

# Maternal protein restriction induces alterations in hepatic tumor necrosis factor- $\alpha$ /CYP7A1 signaling and disorders regulation of cholesterol metabolism in the adult rat offspring

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It is well recognized that adverse events *in utero* impair fetal development and lead to the development of obesity and metabolic syndrome in adulthood. To investigate the mechanisms linking impaired fetal growth to increased cholesterol, an important clinical risk factor characterizing the metabolic syndrome and cardiovascular disease, we examined the impact of maternal undernutrition on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )/c-jun *N*-terminal kinase (JNK) signaling pathway and the cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) expression in the livers of the offspring with a protein restriction model. The male offspring with intrauterine growth restriction (IUGR) caused by the isocaloric low-protein diet showed decreased liver weight at birth and augmented circulation and hepatic cholesterol levels at 40 weeks of age. Maternal undernutrition significantly upregulated cytokine TNF- $\alpha$  expression and JNK phosphorylation levels in the livers from fetal age to adulthood. Elevated JNK phosphorylation could be linked to down-regulated hepatocyte nuclear factor-4 $\alpha$  and CYP7A1 expression, subsequently led to higher hepatic cholesterol. This work demonstrated that intrauterine malnutrition-induced IUGR might result in intrinsic disorder in hepatic TNF- $\alpha$ /CYP7A1 signaling, and contribute to the development of hypercholesterolemia in later life.

**Key Words:** perinatal nutrition, TNF- $\alpha$ , JNK, CYP7A1, cholesterol

Intrauterine growth restriction (IUGR) affects 3–10% of pregnancies, and is recognized as the leading cause of intrauterine fetal death and the second leading cause of neonatal death.<sup>(1,2)</sup> Numerous epidemiological studies have demonstrated a strong association between low birth weight and subsequent development of metabolic syndrome, consisting of type 2 diabetes mellitus, obesity, dyslipidaemia, hypertension and coronary artery disease (CHD).<sup>(3–9)</sup> These findings led to the ‘fetal origins of adult disease hypothesis’, which is also described as the ‘developmental origins of health and disease hypothesis’.<sup>(10)</sup> Studies of animal models are consistent with the concept that adverse events *in utero* predispose the offspring toward the later development of metabolic syndrome.<sup>(11)</sup> All these data suggested that although the underlying causes of the metabolic syndrome in humans are complicated and controversial, maternal undernutrition may be a causal factor.

Underlying molecular mechanisms of metabolic programming remains elusive. Poor nutrition *in utero* may alter life-long gene expression in major organs and the character of the genotype–environment interaction in later life.<sup>(10)</sup> There have been ample evidences suggesting that hepatic tissue may play a major role in linking IUGR to the subsequent development of adult dis-

eases.<sup>(12,13)</sup> Various experimental animal models have expanded our knowledge of how poor intrauterine nutrition may influence liver development.<sup>(14,15)</sup> Among these, protein restriction in early life has been widely used as a rat model of metabolic programming to study the impact of prenatal malnutrition upon adult diseases. Maternal protein restriction exerts profound programming effects on major organs and tissues, such as liver, adipose and muscle. This effect is partly mediated through affecting the epigenetic modification of genes that regulate metabolism and transportation of glucose and lipid.<sup>(16–18)</sup> Our laboratory also developed an IUGR model in rats by maternal protein restriction, whereas the animals showed disturbed hepatic metabolism, in line with other studies.<sup>(19)</sup>

A large amount of evidence has recently suggested that birth weight is an independent risk factor for hypercholesterolemia, a proven risk factor for the CHD.<sup>(20,21)</sup> In the liver, cholesterol is converted to bile acids by a pathway consisting of abundant physiological reactions. Cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) is a liver-specific rate-limiting enzyme of this pathway. Previous studies have revealed programmed changes in the expression of CYP7A1 in IUGR rats caused by protein or iron restriction during pregnancy.<sup>(22,23)</sup> In hepatocytes, CYP7A1 expression could be regulated at the gene transcriptional level by bile acid and proinflammatory cytokines including tumor necrosis factor (TNF)  $\alpha$  and interleukin 1.<sup>(24,25)</sup> TNF- $\alpha$  has been shown to play a pivotal role in the development of coronary artery disease, insulin resistance, type 2 diabetes, and dyslipidemia.<sup>(26)</sup> Experimental study has shown that TNF- $\alpha$  downregulates the expression of hepatic CYP7A1 by activating the c-jun *N*-terminal kinase (JNK) signaling pathway.<sup>(25)</sup> In brief, TNF- $\alpha$  binds to the receptor and phosphorylates the mitogen-activated protein (MAP) kinases JNK in the hepatocytes. Activated JNK inhibits hepatocyte nuclear factor (HNF)4 $\alpha$  expression or phosphorylates HNF-4 $\alpha$  and reduces its DNA binding and transactivation activity, ultimately decreases the transcription rate of CYP7A1. CYP7A1 catalyzes the conversion of cholesterol to two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), both of which are further excreted to bile by bile salt export pump (BSEP).<sup>(27)</sup> Studies of human have demonstrated that higher TNF- $\alpha$  level existed in the cord blood and trophoblasts of IUGR placenta.<sup>(28,29)</sup> Furthermore, studies of rodents have also shown that TNF- $\alpha$  were overproduced in the livers of IUGR offspring.<sup>(30)</sup> Therefore early life programming of the expression of key molecules in the TNF- $\alpha$ /JNK

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signaling pathway may play a role in determining postnatal cholesterol metabolism.

Thus, evidence exists to support the working hypothesis that reprogramming of long-term modifications of the expression of key factors of TNF- $\alpha$ /JNK signaling pathway involved in cholesterol and bile acid metabolism is associated with significant hepatic cholesterol accumulation in the aging IUGR offspring. The aim of the present study was therefore to elucidate the development of hypercholesterolemia and related underlying patterns of gene expression.

## Materials and Methods

**Animals.** All experimental protocols were approved by the Animal Research Committee of China Medical University. Adult Wistar rats were housed under specific pathogen-free conditions in an environmentally-controlled clean room at the Experimental Animal Center (Shengjing Hospital, China Medical University). All animals were individually housed under standard conditions. Food and water were provided *ad libitum* throughout the study. The female rats were mated overnight. The presence of spermatozooids in the vaginal smear was verified the next morning to determine gestational day 0. The rats were then randomly divided into 2 groups: animals in the undernourished group received an isocaloric low-protein diet (8% protein) from day 0 of pregnancy until the birth of their pups as we described previously,<sup>(19)</sup> and control animals were maintained on standard diet (20% protein) during gestation. On the 20th day of gestation (E20), a subset of pups was delivered by caesarian section, the fetal blood was pooled (three or more) in a litter to quantify plasma total cholesterol (TC) and triglyceride (TG) levels, and the fetal livers were dissected out and frozen. IUGR referred to the pups whose birth weight was two standard deviations less than the mean value of the control group. Another subset of rats was allowed to deliver spontaneously. At birth, the litter size was reduced to eight-ten animals. The remaining pups were nourished by their own mothers until weaning at postnatal 3 weeks (PW3), and then fed under normal condition until 40 weeks (PW40). At PW3 and PW40, the pups were killed under ether anesthesia, and livers were dissected, snap frozen in liquid nitrogen, and then stored at  $-70^{\circ}\text{C}$  until use. To avoid any sex and hormonal confounding interference, all the following analyses were performed with male pups only.

**Histological examination of the liver.** The left lobe of the liver specimens were fixed in 4% paraformaldehyde for over 24 h before processing by use of automatic tissue processor (ZT-12M, Wuhan, China) for paraffin embedding. Hematoxylin and eosin staining were performed using standard techniques in 5- $\mu\text{m}$ -thick sections. All images were analyzed with the assistance of image software NISElements BR 2.10 (Nikon, Tokyo, Japan).

**Biochemical analysis.** Plasma TG and TC contents were measured using enzymatic reagent kits from Jiancheng (Nanjing, China) according to the manufacturer's instructions. Liver tissues (200 mg) from the central lobe were homogenized in 2 ml

chloroform-methanol (2:1 vol/vol). The extract was collected, dried, and dissolved in ethanol as described by Folch *et al.*<sup>(31)</sup> Extracted lipids were then examined by colorimetry assay using enzymatic reagent kits for TG and TC (Jiancheng, Nanjing, China), and the result was expressed as  $\mu\text{mol}$  lipid per gram wet weight liver tissue.

**Measurement of liver TNF- $\alpha$ .** One hundred milligrams of liver tissues were homogenized in 1 ml PBS, and protein concentration was determined with a spectrophotometer. Assay for TNF- $\alpha$  was performed by Enzyme Linked Immuno-Sorbent Assay (ELISA) in the liver homogenate in all the groups using a commercially available kit (Invitrogen, Camarillo, CA), following the manufacturer's instructions. TNF- $\alpha$  levels were expressed as picograms per milligram protein.

**RNA isolation and real-time PCR.** Total RNA from liver specimens was isolated at E20, PW3, and PW40 using the TRIzol reagent (Invitrogen), and quantified with the Nanovue instrument (GE Healthcare, Buckinghamshire, England). The RNA strand (2  $\mu\text{g}$ ) was reverse-transcribed into cDNA using the RNA PCR kit (TaKaRa, Dalian, China). The expression of the cDNAs of interest was measured by quantitative real-time PCR using the SYBR Premix Ex Taq kit (TaKaRa) and a Lightcycler detector (Roche, Basel, Switzerland), and was normalized with  $\beta$ -actin used for housekeeping. The results were expressed in arbitrary units relative to the mean value of the control group. The specific primer sets used are listed in Table 1.

**Immunoblot analysis.** The liver tissues were homogenized in the RAPI buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, pH 7.4) with protease inhibitors, and centrifuged at  $12,000 \times g$  at  $4^{\circ}\text{C}$  for 20 min. The protein content was assessed using a BCA Protein Assay Kit (Beyotime, Shanghai, China). Antibodies to HNF-4 $\alpha$ , JNK, and CYP7A1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phosphotyrosine JNK and  $\beta$ -actin were from Abcam (Cambridge, UK). Equal amounts of protein were separated by electrophoresis in 12% SDS polyacrylamide gel and then electroblotted from the gels on a PVDF membrane (Millipore, Billerica, MA) as described by Towbin *et al.*<sup>(32)</sup> After blocking, the membrane was probed with the primary antibody at  $4^{\circ}\text{C}$  overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The immune complexes were visualized using enhanced chemiluminescence reagents (GE Healthcare) and quantified with Quantity One software (Bio-Rad, Richmond, CA).

**Statistical analysis.** All data are presented as means  $\pm$  SEM. Statistical significance was calculated by Student's *t* test using SPSS ver. 17.0 (Chicago, IL). A statistical probability of  $p < 0.05$  was considered significant. The data are presented as a bar graph using the GraphPad Software (San Diego, CA).

**Table 1.** Sequences of primers used in quantitative real-time PCR

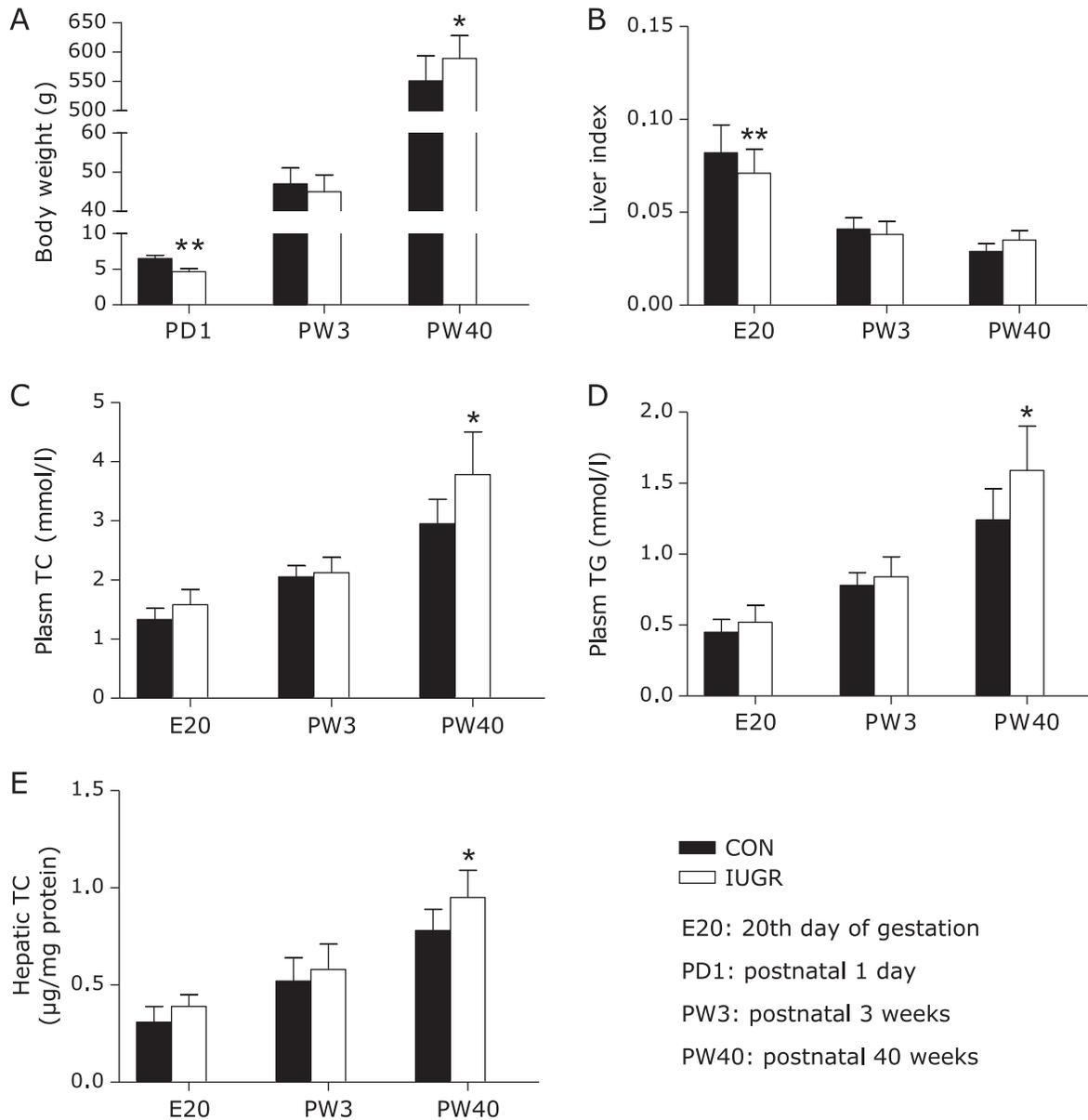
Target genes	Accession No.	Primer sequences (5'-3')	Amplicon size (bp)	Tm ( $^{\circ}\text{C}$ )
TNF- $\alpha$	NM_012675	F: CTCAGATCATCTTCTCAAAA	169	46.9
		R: AGGTACAGCCAATCTGCTAA		52.7
CYP7A1	NM_012942.1	F: TCACGGAAGGGATGTA	202	45.5
		R: GCTTTATGTGCGGTCT		45.3
BSEP	NM_031760.1	F: CAGGCGGAGGCAAGTCTTC	150	58.8
		R: CCCATCAACGTCATCTTGA		60.4
$\beta$ -actin	NM_031144	F: ACTATTGGCAACGAGCGGTT	190	59.9
		R: TGTCAGCAATGCCTGGGTACA		62.1

## Results

**Weight and metabolic files.** In a previous study we reported that protein restriction resulted in lower body weight in near-term fetuses (E20), with no observed change in litter size or sex ratio. In this study, postnatal body weight of IUGR animals was restored to normal during lactation (PW3), whereas it surpassed that of the control group at postnatal 40 weeks because of catch-up growth (Fig. 1A). In agreement with other reports, protein restriction resulted in a significant decrease in fetal liver index (liver weight as a percentage of body weight). However, the restored liver index was comparable with that of the same-aged control animals at PW3 and PW40 (Fig. 1B). Additionally, IUGR group showed a trend towards an augment in plasma TG, TC and

hepatic TC concentration at E20, and a significant increase at PW40 ( $p < 0.05$ ) (Fig. 1C–E).

**Liver histology.** A subset of the liver sections obtained at all three ages was stained with hematoxylin and eosin. In both control and IUGR fetal livers (Fig. 2A and D), there are many immature erythropoietic cells remaining in the perisinusoidal space compared with the livers of adult rats. Hepatic cords are two cells thick, with no lobular architecture observed in the fetal liver, consistent with previous reports.<sup>(33,34)</sup> In contrast, the liver consists mainly of hepatocytes, sinusoidal cells and bile ductile cells after birth, with the hepatocytes arranged as cords that are one cell thick. At day 20 of gestation, the livers of IUGR appeared histologically normal, and this remained the case at 3 weeks of age (Fig. 2B and E) without significant difference was detected



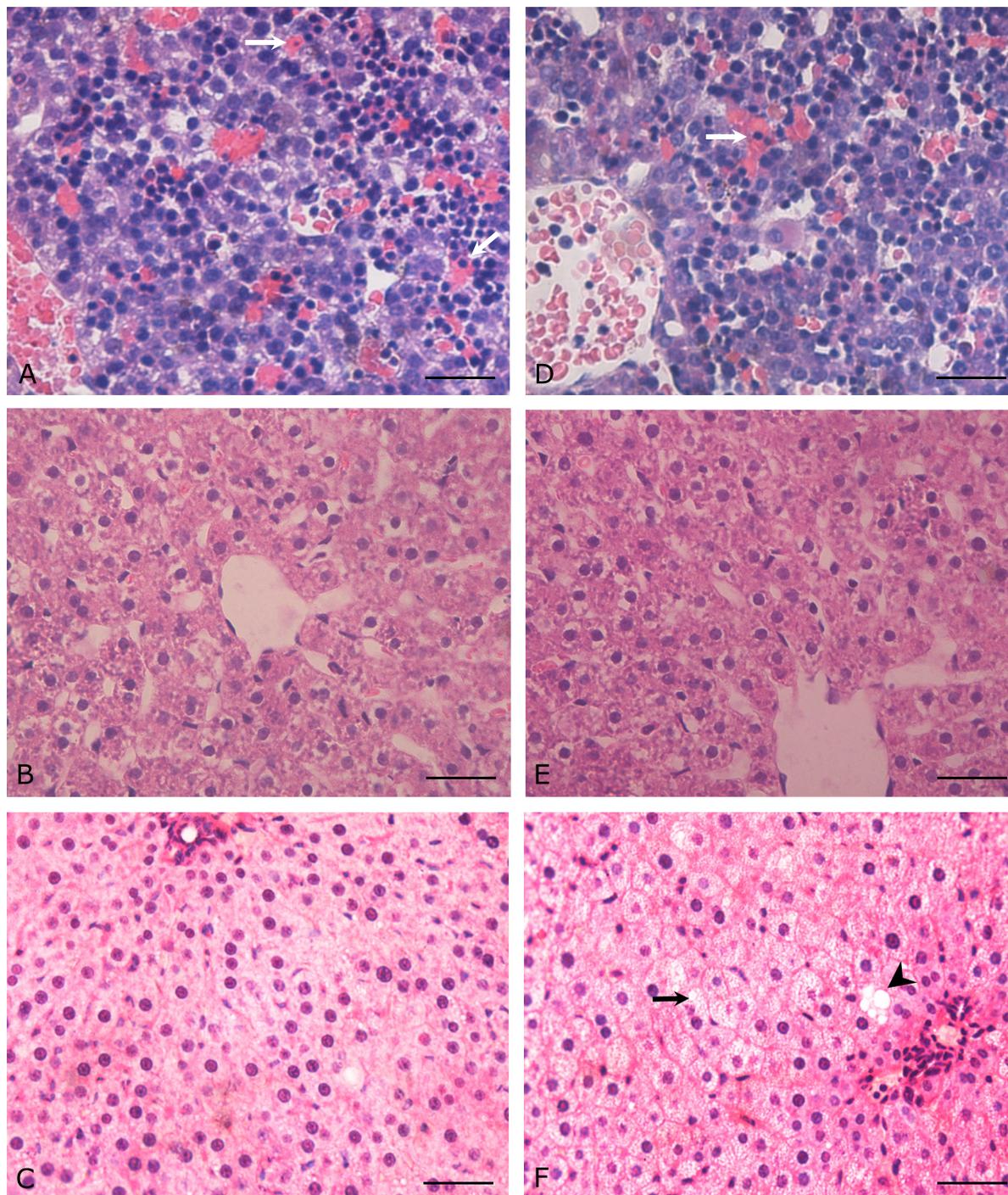
**Fig. 1.** The influence of maternal protein restriction on offspring liver growth and metabolic profile. (A) Body weight of offspring rats at postnatal day 1 (PD1) (control,  $n = 93$ ; and IUGR,  $n = 108$ ), PW3 (control,  $n = 52$ ; and IUGR,  $n = 48$ ) and PW40 (control,  $n = 10$  and IUGR,  $n = 12$ ). (B) Liver weight is expressed as a percentage of body weight at E20 (control,  $n = 42$ ; and IUGR,  $n = 45$ ), PW3 (control,  $n = 8$  and IUGR,  $n = 8$ ) and PW40 (control,  $n = 8$ ; and IUGR,  $n = 8$ ). (C)–(E) Fasting plasma triglyceride (TG), total cholesterol (TC), and hepatic TC concentrations in all time points examined ( $n = 6$  for both groups). Black bars, control group; white bars, IUGR group. Data are shown as means  $\pm$  SE. Significant differences compared with controls of same age are indicated by \* $p < 0.05$  and \*\* $p < 0.01$ .

between two groups. By PW40, however, IUGR animals exhibited mild hepatic steatosis with many microvesicular and a few macrovesicular deposits (Fig. 2C and F).

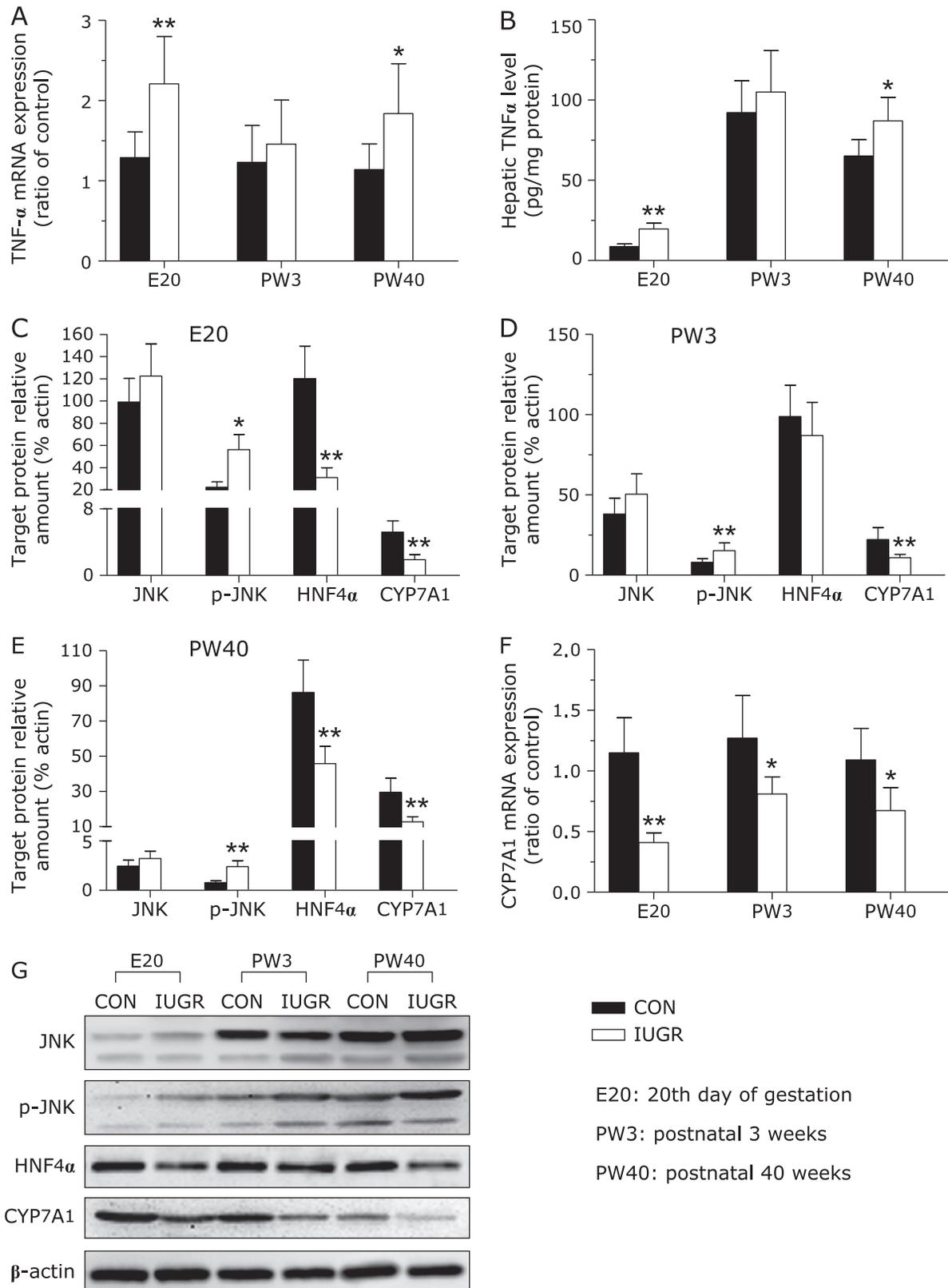
**Effect of maternal malnutrition on the hepatic TNF- $\alpha$ .** Maternal undernutrition significantly upregulated the TNF- $\alpha$  mRNA levels in the livers of IUGR fetal rats ( $p < 0.05$ ), which restored at PW3, but resumed to higher expression level at PW40 (Fig. 3A). To obtain further information of the protein content

difference, we analyzed the cytokine TNF- $\alpha$  using ELISA. In general, mRNA and protein levels of TNF- $\alpha$  were comparable. Compared with the control group, hepatic TNF- $\alpha$  cytokine content was upregulated by 126.52% and 33.85% in the IUGR rats at E20 and PW40, respectively ( $p < 0.05$ ) (Fig. 3B).

**Effect of maternal malnutrition on the JNK/HNF/CYP7A1 signaling pathway.** Cytokine TNF- $\alpha$  regulates CYP7A1 expression through the JNK signaling pathway. We further



**Fig. 2.** Evidence of hepatic steatosis in adult IUGR offsprings. HE staining was performed for the sections from rat livers at different developmental stages. (A)–(C) show representative sections from CON rats (40 $\times$  original magnification) (A) E20, (B) PW3, (C) PW40. (D)–(F) show sections from IUGR rats (D) E20, (E) PW3, (F) PW40. White arrow indicates immature erythrocytes; black arrow indicates microvesicular fat droplets; black arrow head indicates macrovesicular fat droplet. Scale bars = 50  $\mu$ m.



**Fig. 3.** The influence of IUGR on key molecules of TNF- $\alpha$ /CYP7A1 signaling pathway in rat livers at different developmental stages. (A) TNF- $\alpha$  mRNA levels in livers were analyzed by quantitative real-time PCR. Values are expressed relative to same-aged control. (B) TNF- $\alpha$  protein content was measured by ELISA. (C)–(E) Densitometric analysis of total JNK, phospho-JNK, HNF4 $\alpha$  and CYP7A1 protein in rat livers at E20 (C), PW3 (D) and PW40 (E). (F) Hepatic mRNA expression of CYP7A1 at different developmental stages. (G) Representative Western blotting analyses of key molecules of TNF- $\alpha$ /CYP7A1 signaling pathway in rat livers. All protein expression levels were normalized relative to the expression level of  $\beta$ -actin. Data are presented as means  $\pm$  SE for 6 observations per group. Black bars, control group; white bars, IUGR group. Significant differences compared with controls of same age are indicated by \* $p$ <0.05 and \*\* $p$ <0.01.

investigated the expression and activity of hepatic JNK in the IUGR offspring. Overproduction of TNF- $\alpha$  resulted in a remarkable increase in phosphorylation (Thr183/Tyr185) of JNK ( $p < 0.05$ , Fig. 3). However no differences were observed in total JNK content at all time-points examined. To extend this study, we further measured HNF-4 $\alpha$  and CYP7A1 expressions in the livers. In the IUGR group, there was a significant downregulation in hepatic HNF-4 $\alpha$  expression at E20, with no significant change at PW3, and a significant decrease at PW40 compared with the control group ( $p < 0.05$ , Fig. 3). This was accompanied by a statistically significant decrease in hepatic CYP7A1 expression at all time points of investigation ( $p < 0.05$ , Fig. 3).

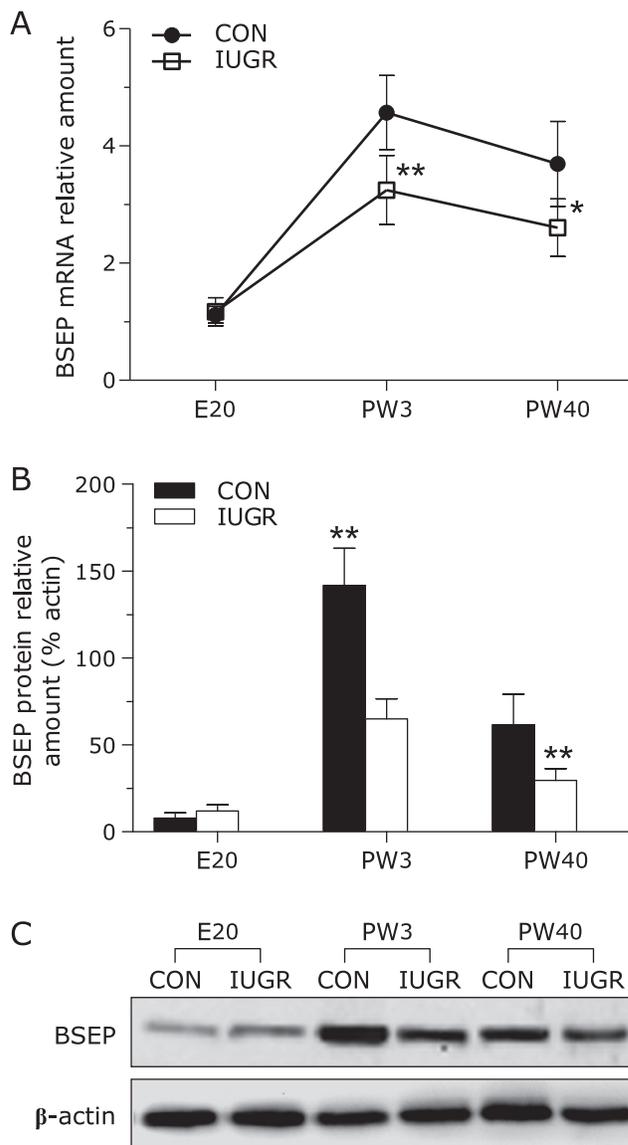
**BSEP in the livers of offspring.** The expression patterns of BSEP mRNA in both groups were shown in Fig. 4A. In both groups, hepatic BSEP mRNA levels were remarkably low before birth, but increased at 3 weeks and 40 weeks of age. Compared to the control group, in the IUGR group there were remarkable decrease in BSEP mRNA levels at PW3 and PW40, with no significant change at E20. Consistent with the PCR data, by western hybridization, there were significant declines in BSEP protein expression at PW3 and PW40, when comparing IUGR with the control group (Fig. 4B and C).

## Discussion

The Barker's "fetal origins of adult disease hypothesis" implicates that fetal *in-utero* environment has a profound impact on one's risk for major chronic diseases. Clinical studies have demonstrated that adverse intrauterine environment that results in IUGR can also alter or "program" the physiological processes leading to the future adult diseases, such as coronary artery disease, hypertension, type 2 diabetes, and obesity. With regard to cholesterol, Forsdahl was the first to suggest a link between development and subsequent hypercholesterolemia in 1978.<sup>(35)</sup> To date, mounting evidence have confirmed there was apparent association between low birth weight and postnatal higher cholesterol, independent of social circumstance, current body weight, cigarette smoking, and alcohol intake.<sup>(20,36)</sup>

Various animal models, regardless of the type of intrauterine injury, have also demonstrated that IUGR could permanently alter offspring cholesterol homeostasis. However the mechanisms underlying these alterations remain largely unknown. Undoubtedly, elucidating the molecular mechanisms in this process will facilitate researchers to identify early life dietary and/or drug intervention strategies for the prevention of these metabolic diseases. In a well established IUGR model, we investigated the effect of protein-malnutrition during pregnancy on offspring cholesterol metabolism. Considering there was stronger association between IUGR and higher cholesterol in human males compared with females,<sup>(37)</sup> All the analyses were performed with male pups only. In keeping with previous reports, we found that birth weight of male pups from protein restriction dams were significantly lowered, however it exceeded that of control pups at postnatal 40 weeks because of catch-up growth. Furthermore, the liver index (liver weight as a percentage of body weight) was lowered at birth, but restored at PW3 and PW40 compared to the control animals. The IUGR group had hypercholesterolemia and significantly augmented accumulation of hepatic cholesterol in aging animals. Higher cholesterol concentration in hepatic was linked with aberrant gene expression and/or kinase activity in the TNF- $\alpha$ /CYP7A1 signaling pathway, which regulates the bile acid biosynthesis.

Cholesterol is an important biological molecule for membrane structure as well as serving as a precursor for the biosynthesis of the steroid hormones, bile acids, and vitamin D. However, an excessive accumulation of this molecule has profound negative impacts on cardiovascular disease. The biosynthesis of the bile acids is the predominant pathway of cholesterol elimination in



**Fig. 4.** The effect of maternal undernutrition on the expression of bile salt export pump (BSEP) gene in rat livers. (A) BSEP mRNA levels in both groups were determined by quantitative real-time PCR, and results were expressed relative to the mean value of the E20 control. (B) and (C) Representative Western blotting and densitometric analysis of BSEP protein expression in the livers of both groups. BSEP protein expression level was normalized relative to the expression of  $\beta$ -actin. Data are presented as means  $\pm$  SE for 6 observations per group. Black bars, control group; white bars, IUGR group. Significant differences compared with controls of same age are indicated by \* $p < 0.05$  and \*\* $p < 0.01$ .

liver, which is called the classic pathway. CYP7A1, the first and rate-limiting enzyme in the bile acid synthetic pathway is expressed normally only in hepatocytes and is highly regulated. Human deficiency of CYP7A1 manifests with a spectrum of phenotypes ranging from hypercholesterolemic and premature gallstones to premature coronary disease. CYP7A1 gene knockout mice had elevated total cholesterol as well as LDL and severely reduced bile acid synthesis.<sup>(38-41)</sup> Studies of humans and experimental animals have indicated that cytokine TNF- $\alpha$  limits cholesterol elimination from the body by inhibiting the expression and activity of CYP7A1. The mechanism involves the activation of the JNK-dependent signaling pathway that subsequently inhibits the expression and activity of HNF4, ultimately de-

creasing the transcription rate of CYP7A1.<sup>(24)</sup>

Previous works have shown that impaired fetal growth caused by protein or iron restriction in pregnancy leads to higher hepatic cholesterol in fetus or aging animals. These are linked to decreased expression of CYP7A1.<sup>(22,23)</sup> Using a pregnancy protein restriction model, we showed that hepatic CYP7A1 protein amount was persistently reduced from fetal to aged rats, consistent with previous reports. Furthermore we observed higher TNF- $\alpha$  concentration and enhanced phosphorylation level of JNK, but lower HNF4 $\alpha$  expression in the livers of IUGR offspring. These data suggested that intrauterine protein restriction might upregulate cytokine TNF- $\alpha$  expression; subsequently activate JNK signaling cascade, and eventually diminish the HNF4-mediated transcription activation of CYP7A1.

TNF- $\alpha$  is a multifunctional cytokine that in the liver acts as a cytotoxic agent in many types of hepatic injury. Kupffer cells, the resident macrophages which constitute 15% of the liver cell population, are major sources of TNF- $\alpha$  and other inflammatory cytokines.<sup>(42)</sup> Pretranscription regulation of TNF- $\alpha$  gene expression within liver follows activation of extracellular signal-regulated kinase (ERK) signaling pathway by growth factors and cytokines, as well as reactive oxygen species (ROS).<sup>(43–46)</sup> Studies have demonstrated that placental insufficiency induces oxidative stress in the offspring liver that creates a self-perpetuating process in which overproduction of ROS,<sup>(47)</sup> and increases hepatic ERK1/2 phosphorylation.<sup>(15,48)</sup> Thus it is possible that IUGR-induced oxidative stress activates ERK signaling and increases the expression of TNF- $\alpha$  in liver. Further studies are needed to confirm this speculation. After released from Kupffer cells, TNF- $\alpha$  activates TNF receptors on hepatocytes to induce JNK and inhibitor kappa beta kinase (IKK) activation, as well as more ROS production. Besides limiting cholesterol elimination, activation of JNK by TNF- $\alpha$  impairs insulin action by inhibiting the activity of insulin receptor substrate (IRS)-1.<sup>(49)</sup> Studies of several animal models showed deactivation of IRS-1 in IUGR offspring livers,<sup>(48,50)</sup> thence the potential relationship between TNF- $\alpha$  expression and the insulin signaling in the IUGR liver deserves further investigation.

BSEP, a member of the ATP-binding cassette (ABC) family of transporters (identified as ABCB11) is responsible for exporting bile acids out of hepatocytes as part of the hepatic detoxification process, while bile acids stimulate hepatic BSEP gene expression in turn.<sup>(51)</sup> Exposure to TNF- $\alpha$  caused downregulations of both BSEP mRNA and protein levels in rat hepatocytes. Administra-

tion of LPS *in vivo* triggered release of the proinflammatory cytokines including TNF- $\alpha$  and caused the decrease of BSEP protein in rat liver.<sup>(52)</sup> We observed similar expression pattern for BSEP and TNF- $\alpha$  gene in the livers of IUGR animals. Thus we speculated that the mechanism responsible for aberrant BSEP expression in IUGR animals maybe caused directly by increased TNF- $\alpha$  outproduction, which invites further investigation.

In short, this work demonstrated that intrauterine malnutrition-induced IUGR led to enhanced cholesterol in later life, through reprogramming of lifelong expression of key factors involved in the regulation of cholesterol and bile acid metabolism. The fundamental mechanisms underlying these changes and their influence on the metabolic syndrome remain unclear. Given the plasticity of the liver in fetal and neonatal stages, understanding the effects of intrauterine malnutrition on cholesterol dysregulation may be potentially helpful for developing early life dietary and drug treatment strategies to reduce the incidence of the metabolic syndrome in adulthood.

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## Abbreviations

BSEP	bile salt export pump
CYP7A1	cholesterol 7 $\alpha$ -hydroxylase
ERK	extracellular signal-regulated kinase
HNF4 $\alpha$	hepatocyte nuclear factor 4 $\alpha$
IKK	inhibitor kappa beta kinase
IL-1 $\beta$	interleukin-1 $\beta$
IRS	insulin receptor substrate
IUGR	intrauterine growth restriction
JNK	c-Jun N-terminal kinase
TNF- $\alpha$	tumor necrosis factor $\alpha$
ROS	reactive oxygen species

## Conflict of Interest

No potential conflicts of interest were disclosed.

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