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Article

Overexpression of FoxO1 Causes Proliferation of Cultured Pancreatic β Cells Exposed to Low Nutrition†

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ABSTRACT: Multiple lines of evidence have shown that the functional defect of pancreatic β cells is the root cause of type 2 diabetes. FoxO1, a key transcription factor of fundamental cellular physiology and functions, has been implicated in this process. However, the underlying molecular mechanism is still largely unknown. Here, we show that the overexpression of FoxO1 promotes the proliferation of cultured pancreatic β cells exposed to low nutrition, while no change in apoptosis was observed compared with the control group. Moreover, by using two specific inhibitors for PI3K and MAPK signaling, we found that FoxO1 might be the downstream transcription factor of these two pathways. Furthermore, a luciferase assay demonstrated that FoxO1 could regulate the expression of *Ccnd1* at the transcription level. Collectively, our findings indicated that FoxO1 modulated by both MAPK and PI3K signaling pathways was prone to cause the proliferation, but not the apoptosis, of pancreatic β cells exposed to low nutrition, at least partially, by regulating the expression of *Ccnd1* at the transcription level.

FoxO proteins, mainly consisting of FoxO1, FoxO3a, and FoxO4, are transcription factors of the forkhead family characterized by the presence of a "winged-helix" DNA-binding domain termed the forkhead box (*1*, *2*). These proteins are strikingly conserved from worms to humans (*3*). Multiple molecular mechanisms, including growth factors and insulin, regulate FoxO transcriptional activity. FoxO proteins have also been shown to critically control a variety of fundamental cellular processes, including metabolism, cell differentiation, proliferation, apoptosis, cell cycle arrest, and other reactions in response to cellular stress (*4*).

The pancreatic β cell is one of the most important target tissues of FoxO signaling (*5*). As is well-known, this cell secretes insulin in response to hyperglycemia, contributing to glucose homeostasis as well as the coordination of metabolism in the body (*4*, *6*). In the adult pancreas, FoxO1 is exclusively expressed in human islet β cells (*7*). Of the three subtypes, FoxO1 is most predominantly found in isolated mouse islets and the βTC-3 β cell. In mouse islets, FoxO3 is expressed at a lower level while FoxO4 is conspicuously undetectable (*8*). Depending on different cell conditions, FoxO1 shuttles between the cytosol and nucleus. It has been reported that an elevated level of expression of the insulin receptor results in accelerated exclusion of FoxO1 from the nucleus to the cytosol under a niche of low nutrients. Generally, inactivated FoxO1 is phosphorylated and always remains in cytosol, while dephosphorylated FoxO1 is activated and performs its functions after being shuttled into the nucleus. A constitutively active FoxO1 has been deemed to inhibit proliferation and promote apoptosis of pancreatic β cells in vitro and in vivo (*9*); however, here we find that overexpression of FoxO1 caused proliferation, but not apoptosis, in cultured pancreatic β cells exposed to low nutrition.

In this study, we explore the underlying mechanism of FoxO1 which induced proliferation in the pancreatic β cells exposed to low nutrition in vitro. We found that low nutrition tended to inhibit FoxO1 through both MAPK and PI3K signaling. In turn, inactivated FoxO1 promoted cell proliferation, at least partially, via transcriptionally regulating the expression of *Ccnd1* in cultured pancreatic β cells.

MATERIALS AND METHODS

Chemicals. The selective MEK inhibitor PD98059 and the selective PI3K inhibitor LY294002 were purchased from Sigma (St. Louis, MO). The mouse monoclonal antibody against the HA tag was obtained from Beyotime Co. Ltd., and the mouse p53 monoclonal antibody was purchased from Sigma. The Access Quick RT-PCR System and luciferase assays were purchased from Promega (Madison, WI). Glucose was obtained from Sigma, and the insulin antibody was purchased from Abcam (Cambridge, MA). The RAT/MOUSE insulin ELISA kit was ordered from Linco Research (St. Charles, MO).

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Plasmids and Cells. Hemaglutinin-tagged FoxO1 and FoxO1ADA (constitutively active FoxO1, CAFoxO1) in the pCMV5 vector were gifts from D. Accili (Columbia University, New York, NY). The dominant-negative FoxO1 (DNFoxO1) that retains the DNA-binding domain (amino acid residues 1−256) but lacks the transactivation domain was cloned into the multiple-clone site of pcDNA3.1. For luciferase assays, the promoter of *Ccnd1*, was cloned and inserted into the multiple-clone site of pGL3-Basic (Promega), and the reporter was termed *Ccnd1*-luc. The plasmid of pRL-SV40 (Promega) was a Renilla reporter constructed as an internal control for assessing the efficiency of transfection. NIT-1 cells ordered from ATCC were cultured in low-glucose (5 mmol/L) Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with or without (low nutrition) 10% fetal bovine serum and 100 units/mL streptomycin/penicillin at 37 °C in a humidified atmosphere of 5% CO_2.

BrdU Assay. Cells were fixed with 4% paraformaldehyde in vitro for 30 min at 4 °C and then incubated in HCl (1 N) for 10 min on ice to break open the DNA structure of the labeled cells, followed by a 10 min incubation in HCl (2 N) at room temperature (RT), before being moved to an incubator for 20 min at 37 °C. Immediately after the acid wash, borate buffer (0.1 M) was added to buffer the cells for 12 min at RT. The mixture was incubated in 0.1 M PBS (pH 7.4), 1% Triton X-100, glycine (1 M), and 5% normal goat serum for 1 h and then incubated at RT overnight with anti-BrdU. Samples were then treated with a variety of secondary antibodies to visualize the anti-BrdU-labeled cells.

MTT Assay. For the MTT assay, NIT-1 cells (pancreatic β cells) were cultured in six-well plates and treated with different factors. After 24 h, NIT-1 cells were seeded in 96-well plates at a density of 5×10^3 cells/well. A total of 20 μL of MTT [3-(4,5)-dimethylthiazozy1-3,5-diphenyltetrazolium bromide] was added to each well of 96-well plates 24 h later at a concentration of 5 mg/mL. The cells were incubated at 37 °C for 4 h; the supernatant was completely discarded, and 150 μL of DMSO was added to each well. Finally, the absorbance of each well at 492 nm was measured with a monitor of the enzyme-linked immunosorbent.

TUNEL Staining. TUNEL staining was performed using the DeadEnd Colorimetric TUNEL System (Promega). Cells were fixed with a 4% paraformaldehyde solution at RT for 25 min. Then cells were permeabilized when the slides were immersed in a 0.2% Triton X-100 solution in PBS at RT for 5 min. Excess liquid was removed, and the cells were covered with 100 μL of equilibration buffer at RT for 10 min. Next, the equilibrated areas were blotted with tissue paper, and 100 μL of rTdT reaction mix was added on a slide. The slides were incubated at 37 °C for 60 min inside a humidified chamber to allow the end labeling reaction to occur. The reactions were terminated when the slides were immersed in 2×SSC in a Coplin jar for 15 min at RT. The endogenous peroxidases were blocked when the slides were immersed in 0.3% hydrogen peroxide in PBS for 5 min at RT; 100 μL of diluted horseradish peroxidase (HRP) was added to each slide, and the slides were incubated for 30 min at RT. Finally, 100 μL of a DBA solution was added to each slide, and the slides were developed for ~10 min. Slides were mounted in 100% glycerol, and staining was observed with a light microscope as described previously (*10*).

Western Blotting. The cells were harvested, washed with PBS, and lysed using RIPA buffer with protease inhibitors for 30 min on ice. The total protein (20 μg) was analyzed via 12% sodium dodecyl sulfate−polyacrylamide gel electorphoresis (SDS−PAGE) and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in Tris-buffered saline Tween 20 (TBS-T) with 5% bovine serum albumin (BSA) for 1 h at RT. It was then incubated with the primary antibodies overnight at 4 °C. The membrane was finally incubated with the goat anti-rabbit HRP-conjugated secondary antibodies for 1 h at RT. Immunoreactive bands were identified using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and exposed to X-rays film (Kodak, Rochester, NY) as described previously (*11*). Measurements were taken in triplicate using β-actin as an internal control.

Semiquantitative RT-PCR. To check the mRNA levels of FoxO1 and the genes related to apoptosis, semiquantitative RT-PCR was performed in triplicate. Total RNA was extracted with Trizol Reagent (Invitrogen), and cDNA was synthesized with the AccessQuick RT-PCR System (Promega). Semiquantitative RT-PCR was performed according to the protocol using the following primers: FoxO1, 5′-ACCGCCAAACAVCCAGTCT-3′ (sense) and 5′-CACCCATCCTACCATAGCC-3′ (antisense); Actb, 5′-GAAATCGTGCGTGACATCAAAG-3′ (sense) and 5′-TGTAGTTTCATGGATGCCACAG-3′ (antisense); Casp3, 5′-TCTGACTGGAAAGCCGAAACT-3′ (sense) and 5′-CCAGGTGCTGTAGAGTAAGCATA-3′ (antisense); Bcl2l11, 5′-GGAGATACGGATTGCACAGGA-3′ (sense) and 5′-TTCAGCCTCGCGGTAATCATT-3′ (antisense); Bad, 5′-TGAGCCGAGTGAGCAGGAA-3′ (sense) and 5′-GCCTCCATGATGACTGTTGGT-3′ (antisense); Bcl2, 5′-GTCGCTACCGTCGTGACTTC-3′ (sense) and 5′-CAGACATGCACCTACCCAGC-3′ (antisense). β-Actin was used for normalization. The PCR amplification profile was as follows: 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min.

Luciferase Assay. For the *Ccnd1*-luc expression assays, NIT-1 cells were seeded at a density of 1×10^5 cells/well in six-well

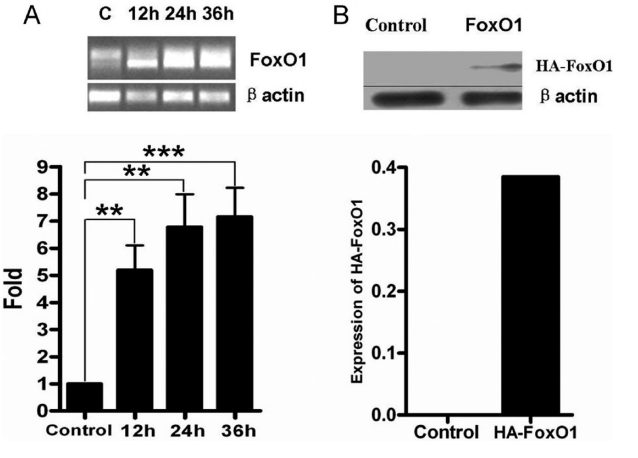

FIGURE 1: Overexpression of FoxO1 detected by RT-PCR and Western blotting in NIT-1 cells. (A) The mRNA level of overexpressed FoxO1 was detected by RT-PCR after 12, 24, and 36 h, and the mRNA of cells that were not transfected with FoxO1 acted as a control and β-actin as an internal control. (B) As HA-FoxO1 was overexpressed in NIT-1 cells, an anti-HA monoclonal antibody was used to detect the expression of ectopic FoxO1 at the protein level by Western blotting 24 h later. An asterisk means $p < 0.05$.

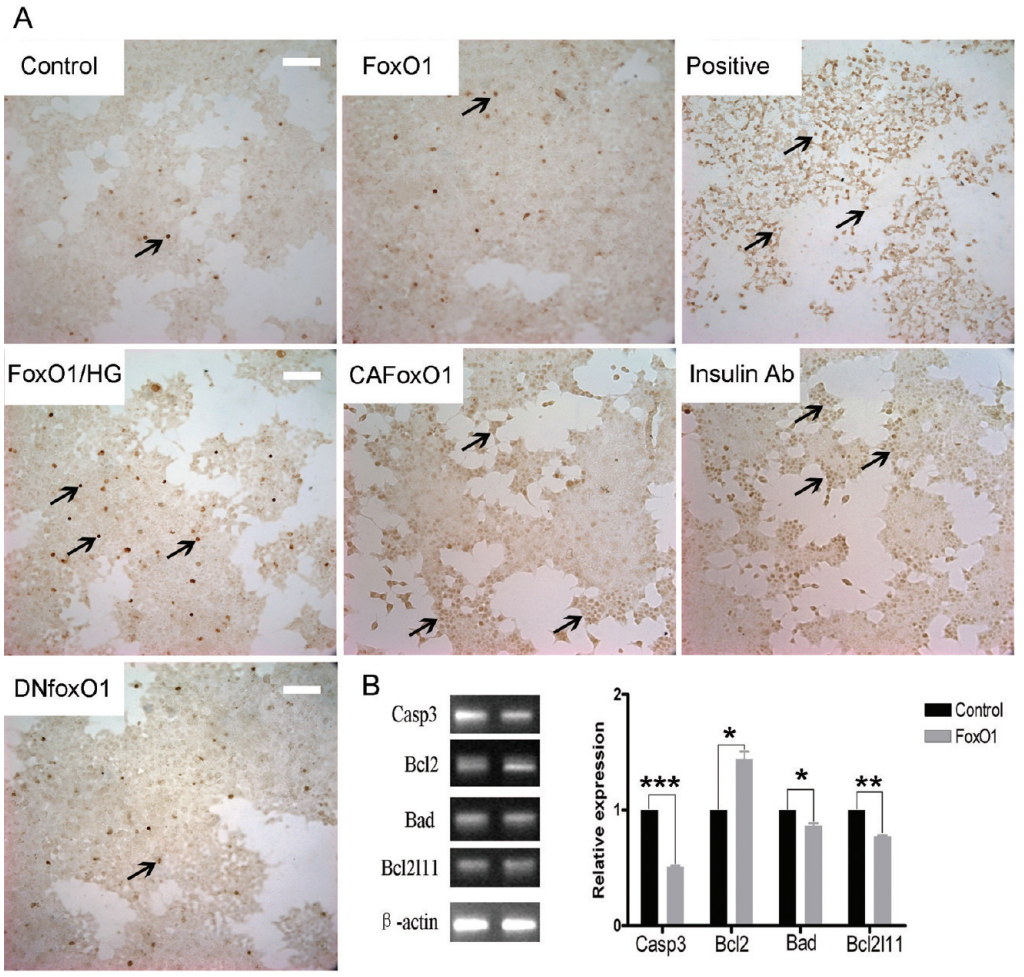

FIGURE 2: Overexpression of FoxO1 does not affect the apoptosis of cultured pancreatic β cells. (A) TUNEL staining was employed to check the apoptosis of NIT-1 cells. The brown spots represent the apoptotic cells, and no significant difference was observed between the control group and the FoxO1 treatment group as well as the DNFoxO1 group (middle lane). However, the CAFoxO1, FoxO1/HG, and insulin Ab groups (middle panel) possessed significantlymore apoptotic cells than the control group. The positive group was treated with DNase I. (B) The mRNA levels of the four genes mainly involved in apoptosis were detected by RT-PCR 24 h after FoxO1 was transfected. Casp3, Bcl2l11, and Bad were downregulated, and Bcl2 was upregulated remarkably. An asterisk means $p < 0.05$.

plates and cotransfected with 1 μg of reporter *Ccnd1*-luc together with 1 μg of the relevant expression vector, or the corresponding empty vector. For experiments conducted in the exponentially growing cells, luciferase activity was measured 24 h after transfection. Normalization was achieved by cotransfecting 0.3 μg of pRL-SV40, a Renilla reporter construct used as an internal control for the efficiency of transfection. Luciferase and Renilla activities were measured according to the Promega protocol. The reporter luciferase activities were normalized versus the Renilla activity (*12*).

Measurement of Insulin Secretion. The NIT-1 cells were seeded in a six-well plate and transfected with various plasmids until the confluence of these cells was up to 70%. After 24 h, the culture medium was collected, cells were removed by centrifugation, and the concentration of insulin was measured with a rat/mouse insulin ELISA kit. The protein of these cells was collected to normalize the insulin level. The concentration of proteins was measured using the Bradford assay.

Statistical Analysis. Data are presented as means ± the standard deviation (SD) or means ± the standard error of the mean (sem). Statistical significance for comparisons between groups was determined using a Student's paired *t* test in statistical software Prism 4.0, and $P < 0.05$ was taken to be the level of significance.

RESULTS

Overexpression of FoxO1 Does Not Affect the Apoptosis of Cultured Pancreatic β Cells. To preliminarily assess the effects of FoxO1 on pancreatic β cells in vitro, we transiently transfected NIT-1 cells exposed to low nutrition with the plasmids of HA-FoxO1, CAFoxO1, and DNFoxO1. The expression of HA-FoxO1 was detected by RT-PCR and Western blotting, as illustrated in Figure 1. As described previously, constitutive activation of FoxO1 may result in apoptosis of pancreatic β cells. Therefore, we employed TUNEL staining to detect the state of cells. Inconsistent with our expectation, we found that the overexpression of FoxO1 and DNFoxO1 did not notably influence the apoptosis of NIT-1 cells exposed to low nutrition, although CAFoxO1 led to apoptosis of NIT-1 cells, as depicted in Figure 2A (middle lane). NIT-1 cells that were under conditions of high glucose (25 mmol/L) and insulin antibody encountered apoptosis (Figure 2A, middle panel). To further reveal the molecular mechanism of the result, RT-PCR was performed to test the mRNA levels of four genes (Casp3, Bcl2l11, Bad, and Bcl2) that are closely related to apoptosis (Figure 2B). It has been suggested that Casp3, Bcl2l11, and Bad play essential roles in the execution phase of cell apoptosis and that Bcl2 inhibits the apoptosis via inactivating the caspases which carry

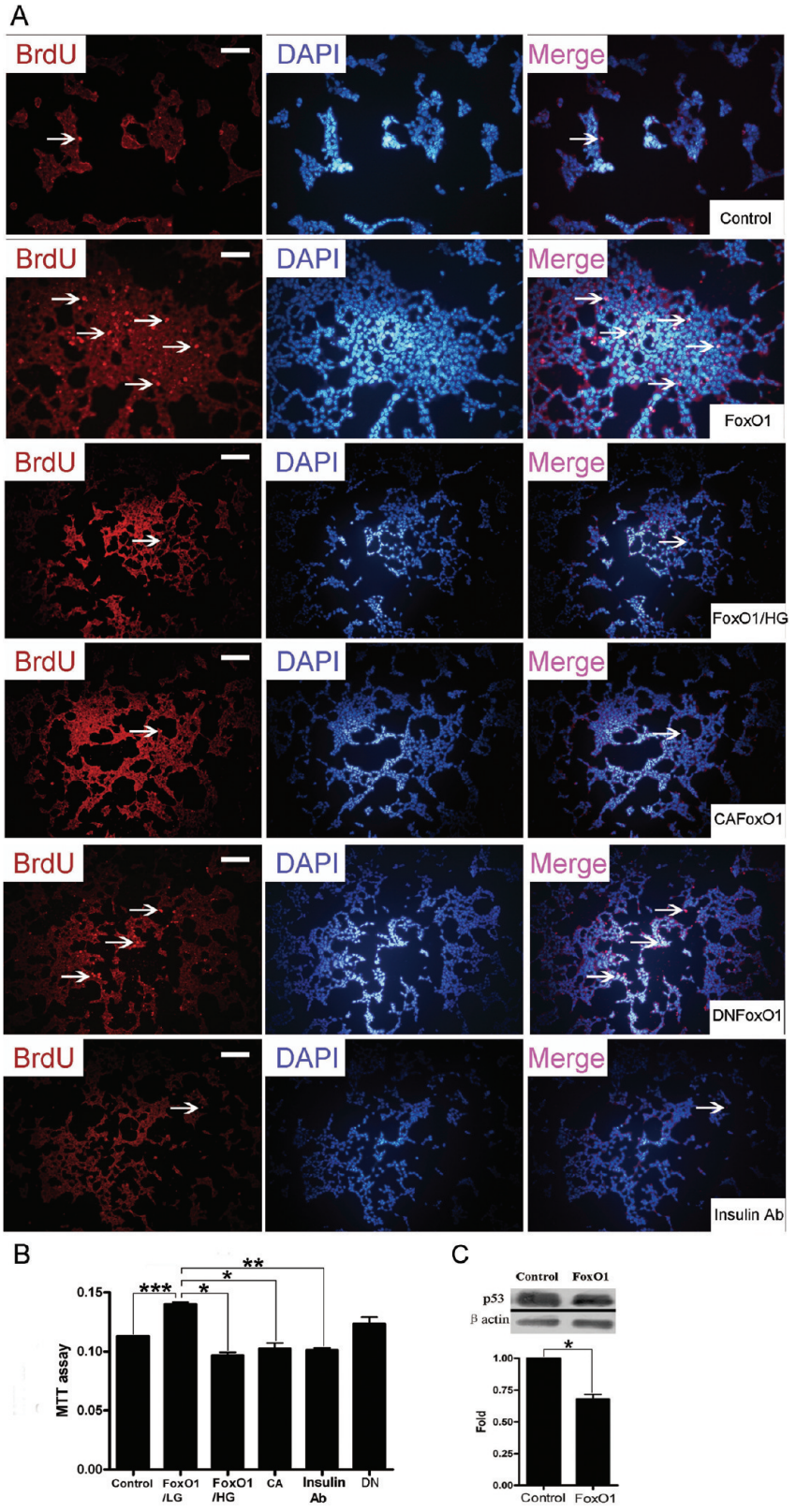

FIGURE 3: Overexpression of FoxO1 results in the proliferation of NIT-1 cells. (A) The proliferation of NIT-1 cells was assessed by the BrdU assay. The nuclei of cells incorporated by BrdU present as red spots; the nuclei were stained with DAPI. In the merged images, the pink spots are the cells incorporated by BrdU (indicated by a white arrow). The cells transfected with Lipofectamine as a control (first panel) and the cells transfected with FoxO1 were more proliferative (second panel) than the control group. Also, the DNFoxO1 group promoted the proliferation of NIT-1 cells, but the CAFoxO1, FoxO1/HG, and insulin Ab groups did not promote the proliferation compared with the control group. (B) To further confirm the proliferation of NIT-1 cells, the MTT assay was subsequently conducted. The cells transfected with FoxO1 displayed significantly higher absorbance than the control, FoxO1/HG, CAFoxO1, and insulin Ab groups. (C) The expression of p53 measured by Western blotting was significantly downregulated by FoxO1 (β-actin also as an internal control). BrdU images from the first panel to the sixth panel represented control, FoxO1, FoxO1/HG, CAFoxO1, DNFoxO1, and the insulin antibody, respectively. An asterisk means $p < 0.05$.

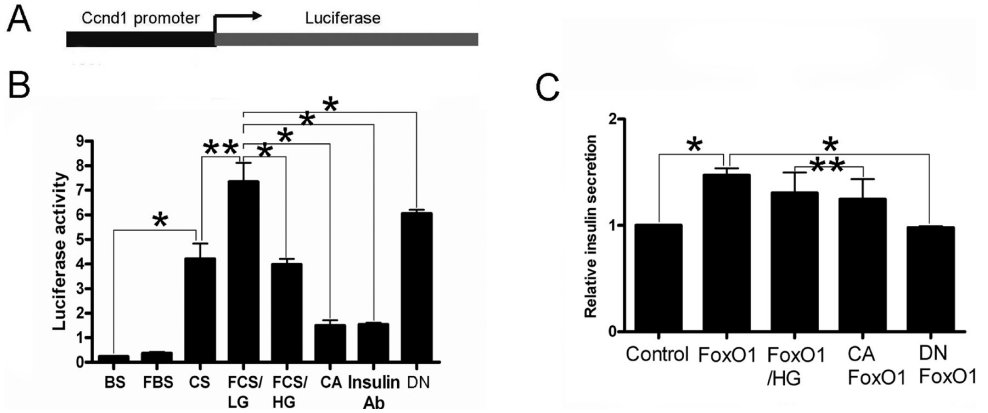

FIGURE 4: FoxO1 regulates the cell cycle of NIT-1 cells and promotes the secretion of insulin. (A) Schematic diagram of the *Ccnd1*-luc vector. (B) HA-FoxO1 and *Ccnd1*-luc were transiently cotransfected into NIT-1 cells, and pRL-SV40 was transfected simultaneously as an internal control. Luciferase activity was measured 24 h post-transfection, normalized to that of Renilla, and compared with the control. The luciferase activity of FoxO1/LG was markedly higher than that of FoxO1/LG, CAFoxO1, and insulin antibody groups, but the DNFoxO1 group was not significantly different from the FoxO1/LG group. Experiments were repeated in triplicate, and the results are expressed as means ± SD: BS, pGL3-basic with pRL-SV40; FBS, FoxO1 with pGL3-basic and pRL-SV40; CS, *Ccnd1*-luc with pRL-SV40; FCS, FoxO1 with *Ccnd1*-luc and pRL-SV40; LG, low glucose; HG, high glucose; CA, constitutively active FoxO1; Insulin Ab, insulin antibody; DN, dominant-negative FoxO1. (C) The level of insulin in the culture medium was measured with the RAT/MOUSE insulin ELISA kit. The cells transfected with FoxO1 secreted significantly more insulin than the control or the cells transfected with DNFoxO1. Insulin levels were normalized by total protein content. An asterisk means $p < 0.05$.

out the process. Stimulated by the overexpression of FoxO1, Casp3, Bcl2l11, and Bad were significantly downregulated and Bcl2 was upregulated, suggesting that FoxO1 is prone to causing cell proliferation. These findings indicate that overexpression of FoxO1 may not influence the apoptosis of cultured pancreatic β cells exposed to low nutrition but probably tends to promote proliferation.

Overexpression of FoxO1 Results in Proliferation of NIT-1 Cells. According to the results from Figure 2, the changes detected in the four genes implied that FoxO1 may cause pancreatic β cell proliferation. Subsequently, the BrdU assay was employed to evaluate the effect of FoxO1 on NIT-1 cell proliferation. In Figure 3A, NIT-1 cells transfected with FoxO1 (second panel) and DNFoxO1 (fifth panel) were significantly more proliferative than the vehicle control (first panel), indicating that the overexpression of FoxO1 promoted the proliferation of NIT-1 cells exposed to low nutrition. The result that DNFoxO1-transfected cells seemed more proliferative is comparable with previous reports (*9*, *13*), but the cells transfected with CAFoxO1 (fourth panel) did not cause proliferation; neither did the cells cultured under the condition of high glucose (third panel) or treated with the insulin antibody (sixth panel). The MTT assay was conducted to further confirm this observation (Figure 3B). On the basis of these two assays, we postulated that FoxO1 may cause proliferation of NIT-1 cells exposed to low nutrition. Additionally, as a repressor of proliferation, the expression of p53 was detected by Western blotting (Figure 3C). Consistent with the cell proliferation, the expression level of p53 in the group with overexpression of FoxO1 was significantly lower than the control. These results suggested that the overexpression of FoxO1 may promote proliferation of cultured pancreatic β cells exposed to low nutrition.

FoxO1 Regulates the Proliferation of Pancreatic β Cells through Ccnd1 Regulation at the Transcription Level. It is known that cyclinD1, encoded by *Ccnd1*, is required for the transition from the G_1 to the S phase and functions as one of the central target genes for proliferative signaling in the G_1 phase. Moreover, the activation of *Ccnd1* at the transcription level represents one of the earliest cell cycle-related events occur during the G_1 to S phase transition, but activation of FoxO1 may repress the expression of *Ccnd1* (*14*, *15*). To explore the underlying mechanism of cell proliferation modulated by FoXO1, we tested the activity of the *Ccnd1* promoter with a luciferase assay in NIT-1 cells exposed to low nutrition, in which FoxO1 was overexpressed. A schematic diagram of the *Ccnd1* promoter-driven luciferase construct is given in Figure 4A. As shown in Figure 4B, the results show that overexpression of FoxO1 strikingly increased the luciferase activity of the *Ccnd1* promoter, implying that overexpression of FoxO1 was able to upregulate the transcriptional activity of the *Ccnd1* promoter. The cells transfected with DNFoxO1 possessed high luciferase activity as well, but the NIT-1 cells that were treated with high glucose and insulin antibody presented a low luciferase activity. These findings indicate that cell proliferation induced by FoxO1 may be mediated, at least partially, by regulation of the expression of *Ccnd1* at the transcription level. It had been reported that activation of FoxO1 inhibits the expression of *Ccnd1* (*14*, *15*) and elevates the level of secretion of insulin (*16*, *17*). Here we found that overexpression of FoxO1 enhanced the luciferase activity of the *Ccnd1* promoter, so we suspected that FoxO1-overexpressing NIT-1 cells may promote the secretion of insulin in an autocrine fashion and inactivate FoxO1. An ELISA was used to measure the concentration of insulin in culture medium. As shown in Figure 4C, overexpression of FoxO1 indeed increased the concentration of insulin.

FoxO1 Is Modulated by both PI3K and MAPK Signaling. To clarify the pathways involved in the process, we first used PD98059, a selective inhibitor for MEK, to determine whether the MAPK signaling mediates the proliferative efforts of FoxO1. The FoxO1, *Ccnd1*-luc, and pRL-SV40 plasmids were cotransfected into NIT-1 cells with serum-free medium which was replaced with serum-free medium with 50 μmol/L PD98059 6 h later. Then another 24 h incubation was required before the activity of the *Ccnd1* promoter was measured. As shown in Figure 5A, the activity of the *Ccnd1* promoter markedly decreased after the MAPK pathway was blocked. Since PI3K

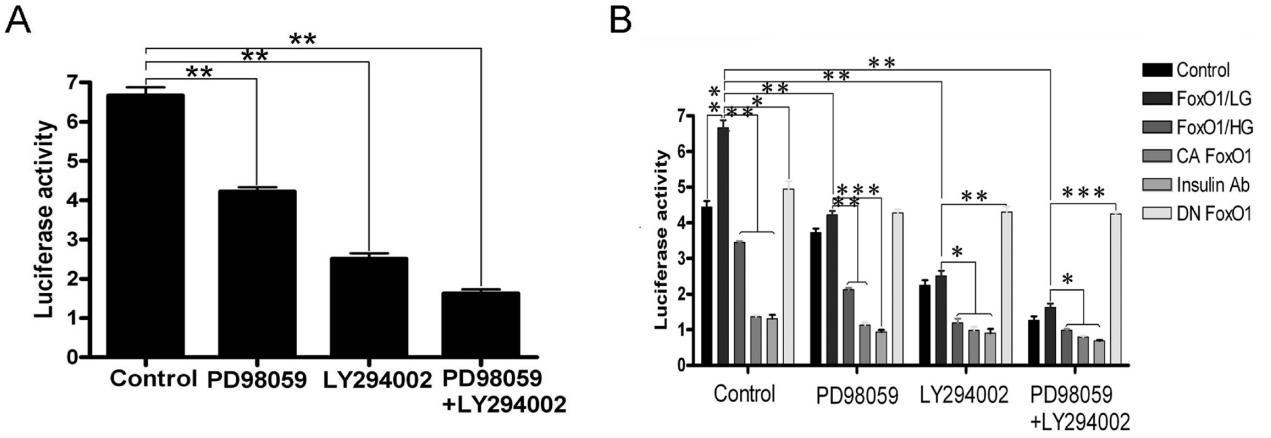

FIGURE 5: FoxO1 is regulated by both PI3K and MAPK pathways. (A) FoxO1 was overexpressed prior to the addition of inhibitors. The activity of the *Ccnd1* promoter was reduced while MAPK and PI3K signaling was inhibited. PD98059 and LY294002 are inhibitors of MEK and PI3K, respectively. (B) Conversely, NIT-1 cells were treated with inhibitors of the signaling in advance. First, these cells were seeded in the medium containing PD98059; the activity of the *Ccnd1* promoter decreased among the FoxO1/LG, FoxO1/HG, CAFoxO1, and insulin antibody groups. However, the overexpression of FoxO1 retained more luciferase activity than other groups, and the activity of luiferase was not affected by DNFoxO1 transfection. LY294002 also resulted in a similar reduction in luciferase activity as well. Significantly, the double inhibition of pathways led to the decline of the activity of the *Ccnd1* promoter with the same trend described previously. An asterisk means $p < 0.05$.

signaling is another canonical pathway for FoxO1, and it is reported that FoxO1 is negatively regulated by AKT activation (*18*, *19*), which is also activated by PI3K, we tried to determine the effect of PI3K signaling on FoxO1 by using LY294002 (50 μmol/L), a selective inhibitor for PI3K. As described previously, LY294002 was added into the serum-free medium for 24 h, and the activity of the *Ccnd1* promoter was measured with the luciferase assay (Figure 5A). Similarly, the promoter activity notably decreased. Therefore, we postulated that the activity of the *Ccnd1* promoter modulated by FoxO1 was mediated by both MAPK and PI3K signaling pathways, and it seems that the PI3K signaling may affect more than MAPK signaling on FoxO1 (Figure 5A). Both of the inhibitors were employed to inhibit these two pathways at the same time; the activity of the promoter was apparently lower than that of a single signaling blocked group. When NIT-1 cells were incubated with inhibitors of MAPK and PI3K individually or simultaneously for 12 h in advance and then cotransfected with FoxO1, *Ccnd1*-luc, and pRL-SV40 plasmids, the activity of luciferase presented the same decreasing trend as before (Figure 5B). While the cells that were under conditions of CAFoxO1 transfection, high glucose, and insulin antibody sustain less luciferase activity than the FoxO1 overexpression group, the luciferase activity of a population of NIT-1 cells that were transfected with DNFoxO1 was not affected. In summary, these findings suggest that FoxO1 as a transcriptional regulator of *Ccnd1* may be modulated by both MAPK and PI3K signaling (Figure 6).

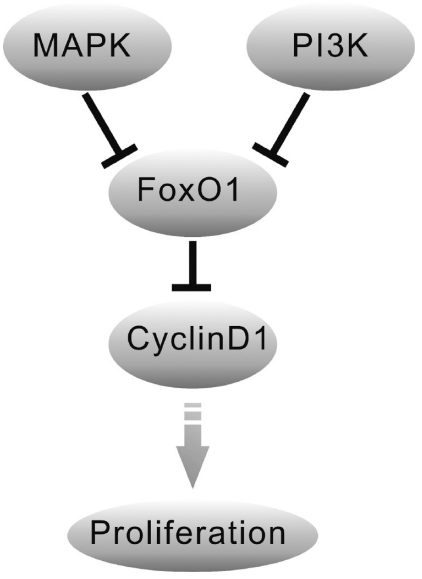

FIGURE 6: Schematic diagram illustrating how FoxO1 modulated by both MAPK and PI3K signaling predisposes cultured pancreatic β cells to cell proliferation, in part, via regulating the expression of *Ccnd1* at the transcription level.

DISCUSSION

Previously, several lines of evidence have shown that constantly activated FoxO1 is competent to inhibit cell proliferation (*20*) and promote apoptosis in pancreatic β cells (*7*, *9*, *21*, *22*). Constitutively active FoxO1 represses cell proliferation via cell cycle arrest at the G_1 phase. Under this condition, the expression levels of a large number of genes, such as $p27^{kip1}$, p21, p57, etc. (*20*), mainly involved in cell cycle progression are all upregulated. Meanwhile, the expression levels of other genes, for example, Bim and Bcl-x_L, closely related to apoptosis are upregulated as well (*9*, *14*). These factors predispose pancreatic β cells to apoptosis. However, in our study, we found that overexpression of FoxO1 in the cultured pancreatic β cells exposed to low nutrition did not induce apoptosis compared with the control group by the TUNEL assay (Figure 2A). Furthermore, the expression levels of four genes were significantly changed; the mRNA levels of Casp3, Bcl2l11, and Bad were markedly downregulated, while the mRNA level of Bcl2 was augmented remarkably (Figure 2B). Since Casp3 is believed to play a central role in the execution phase of cell apoptosis and Bcl2 has been thought to prevent apoptosis by inactivation of the caspases which execute the process, we wonder whether overexpression of FoxO1 would lead to proliferation, but not apoptosis, in NIT-1 cells exposed to low nutrition. Hence, the BrdU assay was conducted to assess cell proliferation. As depicted in Figure 3A, overexpression of FoxO1 promoted proliferation in pancreatic β cells exposed to low nutrition. To further confirm this finding, the MTT assay was employed to count the relative amount of cells. In agreement with the

observation mentioned above, cell proliferation was indeed amplified, after FoxO1 was overexpressed in pancreatic β cells exposed to low nutrition (Figure 3B). It is well-known that p53 induces growth arrest by holding the cell cycle at the G_1 to S phase regulation point and initiates apoptosis if the DNA damage proves to be irreparable. Thus, the protein level of p53 was also checked by Western blotting. As expected, the expression of p53 was obviously downregulated. Collectively, we showed here that FoxO1 does not cause apoptosis; instead, it results in proliferation of pancreatic β cells exposed to low nutrition in vitro.

To elucidate the underlying mechanism of FoxO1 inducing cell proliferation, we subsequently detected the activity of the *Ccnd1* promoter. *Ccnd1*, encoding cyclinD1 which is required for progress of the G_1 to S phase transition, serves as one of the critical target genes for proliferative signaling in the G_1 phase (*23*). Moreover, transcriptional induction of *Ccnd1* represents one of the earliest cell cycle-related events during the G_1 to S phase transition (*24*−*26*). CyclinD1 is capable of shortening the G_1 phase of the cell cycle, suggesting that it may be rate-limiting for G_1 phase progression (*27*−*29*). The induction of *Ccnd1* mRNA in response to cell cycle progression and the stimulation of growth factor is very quick (*30*−*32*) and likely regulated at the transcription level (*33*−*36*). In this study, we demonstrate that overexpression of FoxO1 promotes the activity of the *Ccnd1* promoter, as illustrated in Figure 4. This implies that FoxO1 induces cell proliferation, at least partially, through regulating the expression of *Ccnd1* at the transcription level.

ERK/MAPK signaling, known as a pivotal regulator of the G_1 to S phase transition, is associated with the induction of positive regulators of the cell cycle and the inactivation of antiproliferative genes (*37*). MAPK signaling is shown to phosphorylate and regulate FoxO1 (*38*). On the other hand, ERK1/2 signaling is implicated to be closely related to the expression of *Ccnd1* (*25*, *37*). Therefore, we first assessed ERK/MAPK signaling. Second, we detected PI3K/AKT signaling. PI3K/AKT signaling functions as another key negative regulator of FoxO1. The phosphorylation of PI3K is able to impair the DNA binding ability of FoxO1 (*14*), translocate FoxO1 from the nucleus to the cytosol, and degrade FoxO1 by ubiquitination, eventually causing a decreased level of expression of FoxO1-upregulated genes and an increased level of expression of its downregulated genes (*21*, *39*−*41*). As shown in Figure 5, both MAPK and PI3K signaling may modulate the function of FoxO1 in cell proliferation under the condition of low nutrition.

In this study, it seems that we realized some results that contradict previous reports. We assumed that activation of FoxO1 may repress the expression of *Ccnd1* (*14*, *15*), but the exclusion of FoxO1 may abrogate its role as a repressor, which could elevate the level of expression of *Ccnd1* spontaneously. As a result, it may accelerate the speed of the G_1 to S phase transition and finally promote the proliferation of NIT-1 cells. Predominantly, the medium we used here contained low levels of nutrients, and FoxO1 may retain and keep its activity in cellular nuclei (*42*). It is reported that FoxO1 can elevate the level of secretion of insulin (*16*, *17*) and enhance the expression of the insulin receptor (*42*). When we overexpressed wild-type FoxO1 in NIT-1 cells, a great quantity of FoxO1 promoted insulin secretion (Figure 4C); in turn, insulin may activate both MAPK and PI3K pathways, and FoxO1 may be quickly phosphorylated by ERK1/2 and AKT. FoxO1, therefore, loses its function by being excluded from the nuclei of NIT-1 cells. The exclusion of FoxO1 can also be confirmed by the activation of *Ccnd1*; thus, the proliferation of these cells was observed (Figure 6). Moreover, DNFoxO1 contains only the DNA binding domain and lacks the transactivation domain. It plays a role of the inactive FoxO1 and has been reported to resist the apoptosis of islets (*13*). The three phosphorylation sites (Thr24, Ser253, and Ser316) of CAFoxO1 were mutated so that CAFoxO1 can constantly remain in nuclei and at the state of activation.

In summary, we found that overexpression of FoxO1 is prone to causing cell proliferation, but not apoptosis, in cultured pancreatic β cells exposed to low nutrition. Both MAPK and PI3K signaling may mediate the cell proliferation modulated by FoxO1 in cultured β cells exposed to low nutrition. FoxO1-induced cell proliferation may be modulated, at least partially, by *Ccnd1* at the transcription level. As is well-known, type 2 diabetes is mainly attributed to impaired β cell functions (*43*). Therefore, any factor that could reverse or prevent β cell failure would play a role in the treatment of diabetes. Hence, this study could also imply that FoxO1 could serve as a potential therapeutic target within the clinical application for type 2 diabetes. Taken together, these findings provide us with new insights into the underlying mechanism of proliferation and therapeutic target in pancreatic β cells.

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