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17β-Estradiol Promotes Islet Cell Proliferation in a Partial Pancreatectomy Mouse Model

Tingting Wu,[1,2] Jinyong Xu,[1,2] Shengchun Xu,[2] Lianzhong Wu,[2] Youyu Zhu,[2] Guangwu Li,[1,2] and Zhenhua Ren[1,2,3]

[1]Department of Neurobiology, School of Basic Medicine, Anhui Medical University, Hefei, Anhui 230032, China; [2]Department of Anatomy, School of Basic Medicine, Anhui Medical University, Hefei, Anhui 230032, China; and [3]Cell Therapy Center, Xuanwu Hospital, Capital Medical University, Beijing 100053, China

17β-Estradiol (E2) is a multifunctional steroid hormone in modulating metabolism *in vivo*. Previous studies have reported that E2 could promote insulin secretion and protect β cells from apoptosis. In this study, the partial pancreatectomy (PPx) model was used to study the role of E2 in islet cell proliferation. The animals were divided into four groups, including sham control, PPx model, E2, and E2 plus estrogen antagonist (E2 plus ICI) groups. In the E2 group, 5-bromo-2′-deoxyuridine– and Ki67-positive cells significantly increased after PPx, and the protein expression of forkhead transcription factor M1, cyclin A2, cyclin B1, and cyclin E2 also significantly increased in the isolated islets. The messenger RNA expression of cyclin A2 and cyclin B2 increased in E2 treatment group. Additionally, the effects of E2 on the PPx mice were partially blocked by estrogen antagonist ICI182,780. The results indicated that E2 significantly promoted islet cell proliferation in PPx model mice, and it upregulated the expression of cell cycle genes. In conclusion, E2 treatment is beneficial for islet cell proliferation in adult mice after PPx. A partial pancreatectomy in mice may be an attractive model for the study of islet cell proliferation.

Freeform/Key Words: islet, cell proliferation, partial pancreatectomy, 17β-Estradiol, diabetes mellitus

Diabetes is primarily characterized by hyperglycemia, mainly due to the absence of β cells resulting in insufficient production of insulin in the body. Patients with long-term hyperglycemia of diabetes tend to have various chronic tissue damage and dysfunction, such as retinopathy [1], nephrotoxicity [2], cardiovascular disease [3], and so on. Thus, diabetes is one of the leading diseases that severely threaten human health following tumor, cardiovascular, and cerebrovascular diseases. Recently, studies have shown an increase in the incidence of diabetes worldwide [4]. Therefore, there is an urgent need to find effective and safe drugs for the prevention and treatment of diabetes in the clinic.

The islet is a crucial endocrine pancreas tissue that includes mainly four types of cells, namely, α, β, δ, and pancreatic polypeptide cells. β Cells are most abundant in the islets, accounting for ~60% to 80%. Producing insulin is the most important function of β cells. Insulin, the only hormone to reduce glucose in the body, plays a vital role in maintaining blood glucose homeostasis. Traditionally, sufficient numbers of functional β cells are required to promote the secretion of insulin and control optimal glucose homeostasis [5, 6]. Various evidence [7–9] demonstrates that adult mammalian β cells acquire and supply functional β

Abbreviations: E2, 17β-estradiol; BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; FOXM1, forkhead transcription factor M1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPER, G protein–coupled estrogen receptor; IL, interleukin; INS1, insulin 1; INS2, insulin 2; mRNA, messenger RNA; ngn3, neurogenin 3; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDX-1, pancreatic duodenal homeobox 1; PPx, partial pancreatectomy; SEM, standard error of the mean; TNF, tumor necrosis factor.

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cell mass by self-replication, neogenesis, or transplantation. Dor *et al.* [7] showed that terminally differentiated β cells still have proliferation potential, and new β cells mainly derive from the pre-existing β cell replication or mitosis. Additionally, cellular reprogramming in adult pancreas is used to provide a strategy for regenerating functional β cell mass [10]. Researchers generally think that β cell proliferation is an important way for regeneration of pancreatic β cells [11, 12].

Recent studies have considered that β cell regeneration or expansion by the application of hormones or growth factors is a promising way to improve the symptoms of diabetes [13, 14]. 17β-Estradiol (E2), a well-known steroid hormone, plays a vital role in regulating various physiological and pathological types of metabolism *in vivo*, such as glucolipid metabolism and energy balance. Santos *et al.* [15] demonstrated that E2 can improve pancreatic β cell dysfunction in ovariectomized mice and reduce hepatic insulin degradation. A series of similar studies showed that E2 can promote insulin secretion and protect β cells from apoptosis [16–18]. Epidemiological research has also found that the incidence of diabetes in women is lower than in men, and postmenopausal women using estrogen replacement therapy can significantly reduce the incidence of type 2 diabetes [19]. Studies have shown that estrogen receptors (ERs), including ERα, ERβ, and G protein–coupled ER (GPER), are associated with glucose metabolism [20, 21]. It has been reported that ERα is considered a key regulator involved in insulin biosynthesis [22], and activation of ERα by hyperglycemia can protect β cells from oxidative injury [23].

Partial pancreatectomy (PPx) is a common model in the study of β cell regeneration [24, 25]. In the model, the splenic lobe of the pancreas (tail) is surgically removed, and the duodenal part (head) is reserved. The source of endocrine cells remains controversial. In most of previous studies, PPx mice have been used as a model of β cell replication [26, 27]. However, in other studies, such mice have been used as a neogenesis model of endocrine progenitors within ducts [28]. By now, PPx as a β cell replication model is accepted owing to the evidence of genetic lineage tracing and measures of DNA replication [26, 27, 29, 30]. In this study, the PPx model was used to investigate the effects of E2 on islet cell proliferation in adult mice.

1. Materials and Methods

A. Animals

All procedures involving the use of live animals as described in this study were approved by the Institutional Animal Care and Use Committee of the Anhui Medical University and Capital Medical University, strictly following the Ethical Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and to ameliorate any distress.

Male C57BL/6 mice, 8 weeks of age, were obtained from the Animal Center Laboratory of Beijing and maintained in the Medical Experimental Animal Center of Anhui Province (China). Animals were exposed to a 12-hour light/12-hour dark cycle with temperature of 22 ± 1°C and relative humidity of 60% ± 5%, and they had free access to standard laboratory chow and water.

B. Experimental Model

After ~1 week of acclimatization, the animals were divided into four groups, including a sham control group (n = 8), PPx model group (n = 8), E2 treatment group (n = 8), and E2 plus estrogen antagonist (ICI 182,780) (E2+ICI) group (n = 8). Procedures of the experiments are shown in Fig. 1, including the following: (1) 1 week before the surgery, mice of the E2 and E2+ICI groups were implanted with 0.5 mg of E2 pellets (Innovative Research of America, Sarasota, FL) in a slow-release form (21 days) at the nape of the neck, and placebo pellets were used in mice of sham and PPx groups. (2) Partial pancreatectomy was performed in animals of the PPx, E2, and E2+ICI groups using a method described previously [31, 32]. Briefly, the

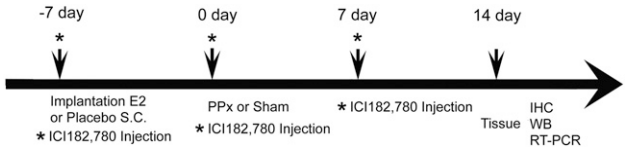

Figure 1. Schematic diagram for the experimental procedures. There were four experimental groups: sham group, PPx group, E2 group, and E2+ICI group. One week before the surgery, the mice of the E2 and E2+ICI groups were implanted with 0.5 mg of E2 pellets, and placebo pellets were used in mice of the sham and PPx groups. Partial pancreatectomy was performed in the animals of PPx, E2, and E2+ICI group mice. The sham group of mice underwent a sham operation. Two weeks after the operation, the mice were euthanized, and the pancreas tissues were dissected and processed for histological and biochemical analysis. For the E2+ICI group, the mice received subcutaneous injection of ICI182,780 once a week for three times. Before euthanasia, BrdU was intraperitoneally injected in the mice twice a day.

mice were anesthetized, and a midline abdominal incision was made. The splenic lobe and mesenteric part of the pancreas were removed by gentle abrasion with cotton applicators, with care being taken to leave the major vessels intact. The duodenal part of the pancreas was retained, and the excised portion accounted for 65% of the total volume of the pancreas. The sham group mice (sham operation) underwent a laparotomy and gentle rubbing of the tissue. (3) Two weeks after the operation, the mice were euthanized, and the pancreatic tissues were dissected and processed for histological and biochemical analyses. For mice of the E2+ICI group, subcutaneous injection of ICI182,780 (5 mg per mouse) (Tocris Cookson, Northpoint, UK) was accepted once a week for three times. Before euthanasia, 5-bromo-2′-deoxyuridine (BrdU; 50 mg/kg) (Sigma-Aldrich, Louis, MO) was intraperitoneally injected in the mice twice a day. Exemestane (Sigma-Aldrich) was administered subcutaneously at a dosage of 5 mg [500 μL consisted of 0.3% hydroxypropyl cellulose in phosphate-buffered saline (PBS)] for each injection. The injection time is shown in Fig. 5d. The total dose was 25 mg per animal.

C. Determination of E2 Concentration

The mice were anesthetized, blood was obtained from the retro-orbital plexus, and plasma was separated. The E2 concentration in the plasma was measured using an enzyme-linked immunosorbent assay (ELISA) kit (estradiol ELISA kit; Abcam, Cambridge, MA) according to the manufacturer's protocol. Briefly, 25 μL of samples, standard, or control was added into their respective wells, and then 200 μL of E2– horseradish peroxidase conjugate was added to each well. After incubation for 2 hours at 37°C, the content of the wells was aspirated, and each well was rinsed three times with 300 μL of diluted washing solution. Tetramethylbenzidine substrate solution (100 μL) was added into all wells. After 30 minutes of incubation at room temperature in the dark, 100 μL of stop solution was added into all wells and the microplate was shaken gently. The absorbance of the sample was measured at 450 nm within 30 minutes.

D. Histological Analysis, Immunohistochemistry, and Immunofluorescent Staining

For histological analysis, samples from pancreas tissue in mice were fixed in 4% para-formaldehyde overnight at 4°C. Pancreatic tissue blocks were sectioned into 10-μm slices. The sections were stained with hematoxylin and eosin reagent (Sigma-Aldrich). The images were captured using an Olympus BX51 microscope. At least 20 islets from the duodenal lobe of pancreas (four animals for all experimental groups) were analyzed. The percentage of area occupied by the islets in total pancreatic tissue was calculated by an image analysis system (Image Lab 5.2; Bio-Rad Laboratories, Hercules, CA) as described previously [33]. The number of islets was counted by randomly choosing 20 fields in areas of interest under ×100 magnification as described previously [34].

The procedure for immunohistochemistry was previously described and included some modifications [35]. Sections were permeabilized with 0.03% Triton X-100 for 40 minutes,

followed by blocking with 5% goat serumin PBS at room temperature for 1 hour. The slides were treated with rabbit anti-Ki67 (1:1000; Cell Signaling Technology, Danvers, MA) and rabbit anti-ERα (1:800; Abcam) overnight at 4°C. After washing with PBS, slides were incubated with biotin-conjugated goat anti-rabbit or mouse secondary antibody (1:800) for 1 hour at room temperature and followed by PBS washes. The slides were incubated in the avidin–biotin–peroxidase complex for 1 hour at room temperature. After rinsing, the slides were developed in 0.05% 3,39-diaminobenzidine (Sigma-Aldrich) containing 0.003% H_2O_2 in PBS. The images were recorded using an Olympus BX51 microscope. Negative controls were performed by omitting the primary antibody. Ki67-positive cells were counted at ×40 magnification. Twenty randomly selected sections covering at least 1000 cells were counted. Four animals were analyzed for each group.

For the immunofluorescence staining, the procedure was previously described and included some modifications [36]. Briefly, after blocking with 1% bovine serum albumin and 0.5% Triton X-100 in PBS for 1 hour, the slides were incubated with a mouse anti-BrdU antibody (1:400; Sigma-Aldrich), mouse anti-insulin (1:800; Sigma-Aldrich), or rabbit anti-ERα (1:200) overnight at 4°C. After rinsing in PBS, the slides were incubated with Alexa Fluor 488–conjugated anti-rabbit or Alexa Fluor 594–conjugated anti-mouse IgG in the dark at room temperature for 1 hour. After rinsing, the slides were covered with mounting media with 4′,6-diamidino-2-phenylindole (DAPI). The images were captured using an Eclipse TE2000 microscope (Nikon, Tokyo, Japan) and SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI). Negative controls were performed by omitting the primary antibody. The details of antibodies used for staining are shown in Table 1.

E. Islet Isolation and Western Blot Analysis

The pancreas tissue was dissected and the islets were isolated with collagenase (Sigma-Aldrich), as previously reported [37]. The protein of isolated islets was extracted and put on immunoblotting analysis as previously described [35]. Briefly, tissues were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 0.5% Nonidet P-40, 0.25% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin. Homogenates were centrifuged at 20,000 × *g* for 30 minutes at 4°C and the supernatant fraction was collected. After determining protein concentration, aliquots of the protein samples (30 μg) were separated on a sodium dodecyl sulfate–polyacrylamide gel

Table 1. Antibody Table

Protein Target Antigen	Clone ID	Name of Antibody	Manufacturer, Catalog Number	Species Raised (Monoclonal or Polyclonal)	Dilution Used	Antibody ID
Ki67	D3B5	Ki67	Cell Signaling Technology, 12202	Rabbit, monoclonal	1:1000	RRID:AB_2620142
ERα	E115	ERα	Abcam, ab32063	Rabbit, monoclonal	1:1000	RRID:AB_732249
BrdU		BrdU	Sigma-Aldrich, B2531	Mouse, monoclonal	1:400	RRID:AB_476793
Insulin	K36AC10	Insulin	Sigma-Aldrich, I2018	Mouse, monoclonal	1:800	RRID:AB_260137
FOXM1	C-20	FoxM1	Santa Cruz Biotechnology, sc-502	Rabbit, polyclonal	1:200	RRID:AB_631523
Cyclin A2	BF683	Cyclin A2	Cell Signaling Technology, 4656	Mouse, monoclonal	1:1000	RRID:AB_10691320
Ccnb1		Cyclin B1	Santa Cruz Biotechnology, sc-70898	Mouse, monoclonal	1:200	RRID:AB_2275448
Ccnb2	A-2	Cyclin B2	Santa Cruz Biotechnology, sc-28303	Rabbit, monoclonal	1:200	RRID:AB_627340
Ccnd2	D52F9	Cyclin D2	Cell Signaling Technology, 3741	Rabbit, monoclonal	1:1000	RRID:AB_2070685
Ccne2		Cyclin E2	Cell Signaling Technology, 4132	Rabbit, polyclonal	1:1000	RRID:AB_2071197
α-Tubulin	DM1A	α-Tubulin	Sigma-Aldrich, T6199	Mouse, Monoclonal	1:5000	RRID:AB_477583

by electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% bovine serum albumin in 0.01 M PBS (pH 7.4) and 0.05% Tween 20 at room temperature for 1 hour. Subsequently, the membranes were probed with antibodies to rabbit anti-ERα (1:1000), mouse anti–cyclin A2 (1:1000; Cell Signaling Technology), mouse anti–cyclin B1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti–cyclin B2 (1:200; Santa Cruz Biotechnology), rabbit anti–cyclin D2 (1:1000; Cell Signaling Technology), rabbit anti–forkhead transcription factor M1 (FOXM1; 1:200; Santa Cruz Biotechnology), rabbit anti–cyclin E2 (1:1000; Cell Signaling Technology), or mouse anti–α-tubulin (1:5000; Sigma-Aldrich) overnight at 4°C. After 0.01 M PBS (pH 7.4) and 0.05% Tween 20 washing, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase. The immune complexes were detected using the western blot system (GE Healthcare, Chalfont St. Giles, UK) and was quantified using the software of Image Lab 5.2 (Bio-Rad Laboratories). The details of antibodies used for immunoblotting are shown in Table 1.

F. RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA from isolated islets was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand complementary DNA was reverse transcribed using SuperScript II (Invitrogen) and oligo(dT) as primers (Promega, Madison, WI). polymerase chain reaction (PCR) was performed on a PTC-100 thermal cycler (Bio-Rad Laboratories) using rTaq polymerase (Takara, Ohtsu, Japan). Quantitative real-time PCR was performed using TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) with a 7900 real-time PCR system (Applied Biosystems). The following TaqMan gene expression assays (Applied Biosystems) were used: Mm00435565_m1 [pancreatic duodenal homeobox 1 (Pdx-1)], Mm00437606_s1 [neurogenin 3 (ngn3)], Mm01950294_s1 [insulin 1 (Ins1)], Mm00731595_gH [insulin 2 (Ins2)], Mm00438063_m1 (cyclin A2), Mm01171453_m1 (cyclin B2), Mm00438070_m1 (cyclin D2), Mm00434228_m1 [interleukin (IL)-1b], Mm00446190_m1 (IL-6), and Mm00443258_m1 [tumor necrosis factor (TNF)-α], Mm99999915_g1 [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], and Mm03928990_g1 (18S). Each quantitative reaction was performed in duplicate. The data were normalized to the value for GAPDH or 18S.

G. Statistical Data Analysis

Quantitative data are expressed as means ± standard error of the mean (SEM). Differences between two groups were analyzed using ANOVA followed by a Dunnett test. Differences in which P was <0.05 were considered statistically significant.

2. Results

A. Effect of E2 Treatment on the Expansion of Islets After a PPx

Two weeks after a partial pancreatectomy, the percentage of area occupied by the islets in total pancreatic tissue using hematoxylin and eosin staining was calculated [Fig. 2(a)]. Compared with sham control animals, the percentage of islet area significantly increased in PPx group mice [Fig. 2(a) and 2(b)]. The number of islets was also counted by randomly choosing 20 fields in duodenal lobe of pancreas under ×10 magnification. There was no difference in the number of islets between all groups of mice [Fig. 2(c)]. Compared with PPx model group mice, E2-treated mice had a significant increase in the percentage of islet area [Fig. 2(b)]. We also evaluated the impact of estrogen antagonist (ICI182,780) on the islets after PPx operation. No statistically significant difference was found in the percentage of islet area between the PPx and E2+ICI groups [Fig. 2(b)]. The results showed that E2 increased the percentage of islet area in the PPx mice, whereas E2 had no effect on increasing the number of islets.

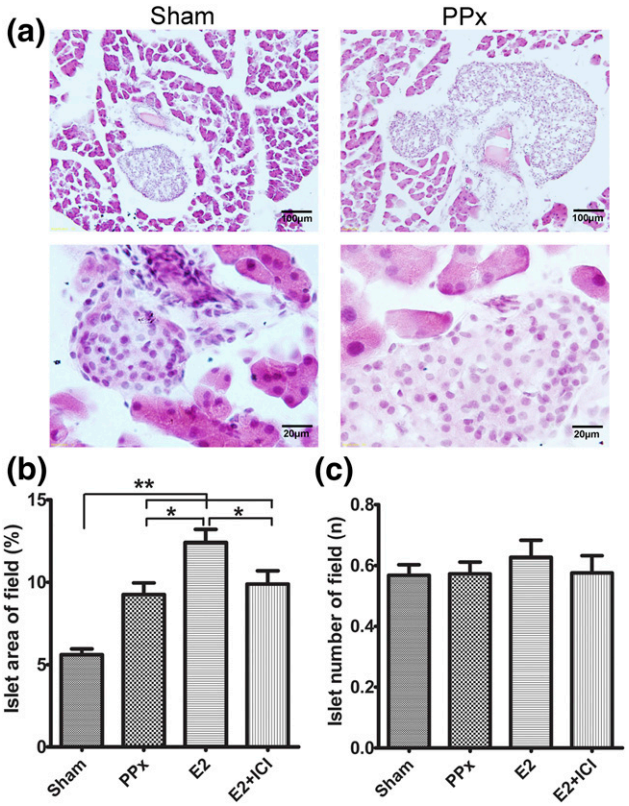

Figure 2. E2 increased the islet size in PPx mice. (a) Two weeks after partial pancreatectomy, the mice were killed, and the pancreatic tissues were processed for hematoxylin and eosin staining (left column was sham group; right column was PPx group). The islet size (b) and number (c) were quantified in each experimental animal group. Each data point was the mean ± SEM of four independent experiments. *$P < 0.05$, **$P < 0.01$.

B. Plasma Estrogen Levels

To determine the persistence of exogenous E2 *in vivo*, the plasma E2 levels were examined using a E2 ELISA kit. We found that the plasma level of E2 was <10 pg/mL in sham mice, as shown in Fig. 3(a). However, the plasma level of E2 was significantly increased in PPx mice, although the content was <20 pg/mL. With the slow release of the sustained-release E2 tablets, we observed that the plasma concentration of E2 remained at a high level in the E2 (157.20 ± 30.63 pg/mL) and E2+ICI (143.60 ± 24.44 pg/mL) groups, far higher than in the control mice.

C. Localization and Expression of ERα in the Islets

As previously reported [16, 23, 38], we now know that β cell survival and glycemic control in various animal models [16, 39] are regulated in part by signaling through ERα [16, 40]. Therefore, ERα has been considered as a main regulator when conducting estrogen intervention. In this study, our aim was to explore ERα subcellular localization in the islets using immunofluorescence staining. Consistent with previous reports [16, 38], ERα was located in the nucleus of islets cells according to fluorescence staining [Fig. 3(b) and 3(c)]. Next, the expression level of ERα was investigated *in vivo* by tissue section staining and immunoblotting. We found the content of ERα significantly increased in the islets of the PPx group mice compared with the sham mice [Fig. 3(c) and 3(d)]. On the contrary, ERα had a reduced expression due to exogenous high concentrations of E2 stimulation [Fig. 3(c) and 3(d)].

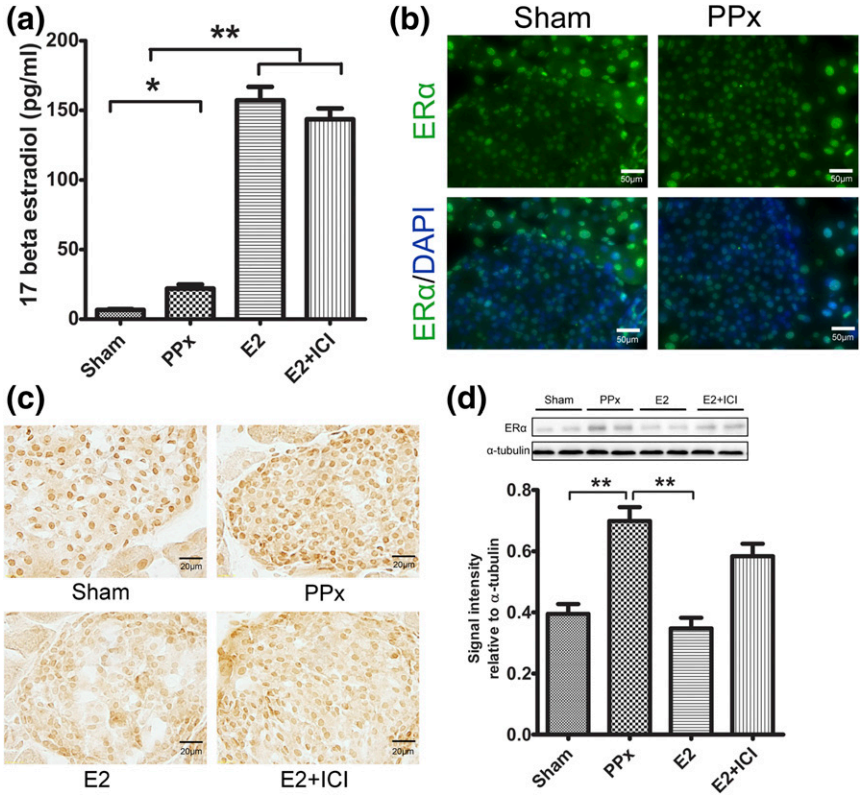

Figure 3. Effect of E2 treatment on ERα expression in PPx mice. (a) Two weeks after E2 or vehicle treatment, mice were euthanized, the plasma was isolated, and the levels of E2 were determined by an ELISA kit. The pancreatic tissues were processed to immunofluorescence staining (b) and immunohistochemistry staining (c) of ERα. (d) The protein expression of ERα was quantified by immunoblotting and normalized to the expression of α-tubulin. Each data point for E2 plasma level was the mean ± SEM of eight independent experiments, and for the protein expression of ERα was four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

D. Effects of E2 on Islet Cell Proliferation

To confirm whether estrogen has a proliferative effect on islet cells *in vivo*, the mice were treated with BrdU and the fluorescent staining of BrdU was performed. As shown in Fig. 4(a), BrdU-positive cells in the islets of residual pancreatic tissue were mainly located in the β cells. BrdU-positive cells were detected infrequently in sham group mice, whereas positive cells were significantly increased in the islets of residual pancreatic tissue after PPx operation [Fig. 4(b)]. Quantitative analysis showed that the rate of BrdU-positive cells in the islets was close to 4% in the PPx group mice [Fig. 4(c)]. In contrast to the PPx model group, the rate of BrdU-positive cells in islets was significantly increased (>6%) in the E2 group. Furthermore, it was observed that estrogen antagonist ICI182,780 significantly reduced the rate of BrdU incorporation [Fig. 4(b) and 4(c)].

To further verify that estrogen promotes cell proliferation, the islet cell proliferation was evaluated by Ki67 staining 2 weeks after PPx operation. There were no Ki67-positive cells observed within the islets of sham mice, whereas a few positive cells were found in the PPx mice [Fig. 5(a) and 5(b)]. Compared with the PPx model group, the Ki67-positive cells in islets of the E2 group mice were significantly increased, and the antagonist ICI182,780 reduced the rate of Ki67-positive cells [Fig. 5(a) and 5(c)]. Additionally, we found that the proliferation rate of pancreatic exocrine cells was much higher than the endocrine cells [Fig. 5(a) and 5(d)]. The increased rates of BrdU incorporation and Ki67-positive cells suggested that E2 promoted islet cell proliferation, whereas ICI182,780, the estrogen receptor antagonist, can partially blunt the effect.

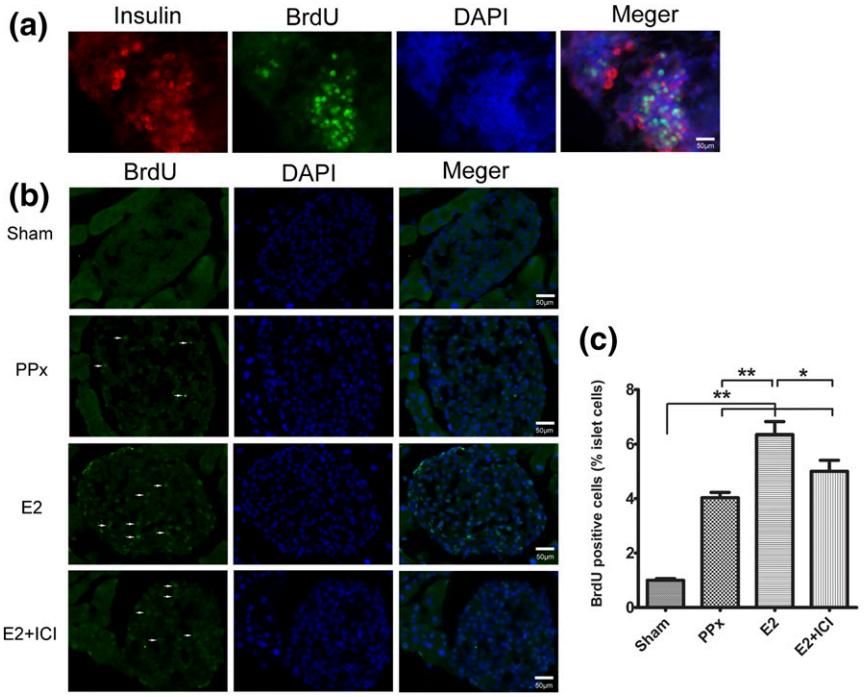

Figure 4. Effect of E2 treatment on the islet cell proliferation by BrdU staining. (a and b) Two weeks after PPx operation, the mice were euthanized and the pancreatic tissues were processed to BrdU staining. (a) Double labeling with insulin (red) and BrdU (green) was performed for the location of BrdU-positive cells within the islets. (b and c) The BrdU-positive cells in the islets were quantified by immunofluorescence staining with BrdU (green) and DAPI (blue). (b) The arrows point to the BrdU-positive cells in the islets. Each data point was the mean ± SEM of four independent experiments. $*P < 0.05$, $**P < 0.01$.

To investigate whether endogenous synthesis of E2 was required for the increased islet cell proliferation after PPx, we subcutaneously administered exemestane, an inhibitor of aromatase, the rate-limiting enzyme for E2 synthesis (Fig. 6), and Ki67 staining was performed. As shown in Fig. 6(a–c), Ki67-positive cells in the islets of residual pancreatic tissue decreased after exemestane administration. The results suggested that endogenous E2 was involved in the islet cell proliferation.

E. Effects of E2 on Cell Cycle Regulators After PPx

To analyze the impact of E2 on the signaling involved in islet cell proliferation, protein expression levels of islets were evaluated 2 weeks after PPx. The results showed that the protein levels of FOXM1 increased in islets after PPx (Fig. 7). Notably, no significant changes in the expression levels of cyclin A2, cyclin B1, cyclin B2, cyclin D2, and cyclin E2 were observed between the sham group and the PPx group (Fig. 7). However, after E2 treatment, the expression levels of FOXM1, cyclin A2, cyclin B1, cyclin D2, and cyclin E2 significantly increased (Fig. 7).

To obtain a comprehensive profile of gene expression, the related gene expression in islets was analyzed after PPx. We found that the messenger RNA (mRNA) levels of cyclin A2 and cyclin B2, but not cyclin D2, increased in the islets of the PPx group mice compared with the sham group [Fig. 8(b)]. Notably, the mRNA levels of cyclin A2 and cyclin B2 in the E2 group increased greatly, and the effect of E2 was blocked by estrogen antagonists [Fig. 8(b)]. Additionally, an increased expression of INS2 was seen in the islets of E2 group mice, and no changes in the expression of PDX-1 and INS1 were observed between all groups [Fig. 8(a)]. We further analyzed the expression of ngn3, a pancreatic endocrine-determining transcription factor, and ngn3 mRNA was not detected in the adult islets (data not shown).

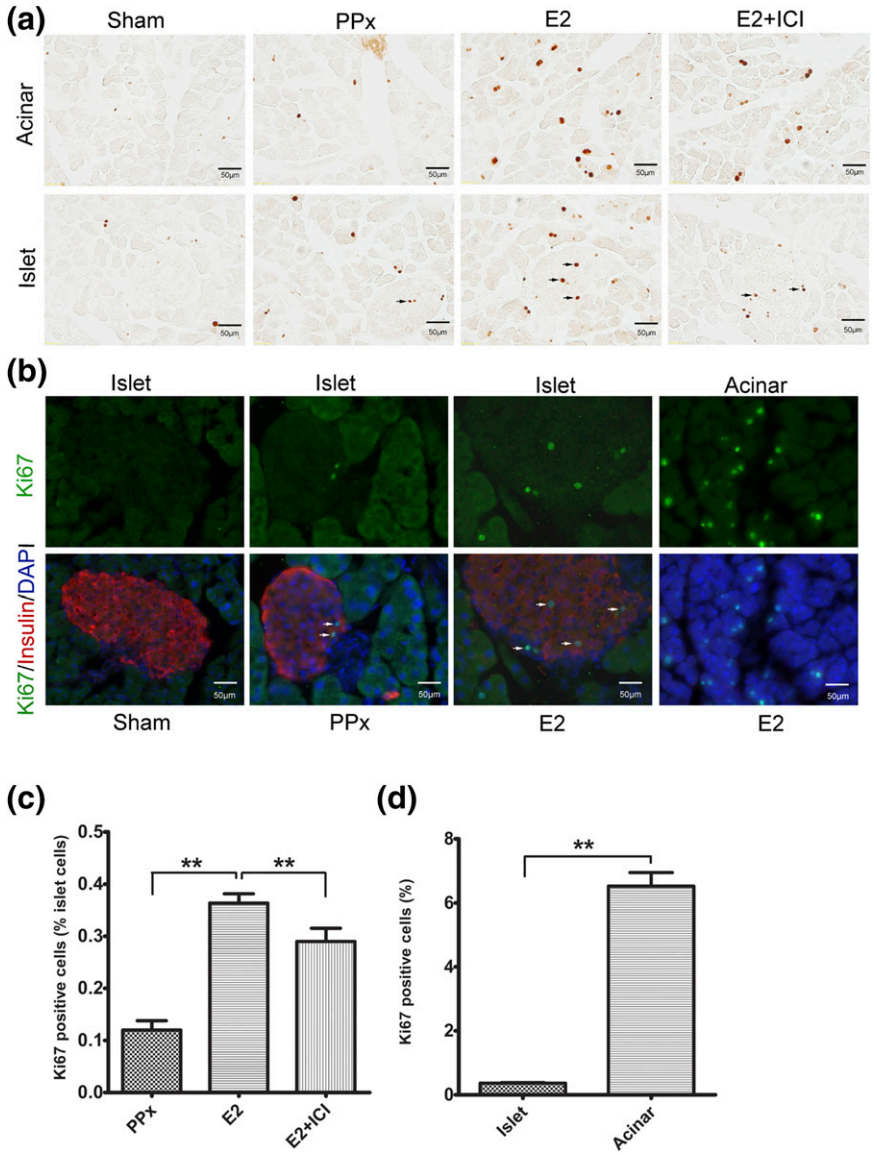

Figure 5. Effect of E2 treatment on the islet cell proliferation by Ki67 staining. (a and b) The pancreatic tissues were processed using immunohistochemistry (a) and immunofluorescence (b) of Ki67 staining. (a) The proliferation of pancreatic acinar cells (upper row) and endocrine islet cells (lower row) were evaluated by immunohistochemistry. (b–d) The Ki67-positive cells were quantified by immunofluorescence staining (Ki67 staining in green, insulin staining in red, and DAPI staining in blue). Each data point was the mean ± SEM of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Additionally, we extended our analysis to inflammatory factors IL-1β, IL-6, and TNF-α to verify whether the inflammatory factors changed in the pancreas regeneration of E2 treatment [Fig. 8(c)]. Indeed, there were no changes relating to mRNA expression of inflammatory factor genes in all groups of mice.

3. Discussion

Adult pancreatic β cells remain in a state of dynamic balance under normal physiological conditions. Insulin produced by β cells is sufficient to maintain glucose homeostasis *in vivo*. Once pancreas injury appears, there will be compensatory mechanisms to restore the function of the pancreas until irreparable deterioration occurs [40]. This compensatory regeneration of

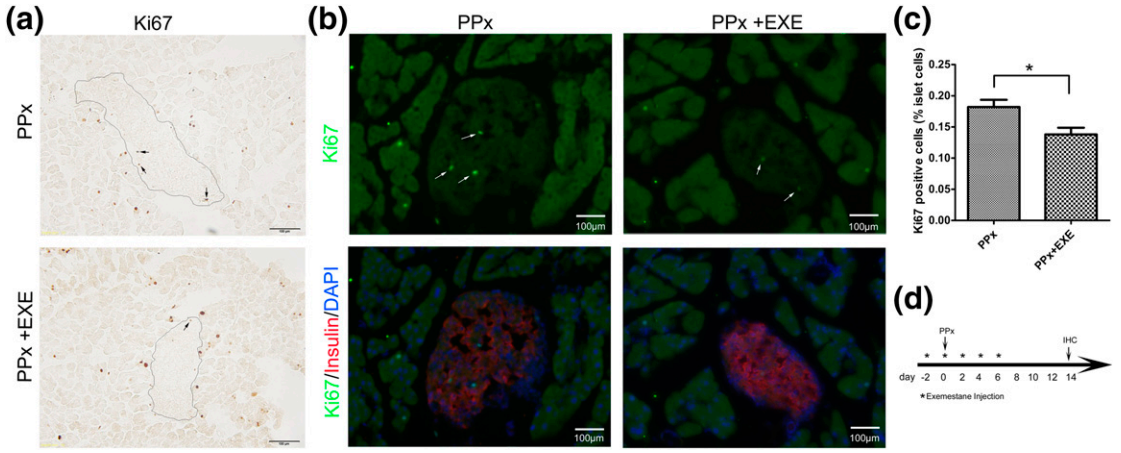

Figure 6. The aromatase inhibitor blunting islet cell proliferation. (a and b) Two weeks after the operation, the pancreas tissues were dissected and processed for immunohistochemistry (a) and immunofluorescence (b) of Ki67 staining. (b and c) The Ki67-positive cells were quantified by immunofluorescence staining (Ki67 staining in green, insulin staining in red, and DAPI staining in blue). (d) Exemestane was administered subcutaneously five times in PPx group mice. Each data point was the mean ± SEM of four independent experiments. $^{*}P < 0.05$.

β cells is achieved mainly through the expansion of β cell mass and the increase of insulin secretion [41]. The PPx model in rodents is currently one of the most common experimental models used to study β cell regeneration [29]. After PPx, the substantial β cell mass reduces in a short period of time, resulting in compensatory regeneration *in vivo* in mice.

It is widely thought that the antidiabetic effect of E2 is mainly attributed to the activation of ERs [22, 42]. Alonso-Magdalena *et al.* [20] demonstrated that E2 has played a significant role in the regulation of insulin synthesis, whereas there was no E2 effect in increasing insulin content in ERα knockout mice, indicating a predominant ERα effect. In this study, we found

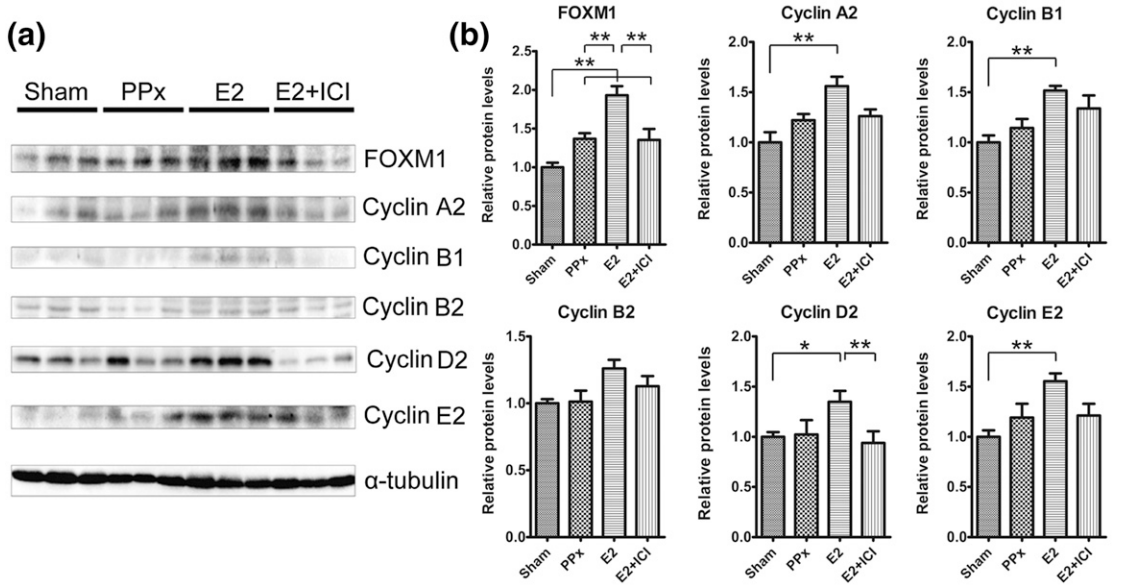

Figure 7. Effect of E2 treatment on the cell cycle regulators. (a) Two weeks after the PPx surgery, the mice were killed, and the isolated islets were processed for immunoblotting analysis of FOXM1, cyclin A2, cyclin B1, cyclin B2, cyclin D2, and cyclin E2. (b) The protein expression was quantified and normalized to the expression of α-tubulin. Each data point was the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

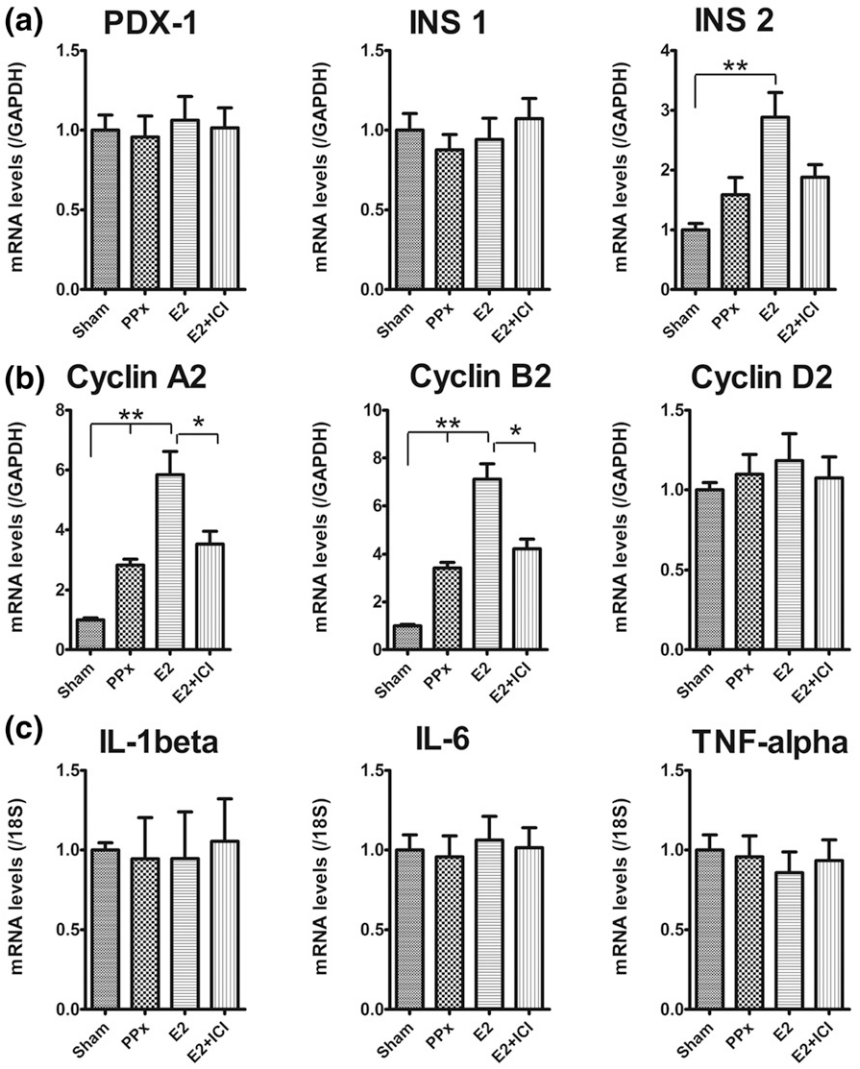

Figure 8. Effect of E2 treatment on mRNA expression in the islets. (a–c) The isolated islets were analyzed for the mRNA expression of PDX-1, INS1, INS2, cyclin A2, cyclin B2, cyclin D2, IL-1β, IL-6, and TNF-α by quantitative real-time PCR. The gene expression was quantified and normalized to the expression of GAPDH or 18S. Each data point was the mean ± SEM of three animals. *$P < 0.05$, **$P < 0.01$.

that ERα was expressed in the nucleus of islet cells. After the PPx operation, the protein expression of ERα was upregulated in the isolated islets. When exogenous E2 was implanted into the PPx mice, we found that the ERα expression was downregulated in the E2 group mice. These findings suggested that a high concentration of exogenous E2 may partially inhibit the expression of ERα.

Previous studies have demonstrated that β cell replication or neogenesis remains at a low level in mice or surgical pancreatic frozen sections [43]. In contrast, many studies have argued that there is rarely replication of β cells in the adult human pancreas under normal physiological conditions [44]. At present, researchers summarize three main molecular mechanisms of pancreas regeneration: (1) self-replication derived from pre-existing β cells [45]; (2) exocrine or endocrine pancreas cell conversion [10, 28, 46], such as the differentiation of ductal epithelial cells, and the transformation of precursor cells or exocrine acinar cells; and (3) directional differentiation from stem cells or progenitor cells [47–49], which has been proposed as a desired mechanism of pancreas regeneration.

Our previous studies demonstrated that E2 regulates human islet-derived precursor cell proliferation and differentiation into the cells that secrete insulin in response to glucose [50].

Yuchi *et al.* [38] demonstrated that exogenous E2 through ERα can drive β cell replication and formation during embryonic development and in the severely injured adult mouse pancreas, and the inhibitor of endogenous E2 synthesis blunted the activation of the β cell cycle. In this study, we found that the islet size increased 2 weeks after PPx, and E2 treatment significantly increased the islet size in the remaining part compared with PPx model mice. In line with the previous report [38], our data suggested that E2 promoted islet cell proliferation by increasing islet size.

Islet regeneration is an interactive process of multiple genes and factors. Cell cycle factors are critical regulators in the control of cell proliferation [51]. Previous studies have shown that FOXM1 is required for the proliferation of β cells after a pancreatectomy or pregnancy [52, 53]. In this study, we found that the expression of FOXM1 increased in the islets after PPx, which was consistent with the findings of a previous study [32], and notably E2 treatment upregulated the levels of FOXM1, cyclin A2, cyclin B1, cyclin D2, and cyclin E2. Alternatively, to obtain functional β cells, multiple genes and transcription factors are associated within β cell differentiation and maturation. A previous study showed that the expression level of insulin genes increases in the islet decompensation period [41]. Ngn3, another important transcription factor, regulated islet formation and β cell maturation [10, 38, 48]. In this study, we found that INS2 expression increased in the islets of E2 group mice. Otherwise, the expression of the ngn3 gene was not detected in the PPx mouse model, which was consistent with a previous report [47].

In conclusion, inadequate β cell mass is an essential component of all forms of diabetes, and many investigators attempt to seek breakthroughs to cure diabetes mellitus by stimulating islet regeneration. Our study reveals a beneficial role of E2 in response to islet regeneration in PPx mice. Therefore, these findings suggest that E2 may be a beneficial candidate in the treatment of diabetic patients.

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Address all correspondence to: Zhenhua Ren, PhD, Department of Anatomy, School of Basic Medicine, Anhui Medical University, 81 Meishan Road, Hefei, Anhui 230022, China. E-mail: renzhenhua1975@163.com.

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