Original Paper

Colonic PDGFR α Overexpression Accompanied Forkhead Transcription Factor FOXO3 Up-Regulation in STZ-Induced Diabetic Mice

Hongli Lu^a Chunmei Zhang^a Nina Song^a Chen Lu^a Ling Tong^a Xu Huang^a
Young-chul Kim^b Jie Chen^c Wenxue Xu^a

^aDepartment of Physiology, School of Medicine, Shanghai Jiao Tong University, Shanghai, China;

^bDepartment of Physiology, College of Medicine, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea; ^cDepartment of Pediatric Surgery, Xin Hua Hospital, Affiliated to Shanghai JiaoTong University School of Medicine, Shanghai, China

Key Words

Diabetes mellitus • FOXO3 • PDGFR α • Colonic dysmotility

Abstract

Background: Colonic transit disorder-induced constipation is a major complication in diabetic patients. PDGFR α ⁺ (platelet-derived growth factor receptor α -positive) cells play critical roles in the inhibitory regulation of colonic motility, and FOXO3 (forkhead transcription factor 3) has a broad range of biological functions. The present study was designed to investigate the relationship between FOXO3 and PDGFR α ⁺ cell proliferation in streptozotocin (STZ)-induced diabetic mice. **Methods:** The major experimental techniques used in this paper are immunohistochemistry, quantitative RT-PCR and Western blotting for the evaluation of specific protein expression; ChIP assay for identifying the interaction between FOXO3 protein and the PDGFR α promoter; and lentiviral transfection for the overexpression of short hairpin RNAs (shRNAs) to down-regulate FOXO3. **Results:** In proximal colonic smooth muscle tissue of STZ-induced diabetic mice, there was a significant increase in PDGFR α and Ki67 immunoreactivity. PDGFR α mRNA and protein expression levels were both significantly increased in colonic smooth muscle tissue, but PDGFR β expression was unchanged. Meanwhile, the expression of PDGF ligands, including both PDGF α and PDGF β , was significantly increased in diabetic colonic smooth muscle tissue. In whole cell and nuclear extracts, the expression of FOXO3 protein was also significantly increased; however, the expression of P-FOXO3 (phosphorylated FOXO3) protein was significantly decreased. When NIH cells were incubated with 50 mmol/L glucose for 12 h, 24 h and 48 h, the expression of PDGFR α significantly increased, and in whole cell and nuclear extracts, the expression of FOXO3 protein was significantly increased. However, the expression of P-FOXO3 protein was significantly decreased. FOXO3 could bind to a site on the PDGFR α promoter, and the basal expression of PDGFR α was significantly reduced when endogenous FOXO3 expression was knocked down with FOXO3 short hairpin RNA (shRNA) in NIH cells. The expression of phosphorylated Akt was significantly down-regulated in diabetic

Wenxue Xu,
and Jie Chen

Department of Physiology, Medical College, and Department of Pediatric Surgery,
Xin Hua Hospital, Shanghai Jiao Tong University, Room 328, Shanghai (China)
E-Mail wenxiexu@sjtu.edu.cn, jiechen1974@163.com

colonic muscle tissue. **Conclusions:** These results suggest that diabetes-induced colonic PDGFR α cell proliferation is mediated by FOXO3 up-regulation. FOXO3 up-regulation may be induced by inhibiting the PI3K/Akt signaling pathway in STZ-induced diabetic mice. PDGFR α cell proliferation could be a new target for clinical therapy of diabetes-induced colonic transit disorder.

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Introduction

Gastrointestinal (GI) dysfunction is very common in patients with diabetes, and the cause is multifactorial. Approximately 75% of patients with diabetes experience some form of GI dysfunction. The major complaints of those patients include dysphagia, reflux, early satiety, nausea, abdominal pain and constipation [1-3], among which constipation is a common symptom of the lower GI system [4-6]. GI motility requires interactions among the enteric nervous system, extrinsic autonomic nerves, smooth muscles, PDGFR α fibroblasts and interstitial cells of Cajal (ICCs). In many reports, these GI symptoms have been hypothesized to be the results of abnormal GI motility, which in turn could be a manifestation of diabetic autonomic neuropathy or loss of ICCs [7, 8]. However, only a few studies on the mechanism underlying colonic symptoms in diabetes models have been conducted. In some diabetic animal models, colonic contractions have been shown to be decreased compared with those in normal animals [9-11]. Therefore, it is necessary to elucidate why diabetes induces colonic dysmotility and elucidate the pathogenesis of altered colonic motility and transit.

Recently, a study demonstrated that the neuromuscular apparatus of the GI tract is complicated by the presence of ICCs and PDGFR α cells (cells labelled by antibodies against platelet-derived growth factor receptor α), which cluster along the varicose fibers of motor neurons and form gap junctions with smooth muscle cells (SMCs), which in turn form an electrical syncytium known as the SIP syncytium (smooth muscle cells, ICCs and PDGFR α cells form an electrical syncytium) [12]. ICCs provide pacemaker activity via ANO1, provide propagation pathways for slow waves and transduce inputs from motor neurons, but PDGFR α cells induce the hyperpolarization of smooth muscle via SK3 [13]. Smooth muscle cells are electrically coupled to ICCs and PDGFR α cells, forming an integrated unit. However, it is not clear whether diabetes induces colonic dysmotility and whether colonic dysmotility is caused by changes in PDGFR α cell function.

Previous studies have indicated that FOXO transcription factors have a broad range of biological functions, including the regulation of cell proliferation, apoptosis and differentiation as well as the regulation of life span and energy metabolism [14, 15]. FOXO transcription factors (FOXOs) are evolutionarily conserved regulators of longevity, and they are inhibited by the insulin/insulin-like growth factor (IGF) signaling pathway. In mice, FOXOs were originally implicated in insulin/insulin-like growth factor signaling (IIS), which results in decreased activity and/or expression of FOXOs [16, 17]. IIS activates phosphoinositide-3-kinase (PI3K), leading to the activation of Akt; subsequently, Akt phosphorylates FOXO, resulting in its exclusion from the nucleus [18, 19]. Previous studies have also demonstrated that the insulin/insulin-like growth factor signaling (IIS) pathway is down-regulated, inducing gastric smooth muscle atrophy and hyperglycemia in the diabetic mice [20, 21]. Hyperglycemia is known to increase oxidative stress, which is implicated in various disorders, because continuous hyperglycemia increases advanced glycation end products (AGEs) and induces ROS production [22], and oxidative stress leads to the increased activity or expression of FOXOs. Recently, using gene chip technology in STZ-induced diabetic mice, we observed that the mRNA levels of FOXO3 transcription factors were up-regulated in gastric muscle tissue (data not shown).

Therefore, to elucidate the mechanism of diabetes-induced colonic dysmotility in the present study, the relationship between FOXO3 transcription factors and PDGFR α cell proliferation was investigated in the colonic smooth muscle tissue of STZ-induced diabetic mice and in NIH/3T3 cells.

Materials and Methods

Ethics

This study was conducted in accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the Science and Technology Commission of the P.R.C. (STCC Publication No. 2, revised 1988). The protocol was approved by the Committee on the Ethics of Animals at Shanghai Jiao Tong University School of Medicine (Permit Number: Hu 686-2009).

Animals and the STZ-induced diabetic mouse model

Adult male ICR mice, aged 5 weeks old and weighing 30 ± 2 g, were used for this study. Eighty mice were fasted overnight and were randomly divided into two groups: a control group ($n = 40$) and a diabetic group ($n = 40$). Diabetes was induced by a single intraperitoneal injection (200 mg/kg) of STZ (Sigma Aldrich, St. Louis, MO, USA) dissolved in ice-cold 0.1 mol/L citrate buffer. Control mice were intraperitoneally administered the same volume of 0.1 mol/L citrate buffer. The mice were housed at a constant temperature (20–25°C) under a 12 h light/dark cycle with free access to water and food. Their blood glucose levels were measured 1 week after STZ injection and were reexamined 2 months later to confirm diabetes (blood glucose >16.7 mmol/L).

NIH/3T3 fibroblast cell culture

NIH/3T3 fibroblast cells were acquired from the Cell Bank of the Chinese Academy of Sciences and were placed into plates containing Dulbecco's modified Eagle medium (DMEM containing 25 mmol/L glucose; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated bovine serum (BS), 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were then incubated at 37°C in an incubator containing 5% CO₂. After one week in culture, the cells displayed fibroblast-shaped morphology. NIH/3T3 cells between the fourth and sixth passages were used in the study. NIH/3T3 cells were treated with 25 mmol/L glucose (control) or 50 mmol/L glucose (high glucose) for 12 h, 24 h and 48 h, and subsequently, the expression of PDGFR α , FOXO3, and phosphorylated FOXO3 (p-FOXO3) was detected.

Immunohistochemistry

The mice were sacrificed by cervical dislocation, and the proximal colons were placed in Krebs's solution (mM): NaCl 118.5; KCl 4.5; MgCl₂ 1.2; NaHCO₃ 23.8; KH₂PO₄ 1.2; glucose 11.0; CaCl₂ 2.4 and pH 7.4 after being bubbled with 95% O₂ and 5% CO₂. The proximal colon was opened, and the colon contents were washed away. Tissue samples were fixed with 4% ice-cold paraformaldehyde for 6–8 h, dehydrated in 20% sucrose, embedded as frozen tissue blocks and cut into 8–10- μ m thickness frozen sections at room temperature. Samples were incubated in 0.1 M phosphate-buffered saline (PBS) containing 10% normal goat serum for 2 h at room temperature to block non-specific binding and incubated with goat anti-PDGFR α antibody (1:200; AF1062, R & D systems, USA) and rabbit anti-Ki67 antibody (1:50; GB13030, Wuhan goodbio technology, China) mixed with Triton-X100 (0.5%, Sigma Aldrich, St. Louis, MO, USA) at 4°C for 24 h. Samples were washed in 0.1 M PBS for 30 min and then incubated at room temperature with cy3- conjugated anti-goat IgG (1:300; GB21404, Wuhan goodbio technology, China), Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:100, Jackson Immuno Research, USA) and DAPI for 2 h. Images were acquired using a confocal laser scanning microscope (Leica TCS SP8, Germany).

Quantitative RT-PCR

The mice were sacrificed by cervical dislocation, and the mucosa and submucosa were removed via sharp dissection in aerated (95% O₂ and 5% CO₂) Krebs solution with the following composition (mM): NaCl 118.5; KCl 4.5; MgCl₂ 1.2; NaHCO₃ 23.8; KH₂PO₄ 1.2; glucose 11.0; and CaCl₂ 2.4. Total RNA was extracted from the proximal colon with TRIzol Reagent (Sigma) following the manufacturer's instructions and was processed for reverse transcription using an AMV First Strand cDNA Synthesis Kit (B532445, Sangon Biotech, China). SYBR Green-based quantitative PCR was performed using the SYBR Green PCR Master Mix (B639271, Sangon Biotech, China). The sequences of these primers were as follows:

PDGFR α forward: 5' GTAACACCAGCAGCGTCAAGT 3', PDGFR α reverse: 5' GATGGTCTGGGTTTCAGGTTG 3' (amplicon size: 168 bp);

PDGFR β forward: 5' ACTCCATCCGCTCCTTTGA 3', PDGFR β reverse: 5' TTGCACTCGGCGATTACAG 3' (amplicon size: 187 bp);

GAPDH forward: 5' GGTGTCTCCTGCGACTTCA 3'; GAPDH reverse: 5' TGGTCCAGGGTTTCTTACTCC 3' (amplicon size: 183 bp);

PDGFR α forward: 5' CTTCTGTAACTGACACGCTCC 3'; PDGFR α reverse: 5' TCCACATCACCCAAGTCTCT 3' (amplicon size: 117 bp);

PDGFR β forward: 5' AGAAGTAGCGAGAAGCAAGCCT 3'; PDGFR β reverse: 5' GCAGTATTCGTGATGATGATAGAT 3' (amplicon size: 132 bp);

FOXO1 forward: 5' GGAGGCAAGAGCGGAAAAT 3'; FOXO1 reverse: 5' GCCACTTAGAAAAGTGAACCC 3' (amplicon size: 117 bp);

FOXO3 forward: 5' ACTGAGGAAAGGGGAAATGG 3'; FOXO3 reverse: 5' CAAAGGTGTCAAGCTGTAAACG 3' (amplicon size: 123 bp); and

FOXO4 forward: 5' CACTCGCCAGATCTACGAA 3'; FOXO4 reverse: 5' TTGTGAACCTTGATGAACCTTGC 3' (amplicon size: 139 bp).

The amount of target gene relative to an endogenous control was determined using the Δ CT method.

Western blot analysis

The mice were sacrificed by cervical dislocation, and the mucosa and submucosa were removed via sharp dissection in aerated (95% O₂ and 5% CO₂) Krebs solution with the following composition (mM): NaCl 118.5; KCl 4.5; MgCl₂ 1.2; NaHCO₃ 23.8; KH₂PO₄ 1.2; glucose 11.0; and CaCl₂ 2.4. Protein samples were extracted from cultured NIH/3T3 cells and proximal colon smooth muscle layers. The samples were lysed in radioimmunoprecipitation assay (RIPA) buffer (1:100; P0013, Beyotime Chemical Co., Jiangsu, China) and Phenylmethanesulfonylfluoride (PMSF) buffer (Beyotime Chemical Co., Jiangsu, China) containing protease inhibitor cocktail (P8340, Sigma-Aldrich, China). Nuclear proteins were extracted using a Nuclear Protein Extraction Kit (Beyotime Institute of Biotechnology, China). The suspension was centrifuged at 12,000 g for 15 min at 4°C, and the protein concentration of the supernatant was determined using the Bradford method. Proteins (40 μ g/lane) were then subjected to 10% or 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred from the polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane using a Semi-Dry Transblot unit (Bio-Rad). The PVDF membranes were blocked in 5% non-fat dry milk for 120 min and then incubated with the following antibodies overnight at 4°C: rabbit anti-PDGFR α antibody and rabbit anti-PDGFR β antibody (1:1 000; #3164, #4564, Cell Signaling Technology, USA); rabbit anti-protein kinase B (anti-Akt), phospho-Akt (ser473) rabbit mAb and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb (1:1 000; #4691, #4060, #2118, Cell Signaling Technology, USA); and rabbit anti-FOXO3 antibody, rabbit phospho-FOXO3 (ser253) antibody and β -Actin rabbit mAb (1:1 000; #2497, #9466, #8457, Cell Signaling Technology, USA). The blots were subsequently washed five times (5 min each wash) with Tris-HCl-buffered saline (TBS)/0.1% Tween-20 and then incubated with horseradish peroxidase (HRP)-linked goat anti-rabbit or anti-mouse secondary antibody for 120 min at room temperature. The detection of protein was achieved using an enhanced chemiluminescence agent (ECL reagents). Images from each Western blot were analyzed using Quantity One software (Bio-Rad).

Chromatin immunoprecipitation assay (ChIP)

Groups of NIH/3T3 cells grown to confluence on 10-cm culture dishes were serum-starved for 24 h. The cells were then incubated with 1% formaldehyde for 10 min to crosslink the DNA-binding proteins to DNA. The reaction was stopped with 0.125 M glycine. The cells were collected in PBS containing protease inhibitor cocktail, pelleted and lysed in the presence of SDS and protease inhibitor cocktail. The lysate was sonicated to shear genomic DNA. The supernatant from each group was brought to a volume of 150 μ l by the addition of SDS lysis buffer and further diluted to a total volume of 1.5 ml using ChIP dilution buffer containing protease inhibitor cocktail. The samples (1.4 ml of the above) were precleared with protein A agarose/salmon sperm DNA by incubating for 30 min at 4°C, and then, the supernatant was collected. Immunoprecipitation was achieved by the addition of FOXO3 antibody or isotope-matched IgG and incubation for 16 h at 4°C. After the addition of protein A agarose/salmon sperm DNA, the sample was incubated for 1 h at 4°C followed by centrifugation. The pellet was washed sequentially with low-salt immune complex wash buffer, high-salt immune complex wash buffer, LiCl immune complex wash buffer and TE buffer. After elution, cross-linking was reversed by incubating with 5 M NaCl at 65°C for 4 h. DNA was extracted and purified using the DNeasy extraction kit (Qiagen, Valencia, CA). A 2- μ l quantity of each immunoprecipitated sample and the

remaining 100 μ l of lysate (input material) were used for PCR amplification. The PCR primers for the FOXO3 binding site on the PDGFR α gene were as follows: sense: 5' TTGGGTCCCTGGTTTGTG 3' and antisense: 5' GTCCTGCCTTTGTTTTCGG 3'. The primers for the housekeeping gene β -actin were as follows: sense 5' GATCTGGCACCACACCTTCT 3' and antisense 5' GGGTGTGAAGGTCTCAA 3'. The PCR reaction included denaturing for 5 min at 95°C followed by 35 cycles of denaturation (95°C for 30 sec), annealing (60°C for 30 sec) and extension (72°C for 15 sec) and a final extension at 72°C for 7 min. The PCR products were electrophoresed on 1.5% agarose gels, and the intensity of the products was quantified with Quantity One. The ratio of the PDGFR α promoter fragment in the immunoprecipitants to that in the input material was then plotted.

Lentiviral shRNA production and infection

Scramble shRNA (sequence: GATCCGTTCTCCGAACGTGTCACGTTTCAAGAGAAAGCTGACACGTTCCGAGAACTTTTGG) and two FOXO 3 shRNAs (sequence 1: GATCCGCAAGCCGTGCCTTGTCAAATTTCAAGAGAAATTTGACAAGGCACGGCTGCTTTTGG; and sequence 2: GATCCGGCTCACTTTGTCCAGATCTTCAAGAGAAAGATCTGGGACAAAGTGAGCCTTTTGG) were cloned into an LV3 vector (Sangon, Shanghai) carrying an ampicillin resistance gene. LV3 scramble shRNA vector, FOXO 3 shRNA1 (with gene plasmid sequence 1 added) and FOXO 3 shRNA2 (with gene plasmid sequence 2 added) were co-transfected into 293T cells using linear polyethyleneimine (PEI) (Polysciences Inc.). The medium was replaced 8 h post-transfection, and lentiviral supernatants were harvested at 48 h. NIH/3T3 fibroblast cells were plated at 2×10^5 cells/well on 6-well plates overnight, and lentiviral supernatants were added in 5 μ g/mL polybrene (Sigma-Aldrich) and spun at 2,500 rpm and 32°C for 90 min. The cells were incubated for another 4 h in a 37°C incubator. After changing the media, infected NIH/3T3 cells were cultured for 72 h or 96 h. Then, the cells were ready for the subsequent experiments.

Statistical analysis

The data are presented as the mean $\pm$ standard error (SE). Statistical analyses were performed using one-way analysis of variance (ANOVA) with Bonferroni's post hoc test for comparisons between groups or, when appropriate, using Student's unpaired t-test. Differences were considered to be statistically significant at $p < 0.05$.

Results

Body weight and blood glucose

Mice were used 8 weeks after STZ injection. The blood glucose levels of the diabetic mice (28.2 ± 1.2 mmol/L, $n = 40$) were approximately 3 times higher than those of the control mice (6.7 ± 0.5 mmol/L, $n = 40$, $p < 0.05$). The body weight of diabetic mice (24 ± 1.2 g, $n = 40$) was significantly decreased compared to that of control mice (38 ± 1.1 g, $n = 40$, $p < 0.05$).

PDGFR α and PDGFR β expression in proximal colon smooth muscle tissue

The SIP syncytium plays an important role in the regulation of colonic transit function, and PDGFR α cells are a very important component of the SIP syncytium, exerting an inhibitory effect on the regulation of smooth muscle motility by activating SK3 channels. Because diabetes slowed colonic transit, the expression of PDGFR α in colonic muscle tissue was observed. Meanwhile, Ki67 expression varies in different cell cycle phases, so the expression of Ki67 in colonic muscle tissue was observed in STZ-induced diabetic mice. Compared with control mice, diabetic mice showed a significant increase in PDGFR α and Ki67 immunoreactivity. To quantify the expression of PDGFR α and Ki67 in smooth muscle layers, the double-positive staining area was measured and was found to be significantly increased using lasx software (Fig. 1A $n = 4$, $p < 0.05$). Real-time PCR and western-blotting showed that the PDGFR α mRNA and protein levels were significantly increased in the proximal colon, respectively (Fig. 1B and C, $n = 6$, $p < 0.05$). The expression of PDGF ligands, including PDGF α and PDGF β , was significantly increased in STZ-induced diabetic mice (Fig. 1D and F, $n = 6$, $p < 0.05$). However, PDGFR β mRNA and protein levels were not significantly changed in STZ-induced diabetic mice (Fig. 1E and G, $n = 6$, $p < 0.05$).

Fig. 1. Expression of PDGFR α and PDGFR β in control and diabetic mice. **A:** Immunofluorescence staining revealed different expression levels of PDGFR α and Ki67 between control and diabetic proximal colons (Bar = 100 μ m). For the quantification of PDGFR α and Ki67 expression in smooth muscle layers, the double-positive staining area was measured using lasx software (* p < 0.05, n = 4). **B:** Western blot revealed different expression levels of PDGFR α between control and diabetic proximal colon smooth muscle layers. A representative blot and densitometric quantification (% GAPDH normalized to controls) are depicted. **C:** Expression of PDGFR α mRNA. **D:** Expression of PDGFR α mRNA. **F:** Expression of PDGFR β mRNA in the proximal colon smooth muscle. Data are presented as the mean $\pm$ SE. * p < 0.05, n = 6. **E:** Western blot revealed different expression levels of PDGFR β between control and diabetic proximal colon smooth muscle layers. A representative blot and densitometric quantification (% GAPDH normalized to control) are depicted. **G:** Expression of PDGFR β mRNA in proximal colon smooth muscle. Data are presented as the mean $\pm$ SE, P > 0.05, n = 6.

The expression of FOXO3 in the proximal colon

FOXO transcription factor mRNA expression levels in the proximal colon. It is well known that the FOXO transcription factors have a broad range of biological functions; however, the contributions of the mammalian FOXO family members (FOXO1, 3, 4 and 6) have not been fully investigated [23], especially their involvement in diabetes-induced colonic motility disorders. Therefore, the expression of FOXO1, FOXO3 and FOXO4 was measured in the proximal colon smooth muscles of STZ-induced diabetic mice. Compared with normal mice, the mRNA level of FOXO3 was significantly increased in the proximal colon of diabetic mice (Fig. 2A, n = 8, p < 0.05), whereas there was no significant difference in FOXO4 and FOXO1 mRNA levels between normal and diabetic mice (Figs. 2B and C, n = 8, P > 0.05). These results suggest that FOXO3 alone is up-regulated in the colonic smooth muscle tissue of diabetic mice, implying a potential regulatory role for FOXO3 in the proximal colon of diabetic mice.

Fig. 2. Expression of FOXO 1, 3 and 4 mRNA in control and diabetic mice. A: Expression of FOXO 3 mRNA in proximal colon smooth muscle. Data are presented as the mean $\pm$ SE. * $p < 0.05$, $n = 8$. B: Expression levels of FOXO 4 mRNA in proximal colon smooth muscle. Data are presented as the mean $\pm$ SE, $P > 0.05$, $n = 8$. C: Expression levels of FOXO 1 mRNA in proximal colon smooth muscle. Data are presented as the mean $\pm$ SE, $P > 0.05$, $n = 8$.

Fig. 3. FOXO3 phosphorylation and nuclear localization in the proximal colon. (A) The expression of FOXO3, (B) nuclear FOXO3, (C) P-FOXO3 (phosphorylated FOXO3) and (D) nuclear P-FOXO3 protein in proximal colon smooth muscle. Western blot revealed different expression levels of (A) FOXO 3, (B) nuclear FOXO3, (C) P-FOXO3 (phosphorylated FOXO3) and (D) nuclear P-FOXO3 protein between control and diabetic proximal colon smooth muscle layers. A representative blot and densitometric quantification (% GAPDH normalized to control) are depicted. Data are presented as the mean $\pm$ SE. * $p < 0.05$, $n = 6$.

FOXO3 phosphorylation and nuclear localization in the proximal colon. To further confirm the role of FOXO3 in the proximal colon of diabetic mice, FOXO3 phosphorylation and nuclear localization were investigated. Compared with normal mice, the expression of FOXO3 protein in whole cell and nuclear extracts from proximal colonic smooth muscle tissue of STZ-induced diabetic mice was significantly increased (Fig. 3A and B, $n = 6$, $P < 0.05$), but the expression of p-FOXO3 (phosphorylated FOXO3) protein was significantly decreased (Fig. 3C and D, $n = 6$, $P < 0.05$). Since FOXO3 dephosphorylation was required for the protein to be translocated into the nucleus to exert its role as a transcription factor, these results suggested that FOXO3 is involved in the up-regulation of PDGFR α in the proximal colon of diabetic mice.

PDGFR α and FOXO3 expression in NIH/3T3 cells

Because NIH/3T3 cells were originally established from primary mouse embryonic fibroblast cells, PDGFR α cells were referred to as fibroblasts or "fibroblast-like cells" (FLC) by

Fig. 4. Expression levels of PDGFR α mRNA and protein in NIH/3T3 cells. A: Expression of PDGFR α mRNA after the NIH/3T3 cells were incubated with 50 mmol/L glucose for 12 h, 24 h and 48 h. Western blot revealed different expression levels of PDGFR α after the NIH/3T3 cells were incubated with 50 mmol/L glucose for 12 h, 24 h and 48 h. A representative blot and densitometric quantification (% GAPDH normalized to control) are depicted in (B). Data are presented as the mean $\pm$ SE. *p < 0.05, n = 8.

Fig. 5. FOXO3 phosphorylation and nuclear localization in NIH/3T3 cells(A) The expression of FOXO3, (B) nuclear FOXO3, (C) P-FOXO3 (phosphorylatedFOXO3) and (D) nuclear P-FOXO3 protein in NIH/3T3 cells. Western blot revealed different expression levels of (A) FOXO 3, (B) nuclear FOXO3, (C) P-FOXO3 (phosphorylatedFOXO3) and (D) nuclear P-FOXO3 protein after the NIH/3T3 cells were incubated with 50 mmol/L glucose for 12 h, 24 h and 48 h. A representative blot and densitometric quantification (% GAPDH normalized to control) are depicted. Data are presented as the mean $\pm$ SE. *p < 0.05, n = 6.

morphologists. Moreover, NIH/3T3 cells also express PDGFR α and FOXO3 protein, making it suitable as an *in vitro* model with which to test the interaction between PDGFR α and FOXO3. Hyperglycemic conditions in the diabetic mice increase oxidative stress, so the NIH/3T3 cells were incubated with 50 mmol/L glucose for 12 h, 24 h or 48 h as an *in vitro* model of diabe-

Fig. 6. FOXO3 binding site in the PDGFR α promoter in NIH/3T3 cells. After immunoprecipitation using the FOXO3 antibody, the PDGFR α DNA was amplified in the cell lysate of NIH/3T3 cells. At the same time, the control group with isotype-matched IgG did not pull down PDGFR α DNA. In the non-immunoprecipitated input samples, PDGFR α DNA showed similar amplification levels among the groups. Data are presented as the mean $\pm$ SE. * p < 0.05, n = 3.

Fig. 7. FOXO3 shRNA abolished the basal expression of PDGFR α . Aa: Expression of FOXO3 mRNA after the NIH/3T3 cells were incubated with four FOXO3 shRNAs: shRNA1, 2, 3 and 4. Western blot revealed different expression levels of FOXO3. A representative blot and densitometric quantification (% GAPDH normalized to control) are depicted in (Ab). B: Expression of PDGFR α protein in NIH/3T3 cells 72 h and 96 h after FOXO3 shRNA 1 and shRNA 2 infection. A representative blot and densitometric quantification (% GAPDH normalized to control) are depicted in (B). Data are presented as the mean $\pm$ SE. * p < 0.05, n = 4.

tes, leading to the significant increase in PDGFR α mRNA and protein levels (Fig. 4A and 4B, n = 8, p < 0.05). Accompany the up-regulation of PDGFR α , the expression of FOXO3 protein in whole cell and nuclear extracts was significantly increased (Fig. 5A and 5B, n = 6, p < 0.05); however, the expression of P-FOXO3 protein in whole cell and nuclear extracts was significantly decreased (Fig. 5C and 5D, n = 6, p < 0.05). These data were consistent with the findings in the proximal colon of diabetic mice, suggesting that high glucose promotes PDGFR α and FOXO3 expression in NIH/3T3 cells.

FOXO3 binding site in the PDGFR α promoter in NIH/3T3 cells

To study the direct relationships between FOXO3 and PDGFR α expression, we performed chromatin immunoprecipitation assays to determine if FOXO3

Fig. 8. The PI3K/Akt signaling pathway in control and diabetic mice. A: Expression of AKT protein in proximal colon smooth muscle. A representative blot and densitometric quantification (% GAPDH normalized to control) are depicted. Data are presented as the mean $\pm$ SE. $P > 0.05$, $n = 6$. B: Expression of P-AKT protein in proximal colon smooth muscle. A representative blot and densitometric quantification (% GAPDH normalized to control) are depicted. Data are presented as the mean $\pm$ SE. * $p < 0.05$, $n = 6$.

directly binds to the PDGFR α promoter. Antibodies against FOXO3 specifically immunoprecipitated the PDGFR α promoter from NIH/3T3 cell chromatin (Fig. 6, $n = 3$, $p < 0.05$), whereas the PDGFR α promoter was not detectable in control isotype-matched IgG immunoprecipitants. However, in the non-immunoprecipitated input samples, PDGFR α DNA showed similar amplification levels among all groups.

FOXO3 shRNA abolished the basal expression of PDGFR α

To determine the causal relationships between FOXO3 transcription factors and PDGFR α expression, we designed four FOXO3 shRNAs named shRNA1, 2, 3 and 4, which effectively reduced FOXO3 expression to different extents (Fig. 7A). Because FOXO3 shRNA 1 and 2 caused the greatest decrease in FOXO3 levels, they were used to determine the effects of FOXO3 knockdown on PDGFR α expression. The results showed that PDGFR α expression was significantly decreased after incubation for 72 h and 96 h (Fig. 7B, $n = 4$, $p < 0.05$). The data suggested that the FOXO3 transcription factor might be associated with PDGFR α expression in NIH/3T3 cells.

p-Akt expression in the diabetic colon

The PI3K/Akt pathway is a central downstream effector of growth factor receptors. In the present study, we investigated whether the PI3K/Akt signal pathway was involved in FOXO3 transcription factor expression in the colonic smooth muscle tissue of diabetic mice. The results showed that Akt phosphorylation was significantly reduced in the diabetic group (Fig. 8B, $n = 6$, $p < 0.05$), whereas Akt protein expression was not significantly changed (Fig. 8A, $n = 6$, $P > 0.05$). Phosphoinositide-3-kinase (PI3K) activation leads to the activation of Akt, which phosphorylates FOXO, resulting in its exclusion from the nucleus, which in turn regulates FOXO3 transcriptional activity and localization [18, 19]. These results suggest that the PI3K/Akt signaling pathway may be involved in FOXO3 transcription factor up-regulation in diabetic mice.

Discussion

There have been many reports of upper GI symptoms in diabetic patients. In recent reports, colonic dysmotility occurs with diabetes, and many patients exhibit constipation [5]. Gastroparesis also frequently affects diabetic patients and is a well-recognized complication

of diabetes [24-26]. However, few studies have been conducted to elucidate the mechanism underlying colonic symptoms in diabetes models. Major GI motility patterns were traditionally believed to be mainly determined by myogenic and neurogenic mechanisms. More recently, interstitial cells have been recognized to contribute to important regulatory functions in GI motor activity. At least 2 types of resident interstitial cells (ICC and PDGFR α ⁺ cells) lie in close proximity to the terminals of enteric motor neurons. Together with smooth muscle cells, ICCs and PDGFR α ⁺ cells form an electrical syncytium (SIP syncytium) and integrate inputs from enteric motor neurons [27, 28]. ICCs provide pacemaker activity via ANO1, provide propagation pathways for slow waves and transduces inputs from motor neurons. Loss of interstitial cells of Cajal (ICCs) correlates with the pathology of diabetic gastroparesis [13, 29]. In contrast, PDGFR α ⁺ cells express both P2Y1 receptors and SK3 channels, which are fundamental components of the inhibitory responses to purines in the gut [30, 31], and thus, they exert inhibitory regulation on colonic motility.

Platelet-derived growth factor (PDGF) family proteins exist as several disulfide-bonded, dimeric isoforms (PDGF AA, PDGF AB, PDGF BB, PDGF CC and PDGF DD) that bind in a specific pattern to two closely related receptor tyrosine kinases: PDGF receptor α (PDGFR α) and PDGF receptor β (PDGFR β). PDGFR α and PDGFR β share 75% to 85% sequence homology between their two intracellular kinase domains, whereas the kinase inserts and the carboxy-terminal tail regions display a lower level (27% to 28%) of homology [32, 33]. PDGFR α ⁺ cells have receptors for purinergic neurotransmitter (P2Y1R) agonists that hyperpolarize PDGFR α ⁺ cells to the equilibrium potential for K⁺ [34]. Thus, it appears that SMCs mediate the fast inhibitory junction potential (IJP) elicited by purinergic nerve stimulation in the gut [35]. As a result, the number of PDGFR α ⁺ cells are increased, which might induce functional disorders of colonic motility and transit. In the present study, we first investigated the expression of PDGFR α in the proximal colonic smooth muscle tissue of diabetic mice. Our results demonstrated that compared with the control group, diabetic mice showed a significant increase in PDGFR α and Ki67 immunoreactivity (Fig. 1A n = 4, $p < 0.05$). Ki67 is a nuclear antigen, and cells express Ki67 during the G1, S and G2 mitotic phases but not during the resting phase G0 [36]. Furthermore, diabetes induced significantly up-regulated PDGFR α mRNA and protein expression but did not affect the expression of PDGFR β . The observed diabetes-induced increases in PDGFR α in the proximal colon could result from increased expression of PDGFR α or increased numbers of PDGFR α ⁺ cells. Evidence indicates that PDGF binding to the PDGFR α receptor stimulates cell proliferation [37]. Our results demonstrated that the expression of PDGF ligands, including PDGF α and PDGF β , were significantly increased in STZ-induced diabetic mice, and at the same time, diabetic mice showed a significant increase in PDGFR α and Ki67 immunoreactivity and in the double-positive staining area in the smooth muscle layers, suggesting that the elevated PDGFR α expression may reflect an increase in the number of PDGFR α ⁺ cells. This would create an imbalance between ICCs and PDGFR α ⁺ cells in SIP, which may result in impaired colonic motility.

The forkhead/winged helix box group O (FOXO) proteins are a set of evolutionarily conserved transcription factors that act as a central integration hub for many important cellular stimuli. In mammals, there are four FOXOs, FOXO1, 3, 4 and 6 [23, 38], and each FOXO can regulate different genes, depending on the cell type [39], to control cell proliferation, apoptosis and differentiation. They also regulate life span and energy metabolism [40, 41]. In *in vivo* studies, our results showed that FOXO3 expression levels were significantly increased, whereas p-FOXO3 expression was decreased in whole cell and nuclear extracts. PI3K/Akt activation leads to the nuclear exclusion of FOXO proteins and thus the inactivation of their transcriptional activities [42-44]. In the present study, we found that the Akt phosphorylation levels were significantly suppressed in diabetic mice. This was in agreement with the increased expression of FOXO3 protein in whole cell and nuclear extracts and the decreased expression of p-FOXO3. In the diabetic mice, the deactivation of phosphoinositide-3-kinase (PI3K) leads to the deactivation of Akt. This causes FOXO to be dephosphorylated, which leads to its translocation into the nucleus to exert its role as a transcription factor [45-47]. This is possible that the nuclear translocation of FOXO3 may be caused by inhibition of the

PI3K/Akt signaling pathway in STZ-induced diabetic mice. However, because hyperglycemia also exists in the *in vivo* models, it is also possible that the nuclear translocation of FOXO3 may also be due to oxidative stress caused by hyperglycemia.

When NIH cells were incubated with 50 mmol/L glucose for 12 h, 24 h and 48 h, the expression of PDGFR α was significantly increased, and FOXO3 protein expression in the whole cell and nuclear extracts was significantly increased; however, the expression of P-FOXO3 in whole cell and nuclear extracts was significantly decreased. Due to the hyperglycemic conditions present in both the *in vivo* and *in vitro* models, oxidative stress is increased. In response to oxidative stress, FoxO proteins dephosphorylate and translocate from the cytoplasm to the nucleus, thus contributing to increased FoxO activity and promoting FoxO target gene-encoded anti-oxidative enzymes [48]. The ChIP assay showed the direct binding of FOXO3 to the PDGFR α promotor, and the basal expression of PDGFR α was significantly reduced when endogenous FOXO3 expression was knocked down with FOXO3 short hairpin RNA (shRNA) in NIH cells, suggesting that the translocation of FOXO3 into the nucleus to function as a transcription factor is involved in the up-regulation of PDGFR α expression.

In summary, the diabetes-induced increase in PDGFR α cells may be mediated by FOXO3 up-regulation via the inhibition of the PI3K/Akt signaling pathway in STZ-induced diabetic mice. It is also possible that the nuclear translocation of FOXO3 may also be due to oxidative stress caused by hyperglycemia. PDGFR α cells are a very important component of SIP, which has an inhibitory effect on colonic motility via the activation of SK3. When PDGFR α cells are increased in the proximal colon, the balance between ICCs and PDGFR α cells in the SIP is destroyed, which may result in colonic motility and transit disorders. Therefore, PDGFR α cells might be a new target for clinical therapy of diabetes-induced colonic transit disorders.

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Disclosure Statement

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