

# Dynamic Changes of Betatrophin and Its Potential Effect on $\beta$ -cells in Spontaneously Developed or Streptozocin-induced Diabetes Monkeys

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## Abstract

Effects of betatrophin on  $\beta$ -cell proliferation and insulin secretion have been reported controversially in rodent studies. This study was to investigate the dynamic changes of betatrophin and its potential effect on  $\beta$ -cell function in non-human primates (NHPs). Blood betatrophin levels in naturally developed diabetes cynomolgus monkeys (n=65) significantly correlated with their body weights and blood glucose levels in the females (n=20) and with insulin and C-peptide levels in the males (n=45). Liver expression of betatrophin mRNA was markedly higher in spontaneously developed diabetes monkeys than in normoglycemic ones. Its liver expression correlated well with islet size and insulin content in normoglycemic cynomolgus monkeys and also well in spontaneously hyperglycemic (>200 mg/dL) cynomolgus monkeys with high blood insulin level, but not well in those with very low blood insulin content. Both blood glucose and betatrophin increased dramatically in normoglycemic rhesus monkeys (n=7) after streptozocin (STZ) administration. Blood insulin initially increased on day 1 after STZ dosing and then decreased dramatically. Two NHPs with blood glucose back to ~80 mg/mL on day 14 after STZ injection showed recovery of insulin secretion. In another two STZ-treated NHPs with blood glucose >250 mg/dL, apparent proliferation of both  $\beta$ -cells and non- $\beta$ -cells was observed in their islets. The rest NHPs with more completed  $\beta$ -cell destruction after STZ dosing showed much higher blood glucose (>300 mg/dL) with very low insulin and no obvious  $\beta$ -cell proliferation. Our results indicate that NHP liver expresses abundant betatrophin mRNA. As its levels correlated well with islet size and insulin content in some diabetic NHPs, it might play a role in  $\beta$ -cell proliferation. Our data are consistent with the clinical finding of the elevation of circulating betatrophin in patients with type 2 diabetes. In addition, betatrophin-enhanced  $\beta$ -cell proliferation seems requiring a minimal base level of pancreatic  $\beta$ -cells and islets.

**Keywords:** Betatrophin;  $\beta$ -cell; Insulin; Streptozocin; Nonhuman primate

## Introduction

Diabetes results from dysfunctional carbohydrate metabolism due to a relative deficiency of insulin. The world prevalence of diabetes among adults (aged 20-79 years) was 6.4%, affecting 285 million adults, in 2010, and will increase to 7.7% and 439 million adults by 2030. Between 2010 and 2030, there will be a 69% increase in numbers of adults with diabetes in developing countries and a 20% increase in developed countries [1]. It has become a major threat to human health.

Though diabetes is treated with antidiabetic drugs or subcutaneous insulin injection, these treatments do not provide the same degree of glycemic control as functional pancreatic  $\beta$ -cells and do not prevent the debilitating consequences of the disease. Treatments that replenish  $\beta$ -cell mass in diabetic patients could allow for the long-term restoration of normal glycemic control and thus represent a potentially curative therapy. Despite the fact that the primary causes for type I and type II diabetes mellitus (T2DM) differ, all diabetes will benefit from treatments that replenish their  $\beta$ -cell mass.

Regeneration of  $\beta$ -cells is usually extremely low except some special conditions like acute injury and pregnant in rodents and humans which imply the existence of  $\beta$ -cell regeneration mechanism under certain specific physiopathological conditions [2-7]. Researchers found mouse  $\beta$ -cells are the products of uniform self-renewal. It is lowered by a replication refractory period that prevents  $\beta$ -cells from immediately redividing [2].  $\beta$ -cell progenitors can be activated in injured adult mouse pancreas. Differentiation of the adult progenitors is Ngn3 dependent

and gives rise to all islet cell types, including glucose responsive  $\beta$ -cells. Multipotent progenitor cells exist in the pancreas of adult mice and can be activated autonomously to increase the functional  $\beta$ -cell mass by differentiation and proliferation [3]. Ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into  $\beta$ -cells [4]. The evidence suggests  $\beta$ -cell regeneration is a process which can be induced.

Betatrophin, also called angiopoietin-like protein 8 (ANGPTL8) [8] lipasin [9-11], is a polypeptide and essentially secreted by liver or adipose tissue, and plays a major role on stimulating cell proliferation of islet cells and enhancing insulin producing in rodents [11-15]. However, the late studies in rodents found ANGPTL8 does not stimulate beta cell replication [16-18]. Human study showed that betatrophin concentrations were approximately 40% higher or doubled

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in patients with type I and type II diabetes compared with controls [19-23]. Betatrophin concentrations are dramatically increased during pregnancy and are significantly higher in gestational diabetes mellitus [24]. It has been known that diabetic pancreas pathophysiology differs in rodents from in humans greatly, but nonhuman primates (NHPs) are more close to humans [25-27]. Numerous studies have used spontaneously developed or streptozocin (STZ)-induced diabetic monkeys to investigate diabetes progression and therapy [28-32].

A spontaneously developed diabetic model gives us an insight of the naturally occurring process of diabetes in NHPs, but it may take years to show the phenotype and raise the difficulty to monitor the disease progress. While STZ-induced diabetes can be initiated in a short period of time and give us a dynamic process from non-diabetes to diabetes in a short duration. The change of blood betatrophin concentration and its correlation with other biological responses might provide important information for diabetes research and therapy. This study was to evaluate the dynamic changes of liver betatrophin transcription and its plasma level in spontaneously developed or STZ-induced diabetes monkeys. In addition, the correlations of betatrophin levels with islet sizes, insulin contents and blood glucose levels were examined.

## Methods

Experiments were performed in cynomolgus ( $n=65$ , 20 females with the age of  $20.3 \pm 0.9$  years and 45 males with the age of  $12.8 \pm 0.7$  years) and rhesus ( $n=8$ , 4 females with their ages from 7 to 15 years and 4 males with their ages of 7 years) monkeys. These monkeys were individually housed and maintained in our animal facility in accordance with the guidelines approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals had continuous access to water ad libitum and controlled access to food according to their body weights. Room temperature was maintained at  $\sim 21^\circ\text{C}$ . The animals were maintained on a 12 hr light/dark cycle with lights off from 7 PM to 7 AM. The monkeys were maintained with a complete, nutritionally balanced diet (Beijing Keao Xieli Feed Co., LTD, Beijing, China) and enriched with seasonal fruits and vegetables. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Crown Bioscience, Inc.

## Streptozocin treatment and diabetes induction

Seven normoglycemia rhesus monkeys were intravenously injected with STZ (Lot S0130, Sigma, USA) at 35 mg/kg (35 mg/mL dissolved in the PBS, phosphate buffer saline) once for diabetes induction and one animal was treated with the neutral PBS as control. The process of induction was similar to others reported in STZ monkey models [29,30,32]. The experimental animals were euthanized on day 7 ( $n=5$ ) and day 14 ( $n=3$ ) after STZ or vehicle injection.

## Plasma and tissue collection

After an animal was fasted overnight for approximately 16 hours, 2 to 3 mL whole blood was collected into K2-EDTA tube. The tubes were inverted for 10 times immediately then placed on ice. Plasma was separated by centrifugation at 3000 rpm for 15 min at  $4^\circ\text{C}$ . The plasma was stored at  $-80^\circ\text{C}$  until analysis.

For tissue collection, the animals were anesthetized with intramuscular injection of 15 mg/kg ketamine (Fujian Gutian Pharma, China) initially and then added 5 mg/kg as needed. The animal euthanasia was conducted with intravenous injection of sodium pentobarbital (Guangzhou Sile, China) at 100 mg/kg. All the tissues or organs collected were processed in 10 times volume of 10% formalin or

RNALater separately for later in vitro experiments. Tissues in formalin were incubated on a shaker for 24 hours and then washed 3 times with PBS. RNA later samples were incubated at  $4^\circ\text{C}$  overnight and then stored at  $-80^\circ\text{C}$  after pipetting the RNA later liquid into cryopreserved vials.

Serum glucose, insulin, Triglycerides (TG), high density lipoproteins (HDL), low density lipoproteins (LDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and Apolipoprotein B (APOB) were analyzed in the clinical lab (The First People's Hospital of Taicang, Taicang, Jiangsu Province, China). Betatrophin was tested with a monkey specific ELISA kit (Aviscera Bioscience, Santa Clara, CA, USA; EIAAB, Wuhan, China).

## Immunohistochemistry

The paraffin embedded sections were deparaffinized/rehydrated in xylene followed by ethanol and PBS serial rehydration. Antigen retrieval was completed in 0.01 M citrate buffer (pH 6.2) for 30 min in boiling water. Sections were treated in 1% triton X-100 for 20 min and then blocked in PBS with 3% goat serum and 0.03% Tween-20 for 30 min. Incubation with the primary antibody (mouse anti-insulin monoclonal and rabbit anti-Ki67 polyclonal antibody) being diluted in 1% goat serum and 0.03% Tween-20 was performed at room temperature for 2 hours in a wet chamber. Three times washed with PBST (PBS with 0.03% Tween-20) were performed following incubation. Slides were incubated in PBS with Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 555 donkey anti-rabbit IgG, 0.5  $\mu\text{g}/\text{mL}$  DAPI, 1% goat serum and 0.03% Tween-20 for 30 min in a wet chamber protected from light. After 3 times washed with PBST, slides were mounted with flumromount (Sigma, USA) for reading by microscope (Olympus BX51, Japan).

## Quantitative polymerase chain reaction

Total RNA from the organs/tissues was extracted using RNAeasy plus mini kit (Qiagen), and genomic DNA was digested using RNase free DNase. For real-time PCR analysis, cDNA was synthesized using Superscript III First Strand cDNA synthesis kit (Invitrogen). Amplification of Betatrophin and the internal control of GAPDH were conducted on Stratagene Mx3005p (Agilent Technologies) by using the SYBR-green reaction system.

## Intravenous glucose tolerance test (ivGTT)

To evaluate  $\beta$ -cell function ivGTT was performed in the experimental monkeys according to the method reported previously [33,34]. The animals were fasted for overnight 16 hrs and then anesthetized with an initial dose of ketamine at 10 mg/kg (i.m.) plus one additional dose of 5 mg/kg during the procedure when needed. The monkey was heated with a circulating water pad ( $37^\circ\text{C}$ ) to prevent body temperature drop. The cephalic and/or saphenous veins were cannulated separately for glucose infusion and blood collection. Glucose (0.25 g/kg=0.5 mL/kg of 50% dextrose) was intravenously infused during 30 sec and the system was then flushed with 5 mL heparinized saline to push in the residual glucose. Blood was collected from a different vein into heparinized tubes pre-chilled on ice immediately before and at 3, 5, 7, 10, 15, 20, 30 and 60 min after glucose infusion. Plasma was then separated and stored at  $-80^\circ\text{C}$  for subsequent assays lately.

## Data Analysis

Data were expressed as mean  $\pm$  standard error of the mean. Statistical significance for multiple observation parameters in the same group was determined by One-way Analysis of Variance. If statistical significance of differences was detected, then Tukey's Multiple

Comparison Test (GraphPad Software, Inc., La Jolla, CA, USA) was also conducted to determine the significance. The comparison between diabetic and normoglycemic groups was tested by the un-paired t-test. Statistical significance was considered if p value was  $<0.05$ .

## Results

### Correlation between blood betatrophin and other parameters in spontaneously developed diabetic monkeys

To investigate if the gender of our housed monkeys affected blood betatrophin level and other parameters, blood samples were collected from overnight fasted adult diabetes cynomolgus monkeys under conscious condition. Compared with the male animals, the plasma levels of glucose, HbA1c, TC and TG were significantly higher in the housed female monkeys (Figure 1). The body weights were significantly

lower in the females than in the males, but the plasma levels of betatrophin, insulin, C-peptide, HDLc and LDLc were not significantly different between the males and females (Figure 1). It is interesting that the plasma betatrophin levels correlated positively with the plasma glucose levels and negatively with the body weights in the female monkeys ( $p < 0.05$ ,  $n = 20$ , Figure 2B and 2E) and correlated negatively with the plasma insulin and C-peptide levels in the males ( $p < 0.05$ ,  $n = 45$ , Figure 2C and 2G). However, other parameters measured, such as age, plasma TC, HbA1c and TG levels, were not significantly correlated with plasma betatrophin levels in both males and females ( $p > 0.05$ , Figure 2A, 2D, 2F, 2H).

To understand if insulin treatment affected betatrophin production, plasma betatrophin and other metabolic parameters were measured and compared between insulin-dependent and insulin-independent diabetes cynomolgus monkeys. The insulin-dependent monkeys were obviously much older than non-insulin-dependent ones ( $p < 0.01$ , Figure 3). Compared with the non-insulin dependent animals, the insulin-dependent monkeys showed significantly higher levels of plasma betatrophin, glucose, HbA1c, insulin, TC and TG (Figure 3). The parameters of body weight, C-peptide, HDLc and LDLc were not significantly different among the animals with or without insulin treatment (Figure 3).

### Betatrophin expression in various tissues from diabetic monkeys

To determine the expression levels of betatrophin in different tissues, 6 cynomolgus monkeys with various blood glucose levels were sacrificed (Table 1) and multiple organs and tissues were collected. Figure 4A shows betatrophin mRNA expression levels in different NHP tissues. Clearly, betatrophin mRNA dominantly expressed in the liver of the monkeys. In the adult monkeys with blood glucose  $< 90$  mg/dL, the levels of betatrophin mRNA expressed in the liver correlated

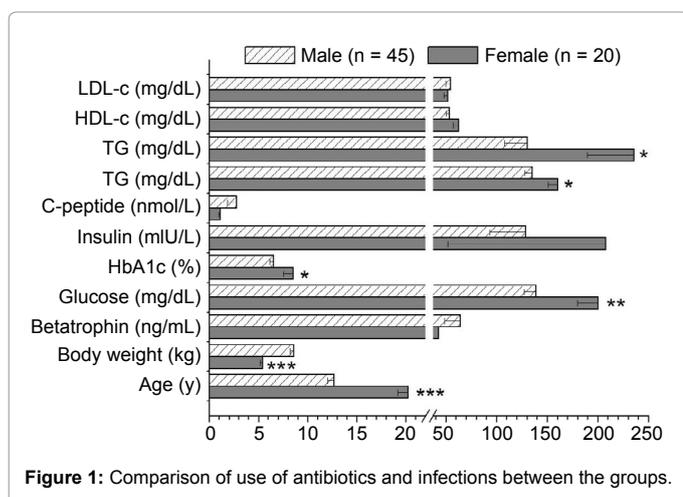


Figure 1: Comparison of use of antibiotics and infections between the groups.

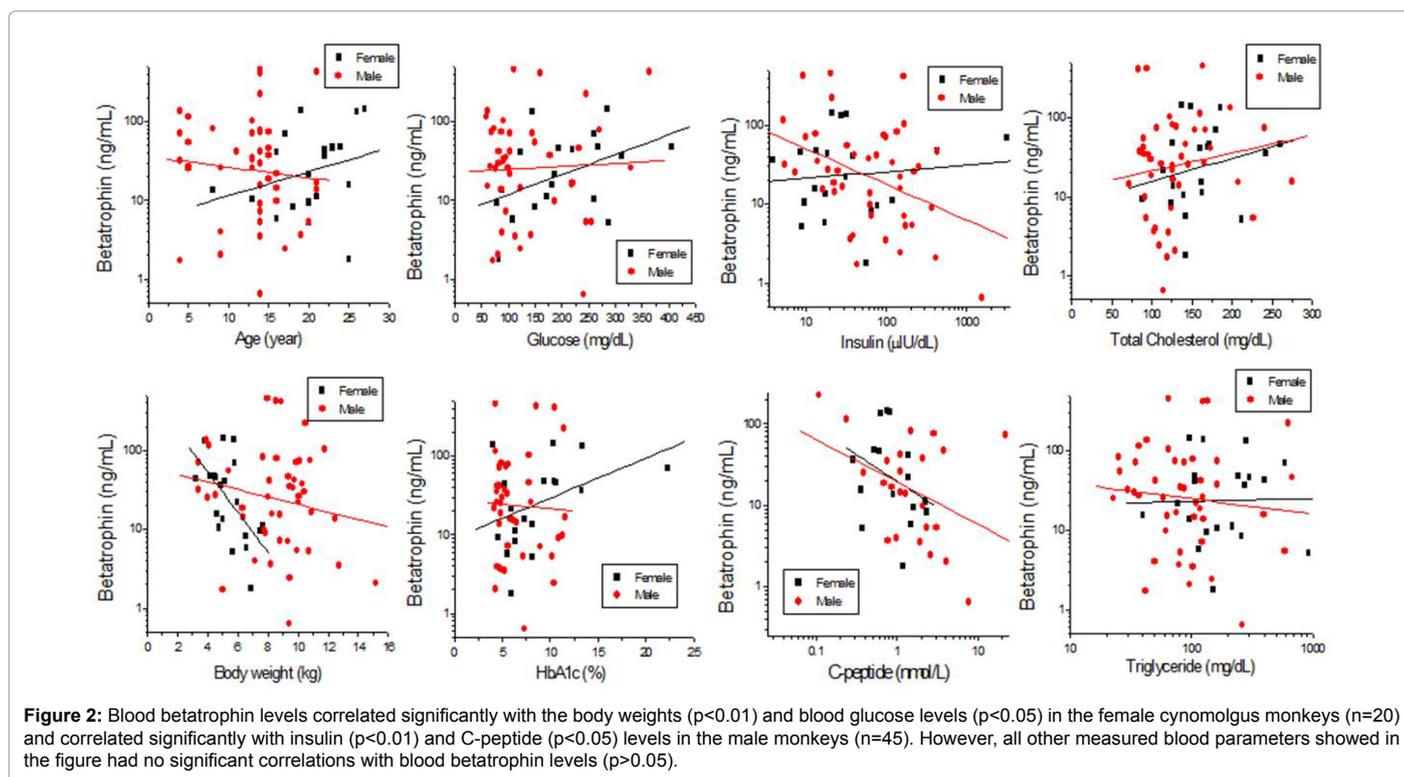
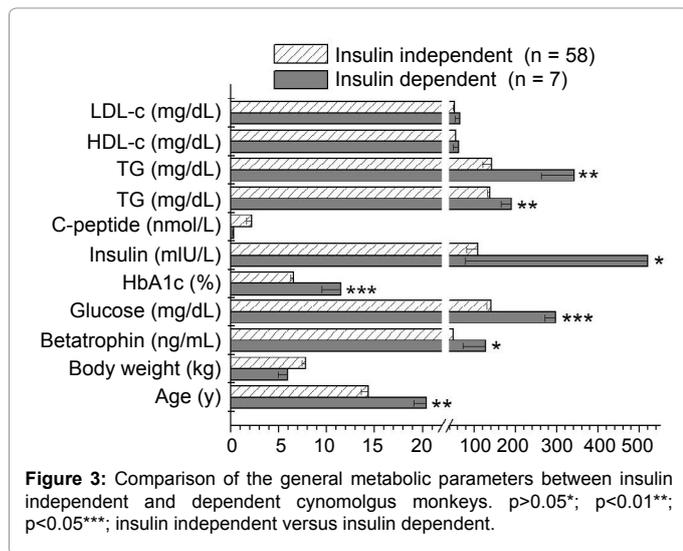


Figure 2: Blood betatrophin levels correlated significantly with the body weights ( $p < 0.01$ ) and blood glucose levels ( $p < 0.05$ ) in the female cynomolgus monkeys ( $n = 20$ ) and correlated significantly with insulin ( $p < 0.01$ ) and C-peptide ( $p < 0.05$ ) levels in the male monkeys ( $n = 45$ ). However, all other measured blood parameters showed in the figure had no significant correlations with blood betatrophin levels ( $p > 0.05$ ).



**Figure 3:** Comparison of the general metabolic parameters between insulin independent and dependent cynomolgus monkeys.  $p > 0.05^*$ ;  $p < 0.01^{**}$ ;  $p < 0.05^{***}$ ; insulin independent versus insulin dependent.

Animal ID	Age (year)	Body weight (kg)	Fasting glucose (mg/dL)	Fasting insulin (mIU/L)
V03	12	4.2	77	15
J01	19	8.2	57	46
D05	22	14.1	87	122
G01	15	8.6	250	301
M04	22	6.1	67	127
V04	20	3.6	212	3

Note: Monkeys M04 and V04 were female and all of others were male.

**Table 1:** Characteristics of the cynomolgus monkeys enrolled in the experiment.

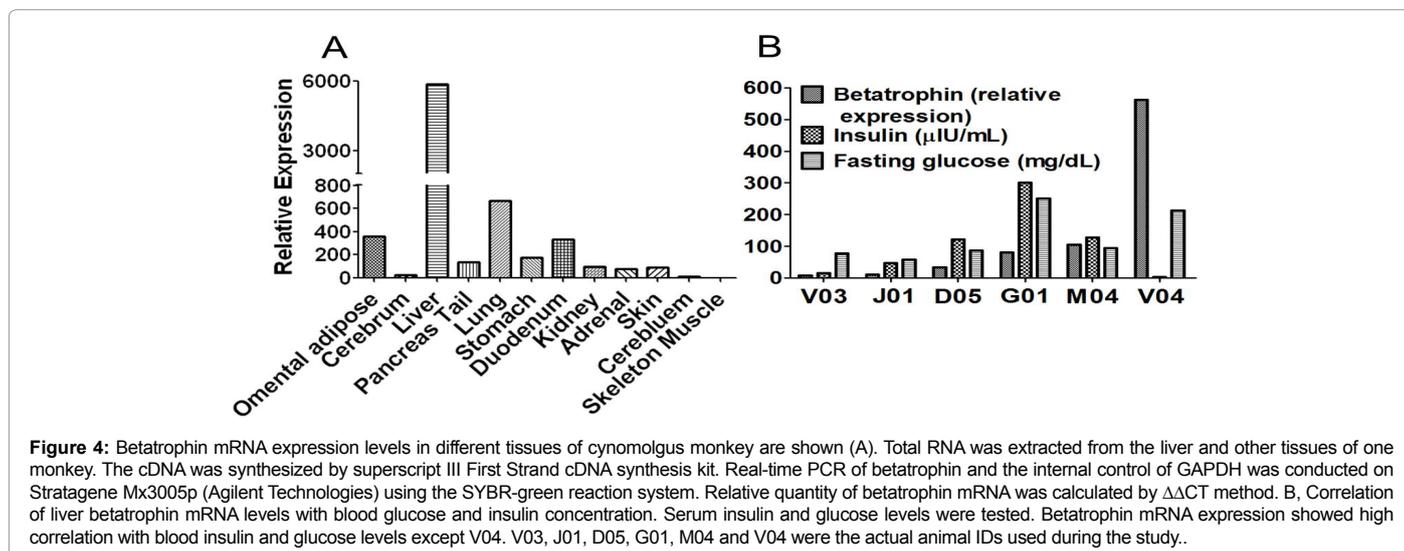
well with the islet size and insulin content (Figures 4B and 5). However, compared with the normoglycemia animals, the diabetic monkeys expressed markedly higher betatrophin mRNA in their livers. Two different correlations were observed between liver betatrophin mRNA levels and islet sizes in the diabetic monkeys. The betatrophin mRNA level in the liver correlated well with the islet size in the animal G01 who had hyperglycemia (>200 mg/dL) and high blood insulin (Figures 4B and 5C). However, another hyperglycemia animal V04, who was older and suffered from diabetes for a longtime, with extremely high

betatrophin mRNA expression and very low blood insulin did not show the increase in islet size (Table 1, Figures 4B and 5D).

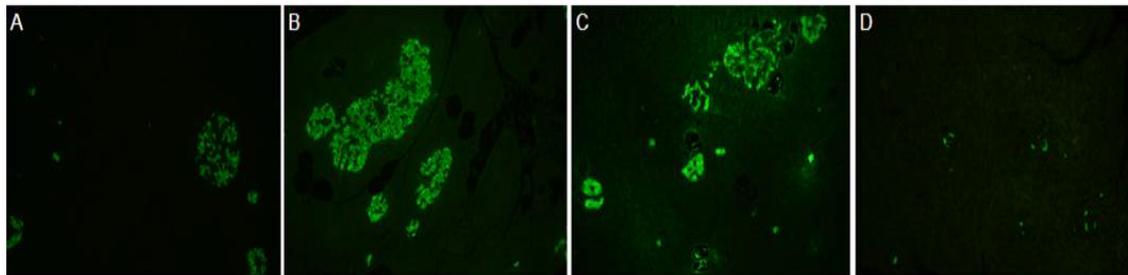
### Dynamic changes of betatrophin in STZ-induced diabetic monkeys

The above data from the spontaneously developed diabetes cynomolgus monkeys suggest that betatrophin had certain correlations with diabetes development and  $\beta$ -cell function. However, the progress of naturally developed diabetes in NHPs takes a longtime and is hardly to predict or separate the stages. The streptozocin-induced diabetes model in various monkey colonies has been characterized and used in many metabolic studies [29,32,35]. Therefore, in order to investigate the dynamic changes of betatrophin and  $\beta$ -cells, a STZ-induced diabetes model was conducted in rhesus monkeys (Table 2). Seven of eight normoglycemic rhesus monkeys were administered with 35 mg/kg STZ, because one study showed that monkeys treated with both 100 and 68 mg/kg of STZ exhibited continuous hyperglycemia, which coincided with a nearly complete loss of islet  $\beta$ -cells [28]. In order to preserve some  $\beta$ -cells, a relatively low dose of STZ was thus applied in our study. The blood betatrophin and glucose levels were markedly increased after STZ treatment. Blood insulin initially increased on day 1 after STZ and then decreased (Figure 6A). Hyperglycemia and hypo-insulin were successfully induced after STZ treatment except one monkey was died of high plasma glucose (594 mg/dL) and ketone (9 mM/L) 1 day after STZ administration. Both plasma glucose and betatrophin increased dramatically with a decrease in insulin (Figure 6A). Plasma betatrophin markedly increased in one to two days with glucose increase and insulin decrease after STZ injection. Intravenous GTT in the STZ-treated monkey after overnight fasting showed a typical diabetic response with almost no insulin secretion during ivGTT (Figure 6B).

Immunohistochemical staining of islets showed no obvious  $\beta$ -cell proliferation in the vehicle-treated monkey (Figure 7A). However, the islets in two STZ-treated NHPs with partial recovery of blood glucose, from 120 mg/dL on day 1 after STZ injection to 80 mg/dL on day 7 and 65 mg/dL on day 14 after STZ dosing (Figure 7B), clearly showed  $\beta$ -cell regeneration. In another two STZ-treated animals with higher blood glucose, >300 mg/dL, even more  $\beta$ -cell proliferation was observed in their islets (Figure 7C), but no recovery of blood glucose on day 7. The islets in the remaining two STZ-treated animals with blood glucose



**Figure 4:** Betatrophin mRNA expression levels in different tissues of cynomolgus monkey are shown (A). Total RNA was extracted from the liver and other tissues of one monkey. The cDNA was synthesized by superscript III First Strand cDNA synthesis kit. Real-time PCR of betatrophin and the internal control of GAPDH was conducted on Stratagene Mx3005p (Agilent Technologies) using the SYBR-green reaction system. Relative quantity of betatrophin mRNA was calculated by  $\Delta\Delta CT$  method. B, Correlation of liver betatrophin mRNA levels with blood glucose and insulin concentration. Serum insulin and glucose levels were tested. Betatrophin mRNA expression showed high correlation with blood insulin and glucose levels except V04. V03, J01, D05, G01, M04 and V04 were the actual animal IDs used during the study.

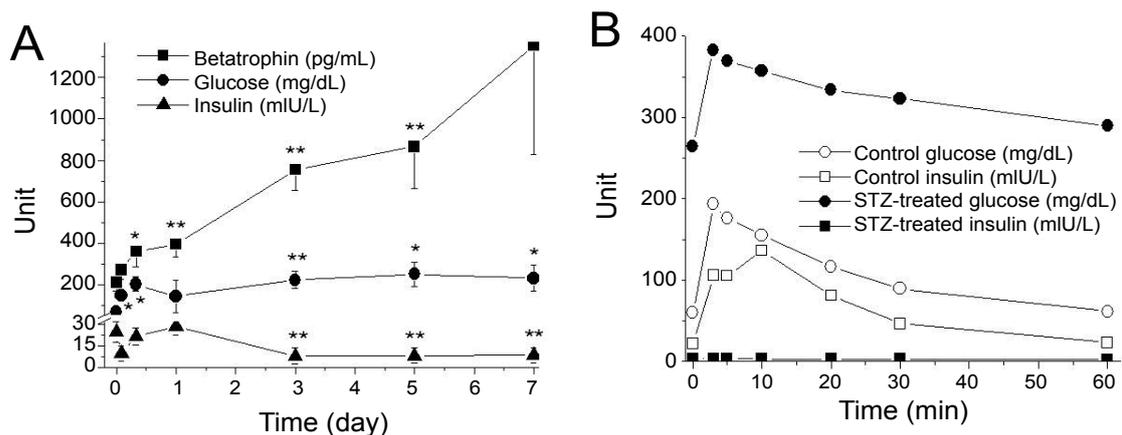


**Figure 5:** Correlation between liver betatrophin mRNA levels and islets quantities and sizes in the pancreas (insulin immunohistochemistry, 10X). Betatrophin relative expression in liver was analyzed by real-time PCR. Paraffin section was stained by mouse anti-insulin antibody. Normal betatrophin level with some small islets in normal monkey V03 (A). High betatrophin level with increased islets number in prediabetic monkey M04 (B). High betatrophin level with expand islets in diabetic monkey G01 (C). High level betatrophin with rare beta cells in pancreas in severe diabetic monkey V04 (D).

Group		Vehicle				STZ			
Monkey ID		I04	H03	N02	P02	G04	C03	D03	G03
Gender		F	M	F	F	F	M	M	M
Age		13	7	7	7	15	7	7	7
Body weight (kg)		6.7	7.0	6.5	6.3	11.4	10.4	12.0	8.7
Day 0	Insulin (mIU/L)	45	3	27	18	52	12	47	13
	Glu (mg/dL)	68	57	51	77	76	57	81	68
	Betatrophin (pg/mL)	107	425	258	201	178	125	166	116
Day 7	Insulin (mIU/L)	21.3	7.4	29.2	1.3	3.2	2.9	3.2	0.0
	Glu (mg/dL)	75	80	90	604	366	361	264	359
	Betatrophin (pg/mL)	219	997	1072	457	972	850	436	3415
Day 14	Insulin (mIU/L)	25	7	14					
	Glu (mg/dL)	68	75	55					
	Betatrophin (pg/mL)	193	632	559					

Note: Day 0=pre-treatment; Day 7 and Day 14=the days after treatment.

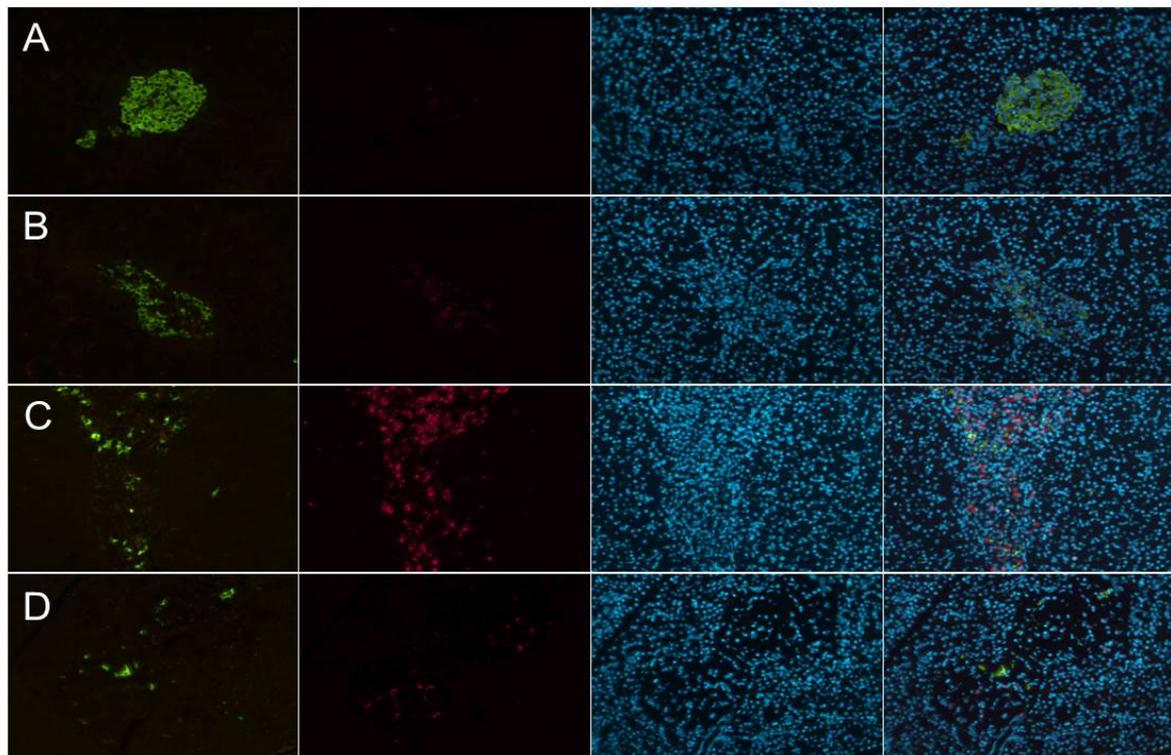
**Table 2:** General information of the animals enrolled for STZ treatment.



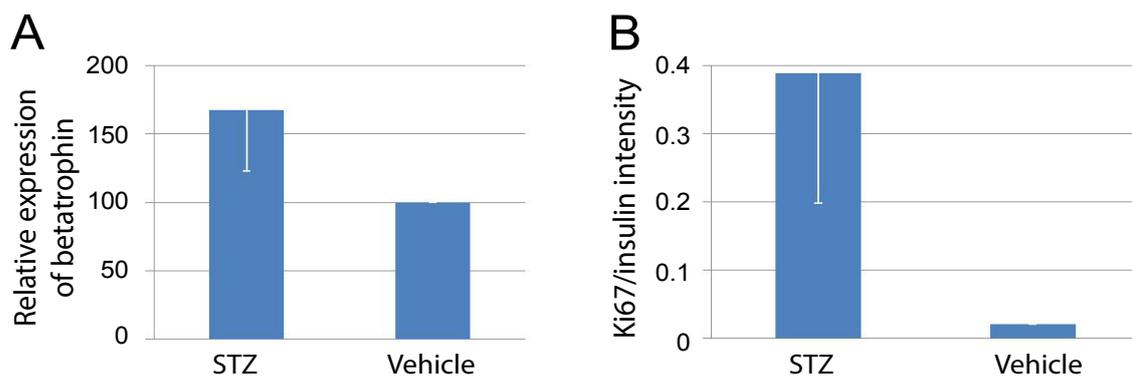
**Figure 6:** Effects of STZ on blood betatrophin, glucose and insulin in NHPs. (A) Elevation of blood glucose and betatrophin during diabetic induction after STZ injection on day 0. (B) Changes of blood glucose and insulin during ivGTT conducted before (Control) and on day 7 after STZ injection (STZ-treated) in one NHP.  $p < 0.05^*$ ;  $p < 0.01^{**}$ ; versus day 0 for glucose and betatrophin; versus day 1 for insulin.

$>300$  mg/dL, whose  $\beta$ -cells were severely destroyed, showed little  $\beta$ -cell proliferation (Figure 7D). In addition, betatrophin mRNA relative expression in the liver was 50% higher in STZ-treated group than in the vehicle one (Figure 8A). The  $\beta$ -cell proliferation was approximately

20 times higher in the STZ-treated group than in the vehicle animal (Figure 8B). To examine the dynamic changes of blood glucose and betatrophin, the experimental duration was extended to 14 days in 3 animals with repeated ivGTTs. The vehicle animal had no obvious



**Figure 7:** Immunohistochemical staining of paraffin-sectioned pancreatic islets (20X) against insulin, Ki67 and DAPI. (A) Islets from the vehicle treated animal showed no obvious  $\beta$ -cell proliferation. (B) Moderate  $\beta$ -cell proliferation was shown in islets of two STZ-induced hyperglycemic monkeys with recovery of blood glucose, from 120 mg/dL on day 1 to 80 mg/dL on day 7 and then to 65 mg/dL on day 14, respectively, after STZ administration. (C) Obvious  $\beta$ -cell proliferation in islets was observed in other 2 STZ-treated animals with hyperglycemia constantly above 300 mg/dL on day 7 after STZ dosing. (D) Islets from the rest of 2 STZ-treated animals with constant >300 mg/dL blood glucose and severe destruction of islets showed rare  $\beta$ -cell proliferation on day 7 after STZ injection.

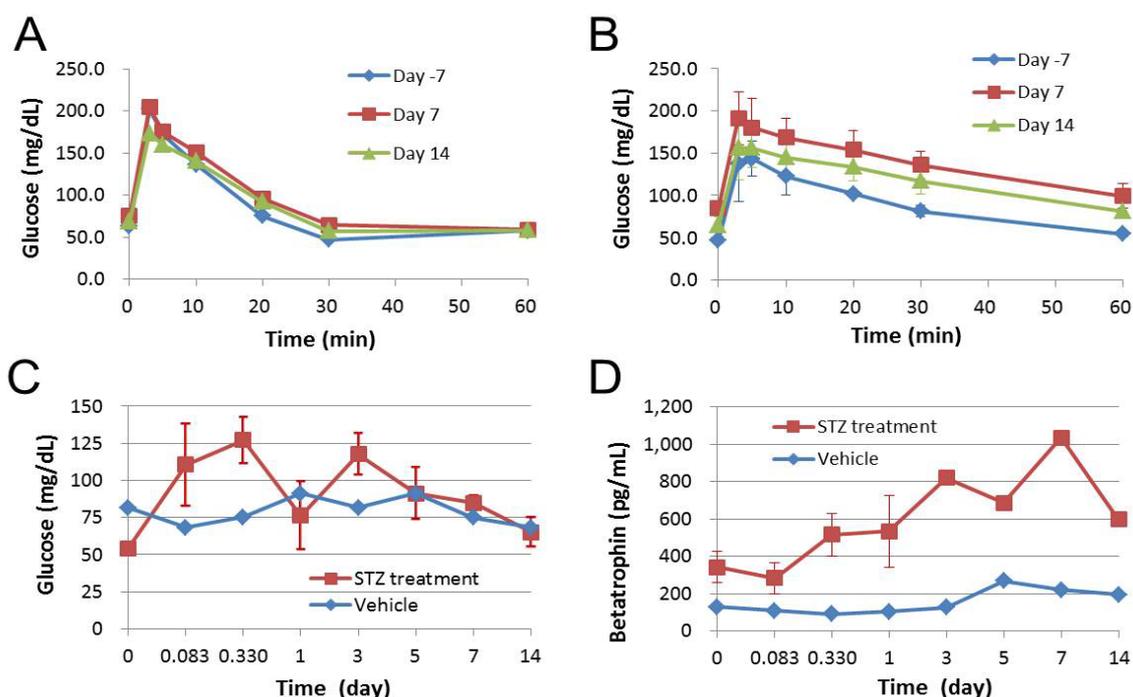


**Figure 8:** Betatrophin relative expression in liver and  $\beta$ -cell proliferation in pancreas after STZ treatment. (A) Betatrophin mRNA expression in liver in vehicle (n=1) and STZ-treated animals (n=7). Total RNA was extracted from liver tissue and then reversely transcribed into single strand cDNA. Betatrophin mRNA relative expression levels (GAPDH as internal control) were conducted by real-time PCR. (B) Beta-cell proliferation rate comparison among the vehicle (n=1) and STZ-treated animals (n=7). IHC of the antibody and conjugated dye against insulin or Ki67 was performed in the pancreas paraffin sections and staining intensity was quantified with the software Image J (National Institutes of Health, USA).

change on ivGTT during the study (Figure 9A), but the STZ-treated animals recovered toward the pre-treated level on day 14 (Figure 9B). The blood glucose decreased consecutively after the initial increase in 3 days after STZ treatment (Figure 9C) and betatrophin decreased at the end of the 2nd week after STZ injection (Figure 9D). Our data may explain, at least partially, why circulating betatrophin level is elevated in patients with type 2 diabetes.

## Discussion

The main finding of this study is that liver expressed abundant mRNA of betatrophin in NHPs. The expression levels correlated well with blood betatrophin contents and islet sizes in most naturally developed diabetic cynomolgus monkeys. In addition, blood betatrophin levels showed dynamic changes during the process of STZ-induced diabetes



**Figure 9:** Changes of blood betatrophin and glucose in STZ-treated animals. Three times of ivGTT were conducted under anesthesia on day -7, 7 and 14 in STZ (n=2) or vehicle (n=1) treated animals with overnight (~16 hrs) fast. The ivGTT curves overlapped well in the vehicle control (A). However, the curve of ivGTT in two STZ treated animals shifted up markedly on day 7 after STZ-treatment and returned toward to the baseline with slower glucose clearance rate (compared with day -7) on day 14 after STZ administration (B). The time course of blood glucose showed no change in vehicle animal (n=1), but in STZ-treated animals (n=2) showed the initial increase within day 1 and 2nd increase on day 3 and then descended continuously toward the pretreatment level (C). Compared with the vehicle animal, blood betatrophin in the STZ-treated animals increased gradually from day 1 until its peak on day 7 after STZ administration and then declined toward the pre-treatment level (D).

in rhesus monkeys. Beta-cell proliferation or regeneration was observed in diabetic animals with high blood betatrophin. However, such regeneration seems requiring a base number of pancreatic islets or  $\beta$ -cells, which means that the betatrophin effect on  $\beta$ -cell regeneration was greatly diminished in animals with little functional islets and  $\beta$ -cells. The main cause in most diabetes patients is insulin resistance and eventually  $\beta$ -cell failure or depletion. An effective approach to cure diabetes is thus to recover pancreatic function via replenishing the lost or dysfunctional  $\beta$ -cells.

Betatrophin, a polypeptide, primarily produced in the liver showed the enhancement effect on  $\beta$ -cell regeneration/proliferation and insulin secretion in early rodent study [12,36], but such effect was not disapproved in late studies [16-18]. The islets between rodents and primates exists obvious differences in the structure and signaling pathway. Insulin-immunoreactive, glucagon-immunoreactive and somatostatin-immunoreactive cells were found all randomly distributed in human and monkey islets. In contrast, insulin-containing cells were located in the core, and glucagon- and somatostatin-containing cells in the mantle of mouse islets [25, 26]. Such differences made our current study highly valuable as betatrophin also dominantly expressed and produced in the liver and increased dramatically in diabetes NHPs. In addition, betatrophin potentially enhanced  $\beta$ -cell regeneration in diabetes monkeys (Figures 4 and 5). It is interesting that blood betatrophin level was similar in the diabetic males to females (Figure 1), even though the female monkeys were older with significantly higher levels of blood glucose, TG and TC. However, compared with the insulin-independent monkeys, the blood betatrophin level was significantly higher in the

group of insulin-dependent monkeys (Figure 3). The group with insulin-dependent diabetes had significantly older age and higher levels of blood glucose, HbA1c, TG and TC, which might be the causes leading to higher blood betatrophin [37]. This phenomenon is similar to the clinical finding that elevated betatrophin levels were measured in the patients with type 2 diabetes [19,20,23,38]. Meta-analysis showed that circulating betatrophin level of T2DM patients is higher than that of nondiabetic adults in the non-obese population, but not in the obese population [39]. Betatrophin positively correlated with age in the controls and with HbA1c in type 2 diabetes patients [21,22]. In addition, plasma betatrophin concentrations in diabetes patients were positively correlated with duration of Type 2 diabetes and negatively correlated with increments of C-peptide concentration and creatinine clearance [40]. However, one recent study showed betatrophin and irisin were not associated with  $\beta$ -cell function in previously diagnosed T2DM patients [41].

In the current study, the relative expression level of betatrophin in the liver was highly correlated with the plasma glucose level and rate of new cell regeneration in NHPs. In adult monkeys with blood glucose <90 mg/dL, the liver mRNA levels of betatrophin correlated well with the islet sizes and insulin contents. However, the diabetic animals expressed markedly higher betatrophin mRNA in their livers showed two different correlation patterns between liver betatrophin mRNA levels and islet sizes. In hyperglycemic NHPs, the betatrophin mRNA levels in the liver correlated well with the islet sizes in the animals with high blood insulin, but not correlated well in those with very low blood insulin (Figures 4 and 5). Immunohistological analysis of the pancreas showed that the islets in the diabetic animals with high blood insulin

were larger and contained more insulin, whereas the islets in those diabetic NHPs with low blood insulin showed much less  $\beta$ -cells and less insulin. High betatrophin seemed ineffective in animals at late-stage diabetes with very low insulin, probably due to dysfunction or depletion of  $\beta$ -cells. Plasma betatrophin levels correlated well with plasma glucose levels in the female monkeys and correlated well with insulin levels in the male monkeys (Figure 2). Such differences could be age-related as the males were significantly younger than the females (Figure 1). Therefore, the diabetic stage in the females could be more advanced. In rodents, an increase in hepatic betatrophin gene expression stimulated mouse  $\beta$ -cell replication [42]. However, Gusarova et al. reported that  $\beta$ -cell proliferation was not affected by the lack of ANGPTL8 in response to diet induced insulin resistance or the S961 insulin receptor antagonist treatment in ANGPTL8 knockout mice [18]. The results from two recent studies also demonstrate that ANGPTL8 does not stimulate significant  $\beta$ -cell proliferation [16,17]. In addition, human  $\beta$ -cells were completely unresponsive to betatrophin stimulation. These results questioned whether betatrophin can be developed as a therapeutic approach for treating human diabetes [42]. Betatrophin levels were also affected by other parameters, such as triglycerides [38,43]. In this study, TG and TC were also significantly higher in naturally developed diabetes NHPs, which might also be the factors affecting betatrophin levels (Figures 1 and 3) [44].

Immunohistological analysis of the pancreas showed that the islets in the diabetic animals with high blood insulin were larger and contained more insulin, whereas the islets in those diabetic NHPs with low blood insulin were smaller and contained less insulin. Our results indicate that betatrophin is expressed in NHP liver and might play an important role in  $\beta$ -cell regeneration at the early stage of diabetes in diabetic monkeys with high blood insulin, because its levels correlated well with islet sizes and insulin contents. However, high betatrophin could be ineffective in animals at late-stage diabetes with very low insulin, probably due to depletion of  $\beta$ -cells. Elevated triglycerides accompanied with increased betatrophin may also be a clue to explore the mechanism of betatrophin triggered  $\beta$ -cells replenishment.

STZ-induced hyperglycemia in various species have been used for biomedical research [45-47]. If the STZ dose was 60 mg/kg or higher, it easily induces marked hyperglycemia [30,32,35]. In this study single injection of 35 mg/kg STZ in adult normoglycemia NHPs successfully induced hyperglycemia/hypoinsulinemia (Table 2) to mimic acutely progressed diabetes and then the dynamic changes of blood betatrophin were examined. In STZ-induced rhesus monkeys with normal blood glucose less than 80 mg/dL, blood betatrophin and glucose levels increased dramatically after STZ-induced  $\beta$ -cell destruction. In STZ-treated NHPs except one animal died on the 2nd day after STZ treatment, blood betatrophin increased dramatically and proliferation of pancreas cells show high correlation with the increased plasma betatrophin especially in 2 animals with partially recovered blood glucose level, from 120 mg/dL to 80 mg/dL 7 days after STZ treatment. In another 2 STZ-treated animals with higher blood glucose, >250 mg/dL, more cell proliferation was observed in none  $\beta$ -cell area in the islets even though obvious increased  $\beta$ -cell proliferation was found. The rest 2 STZ treated animals whose  $\beta$ -cells were severely destroyed showed little  $\beta$ -cell proliferation. In addition, rare proliferated  $\beta$ -cells or  $\alpha$ -cells were observed in the vehicle treated animal (Figure 7). While, elevated triglycerides were also found in the STZ-treated animals accompanied with increased betatrophin.

Blood betatrophin concentrations changed dynamically and highly correlated with the blood glucose levels during the STZ induction process. Betatrophin and glucose levels increased initially

and then subsided lately after STZ administration, which indicates there might be some feedback stimulation and inhibition. The relative expression of liver betatrophin increased by over 50% in STZ treated animals compared with vehicle animal (Figure 8A). The level of  $\beta$ -cell regeneration was likely different at various diabetes stages and damaged levels of the pancreas. Beta cell proliferation rate is not high in the pancreas with severely destroyed islets. The data demonstrate that blood betatrophin rose parallelly with glucose elevation during STZ induction of diabetes in NHPs. Such increase in blood betatrophin led to  $\beta$ -cell regeneration in some animals, 5 out of 7, but not in those NHPs with severely destroyed islets, 2 out of 7. In 2 STZ-induced animals blood glucose went back toward their pre-STZ levels 7 to 14 days after STZ dosing. Their ivGTT tests on day 14 also had their glucose clearances similar to those before STZ treatment (Figure 9B).

Beta-cell replication is extremely low under normal condition and is elevated by 4-fold during gestation, 2- to 4.5-fold with high glucose infusion, and 4-fold in a  $\beta$ -cell ablation model [7,48,49]. Compared with the control,  $\beta$ -cell regeneration increased multiple times in most STZ-treated NHPs, which suggests the potential effect of betatrophin on  $\beta$ -cell replenishment (Figures 7 and 8). However, either STZ-induced or spontaneously developed diabetes animals with mostly destroyed islets had little  $\beta$ -cell regeneration. These results suggest that certain number of  $\beta$ -cells are needed for betatrophin to trigger off obvious regeneration or more  $\beta$ -cell death overrides the regenerated ones. Therefore, the mass of  $\beta$  cells represents the balance between  $\beta$ -cell growth (differentiation and replication) and death (apoptosis). Even with high concentration of betatrophin, this balance may not be reached if the number of  $\beta$ -cells is too low, which possibly explain why some NHPs at late stage diabetes have high betatrophin, but show little  $\beta$ -cell regeneration (Figures 5, 7 and 8). In adults,  $\beta$ -cell regeneration can result from existing  $\beta$ -cells or precursor cells. Also,  $\beta$ -cell regeneration can be achieved from exocrine cells and proliferation of ducts and their subsequent differentiation into new pancreatic lobes [50-52]. In our study, most of the Ki67 staining was located in islets but not ductile which indicates the new generated  $\beta$ -cells were from replication of existing  $\beta$ -cells or precursor cells [53]. The mechanism how betatrophin induces  $\beta$ -cell regeneration is still unclear. Some studies showed that betatrophin involved in irisin stimulated  $\beta$ -cell regeneration at the downstream of the signal pathway [54,55]. In addition, pregnancy in mice stimulated proliferation of maternal pancreatic islet  $\beta$ -cells accompanied by reduced islet levels of menin and its targets, and prolactin repressed islet menin levels and stimulated beta-cell proliferation [7]. It is unclear if betatrophin-induced  $\beta$ -cell regeneration in NHPs also involved menin or prolactin.

Our study demonstrates the dynamic changes of betatrophin and its potential effects on  $\beta$ -cell regeneration in both spontaneously developed and STZ-induced diabetic monkeys. However, there are several limitations on the interpretation of the results. Firstly, blood betatrophin were measured in a large group of spontaneously diabetes monkeys (Figures 1-3, n=65), but the immunohistological data were obtained from very limited number of animals (Figures 4 and 5, n=6). Therefore, some distortion potentially occurred when various correlations were analyzed. Secondly, the animal number and duration of STZ experiment were relatively small, especially for control (n=1), and short (only 1 week for baseline and 2 weeks after STZ treatment) with limited sampling points, including tissue collection. Thirdly, there is a lack of mechanistic experiments. Therefore, the data were relatively incomplete and hard to make statistical analysis and conclusion. More studies are required to delineate why betatrophin likely loses its effects on  $\beta$ -cells at the late stage of diabetes in NHPs, which may provide

vital information for understanding the role of this polypeptide during disease development.

In conclusion, spontaneously developed or STZ-induced diabetic NHPs had high blood betatrophin which might enhance  $\beta$ -cell proliferation in most cases. Betatrophin effect on  $\beta$ -cell proliferation could be via stimulating the remained  $\beta$ -cells to replicate. Therefore, a minimal base level of pancreatic  $\beta$ -cells or islets might be required for betatrophin effect on  $\beta$ -cell replication. The increased circulating betatrophin could contribute to the lately occurred recovery from STZ-induced hyperglycemia toward the pre-STZ glucose level. Further study is required to find the solid correlation between betatrophin and  $\beta$ -cell replication in NHPs.

## Declaration

Ethics approval and consent to participate: This is not a human study. Ethics approval and consent are thus not applicable. The study protocol and experimental procedures for suing the animals were approved by the IACUC of Crown Bioscience Inc with the members outside of the company.

**Consent for publication:** All the authors have read and approved the manuscript for submission and their consents are available if requested.

**Availability of data and material:** All the materials and relevant raw data supporting our findings can be found in Tables and Figures in the manuscript and are freely available to readers or scientists wishing to use them for non-commercial purposes.

**Competing interests:** All of the authors are employee of Crown Bioscience, Inc., except J Wang who is employee of Taicang Affiliated Hospital of Soochow University. The authors declare no conflict of interest in this study.

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**Authors' contributions:** Y Liu, X Wang, B Wang and G Sun conducted the study. Y Liu, F Gregoire, K Chng, J Wang, Y (Jim) Wang and Y-F Xiao participated study design and manuscript preparation.

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