Cell Stress and Chaperones (2012) 17:495–505
DOI 10.1007/s12192-012-0326-6

ORIGINAL PAPER

Impairment of cellular immunity is associated with overexpression of heat shock protein 70 in neonatal pigs with intrauterine growth retardation

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Received: 4 November 2011 / Revised: 10 January 2012 / Accepted: 11 January 2012 / Published online: 24 January 2012

Abstract Neonates with intrauterine growth retardation (IUGR) are susceptible to decreases in cellular immunity. In recent years, a growing body of evidence indicates that Hsp70 may serve as a danger signal to the innate immune system and promote receptor-mediated apoptosis. Using neonatal pigs with IUGR, we investigated immune function of pigs and expression of heat shock protein 70 (Hsp70), nuclear factor-kappa B (NF-κB), and forkhead box O 3a (FoxO3a) in the intestinal tract. Samples from the blood, duodenum, jejunum, and ileum of normal body weight (NBW) piglets and IUGR piglets were collected at day 7 after birth. Furthermore, to test whether Hsp70 is associated with regulation of NF-κB and FoxO3a, *Hsp70* was silenced using small RNA interference (siRNA) in IEC-6 cells. Body and intestinal weights were lower in IUGR piglets than in NBW piglets ($p<0.05$). Proliferation of peripheral blood lymphocytes was decreased ($p<0.05$) in IUGR piglets. Cytokine concentrations (IFN-γ, IL-4, IL-10, IL-1, and IL-8) were lower in serum of IUGR piglets. The levels of IFN-γ and IL-10 were decreased ($p<0.05$) in the ileum of IUGR piglets, but IL-4 was increased ($p<0.05$). The expressions of Hsp70 and FoxO3a were increased, and NF-κB activity was downregulated in IUGR piglets ($p<0.05$). Furthermore, siRNA-mediated Hsp70 downregulation increased NF-κB activity, inhibited expression of FoxO3a, and decreased cell apoptosis. In contrast, overexpression of Hsp70 inhibited NF-κB activation. In conclusion, IUGR impairs immune functions in neonatal pigs. An inefficient immunity in IUGR piglets is associated with overexpression of Hsp70, which impairs NF-κB signaling and upregulates FoxO3a expression.

Keywords Heat shock protein 70 · IUGR · Immune function · Piglet

Abbreviations

Hsp70	Heat shock protein 70
Hsp	Heat shock proteins
IUGR	Intrauterine growth retardation
NBW	Normal body weight
NF-κB	Nuclear factor-kappa B
FoxO3a	Forkhead box O 3a
IKK	IκB kinase
siRNA	Small RNA interfence
LPS	Lipopolysaccharide
ConA	Concanavalin A

Introduction

Intrauterine growth retardation (IUGR) is defined as impaired growth and development of the mammalian embryo/fetus or its organs during gestation (Wu et al. 2006). With 5–10% of human infants affected, it is considered a major problem in human medicine and animal production (McMillen and Robinson 2005). IUGR places the newborn infant at considerable risk for increased mortality and morbidity; the latter includes poor physical growth, permanent

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maldevelopment, and increased susceptibility to infection (Pallotto and Kilbride 2006). IUGR also affects development and function of the immune system in the newborn infant (Cromi et al. 2009; Briana et al. 2010). Changes in thymus size and histopathology have been observed both in IUGR infants and in animal models of IUGR (Lang et al. 2000; Cromi et al. 2009). In IUGR, there are low levels of IgG, low numbers of B cells, and low percentage as well as numbers of T cells in the cord blood or thymus (Manerikar et al. 1976; Contreras et al. 2011). Furthermore, IUGR leads to altered cytokine profiles in the placenta and fetus (Hahn-Zoric et al. 2002; Amu et al. 2006). Although several studies have examined immune function in IUGR infants or animal models of IUGR, most of these were conducted on the placenta, fetus, umbilical cord blood, or infant plasma. The intestine is an important location for digestion and absorption of nutrients, and the gut-associated lymphoid tissue is the largest immune organ of the body. Moreover, the intestinal barrier is involved in the first few steps of postnatal immune system maturation (D'Inca et al. 2010), and lymphocytes first appear in the intestinal subepithelium early in development (Veereman-Wauters 1996). Notwithstanding the importance of the intestinal tract, neither the effects of IUGR on the intestinal immune system nor the molecular mechanisms underlying these effects in neonates have been elucidated.

Heat shock proteins (Hsp) are a family of stress-responsive proteins present in every organism from bacteria to man. According to their molecular size, Hsp are classified into four major families, namely, small Hsp, Hsp60, Hsp70, and Hsp90 (Watanabe et al. 2004). Within the Hsp family, Hsp70—a molecular chaperone involved in the folding of nascent and misfolded protein under normal conditions—is one of the most abundant and best-characterized proteins. Many studies have shown that Hsp70 protects the cells from various stresses (Oyake et al. 2006). However, in recent years, a growing body of evidence suggests that extracellular Hsp70 may serve as a danger signal to the innate immune system and that it may contribute to the establishment of autoimmune diseases (Millar et al. 2003; Davies et al. 2006; Luo et al. 2008). It has previously been shown that Hsp70 regulates cytokine expression in blood mononuclear cells (Campisi et al. 2003; Dokladny et al. 2010). Indeed, recombinant human Hsp70 stimulates the production of pro-inflammatory cytokines such as TNF-α and IL-6 in human monocytes and macrophages (Asea et al. 2000). In contrast, overexpression of Hsp70 in animals is sufficient to inhibit LPS-induced increases in cytokine expression (Dokladny et al. 2010). Elevated Hsp70 also inhibits cellular proliferation and promotes TNF-mediated apoptosis by impairing NF-κB survival signaling in HeLa and 293 cells (Wei et al. 1995; Ran et al. 2004). Interestingly, we found that the expression of Hsp70 was significantly increased in the intestine of IUGR piglets at birth (Zhong et al. 2010). Therefore, we hypothesized that impairment of cellular immunity in IUGR neonates may be associated with expression of Hsp70. By using the newborn piglet, a widely used animal model for studying physiology and nutrition of the human infant, we investigated the effects of IUGR on the immune function of the intestine of neonatal pigs and the underlying molecular mechanisms.

Material and methods

Animals and treatment

Twenty piglets born from Large White × Landrace sow crosses were obtained from Jiangsu Kangle Agricultural and Pastoral Co., Ltd. (Jiangsu, China). Pregnant sows were fed daily with a 2-kg gestating diet during the entire period of pregnancy and had free access to drinking water. The diet met the NRC (1998) requirements for nutrients. At the term of birth (day 114 of gestation), ten pairs of normal body weight (NBW) piglets and IUGR piglets taken from ten litters were separated from their dams without suckling and were distributed equally into two groups: NBW group and IUGR group. A piglet was defined as IUGR when its birth weight was 2 SD below the mean birth weight of the total population, while a piglet was defined as NBW when its birth weight was within 1 SD of the mean birth weight of the total population (Wang et al. 2005). Newborn piglets were bottle-fed with equal volumes (220 ml/kg body weight of infant formula) for 7 day at 3-h intervals. Infant formula (12% wt/v) was freshly prepared using cow's milk powder (Fernleaf, New Zealand) and boiled water (37°C) (Table 1). Room temperature was maintained at 25°C with supplemental heat by radiant heaters suspended above the cages to maintain the surrounding temperature between 30°C and 32°C. The experimental protocol was in accordance with the guide for the "Care and Use of Laboratory Animals" prepared by the Institutional Animal Care and Use Committee, Nanjing Agricultural University, China.

Sample collection

At the end of the feeding trial, five NBW piglets and five IUGR piglets were randomly selected to be euthanized as previously described (Zhong et al. 2011). Plasma was obtained after centrifugation at 3,000× g for 15 min at 4°C and stored at −80°C. The duodenum, jejunum, and ileum were weighed, and their lengths were measured after careful removal of luminal contents and rinsing with saline. One sample of approximately 1 cm in length from each intestinal segment was stored for histological analyses. The sample was fixed in 4% paraformaldehyde in 100-mmol/l phosphate

Table 1 Composition of infant formula fed to piglets

Ingredient	Per 1 -L milk
Protein[a] (g)	16.50
Fat (g)	36.30
Linoleic Aacid (mg)	6,500.00
Ducosahexenoic acid (mg)	59.40
Arachidonic Acid (mg)	92.40
Carbohydrate[b] (g)	73.00
Minerals premix[c] (g)	2.54
Vitamins premix[d] (g)	2.32

Infant formula concentrations from manufactures

[a] The protein source is whey protein concentrate

[b] The carbohydrate source is lactose

[c] Mineral premix contained: 580-mg/l calcium, 480-mg/l phosphorus, 40-mg/l magnesium, 11-mg/l iron, 10-mg/l zinc, 60-μg/l manganese, 420-μg/l copper, 89-μg/l iodine, 19.1-μg/l selenium, 360-mg/l sodium, 640-mg/l potassium, and 420-mg/l chloride

[d] Vitamin premix contained: 730-μg/l retinol, 210-μg/l β-carotene, 11.2-μg/l cholecalciferol, 9.2-mg/l α-tocopherol, 92-μg/l bisulfate menadione complex, 730-g/l thiamin, 1,120-g/l riboflavin, 554-g/l vitamin B-6, 1.98-g/l vitamin B-12, 5,280-μg/l niacin, 86-μg/l folic acid, 376-μg/l pantothenic acid, 21-μg/l biotin, 80-mg/l ascorbic acid, 110-mg/l choline, and 33-mg/l inositol

buffer (pH 7.4) for 24 h. The mucosa of the rest of the intestine was scraped off with a glass slide and immediately placed in liquid nitrogen for protein analysis.

Lymphocyte proliferation

Lymphocyte proliferation was measured by using a colorimetric assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO, USA] in cultures of purified peripheral blood mononuclear cells (Wang et al. 2008). Briefly, peripheral blood mononuclear cells were isolated from heparinized blood by density gradient centrifugation at 3,000× g for 20 min at room temperature. The mononuclear cells were washed three times in RPMI-1640 culture medium and then suspended in RPMI-1640 culture medium containing 10% (v/v) heat-inactivated fetal calf serum, 100-U/ml penicillin, 100-mg/ml streptomycin, and 25-mmol/l HEPES buffer (Sigma, St. Louis, MO, USA). After a final wash, cell activity was detected by trypan blue dye exclusion, and cell concentrations were adjusted to 2×10^6 cells/ml culture medium. Then, 100 μl of cell suspension was added into 96-well microtiter plates in a total culture volume of 200 μl.

LPS (*Escherichia coli* 0111:B4; Sigma, St. Louis, MO, USA) at 15 μg/ml of final concentration was added per well for stimulating B-lymphocyte proliferation, and concanavalin A (ConA; Sigma, St. Louis, MO, USA) at 25 μg/ml per well was used to stimulate T-lymphocyte proliferation. Wells without mitogen were used as control. All tests were carried out in triplicate. The plates were incubated at 37°C in a 5% CO_2 incubator for 66 h. After incubation, 10 μl of MTT solution dissolved in PBS at 5 mg/ml was added to all wells at a final concentration of 5 μg/ml; after which, the plates were incubated at 37°C for another 6 h. Following incubation, 100 μl of a 10% SDS–HCl solution was added to lyse the cells and solubilize the MTT crystals. The plates were read at 570 nm on an automated microplate reader (Model 550; Bio-Rad, Hercules, California, USA). Lymphocyte proliferation was expressed as a stimulation index and calculated according to the following formula for results of triplicate assays: Stimulation index = (Absorbance value for mitogen-stimulated cultures)/(absorbance value for non-stimulated cultures).

Cytokine analysis by ELISA

Frozen intestine samples were homogenized in cold solution (200 mg of tissue per ml) as previously described (Zhong et al. 2010). The homogenate was centrifuged at 1,200× g for 15 min at 4°C. The supernatant fluid was collected, and total protein was determined by the BCA method. The concentrations of the cytokines (IFN-γ, IL-4, IL-10, IL-1, and IL-8) in the serum and intestine were determined using commercially available pig ELISA kit according to the manufacturer's instructions (RapidBio, Calabasas, CA, USA). The sensitivities of the IFN-γ, IL-4, IL-10, IL-1, and IL-8 assays were 30, 5, 10, 10, and 10 pg/ml, respectively. Samples were assayed in triplicate. Results were detected using a microplate ELISA reader at 450 nm and were expressed as picograms per milliliter in the serum and picograms per microgram protein in the intestine.

Cell culture

The IEC-6 cell was used as a substitute mammalian cell line since we were not able to obtain a porcine intestinal epithelial cell for the present study. The IEC-6 cell line was obtained from the American Type Culture Collection (ATCC) at passage 11 and was maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented with 5% heat-inactivated FBS (Gibco, Grand Island, NY, USA), 4-mg/l insulin, 100-IU/ml, and 100-IU/ml streptomycin at 37°C in 5% CO_2. Cells were divided into five groups with six replicate wells per group: control group, LPS group, LPS+heat group, LPS+siRNA group, and LPS+heat+siRNA group.

Hsp70 knockdown using siRNA

The *Hsp70* small interfering RNA (siRNA) and FAM negative control siRNA were purchased from Shanghai Genepharma RNAi Company (Shanghai, China). The siRNA

duplexes targeted to rat *Hsp70* gene sites were 5′-TTCTCCTTCCCTGTATAATCT-3′, and the sequences of the *Hsp70*-specific siRNA were 5′-CUCCUUCCCU GUAUAAUCUTT -3′ and 5′-AGAUUAUACAGGGAA GGAGTT-3′. Each siRNA was transfected into IEC-6 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h after transfection, cells were treated with LPS (*E. coli* 0111:B4, Sigma, St. Louis, MO, USA) at concentrations of 1,000 ng/ml for 24 h or subjected to 41°C heat shock in a water bath for 1 h and then recovery incubation at 37°C for 23 h. The treated cells were harvested and processed for western blotting and apoptosis analysis.

Protein immunoblot analysis

The total cellular protein, cytoplasmic, and nuclear proteins were isolated from IEC-6 cells and 200 mg of frozen intestine tissues using a total protein extraction kit and a cytoplasmic and nuclear protein extraction kit (Beyotime Biotechnology, Haimen, Zhejiang, China) for determination of Hsp70, IκB, NF-κB, and FoxO3a. The protein concentrations were determined using the BCA protein assay kit according to the protocol provided by the manufacturer (Beyotime Biotechnology, Haimen, Zhejiang, China). A total of 40 μg of protein and 5× sample buffer (0.25-mol/l Tris–HCl, pH 6.8, 0.5-mol/l β-mercaptoethanol, 10% SDS, 0.5% solution of bromophenol blue, and 50% glycerol) were combined. Subsequently, the samples were boiled for 5 min and were electrophoretically resolved by 10% SDS–PAGE (Hou et al. 2010). Protein was then transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk–PBS with 0.1% Tween 20 for 1 h at room temperature before overnight incubation at 4°C with mouse monoclonal anti-Hsp70 (#SPA-810, Stressgen, San Diego, CA, USA), rabbit polyclonal anti-p65 (Bioworld, Dublin, OH, USA), mouse polyclonal anti-IκBα (Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-FoxO3a (Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-β-actin antibody (Cell Signaling Technology, Beverly, MA, USA), or mouse monoclonal anti-α-tubulin antibody (Sigma, St. Louis, MO, USA) (1:1,000 dilution in 5% skim milk–PBS with 0.1% Tween 20). The membranes were then washed thrice in 1× PBS with 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody diluted 1:3,000 in 5% skim milk–PBS with 0.1% Tween 20 for 1 h at room temperature. Following successive washes, the membranes were developed using an ECL kit. The blots were exposed to X-ray film in a cassette equipped with a Dupont Lightning Plus intensifying screen. The films were scanned using a Microtek ScanMaker V scanner connected to a computer. Images were obtained for Adobe Photoshop and quantitated using the ImageJ software.

IκB kinase (IKK) assay

The IKK activity was assessed using an in vitro kinase assay as previously described (Yoo et al. 2000). Rabbit polyclonal anti-IKKα/β antibody (Bioworld, Dublin, OH, USA) was used to immunoprecipitate the IKK complex for determination of IKK activity. The kinase reaction products were subjected to SDS–PAGE in 10% gels, followed by transfer to a nitrocellulose membrane and autoradiography.

Apoptosis analysis

Surface exposure of phosphatidylserine in apoptotic cells was quantitatively detected using annexin V–FITC/propidium iodide (PI) apoptosis detection kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The treated cells in six-well culture plates were harvested and washed twice in PBS. Cells were resuspended in 1× annexin-binding buffer. The cell suspension was stained with Alexa Fluor 488-conjugated annexin V and PI. After incubation for 10 min at room temperature in the dark, the samples were immediately analyzed by FACS (Epics Altraii, Beckman, CA, USA).

Statistical analysis

The data on body weights, intestine weights, stimulation index, and cytokine concentrations were analyzed by *t*-test using SPSS (Version 16.0; SPSS). The expressions of Hsp70, NF-κB, and FoxO3a in intestine were analyzed as a mixed model with treatment, intestinal site, and treatment × intestinal site as fixed effects as well as piglet as random effect using the PROC MIXED procedure of SPSS and then were analyzed by *t*-test. In vitro data were analyzed by one-way ANOVA. The least significant

Table 2 Body and intestine weight of newborn piglets with IUGR and with NBW

	NBW	IUGR
Body weight at day 0	1.57±0.02	0.80±0.02
Body weight at day 7 (kg)	1.93±0.07	1.06±0.06*
Percent of body weight at day 7 (%)		
Duodenum	0.16±0.01	0.13±0.01
Proximal jejunum	1.03±0.03	0.85±0.05*
Distal jejunum	1.06±0.02	0.76±0.08*
Ileum	2.02±0.06	1.51±0.11*

Values are means±SEM, $n=5$ per treatment. Mean differences between values for IUGR piglets and NBW piglets are indicated by *$p<0.05$

Table 3 Stimulation index for peripheral blood lymphocytes in NBW piglets and IUGR piglets

Piglets	NBW	IUGR
ConA	1.142±0.049	1.023±0.044*
LPS	1.221±0.041	1.071±0.042*

Values are means±SEM, n=5 per treatment. Mean differences between values for IUGR piglets and NBW piglets are indicated by *p<0.05

difference (LSD) test was applied as a posthoc test. Differences were considered significant at p<0.05.

Results

Body weight and intestinal weight

Body weight of newborn piglets with IUGR was 48% lighter than NBW piglets at day 7 (p<0.05). The relative weight of the duodenum, jejunum, and ileum in the IUGR piglets was lower than those in the NBW piglets (p<0.05) (Table 2).

Proliferation of peripheral blood lymphocytes

Proliferation of peripheral blood lymphocytes induced by ConA and LPS was decreased in IUGR piglets as compared with NBW piglets (p<0.05) (Table 3).

Cytokine concentrations

The concentrations of IFN-γ, IL-4, IL-10, and IL-1 in the serum of IUGR piglets were lower than those in the serum of NBW piglets (p<0.05). Production of IL-8 was also decreased in the plasma of IUGR piglets (p=0.08) (Table 4). There was no difference in the concentrations of IFN-γ, IL-4, IL-10, and IL-1 in the jejunum between the NBW and IUGR piglets (Table 4). The levels of IFN-γ and IL-10 in the ileum of the IUGR piglets were lower than those in the ileum of the NBW piglets (p<0.05). However, concentrations of IL-4 were increased in the IUGR piglets (p<0.05). IL-8 was undetectable in the jejunum and ileum.

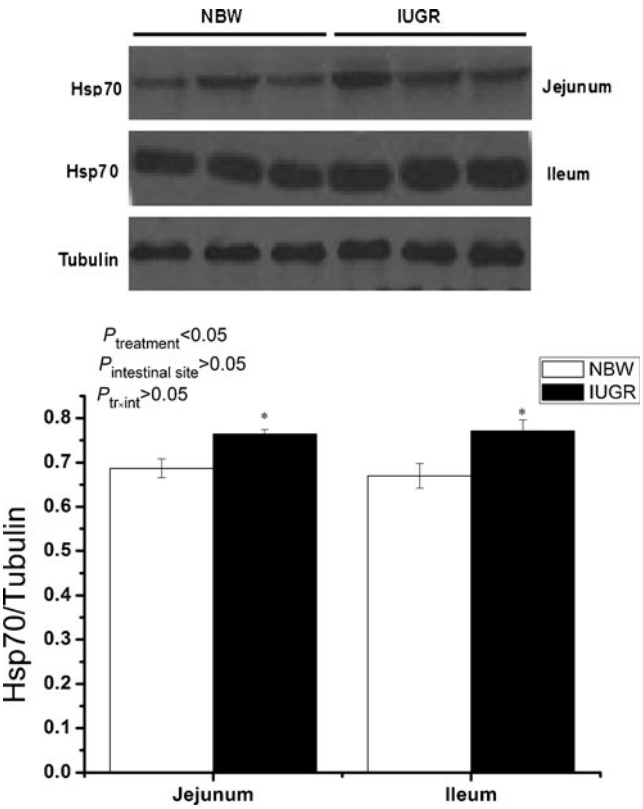

Fig. 1 Expression of Hsp70 in the jejunum and ileum of NBW piglets and IUGR piglets. Values are means±SEM, n=5 per treatment. Mean differences between values for IUGR piglets and NBW piglets are indicated by *P<0.05. $P_{treatment}$ P value for treatment, $P_{intestinal\ site}$ P value for intestinal site, $P_{tr\times int}$ P value for interaction between treatment and intestinal site

Expression of Hsp70, NF-κB, and FoxO3a in the intestine

The expressions of Hsp70, NF-κB, and FoxO3a were affected by treatment (p<0.05) (Figs. 1, 2, and 3). Intestinal site did not affect the expression of Hsp70 and NF-κB, but

Table 4 The cytokine concentrations in serum and intestine of NBW piglets and IUGR piglets

Item	Serum		Jejunum		Ileum	
	NBW pg/ml	IUGR	NBW pg/mg protein	IUGR	NBW pg/mg protein	IUGR
IFN-γ	185±9	130±11*	7.46±0.49	8.97±0.92	9.22±0.49	7.62±0.92*
IL-4	1491±42	968±23*	39.94±2.60	45.72±3.21	32.32±2.81	42.37±1.61*
IL-10	80.9±11.8	43.7±2.9*	4.63±0.45	3.91±0.98	4.52±0.27	3.71±0.15*
IL-1	98.07±3.07	79.84±3.05*	3.95±0.37	4.51±0.68	4.09±0.23	3.93±0.24
IL-8	95.53±5.77	88.53±2.33	not detected	not detected	not detected	not detected

Values are means±SEM, n=5 per treatment. Mean differences between values for IUGR piglets and NBW piglets are indicated by *p<0.05

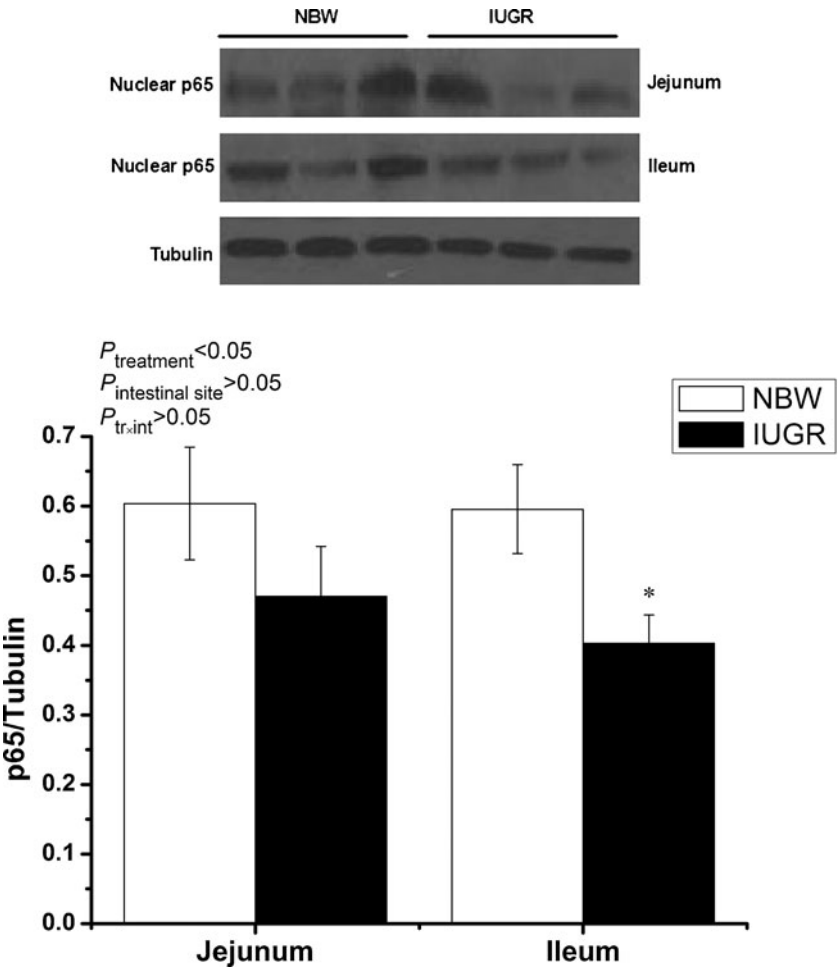

Fig. 2 Expression of the NF-κB p65 subunits in the jejunum and ileum of NBW piglets and IUGR piglets. Values are means ±SEM, $n=5$ per treatment. Mean differences between values for IUGR piglets and NBW piglets are indicated by $^*P<0.05$. $P_{treatment}$ P value for treatment, $P_{intestinal\ site}$ P value for intestinal site, $P_{tr\times int}$ P value for interaction between treatment and intestinal site

affected expression of FoxO3a. There was no interaction between treatment and intestinal site.

The expression of Hsp70 in the jejunum and ileum of IUGR piglets was higher than that in jejunum and ileum of NBW piglets ($p<0.05$) (Fig. 1). Compared with NBW piglets, the expression of the NF-κB p65 subunit was decreased in the jejunum ($p=0.055$) and ileum ($p<0.05$) of the IUGR piglets (Fig. 2). Furthermore, the expression of FoxO3a in the jejunum and ileum of IUGR piglets was increased as compared with the NBW piglets ($p<0.05$) (Fig. 3).

Expression of IKKα/β, IκB, NF-κB, and FoxO3a in IEC-6 cells

Compared with the control group, IKKα/β activity in IEC-6 cells was induced by stimulation of LPS ($p<0.05$) (Fig. 4a). IKKα/β activity in the LPS+heat group was inhibited by overexpression of Hsp70 ($p<0.05$). Interestingly, the expression of the latter was induced by heat shock (data not shown). On the other hand, IKKα/β activity in the LPS+siRNA group and LPS+heat+siRNA group (i.e., groups in which *Hsp70* expression had been silenced) was higher than that in the LPS+heat group ($p<0.05$). Compared with the control group, the expression of IκB was reduced by stimulation of LPS ($p<0.05$) (Fig. 4b). After heat shock, the levels of IκB were increased in the LPS+heat group ($p<0.05$). After siRNA-mediated *Hsp70* gene silencing, the concentrations of IκB in IEC-6 cells decreased ($p<0.05$). Activity of NF-κB in the LPS group was increased compared with the control group ($p<0.05$) (Fig. 4c). There was a tendency ($p=0.063$) toward the reduced expression of NF-κB in the heat+LPS group as compared with the LPS group. After *Hsp70* knockdown, the expression of NF-κB in IEC-6 cells was increased ($p<0.05$). In addition, the expression of FoxO3a in IEC-6 cells was induced by stimulation of LPS ($p<0.05$) (Fig. 4d). There was no difference in the expression of FoxO3a in IEC-6 cells between the LPS and the LPS+heat groups. After *Hsp70* knockdown, the expression of FoxO3a in IEC-6 cells was lower than that in all of the other groups ($p<0.05$).

Apoptotic cells

IEC-6 cells in the LPS group had a higher apoptosis rate (of up to 41.8%) compared with the control group ($p<0.05$) (Fig. 5).

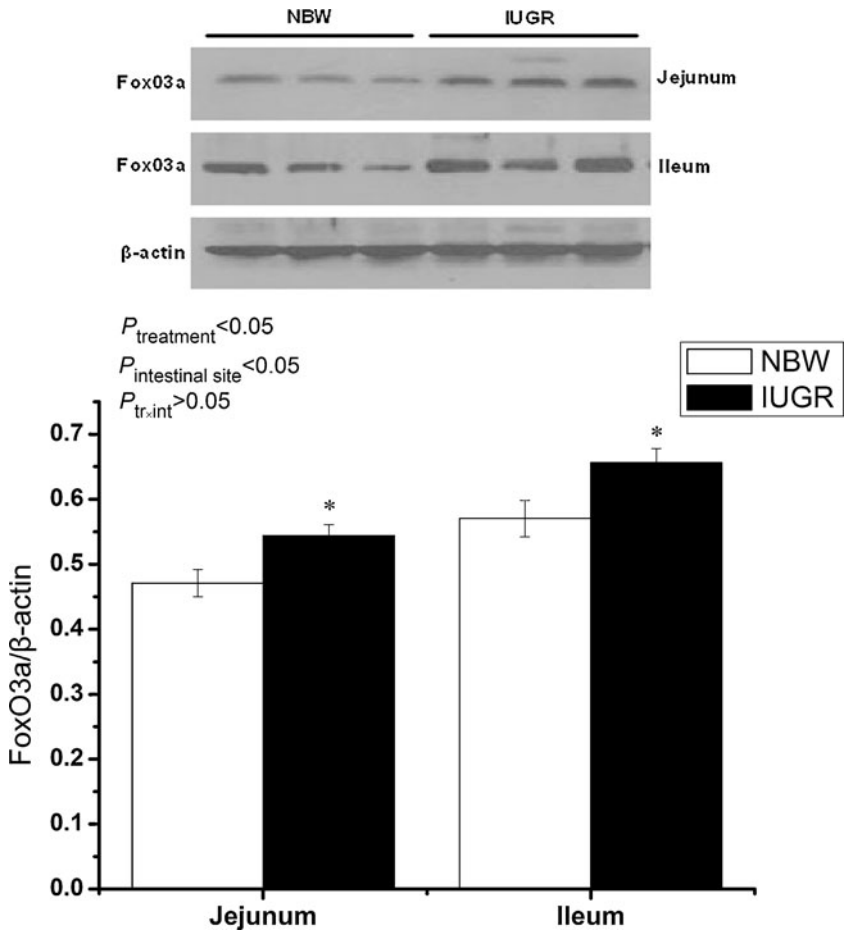

Fig. 3 Expression of FoxO3a in the jejunum and ileum of NBW piglets and IUGR piglets. Values are means±SEM, $n=5$ per treatment. Mean differences between values for IUGR piglets and NBW piglets are indicated by $^*P<0.05$. $P_{treatment}$ P value for treatment, $P_{intestinal\ site}$ P value for intestinal site, $P_{tr\times int}$ P value for interaction between treatment and intestinal site

After the heat shock, the apoptosis rate of IEC-6 cells in the LPS+heat group was lower than that in the LPS group, but it was still higher than that in the control group ($p<0.05$). The apoptosis rate of IEC-6 cells was also decreased upon *Hsp70* gene silencing as compared with the LPS and the LPS+heat group.

Discussion

IUGR has been linked to deficits in several aspects of adaptive immunity, to involution of lymphoid tissues such as the thymus (Amu et al. 2006; Cromi et al. 2009; Contreras et al. 2011), and to postnatal development of atopy and autoimmune disease (Godfrey et al. 1994). Here, we showed that IUGR inhibited the proliferation of peripheral blood lymphocytes, decreased the concentrations of cytokines, increased the expression of Hsp70 and FoxO3a, and downregulated the activity of NF-κB in the intestine of neonatal piglets ($n=5$). Furthermore, downregulation of Hsp70 using siRNA in rat IEC-6 cells increased translocation of NF-κB from the cytoplasm to the nucleus, inhibited FoxO3a expression, and decreased cell apoptosis in vitro. In contrast, overexpression of Hsp70 inhibited NF-κB activation.

Schuit et al. (1982) reported that T- and B-lymphocyte function is persistently impaired in infants with IUGR. At birth, T and B peripheral blood lymphocytes in infants and piglets with IUGR are indeed decreased (Schuit et al. 1982; Lin et al; 2009), and delayed cutaneous hypersensitivity to PHA is diminished (Ferguson 1978). In the present study, ConA- and LPS-stimulated proliferation of peripheral blood lymphocytes in IUGR piglets was significantly decreased compared with NBW piglets, indicating insufficient postnatal immunity in the former.

Cytokines mediate epithelial alterations associated with immune activation (Pie et al. 2004; Garrett et al. 2010). Changes in cytokine production occur under stress condition (Frossi et al. 2007), bacteria infection (Couper et al. 2008), or malnutrition (Chatraw et al. 2008). In the present study, the serum concentration of cytokines (IFN-γ, IL-1, IL-4, and IL-10, but not IL-8) was decreased in IUGR piglets. Amu et al. (2006) also found lower levels of IL-1 and IL-10 in the serum of mothers and infants with IUGR as compared with the non-IUGR infants. Expression of IL-4 in cord blood has also been found to be lower in IUGR infants than in controls (Omu et al. 2004). These results indicated that IUGR impaired cytokine expression in neonates. In addition, there was no difference in the concentrations of

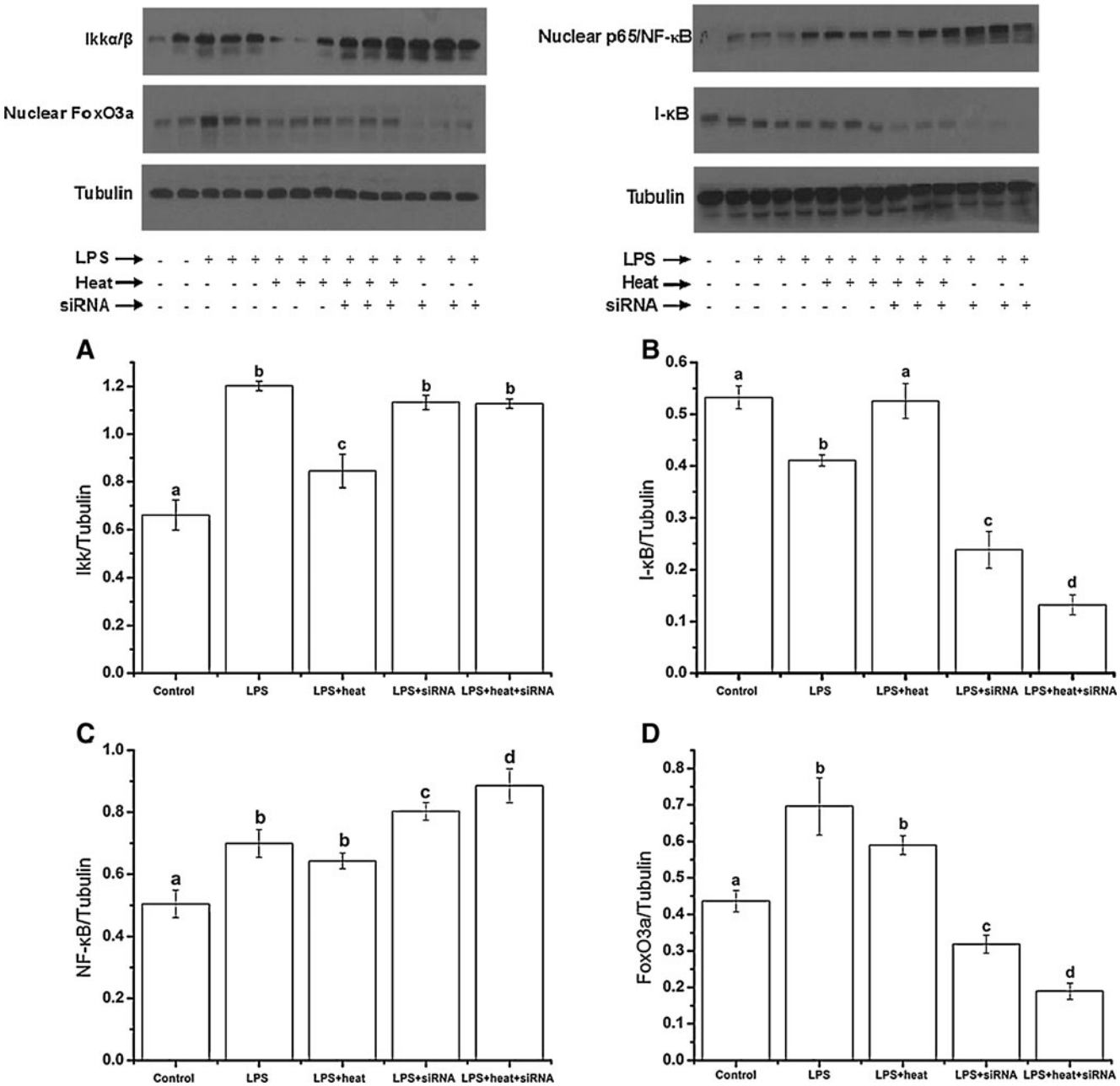

Fig. 4 Activity of IKK (**a**), concentrations of IκB (**b**), activity of NF-κB (**c**), and expression of FoxO3a (**d**) induced by LPS, heat shock, and siRNA-mediated *Hsp70* gene silencing in IEC-6 cells. Values are means±SEM, $n=6$ per treatment. Means without a common letter differ ($P<0.05$)

cytokine in the jejunum between NBW piglets and IUGR piglets. Nonetheless, the levels of IFN-γ and IL-10 in the ileum of IUGR piglets were significantly lower than that in the NBW piglets. The differences in cytokine expression between the jejunum and ileum imply that the expression of these cytokines is site specific. The site-specific effect might be linked to particular lymphoid structures or the number of lymphocytes in the small intestine (Pie et al. 2004) or linked to the microbiota since differences in intestinal bacteria may induce molecular changes (D'Inca et al. 2010). Furthermore, in contrast to other serum cytokines, the level of IL-4 was increased in the intestine of IUGR piglets compared with NBW piglets. IL-4 has multiple immunoregulatory functions (Boniface et al. 2008; Geissmann et al. 2010). In addition, IL-4 participates in intestinal integrity and regulates intestinal water and ion transport (Greenwood-Van Meerveld et al. 2000). Previous studies demonstrated that IL-4 increased mucosal permeability, decreased glucose absorption, and decreased chloride secretion in the murine intestine (Madden et al. 2002). Therefore, higher levels of IL-4 might adversely affect gastrointestinal function, including cell renewal and

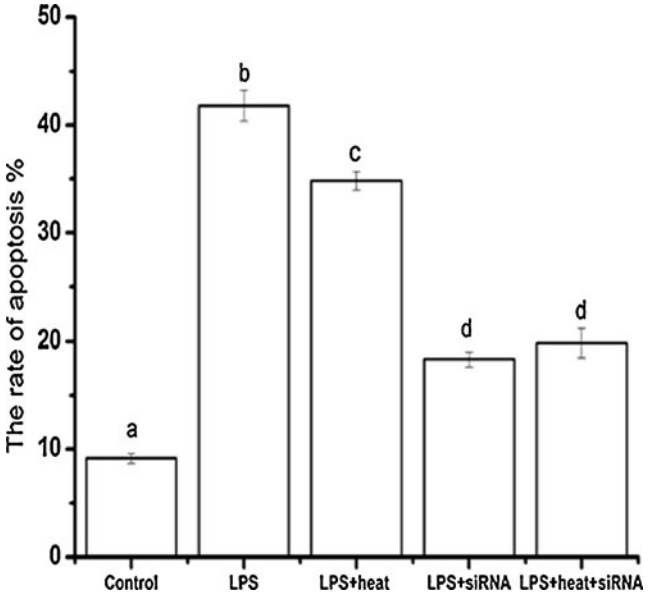

Fig. 5 Apoptosis induced by LPS, heat shock, and siRNA-mediated *Hsp70* gene silencing in IEC-6 cells. Values are means±SEM, $n=6$ per treatment. Means without a common letter differ ($P<0.05$)

morphological changes in the intestinal epithelium of IUGR piglets at day 7 after birth.

Under non-stressful conditions, Hsp70 is constitutively expressed to assist protein folding and degradation, transport across cellular membranes, and assembly into macromolecular structures (Mayer and Bukau 2005). Under stressful situations, expression of Hsp70 is upregulated. Previous studies have indicated that IUGR is associated with expression of Hsp in human placentas (Hnat et al. 2005; Liu et al. 2008). Liu et al. (2008) demonstrated that *Hsp70* mRNA and protein are increased in placental vascular disease induced by IUGR compared with normal pregnancies. In a previous study, we found that IUGR upregulates the expression of *Hsp70* mRNA and proteins in the intestine of neonatal piglets at birth (Zhong et al. 2010). In the present study, we demonstrated that the expression of Hsp70 was increased in the jejunum and ileum of IUGR piglets at day 7 after birth.

In addition to its chaperone functions in protein folding and assembly, Hsp70 is a potent activator of the innate immune system and of the activation and maturation of dendritic cells (Tsan and Gao 2004). However, in recent years, a growing body of evidence suggested that Hsp70 may serve as a danger signal to the innate immune system (Millar et al. 2003; Davies et al. 2006; Luo et al. 2008). It has been previously shown that induction of *Hsp70* gene transcription can suppress cytokine gene transcription. Indeed, overexpression of Hsp70 in rats is sufficient to inhibit LPS-induced increases in cytokine expression (Dokladny et al. 2010). Furthermore, even though Hsp70 is known to protect cells by interfering with the mitochondrial apoptotic pathway, there is growing evidence that overexpression of Hsp70 can also promote receptor-mediated apoptosis (Bulut et al. 2005). For instance, elevated Hsp70 inhibits cellular proliferation and promotes TNF-mediated apoptosis by impairing NF-κB survival signaling in HeLa and 293 cells (Wei et al. 1995; Ran et al. 2004). Elevated Hsp70 promotes TCR/CD3- and Fas/Apo-1/CD95-mediated Jurkat T-cell apoptosis (Senf et al. 2008). Interestingly, IUGR is associated with increased apoptosis in the intestines of neonatal pigs (D'Inca et al. 2010). Therefore, these results suggested that immune dysfunction might be associated with overexpression of Hsp70 in cells or animals.

NF-κB is an essential transcription factor that regulates transcription of many genes involved in immune function, inflammation, and control of cell proliferation, growth, and apoptosis (Dokladny et al. 2010). NF-κB normally exists as an inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 and p65 subunits, bound to inhibitory proteins of the IκB. Induction of NF-κB is mediated by increased activity of the IκB kinase (IKK) complex. The activation of the IKK complex causes the phosphorylation of IκB and then leads to the ubiquitination and degradation of IκB, the dissociation of the NF-κB/IκB complex, resulting in nuclear translocation of NF-κB, subsequently inducing a variety of genes encoding signaling proteins (Alexander et al. 2002). In the present study, nuclear NF-κB p65 subunits were decreased in the intestine of IUGR piglets. Baserga et al. (2010) also reported that IUGR significantly decreased NF-κB p65 binding to the 11β-hydroxysteroid dehydrogenase type 2 promoter, resulting in hypertension in the kidney of rats at day 0. Notably, upregulation of Hsp70 can impair NF-κB survival signaling (Ran et al. 2004; Dokladny et al. 2010). Dokladny et al. (2010) found that Hsp70 inhibits LPS-induced NF-κB p65 nuclear translocation and IκBα degradation in vivo. It was reported that the ability of Hsp70 to promote TNF-mediated apoptosis was attributed to binding with IKK, blocking phosphorylation of IKK, and impairing NF-κB survival signaling (Ran et al. 2004).

FoxO3a is an important modulator of cell apoptosis and immune function (Jonsson and Peng 2005). The unphosphorylated, transcriptionally active form of FoxO3a translocates to the nucleus and induces the transcription of genes encoding pro-apoptotic and anti-proliferative proteins such as FasL, Bim, and p130 (Barreyro et al. 2007). Since IUGR is associated with increased apoptosis in the intestine of neonatal pigs (D'Inca et al. 2010) and FoxO3a regulates apoptosis, it could be suggested that FoxO3a may play a role in intestinal atrophy and impairment of immune function in IUGR neonates. Furthermore, the interaction of Hsp70 with FoxO3a has been shown (Kim et al. 2005; Senf et al. 2008). Therefore, we speculated that the pro-apoptotic function of Hsp70 could be associated with FoxO3a. In the

present study, we found that the expression of FoxO3a in the jejunum and ileum of IUGR piglets was significantly increased. Interestingly, it has been verified that IKK induces ubiquitination and degradation of FoxO3a through the proteasome pathway (Hu et al. 2004). As described previously, overexpression of Hsp70 can block phosphorylation of IKK and impair NF-κB signaling. Thus, it seems to be plausible that Hsp70 protein may be involved in the regulation of FoxO3a.

Taken together, we speculated that Hsp70 overexpression inhibits IKK activities, subsequently impairing NF-κB activity and upregulating FoxO3a expression in the intestine of IUGR piglets. To test this hypothesis, the expression of Hsp70 gene was silenced using siRNA, and the overexpression of Hsp70 was induced by heat shock in epithelial cells, which play important roles in immune function and are a predominant part of the mucosal immune system. The present data indicated that downregulation of Hsp70 using siRNA can increase LPS-induced IKK activity, degradation of IκB, and translocation of NF-κB from the cytoplasm to the nucleus and inhibit expression of FoxO3a as well as decrease IEC-6 cell apoptosis. In contrast, overexpression of Hsp70 inhibited NF-κB activation in IEC-6 cells.

In conclusion, IUGR altered immune functions in neonatal pigs. An inefficient immunity in postnatal IUGR piglets is associated with overexpression of Hsp70, which impairs NF-κB survival signaling and upregulates FoxO3a expression. The present results set the stage for future investigation that can address interventions, such as dietary modifications, which could modulate the epigenetic response to the IUGR insult.

Acknowledgement This project was supported by a grant from the National Natural Science Foundation of China (no. 30972116), the Youth Sci-Tech Innovation Fund NJAU (no. KJ2011010), the Specialized Research Fund for the Doctoral Program of Higher Education of China (no. 20110097120033), and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. We would like to thank Yuanxiao Wang and Jianjun Wang for their assistance regarding sample collection.

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