

## Original Article

# Maternal protein restriction induces alterations in Nkx2.2 expression in pancreatic $\beta$ cells of intrauterine growth restriction rats

Dan Zhang<sup>1</sup>, Xiaomei Liu<sup>2</sup>, Min Yang<sup>1</sup>, Shi Tang<sup>1</sup>, Ying Xin<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Shengjing Hospital, China Medical University, Shenyang, China; <sup>2</sup>Key Laboratory of Obstetrics and Gynecology of Higher Education of Liaoning Province, Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University, Shenyang, China

Received June 1, 2018; Accepted August 4, 2018; Epub December 15, 2018; Published December 30, 2018

**Abstract:** Intrauterine growth restriction (IUGR) is an independent contributor to the risk of developing metabolic syndrome, and type 2 diabetes mellitus, in adulthood. Nkx2 homeobox 2 (Nkx2.2) is involved in pancreatic  $\beta$  cell development, but its role in metabolic syndrome after IUGR is unclear. To characterize the mechanism of Nkx2.2 in IUGR, we examined Nkx2.2 expression in pancreatic  $\beta$  cells of an IUGR rat model. The IUGR rat model was established by supplying a low protein isocaloric diet throughout gestation. Metabolism index, plasma glucose and insulin concentrations were then determined. A series of pancreas development indices, including the number and size of islets and  $\beta$  cells, were determined at four developmental stages: N1 (at birth), postnatal week 3 (weaning stage), 12 weeks (early adult stage), and 40 weeks (old age). Nkx2.2 expression in their pancreatic  $\beta$  cells was analyzed by double immunofluorescence analysis, Western blotting, and quantitative polymerase chain reaction. The 40-week rats were studied by hyperinsulinemic-euglycemic clamp method. The IUGR rats exhibited some morphological and functional aberrations in the pancreas at birth and at postnatal weeks 12 and 40. They also exhibited hyperglycemia, hyperinsulinemia, and insulin resistance at postnatal weeks 40 as well as significantly decreased Nkx2.2 expression in pancreatic  $\beta$  cells at birth and postnatal weeks 40. Maternal protein restriction hindered Nkx2.2 expression in mature  $\beta$  cells of IUGR rats. This might contribute to the development of metabolic syndrome in later life.

**Keywords:** Maternal protein restriction, pancreatic  $\beta$  Cells, NK2 homeobox 2, intrauterine growth restriction, metabolic syndrome

## Introduction

Intrauterine growth restriction (IUGR), one of the most common embryo development disorders, is defined as the failure of a fetus to achieve its genetic potential for size [1]. This condition affects 3%-10% of pregnancies and is recognized as a major cause of fetal and neonatal morbidity and mortality [2]. In particular, there is an intimate connection between IUGR and metabolic syndrome, whereby IUGR newborns are strongly predisposed to developing metabolic syndrome in later life [3]. Metabolic syndrome is a group of diseases characterized by insulin resistance conditions, including obesity, hypertension, hyperlipidemia, coronary heart disease, type 2 diabetes mellitus, and abnormal glucose tolerance in later life [4]. The

incidence of metabolic syndrome is increasing worldwide, and the condition is now often diagnosed in younger patients [5]. However, its mechanism has not yet been elucidated. Therefore, studies in animal models provide important information. Maternal undernutrition, a primary cause of IUGR, is widely employed for inducing IUGR in rodents. Specifically, IUGR is produced when dams are fed a low protein isocaloric diet throughout gestation [6].

In IUGR rats, reduction in fetal weight is observed as early as embryonic day 14 [6]. An IUGR fetal pancreas shows decreased insulin secretion, small islet size, low islet density, low  $\beta$  cell number, and decreased pancreatic weight and insulin content [7, 8]. When IUGR rats are

maintained on the same low protein diet during sucking, weaning, and adulthood, fasting insulin levels remain low in the presence of normal blood glucose levels [9]. The IUGR rats exhibit impaired glucose tolerance at 10 weeks, along with a relatively low insulin response [7], and peripheral tissues exhibit obvious insulin resistance at 15 weeks [10]. The effect of maternal malnutrition on the pancreas seems to be persistent, and deficiencies occurring *in utero* or soon after birth will negatively affect pancreatic  $\beta$  cells, thus contributing to glucose intolerance and insulin resistance later in life [11].

The development and differentiation of pancreatic  $\beta$  cells involves sequential modifications in gene expression that is controlled by a cascade of transcription factors, including neurogenin 3 (Ngn3), NeuroD/Beta2, paired box 4 (Pax4), Nkx2 homeobox 2 (Nkx2.2), and Nkx6 homeobox 1 (Nkx6.1) [12]. Nkx2.2 is a member of the mammalian NK2 homeobox transcription factor family, which is most homologous to the *Drosophila* NK2/ventral nervous system defective gene. *Nkx2.2* was originally identified as a gene expressed in the ventral regions of the developing vertebrate central nervous system. Patients with homozygous *NKX2.2* nonsense or frameshift mutations exhibit severe defects in insulin secretion and present with diabetes at an early age, without any features of pancreatic exocrine dysfunction [13]. *Nkx2.2*-null mice developed severe hyperglycemia and died shortly after birth [14]. Immunohistochemical findings revealed that the mutant embryos lacked insulin-producing  $\beta$  cells and had relatively few glucagon-producing  $\alpha$  and pancreatic polypeptide (PP) cells [14]. Remarkably, in *Nkx2.2* mutant mice, there remains a large population of islet cells that do not produce any of the four endocrine hormones. These cells express some  $\beta$ -cell markers, such as islet amyloid polypeptide and Pdx1, but lack other definitive  $\beta$ -cell markers [14].

*Nkx2.2* expression in the mouse embryonic pancreatic epithelium begins on embryonic day 9.5, when the dorsal pancreatic bud evagination begins to form [12]. Between embryonic days 13.5 and 18.5, which is the peak period of  $\beta$  cell neogenesis, *Nkx2.2* is expressed in a subset of incompletely differentiated endocrine precursor cells that co-express Ngn3 [12]. *In vitro* and *in vivo* data suggested that *Nkx2.2*

mediates these early cell-fate decisions by functioning both as a repressor and activator of transcription, depending on the context of development [15, 16]. In the mature pancreas, *Nkx2.2* expression is restricted to differentiated endocrine cells, including  $\alpha$ ,  $\beta$ , and PP cells, except  $\delta$  cells [14]. In mature islets, the function of *Nkx2.2* is to maintain normal islet morphology and optimize insulin gene expression and glucose-stimulated insulin secretion [12, 14]. Therefore, *Nkx2.2* is expressed during at least three distinct stages of islet cell differentiation involving the initial broad pancreatic precursor population, a subset of Ngn3-expressing islet progenitor cells, and in differentiated islet cells [17]. Therefore, *Nkx2.2* is thought to be required for the final differentiation of pancreatic  $\beta$  cells, and, in its absence,  $\beta$  cells are trapped in an incompletely differentiated state.

Most studies have focused on *Nkx2.2* expression in embryos [18]. However, studies on *Nkx2.2* expression in postnatal individuals are rare. *Nkx2.2* is thought to be involved in pancreatic  $\beta$  cell development and function in IUGR individuals and so, we hypothesized that *Nkx2.2* is likely to have a role in the development of metabolic syndrome after IUGR. However, no study on this subject has been reported. This study aimed to characterize the role of *Nkx2.2* in the development of pancreatic  $\beta$  cells of IUGR rats.

### Materials and methods

#### *Rats with IUGR*

All animal procedures were reviewed and approved by the Laboratory Animal Ethics Committee of China Medical University. 20 outbred Wistar rats (10-12 weeks of age; body weight, 230-260 g) were purchased from the Animal Laboratory, Experimental Research Center, Shengjing Hospital, China Medical University. Rats were housed under specific pathogen-free conditions in an environmentally controlled clean room at the Animal Laboratory.

Maternal protein restriction was used to develop IUGR in these rats, as previously described [19]. While ten animals were maintained on standard chow (20% protein) during gestation, ten undernourished animals received a low protein isocaloric diet (8% protein) until delivery. The feed was purchased from the Institute of

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Zoology, Chinese Academy of Sciences. The offspring of rats placed on a normal diet were assigned to the control group. Among rats birthed by mothers placed on the low protein diet, those with a newborn weight lower than 2 × standard deviation of the newborn weight of control rats were assigned to the IUGR group.

### *Sample collection*

Within 12 hours of birth, blood samples from both the control group and IUGR group rats were pooled to determine the levels of blood glucose and insulin in the two groups, and the pancreas was quickly dissected and weighed. The pancreas of one rat from each group was harvested for double immunofluorescence staining, while the other were used for isolation and purity analysis of pancreatic islets.

The remaining eight pups were fed by their own mothers until weaning at PW3 and then fed under normal conditions until PW12 and PW40. To exclude the influence of estrogen, male offspring (n = 6; one pup each from 6 rats) of different ages (PW3, PW12, and PW40) were selected as study subjects from the control and IUGR groups. Before sample collection, these rats were fasted for 12 hours from the preceding night until morning. They were then sacrificed under ether anesthesia, and blood samples were collected through cardiac puncture for detecting glucose and insulin levels. As in the case of newborn rats, the pancreas of one rat from each litter was harvested for double immunofluorescence staining, while the other were used for isolation and purity analysis of pancreatic islets.

### *Isolation and purity analysis of pancreatic islets*

Pancreatic islets of newborn rats (n = 10; 10 rats from 6 pups) from the two groups were isolated as described previously using collagenase P (Roche, Penzberg, Germany) [20]. The islets were rinsed, handpicked using the spearhead of a pipet, counted, and photographed. Under a dissecting microscope, the islets appeared round/oval and white/gray and were easily separated from the acini. This selection process for islets was repeated three times. The final suspension was centrifuged at 250 × g at 4°C for 1 minute, and the precipitate was stored at -80°C for further use.

Pancreatic islets of PW3, PW12, and PW40 rats (n = 6; 6 rats from 6 pups) from the two groups were isolated as described previously, briefly, the pancreas was distended by injecting 3 ml of collagenase (2 mg/mL; type XI; Sigma-Aldrich) through the common bile duct [20]. The islets were then handpicked under a dissecting microscope, counted, and photographed as described for newborn islets and stored at -80°C for further use.

Islets were identified by dithizone (DTZ; Sigma-Aldrich) staining. Islet suspension samples (1 mL) were incubated with 10 µL DTZ solution at 37°C for 10-15 minutes. The islets appeared scarlet under a stereoscopic microscope.

### *Hyperinsulinemic-euglycemic clamp*

Postnatal week 40 rats from the IUGR (n = 7) and control (n = 9) groups were anesthetized and cannulated through a midline incision on the ventral aspect of the neck as previously described [21]. For chronic cannulation, polyethylene catheters (PE-50; Cay Adams, Boston, MA, USA) were inserted into the left carotid artery and right jugular vein. Each catheter was extended with a segment of silastic tubing (length, 3 cm; internal diameter, 0.02 inches; Care Express Products, Inc., NY, USA), brought around the neck subcutaneously, and passed through a small skin incision at the base of the neck. After surgery, the rats were allowed 3-4 days of recovery, with at least 2 consecutive days of weight gain before clamp experiments.

Clamping maintained blood glucose concentrations at 5.3 mM during the 2 hour infusion period, while steady state insulin infusion at 10 mIU/kg/min was achieved by infusion of 20% glucose at a variable rate. Infusion procedures were performed as previously described [21]. Blood samples were collected before clamping and every 10 minutes during the last 30 minutes of each experiment. Plasma glucose concentrations were determined by the glucose oxygenase method using a glucose analyzer (BIOSEN 5030, Germany). Plasma insulin was analyzed using enzyme-linked immunosorbent assay kits (R & D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

Modified β-cell function index (MBCI) was determined using the following equation: MBCI =

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(fasting plasma insulin concentration  $\times$  fasting blood glucose concentration)/(blood glucose concentration 2 hours after glucose loading + blood glucose concentration 1 hour after glucose loading -  $2 \times$  fasting blood glucose concentration) [22]. Steady state blood glucose (SSBG), the mean blood glucose concentration at the steady state, indicated the stability and comparability of the clamp experiment. In this study, SSBG was determined as the mean value of thirteen glucose concentration measurements after achieving the steady state. The steady state glucose infusion rate (SSGIR), the mean glucose infusion rate within 60 minutes after achieving the steady state, was used to evaluate the insulin sensitivity of peripheral tissues and was determined using the following equation: SSGIR (mg/kg/min) = mean glucose infusion rate in steady state (mL/h)/60 (min)  $\times$  200/body weight (kg) [23].

### *Immunofluorescence staining*

Pancreas specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into 2- $\mu$ m thickness. Some tissue sections were evaluated by immunofluorescence staining with mouse anti-rat insulin monoclonal antibodies (1:50 dilution; Wuhan Boster Biological Technology, Wuhan, China) and biotinylated goat-anti-mouse immunoglobulin G (1:32 dilution; Wuhan Boster Biological Technology). For each section of the pancreas, consecutive images of adjacent non-overlapping areas of the entire section were acquired with a fluorescence microscope. Image analysis software (NIS-Elements BR 2.10, Nikon, Tokyo, Japan) was used to quantify insulin-positive areas as well as the area of the entire pancreatic section. For animals of each age group, 100  $\times$  magnification images of up to five sections (each containing at least five islets) of each pancreas were chosen for evaluation of immune-positive areas. In both groups, the pancreas of up to eight rats was examined from each age group.

Pancreatic  $\beta$ -cell fraction was calculated by dividing the insulin positive cell area by the total pancreatic tissue area in the entire section [24]. The pancreatic  $\beta$  cell mass was calculated by multiplying the  $\beta$  cell fraction with the weight of the pancreas [25]. For each specimen, ten fields were chosen within which the

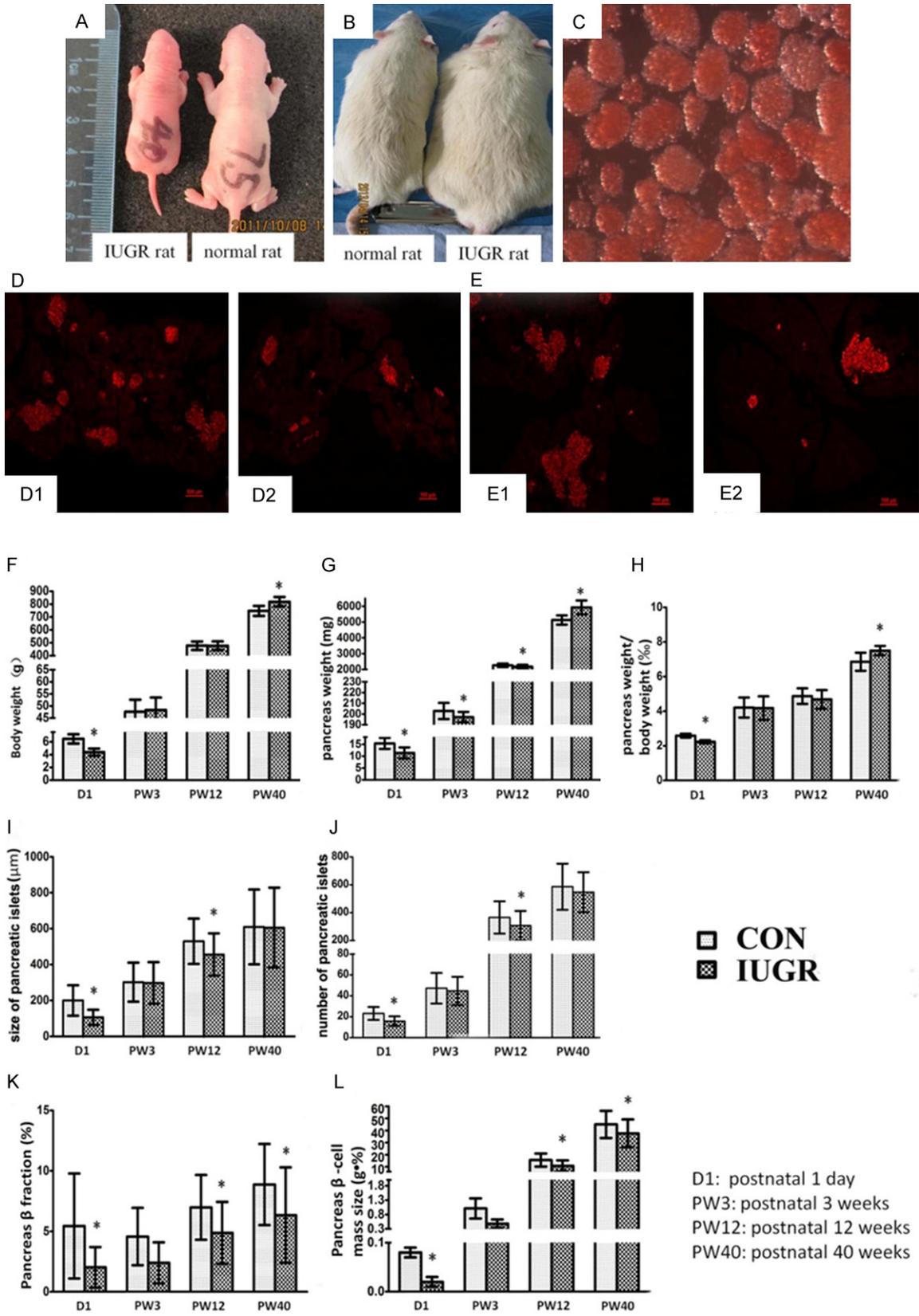
diameters of islets were measured [26]. The mean value of islet diameters was considered as the islet size [27].

Double immunofluorescence visualized Nkx2.2 expression. Primary and secondary antibodies were used in the following combinations: mouse anti-rat insulin monoclonal antibody (1:50 dilution; Wuhan Boster Biological Technology); rabbit anti-rat Nkx2.2 antibody (1:100 dilution; sc-25404, Santa Cruz Biotechnology, Santa Cruz, CA, USA); biotinylated goat anti-mouse immunoglobulin G (1:32 dilution; Wuhan Boster Biological Technology); and Alexa-488 donkey anti-rabbit immunoglobulin G (1:300 dilution; Life Technologies Corporation, USA). For negative controls, primary antibodies were substituted with PBS. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). Images were acquired using a confocal laser scanning microscope (CE1, Nikon, Tokyo, Japan). The image analysis software, NIS-Elements BR 2.10 (Nikon, Tokyo, Japan), was used to analyze the localization of insulin and Nkx2.2. For each slide, images acquired at 200  $\times$  and 400  $\times$  magnifications were chosen for evaluating the area of positive cells.

### *RNA isolation and real-time PCR*

Total RNA was isolated from pancreatic islet specimens of newborn, PW3, PW12, and PW40 rats using the TRIzol reagent (Invitrogen). The quantity and quality of total RNA were determined by spectrophotometry (Nanovue spectrophotometer; GE Healthcare, Buckinghamshire, England). Total RNA (2  $\mu$ g) was reverse-transcribed to cDNA using a reverse transcription system (TaKaRa, Dalian, China) and amplified using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green polymerase chain reaction (PCR) reagents (TaKaRa). Primers for Nkx2.2 and  $\beta$ -actin genes were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA). The primer sequences were as follows: Nkx2.2, forward: 5'-CAGCCTCATCCGTCTCAC-3'; reverse: 5'-GGCGTCACC TCCATACCT-3'; and  $\beta$ -actin, forward: 5'-ACTATTGGCAACGAGCG GTT-3'; reverse: 5'-TGTCAGCAATGCCTGGGTACA-3'. All samples were amplified in triplicate. The reaction

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**Figure 1.** The effect of maternal protein restriction on the pancreas development of IUGR rats. (A) Representative appearance of newborn rats: IUGR rat (left one, birth weight: 4.0 g); the control rat (right one, birth weight: 7.5 g).

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(B) Representative appearance of PW40 rats: IUGR rat (right one, body weight: 810 g); the control rat (left one, body weight: 710 g). (C) Pancreatic islets exhibited a scarlet round/oval appearance after staining with DTZ. (D, E) Immunofluorescence study for insulin expression (red) on paraffin sections of pancreas in control (D1, E1) and IUGR (D2, E2) rats at newborn (D1, 2) and PW40 (E1, 2) (original magnification x40). (F-L) A series of indexes relating to metabolic syndrome, including body weight (F), pancreas weight (G), pancreas weight/body weight ratios (H), size (I) and number (J) of pancreatic islets, and pancreatic  $\beta$ -cell fraction (K) and mass size (L) of control and IUGR rats at four important ages. (F-J) Normally distributed data were presented as the mean  $\pm$  standard error of the mean. (K, L) Nonparametric distributed data were presented as  $M \pm Q_{U-L}$ . CON, control; IUGR, intrauterine growth restriction. \* $P < 0.05$  vs control.

conditions were: 95°C for 10 seconds then 95°C for 10 seconds and 56°C for 34 seconds, for 40 cycles.  $\beta$ -actin was used as the reference gene for normalization, and the relative quantities of transcripts were calculated using the  $2^{-\Delta\Delta CT}$  formula [28].

### Western blotting

Immunoblotting was performed as previously described [19]. Briefly, pancreatic islets were homogenized in the RAPI buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], sodium orthovanadate, sodium fluoride, and ethylene diamine tetraacetic acid, pH 7.4) containing protease inhibitors and centrifuged at  $12,000 \times g$  at 4°C for 20 minutes. Protein content was assessed using a bicinchoninic acid assay kit (BCA Protein Assay Kit; Beyotime, Shanghai, China). After heating, equal quantities of protein were electrophoresed on 12% precast SDS-polyacrylamide gels and then electroblotted from the gels onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated overnight at 4°C with primary antibodies [rabbit anti-rat Nkx2.2 (1:100 dilution; Santa Cruz Biotechnology)] or rabbit anti- $\beta$ -actin (1:5000 dilution; Abcam, Cambridge, UK). They were then incubated at room temperature for 2 hours with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling, Beverly, MA, USA). Immune complexes were visualized using enhanced chemiluminescence reagents (GE Healthcare) and quantified using the Quantity One software (Bio-Rad, Hercules, CA).

### Statistical analysis

Normally distributed data are presented as the mean  $\pm$  standard error of the mean, while non-parametric distributed data are presented as  $M \pm Q_{U-L}$ . The statistical significance was calculated using the Student's *t*-test by SPSS 17.0 (Chicago, IL). Values of  $P < 0.05$  were consid-

ered statistically significant. The data were presented as bar graphs using GraphPad software (GraphPad, San Diego, CA, USA).

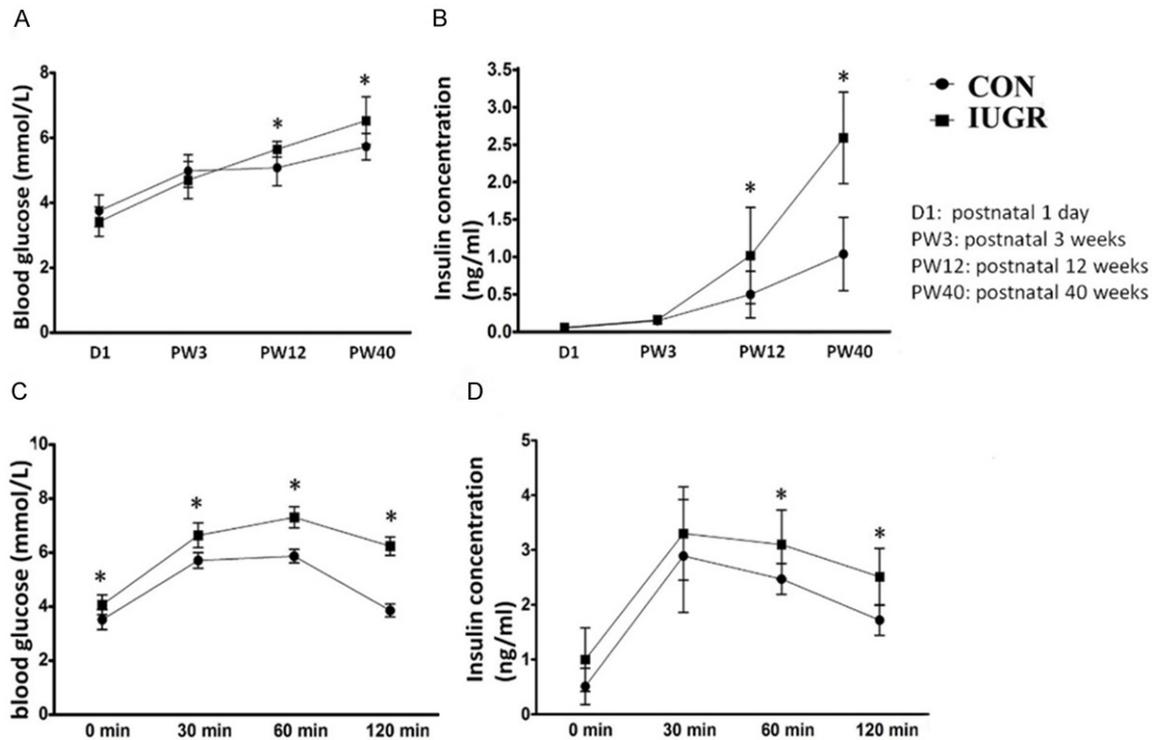
## Results

### *The effect of maternal protein restriction on pancreatic development in IUGR rats*

In this study, pregnant rats of both the low protein and normal diet groups experienced a mean gestation period of 21.5 days. Pregnant rats of the normal diet group birthed 121 pups, while those of the low protein diet group birthed 105. Therefore, there was no significant difference in litter size between the two groups. The mean newborn body weight of the control rats was  $6.45 \pm 0.72$  g. Among rats birthed by mothers placed on the low protein diet, those with newborn weight  $< 5$  g were considered IUGR rats. The mean weight of the IUGR group was  $4.39 \pm 0.56$  g. The incidence of IUGR in the maternal protein restriction group was 63.8% (67/105).

Relative to control rats, IUGR rats exhibited some differences in body, pancreas weight, and pancreas weight/body weight ratio at different developmental points because of maternal protein restriction (**Figure 1F, 1G**). The newborn body weight in the IUGR group was significantly lower than that in the control group ( $P < 0.05$ ) (**Figure 1A, 1F**). Most rats in the IUGR group experienced catch-up growth, so there was no significant difference in body weight between the control and IUGR groups at PW3 and PW12. By PW40, the body weight of IUGR rats exceeded that of the control rats ( $P < 0.05$ ) (**Figure 1B**). At birth, PW3, and PW12, the IUGR rats exhibited noticeably lower pancreas weight than the control rats ( $P < 0.05$ ). At PW40, the pancreas weight of IUGR rats was greater than that of the control rats ( $P < 0.05$ ) (**Figure 1G**). Relative to the control group, the pancreas weight/body weight ratios of the IUGR group were lower at birth ( $P < 0.05$ ) and higher at PW40 ( $P < 0.05$ ) (**Figure 1H**).

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**Figure 2.** Effect of maternal protein restriction on the pancreas islet function of IUGR rats. (A, B) The changes in fasting glucose concentrations (A) and insulin concentrations (B) in the control and IUGR rats at different developmental points. (C, D) The changes of glucose concentration (C) and insulin level (D) of IUGR and control rats at PW40 by hyperinsulinemic-euglycemic clamp ( $n = 7$  per group). CON, control; IUGR, intrauterine growth restriction. Significant differences compared with controls of same age are indicated by \* $P < 0.05$ .

Pancreatic islets exhibited a scarlet round/oval appearance after staining with DTZ (**Figure 1C**). Intrauterine protein restriction had an influence on both islet size and number. At birth and PW12, the pancreatic islet size and number of IUGR rats were lower than those of control rats ( $P < 0.05$ ). However, there was no significant intergroup difference in pancreatic islet size or number at PW3 or PW40 (**Figure 1I, 1J**).

Pancreatic  $\beta$  cells with specific immunoreactivity to insulin appeared red upon immunofluorescence staining (**Figure 1D, 1E**). At birth, PW12, and PW40, the pancreatic  $\beta$ -cell fraction and  $\beta$ -cell mass from IUGR rats were lower than those from control rats ( $P < 0.05$ ) (**Figure 1K, 1L**).

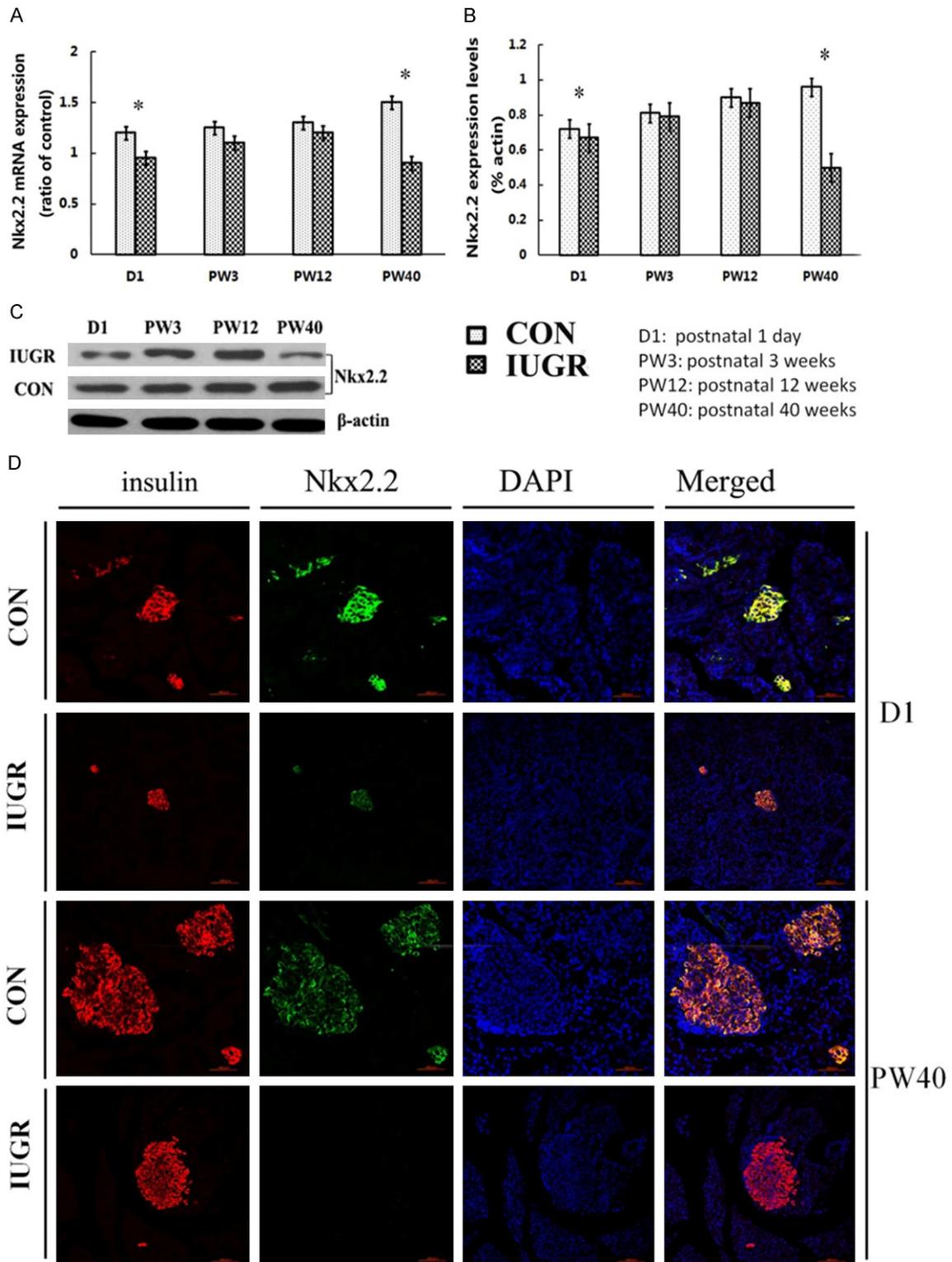
### Effect of maternal protein restriction on pancreatic islet function in IUGR rats

Maternal protein restriction resulted in some differences in blood glucose and insulin concentrations between the control and IUGR rats at different developmental points. At PW12 and

PW40, the fasting glucose and insulin concentrations of IUGR rats were greater than those of the control rats ( $P < 0.05$ ). However, there were no significant intergroup difference in this regard at birth or PW3 (**Figure 2A, 2B**).

The hyperinsulinemic-euglycemic clamp technique was used to confirm whether IUGR rats developed insulin resistance at PW40. At each developmental point, IUGR rats exhibited greater glucose concentrations than control rats ( $P < 0.05$ ). Additionally, insulin levels of IUGR rats at 60 and 120 minutes after glucose loading exceeded those of control rats ( $P < 0.05$ ) (**Figure 2C, 2D**). These results indicated that IUGR rats had impaired pancreatic  $\beta$  cell function at PW40. In comparison with control rats ( $22.20 \pm 4.01 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ), IUGR rats ( $9.96 \pm 2.56 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) exhibited lower SSGIR, which suggested that the latter had developed insulin resistance at PW40. There was no significant difference in SSBG between the IUGR ( $5.50 \pm 0.21 \text{ mmol/L}$ ) and control rats ( $5.46 \pm 0.19 \text{ mmol/L}$ ). These results also confirmed the stability and comparability of the clamp test.

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**Figure 3.** Effect of maternal protein restriction on Nkx2.2 expression in the pancreatic  $\beta$  cells in the control and IUGR rats. A. Nkx2.2 mRNA levels in both groups were detected by quantitative real-time PCR. Values are expressed relative to same-aged control. B. Densitometric analysis of Nkx2.2 protein in rat pancreatic islets at newborn, PW3, PW12, and PW40. Protein expression levels were normalized relative to the expression level of  $\beta$ -actin. C. Representative Western blotting analyses of Nkx2.2 in rat pancreatic islets of the two groups. D. Representative sections showed Nkx2.2 (green) expression in the insulin-positive cells (red) by double immunofluorescence staining in the

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control and IUGR rats at birth and PW40 (original magnification x200). Data are presented as means  $\pm$  SE for 6 observations per group. Significant differences compared with controls of same age are indicated by \* $P < 0.05$ .

### *The effect of maternal protein restriction on Nkx2.2 expression in pancreatic $\beta$ cells*

Real-time PCR showed Nkx2.2 mRNA expression levels showed that maternal protein restriction caused a significant downregulation of Nkx2.2 mRNA levels in pancreatic islets of IUGR neonatal rats ( $P < 0.05$ ). The mRNA levels were restored at PW3 and PW12 but reduced to almost zero at PW40 (**Figure 3A**).

Nkx2.2 expression was also evaluated by Western blotting (**Figure 3C**). Nkx2.2 levels in the pancreatic islets of control rats increased with age. At birth, Nkx2.2 levels in the pancreatic islets of IUGR rats were significantly lower than those of control rats ( $P < 0.05$ ). At PW3 and PW12, although the pancreatic islet Nkx2.2 levels of IUGR rats were still lower than those of control rats, the difference was not significant. At PW40, Nkx2.2 was not detected in pancreatic islets of IUGR rats ( $P < 0.05$ ) (**Figure 3B**).

Double immunofluorescence staining revealed Nkx2.2 expression in the cytoplasm of pancreatic  $\beta$  cells in control rats of different ages. In IUGR rats, Nkx2.2 was expressed in pancreatic  $\beta$  cells at birth, PW3, and PW12, with expression patterns similar to those in control rats of corresponding ages. At PW40, Nkx2.2-positive pancreatic  $\beta$  cells were observed in the control group but not in the IUGR group (**Figure 3D**).

### **Discussion**

There is increasing experimental and epidemiological evidence that IUGR increases the risk of developing metabolic syndrome in later life [2-4, 29, 30], with most studies suggesting the reason to be a combination of increased risks of insulin resistance and impairment of  $\beta$  cell development and function [7, 10]. The aim of this study was to investigate the role of Nkx2.2 in development of pancreatic  $\beta$  cells of IUGR rats, to see if this might be important for the development of metabolic syndrome after IUGR. The results show that as expected IUGR rats develop impaired pancreatic  $\beta$  cell development and progressive  $\beta$  cell dysfunction. Alongside this there was decreased Nkx2.2 protein expression levels in the pancreatic islets of IUGR rats compared to control rats at

birth and week 40, with similar results were seen at the mRNA level. This suggests that low expression levels of Nkx2.2 in IUGR may have a role in the development of metabolic syndrome in later life and this relationship deserves further study.

It is now becoming better understood how nutrition during early life has implications for the development of metabolic disease later in life. For example, a previous study has shown that tumor necrosis factor- $\alpha$ /c-jun N-terminal kinase signaling and cholesterol 7 $\alpha$ -hydroxylase expression in the livers of IUGR rats may contribute to hypercholesterolemia later in life [19], while alterations in mitochondrial fatty acid  $\beta$ -oxidation might explain the progressive impairment in energy homeostasis and insulin sensitivity [31]. There is a suggestion that alterations in adipocyte cell size impairs several insulin-signaling proteins' expression levels through post-transcriptional mechanisms [32]. Nkx2.2 has been identified as a crucial regulator for pancreatic endocrine, cell specification and differentiation in developing rat embryos. In postnatal animals, Nkx2.2 expression is restricted to mature islet cells [15]. Nkx2.2 performs essential roles in islet-cell development and functions as a cofactor for targeting genes. Recent studies have reported that Nkx2.2 expression is initiated by a cascade of transcription factors (including Ngn3, Pdx1, Beta2, and Pax4) and maintained by a network of interdependent signals. Only a few transcriptional targets of Nkx2.2 have been identified to date, including Ins2, NeuroD1, and MafA [15, 33]. Nkx2.2 consensus sites are also present in several regions on the Nkx2.2 promoter, and it has been speculated that Nkx2.2 can control its own transcription through a feedback loop [17]. In the present study, Nkx2.2 was expressed in pancreatic  $\beta$  cells of control rats from the neonatal stage to old age, and the expression levels increased with age. However, in IUGR rats, Nkx2.2 expression in pancreatic  $\beta$  cells was relatively low at birth, indicating that maternal protein restriction might have hindered Nkx2.2 expression in fetal pancreatic  $\beta$  cells. In addition, Nkx2.2 expression was significantly reduced in pancreatic  $\beta$  cells of IUGR rats at PW40. These results demonstrate that

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the effect of maternal protein restriction on Nkx2.2 expression in pancreatic  $\beta$  cells persists until old age. In a previous study, Nkx2.2 expression in islets was shown to have distinctly decreased in adult monkeys that developed obesity and insulin resistance after 24 months of feeding on a high fat/sugar diet [34].

This study shows similar results to other studies regarding the development of metabolic syndrome. Our findings reveal some aberrations in IUGR rats at four different developmental ages, which indicated that maternal protein restriction resulted in impaired pancreatic  $\beta$  cell development and progressive  $\beta$  cell dysfunction. The aberrations observed in the fetal pancreases of IUGR rats in the present study were consistent with those reported in other studies [7, 10]. In previous studies, the replication rate of pancreatic  $\beta$  cells was shown to have decreased because of lengthening of the cell cycle and reprogramming of cell kinetics, and the apoptosis rate was shown to have increased in pancreatic  $\beta$  cells of fetal IUGR rats [35]. This reduced the replication rate of pancreatic  $\beta$  cells and increased their apoptosis rate and could potentially have contributed to the relatively low pancreatic  $\beta$ -cell mass of IUGR rats in the present study. In a previous study, these abnormalities were still apparent when IUGR fetal rats were withdrawn from an intrauterine environment and allowed to grow for 7 days [36]. In the present study, the number and size of both islets and  $\beta$  cells at PW12 were lower in IUGR rats than in control rats. Moreover, at PW12, IUGR rats exhibited hyperglycemia and hyperinsulinemia. Yuan et al. observed a markedly diminished glycaemic response to insulin load in 15-week-old IUGR rats, relative to a control group [10]. It was suggested that IUGR rats showed a progressive loss of  $\beta$ -cell function with age.

Our results show that IUGR rats experienced catch-up growth, especially after PW12. Postnatal catch-up growth was also reported in children with low birth weight in a prospective cohort study by Ong et al. [37]. A survey in Finland showed that, besides low birth weight, catch-up growth between birth and 7 years of age was also an important risk factor for development of insulin resistance and type 2 diabetes [38]. Catch-up growth has been suggested

to be one of the causes of insulin resistance and type 2 diabetes in IUGR individuals [38].

In the present study, the impact of intrauterine protein restriction on the pancreas of older rats was explored. At PW40, IUGR rats were fat, with remarkably high adipose tissue deposits in the abdomen, especially around the kidneys. The body weight of these rats significantly exceeded those of control rats. Additionally, at PW40, the pancreas weight and pancreas weight/body weight ratio of IUGR rats were both greater than those of control rats. However, there was no significant difference between the two groups in islet number or size. These intergroup differences might be attributable to reprogramming of pancreatic structures in IUGR rats. At PW40, the size of pancreatic structures (except islets) such as the acinus and duct increased in IUGR rats, while the pancreatic  $\beta$  cell number and size decreased. This might have resulted from reprogramming of cell types in the islets, such as increased  $\alpha$  cell number. In a previous study involving monkeys, the number of  $\alpha$  cells increased significantly with a high fat/sugar diet, at the expense of  $\beta$  cells [34]. Moreover, in the present study, IUGR rats developed hyperglycemia and hyperinsulinemia at PW40. Additionally, the results of the hyperinsulinemic-euglycemic clamp test showed that PW40 IUGR rats developed insulin resistance, which is central to the metabolic syndrome. At PW40, IUGR rats were unable to adaptively enhance  $\beta$  cell production in response to increasing metabolic demand and consequent insulin resistance. These findings in PW40 IUGR rats provided strong evidence that impairment of pancreatic development and pancreatic dysfunction persisted from birth to old age, even in the presence of metabolic syndrome. These results support the well-known “thrifty phenotype hypothesis”, which states that changes in intrauterine nutritional environment cause alterations in islet  $\beta$  cell development and function, which have lifelong effects and predispose rats to metabolic syndrome in later life.

This study showed that the expression pattern of Nkx2.2 in IUGR rats was impaired from neonatal to old age, suggesting that maternal protein restriction hindered Nkx2.2 expression in mature  $\beta$  cells. Changes in Nkx2.2 expression induced by intrauterine protein restriction

might contribute to the development of adult metabolic syndrome.

### Acknowledgements

The study was supported by the National Natural Science Foundation of China (No. 81571449).

### Disclosure of conflict of interest

None.

**Address correspondence to:** Ying Xin, Department of Pediatrics, Shengjing Hospital, China Medical University, Shenyang 110004, China. Tel: +86-18940251876; Fax: +86-24-96615-52311; E-mail: xin.ying168@live.cn

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