Prenatal Glucocorticoid Administration Accelerates Maturation in the Hepatocytes of Fetal Rats

Tsukasa Kobayashi (✉ kobayashit@marianna-u.ac.jp)

Saint Marianna University School of Medicine: Sei Marianna Ika Daigaku <https://orcid.org/0000-0002-9871-169X>

Yuko Takeba

Saint Marianna University School of Medicine: Sei Marianna Ika Daigaku

Yuki Ohta

Saint Marianna University School of Medicine: Sei Marianna Ika Daigaku

Masanori Ootaki

Saint Marianna University School of Medicine: Sei Marianna Ika Daigaku

Keisuke Kida

Saint Marianna University School of Medicine: Sei Marianna Ika Daigaku

Minoru Watanabe

Saint Marianna University School of Medicine: Sei Marianna Ika Daigaku

Taroh Iiri

Saint Marianna University School of Medicine: Sei Marianna Ika Daigaku

Naoki Matsumoto

Saint Marianna University School of Medicine: Sei Marianna Ika Daigaku

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Abstract

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

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

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

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

Introduction

The prevalence rates of low birth weight and preterm delivery infants are high among developed countries, especially, in Japan. Therefore, the importance of research focusing on the fetal and childhood periods is advocated (Sata 2016). Preterm birth occurs before the physiological rise in endogenous glucocorticoid (GC). GC action is an important in the structural and functional maturation of various organs (Khulan et al. 2016; Rog-Zielinska et al. 2014).

Prenatal GC administration in mothers with babies at risk of being preterm delivery between 26 and 32 weeks gestation in commonly is used to accelerate the pulmonary circulation and reduce mortalities (Hayes et al. 2008). We previously demonstrated that prenatal GC administration growth with development of cardiac functions in premature infant model rats (Sakurai et al. 2019; Mizuno et al. 2010; Tsuzuki et al. 2009).

The neonatal liver is generally immature and develops the structure and function during the early postnatal period. However, preterm infants with immature liver function are subject to risk of hypoglycemia, hyperbilirubinemia, cholestasis, bleeding, and impaired drug metabolism (Grijalva and Vakili 2013). Furthermore, low birth weight and premature birth infants often develop physiological jaundice with immature fetal liver function (Grijalva and Vakili 2013; Woodgate and Jardine 2015).

The role of GC has been demonstrated in association with differentiation of hepatocytes and the bile duct (Baribault and Marceau N 1986; Monga et al. 2001). Dexamethasone (DEX) is used for differentiation of

hepatic stem progenitor cells established from induced pluripotent stem (iPS) cells to hepatocyte maturation (Snykers et al. 2009).

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

Therefore, we predicted that prenatal GC administration would promote maturation of the liver during fetal development. The fetal liver cells are structural immature and have many stem cells. Various factors can be linked to maturation in the fetal period (Shiojiri 1997).

The expression levels of various enzymes, such as *tyrosine aminotransferase* (TAT), G6Pase (Glucose 6-phosphatase), and uridine diphosphate glucuronosyltransferase (UGT) 1A, increase rapidly from the late embryonic period to birth (Kamiya et al. 1999; Asahina et al. 2004; Kishi et al. 2008).

In this study, we aimed to elucidate the process of differentiation and maturation of hepatocytes that accompanies growth in the fetal liver.

Materials And Methods

1) Animal experiments

Based on previous reports, we determined the dose of GC (Tsuzuki et al. 2009; Mizuno et al. 2010; Sakurai et al. 2019). DEX (Fujifilm Wako Pure Chemical, Osaka, Japan) dissolved in sesame oil (Kanto Chemical, Tokyo)—at doses of 1.0 and 2.0 mg/kg—was administered subcutaneously on days 17 and 19 of the gestation period of the 8 weeks-old Wistar pregnant rats. The 8-week old pregnant rats have purchased from CLEA (Tokyo, Japan). Treatment of 2 doses of DEX given every 24 hours has been performed in clinical practice. Samatani et al. have been described the optimal regimen of DEX for fetal lung maturation during late gestation by pharmacokinetic/pharmacodynamics (PK/PD) simulation, when they injected the dose of total amount 6μM/kg in pregnant rats (Samtani et al. 2006a, b). We determined the dose of DEX by referring to these investigations. After mating, the vaginal smear was taken every morning, the day of finding sperm was designated as day 1 of pregnancy. The pregnant rats give birth to 10–14 neonates on day 22 of gestation. On day 19 and 21 fetal rats were delivered by cesarean section and liver samples were extracted under inhaled isoflurane anesthesia. To evaluate the change the factor with growth, liver samples were also extracted from spontaneously delivered 1-day-old neonates, 3-day-old neonates, 5-day-old neonates, and 8-week-old-adult rat. The 8-week-old adult rats were male.

Rats were kept at a constant temperature ($23 \pm 1^\circ\text{C}$), constant humidity ($55 \pm 5\%$), artificial 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).

After mating, the vaginal smear was taken every morning, the day of finding sperm was designated as day 1 of pregnancy. We selected equally or randomly the respective fetuses born out of three pregnant rats. The liver samples were used from different pregnant rats.

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 ($\times 400$).

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® sepaSol-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 (HNF4 α) 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 CSA® Biotin-free Tyramide Signal Amplification System (Dako, Carpinteria, CA, USA) was used to demonstrate immunoreactivity according to the manufacturer's instructions. The chromogenic substrate 3,3'-diaminobenzidine was used. The sections were then counterstained with hematoxylin.

The OLYMPUS CX41 upright microscope (Olympus, Tokyo, Japan) was used to acquire immunohistochemistry digital images. ImageJ 1.49v. processing software was used to calculate the density of the positive area.

5) HNF4α enzyme-linked immunosorbent assay (ELISA)

HNF4α protein levels were measured using Rat HNF4A/HNF4 ELISA Kit (LifeSpan Biosciences, WA, USA) according to the manufacturer's instructions. The tissues samples were prepared using CellLytic™ MT cell lysis reagent for mammalian tissues (Sigma-Aldrich, MO, US), and were quantified using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, CA, USA). Absorbance was measured at a wavelength of 450 nm using Viento nano (DS Pharma Biomedical, Osaka, JAPAN)

6) Statistical analysis

Results are shown as mean ± standard error of mean (SEM). JMP pro 13 (SAS Institute Inc., Cary, NC, USA) was used to perform the statistical analyses. All mRNA expression levels were analyzed statistically using non-parametric test, Steel test. Evaluation of protein levels was analyzed using Dunnett's test from the reason that the sample size is small. To compare the characteristics in the respective group, two-way ANOVA was used for the continuous variables, such as DEX treatment × age × measure.

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).

The cyclin B mRNA levels was significantly decrease in 19 groups with DEX 1.0 mg/kg administration. However, Ki-67 and CDK1 mRNA levels were not significantly changed. (Untreated group vs. DEX 1mg: Ki-67, 1.0 ± 0.2 vs. 0.53 ± 0.1 , $P = 0.075$; cyclin B, 1.0 ± 0.07 vs. 0.55 ± 0.09 , $P = 0.014$; CDK1, 1.0 ± 0.07 vs. 0.74 ± 0.11 , $P = 0.17$). Cyclin B mRNA levels decreased in 21F with administration of DEX 1.0 mg/kg (0.56-fold) compared with that of the untreated group. And CDK1 mRNA levels decreased in 21F with administration of DEX 1.0 mg/kg (0.43-fold) and 2.0 mg/kg (0.42-fold) compared with that of the untreated group.

3) Thy-1 and Dlk1 expressions

High levels of Thy-1 (Fig. 3A) and Dlk1 (Fig. 3E) mRNA expression were observed in 19F and the levels gradually decreased during growth.

Thy-1 mRNA levels tended to decrease in 21F with administration of DEX 1.0 mg/kg (0.43-fold) and 2.0 mg/kg (0.42-fold) compared with that of the untreated group; however, Dlk1 mRNA levels in the liver were unchanged after administration of prenatal DEX (Fig. 3B, F).

Thy-1 protein positive cells also decreased in 21F with administration of DEX 2.0 mg/kg ($28.7 \pm 2.6\%$) compared with the untreated group ($42.3 \pm 1.4\%$) (Fig. 3C, 3D).

4) Alpha-fetoprotein (AFP) and albumin mRNA and protein levels

In untreated groups, AFP mRNA levels gradually decreased from 19F to adult rats (Fig. 4A). Although AFP levels mRNA were increased in 19F with DEX 2.0 mg/kg administration, those were unchanged in the 21F with administration of DEX (Fig. 4B).

In contrast, albumin mRNA levels in the liver of 19F were low, and the levels gradually increased during growth (Fig. 4C, E, F).

Albumin mRNA (5.79-fold) and protein levels increased in the 19F with administration of DEX 2.0 mg/kg compared with the untreated group (Fig. 4D, E, G).

G6Pase and TAT mRNA levels in livers of 8W rats were higher than those of 19F rats; however, prenatal DEX administration did not affect 19F and 21F (Fig.S2).

5) HGF mRNA levels and protein levels

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

Administration of 1.0 mg / kg DEX in the 19F group increased 1.6-fold, and administration of 2.0 mg / kg DEX in the 21F group increased 2.9-fold compared with that of respective untreated groups (Fig. 5B).

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

6) HNF4α mRNA and protein levels.

HNF4α is involved in maturation of hepatocytes. HNF4α mRNA and protein levels in adult rats were higher than those in 19F rats (Fig. 6A, C).

Prenatal DEX administration significantly increased HNF4α mRNA levels both in the 19F (DEX 1.0 mg/kg, 1.6-fold: 2.0 mg/kg, 3.7-fold) and 21F (DEX 1.0 mg/kg, 3.2-fold: 2.0 mg/kg, 2.9-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).

7) Inter-treatment and age interacted

The obtained all data was compared using a two-way ANOVA variance test to detect any differences between groups, considering in the age, DEX treatment, and both factors.

The effect of age or DEX treatment was significant difference in the fetal hepatocyte size by two-way ANOVA. However, there was not significant difference between the hepatocyte size and both of the age and DEX (Table 2).

Significant difference between mRNA levels of Ki-67, Dlk1, TAT, and G6Pase and age was observed. However, there was no significant difference between those mRNA levels both of age and DEX treatment. The mRNA levels of cyclin B, CDK1, AFP, albumin, HGF, and HNF4 α showed significant differences between age or DEX treatment. There were not a significant differences between the respective gene expressions and both factors of the age and DEX treatment. In, Thy1, mTOR and Largen mRNA levels, there was no significant difference in the effects of age and DEX respectively. (Table 3).

The protein levels of albumin, HGF, and HNF4 α had differences between the age and DEX treatment. Under the influence of both factors of age and DEX treatment, was greater than the one age or DEX treatment (Table 4).

These results indicate that effects of the age and DEX treatment on gene expressions and protein production are independent.

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). We have previously confirmed the expression of MRP2 and albumin by prenatal GC administration (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). The results of the two-way ANOVAs (DEX treatment x age) presented significant main effects for DEX treatment and age but no interaction. Therefore, We cannot conclude that the increase in cell size due to DEX administrate is different between 19F and 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 significantly change Ki-67 mRNA expression i.e., DEX administration may have a little affect cell proliferation in fetal hepatocytes. However, another cell proliferation markers (ex. PCNA, MCM-2, and BrdU) need to evaluate in the future. Hepatocyte proliferation and differentiation for functional growth occur simultaneously during late mammalian gestation. Gruppuso et al. (1999) indicated the presence of independent signaling pathways for control of proliferation and differentiation in developing hepatocytes. The cell cycle of hepatocytes is controlled from G(0) to mitosis by the regulation of cyclins and CDK. The cyclin B1 protein forms a complex with 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. (2004) reported that Dlk-1 expression is strongest at murine fetal day 10.5 and is downregulated after fetal day 16.5. 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 G6Pase 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 G6Pase 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 G6Pase, 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 G6Pase, 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 (HNF4α) has essential roles in structure formation and function maturity of the hepatocyte (Li et al. 2000). HPCs are negative for HNF4α. They start expressing this transcription factor when they differentiate into hepatocytes (Parviz et al. 2003). The HNF4α 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).

HNF4α 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. (2006) have reported that continuous administration of prenatal DEX increases HNF4α mRNA expression in the rat liver. Our results in the animal model are close to clinical therapeutics and are consistent with those obtained by Nyirenda et al. (2006). Thus, increase in hepatic HNF4α expression relates to liver maturation.

The obtained all data was compared using a two-way ANOVA variance test to detect any differences between groups, considering in the age, DEX treatment, and both factors. Almost the gene and protein levels, except for some gene and protein were significant differences between the age or DEX treatment. These results indicate that effects of fetal age and DEX treatment on gene expression and protein production are independent. There is little effect on interaction between the age and DEX treatment.

Prenatal GC is used on late birth at < 36 week's gestation. Pregnant rats are normally deliver on gestational day 22, ie., full term birth. We examined day 17 and 19 of gestation period because at this gestational age the rats are premature enough to satisfy the definition of premature enough to satisfy the definition of prematurity yet mature enough to that the majority of them are able to survive without the need for supportive care (ex, mechanical ventilation) (Chen Y et al. 1994). In animal model, the remarkable maturation in the heart and the lung were demonstrated in our studies by prenatal GC administration. Therefore, we think the importance whether antenatal GC acts other organs including the liver in this study. Although, this study has not showed whether prenatal GC acts through the glucocorticoid receptor and other factors including the metabolic enzymes in liver function, we demonstrated antenatal GC contributes the maturation of liver. This knowledge is new. In the future, it is

necessary to investigate the liver function and adverse effects of prenatal glucocorticoid administration. We attempt to obtain a new interpretation about before 17-day fetuses in the further study.

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.

Declarations

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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

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

Not applicable

Consent for publication

Not applicable

References

1. Agnew EJ, Ivy JR, Stock SJ, Chapman KE (2018) Glucocorticoids, antenatal corticosteroid therapy and fetal heart maturation. *J Mol Endocrinol* 61:R61–R73. <https://doi.org/10.1530/JME-18-0077>
2. Asahina K, Fujimori H, Shimizu-Saito K, Kumashiro Y, Okamura K, Tanaka Y, Teramoto K, Arii S, Teraoka H (2004) Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells. *Genes Cells* 9:1297–1308. <https://doi.org/10.1111/j.1365-2443.2004.00809.x>
3. Baribault H, Marceau N (1986) Dexamethasone and dimethylsulfoxide as distinct regulators of growth and differentiation of cultured suckling rat hepatocytes. *J Cell Physiol* 129:77–84. <https://doi.org/10.1002/jcp.1041290112>
4. Bhutani VK, Wong RJ (2013) Bilirubin Neurotoxicity in Preterm Infants: Risk and Prevention. *J Clin Neonatol* 2:61–69. <https://doi.org/10.4103/2249-4847.116402>
5. Chen Y, Whitney PL, Frank L. (1994) Comparative responses of premature versus full-term newborn rats to prolonged hyperoxia. *Pediatr Res*. 35: 233-237. <https://doi.org/10.1203/00006450-199402000-00023>.
6. Dean S, Tang JI, Seckl JR, Nyirenda MJ (2010) Developmental and tissue-specific regulation of hepatocyte nuclear factor 4-alpha (HNF4-alpha) isoforms in rodents. *Gene Expr* 14:337–344. <https://doi.org/10.3727/105221610x12717040569901>
7. Derman E, Krauter K, Walling L, Weinberger C, Ray M, Darnell JE Jr. (1981) Transcriptional control in the production of liver-specific mRNAs. *Cell* 23:731–739. [https://doi.org/10.1016/0092-8674\(81\)90436-0](https://doi.org/10.1016/0092-8674(81)90436-0)
8. Fiegel HC, Park JJ, Lioznov MV, Martin A, Jaeschke-Melli S, Kaufmann PM, Fehse B, Zander AR, Kluth D (2003) Characterization of cell types during rat liver development. *Hepatology* 37:148–154. <https://doi.org/10.1053/jhep.2003.50007>
9. Grijalva J, Vakili K (2013) Neonatal liver physiology. *Semin Pediatr Surg* 22:185-189. <https://doi.org/10.1053/j.sempedsurg.2013.10.006>
10. Gruppuso PA, Bienieki TC, Faris RA (1999) The Relationship between Differentiation and Proliferation in Late Gestation Fetal Rat Hepatocytes. *Pediatr Res* 46:14–19. <https://doi.org/10.1203/00006450-199907000-00003>

11. Hayes EJ, Paul DA, Stahl GE, Seibel-Seamon J, Dysart K, Leiby BE, Mackley AB, Berghella V (2008) Effect of antenatal corticosteroids on survival for neonates born at 23 weeks of gestation. *Obstet Gynecol* 111:921–926. <https://doi.org/10.1097/AOG.0b013e318169ce2d>
12. Jochheim A, Hillemann T, Kania G, Scharf J, Attaran M, Manns MP, Wobus AM, Ott M (2004) Quantitative gene expression profiling reveals a fetal hepatic phenotype of murine ES-derived hepatocytes. *Int J Dev Biol* 48:23–29. <https://doi.org/10.1387/ijdb.15005571>
13. Kamiya A, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K, Taga T, Yoshida K, Kishimoto T, Miyajima A (1999) Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. *EMBO J* 18:2127–2136. <https://doi.org/10.1093/emboj/18.8.2127>
14. Kamiya A, Kinoshita T, Miyajima A (2001) Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways. *FEBS Lett* 492:90–94. [https://doi.org/10.1016/s0014-5793\(01\)02140-8](https://doi.org/10.1016/s0014-5793(01)02140-8)
15. Khulan B, Liu L, Rose CM, Boyle AK, Manning JR, Drake AJ. (2016) Glucocorticoids accelerate maturation of the heme pathway in fetal liver through effects on transcription and DNA methylation. *Epigenetics* 11:103–109. <https://doi.org/10.1080/15592294.2016.1144006>
16. Kishi M, Emi Y, Sakaguchi M, Ikushiro S, Iyanagi T (2008) Ontogenic isoform switching of UDP-glucuronosyltransferase family 1 in rat liver. *Biochem Biophys Res Commun* 377:815–819. <https://doi.org/10.1016/j.bbrc.2008.10.043>
17. Li J, Ning G, Duncan SA (2000) Mammalian hepatocyte differentiation requires the transcription factor HNF-4 α . *Genes Dev* 14:464–474. <https://doi.org/10.1101/gad.14.4.464>
18. Masson NM, Currie IS, Terrace JD, Garden OJ, Parks RW, Ross JA (2006) Hepatic progenitor cells in human fetal liver express the oval cell marker Thy-1. *Am J Physiol Gastrointest Liver Physiol* 291:G45–G54. <https://doi.org/10.1152/ajpgi.00465.2005>
19. Mizuno M, Takeba Y, Matsumoto N, Tsuzuki Y, Asoh K, Takagi M, Kobayashi S, Yamamoto H (2010) Antenatal Glucocorticoid therapy accelerates ATP production with creatine kinase increase in the growth-enhanced fetal rat heart. *Circ J* 74:171–180. <https://doi.org/10.1253/circj.cj-09-0311>
20. Monga SPS, Tang Y, Candotti F, Rashid A, Wildner O, Mishra B, Iqbal S, Mishra L (2001) Expansion of Hepatic and Hematopoietic Stem Cells Utilizing Mouse Embryonic Liver Explants. *Cell Transplant* 10:81–89. <https://doi.org/10.3727/000000001783986945>
21. Nava S, Westgren M, Jaksch M, Tibell A, Broomé U, Ericzon, BG, Sumitran-Holgersson S (2005) Characterization of cells in the developing human liver. *Differentiation* 73:249–260. <https://doi.org/10.1111/j.1432-0436.2005.00019.x>
22. Nishina H (2012) hDlk-1: a cell surface marker common to normal hepatic stem/progenitor cells and carcinomas. *J Biochem* 152:121–123. <https://doi.org/10.1093/jb/mvs069>
23. Nyirenda MJ, Dean S, Lyons V, Chapman KE, Seckl JR (2006) Prenatal programming of hepatocyte nuclear factor 4 α in the rat: A key mechanism in the 'foetal origins of hyperglycaemia'? *Diabetologia* 49:1412–1420. <https://doi.org/10.1007/s00125-006-0188-5>

24. Ozdemir H, Guvenal T, Cetin M, Kaya T, Cetin A (2003) A placebo-controlled comparison of effects of repetitive doses of betamethasone and dexamethasone on lung maturation and lung, liver, and body weights of mouse pups. *Pediatr Res* 53:98–103. <https://doi.org/10.1203/00006450-200301000-00017>
25. Panduro A, F Shalaby, D A Shafritz (1987) Changing patterns of transcriptional and post-transcriptional control of liver-specific gene expression during rat development. *Genes Dev* 1:1172–1182. <https://doi.org/10.1101/gad.1.10.1172>
26. Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA (2003) Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. *Nat Genet* 34:292–296. <https://doi.org/10.1038/ng1175>
27. Porter LA, Donoghue DJ (2003) Cyclin B1 and CDK1: nuclear localization and upstream regulators. *Prog Cell Cycle Res* 5:335–347.
28. Rog-Zielinska EA, Richardson RV, Denvir MA, Chapman KE. (2014) Glucocorticoids and foetal heart maturation; implications for prematurity and foetal programming. *J Mol Endocrinol* 52:R125-R135. <https://doi.org/10.1530/JME-13-0204>
29. Sata F. (2016) Developmental Origins of Health and Disease (DOHaD) and Epidemiology. *Jpn J Hyg* 71:41–46. (In Japanese) <https://doi.org/10.1265/jjh.71.41>
30. Samtani MN, Pyszczyński NA, Dubois DC, Almon RR, Jusko WJ. (2006a) Modeling glucocorticoid-mediated fetal lung maturation: I. Temporal patterns of corticosteroids in rat pregnancy. *J Pharmacol Exp Ther* 317:117-126. <https://doi.org/10.1124/jpet.105.095851>
31. Samtani MN, Pyszczyński NA, Dubois DC, Almon RR, Jusko WJ (2006b) Modeling glucocorticoid-mediated fetal lung maturation: II. Temporal patterns of gene expression in fetal rat lung. *J Pharmacol Exp Ther* 317:127-138. <https://doi.org/10.1124/jpet.105.095869>
32. Sakurai K, Osada Y, Takeba Y, Mizuno M, Tsuzuki Y, Ohta Y, Ootaki M, Iri T, Aso K, Yamamoto H, Matsumoto N (2019) Exposure of immature rat heart to antenatal glucocorticoid results in cardiac proliferation. *Pediatr Int* 61:31–42. <https://doi.org/10.1111/ped.13725>
33. Sell S (2008) Alpha-fetoprotein, stem cells and cancer: how study of the production of alpha-fetoprotein during chemical hepatocarcinogenesis led to reaffirmation of the stem cell theory of cancer. *Tumour Biol* 29:161–180. <https://doi.org/10.1159/000143402>
34. Shiojiri N (1997) Development and differentiation of bile ducts in the mammalian liver. *Microsc Res Tech* 39:328–335. [https://doi.org/10.1002/\(SICI\)1097-0029\(19971115\)39:4<328::AID-JEMT3>3.0.CO;2-D](https://doi.org/10.1002/(SICI)1097-0029(19971115)39:4<328::AID-JEMT3>3.0.CO;2-D)
35. Smith LM, Qureshi N, Chao CR (2000) Effects of single and multiple courses of antenatal glucocorticoids in preterm newborns less than 30 weeks' gestation. *J Matern Fetal Med* 9:131–135. [https://doi.org/10.1002/\(SICI\)1520-6661\(200003/04\)9:2<131::AID-MFM9>3.0.CO;2-M](https://doi.org/10.1002/(SICI)1520-6661(200003/04)9:2<131::AID-MFM9>3.0.CO;2-M)
36. Snykers S, De Kock J, Rogiers V, Vanhaecke T (2009) In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem Cells* 27:577–605. <https://doi.org/10.1634/stemcells.2008-0963>

37. Takeba Y, Sakurai K, Osada Y, Mizuno M, Tsuzuki Y, Ohta Y, Ootaki M, Watanabe M, Machida T, Watanabe D, Nakamura Y, Kobayashi T, Iiri T, Naoki Matsumoto (2018) Production of Bilirubin Transporters, Multidrug Resistance-Associated Protein 2 in the Fetal Rat Liver with Antenatal Glucocorticoid Administration. *St. Marianna Med J* 45: 281–287. (In Japanese)
38. Tanimizu N, Tsujimura T, Takahide K, Kodama T, Nakamura K, Miyajima A (2004) Expression of Dlk/Pref-1 defines a subpopulation in the oval cell compartment of rat liver. *Gene Expr Patterns* 5:209–218. <https://doi.org/10.1016/j.modgep.2004.08.003>
39. Tsuzuki Y, Takeba Y, Kumai T, Matsumoto N, Mizuno M, Murano K, Asoh K, Takagi M, Yamamoto H, Kobayashi S (2009) Antenatal glucocorticoid therapy increase cardiac α -enolase levels in fetus and neonate rats. *Life Sci* 85:609–616. <https://doi.org/10.1016/j.lfs.2009.06.017>
40. Woodgate P, Jardine LA. (2015) Neonatal jaundice: phototherapy. *BMJ Clin Evid*. 2015: 0319.
41. Wynne K, Rowe C, Delbridge M, Watkins B, Brown K, Addley J, Woods A, Murray H (2020) Antenatal corticosteroid administration for foetal lung maturation. *F1000 Faculty Rev* 219. <https://doi.org/10.12688/f1000research.20550.1>
42. Zhang W, Li W, Liu B, Wang P, Li W, Zhang H (2012) Efficient generation of functional hepatocyte-like cells from human fetal hepatic progenitor cells in vitro. *J Cell Physiol* 227:2051–2058. <https://doi.org/10.1002/jcp.22934>

Tables

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	TCGATCGGTTCATGCAGGAC	60	209
	Reverse	GCGACCCAGACTGAAGTTCA		
CDK1	Forward	GCCAGAAGTAGAGTCCCTGC	60	270
	Reverse	AGAGCCAACAGTAAACGCCA		
Thy-1	Forward	CCGCGTCAACCTTTTCAGTG	60	249
	Reverse	ATGAAGTCCGTGGCTTGGAG		
DLK1	Forward	CCCTGTGTGAGAAGTGCGTA	60	207
	Reverse	CAGAGAATCCAGGGGTGCAG		
AFP	Forward	ATTTGCCACGAGACGGAAC	60	273
	Reverse	TGCCTTGTCATACTGAGCGG		
Albumin	Forward	AAGAGAAAGCACTGGTCGCA	60	252
	Reverse	TGGCCTGGTTCTCACACATG		
HGF	Forward	GGCCATGGTGCTACACTCTT	60	206
	Reverse	CTTGTGGGGGTACTGCGAAT		
HNF4a	Forward	AGTGTCGTTACTGCAGGCTC	60	281
	Reverse	GTACTTGGCCCATTCGACCA		
HPRT	Forward	GCCCCAAAATGGTTAAGGTTG	60	176
	Reverse	TCCACTTTCGCTGATGACACA		

CDK1: cyclin-dependent kinase 1, Thy-1: Thymus cell antigen 1, DLK1: , AFP: a-fetoprotein, HGF: hepatocyte growth factor, HNF4a: Hepatocyte nuclear factor 4 of alpha, HPRT: hypoxanthine phosphoribosyltransferase

Table 2. ANOVA summary table for the cell size

	Age		DEX treatment		Age × DEX	
	<i>F</i>	<i>p</i> -value	<i>F</i>	<i>p</i> -value	<i>F</i>	<i>p</i> -value
Cell size	65.77	<0.001*	9.007	0.0052*	0.519	0.4765

Table 3. ANOVA summary table for the target gene expression.

	Age		DEX treatment		Age × DEX	
	<i>F</i>	<i>p</i> -value	<i>F</i>	<i>p</i> -value	<i>F</i>	<i>p</i> -value
Ki-67	8.940	0.0049*	0.018	0.8952	0.187	0.6681
Cyclin B	22.61	< .0001*	6.632	0.0140*	0.117	0.7347
CDK1	88.49	< .0001*	8.950	0.0049*	0.542	0.4661
Thy1	3.750	0.0603	0.944	0.3374	1.386	0.2464
DLK1	14.74	0.0005*	2.759	0.1050	0.733	0.3974
AFP1	9.149	0.0044*	4.428	0.0420*	0.927	0.3416
Albumin	16.95	0.0002*	5.160	0.0289*	0.894	0.3504
HGF	16.88	0.0002*	9.026	0.0047*	3.206	0.0813
HNF4a	79.10	<.0001*	28.62	<.0001*	6.019	0.0189*
mTOR	1.089	0.3034	0.212	0.6481	0.160	0.6918
Largen	0.286	0.5956	0.149	0.7016	0.507	0.4807
G6Pase	10.51	0.0025*	0.531	0.4708	0.314	0.5784
TAT	8.957	0.0049*	0.810	0.3741	0.837	0.3661

Table 4. ANOVA Summary table for the target protein levels.

	Age		DEX treatment		Age × DEX	
	<i>F</i>	<i>p</i> -value	<i>F</i>	<i>p</i> -value	<i>F</i>	<i>p</i> -value
Thy1	0.000	0.9955	11.93	0.0014*	12.16	0.0013*
Albumin	5.061	0.0303*	5.425	0.0253*	4.783	0.0350*
HGF	80.31	< .0001*	2.322	0.1359	1.607	0.2126
HNF4a	40.20	< .0001*	25.37	< .0001*	6.284	0.0166*
HNF4a by ELISA	23.08	0.0001*	34.26	<.0001*	0.236	0.6325

ELISA: Enzyme-Linked Immuno Sorbent Assay

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. Fetal liver, neonatal liver, and adult rat liver tissues: $n = 3$. Two non-adjacent locations were photographed, for each tissue specimen (C). * $P < 0.05$, ** $P < 0.01$, 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). Fetal liver, neonatal liver, and

adult rat liver sample (mRNA): n = 7. *P < 0.05 and **P < 0.01 vs. the respective 19 fetus group. †P < 0.05 and ††P < 0.01 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). Fetal, neonatal, and adult rat mRNA sample: n = 7. 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 specimen: n = 4. 21F, 1N, and 8W rat tissues specimen: n = 3. Two non-adjacent locations were photographed, for each tissue specimen (D). **P < 0.01 vs. the respective 19 fetus group. †P < 0.05 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). Fetal, neonatal, and adult rat mRNA sample: n = 7. 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 specimen: n = 4. 21F, 1N, and adult rat tissues specimen: n = 3. Two non-adjacent locations were photographed, for each tissue specimen (F) and (G). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the respective 19 fetus group. †P < 0.05 and ††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). Fetal, neonatal, and adult rat mRNA sample: n = 7. 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 specimen: n = 4. 21F, 1N, and 8W rat tissues specimen: n = 3. Two non-adjacent locations were photographed, for each tissue specimen (D). *P < 0.05 vs. the respective 19 fetus group. †P < 0.05, ††P < 0.01, and †††P < 0.001 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). Fetal, neonatal, and adult rat mRNA sample: n = 7. ELISA of HNF4α protein production in liver tissue after prenatal DEX (C) and (D). Fetal, neonatal, and adult rat protein extract sample: n = 4. 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 specimen: n = 4. 21F, 1N, 8W rat tissues specimen: n = 3. Two non-adjacent locations were photographed, for each tissue specimen (F). **P < 0.01 and ***P < 0.001 vs. the respective 19 fetus group. †P < 0.05, ††P < 0.01 and †††P < 0.001 vs the respective untreated group

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