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# Effect of FIGF overexpression on liver cells transforming to insulin-producing cells

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Limitation in the number of insulin-producing pancreatic β-cells is a typical feature of diabetes. It has been indicated that activating pancreatic transcription factors can promote the transformation of hepatocytes into insulin-secreting β-like cells, indicating that direct hepatocyte differentiation seems promising as a treatment for diabetes. Nevertheless, the reprogramming efficiency still remains low. Our previous study found that the expression of c-fos-induced growth factor (FIGF) was increased in the pancreatic tissues in partial pancreatectomy mice compared to that in normal mice. Here, we observed that treatment with Ad-FIGF was found to enhance MafA and Ngn3-induced reprogramming of BNL CL.2 cells to β-like cells with the ability of secreting insulin. And FIGF overexpression increased the levels of histone H3/H4 acetylation at MafA and Ngn3 promoter regions in BNL CL.2 cells. Importantly, *in vivo* study further confirmed that forced expression of FIGF facilitated the insulin expression and decreased the blood glucose levels in STZ mice. These results strengthen the possibility of developing cell-based therapies for diabetes through utilizing β-like cells derived from non-insulin-secreting cells.

**Keywords.** C-fos-induced growth factor (FIGF); diabetes; histone H3/H4 acetylation; MafA and Ngn3

**Abbreviations:** Ngn3, Neurogenin-3; FIGF, C-fos-induced growth factor; VEGF-D, Vascular endothelial growth factor-D; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; PFA, Paraformaldehyde; BSA, Bovine serum albumin; RIPA, Radioimmunoprecipitation assay; STZ, Streptozotocin

## 1. Introduction

Diabetes is a metabolic disease characterized by hyperglycemia,which is mainly caused by insulin secretion defection or/and impaired biological function. Chronic hyperglycemia during diabetes could leads to many other diseases like cerebrovascular disease, coronary artery disease and chronic kidney disease without proper treatment. So far, the most common therapy for diabetes is insulin treatment, which may result in severe hypoglycemia (Kaczorowski *et al.* 2010). Hence, generating insulin-producing β-like cells may be a promising therapy for diabetes.

MafA, a member of MAF basic leucine zipper transcriptional activators, was identified as the transcription factor of insulin biosynthesis by binding to the C1/RIPE3b element (Docherty *et al.* 2005). MafA plays a fundamental role in the maturation of β-cells. Matsuoka *et al.* demonstrated that preserving MafA expression in diabetic islet-cells improves

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glycemic control *in vivo* (Matsuoka *et al.* 2015). Nishimura *et al.* reported that knockout of MafA in β-cells results in a deeper loss of cell identity, which is involved in diabetes pathology (Nishimura *et al.* 2015). Neurogenin-3 (Ngn3), one of the pancreatic transcription factors which participate in β-cell development, is also certified to promote early and ectopic development of pancreatic β-cells (Oropeza and Horb 2012). Therefore, activation of MafA and Ngn3 may be a promising strategy for generating insulin-producing β-like cells.

C-fos-induced growth factor (FIGF), also as known as vascular endothelial growth factor-D (VEGF-D), is a member of VEGF family of secreted glycoproteins (Orlandini *et al.* 1996). VEGF-D is recognized by VEGF receptors (VEGFR)-2 and -3 to exert angiogenic and lymphangiogenic functions (Achen *et al.* 1998; Davydova *et al.* 2016; Jauhiainen *et al.* 2011). VEGF-D plays vital roles in both physiological and pathological conditions. For example, it is reported that VEGF-D not only stimulates the proangiogenic phenotype of endothelial cells, but also modulates the antioxidant potential of cells to ensure their survival in the tumor microenvironment (Papiewska-Pająk *et al.* 2017). It is also proved that VEGF-D enhances oedema in response to hyperoxia in mice, and VEGF-D signalling facilitates vascular leak in human hyperoxic acute lung injury (Sato *et al.* 2016). In addition, studies indicated that VEGF-D promotes tumor lymphangiogenesis in various human cancers (Honkanen *et al.* 2016; Morfoisse *et al.* 2016; Sun *et al.* 2015). Our previous study found that FIGF mRNA expression was significantly increased in pancreatic excision mice, and overexpression of FIGF elevated the insulin 1 expression and insulin secretion in MS 1 cells (Teng *et al.* 2012), indicating that FIGF could be a promising regulator in β-cell regeneration. However, the precise mechanism of FIGF in β-cell regeneration needs further investigation.

In the present study, we initially found that the expression of FIGF was positively correlated with insulin expression. Besides, overexpressing of FIGF in BNL CL.2 cells enhanced the insulin expression by indirectly activation of MafA and Ngn3 via histone modification. Furthermore, *in vivo* study further confirmed that forced expression of FIGF facilitated the insulin expression and relieved hyperglycemia by up-regulating the MafA and Ngn3 expression in STZ mice. These findings suggested that FIGF facilitates the generation of insulin-producing β-like cells derived from BNL CL.2 cells through indirectly activation of MafA and Ngn3 via histone modification and may therefore be of use in cell-based therapies for diabetes.

## 2. Materials and methods

### 2.1 *Cell culture and lentivirus transduction*

BNL CL.2 normal mouse liver cell line was obtained from the Stem cell institute, General Hospital of Ningxia Medical University (Ningxia, China). The cells were regularly grown in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, USA) containing 10% fetal bovine serum (FBS, Hyclone, USA) and 1% penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were maintained in a 37°C incubator with a humidified atmosphere of 5% $CO_2$.

Ad-FIGF and Ad-GFP were prepared by Stem cell institute, General Hospital of Ningxia Medical University (Ningxia, China). BNL CL.2 cells ($1.2\times10^6$/well) were cultured in 6-well cell culture plate overnight and then transduced with purified Ad-FIGF (1 μL) and Ad-GFP (1 μL) with fresh culture media separately. Two hours later, culture media was changed for further culture, and seventy-two hours later, cells were harvested for immunofluorescence staining to detect the efficiency of lentivirus transduction.

### 2.2 *Immunofluorescence staining*

BNL CL.2 cells transduced with Ad-FIGF or Ad-GFP were washed with PBS for three times, and were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, USA) for 30 min at room temperature. After washing with PBS, cells were then permeabilized with 0.1% Triton X-100 (Sigma Aldrich, USA) for 15 min. Straight after, cells were washed with PBS and blocked with 5% bovine serum albumin (BSA, USB Corporation) for 30 min, incubated with primary antibodies overnight at 4°C and secondary antibodies for at room temperature for 2 h. Cells finally were visualized with a Zeiss LSM 780 confocal microscope (Carl Zeiss, Thornwood, NY, USA).

### 2.3 *RNA isolation and qRT-PCR*

Total RNA was isolated by TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol and quantified with at 260 and 280 nm a spectrophotometer. The PrimeScript$^{TM}$RT reagent Kit with gDNA Eraser (Takara, Beijing, China) was employed to synthesize cDNA by 250 ng of total RNA. Quantitative PCR was conducted using a TB Green$^{TM}$ Premix Ex Taq$^{TM}$a (Takara). qRT-PCR analysis was conducted with the qTOWER3G (Analytic Jena, Germany). β-actin was employed as an endogenous control. The relative expression level of target genes was calculated using the comparative Ct method. All the qRT-PCR experiments were performed at least 3 times. The primer sequences (forward and reverse) were designed as follows: β-actin, forward, 5′-AGGTCGG AGTCAACGGAT-3′ and reverse, 5′-TCCTGGAAGATG GTGATG-3′; FIGF, forward, 5′-ACAAGCACCTCCTACA TCTC-3′ and reverse, 5′-GTGGGCAAGCACTTACAA C-3′; insulin 2, forward, 5′-ACCCCACCCAGGCTTTTG-3′ and reverse, 5′-CACTTCACGGCGGGACAT-3′; Ngn3,

forward, 5′-AAAGCGAGTTG-GCACTAAGCA-3′ and reverse, 5′-CGTCTGG-GAAGGTGGGAAGTA-3′; MafA, forward, 5′-AGGAGGAGGTCATCCGACTG-3′ and reverse, 5′-CTTCTCGCTCTCCAGAATGTG-3′.

### 2.4 *Western blotting*

Western blotting was performed as described in a previous study (Jung *et al.* 2018). The BNL CL.2 cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing a complete protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were determined using Coomassie protein assay reagent (Thermo Scientific, Waltham, MA, USA). Protein extracts were separated by 10% SDS-polyacrylamide gels, and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore Immobilon, Burlington, MA, USA). After blocking with 5% skim milk for an hour at room temperature, immunoblots were probed with primary antibodies against overnight against FIGF (ab155288; Abcam, Shanghai, China), insulin (RRID: AB_2282474; Santa Cruz, Texas, USA), MafA (ab86020; Abcam, Shanghai, China), Ngn3 (ab176124; Abcam, Shanghai, China), and b-actin (TA-09; ZSGB, Beijing, China). Straight after, immunoblots were incubated with secondary antibodies conjugated with horseradish peroxidase (Leinco Technology, St. Louis, MO, USA) for an hour at room temperature, and proteins were detected using a gel documentation system (Gel Doc 1000, Bio-Rad Laboratories, Hercules, CA, USA).

### 2.5 *Histone extraction and Histone H3/H4 acetylation analysis*

Histone extracts were performed from BNL CL.2 cells by using EpiQuik$^{TM}$ Nuclear Extraction kit (Epigentek, Farmingdale, NY, USA). In brief, cells were harvested and pelleted by centrifugation at 1000 rpm for 5 min at 4°C. Cells were resuspended in GF1 buffer and lysed on ice, and then cells were centrifuged at 12000 rpm for 30 sec. Subsequently, GF2 buffer was added into the cells and incubated on ice for 5 min. Then cells were centrifuged at 12000 rpm for 5 min, and the supernatant fraction was carefully removed to a new vial. 1/4 volume of TCA buffer was added into the supernatant fraction and incubated on ice for 5 min, and centrifuged at 12000 rpm for 2 min. Cell pellet were resuspend in 3 of extraction buffer (0.5N HCl + 10% glycerol) and incubated on ice for 30 min. Supernatant fraction was removed to a new vial after centrifuging. Eight volumes of acetone were add and maintained at −20°C overnight. After centrifuging, the pellet was dissolve in distilled water. Quantify the protein concentration of each sample.

Histone H3/H4 acetylation analysis were performed using EpiQuik Total Histone H3 Acetylation Detection Fast Kit (Colorimetric) and EpiQuik Total Histone H4 Acetylation Detection Fast Kit (Colorimetric) (Epigentek, USA) following the manufacturer's protocol 72 h after Ad-FIGF treatment as previously described (Kocic *et al.* 2014). Specific buffers GF3 and GF4 were added into each well, together with the 2 μg of the histone extract into the sample wells. GF5 buffer was added into the well and incubated for 30 min, and then the wells were washed with GF3 buffer for three times. GF7 buffer was dilute and added into the wells and incubated for 1 h, and then the wells were washed with GF3 buffer for three times. The diluted GF8 buffer was added and incubated at room temperature for 30 min. After washing, GF9 buffer was added to each well and incubated at room temperature for 10 min. Finally, GF10 buffer was added, and the obtained color was read on a microplate reader at 490 nm. The calculation formula is as follows: $\text{H3/4 acetylation} = (\text{Sample OD} - \text{blank OD})/(\text{standard OD} - \text{blank OD}) \times 100\%$.

### 2.6 *ChIP assay*

The ChIP assay was performed using EZ ChIP chromatin immunoprecipitation kit (Millipore) as per the manufacturer's instructions. Briefly, chromatin from BNL CL.2 cells infected with Ad-FIGF or Ad-GFP was sonicated with a Diagenode Bioruptor (UCD-200TM-EX; Diagenode Inc.) at high power cycles of 30′-on and 30′-off for 10 min. After sonication, cell lysate underwent centrifugation, and 100 μl of supernatant was collected for each immunoprecipitation. Before immunoprecipitation, ten percent of DNA sample was preserved as an input control. Primary antibodies against antiacetyl Anti-acetyl-Histone H3 (Lys9) antibody (07-352, Millipore), Anti-Histone H4 (tri methyl K20) antibody (ab9053, Abcam), and rabbit IgG (Invitrogen) were used for immunoprecipitation. The detailed information is in the product link: Anti-acetyl-Histone H3 (Lys9) antibody 232 (07-352, Millipore) (*https://www.merckmillipore.com/CN/zh/product/Anti-acetyl-Histone-H3-Lys9-Antibody,MM_NF-07-352?bd=1*); Anti-Histone H4 (tri methyl K20) antibody (ab9053, Abcam) (*https://www.abcam.cn/histone-h4-tri-methyl-k20-antibody-chip-grade-ab9053.html#top-400*). All DNA samples, including the input DAN and antibody-bound chromatin were reversecross-linked, purified, and then subjected to a PCR. PCR products were visualized by autoradiography after electrophoresis on 2% agarose gels.

### 2.7 *Animal experiments*

Six- to eight-week-old male C57BL/6 mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. A total of 25 mice were kept in separate standard cages and maintained in a controlled temperature (25 ± 2°C), with a humidity of 50% ± 20% under a 12 h/12 h light/dark cycles. After acclimatising for a week, all mice were injected intraperitoneally with streptozotocin STZ

(Sigma, USA) at a dose of 50 mg/kg in 0.1 M sodium citrate buffer for 7 days continuously. The blood glucose levels of mice were detected by a glucometer (Johnson & Johnson, USA), and blood glucose level > 16.7 mmol/L was considered as type I diabetes.

Next, 40 μl Ad-FIGF or Ad-GFP (40 μl, MOI = $1.1 \times 10^{13}$) was transplanted into STZ-induced diabetic mice via tail vein injection. One week after infection, six of the mice were sacrificed, and the pancreases were harvested for immunofluorescence. Blood glucose levels were measured every week for 3 weeks after Ad-FIGF or Ad-GFP injection. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of General Hospital of Ningxia Medical University.

### 2.8 *Statistical analysis*

Data analyses were performed using the SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). All analyses were done for at least three times. One-way ANOVA or Student's $t$ test were used to monitor the difference between groups. Spearman's correlation analysis was conducted to verify the association between FIGF and insulin. All experimental values were shown as the mean ± SD. $p < 0.05$ was considered significance for all tests.

## 3. Results

### 3.1 *FIGF expression is elevated in mice with partial pancreatectomy*

In our previous study, we constructed a partial pancreatectomy mice model to find out the differentially expressed genes by whole genome microarray analysis. We compared the differentially expressed genes in mice 48 h after the partial pancreatectomy with normal mice and observed that the mRNA expression of c-fos-induced growth factor (FIGF) was highly expressed in pancreatic tissues in partial pancreatectomy mice compared to that in normal mice (figure 1), indicating that FIGF may play an important role in diabetes.

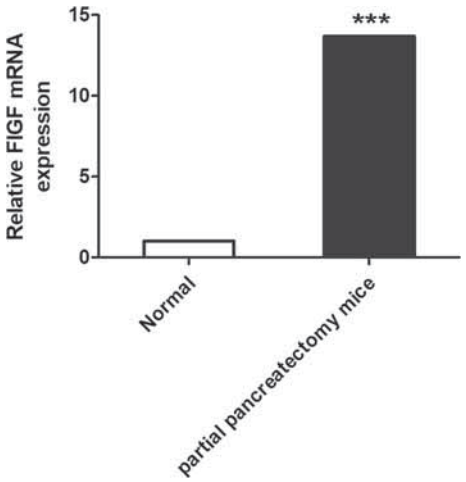


**Figure 1.** FIGF expression is elevated in mice with partial pancreatectomy. The mRNA expression of FIGF was 13.69 higher in the pancreatic tissues in partial pancreatectomy mice compared with that in normal mice.

### 3.2 *FIGF promotes insulin expression in BNL CL.2 cells*

To investigate the role of FIGF in the regulation of insulin expression in non-insulin producing cells, BNL CL.2 cells were employed. Adenoviral vector encoding FIGF, called Ad-FIGF and its negative control, Ad-GFP were introduced into BNL CL.2 cells respectively. Immunofluorescence of GFP shown in figure 2A illustrated that the method we employed transduced Ad-FIGF or Ad-GFP cells successfully. To further confirm the transduction and check the efficiency of Ad-FIGF, qRT-PCR and western blot conducted, and the results proved that BNL CL.2 cells transduced with Ad-FIGF expressed much higher FIGF than that transduced with Ad-GFP (figure 2B). As expected, immunofluorescence staining for insulin demonstrated that insulin expression was notably enhanced in Ad-FIGF-treated cells compared with Ad-GFP-treated cells (figure 2C). qRT-PCR and western blot analysis further confirmed that the mRNA and protein levels of insulin were significantly increased by overexpression of FIGF (figure 2D). In consistent, the ELIAS results demonstrated that the insulin expression in the medium of cells overexpressing FIGF was enhanced (figure 2E), indicating that FIGF promotes insulin expression in BNL CL.2 cells.

### 3.3 *FIGF enhances transcription of MafA and Ngn3 via H3/H4 histone modification*

To gain insight into the molecular details by which FIGF altered the phenotype of BNL CL.2 cells, we firstly analyzed the transcription level of MafA and Ngn3 genes in BNL CL.2 cells by qRT-PCR. Results showed that the mRNA level of MafA and Ngn3 were increased in Ad-FIGF treated cell (figure 3A). Congruously, the protein levels of MafA and Ngn3 were evidently promoted in FIGF-overexpressed BNL CL.2 cells detected by western blotting (figure 3B). In addition, we examined the acetylation status of the histone to verify the epigenetic regulatory effects exerted by FIGF in BNL CL.2 cells. Histone H3/H4 acetylation analysis demonstrated that the levels of histone H3/H4 acetylation were up-regulated in FIGF-overexpressed BNL CL.2 cells (figure 3C-D). Furthermore,

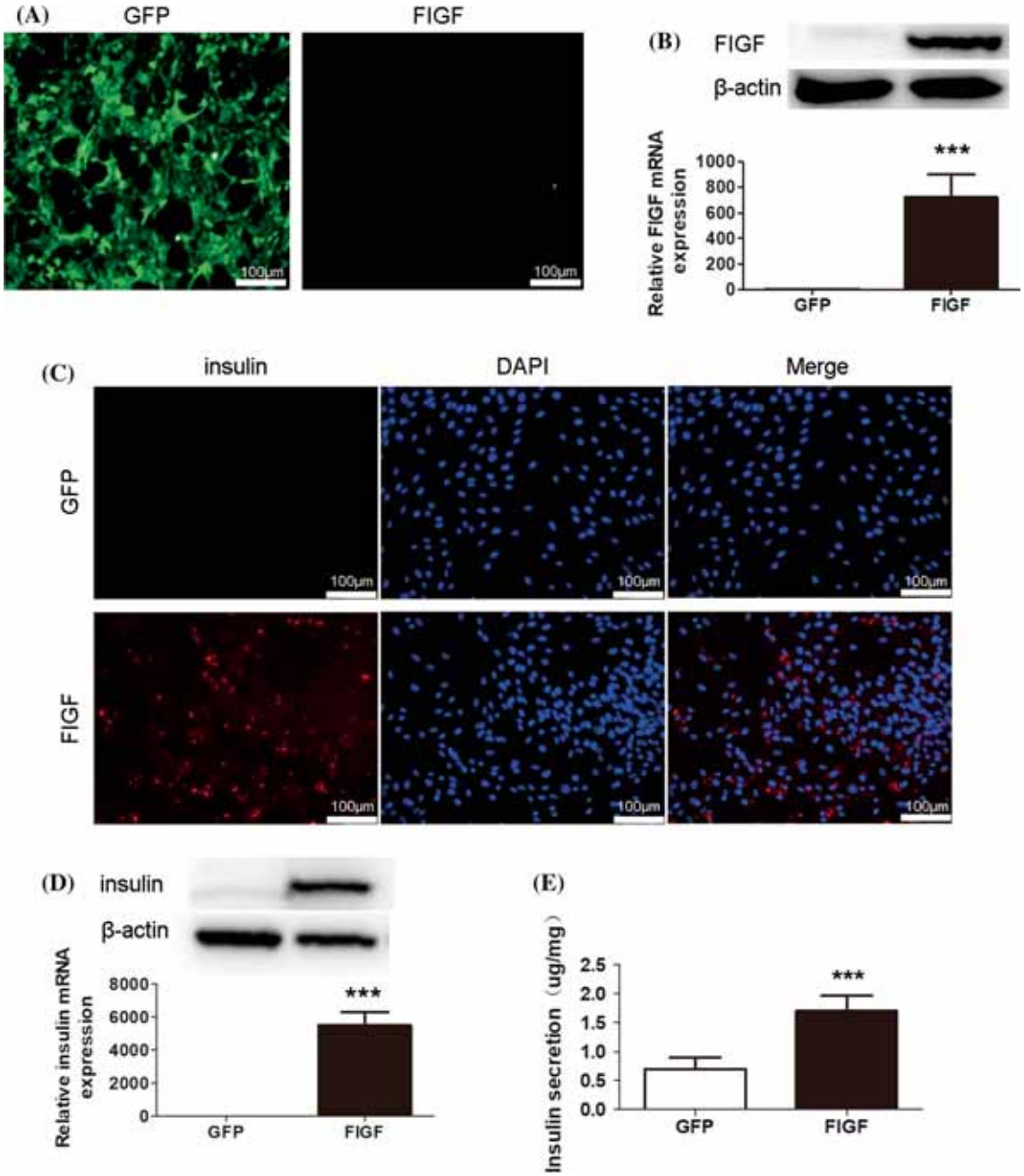


**Figure 2.** FIGF promotes insulin expression in liver cells. (**A**) Immunofluorescence verified that BNL CL.2 cells were successfully transduced with Ad-FIGF or Ad-GFP. Bars = 100 μm. (**B**) qRT-PCR and western blotting showed that BNL CL.2 cells transduced with Ad-FIGF expressed much higher FIGF mRNA and protein levels than that transduced with Ad-GFP. (**C**) Immunocytochemistry staining exhibited increased insulin expression in Ad-FIGF-treated cells compared with Ad-GFP-treated cells. Bars = 100 μm. (D) The mRNA and protein levels of insulin-1 were examined by qRT-PCR and western blot analysis in FIGF-overexpressed BNL CL.2 cells and negative control. (**E**) The secretion of insulin-1 in culture media were examined by ELISA in FIGF-overexpressed BNL CL.2 cells and negative control.

the ChIP analysis showed a significant enhancement in H3/H4 acetylation in the MafA and Ngn3 promoters following Ad-FIGF treatment (figure 3E-F). Hence, the above results testified that FIGF enhances the transcription of MafA and Ngn3 via increasing H3/H4 histone modification in BNL CL.2 cells.

### 3.4 *FIGF facilitates the insulin expression in vivo*

To assess the function of FIGF *in vivo*, mice were intraperitoneally injected with streptozotocin (STZ) to imitate a type I diabetic state. Blood glucose levels were measured to ensure drug efficacy. Afterwards, Ad-FIGF

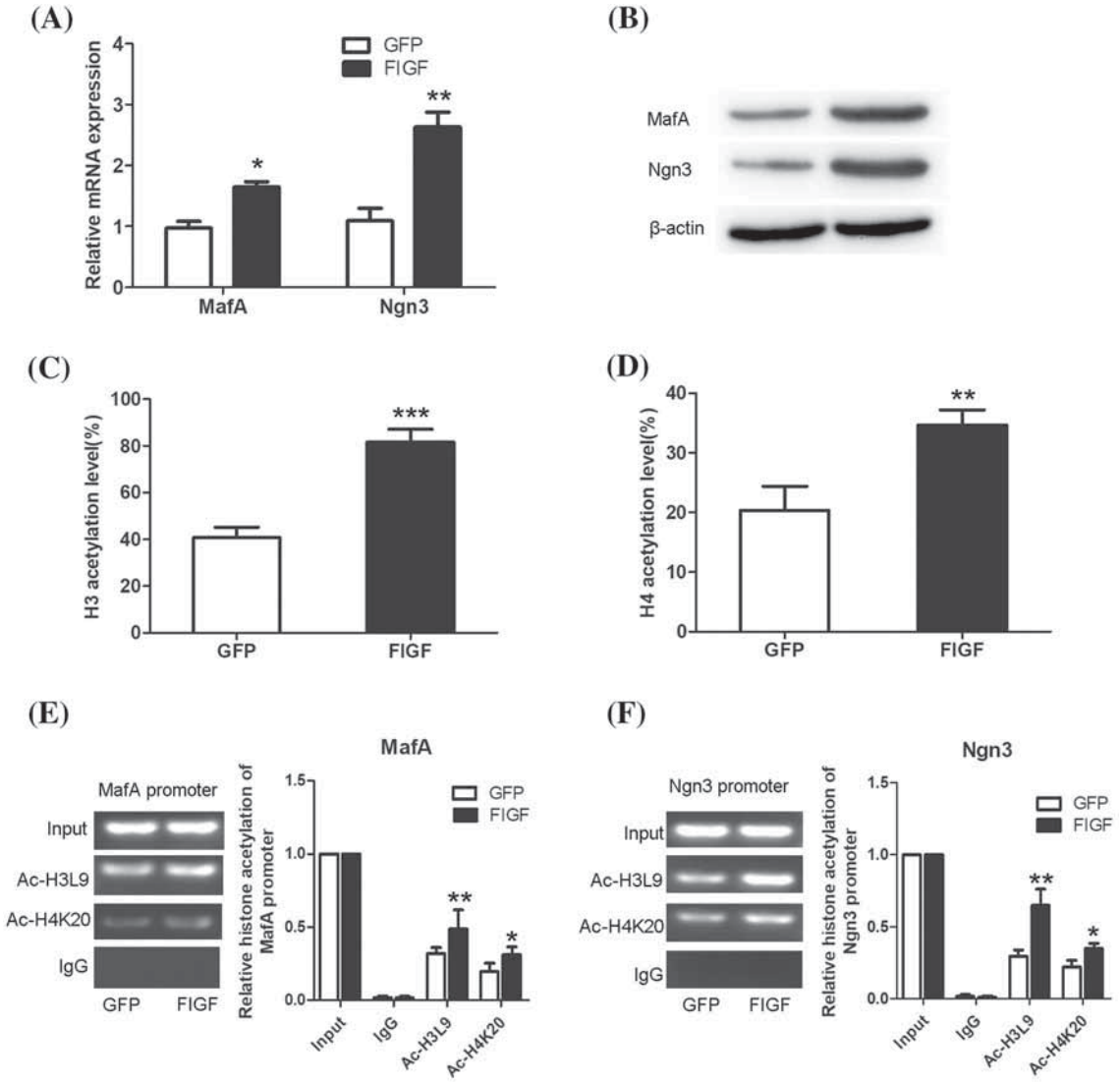


**Figure 3.** FIGF enhances transcription of MafA and Ngn3 via H3/H4 histone modification in BNL CL.2 cells. (**A**) The transcription level of MafA and Ngn3 genes in BNL CL.2 cells were tested by qRT-PCR. (**B**) The protein expression of MafA and Ngn3 were detected by western blotting in BNL CL.2 cells. All band densities were normalized to β-actin. (**C**, **D**) Histone H3/H4 acetylation in Ad-FIGF treated and Ad-GFP treated CL.2 cells (72 h after treatment). (**E**, **F**) Histone H3/H4 acetylation at MafA and Ngn3 promoter regions were conducted with ChIP and Q-PCR assay in in Ad-FIGF treated and Ad-GFP treated CL.2 cells.

and empty vector were injected into mice via tail vein, respectively. After infection, the level of blood glucose was measured every week. As shown in figure 4A, Ad-FIGF injection gradually decreased the blood glucose levels and showed significant difference in day 14 after injection compared with Ad-GFP injection. In addition, the serum insulin was increased in Ad-FIGF injected mice (figure 4B). Meanwhile, the immunofluorescence results illustrated that the expression of insulin were evidently elevated in pancreatic tissues in Ad-FIGF-treated STZ group (figure 4C). Taken together, our results demonstrated that FIGF could facilitate the insulin expression and help to moderate hyperglycemia in STZ mice.

## 4. Discussion

This study demonstrated that treatment with Ad-FIGF can enhance the generation of β-like cells from BNL CL.2 cells *in vitro* and increased the insulin secretion to stabilize levels of blood glucose *in vivo*, which enabled its application for treating diabetes.

The main function of FIGF is promoting angiogenesis and lymphangiogenesis. Adenovirus-mediated gene transfer of human VEGF-D enhances transient angiogenic effects in both mouse hind limb muscle (Kholová *et al.* 2007) and porcine heart (Rutanen *et al.* 2004). VEGF-C and -D are reported to participate in lymphangiogenesis in mouse unilateral ureteral obstruction (Lee *et al.* 2013). Interestingly,

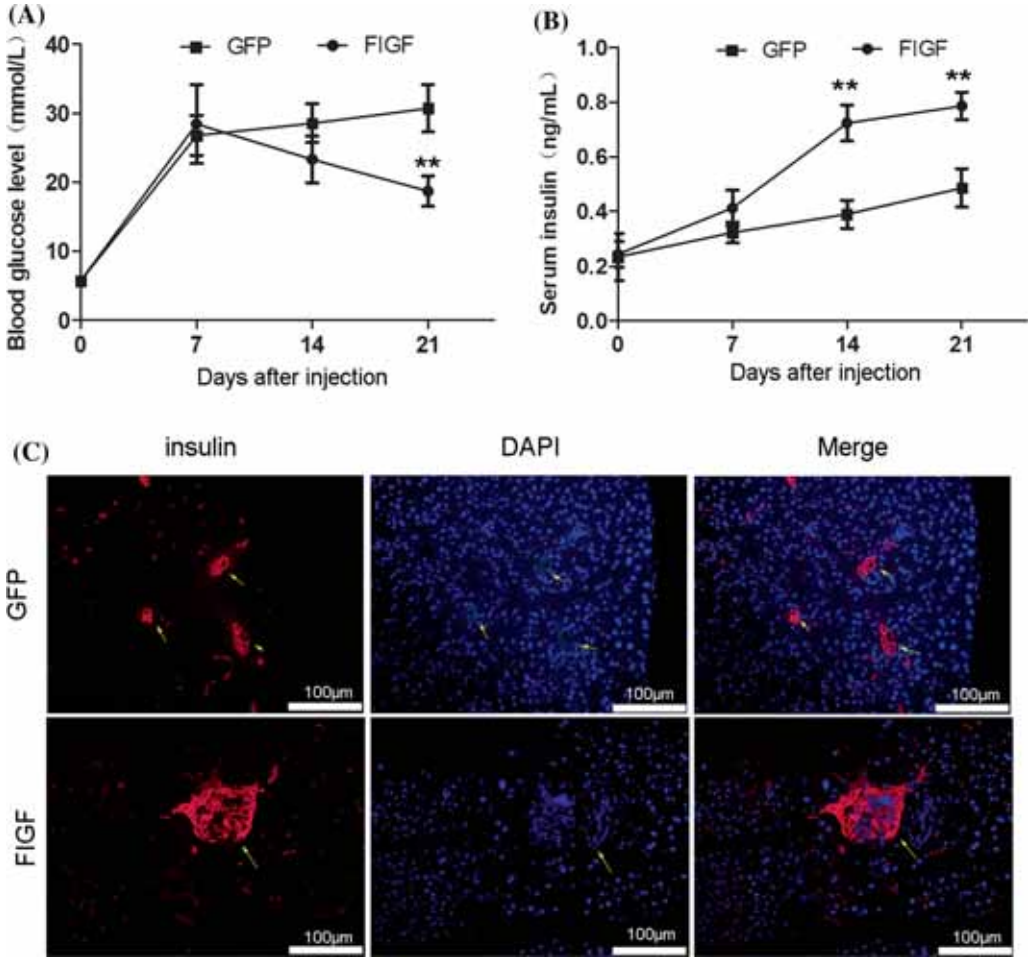


**Figure 4.** FIGF facilitates the insulin expression *in vivo*. (**A**) Blood glucose level was measured after adenovirus vector injection in STZ and STZ+FIGF groups every week. (**B**) The expressions of insulin-1 in serum were determined in mice of STZ and STZ+FIGF groups. (**C**) Immunofluorescence staining of insulin in pancreatic tissues harvested from mice of STZ and STZ+FIGF groups. Bars = 100 μm. The yellow arrow indicates the islets.

VEGF-D gene transfer is verified to induce efficient angiogenesis in the presence of severe diabetes (Roy *et al.* 2010). More importantly, FIGF was found to play an important role in β-cell regeneration (Teng *et al.* 2012). Nevertheless, the cell reprogramming function of FIGF needs further investigation. In this study, *in vitro* assays showed that overexpressing of FIGF in BNL CL.2 cells enhanced the insulin expression, and *in vivo* assays further confirmed that injection of Ad-FIGF in STZ mice stimulated the expression of insulin and stabilize the blood glucose, demonstrating that FIGF facilitates insulin expression in BNL CL.2 cells and has a great potential for diabetes treatment.

Recently, great efforts have been made to differentiate non-insulin-secreting-cells to β-like cells by transfection of pancreas-specific transcription factors. Xu *et al.* indicated that combined expression of Pdx1 and MafA with either Ngn3 or NeuroD promotes the reprogramming of mouse embryonic stem cells into insulin-producing cells (Xu *et al.* 2013). You *et al.* demonstrated overexpression of PDX-1, NeuroD and MafA induces the reprogramming of porcine neonatal pancreas cell clusters and adult pig pancreatic cells into β-like cells (You *et al.* 2011). Chang *et al.* proved that PDGF facilitates direct lineage differentiation of Hepatocytes to functional β-like cells induced by Pdx1 and Ngn3 (Chang *et al.* 2016). Consistently, our results showed that forced expression of FIGF improved the protein levels of MafA and Ngn3 in BNL CL.2 cells. More importantly, Ad-FIGF treatment evidently elevated the insulin expression in pancreatic tissues in STZ mice. Furthermore, it was verified that FIGF overexpression up-regulated the histone H3/H4 acetylation in BNL CL.2 cells. These phenomena emphasized that FIGF facilitates insulin expression by up-regulating the MafA and Ngn3 expression and H3/H4 histone modification *in vitro* and *in vivo*.

In conclusion, we have demonstrated that FIGF facilitates the insulin expression of BNL CL.2 cells by promoting the MafA and Ngn3 expression via enhancing the histone H3/H4 acetylation. These findings support the assumption of employing β-like cells derived from non-insulin-secreting cells for the cell-based treatment for patients with diabetes.

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