

**ORIGINAL ARTICLE**

# Long-term case study of a Wuzhishan miniature pig with diabetes

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**Abstract**

**Background:** Miniature pigs are attractive animal models for exploring diabetes because they are similar to humans in terms of physiological structure and metabolism. However, little is known about the complications of diabetes in pigs.

**Methods:** In this study, a 28-month observation of a Wuzhishan miniature pig with streptozotocin (STZ)-induced (120 mg/kg) diabetes was conducted, to investigate diabetes-related complications and the possibility of self-recovery in miniature pigs. Blood glucose, serum and urinary biochemistry was measured, and histopathologic examinations of eyes, kidney and pancreas were made.

**Results:** During the observation, diabetic complications of eyes and kidney were observed. The eye complications included bilateral cataracts in the 15th month and degeneration of inner retina and microaneurysm in the 28th month. Kidney complications included glomerular mesangial expansion, focal segmental glomerular sclerosis, and renal tubular epithelial degeneration, but no proteinuria was observed. By 28 months after the application of STZ, with no treatment given, blood glucose had recovered and the number of pancreatic islet beta-cells had increased significantly.

**Conclusions:** We showed that the STZ-induced diabetes model in miniature pigs could accurately mimic the pathological changes of human diabetes, and that pancreatic islet beta-cell regeneration did occur in an adult miniature pig, providing a new means for exploring diabetic complications and pancreatic islet beta-cell regeneration.

**KEYWORDS**

beta-cell regeneration, diabetes-related complications, pig

**1 | INTRODUCTION**

Diabetes-related complications are the main cause of disability, death, and increased medical expenditure in patients with

diabetes. The incidence of complications is highly related to the patient's age, gender, systolic blood pressure, etc, with the duration of the disease considered to be one of the key influencing factors.<sup>1</sup> For example, diabetic nephropathy (DN) usually occurs

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in a susceptible population with diabetes onset at between 15 and 25 years.<sup>2,3</sup> Current experimental data on diabetes originate mainly from rodent models, and studies on the pathogenesis of diabetes-related complications and drug testing are performed mainly in rodents.<sup>4-6</sup> However, the metabolic mechanisms in rodents and humans are significantly different, limiting the clinical relevance of these research results. Several clinical trials on new drugs targeting advanced DN have shown disappointing results in recent years.<sup>7-9</sup>

Pigs are generally metabolically similar to humans, with similar gastrointestinal structure and nutrient uptake mechanisms. Pancreas development and morphology in the pig is also quite similar to that of humans. Moreover, the insulin of pigs and humans differs by only one amino acid.<sup>10-12</sup> Therefore, pigs are attractive animal models for exploring diabetes. Streptozotocin (STZ) has selective chemotoxicity on pancreatic islet beta-cells and is commonly used to experimentally induce insulin-deficient diabetes.<sup>13,14</sup> A rapid intravenous injection of more than 100 mg/kg STZ can cause porcine insulin-deficient diabetes, which persists for more than 3 years.<sup>10,15-18</sup>

Some studies have shown that sensitivities to STZ varied greatly in pigs. It is speculated that this difference relates to genetic factors.<sup>18,19</sup> Liu *et al*<sup>19</sup> found that the sensitivities of different breeds of pigs to STZ is different; the application of a dose of 150 mg/kg successfully induced diabetes in Landrace pigs, but a dose of 200 mg/kg was required to induce diabetes in Gottingen miniature pigs. We compared the sensitivities of the three most commonly used Chinese miniature pigs (Bama, Wuzhishan, and Guizhou) to STZ-induced diabetes. Of these, Wuzhishan miniature pigs were relatively sensitive to STZ; a dose of 120 mg/kg could cause insulin-deficient diabetes. In this study, a Wuzhishan miniature pig with STZ-induced diabetes was observed for 28 months to investigate the possibility of self-recovery and diabetes-related complications in these miniature pigs.<sup>20</sup>

## 2 | METHODS

### 2.1 | Ethical statement

This trial was approved by the Laboratory Animal Welfare and Ethics Committee of the PLA General Hospital (Document Number: IACUC-D13009).

### 2.2 | Experimental animals

Three male Wuzhishan miniature pigs (7-8 months old; weighing 20.5-26.5 kg) were selected for the experiments and a fourth Wuzhishan miniature pig (36 months old; weighing 66 kg) was used as a control for the pathological study. The pigs purchased from the Beijing Institute of Animal Husbandry and Veterinary Medicine in the Chinese Academy of Agricultural Sciences. The trial was completed at the animal

experimental center of the PLA General Hospital. The animals were reared in a single cage at room temperature of 20-28°C and a relative humidity of 40%-80%. They were given measured quantities of feed at regular intervals but had free access to drinking water.

### 2.3 | Experimental instruments and reagents

Blood glucose was measured using a Bayer Contour blood glucose meter (Leverkusen, Germany). STZ (S0130) was purchased from Sigma (MA, USA). The antibodies for insulin (ab46716), glucagon (ab10988), and growth hormone release-inhibiting factor (ab22682) were all purchased from Abcam (MA, USA). Fetal bovine serum, primary antibody dilution buffer, goat anti-rabbit secondary antibody, diaminobenzidine chromogenic solution, and neutral balsam were purchased from Zhong Shan Jin Qiao company (Beijing, China).

### 2.4 | Experimental methods

After 1 week of adaptive feeding, three miniature pigs were treated with STZ. After overnight fasting, STZ (120 mg/kg) was injected into the lateral saphenous vein of the hind limb within 4 minutes. Blood glucose values in the ear vein were evaluated with a blood glucose meter every 2 hours within a 24 hour period. Fasting blood glucose was tested every morning for 28 days, at which time two miniature pigs were injected intramuscularly with ketamine hydrochloride (25 mg/kg) to induce anesthesia, followed by an intravenous injection of 3% sodium pentobarbital (6 mg/kg), and sacrifice by femoral artery bloodletting. The results from these two pigs, termed "28 d", were combined to give an average result. The remaining experimental pig had a higher blood glucose level and was further observed for 28 months; resulting data were termed "28 mo". The general clinical manifestations were observed daily, and fasting blood glucose, serum biochemistry, urine, and urinary protein were tested monthly. The experimental pig and the control were sacrificed after 28 months and pathologically examined.

Intravenous glucose tolerance tests (IVGTT) were performed at 0, 15, and 28 months after STZ application as previously reported. A Roche Automated Biochemical Analyzer (Cobas C701; Basel, Switzerland) was used to measure the serum biochemical indices, including creatinine, urea nitrogen and uric acid. A routine urinalysis was performed using a Sysmex automatic urine analyzer (UF-1000i; Kobe, Japan). Urine protein was measured using a Siemens automatic protein analyzer (BN II; Munich, Germany).

After sacrifice, pancreas, kidney, and eyeballs were fixed in 10% neutral formalin. Paraffin sections were made using standard procedures for hematoxylin and eosin (HE) staining. Renal and retinal sections were also stained with periodic acid-Schiff (PAS). Pancreatic tissue sections were made for insulin, glucagon, and somatostatin immunohistochemical staining, and insulin and glucagon immunofluorescence double staining.

### 3 | RESULTS

#### 3.1 | Changes in blood glucose level and glucose tolerance test

In contrast to the control, hypoglycemia was induced in all the miniature pigs after the application of STZ (Figure 1A). Although the blood glucose levels fluctuated greatly within 28 days, sustained rises were observed in 28 d and 28 mo (Figure 1B); 28 mo was observed for 28 months because of the higher fasting plasma glucose at the earlier stage. The 28 mo fasting plasma glucose level was maintained at between 12.4 and 29.2 mmol/L over 22 months after the application of STZ and then began to recover. By the 28th month, it was almost restored to the initial level (Figure 1C).

Intravenous glucose tolerance test results showed that the blood glucose regulation response in the 28 mo miniature pig was rapid before STZ induction; 60 minutes after intravenous glucose administration, the blood glucose had recovered to the initial level. Fifteen months after the application of STZ, the fasting plasma glucose level and the highest blood glucose level of this pig increased significantly after intravenous glucose administration, but the blood glucose regulation response was slower; 120 min after intravenous glucose administration, it had not recovered to the level before administration. By the 28th month, the fasting plasma glucose level of the 28 mo miniature pig had recovered to the pre-test level. However, the response of blood glucose regulation remained slower (Figure 1D).

#### 3.2 | Diabetic eye diseases

Binocular mild cataracts began to appear in 28 mo in the 15th month after STZ injection. Fundus examination showed no significant abnormalities. The extent of the cataracts gradually increased, and the fundus could not be seen by the 28th month (Figure 2A).

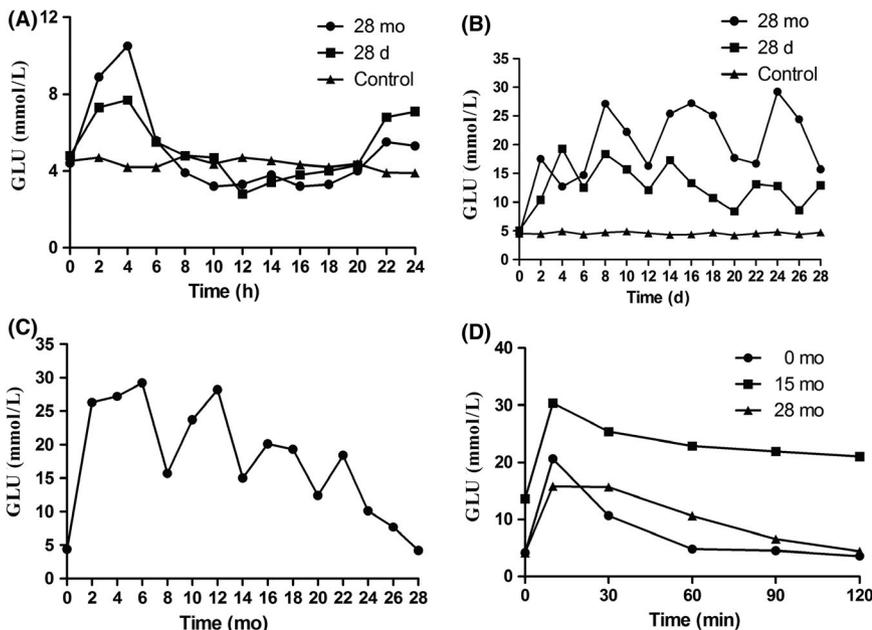
Retinal biopsies revealed significant pathological changes in the retina in the 28th month, including degeneration and micro-aneurysms (Figure 2C,D); these were categorized as moderate nonproliferative diabetic retinopathy according to the international clinical criteria for the severity of diabetic retinopathy.<sup>21</sup> Retinal degeneration occurred, with shrinkage and reduction in the number of the inner nuclear layer cells, reduction in the number of ganglion cells, and vacuolar degeneration of the nerve fiber layer (Figure 2C). These lesions were thought to be related to blood supply disorders caused by retinal microangiopathy.

#### 3.3 | Diabetic nephropathy

During the 28-month observation period, the urea (UN), creatinine (CRE), and uric acid (UA) levels of the 28 mo pig fluctuated within the normal range after the application of STZ (Table 1). Compared with the normal values for serological indices in the miniature pigs (UN,  $2.35 \pm 0.70$  mmol/L; CRE,  $96.67 \pm 16.75$   $\mu$ mol/L; UA,  $4.67 \pm 1.51$   $\mu$ mol/L), only a few values increased slightly at individual time points (19 and 28 months after the application of STZ, UN was 4.39 and 6.47 mmol/L, respectively; 22 and 26 months after the application of STZ, UN was 7 and 8.4  $\mu$ mol/L, respectively). Therefore the renal function of the pig was considered normal during the trial period.

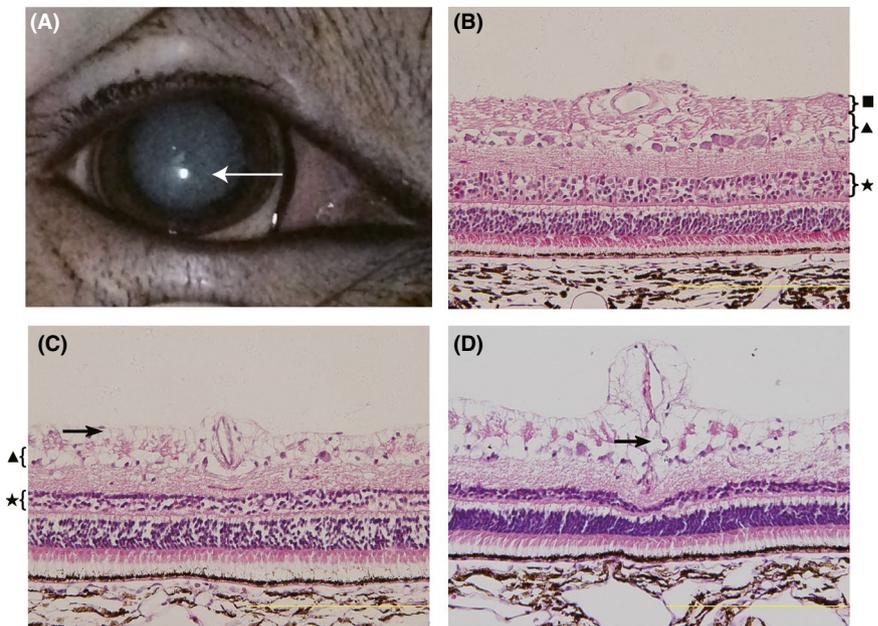
Mild albuminuria in the pig was observed in the 15th month, which then increased slowly. By the 19th month, the urine protein level reached 4.15 g/L, and the urinary microalbumin level reached 4.73 mg/dL. Thereafter, no significant increase was observed; urine protein fluctuated between 2.64 and 4.15 g/L; urinary microalbumin fluctuated between 4.28 and 7.04 mg/dL (Table 1).

Pathological examination of the kidneys showed no obvious abnormality in the control miniature pig and the two 28 d pigs 28 days after the application of STZ, whereas the kidneys of the pig 28 months



**FIGURE 1** Changes in blood glucose levels of Wuzhishan miniature pigs after the application of STZ (120 mg/kg). A, 24 h. B, 28 d. C, 28 mo. D, IVGTT determination

**FIGURE 2** Pathological changes after 28 mo in the eyes of Wuzhishan miniature pigs with STZ-induced diabetes. A, Cataract of the right eye of the diabetic pig. B, Retina of the control pig, 4 mm from the left side of the optic nerve bundle. C, Retina of the diabetic pig, 4 mm from the left side of the optic nerve bundle. ■, nerve fiber layer. ▲, ganglion cell layer. ★, inner nuclear layer. Pyknosis and reduction of cells in the inner nuclear layer and ganglion cell layer were observed. Vacuolar degeneration of the cells in the nerve fiber layer was also found (black arrow). D, Retina of the diabetic pig; irregular expansion of retinal arterioles was found. The arrow points to a microaneurysm. Bar = 200 μm; magnification 200×



**TABLE 1** Examination of serum and urine of Wuzhishan miniature pigs after STZ treatment

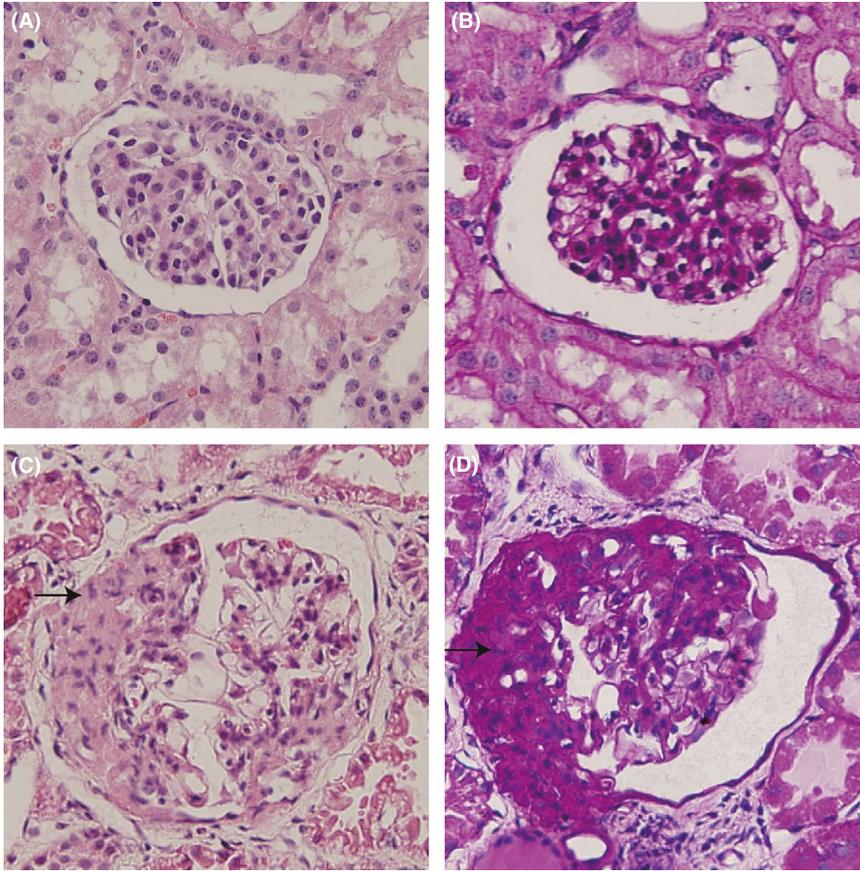
Time (month)	Serum					Urine	
	BUN (mmol/L)	Scr (μmol/L)	UA (μmol/L)	Insulin (μIU/mL)	Glucagon (pg/mL)	PRO (g/L)	mALB (mg/dL)
0	2.35	96.7	4.6	18.53	101.54	0.00	0.00
1	2.47	84.3	3.3	9.58	99.87	0.00	0.00
15	2.67	79.3	1.5	9.63	92.31	0.58	1.25
16	2.83	76.4	3.2	8.96	102.16	0.69	1.48
17	3.52	71.5	4.2	8.19	95.76	1.85	2.64
18	3.60	83.4	3.3	9.32	103.47	2.15	3.82 <sup>a</sup>
19	4.39 <sup>a</sup>	104.6	4.5	9.87	119.25	4.15	4.73 <sup>a</sup>
20	3.54	89.8	3.1	8.56	106.48	2.64	5.64 <sup>a</sup>
21	3.06	91.0	4.6	6.71	98.22	2.96	7.04 <sup>a</sup>
22	2.81	85.9	7.0 <sup>a</sup>	6.13	138.42	2.91	4.28 <sup>a</sup>
23	2.26	64.6	3.6	7.57	135.89	3.14	4.33 <sup>a</sup>
24	2.24	71.0	2.6	10.81	106.75	3.28	4.63 <sup>a</sup>
25	2.12	75.4	4.1	14.06	97.23	3.59	5.82 <sup>a</sup>
26	2.94	95.0	8.4 <sup>a</sup>	17.92	89.06	4.05	4.97 <sup>a</sup>
27	3.38	75.3	2.6	10.73	94.05	3.32	5.23 <sup>a</sup>
28	6.47 <sup>a</sup>	105.5	4.0	11.3	103.84	3.69	5.16 <sup>a</sup>

Abbreviations: BUN, blood urea nitrogen; mALB, microalbumin; PRO, protein; Scr, serum creatinine; UA, uric acid.

<sup>a</sup>Values higher than that of control.

after the application of STZ manifested obvious morphological abnormalities. Glomerular basement membrane (GBM) thickening, mesangial expansion, and sclerosis were observed (Figure 3C,D, black arrow). According to the Tervaert pathologic classification,<sup>22</sup> these features indicated Class IIa DN. Glomerular mesangial expansion was generally visible; stromal hyperplasia and proliferation existed in some of the cells. Renal glomeruli with severe mesangial expansion (the expanded mesangial region exceeded the average width of the glomerular capillary cavity) were distributed mainly between

the renal cortex and the medulla. Five sections of different parts of the kidney were selected. The number of renal glomeruli with severe mesangial expansion among 100 renal glomeruli was determined, with an average of 3.1 ± 0.91%. Sclerosing lesions in the entire renal glomerulus were occasionally seen, but no Kimmelstiel-Wilson lesion was observed. Tubular abnormalities showed basement membrane thickening, vacuolar degeneration in renal tubular epithelial cells, and lumen protein-like substance deposition. Arteriolar hyalinosis was occasionally observed in the glomerular basement.



**FIGURE 3** Pathological changes after 28 mo in the kidneys of Wuzhishan miniature pigs with STZ-induced diabetes. A, HE staining in the control pig. B, PAS staining in the control pig. C, HE staining in the diabetic pig. Hyperplasia of the glomerular mesangium was observed (black arrow); the mesangium was fused with the glomerular capsule, and the area of connective tissue in the glomerulus exceeded the area of the capillary. D, PAS staining in the diabetic pig. Glomerular capillary and renal tubular basement membrane thickening were observed. Hyperplastic mesangium was positively stained by PAS staining (black arrow). Bar = 100  $\mu$ m; magnification 400 $\times$

### 3.4 | Morphology and function of the pancreas

The level of insulin had significantly decreased 28 days after the application of STZ but, excitingly, it had increased 24 months after the application of STZ (Table 1), indicating that the secretion of pancreatic islets was partially restored.

The pancreatic islets of the control miniature pig differed in size and were generally small. HE staining showed that pancreatic islet was shallow; the cells were arranged regularly with clear boundaries with exocrine glands (Figure 4A). Immunohistochemical staining of insulin, glucagon, and somatostatin showed that most pancreatic islets were composed mainly of beta-cells, with other endocrine cells rarely seen (Figure 4B). Some pancreatic islets also contained alpha-cells scattered throughout the pancreatic islets, but these comprised no more than 30% of total cells (Figure 4C). The proportion of delta-cells in pancreatic islets was small (Figure 4D).

In the experimental pigs, the number of pancreatic islets was significantly reduced by the 28th day after the application of STZ. Those remaining were significantly atrophied and collapsed and were squeezed by the surrounding exocrine glands; the boundaries were also irregular. Pancreatic islet cells were irregularly arranged with necrotic and few newly generated cells (Figure 4E). Immunohistochemical staining revealed a significant decrease in the number of beta-cells in the remaining pancreatic islets (Figure 4F), a relative increase in the number of alpha-cells, and a concentrated distribution due to exocrine extrusion (Figure 4G); the number of

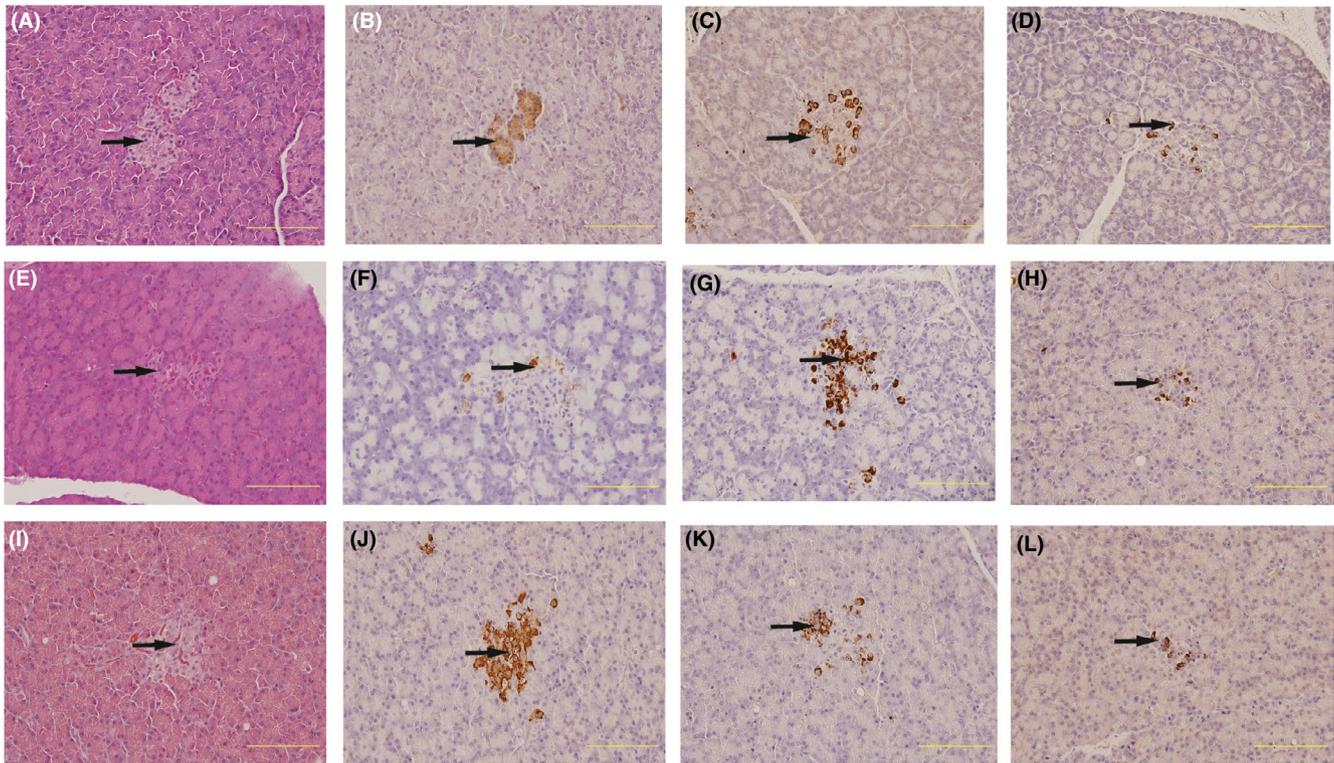
delta-cells was still small (Figure 4H). The pancreatic islets of the two miniature pigs were significantly damaged with similar lesions.

The number of pancreatic islets was still small 28 months after the application of STZ. The remaining pancreatic islets were significantly atrophied and collapsed and were similar in morphology to those seen on the 28th day after the application of STZ. However, no denatured and necrotic cells were observed and the endocrine cells were arranged more regularly than before (Figure 4I). The number of pancreatic islet beta-cells increased to a level similar to the control (Figure 4J). The number of alpha-cells still accounted for the majority of non-beta cells in a small fraction of pancreatic islets (Figure 4K). The number of delta-cells remained small, similar to the proportion on the 28th day after application the application of STZ (Figure 4L). In addition, immunofluorescence was detected in a small percentage of cells co-stained with insulin and glucagon (Figure 5), suggesting that a small number of alpha-cells had transformed into beta-cells.

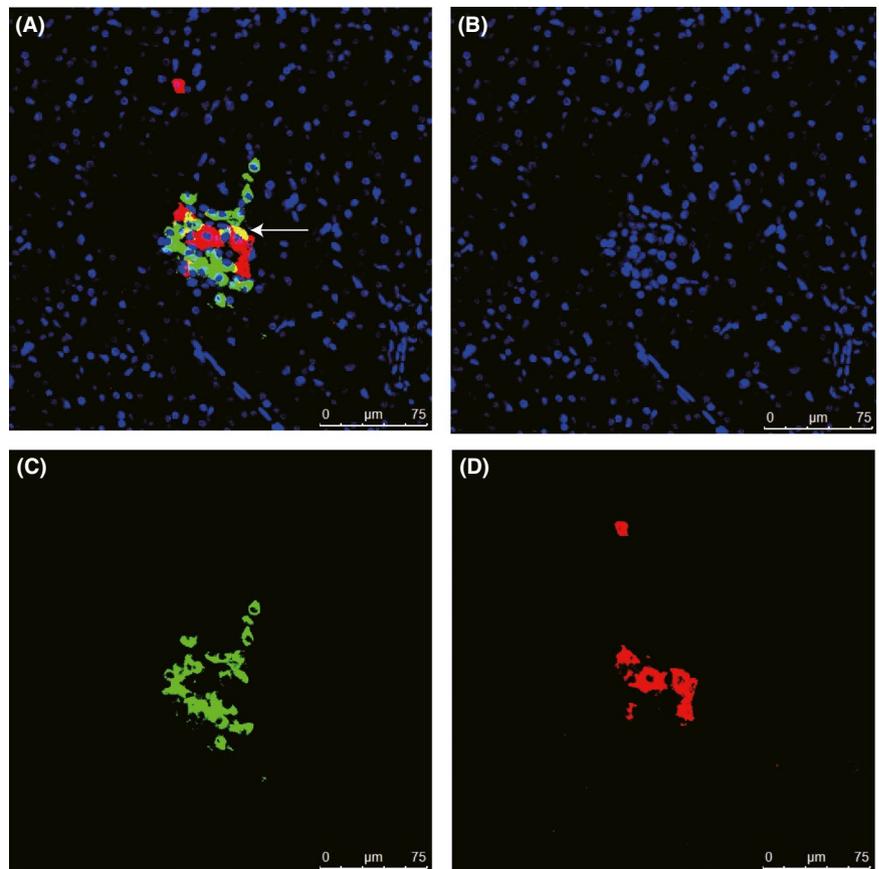
## 4 | DISCUSSION

### 4.1 | Main achievements

This study showed that STZ application (120 mg/kg) can cause insulin-deficient diabetes in Wuzhishan miniature pigs. However, individual differences between pigs existed, including transience or persistence of hyperglycemia. The miniature pig with persistent



**FIGURE 4** Pathological changes in the pancreatic islets of Wuzhishan miniature pigs with STZ-induced diabetes. A-D, Results from the control pig. E-H, Results from the pig with STZ-induced diabetes after 28 d. I-L, Results of the pig with STZ-induced diabetes after 28 mo. A, E and I show HE staining. Black arrow points to pancreatic islet. B, F and J show immunohistochemical staining of insulin. Black arrows represent positive staining. C, G and K show immunohistochemical staining of glucagon. Black arrows represent positive staining. D, H and L show immunohistochemical staining of somatostatin. Black arrows represent positive staining



**FIGURE 5** Immunofluorescence staining of pancreatic islets of a Wuzhishan miniature pig with STZ-induced diabetes after 28 months. A, Merged image from the images in B, C and D, showing, respectively, nucleus (blue), insulin (green), and glucagon (red). The arrow points to the insulin- and glucagon-positive cell, which indicates that the cell is transforming from an alpha-cell (glucagon-positive) into a beta-cell (insulin-positive). B, DAPI staining of nucleus. C, Immunofluorescence staining of insulin. D, Immunofluorescence staining of glucagon

hyperglycemia was able to survive for a long time without treatment and the diabetes was spontaneously alleviated 28 months after the application of STZ. Fasting blood glucose level and glucose tolerance levels (IVGTT) were restored to normal, indicating that the insulin secretion in this pig was sufficient to control glucose metabolism and maintain a stable blood glucose level within a certain range. Pathological examination found that the number and morphology of pancreatic islets in the pig were not completely recovered compared with those on the 28th day after the application of STZ, but the number of beta-cells in pancreatic islets had increased significantly and transformation of alpha-cells into beta-cells was observed. Eye and kidney complications were present during the observation period of 28 months. Bilateral cataracts were observed 15 months after the application of STZ and degeneration of inner retina and microaneurysm were observed 28 months after the application. Urinary microalbumin was detected from the 15th month; glomerular mesangial expansion, focal segmental glomerular sclerosis, and degeneration and necrosis of epithelial cells in renal tubules were observed by the 28th month. These results show that the STZ-induced diabetes model in miniature pigs can provide a new means of studying diabetes-related complications and regeneration of beta-cells in pancreatic islets.

## 4.2 | Diabetes-related complications

Despite great advances in understanding the pathogenesis of diabetes-related complications, limited progress has been made in determining the specific factors leading to complications or predicting the occurrence and progression of complications. One important reason is the lack of animal models that reliably simulate human diseases. Therefore, in 2001, the National Institutes of Health (NIH) initiated the Animal Models of Diabetic Complications Consortium (AMDCC), with the aims of hastening the development and identification of animal models, promoting the development of treatment and prevention strategies for diabetes-related complications, and testing their effectiveness. In recent years, a large number of rodent models for diabetes-related complications have been established, including artificially induced animal models and spontaneous and genetically engineered (gene knockout and transgenic) animal models, which have improved the phenotypic and strain analyses of mice and enhanced understanding of the pathogenesis of diabetes-related complications but, unfortunately, no rodent model has achieved the criteria of AMDCC.<sup>4-6</sup> Recently, miniature pigs have become an attractive animal model for exploring diabetes, especially for studying the pathogenesis of diabetes-related complications, because they are similar to humans in terms of anatomic structure and metabolic mechanisms.

Diabetic nephropathy is the main cause of end-stage renal disease. The imbalance between vascular endothelial cell dysfunction and the angiogenesis response is crucial in the development of the disease.<sup>23,24</sup> Moreover, pathological changes in kidneys may serve as a marker of systemic microvascular injury.<sup>25</sup> Histological

findings in patients with DN include GBM thickening, podocyte foot process widening or detachment, mesangial expansion, mesangial sclerosis, nodular sclerosis/Kimmelstiel-Wilson lesions, and vasculopathy (hyalinosis). Of these, GBM thickening and podocyte foot process widening or detachment are thought to be the early lesions of DN.<sup>22,26-29</sup>

Compared with other animals, the kidney of the miniature pig is more similar to that of humans in anatomy and function. For example, the pig has a multilobular and multipapillary kidney similar to that in humans.<sup>30</sup> Maile *et al*<sup>31</sup> have reported early kidney lesions in Yorkshire pigs 6 months after diabetes onset, including GBM thickening, podocyte depletion, and mesangial expansion, as well as an increased urinary albumin-creatinine (Cr) ratio. Significant lesions were observed in male domestic pigs 15 months following induction of diabetes, including mesangial expansion, podocyte injury, and foamy cytoplasm and hyaline droplets in renal tubular.<sup>32</sup> Marshall *et al*<sup>33</sup> also reported severe DN in Hanford pigs with STZ-induced diabetes. Severe mesangial expansion was noted and nodular swelling of the mesangium and distinct cell proliferation and matrix proliferation were observed in the 23rd month. In the 37th month, more severe mesangial expansion was observed, and hyaline droplets could be seen in the glomerular capsule wall.

In this study, no obvious functional and morphological changes were found in the kidney of miniature pigs 28 days after the application of STZ. By the 15th month, proteinuria occurred, which gradually increased and by the 28th month the morphology of the kidneys showed significant abnormality, and renal function also began to decline (0 month vs 28 months: BUN 2.35 vs 6.47 mmol/L; CRE 96.7 vs 105 mmol/L). The renal lesions were similar to the results of Marshall *et al* but more serious than those in the 6- and 15-month studies, indicating that proteinuria is a hallmark of STZ-induced DN in miniature pigs, with the progress of pathological changes being closely related to the course of diabetes.

In addition, end-stage glomerular lesions (glomerular sclerosis) were found in this study. In the severest cases, the entire glomeruli were sclerotic. However, Kimmelstiel-Wilson lesion was not found, possibly linked to the shorter course of diabetes, since the Kimmelstiel-Wilson lesion is generally considered as indicating a transitional period from early or moderate progression to a more advanced stage of the disease.<sup>34,35</sup> In the Tervaert pathological classification, Kimmelstiel-Wilson lesion is a marker of grade III DN.<sup>22</sup> The presence of at least one Kimmelstiel-Wilson lesion is considered to mark a longer disease course and is a poor clinical indicator of diabetes.<sup>34,36</sup> In addition, severe glomerular lesions were found at the boundary of the renal cortex and the medulla.

Retinopathy is another important complication of diabetes, and is a major cause of blindness in adults worldwide.<sup>37</sup> Diabetic retinopathy affects the microcirculation of the retina, and changes in its histology and pathophysiology lead to visual impairment and blindness.<sup>38</sup> In clinical practice, retinal microaneurysm, hemorrhage, cotton wool spots, and angiogenesis are classic signs of retinal vascular disorders. Prior to these manifestations, histological changes appear, including capillary basement membrane thickening, loss of

periderm cells or ghost residue, and proliferation of capillary endothelial cells, leading to blindness through hemorrhage and tractional retinal detachment.<sup>39</sup>

Pig's eyes are quite similar to human eyes both in size and structure,<sup>40</sup> as are the basic structures of the retina and blood vessels.<sup>41</sup> Hainsworth *et al*<sup>42</sup> first reported retinal capillary basement membrane thickening in Yucatan miniature pigs with alloxan-induced diabetes in the 20th week after induction. Lee *et al*<sup>43</sup> extended the observation period to 32 weeks in Yorkshire pigs with STZ-induced diabetes. They found that all pigs with diabetes had different degrees of cataract, and 75% exhibited retinal capillary basement membrane thickening. However, no other microvascular abnormality was noted. Our 28-month study is the first to show significant microvascular lesions, reaching the microaneurysm stage, in the retina of diabetic pig. In addition, the lack of blood supply caused by the retinal microvascular lesions led to an obvious lesion of the inner retina. The manifestations were pyknosis, reduction of the inner nuclear layer of the retina, ganglion cell loss, and vacuolar degeneration of the nerve fiber layer, which indicated that simple hyperglycemia can cause obvious retinopathy in miniature pigs, and the process of lesion development simulates diabetic retinopathy in humans more closely than that in rodents.

### 4.3 | Regeneration of pancreatic islet beta-cells

Currently, normal blood glucose level can be restored in diabetic patients via beta-cell transplantation (type 1 diabetes) or islet transplantation (type 2 diabetes).<sup>44-46</sup> Since it is difficult to find beta-cell donors, functional recovery or regeneration of endogenous islet beta-cells is an attractive option.

Recent studies have suggested that regeneration of beta-cells in adults occurs via three pathways: proliferation of beta-cells, neogenesis from non-beta cell precursors, and transformation from alpha-cells. These three pathways have been confirmed in rodents.<sup>47-52</sup> Further, it is clear that the enhancement of residual beta-cell replication is the main mechanism of beta-cell damage repair.<sup>52,53</sup> Unfortunately, it is impossible to measure the quality of human pancreatic islet beta-cells *in vivo*. Models involving transplantation of human pancreas into immunodeficient animals also have some limitations and effects could be influenced by many exogenous factors.<sup>54-57</sup> Despite few studies having found any evidence of  $\beta$ -cell replication in adult humans,<sup>58-60</sup> many xenograft models have shown newly activated human pancreatic islet cells in the grafts. However, some scholars believe that these results do not exclude the influence of the microenvironment in mice or the effect of ischemia and temperature changes on proliferation gene expression.

The pancreas of pigs provides a large animal model for the study of pancreatic islet endogenous regeneration. Kim *et al*<sup>12</sup> have reported that the range of islet sizes in humans, pigs and mice closely overlaps. They all exhibit a similar pattern of islet distribution, but the fraction of alpha-, beta- and delta-cells within an islet varies between islets in the different species. Porcine islets have

their own particular characteristics, including absence of large pancreatic islets and a less compact islet structure. In this study of miniature pigs, the number of pancreatic islet beta-cells decreased significantly 28 days after the application of STZ (120 mg/kg), but the insulin level in the blood circulation increased by 24 months after the application. Pathological sections showed that the number of beta-cells had increased significantly by the 28th month. Moreover, without any treatment, the blood glucose had recovered, indicating that pancreatic islet beta-cell regeneration in adult miniature pigs had occurred. Regarding the regeneration mechanism, only a small number of alpha-cells were seen to transform to beta-cells and little neogenesis of beta-cells was observed. We speculate that the generation of beta-cells in the miniature pigs depends mainly on replication; its specific mechanism needs further exploration.

In conclusion, this pig model simulates well the pathogenesis of human diabetes. In this study, severe mesangial dilatation and glomerulosclerosis were detected, while in the majority of other DN models only early renal lesions have been observed. Our study thus provides a new research strategy and a good model for the study of DN lesions. In addition, we report for the first time significant microangiopathy, such as microaneurysm, in diabetic pig retina, providing an important basis for the use of miniature pigs in diabetic retinopathy studies.

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### CONFLICT OF INTEREST

None.

### AUTHOR CONTRIBUTIONS

HC and MN conceived and designed the study. MN, YL, LX, YZ, JY, YJ, XD and HC performed the experiments. HC and MN wrote the paper. YL, LX, YZ, JY, YJ and XD reviewed and edited the manuscript. All authors read and approved the manuscript.

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### REFERENCES

1. Lagani V, Koumakis L, Chiarugi F, Lakasing E, Tsamardinos I. A systematic review of predictive risk models for diabetes complications based on large scale clinical studies. *J Diab Compl.* 2013;27(4):407-413.
2. Caramori ML, Fioretto P, Mauer M. The need for early predictors of diabetic nephropathy risk: is albumin excretion rate sufficient? *Diabetes.* 2000;49(9):1399-1408.
3. Mogensen CE. Microalbuminuria and hypertension with focus on type 1 and type 2 diabetes. *J Intern Med.* 2003;254(1):45-66.
4. Brosius FC, Alpers CE, Bottinger EP, et al. Mouse models of diabetic nephropathy. *J Am Soc Nephrol.* 2009;20(12):2503-2512.



5. Papatheodorou K, Banach M, Edmonds M, Papanas N, Papazoglou D. Complications of diabetes. *J Diabetes Res.* 2015;2015:1-5. <https://doi.org/10.1155/2015/189525>
6. Papatheodorou K, Papanas N, Banach M, Papazoglou D, Edmonds M. Complications of diabetes 2016. *J Diabetes Res.* 2016;2016:1-3. <https://doi.org/10.1155/2016/6989453>
7. Parving H-H, Brenner BM, McMurray JJV, et al. Cardiorenal end points in a trial of aliskiren for type 2 diabetes. *N Engl J Med.* 2012;367(23):2204-2213.
8. de Zeeuw D, Akizawa T, Audhya P, et al. Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease. *N Engl J Med.* 2013;369(26):2492-2503.
9. Fried LF, Emanuele N, Zhang JH, et al. Combined angiotensin inhibition for the treatment of diabetic nephropathy. *N Engl J Med.* 2013;369(20):1892-1903.
10. Larsen MO, Rolin B. Use of the Gottingen minipig as a model of diabetes, with special focus on type 1 diabetes research. *ILAR J.* 2004;45(3):303-313.
11. Ferrer J, Scott WE, Weegman BP, et al. Pig pancreas anatomy: implications for pancreas procurement, preservation, and islet isolation. *Transplantation.* 2008;86(11):1503-1510.
12. Kim A, Miller K, Jo J, Kilimnik G, Wojcik P, Hara M. Islet architecture: a comparative study. *Islets.* 2009;1(2):129-136.
13. Vinerean HV, Gazda LS, Hall RD, Smith BH. Streptozotocin is responsible for the induction and progression of renal tumorigenesis in diabetic Wistar-Furth rats treated with insulin or transplanted with agarose encapsulated porcine islets. *Islets.* 2011;3(4):196-203.
14. Bell RH Jr, Hye RJ, Miyai K. Streptozotocin-induced liver tumors in the Syrian hamster. *Carcinogenesis.* 1984;5(10):1235-1238.
15. Gabel H, Bitter-Suermann H, Henriksson C, Save-Soderbergh J, Lundholm K, Brynger H. Streptozotocin diabetes in juvenile pigs. Evaluation of an experimental model. *Horm Metab Res.* 1985;17(6):275-280.
16. Wilson JD, Dhall DP, Simeonovic CJ, Lafferty KJ. Induction and management of diabetes mellitus in the pig. *Aust J Exp Biol Med Sci.* 1986;64(Pt 6):489-500.
17. Grussner R, Nakhleh R, Grussner A, Tomadze G, Diem P, Sutherland D. Streptozotocin-induced diabetes mellitus in pigs. *Horm Metab Res.* 1993;25(4):199-203.
18. Dufrane D, van Steenberghe M, Guiot Y, Goebbels RM, Saliez A, Gianello P. Streptozotocin-induced diabetes in large animals (pigs/primates): role of GLUT2 transporter and beta-cell plasticity. *Transplantation.* 2006;81(1):36-45.
19. Liu X, Mellert J, Hering BJ, et al. Sensitivity of porcine islet beta cells to the diabetogenic action of streptozotocin. *Transplant Proc.* 1998;30(2):574-575.
20. Chen H, Liu YQ, Li CH, et al. The susceptibility of three strains of Chinese minipigs to diet-induced type 2 diabetes mellitus. *Lab Anim.* 2009;38(11):355-363.
21. Haneda S, Yamashita H. International clinical diabetic retinopathy disease severity scale. *Nihon Rinsho.* 2010;68(Suppl 9):228-235.
22. Tervaert TWC, Mooyaart AL, Amann K, et al. Pathologic classification of diabetic nephropathy. *J Am Soc Nephrol.* 2010;21(4):556-563.
23. Saran R, Robinson B, Abbott KC, et al. US renal data system 2016 annual data report: epidemiology of kidney disease in the United States. *Am J Kidney Dis.* 2017;69(3 Suppl 1):A7-A8.
24. Cumble BC, Hermayer KL. Current concepts in targeted therapies for the pathophysiology of diabetic microvascular complications. *Vasc Health Risk Manag.* 2007;3(6):823-832.
25. Edwards MS, Wilson DB, Craven TE, et al. Associations between retinal microvascular abnormalities and declining renal function in the elderly population: the Cardiovascular Health Study. *Am J Kidney Dis.* 2005;46(2):214-224.
26. Reiser J, Sever S. Podocyte biology and pathogenesis of kidney disease. *Annu Rev Med.* 2013;64:357-366.
27. Weil EJ, Lemley KV, Mason CC, et al. Podocyte detachment and reduced glomerular capillary endothelial fenestration promote kidney disease in type 2 diabetic nephropathy. *Kidney Int.* 2012;82(9):1010-1017.
28. Toyoda M, Najafian B, Kim Y, Caramori ML, Mauer M. Podocyte detachment and reduced glomerular capillary endothelial fenestration in human type 1 diabetic nephropathy. *Diabetes.* 2007;56(8):2155-2160.
29. Mac-Moune Lai F, Szeto C-C, Choi PCL, et al. Isolate diffuse thickening of glomerular capillary basement membrane: a renal lesion in prediabetes? *Mod Pathol.* 2004;17(12):1506-1512.
30. Swindle MM. Swine as replacements for dogs in the surgical teaching and research laboratory. *Lab Anim Sci.* 1984;34(4):383-385.
31. Maile LA, Busby WH, Gollahon KA, et al. Blocking ligand occupancy of the alphaVbeta3 integrin inhibits the development of nephropathy in diabetic pigs. *Endocrinology.* 2014;155(12):4665-4675.
32. Khairoun M, van den Heuvel M, van den Berg BM, et al. Early systemic microvascular damage in pigs with atherogenic diabetes mellitus coincides with renal angiotensin dysbalance. *PLoS ONE.* 2015;10(4):e0121555.
33. Marshall M, Oberhofer H, Staubesand J. Early micro- and macro-angiopathy in the streptozotocin diabetic minipig. *Res Exp Med (Berl).* 1980;177(2):145-158.
34. Hong D, Zheng T, Jia-qing S, Jian W, Zhi-hong L, Lei-shi L. Nodular glomerular lesion: a later stage of diabetic nephropathy? *Diabetes Res Clin Pract.* 2007;78(2):189-195.
35. Mason RM, Wahab NA. Extracellular matrix metabolism in diabetic nephropathy. *J Am Soc Nephrol.* 2003;14(5):1358-1373.
36. Schwartz MM, Lewis EJ, Leonard-Martin T, Lewis JB, Batlle D. Renal pathology patterns in type II diabetes mellitus: relationship with retinopathy. The Collaborative Study Group. *Nephrol Dial Transplant.* 1998;13(10):2547-2552.
37. Sivaprasad S, Gupta B, Crosby-Nwaobi R, Evans J. Prevalence of diabetic retinopathy in various ethnic groups: a worldwide perspective. *Surv Ophthalmol.* 2012;57(4):347-370.
38. Durham JT, Herman IM. Microvascular modifications in diabetic retinopathy. *Curr Diab Rep.* 2011;11(4):253-264.
39. Miwa K, Nakamura J, Hamada Y, et al. The role of polyol pathway in glucose-induced apoptosis of cultured retinal pericytes. *Diabetes Res Clin Pract.* 2003;60(1):1-9.
40. Sanchez I, Martin R, Ussa F, Fernandez-Bueno I. The parameters of the porcine eyeball. *Graefes Arch Clin Exp Ophthalmol.* 2011;249(4):475-482.
41. Bloodworth JM Jr, Gutgesell HP Jr, Engerman RL. Retinal vasculature of the pig. Light and electron microscope studies. *Exp Eye Res.* 1965;4(3):174-178.
42. Hainsworth DP, Katz ML, Sanders DA, Sanders DN, Wright EJ, Sturek M. Retinal capillary basement membrane thickening in a porcine model of diabetes mellitus. *Comp Med.* 2002;52(6):523-529.
43. Lee SE, Ma W, Rattigan EM, et al. Ultrastructural features of retinal capillary basement membrane thickening in diabetic swine. *Ultrastruct Pathol.* 2010;34(1):35-41.
44. Shapiro AMJ, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med.* 2006;355(13):1318-1330.
45. Harlan DM, Kenyon NS, Korsgren O, Roep BO. Immunology of Diabetes 5. Current advances and travails in islet transplantation. *Diabetes.* 2009;58(10):2175-2184.
46. Nath DS, Gruessner AC, Kandaswamy R, Gruessner RW, Sutherland DE, Humar A. Outcomes of pancreas transplants for patients with type 2 diabetes mellitus. *Clin Transplant.* 2005;19(6):792-797.
47. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. *J Clin Invest.* 2007;117(9):2553-2561.
48. Suri A, Calderon B, Esparza TJ, Frederick K, Bittner P, Unanue ER. Immunological reversal of autoimmune diabetes

- without hematopoietic replacement of beta cells. *Science*. 2006; 311(5768):1778-1780.
49. Inada A, Nienaber C, Katsuta H, et al. Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. *Proc Natl Acad Sci USA*. 2008;105(50):19915-19919.
  50. Chung CH, Hao E, Piran R, Keinan E, Levine F. Pancreatic beta-cell neogenesis by direct conversion from mature alpha-cells. *Stem Cells*. 2010;28(9):1630-1638.
  51. Thorel F, Nepote V, Avril I, et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. *Nature*. 2010;464(7292):1149-1154.
  52. Dor Y. beta-Cell proliferation is the major source of new pancreatic beta cells. *Nat Clin Pract Endocrinol Metab*. 2006;2(5):242-243.
  53. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature*. 2004;429(6987):41-46.
  54. Caballero F, Siniakowicz K, Hollister-Lock J, et al. Birth and death of human beta-cells in pancreases from cadaver donors, autopsies, surgical specimens, and islets transplanted into mice. *Cell Transplant*. 2014;23(2):139-151.
  55. Levitt HE, Cyphert TJ, Pascoe JL, et al. Glucose stimulates human beta cell replication in vivo in islets transplanted into NOD-severe combined immunodeficiency (SCID) mice. *Diabetologia*. 2011;54(3):572-582.
  56. Tyrberg B, Ustinov J, Otonkoski T, Andersson A. Stimulated endocrine cell proliferation and differentiation in transplanted human pancreatic islets: effects of the ob gene and compensatory growth of the implantation organ. *Diabetes*. 2001;50(2):301-307.
  57. Kitazawa T, Yokoyama K, Kubota K. Combination therapy of glucagon-like peptide-1 receptor agonists and insulin for patients who developed diabetes after partial pancreatectomy. *J Diabetes Investig*. 2016;7(3):381-385.
  58. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes*. 2003;52(1):102-110.
  59. Köhler CU, Kreuter A, Rozykowski MC, et al. Validation of different replication markers for the detection of beta-cell proliferation in human pancreatic tissue. *Regul Pept*. 2010;162(1-3):115-121.
  60. Gregg BE, Moore PC, Demozay D, et al. Formation of a human beta-cell population within pancreatic islets is set early in life. *J Clin Endocrinol Metab*. 2012;97(9):3197-3206.

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