Prenatal glucocorticoid administration accelerates the maturation of fetal rat hepatocytes

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

Methods and results Dexamethasone (DEX) was administered to pregnant Wistar rats on gestational days 17 and 19 before cesarean section. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine the mRNA levels of albumin, hepatocyte nuclear factor-4 alpha (HNF4α), hepatocyte growth factor (HGF), thymus cell antigen 1 (Thy-1), cyclin B, and Cyclin-dependent kinase 1 (CDK1) in the liver samples. Immunohistochemical staining and enzyme-linked immunosorbent assay were performed to examine protein production. The hepatocytes enlarged because of growth and prenatal DEX administration. Albumin, HNF4α, and HGF levels increased secondary to growth and prenatal DEX administration. The levels of the cell cycle markers cyclin B and CDK1 gradually decreased during growth and with DEX administration.

Conclusions The results suggest that prenatal GC administration leads to hepatocyte maturation via expression of HNF4α and HGF in preterm fetuses.

Keywords Glucocorticoid · Fetus · Liver · Maturation · Development · Cell proliferation

Introduction
Recently, the prevalence of low-birth-weight in Japan has been estimated to be approximately 10%, which is the highest among that in developed countries [1, 2]. Therefore, research focusing on the fetal and childhood periods is advocated. Preterm birth occurs before the physiological increase in endogenous glucocorticoid (GC) levels. GC action is important in the structural and functional maturation of various organs [3, 4].

Prenatal GC administration is commonly performed in mothers at risk of infant delivery between 26 and 32 gestational weeks to accelerate pulmonary circulation and reduce the incidence of mortality [5]. We previously demonstrated that prenatal GC administration promotes the growth and development of cardiac function in preterm infant rats [6]. However, not only the cardiac function, but the neonatal liver is also generally immature and its structure and function develop during the early postnatal period. Preterm human infants with immature liver function are at risk of hypoglycemia, hyperbilirubinemia, cholestasis, bleeding, and impaired drug metabolism. Moreover, low-birth-weight and preterm infants often develop physiological jaundice with immature fetal liver function [7, 8].

Hepatocyte proliferation and differentiation occur simultaneously during late mammalian gestation for liver mass growth [9]. GC has been reported to play a role in the differentiation of hepatocytes and the bile duct [10, 11]. DEX is administered for the differentiation of hepatic stem
progenitor cells established from induced pluripotent stem cells into hepatocytes and their subsequent maturation [12].

Therefore, we hypothesized that prenatal GC administration would promote liver maturation during fetal development. Prenatal GC administration has been widely performed to treat preterm birth-related diseases; however, its clinical use remains controversial owing to the associated toxicity or benefit. Zhang et al. reported that DEX inhibited hepatocyte proliferation via autophagy through the activation of the GC receptor (GR)-forkhead protein O1 (FOXO1) pathway in the fetal mouse liver [13]. Repetitive GC administration has been reported to decrease the liver weight of mice [14]. Prenatal GC administration has often resulted in growth retardation in fetal rodents [15]. These findings suggest that rodents have short gestation periods and exhibit rapid fetal growth. The toxic effects of GC vary according to the dosage, number of doses per day, time of administration in the gestational stage, and the number of administrations. Therefore, whether GC inhibits fetal liver maturation in our established preterm rat model needs to be investigated.

Repetitive DEX administration transiently and/or permanently affects fetal and neonatal growth [14]. Fetal hepatocytes are structurally immature and have several stem cells. Various factors have been associated with liver and hepatocyte maturation in the fetal period. Albumin and alpha-fetoprotein (AFP) gene expressions as proliferative hepatoblast markers appear simultaneously in 17–19-day-old fetal rat hepatocytes. Albumin concentration increases gradually from day 12 of fetal development to after birth in mice [16]. However, AFP levels decline extremely rapidly and become undetectable in adult mice. At a late gestational or perinatal stage, glucose 6-phosphatase (G6Pase) is predominantly expressed in the liver. Tyrosine aminotransferase (TAT) is an excellent enzymatic marker of peri- or postnatal hepatocyte-specific differentiation. These enzymes play important roles in glucose metabolism and are rapidly activated during the neonatal developmental period [17, 18].

The hepatocyte maturation factors induced the expression of mature hepatocyte-specific genes in differentiating progenitor hepatocytes, suggesting that these cells have the potential to become mature hepatocytes. These cells differentiate into hepatocytes and epithelial cells of the bile ducts and subsequently form connective tissue around the portal vein, the GC hormone, and basal layer component [19].

In light of the above, we aimed to clarify the progression of liver maturation during fetal development by analyzing the expression of growth factors and maturation markers. We subsequently demonstrated whether GC activates the expression of these factors for fetal liver maturation.

**Materials and methods**

**Animal experiments**

We determined the dose of GC based on previous reports [6]. Wistar rats were purchased from CLEA (Tokyo, Japan). 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 to 8-week-old pregnant Wistar rats. In clinical practice, two doses of DEX have been administered every 24 h. Samatani et al. administered a total dose of 6 µM/kg in pregnant rats and described the optimal regimen of DEX for fetal lung maturation during the late gestation period using pharmacokinetic/pharmacodynamics simulation [20, 21]. We determined the dose of DEX by referring to these previous literatures. After mating, vaginal smears were obtained every morning. The day of finding sperms in the vaginal smear samples was designated as day 1 of pregnancy. The pregnant rats gave birth to a newborn on day 22 of gestation. On days 19 and 21, fetal rats were delivered by cesarean section, and liver samples were extracted under inhaled isoflurane anesthesia. A part of each liver sample was frozen in liquid nitrogen for mRNA and protein extraction and the other part was fixed in 10% formalin for subsequent histological evaluation. To evaluate the effect of age on liver maturation, the liver samples were also extracted from spontaneously delivered 1-day-old (1 N), 3-day-old (3 N), and 5-day-old (5 N) neonates as well as 8-week-old (8 W) adult rats.

The rats were kept under a constant temperature (23 ± 1 °C) and constant humidity (55% ± 5%) at the Laboratory Animal Care and Management Facility of St. Marianna University School of Medicine. They were provided unlimited drinking water and kept at a 12-h light/12-h dark cycle. This study complied with the “Guiding principles for the care and use of laboratory animals” of The Japanese Pharmacological Society and was approved by the Experimental Animal Research Committee of St. Marianna University Graduate School of Medicine (approval number: 2002008).

**Histological evaluation**

Rat liver samples were preserved in 10% formalin, embedded in paraffin, and cut into 5-µm sections. The sections were stained with hematoxylin and eosin. Tissue specimens of four 19-day fetuses (19F) and three rats (21-day fetuses [21F], 1 N, 3 N, 5 N, and 8 W) were used for the
histological evaluation. For each specimen, two nonadjacent fields were considered for counting the number of hepatocyte under a microscope. The hepatocyte size was determined as the total number of hepatocytes by area of view ($2.8 \times 10^4 \mu m^2$) under a microscope with high magnification ($\times 400$).

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

Total RNA was extracted from the liver tissues using an RNeasy® sepa- sol-RNA I super G kit (Nakarai, Kyoto, Japan). A NanoDrop One/OneC Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentration of total RNA at an optical density of 260 nm. cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diadiagnostic GmbH, Mannheim, Germany). RT-PCR was performed using Step One Plus and Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific). The PCR conditions were as follows: 45 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 30 s, and extension at 72 °C for 40 s.

Gene expression was determined using the relative values of standard curve values. In each sample, the relative value was normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase ($HPRT$). The primers used are shown in Supplemental Table 1 and Supplemental Table 2. The total number of samples in each group was seven.

**Immunohistochemistry**

The liver samples were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was inactivated with 0.1% hydrogen peroxidase. After blocking, the samples were treated with anti-albumin antibody (Proteintech, IL, USA), anti-hepatocyte growth factor (HGF) antibody (Bioss Antibodies, MA, USA), anti-hepatocyte nuclear factor 4 alpha (HNF4α) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-thymus cell antigen 1 (Thy1) antibody (Abcam, Cambridge, UK) overnight at 4 °C. A CSAII Biotin-free Tyramide Signal Amplification System (Dako, Carpinteria, CA, USA) was used to demonstrate immunoreactivity according to the manufacturer’s instructions. 3,3'-diaminobenzidine was used as the chromogenic substrate. The samples were then counterstained with hematoxylin.

The OLYMPUS CX41 upright microscope (Olympus, Tokyo, Japan) was used to acquire immunochemistry digital images. ImageJ version 1.49 software was used to calculate the density of the positive area. Tissue specimens of four fetuses (19F) and three rats (21F, 1 N, 3 N, 5 N, and 8 W) were used for immunohistochemistry analysis. The ratio of positive area in one field was evaluated under a microscope.

**Determination of HNF4α levels**

HNF4α protein levels were measured using Rat HNF4A/HNF4 enzyme-linked immunosorbent assay (ELISA) Kit (LifeSpan Biosciences, WA, USA) according to the manufacturer’s instructions. The tissues samples were lysed using CelLytic™ MT cell lysis reagent for mammalian tissues (Sigma-Aldrich, MO, US) and the total protein was 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). Four samples in each group were used for the ELISA assay.

**Statistical analyses**

The results are presented as mean ± standard error of the mean. JMP pro 13 (SAS Institute Inc., Cary, NC, USA) software was used to perform statistical analyses. The obtained data were compared using two-way analysis of variance (ANOVA) to detect any intergroup differences in age, DEX administration, and an interaction of both these factors. Subsequently, a multiple comparison was performed using the Dunnett’s test.

**Results**

**Interaction effect of age and DEX administration**

Table 1 and Supplemental Table 3 show the ANOVA results of the effect of age and DEX administration on hepatocyte cell size, mRNA expression, and the protein levels of growth factors. The size of hepatocytes showed either the age effect or DEX effect alone, but not the interaction effect of both factors. DEX administration significantly enlarged hepatocytes in a dose-dependent manner. Age had an effect on all mRNA levels except those for Thy-1. Thy-1 protein levels showed an effect in terms of both age and DEX administration. Ki-67, cyclin B, and cyclin-dependent kinase 1 (CDK1) mRNA levels were decreased in 19F and 21F rats with DEX (Ki-67, $P = 0.8952$, cyclin B, $P = 0.0140$; CDK1, $P = 0.0049$). In contrast, delta-like 1 homolog (DLK1), AFP, albumin, and HGF mRNA levels were increased in 19F rats but not in 21F rats with DEX (DLK1, $P = 0.1050$; AFP, $P = 0.0420$; albumin, $P = 0.0289$; HGF, $P = 0.0047$). HNF4α mRNA and protein levels were significantly increased in 19F and 21F rats with DEX (HNF4α mRNA, $P < 0.0001$; HNF4α protein, $P < 0.0001$). Furthermore, these results showed the interaction effect of age and DEX administration. Although HNF4α protein levels measured by ELISA
were also significantly increased in 19F and 21F rats with DEX, the interaction effect of age and DEX administration was not observed (HNF4α [ELISA], P > 0.0001). The reason may be the variation of individual values (Table 1, Supplemental Table. 3).

| Table 1 | Mean, SEM, ANOVA for the cell size, mRNA expression, and protein levels |
|------------------|------------------|------------------|
| **Cell size (µm²)** | 19F, Mean (SEM) | 21F, Mean (SEM) |
| 0 | 105.7 (5.0) | 106.4 (3.6) |
| 1 | 145.0 (13.2) | 145.0 (13.2) |
| 2 | 164.9 (12.3) | 167.5 (14.0) |
| **mRNA** |  |  |
| Ki-67 | 1.00 (0.19) | 0.53 (0.10) |
| Cyclin B | 1.00 (0.08) | 0.55 (0.10) |
| CDK1 | 1.00 (0.07) | 0.74 (0.11) |
| Thy1 | 1.00 (0.08) | 0.50 (0.18) |
| DLK1 | 1.00 (0.08) | 0.88 (0.26) |
| AFP1 | 1.00 (0.07) | 1.50 (0.22) |
| Albumin | 1.00 (0.09) | 3.42 (0.47) |
| HGF | 1.00 (0.05) | 1.62 (0.17) |
| HNF4α | 1.00 (0.07) | 1.64 (0.16) |
| **Protein** |  |  |
| Thy1 (%) | 36.4 (1.4) | 35.6 (1.9) |
| Albumin (%) | 20.2 (5.6) | 46.4 (4.9) |
| HGF (%) | 15.5 (3.1) | 25.4 (1.9) |
| HNF4α (%) | 34.9 (2.7) | 42.8 (1.7) |
| HNF4α by ELISA (pg/µg) | 18.2 (3.2) | 51.7 (6.2) |
| **Age** |  |  |
| DEX treatment |  |  |
| 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 |
| **Cell size (µm²)** | 69.8 (2.7) | 105.7 (5.0) | 106.4 (3.6) | 145.0 (13.2) | 164.9 (12.3) | 167.5 (14.0) |
| mRNA |  |  |  |  |  |  |  |  |
| Ki-67 | 1.00 (0.19) | 0.53 (0.10) | 0.91 (0.28) | 0.36 (0.04) | 0.47 (0.10) | 0.41 (0.05) |
| Cyclin B | 1.00 (0.08) | 0.55 (0.10) | 0.77 (0.14) | 0.56 (0.09) | 0.32 (0.07) | 0.27 (0.07) |
| CDK1 | 1.00 (0.07) | 0.74 (0.11) | 0.83 (0.10) | 0.47 (0.04) | 0.20 (0.03) | 0.20 (0.02) |
| Thy1 | 1.00 (0.08) | 0.50 (0.18) | 1.05 (0.38) | 0.81 (0.22) | 0.35 (0.14) | 0.34 (0.12) |
| DLK1 | 1.00 (0.08) | 0.88 (0.26) | 1.53 (0.34) | 0.40 (0.10) | 0.46 (0.15) | 0.57 (0.20) |
| AFP1 | 1.00 (0.07) | 1.50 (0.22) | 1.49 (0.14) | 0.81 (0.08) | 1.01 (0.07) | 0.99 (0.25) |
| Albumin | 1.00 (0.09) | 3.42 (0.47) | 5.79 (1.44) | 6.40 (2.53) | 10.5 (1.53) | 8.37 (1.41) |
| HGF | 1.00 (0.05) | 1.62 (0.17) | 2.11 (0.41) | 2.36 (0.33) | 4.83 (1.08) | 6.76 (1.96) |
| HNF4α | 1.00 (0.07) | 1.64 (0.16) | 3.69 (0.34) | 3.75 (0.46) | 11.81 (1.03) | 10.99 (1.34) |
| **Protein** |  |  |  |  |  |  |  |  |
| Thy1 (%) | 36.4 (1.4) | 35.6 (1.9) | 42.3 (1.4) | 37.5 (2.7) | 28.7 (2.6) |
| Albumin (%) | 20.2 (5.6) | 46.4 (4.9) | 47.8 (8.7) | 57.2 (3.3) | 46.6 (3.9) |
| HGF (%) | 15.5 (3.1) | 25.4 (1.9) | 49.2 (3.4) | 59.9 (3.3) | 49.4 (4.9) |
| HNF4α (%) | 34.9 (2.7) | 42.8 (1.7) | 43.3 (3.1) | 60.8 (1.6) | 65.1 (2.2) |
| HNF4α by ELISA (pg/µg) | 18.2 (3.2) | 51.7 (6.2) | 34.9 (2.7) | 65.6 (2.8) | 74.4 (12.7) |

**CDK1** cyclin-dependent kinase 1, **Thy-1** Thymus cell antigen 1, **DLK1** delta-like 1 homolog, **AFP** alpha-fetoprotein, **HGF** hepatocyte growth factor, **HNF4α** hepatocyte nuclear factor 4 of alpha, **ELISA** enzyme-linked immunosorbent assay, **SEM** standard error of mean, *P < 0.05
Hepatocyte size

With growth, the size of the hepatocytes gradually increased from the fetuses (19F: 69.8 ± 2.7 µm² and 21F: 145.0 ± 13.2 µm²) to the neonates (1 N: 164.1 ± 13.4 µm²; 3 N: 162.9 ± 5.2 µm²; and 5 N: 231.0 ± 10.2 µm²) to the adult rats (359.1 ± 36.0 µm²) (Fig. 1A, B). Prenatal DEX administration significantly increased the cell size in 19F rats (DEX 1.0 mg/kg: 105.7 ± 4.95 µm²; 2.0 mg/kg: 106.4 ± 3.64 µm²) (Fig. 1C). Pan paniscus proline rich 16 (PRR16) mRNA and target of rapamycin (mTOR) levels were not affected in 19F and 21F rats in terms of the process of growth or prenatal DEX administration (Fig. S1).

Ki-67, cyclin B, and CDK1 mRNA levels

The mRNA expressions of proliferation and cell cycle markers Ki-67, cyclin B, and CDK1 gradually decreased from 19F rats to adult rats. The mRNA levels of cyclin B were significantly decreased in the 19F group following DEX 1.0 mg/kg administration. However, the mRNA levels of Ki-67 and CDK1 were not significantly changed (untreated group vs. DEX 1 mg/kg: cyclin B: 1.0 ± 0.07 vs. 0.55 ± 0.09, P = 0.015 and CDK1: 1.0 ± 0.07 vs. 0.74 ± 0.11, P = 0.127). The mRNA levels of cyclin B decreased in the 21F group following DEX 2.0 mg/kg administration (0.48-fold) compared with that in the untreated group. The mRNA levels of CDK1 decreased in the 21F group with DEX 1.0 mg/kg (0.43-fold) and 2.0 mg/kg (0.42-fold) administration compared with that in the untreated group (Fig. 2).

Thy-1 and Dlk1 mRNA and protein levels

High mRNA expression levels of Thy-1 and Dlk1 were observed in 19F rats, and the levels gradually decreased with growth. The mRNA levels of Thy-1 tended to decrease in 21F rats following DEX 1.0 mg/kg (0.43-fold) and 2.0 mg/kg (0.42-fold) administration compared with that in the untreated group; however, the mRNA levels of Dlk1 in the liver were unchanged after prenatal DEX administration (Fig. S2A, Fig. S2D). The number of Thy-1-protein-positive cells also decreased in 21F rats following DEX 2.0 mg/kg administration (28.7 ± 2.6%) compared with that in the untreated group (42.3 ± 1.4%) (Fig. S2C).
AFP and albumin mRNA and protein levels

In the untreated group, the AFP mRNA levels gradually decreased from 19F rats to adult rats. The AFP mRNA levels did not change in the 19F and 21F rats after DEX administration (Fig. S3A). In contrast, the albumin mRNA levels in the liver of 19F rats were low and gradually increased with growth. The albumin mRNA (5.79-fold) and protein levels increased in 19F rats following DEX 2.0 mg/kg administration compared with that in the untreated group (Fig. S3B–D).

The glucose 6-phosphatase (G6Pase) and TAT mRNA levels in the livers of 8 W rats were higher than those in the livers of 19F rats; however, prenatal DEX administration did not affect G6Pase and TAT mRNA levels in 19F and 21F rats (Fig. S4).

Fig. 2 Ki-67, cyclin B, and CDK1 mRNA and protein levels. Ki-67, cyclin B, and cyclin-dependent kinase 1 (CDK1) mRNA levels were determined in liver tissues in 19F and 8 W rats (A), (C), and (E). Ki-67, cyclin B, and CDK1 mRNA levels were determined in fetal rats after prenatal DEX administration (B), (D), and (F). **P < 0.01 and ***P < 0.001 vs. the respective 19F group. †P < 0.05 and †††P < 0.001 vs. the respective untreated group
HGF mRNA and protein levels

The HGF mRNA levels increased two-fold in the 1 N rats compared with that in the 19F rats. In addition, the administration of 2.0 mg/kg DEX in 21F rats increased the HGF mRNA levels about three-fold as compared to that in the respective untreated group (Fig. 3A). In the immunohistochemistry analysis, HGF production increased in the liver tissue of 19F rats after prenatal DEX administration (C). HGF positive staining intensity was analyzed using NIH image analysis. **P < 0.01 vs. the respective 19F group. †P < 0.05 and †††P < 0.001 vs. the respective untreated group.

HNF4α mRNA and protein levels

HNF4α is involved in hepatocyte maturation. The HNF4α mRNA and protein levels in adult rats were higher than those in 19F rats. HNF4α mRNA levels were significantly increased in 19F rats with 2.0 mg/kg DEX (3.7-fold) and in 21F rats with 1.0 mg/kg (3.2-fold) and 2.0 mg/kg DEX (2.9-fold) compared with that in the untreated group (Fig. 4A). The HNF4α protein levels were also significantly increased in 19F (DEX 2.0 mg/kg, 51.7 ± 6.2 pg/µg) and 21F (DEX 1.0 mg/kg, 65.6 ± 2.8 pg/µg; DEX 2.0 mg/kg, 74.4 ± 12.7 pg/µg) rats after DEX administration compared with that in the untreated group (19F: 18.2 ± 3.2 pg/µg and 21F: 34.9 ± 2.7 pg/µg) (Fig. 4B).
The immunohistochemical staining results showed that the HNF4α protein levels in 19F rats following DEX administration (1.0 mg/kg: 48.4 ± 2.1% and 2.0 mg/kg: 42.8 ± 1.7%) significantly increased compared with that in the untreated group (34.9 ± 2.7%). In addition, DEX administration significantly increased the HNF4α protein levels in 21F rats (1.0 mg/kg: 60.8 ± 1.6% and 2.0 mg/kg: 54.2 ± 1.9%).
Discussion

Preterm infants are at risk of hepatic insufficiency because the immaturity of liver results in a delay in achieving detoxification and synthetic functions [22]. Prenatal GC administration accelerates the development of cardiopulmonary functions thereby reducing respiratory distress syndrome and mortality in preterm infants [23, 24]. GC induces organ maturation and is necessary for sustaining extruterine life [25]. We previously confirmed the expression of MRP2 and albumin following prenatal GC administration in the fetal liver [26]. However, the mechanisms of fetal liver growth following prenatal GC administration remain unclear. Recent studies reported that DEX suppressed growth and induced hepatocytes maturation on the cultured hepatocytes of rats [17, 27]. In this study, we verified that from 19F to 5N, the hepatocyte size gradually increased. Additionally, prenatal DEX administration increased the size of fetal rat hepatocytes. On day 15 of gestation, 30% of fetal rat liver volume consisted of hepatocytes. On day 18, the volumetric densities of hepatocytes and bile canaliculi were increased. After birth, the volumetric densities of hepatocytes increased and occupied approximately 74% of the liver [28]. These data have been already been demonstrated and it is concluded that perinatal changes in hepatocytes size are related to the accumulation and discharge of glycogen and lipid [28]. In humans, the overexpression of PRR16/Largen and mTOR gene is known to be associated with increases in hepatocyte size [29, 30]. However, in the present study, prenatal GC administration did not affect mTOR or PRR16 expression. Although these factors were not directly related to increase in hepatocyte cell size, it is imperative to consider other factors. Processes essential to attaining adequate liver mass and function during the fetal period include activation of a specific cell lineage in early development and cell proliferation or differentiation, accomplished by enzymes and transcription factors. We investigated whether hepatocyte enlargement depends on cell proliferation or differentiation. High mRNA levels of the cell proliferation marker Ki-67 were found in 19F fetal hepatocytes, which gradually decreased with growth. This indicates that the capacity for cell proliferation declines with liver growth. Furthermore, prenatal DEX administration did not change Ki-67 mRNA expression. Hepatocyte proliferation and differentiation for functional growth occur during late mammalian gestation. Gruppuso et al. reported the presence of independent signaling pathways that control the proliferation and differentiation of developing hepatocytes [9]. The cell cycle of hepatocytes is controlled from the G0 phase to the mitotic phase (M) by the regulation of cyclins and CDKs. Cyclin B1 forms a complex with CDK1, and the activation of this complex initiates mitosis [31]. Thus, cyclin B1 is a key marker for M phase initiation. In the present study, cyclin B and CDK1 mRNA levels significantly decreased from 19F to 1N and adult rats. Additionally, prenatal DEX administration decreased cyclin B and CDK1 mRNA levels in 19F and 21F rats. The transcriptional inhibition of the cyclin B–CDK1 complex by prenatal DEX administration limited cell proliferation. Precursor cells in the liver continue to divide and grow until a fully differentiated state is attained. Terminal differentiation leads to cell proliferation arrest and permanent exit from the cell division cycle [32]. Therefore, prenatal GC administration may have suppressed cell proliferation and promoted differentiation into mature hepatocytes.

Premature hepatocytes include hepatic progenitors during hepatocyte development [33]. Thy-1 and Dlk-1 are stem cell markers that can identify stem cell character [34, 35]. With growth, the mRNA levels of Dlk-1 significantly decreased in the fetal liver. Prenatal DEX administration tended to decrease Thy-1 mRNA levels in 21F rats. Tanimizu et al. reported that Dlk-1 expression was strongest at murine fetal day 10.5 and was downregulated after fetal day 16.5. Because Dlk-1 expressions in the livers of 19F and 21F rats were negligible, DEX may not have had any effect [36]. The early rat fetal liver also contains Thy-1-positive cells, which gradually decrease in number with growth. The mRNA and protein levels of Thy-1 were sustained in 21F rats but decreased after birth. Therefore, DEX may have led to the decrease in Thy-1 expression, which suggests that DEX activates transcription or growth factors and leads to hepatocyte differentiation. Hepatocyte differentiation is controlled by a complex network of transcription factors. Yin et al. reported that HNF4α reduced the proportion of stem/progenitor cells and suppressed the expression of AFP and Ki-67 [37].

Hepatic maturation is characterized according to the stage of liver growth with specific genes [38, 39]. Some studies have reported a high level of AFP in fetal rat hepatocytes, and the AFP level gradually decreased with hepatic maturation. In contrast, albumin and G6Pase levels were reported to be increased in mature hepatocytes [16, 40]. In this study, AFP mRNA levels decreased in the liver of 19F rats during growth. Our findings are in agreement with those obtained by Zhang et al. In that study, AFP levels decreased as differentiation progressed in human hepatic progenitor cells (HPCs). Cells exposed to a mixture of oncostatin M, DEX, and HGF gradually exhibited differentiated hepatic functions, including albumin production in vitro [41]. Prenatal DEX administration did not change AFP mRNA levels in the fetal liver. The mRNA levels of albumin, TAT, and G6Pase increased in the liver from fetal rats to neonatal rats. DEX administration only increased
albumin production in 19F rats. HPCs morphologically and functionally differentiate into hepatocytes and cholangiocytes. Freshly isolated HPCs coexpressed G6Pase, glycogen, albumin, and gamma glutamyl transpeptidase and could differentiate into functional liver cells [42]. A combination of HGF, oncostatin M, and DEX induce hepatocyte maturation [43]. HGF in the presence of DEX induced the expression of G6Pase, TAT, and albumin in fetal hepatocytes. HGF appears in the liver in the first few days after birth. We found that the mRNA and protein levels of HGF were increased in the liver of 19F rats after prenatal DEX administration, suggesting that DEX may also have a direct effect on HGF level elevation. HGF resulted in a slight decrease in cell proliferation and activated HNF4α transcription. HGF and epidermal growth factor are regulated in the formation of biliary epithelium and connective tissue [26]. Further research is needed to clarify the underlying mechanism.

HNF4α plays essential roles in the structural formation and functional maturity of hepatocytes [44]. HPCs are negative for HNF4α. They start expressing HNF4α following their differentiation into hepatocytes [45]. HNF4α has two promoters of P1 and P2 isoforms in the mouse liver. Transcription starts via the P2 promoter during fetal life but switches to P1 at birth [46]. We speculate that exposure to DEX in the fetal rat liver suppresses P2 but enhances the expression of transcripts from P1.

The mRNA and protein levels of HNF4α were significantly increased in the liver with growth. Prenatal DEX administration also increased their levels in the liver of 19F and 21F rats. Nyirenda et al. reported that continuous administration of prenatal DEX increased HNF4α mRNA expression in rat livers [47]. Our results are consistent with those obtained by Nyirenda et al. Thus, increase in HNF4α and HGF levels is related to liver maturation. However, other relevant factors might also play a role and will be studied in future research.

A limitation of the present study is that we did not investigate whether prenatal GC administration acts through GC receptor-binding or indirectly for liver maturation. Further studies are necessary to elucidate these mechanisms using isolated fetal hepatocytes. In addition, it is essential to consider the differences in the effects of DEX between men and women. Few studies have reported on sex differences in factors affecting fetal liver differentiation and maturation. Van den Akker et al. reported no difference in GC receptor mRNA levels between boys and girls with sepsis [48]. However, Duma et al. reported a difference in GC therapy between male and female adrenalectomized rats [49]. At present, the efficacy and toxicity of prenatal GC administration remains the subject of future research.

Conclusion

We conclude that hepatocyte differentiation for the maturation of liver begins on day 19 and day 21 of gestation. These results suggest that prenatal GC administration induces hepatocyte differentiation, and liver maturation is achieved via the expression of HNF4α and HGF in preterm fetuses.

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Data availability All data generated or analyzed during this study are included in this article and its supplementary information.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This study complied with the “Guiding principles for the care and use of laboratory animals” of 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.

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