokines. Members of STAT family are latent cytosolic transcription factors with SH2 domains that are phosphorylated by JAKs upon binding to the receptor, enabling them to dimerize and enter the nucleus where they bind specific regulatory sequences to activate or repress transcription of target genes. FoxO1 promoter region contains predicted STAT1 binding sites. To investigate the role of STAT1 on FoxO1 promoter activity in pancreatic $\beta$-cells, we co-transfected STAT1 expression plasmid and luciferase reporter construct containing FoxO1 (-974/-18) promoter or into RINm5F cells. **Figure 3** shows relative luciferase activity for control and STAT1 group. Interestingly, STAT1 decreased the activity of FoxO1 promoter by 12%.

***Effect of ETS oncogene family member (Elk-1)***

Based on sequence analysis, FoxO1 promoter region (-974/-18) contains predicted consensus binding sites for Elk-1 both in activation domain and inhibition domain. Although Elk-1 binding sites exist in the FoxO1 promoter, whether this cis-acting factor participates in the regulation of FoxO1 expression has not been reported so far. To determine whether Elk-1 can regulate FoxO1 expression in pancreatic -cells, we co-transfected RINm5F cells with luciferase reporter construct containing FoxO1 promoter and Elk-1 expression plasmid. **Figure 4** shows relative luciferase activity for control and Elk-1 group. Interestingly, Elk-1 did not affect the FoxO1 (-974/-18) promoter activity.

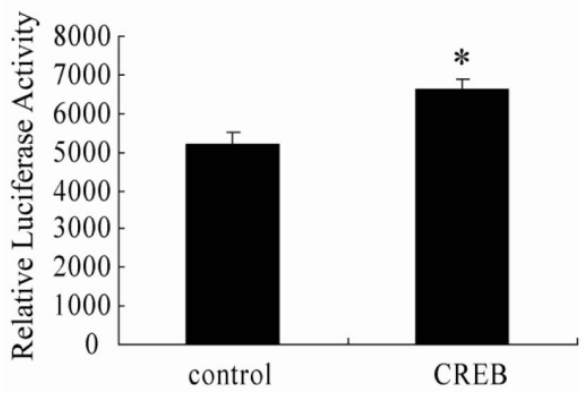


**Figure 2.** Relative luciferase activities for control and CREB groups (mean±SD). The experiments were performed in triplicate and repeated three times independently. *significant difference ($p < 0.5$), compared with the control, using one-way ANOVA plus Tukey's post hoc comparison

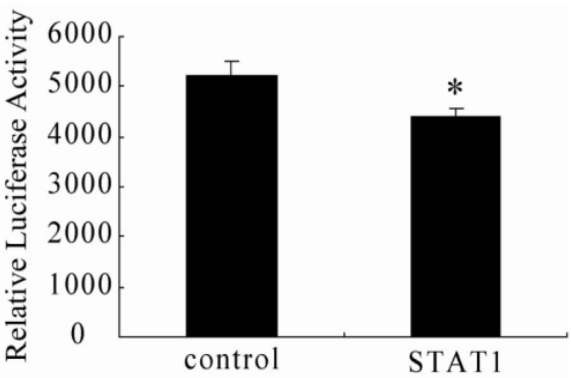


**Figure 3.** Relative luciferase activity for control and STAT1 group (mean±SD). The experiments were performed in triplicate and repeated three times independently. *significantly difference ($p < 0.5$), compared with the control

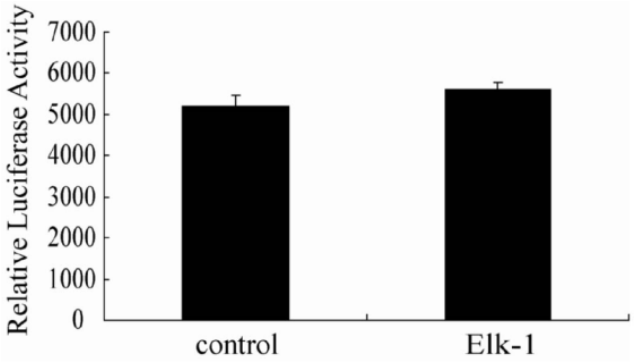


**Figure 4.** Relative luciferase activity for control and Elk-1 group (mean±SD). There is no significant difference ($p < 0.05$) between control and Elk-1 group

## Discussion

Forkhead box O1 (FoxO1) is a member of the mammalian forkhead transcription factors in the O class. It can play a significant role during normal cellular function as well as during progressive disease. Progressive interest in FoxO1 has shown that this transcription factor can promote cell proliferation as well as cell death [13]. Clinical and experimental studies have exemplified the role of FoxO proteins in cellular metabolism and disorders such as diabetes mellitus. FoxO protein can stimulate the insulin-like growth factor binding protein-1 promoter by binding to the insulin-responsive sequence [14]. Insulin and insulin-like growth factor-1 can suppress FoxO1 protein activity through activation of Akt, also known as protein kinase B [14, 15]. Several studies with FoxO1 have also shown that over-expression of this transcription factor in skeletal muscles of mice can lead to reduced skeletal muscle mass and poor glycemic control [16], illustrating that activation of FoxO1 proteins may impair cellular energy reserves. In addition, in a previous study [17], we proved that dexamethasone markedly increased FoxO1 mRNA and protein expression and decreased FoxO1 phosphorylation through the Akt pathway, which resulted in an increase in active FoxO1 in RINm5F cells and isolated rat islets. Many researches implied the important role of FoxO1 in pancreatic $\beta$-cells dysfunction and diabetes development. It is critical to know how basal and induced FoxO1 expression is regulated in -cells.

FoxO1 expression is regulated by the binding of specific transcription factors to cis-acting elements on the FoxO1 promoter. In this study, we investigated the role of three transcription factors on FoxO1 gene promoter activity in RINm5F cells and found that CREB can positively regulate FoxO1 promoter activity. On the contrary, STAT1 plays a negative role on FoxO1 promoter.

The CREB can be regulated by multiple signaling pathways. These includes a variety of signaling cascades such as protein kinase C (PKC), calmodulinkinase (CaMK), extra cellular signal-regulated kinases 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p38MAPK) [18-20]. The present study showed that over-expression of CREB could up-regulate the mouse FoxO1 promoter activity in RINm5F cells. This suggested that stimuli, which activate this pathway, would possibly induce FoxO1 expression followed by -cells dysfunction.

The STAT family encompasses seven mammalian members, designed STAT1, -2, -3, -4, -5a, -5b, and -6. Mitsuru et al. [21] showed that STAT family member STAT5b played a critical role in the growth hormone-regulated inhibition of IGF binding protein-1 gene transcription by impairing the actions of FoxO1 on the IGF binding protein-1 promoter. In the present study, we found that the STAT family member STAT1 played a negative role on FoxO1 promoter activity in RINm5F cells. This finding raised the possibility that activated STAT1 might protect -cells function through inhibiting FoxO1 expression. However, the role of potential STAT1 binding site involved in FoxO1 promoter remains to be determined in further experiments.

ETS family is defined by a highly conserved DNA binding domain that binds to the core consensus sequence GGAA/T and consists of many evolutionarily conserved transcription factors [22]. Transcription factor Elk-1 is a prominent substrate of phosphorylated mitogen-activated protein kinases

(MAPKs) [23]. Kasza et al. [24] indicated that Elk-1 could bind to the serum response factor (SRF) promoter and subsequent regulation of SRF expression occurred in a ternary complex-dependent manner. Although FoxO1 promoter region contains predicted Elk-1 binding site, it has not been clarified whether it really participates in the regulation of FoxO1 expression. Our study showed that over-expression of Elk-1 did not affect the FoxO1 promoter activity, suggesting that Elk-1 might do not regulate the FoxO1 gene in transcription level.

## Conclusion

In pancreatic $\beta$-cells, CREB, STAT1, and Elk-1 transcription factors played different roles in the regulation of FoxO1 gene promoter activity through over-expression studies. This may be helpful for better understanding the transcriptional regulation of FoxO1 in pancreatic $\beta$-cells. In addition, these transcriptional regulators of FoxO1 expression may be potential targets for the prevention of $\beta$-cells damage mediated by dexamethasone.

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