Prenatal Glucocorticoid Administration Accelerates Maturation in The Hepatocytes of Fetal Rats

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**Prenatal Glucocorticoid Administration Accelerates Maturation in the Hepatocytes
of Fetal Rats**

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Category of study: basic science

25 Abstract

26 Preterm and low birth weight infants often experience liver dysfunction after birth because the
27 liver is immature. As such, prenatal glucocorticoid (GC) is clinically administered to pregnant
28 women at risk of preterm birth for maturation of the cardiopulmonary function. However, the
29 effects of prenatal GC administration on the liver remain unclear. We aimed to investigate the
30 effects of prenatal GC administration regarding maturity of the liver in preterm rats.

31 Dexamethasone (DEX) was administered to pregnant Wistar rats on gestational days 17 and 19
32 before cesarean section. Real time polymerase chain reaction (RT-PCR) was then used to analyze
33 the mRNA levels (albumin, HNF4 α , HGF, Thy-1, cyclin B, and CDK1) in the liver samples.
34 Immunohistochemical staining and enzyme-linked immunosorbent assay (ELISA) were used to
35 analyze protein produced.

36 Hepatocyte size enlarged because of growth and administration of prenatal DEX. Albumin,
37 HNF4 α , and hepatocyte growth factor (HGF) increased secondary to growth and administration
38 of prenatal DEX. Cell cycle markers, Cyclin B, and CDK1 gradually decreased during growth
39 and by administration of DEX.

40 These results suggest that prenatal GC administration induces hepatocyte differentiation and
41 achieves maturation via expression of HNF4 α and HGF in premature fetuses.

42

Key words

Glucocorticoid, Fetus, liver, maturation, development, cell proliferation

Declarations

Funding

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Competing Interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generate or analyzed during this study are included in this publish article and its
supplementary information files.

Code availability

Not applicable

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Author' contributions

TK and YT contributed to the study conception and design. Material preparation, experiment, and data collection were performed by TK. TK, YT, YO, MO, and KK contributed analysis and interpretation of data for the manuscript. The first draft of the manuscript was written by TK. YT contributed to assist in the preparation of the manuscript. MW contributed Resources. NM and TI contributed supervision and funding acquisition. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Ethics approval

This study has complied with the “Guiding principles for the care and use of laboratory animals” by The Japanese Pharmacological Society and was approved by the Experimental Animal Research Committee of St. Marianna University Graduate School of Medicine (Approval number: 2002008).

Consent to participate

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79 **Consent for publication**

80 Not applicable

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Introduction

94 The prevalence rates of low birth weight infants and preterm delivery are high among
95 developed countries. Therefore, the importance of research focusing on the fetal and childhood
96 periods is advocated (Sata, 2016). Low birth weight and premature birth infants often develop
97 physiological jaundice with immature fetal liver function.

98 Preterm glucocorticoid (GC) administration in mothers with babies at risk of being delivered
99 preterm is used to accelerate the pulmonary circulation and reduce mortalities (Hayes et al, 2008).
100 We previously demonstrated that prenatal GC administration promotes growth with development
101 of cardiac functions in premature infant model rats (Sakurai et al, 2019; Mizuno et al, 2010;
102 Tsuzuki et al, 2009).

103 In contrast, repeated administration of prenatal GC has been reported to decrease the liver
104 weight of mice (Ozdemir et al, 2003). However, there is no explanation regarding the
105 development of the fetal liver subsequent to prenatal GC administration.

106 The role of GC has been demonstrated in association with differentiation of hepatocytes and
107 the bile duct (Baribault, 1986; Monga, 2001). DEX is used for differentiation of hepatic stem
108 progenitor cells established from induced pluripotent stem (iPS) cells to hepatocyte maturation
109 (Snykers, 2009).

110 Therefore, we predicted that prenatal DEX administration would promote maturation of the liver

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3 111 during fetal development, during which liver cells are immature and have many stem cells.
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6 112 Various factors can be linked to maturation in the fetal period (Shiojiri, 1997).
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10 113 The expression levels of various enzymes, such as tyrosine aminotransferase (TAT), G6 Pase
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12 114 (Glucose 6-phosphatase), and uridine diphosphate glucuronosyltransferase (UGT) 1A, increase
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14 115 rapidly from the late embryonic period to birth (Kamiya, 1999; Asahina, 2004; Kishi, 2008).
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20 116 This study aimed to elucidate the process of differentiation and maturation of hepatocytes that
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22 117 accompanies growth in the fetal liver.
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26 118 27 28 29 119 **Material and Methods**

30 31 32 120 1) Animal experiments

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37 121 Dexamethasone (DEX: Fujifilm Wako Pure Chemical, Osaka, Japan) dissolved in sesame oil
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39 122 (Kanto Chemical, Tokyo)—at doses of 1.0 and 2.0 mg/kg—was administered subcutaneously on
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42 123 days 17 and 19 of the eight weeks gestation period of the Wistar pregnant rats (CKEA, Tokyo
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45 124 Japan). On days 19 and 21 fetal rats were delivered by cesarean section and liver samples were
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48 125 extracted under inhaled isoflurane anesthesia. Liver samples were also extracted from
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51 126 spontaneously delivered 1-day-old neonates and 8-week-old rats.
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56 127 Rats were kept at a constant temperature ($23 \pm 1^{\circ}\text{C}$), constant humidity ($55 \pm 5\%$), artificial
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lighting (6–18 o'clock), and darkness in the Laboratory Animal Care and Management Facility of St. Marianna University School of Medicine. They were kept under free drinking water under a cycle (12 hours light period, 12 hours dark period). This study has complied with the “Guiding principles for the care and use of laboratory animals” by The Japanese Pharmacological Society and was approved by the Experimental Animal Research Committee of St. Marianna University Graduate School of Medicine (Approval number: 2002008).

2) Histological evaluation

Rat liver sections were preserved in 10% formalin, and embedded in paraffin, 5 μm sections. The sections were stained with hematoxylin and eosin (H&E) solution. We evaluated hepatocyte size as the total number of hepatocytes by area of view ($2.8 \times 10^4 \mu\text{m}^2$) under microscope with high magnification (×400).

3) The mRNA extraction and real time reverse transcription - polymerase chain reaction (RT-PCR)

Total RNA in the liver tissue was isolated using cell lysates, a RNeasy® sepaol-RNA I super G kit (Nakarai, Kyoto, Japan). A NanoDrop One / OneC Spectrophotometer (Thermo Fisher

Scientific, Waltham, MA, USA) was used to measure total RNA concentration at an optical density of 260 nm. The cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diadnostic GmbH, Mannheim, Germany). RT-PCR was performed using Step One Plus and Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific). Table 1 shows the used primers. The PCR conditions were 95°C, 20 s (denaturation), 60°C, 30 s (annealing), and 72°C, 40 s (extension) 45 cycles.

Gene expression was determined using the relative values of standard curve values. In each sample the relative value was normalized to the housekeeping gene for hypoxanthine phosphoribosyltransferase (HPRT). Table 1 and Supplement 1 show the used primers.

4) Immunohistochemistry

Liver specimen were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was inactivated with 0.1% hydrogen peroxidase. After blocking, the anti-albumin antibody (Proteintech, IL, USA), anti-hepatocyte growth factor (HGF) antibody (Bioss Antibodies, MA, USA), anti-hepatocyte nuclear factor 4 of alpha (HNF4α) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-thymus cell antigen 1 (Thy1) antibody (Abcam, Cambridge, UK) were treated overnight at 4°C. A CSA11 Biotin-free Tyramide Signal Amplification System (Dako, Carpinteria, CA, USA) was used to demonstrate immunoreactivity

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3 163 according to the manufacturer's instructions. The chromogenic substrate 3,3'-diaminobenzidine
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6 164 was used. The sections were then counterstained with hematoxylin.
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10 165 The OLYMPUS CX41 upright microscope (Olympus, Tokyo, Japan) was used to acquire
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13 166 immunocytochemistry digital images. ImageJ 1.49v. processing software was used to calculate the
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16 167 density of the positive area.
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23 169 5) HNF4 α enzyme-linked immunosorbent assay (ELISA)
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27 170 HNF4 α protein levels were measured using Rat HNF4A/HNF4 ELISA Kit (LifeSpan
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30 171 Biosciences, WA, USA) according to the manufacturer's instructions. The tissues samples were
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33 172 prepared using CelLyticTM MT cell lysis reagent for mammalian tissues (Sigma-Aldrich, MO,
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36 173 US), and were quantified using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, CA,
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39 174 USA). Absorbance was measured at a wavelength of 450 nm using Viento nano (DS Pharma
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50 177 6) Statistical analysis
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53 178 Results are shown as mean $\pm$ standard error of mean (SEM). JMP pro 13 (SAS Institute Inc.,
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56 179 Cary, NC, USA) was used to perform the statistical analyses. The Dunnett's test was used to
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evaluate differences between respective groups. $P < 0.05$ was considered significant.

Results

1) Hepatocyte size

Through growth, the cell size of a hepatocyte gradually increases from the fetuses (19F; $69.8 \pm 2.7 \mu\text{m}^2$, 21F; $145.0 \pm 13.2 \mu\text{m}^2$) to the neonates (1N; $164.1 \pm \mu\text{m}^2$, 3N; $162.9 \pm 5.2 \mu\text{m}^2$, 5N; $231.0 \pm 10.2 \mu\text{m}^2$), to adult rats ($359.1 \pm 36.0 \mu\text{m}^2$) (Fig. 1A, B). Prenatal DEX administration significantly increased the cell size in the 19F group (DEX: 1.0 mg/kg; $105.7 \pm 4.95 \mu\text{m}^2$, 2.0 mg/kg; $106.4 \pm 3.64 \mu\text{m}^2$) (Fig. 1C). Largen mRNA and mTOR levels were not affected in 19F and 21F, the process of growth, or by prenatal DEX administration (Fig. S1).

2) Ki-67, Cyclin B, and CDK1 mRNA levels

The mRNA expressions of proliferation and cell cycle markers Ki-67, cyclin B, and cyclin-dependent kinase 1 (CDK1) mRNA levels gradually decreased from 19F to adult rats during growth (Fig.2A, C, E).

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195 Ki-67 mRNA levels remained unchanged with DEX in 19F and 21F (Fig.2B). Cyclin B and
196 CDK1 mRNA levels tended to decrease in 19F with administration of DEX 1.0 mg/kg, but both

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10 199 3) Thy-1 and DLK1 expressions
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14 200 High levels of Thy-1 (Fig.3A) and DLK1 (Fig.3E) mRNA expression were observed in 19F and
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16 201 the levels gradually decreased during growth.
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20 202 Thy-1 mRNA levels tended to decrease in 21F with administration of DEX 1.0 mg/kg (0.43-fold)
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22 203 and 2.0 mg/kg (0.42-fold) compared with that of the untreated group; however, DLK1 mRNA
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24 204 levels in the liver were unchanged after administration of prenatal DEX (Fig.3B, F).
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29 205 Thy-1 protein positive cells also decreased in 21F with administration of DEX 2.0 mg/kg (28.7
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31 $\pm 2.6\%$) compared with the untreated group ($42.3 \pm 1.4\%$) (Fig.3C, 3D).
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40 208 4) Alpha-fetoprotein (AFP) and albumin mRNA and protein levels
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44 209 In untreated groups, AFP mRNA levels gradually decreased from 19F to adult rats (Fig.4A).
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46 210 AFP mRNA levels were unchanged in 19F and 21F with administration of DEX (Fig.4B).
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50 211 In contrast, albumin mRNA levels in the liver of 19F were low, and the levels gradually
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52 212 increased during growth (Fig.4C, E, F).
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56 213 Albumin mRNA (5.79-fold) and protein levels increased in the 19F with administration of DEX
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214 2.0 mg/kg compared with the untreated group (Fig.4D, E, G).

215 G6 Pase and TAT mRNA levels in livers of 8W rats were higher than those of 19F rats; however,
216 prenatal DEX administration did not affect 19F and 21F (Fig.S2).

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218 5) HGF mRNA levels and protein levels

219 HGF levels increased two-fold in the 1N group compared with the 19F group (Fig.5A).

220 Administration of 2.0 mg/kg prenatal DEX significantly approximately 2-fold increased in 19F
221 in compared with the untreated group (Fig.5B).

222 In immunohistochemical staining, HGF protein levels also showed a significant increase in the
223 19F with DEX administration (1.0 mg/kg, $34.4 \pm 1.5\%$ and 2.0 mg/kg, $25.4 \pm 1.9\%$)
224 compared with the untreated group ($15.5 \pm 3.1\%$). However, HGF protein levels were
225 unchanged in the 21F groups by DEX administration (Fig.5C, D).

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227 6) HNF4 α mRNA and protein levels.

228 HNF4 α is involved in maturation of hepatocytes. HNF4 α mRNA and protein levels in adult rats
229 were higher than those in 19F rats (Fig.6A, C).

230 Prenatal DEX administration significantly increased HNF4 α mRNA levels both in the 19F (DEX

2.0 mg/kg, 3.69-fold) and 21F (DEX 1.0 mg/kg, 3.15-fold; DEX 2.0 mg/kg, 2.93-fold) groups (Fig.6B).

Furthermore, HNF4 α protein levels also significantly increased in the 19F (DEX 2.0 mg/kg, 51.7 ± 6.2 pg/ μ g) and 21F (DEX 1.0 mg/kg, 65.6 ± 2.8 pg/ μ g; DEX 2.0 mg/kg, 74.4 ± 12.7 pg/ μ g) groups by DEX administration compared with the untreated group (19F, 18.2 ± 3.2 pg/ μ g; 21F, 34.9 ± 2.7 pg/ μ g) (Fig.6D).

In immunohistochemical staining, HNF4 α protein levels in 19F by DEX (1.0 mg/kg: $48.4 \pm 2.1\%$, 2.0 mg/kg: $42.8 \pm 1.7\%$) significantly increased compared with the untreated group ($34.9 \pm 2.7\%$). Also, administration of DEX significantly increased HNF4 α protein levels in 21F (1.0 mg/kg: $60.8 \pm 1.6\%$, 2.0 mg/kg: $65.1 \pm 2.2\%$) compared with the untreated group ($43.3 \pm 3.1\%$) (Fig.6E, F).

Discussion

Preterm infants risk hepatic insufficiency because their immaturity results in a delay in achieving detoxification and synthetic functions (Bhutani and Wong, 2013).

Prenatal GC treatment accelerates development of cardiopulmonary functions for reducing respiratory distress syndrome and mortality in premature infants (Wynne et al, 2020; Agnew et al, 2018). GC triggers organ maturation and is necessary for sustaining extrauterine life (Smith et al,

2000). Our previous studies have demonstrated that prenatal GC administration in pregnant rats increased the production of liver transporters and multidrug resistance-associated protein 2 in the preterm fetal liver (Takeba et al, 2018). However, the mechanisms of fetal liver growth by prenatal GC administration remains unclear. In this study, prenatal DEX administration increased the size of hepatocytes in fetal liver and maturation-related factors. This suggests that DEX induces fetal hepatocyte growth. First, we found that hepatocyte size gradually enlarged from 19-day fetuses (19F) to 5-day-old neonates (5N). Additionally, enlargement of hepatocytes was also observed in adult rats. Prenatal DEX administration enlarged hepatocyte size in 19F, but not 21-day fetuses (21F). Processes essential to attaining adequate liver mass and function during the fetal period include activation of specific cell lineage early in development and cell proliferation or differentiation, accomplished by enzymes and transcriptional factors. We therefore studied whether hepatocyte enlargement depends on cell proliferation or differentiation. High levels of cell proliferation marker Ki-67 mRNA were expressed in fetal hepatocytes, and their levels gradually decreased. This indicates that the capacity for cell proliferation declines with liver growth. However, prenatal DEX administration did not change Ki-67 mRNA expression i.e., DEX administration did not affect cell proliferation in fetal hepatocytes. Hepatocyte proliferation and differentiation for functional growth occur simultaneously during late mammalian gestation. Gruppuso et al. indicated the presence of independent signaling pathways for control of

proliferation and differentiation in developing hepatocytes (Gruppuso et al, 1999). The cell cycle of hepatocytes is controlled from G(0) to mitosis by the regulation of cyclins and cyclin-dependent kinases (cdk). The cyclin B1 protein forms a complex with cyclin-dependent kinase 1 (CDK1), and this activation initiates mitosis (Porter and Donoghue, 2003). The mRNA expressions of cyclin B and CDK1 significantly decreased from 19F to 1N to adult rats. Additionally, prenatal DEX administration decreased cyclin B and CDK1 mRNA expressions in 19F and 21F. The transcriptional inhibition of the cyclin B-CDK1 complex by prenatal DEX limits proliferation. Premature hepatocytes include hepatic progenitors during hepatocyte development (Masson et al, 2006). Thy-1 and Delta-like 1 homolog (Dlk)-1 are known as stem cell markers. These markers can identify stem cell character (Fiegel et al, 2003; Nishina, 2012). With growth, the mRNA expressions of Thy-1 and Dlk-1 significantly decreased from the fetal liver. Prenatal DEX administration tended to decrease Thy-1 mRNA levels in 21F. Tanimizu et al. reported that Dlk-1 expression is strongest at murine fetal day 10.5 and is downregulated after fetal day 16.5 (Tanimizu, 2004). Because Dlk-1 expressions in the livers of 19F and 21F were scanty, DEX may not have had any effect. The early rat fetal liver also contains Thy-1-positive cells, which gradually decrease with growth. Thy-1 mRNA and protein levels were sustained until 21F, therefore indicating that DEX may have been influential. Hepatic maturation is characterized according to the stage of liver growth with specific genes

(Derman et al, 1981; Panduro et al, 1987). Some studies have reported a high level of AFP in fetal rat hepatocytes, and the AFP gradually decreased with hepatic maturity. In contrast, albumin or G6 Pase levels are increased in mature hepatocytes (Jochheim et al, 2004; Sell, 2008). Levels of AFP mRNA decreased in 19F liver during growth. Our finding corresponded with that obtained by Zhang et al. The level of AFP decreased as differentiation progressed in human hepatic progenitor cells. Cells exposed to a mixture of oncostatin M, DEX, and HGF gradually featured differentiated hepatic functions including albumin production in vitro (Zhang et al, 2012). Prenatal DEX administration did not change AFP mRNA levels in fetal liver. Albumin, TAT, and G6 Pase mRNA levels increased from the fetal liver to neonatal liver with growth. DEX administration only increased albumin production in 19F. Human hepatic progenitor cells (HPCs) morphologically and functionally differentiate into hepatocytes and cholangiocytes. Freshly isolated HPCs co-expressed glucose-6-phosphatase, glycogen, albumin, and gamma glutamyl transpeptidase, and could differentiate into functional liver cells (Nava et al. 2005). A combination of HGF, oncostatin M, and DEX induces hepatocyte maturation. HGF, in the presence of DEX, induces expression of G6P, TAT, and albumin in fetal hepatocytes (Kamiya et al 2001). HGF appears in the liver in the first few days after birth. We found that HGF mRNA and protein levels were enhanced in the 19F liver with prenatal DEX administration. This result suggests that DEX may have a direct effect on HGF increase. The

mechanism is the subject for future study.

Hepatocyte nuclear factor 4-alpha (HNF4 α) has essential roles in structure formation and function maturity of the hepatocyte (Li et al, 2000) . HPCs are negative for HNF4 α . They start expressing this transcription factor when they differentiate into hepatocytes (Parviz et al, 2003). The HNF4 α gene has two promoters of P1 and P2 isoforms in mouse liver. Transcription starts via P2 promoter during fetal life, but switches to P1 at birth. Exposure to DEX in the fetal rat liver suppresses P2, but enhances the expression of transcripts from P1 (Dean et al, 2010).

HNF4 α mRNA and protein levels significantly increase in the liver with growth. Prenatal DEX administration also increased their levels in 19F and 21F livers. Nyirenda et al. have reported that continuous administration of prenatal DEX increases HNF4 α mRNA expression in the rat liver (Nyirenda et al, 2006). Our results in the animal model are close to clinical therapeutics and are consistent with those obtained by Nyirenda et al. Thus, increase in hepatic HNF4 α expression relates to liver maturation.

This study has not showed whether prenatal GC acts through the glucocorticoid receptor and other factors including the metabolic enzymes in liver function.

Conclusion

These results suggest that prenatal GC administration induces hepatocyte differentiation, and liver maturation is achieved via the expressions of HNF4 α and HGF in premature fetuses.

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Figure Legends

Figure 1. Histology of the hepatocyte in liver tissue with growth.

(A) Histology of hepatocytes on hematoxylin and eosin staining in day-19 fetuses (19F), day-21 fetuses (21F), 1-day-old (1N), 3-day-old (3N), 5-day-old (5N) neonatal, and 8-weeks adult rats (8W) in liver tissue with growth and in day-19 and day-21 fetal rats after prenatal DEX administration. The photos show images at high magnification ($\times 400$), scale bar = 20 μm . The area of $2.8 \times 10^4 \mu\text{m}^2$ at field of view was divided by total hepatocyte number to obtain a hepatocyte size in liver tissues with growth (B), and in the fetal liver rats after DEX (C). Data are representative of three independent experiments.

*P < 0.05, **P < 0.01, and ***P < 0.001 vs. 19F group. ††† P < 0.001 vs the respective untreated group.

Figure 2. Ki-67, Cyclin B, and CDK1 mRNA and protein levels.

Ki-67, Cyclin B, and CDK1 mRNA levels were analyzed in liver tissues in from 19F to 8W rats (A), (C), and (E). Ki-67, Cyclin B, and CDK1 mRNA levels were evaluated in fetal rats after prenatal DEX administration (B), (D), and (F). Data are representative of

seven independent experiments. $**P < 0.01$ and $***P < 0.001$ vs. the respective 19F fetus group. $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ vs the respective untreated group.

Figure 3. Thy1 and DLK1 mRNA and protein levels.

Thy-1 and DLK1 mRNA levels were analyzed in liver tissues from 19F to 8W rats (A) and (E). Thy-1 and DLK1 mRNA levels were evaluated in the 19F and 21F rats after prenatal DEX administration (B) and (F). Data are representative of seven independent experiments. In immunohistochemistry, images of Thy-1 production in liver tissue after prenatal DEX (C). Thy-1 positive staining intensity was analyzed using NIH image analysis. 19F rat tissues: $n = 4$. 21F rat tissues: $n = 3$ (D). $***P < 0.001$ vs. the respective 19F fetus group. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ vs the respective untreated group.

Figure 4. AFP mRNA and albumin mRNA and protein levels.

AFP and albumin mRNA levels were analyzed in liver tissues from 19F to 8W (A) and (C). AFP and albumin mRNA levels were evaluated in 19F and 21F rats after prenatal DEX administration (B) and (D). Data are representative of seven independent experiments. In immunohistochemistry, albumin production increased in liver tissue after

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3 prenatal DEX (E). Albumin positive staining intensity was analyzed using NIH image
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6 analysis. 19F rat tissues: n = 4. 21F, 1N, 5N and adult rat tissues: n = 3 (F) and (G). *P <
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Figure 5. HGF mRNA and protein levels.

HGF mRNA levels were analyzed in liver tissues from 19F to 8W (A). HGF mRNA levels
were evaluated in the 19F and 21F rats after prenatal DEX administration (B). Data are
representative of seven independent experiments. In immunohistochemistry, HGF
production increased in liver tissue of 19F after prenatal DEX (C). HGF positive staining
intensity was analyzed using NIH image analysis. 19F rat tissues: n = 4. 21F rat tissues:
n = 3 (D). **P < 0.01 vs. the respective 19F fetus group. †P < 0.05 ††P < 0.01 vs the
respective untreated group.

Figure 6. HNF4α mRNA and protein levels.

HNF4α mRNA levels were analyzed in liver tissues from 19F to 8W (A). HNF4α
mRNA
levels were evaluated in the 19F and 21F rats after prenatal DEX administration (B). Data

534 are representative of seven independent experiments. ELISA of HNF4 α protein
535 production in liver tissue after prenatal DEX (C) and (D). Data are representative of four
536 independent experiments.

537 In immunohistochemistry, albumin production was analyzed in liver tissue after prenatal
538 DEX (E). HNF4 α and albumin positive staining intensity were analyzed using NIH
image

539 analysis. 19F rat tissues: n = 4. 21F rat tissues: n = 3 (F). *P < 0.05 and ***P < 0.001 vs.
540 the respective 19F fetus group. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the respective
541 19F fetus group. [†]P < 0.05, ^{††}P < 0.01 and ^{†††}P < 0.001 vs the respective untreated group

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543 **Figure Supplement 1. mTOR and Largen mRNA levels.**

544 Largen and mTOR mRNA levels were analyzed in 19F and 21F rats after prenatal DEX
545 administration (A) and (B). Data are representative of seven independent experiments.

546 Largen and mTOR mRNA levels were not affected in 19F and 21F during growth or by
547 prenatal DEX administration.

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549 **Figure Supplement 2. G6 Pase and TAT mRNA levels.**

550 G6 Pase and TAT mRNA levels were analyzed in liver tissues from 19F to 8W (A) and

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3 551 (C). G6 Pase and TAT RNA levels were unchanged in the 19F and 21F rats after prenatal
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6 552 DEX administration (B) and (D). Data are representative of seven independent
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9 553 experiments. *P < 0.05 and ***P < 0.001 vs. the respective 19F fetus group. G6 Pase and
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13 554 TAT mRNA levels in livers of 8W rats were higher than those of 19F rats; however,
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16 555 prenatal DEX administration did not affect 19F and 21F rats.
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Figure 1

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Figure 6

1 **Table 1. Sequence of PCR primers**

Gene		Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon (bp)
Ki-67	Forward	AGCGGCTCTCTTTAACACAGT	60	211
	Reverse	TGACCCCCAAAGGATACACG		
Cyclin B	Forward	TCGATCGGTTTCATGCAGGAC	60	209
	Reverse	GCGACCCAGACTGAAGTTCA		
CDK1	Forward	GCCAGAAGTAGAGTCCCTGC	60	270
	Reverse	AGAGCCAACAGTAAACGCCA		
Thy-1	Forward	CCGCGTCAACCTTTTCAGTG	60	249
	Reverse	ATGAAGTCCGTGGCTTGGAG		
DLK1	Forward	CCCTGTGTGAGAAGTGCCTA	60	207
	Reverse	CAGAGAATCCAGGGGTGCAG		
AFP	Forward	ATTTGCCACGAGACGGAAC	60	273
	Reverse	TGCCTTGTCTACTGAGCGG		
Albumin	Forward	AAGAGAAAGCACTGGTCGCA	60	252
	Reverse	TGGCCTGGTTCTCACACATG		
HGF	Forward	GGCCATGGTGCTACACTCTT	60	206
	Reverse	CTTGTGGGGGTACTGCGAAT		
HNF4 α	Forward	AGTGTCGTTACTGCAGGCTC	60	281
	Reverse	GTAATTGGCCCATTCGACCA		
HPRT	Forward	GCCCCAAAATGGTTAAGGTTG	60	176
	Reverse	TCCAATTTCTGCTGATGACACA		

2 CDK1: cyclin-dependent kinase 1, Thy-1: Thymus cell antigen 1, DLK1:, AFP: a-fetoprotein,

3 HGF: hepatocyte growth factor, HNF4 α : Hepatocyte nuclear factor 4 of alpha

Figures

Figure 1

Histology of the hepatocyte in liver tissue with growth. (A) Histology of hepatocytes on hematoxylin and eosin staining in day-19 fetuses (19F), day-21 fetuses (21F), 1-day-old (1N), 3-day-old (3N), 5-day-old (5N) neonatal, and 8- weeks adult rats (8W) in liver tissue with growth and in day-19 and day-21 fetal rats after prenatal DEX administration. The photos show images at high magnification ($\times 400$), scale bar = 20 μm . The area of $2.8 \times 10^4 \mu\text{m}^2$ at field of view was divided by total hepatocyte number to obtain a hepatocyte size in liver tissues with growth (B), and in the fetal liver rats after DEX (C). Data are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. 19F group. ††† $P < 0.001$ vs the respective untreated group.

Figure 2

Ki-67, Cyclin B, and CDK1 mRNA and protein levels. Ki-67, Cyclin B, and CDK1 mRNA levels were analyzed in liver tissues in from 19F to 8W rats (A), (C), and (E). Ki-67, Cyclin B, and CDK1 mRNA levels were evaluated in fetal rats after prenatal DEX administration (B), (D), and (F). Data are representative of seven independent experiments. **P < 0.01 and ***P < 0.001 vs. the respective 19F fetus group. †P < 0.05 and †††P < 0.001 vs the respective untreated group.

Figure 3

Thy1 and DLK1 mRNA and protein levels. Thy-1 and DLK1 mRNA levels were analyzed in liver tissues from 19F to 8W rats (A) and (E). Thy-1 and DLK1 mRNA levels were evaluated in the 19F and 21F rats after prenatal DEX administration (B) and (F). Data are representative of seven independent experiments. In immunohistochemistry, images of Thy-1 production in liver tissue after prenatal DEX (C). Thy-1 positive staining intensity was analyzed using NIH image analysis. 19F rat tissues: n = 4. 21F rat tissues: n = 3 (D). ***P < 0.001 vs. the respective 19F fetus group. †P < 0.05 and ††P < 0.01 vs the respective untreated group.

Figure 4

AFP mRNA and albumin mRNA and protein levels. AFP and albumin mRNA levels were analyzed in liver tissues from 19F to 8W (A) and (C). AFP and albumin mRNA levels were evaluated in 19F and 21F rats after prenatal DEX administration (B) and (D). Data are representative of seven independent experiments. In immunohistochemistry, albumin production increased in liver tissue after prenatal DEX (E). Albumin positive staining intensity was analyzed using NIH image analysis. 19F rat tissues: n = 4. 21F, 1N, 5N and adult rat tissues: n = 3 (F) and (G). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the respective 19F fetus group. ††P < 0.01 vs the respective untreated group.

Figure 5

HGF mRNA and protein levels. HGF mRNA levels were analyzed in liver tissues from 19F to 8W (A). HGF mRNA levels were evaluated in the 19F and 21F rats after prenatal DEX administration (B). Data are representative of seven independent experiments. In immunohistochemistry, HGF production increased in liver tissue of 19F after prenatal DEX (C). HGF positive staining intensity was analyzed using NIH image analysis. 19F rat tissues: n = 4. 21F rat tissues: n = 3 (D). **P < 0.01 vs. the respective 19F fetus group. †P < 0.05 ††P < 0.01 vs the respective untreated group.

Figure 6

HNF4α mRNA and protein levels. HNF4α mRNA levels were analyzed in liver tissues from 19F to 8W (A). HNF4α mRNA levels were evaluated in the 19F and 21F rats after prenatal DEX administration (B). Data are representative of seven independent experiments. ELISA of HNF4α protein production in liver tissue after prenatal DEX (C) and (D). Data are representative of four independent experiments. In immunohistochemistry, albumin production was analyzed in liver tissue after prenatal DEX (E). HNF4α and albumin positive staining intensity were analyzed using NIH image analysis. 19F rat tissues: n = 4. 21F rat tissues: n = 3 (F). *P < 0.05 and ***P < 0.001 vs. the respective 19F fetus group. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the respective 19F fetus group. †P < 0.05, ††P < 0.01 and †††P < 0.001 vs the respective untreated group