Biochemical and Biophysical Research Communications 382 (2009) 46–50

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of hepatic sexual dimorphism in Alb-DsRed2 transgenic rats

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ARTICLE INFO

Article history:
Received 29 January 2009
Available online 27 February 2009

Keywords:
Sexual dimorphism
Pituitary hormone
Hepatocytes
Transgenic rat

ABSTRACT

We previously created the Alb-DsRed2 transgenic (Tg) rat that specifically expresses the red fluorescent protein, DsRed2, in the liver. Herein, we demonstrate that the DsRed2 expression is sexually dimorphic and exhibits a male-specific pattern. The profiling of sexual dimorphism in DsRed2 expression during pre-pubertal development was investigated using an *in vivo* fluorescent imaging analysis. The DsRed2 expression decreased gradually in both sexes until 28 days after birth. While DsRed2 expression was not persistent in the female liver, the male hepatic expression increased again at 35 days. Sexual dimorphic DsRed2 expression did not change in gonadectomized male and female Tg-rats. However, female hepatic DsRed2 was induced 72 h after the hypophysectomy. Hepatocytes isolated from the female Tg-rats also revealed DsRed2 induction by 96 h in culture. These results suggest that the pituitary hormone suppresses the female hepatic DsRed2 expression causing the sexual dimorphism of DsRed2 expression.

Introduction

Physiological differences between males and females exist in a variety of tissues, including some not usually considered sexually dimorphic. The rodent liver is one example that has provided an excellent model for investigating the complex interplay of hormonal, developmental, and tissue-specific control of gene expression [1,2]. There is a sex-specific pattern of liver gene expression for several cytochrome P450 enzymes involved in steroid and xenobiotic metabolism [3,4]. Early studies of male-specific hepatic gene expression revealed that neonatal exposure to testosterone initiated male-specific pulsatile secretion of growth hormone (GH) from the pituitary [5]. Male-specific gene expression in the adult male liver is maintained by pulsatile GH secretion [6,7]. On the other hand, adult female rats are characterized by a more frequent pituitary GH release and the near-continuous presence of GH in plasma [8]. Many studies have focused on male-specific pulsatile GH-dependent gene activation [9,10]. However, there is little information on the inactivation of male-specific genes in the female liver.

In a previous article we reported the development of the Alb-DsRed2 transgenic (Tg) rat that was designed with liver-specific expression of the red fluorescent protein, DsRed2 [11]. Interestingly, the expression of DsRed2 in this Tg animal is repressed in the female liver during pre-pubertal development, and hepatic DsRed2 is considered a male-specific protein resulting from sexually dimorphic regulation. In the current study, we focused on the regulation of hepatic DsRed2 expression in the Tg rat as a model to explore the repression mechanism of male-specific genes in the female liver. To determine the expression level of hepatic DsRed2, we employed an *in vivo* bio-imaging system based on the detection of fluorescence. The *in vivo* imaging of light-emitting probes, such as fluorescent proteins or firefly luciferase, is a powerful strategy that has enabled a wide range of biological studies to be undertaken in living animals [11–13]. Imaging reporters with light emission in the red to infrared wavelengths (>600 nm) are preferred because of the high transmission of light through tissues at these wavelengths. DsRed2, therefore, is an attractive choice for *in vivo* applications for this reason [14]. Herein, we demonstrate that hepatic DsRed2 expression levels can be evaluated quantitatively using an *in vivo* fluorescent imaging system. The use of this system revealed that pituitary hormone-mediated repression of DsRed2 in the female liver contributed to the sexually dimorphic expression of hepatic DsRed2.

Materials and methods

Experimental animals. Alb-DsRed2 transgenic male and female rats [Wistar-Tg(Alb-DsRed2)34Jmsk], which were established as

Abbreviations: GH, Growth hormone; Tg, transgenic; IVIS, *in vivo* bio-imaging system; Hypox, hypophysectomy; STAT, signal transducer and activator of transcription.

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doi:10.1016/j.bbrc.2009.02.119

reported previously [11], were used in all experiments. To evaluate the effect of gonadal hormones and pituitary hormones on hepatic DsRed2 expression, 5-week-old male and female rats were gonadectomised and 8-week-old male and female rats were hypophysectomised, respectively. The effect of gonadectomy was investigated 3 weeks after the operation and the effect of hypophysectomy was investigated 72 h after the operation. The effectiveness of the hypophysectomy was verified by the absence of pituitaries or fragments at necropsy. All experiments in this study were performed in accordance with the Jichi Medical University Guide for Laboratory Animals.

Quantification of hepatic DsRed2 expression. The animals were anaesthetized by isoflurane and operated upon to expose the liver. DsRed2 expression levels in the liver were quantified *in vivo* using the non-invasive bio-imaging system IVIS™ (Xenogen, Alameda, CA). DsRed2 fluorescence was excited by light at 560 nm, and light emission at 600 nm from the liver tissue was collected using a cooled charge-coupled device camera [14]. Living Image software (Xenogen) was used to quantify the fluorescent signals, which were expressed as digitizing unit (photon/sec/cm^2/steradian). The expression of DsRed2 was also visualized using fluorescent microscopy under 560-nm excitation light.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total hepatic RNA was extracted using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. To eliminate genomic DNA, extracted RNA was incubated with 0.05 U/μl DNase I (Ambion) at 37 °C for 60 min. Hepatic RNA was reverse transcribed in 20-μl reaction mixes containing 1 nmol oligo dT (18mer), 400 U ReverTra Ace (Toyobo, Japan), 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 U human placental RNase inhibitor (Takara, Tokyo, Japan) and 1.1 mM dNTPs, and incubated at 42 °C for 60 min [15]. PCR was performed using specific primer sets for rat CYP2C11 (upstream 5′-TAC TTT CCC TGC CAT TAT TGA TTA CTT CCC TG-3′ belonging to exon 5 of CYP2C11 and downstream 5′-ACG TGT TTC AGC AGC AGC AGG AGT C-3′ belonging to exon 6 of CYP2C11), rat CYP2C12 (upstream 5′-TTC AAC GCA TTC CCT ATT CTT CTG G-3′ belonging to exon 5 of CYP2C12 and downstream 5′-CAA AAG TGC AAA TCT CAG CGT TAA GC-3′ belonging to exon 6 of CYP2C12), and rat actin (purchased from Funakoshi, Japan). The PCR of CYP2C11 was run for 20 cycles (96 °C for 30 s, 64 °C for 50 s and 72 °C for 4 min), the PCR of CYP2C12 was run for 20 cycles (96 °C for 30 s, 59 °C for 50 s and 72 °C for 4 min), and the PCR of rat actin was run for 18 cycles (96 °C for 30 s, 60 °C for 50 s and 72 °C for 4 min) using Takara *Taq* (Takara). Aliquots (10 μl) of PCR products were resolved on 2% (w/v) agarose gels stained with ethidium bromide.

Primary culture for rat hepatocytes. Hepatocytes were prepared as previously described [16]. Briefly, hepatocytes were isolated from 10-week-old rats using a two-step collagenase perfusion method and centrifuged at 50*g* for 1 min. The cell pellet was used as a fraction of parenchymal hepatocytes. Two million hepatocytes were resuspended in 2 ml of hepatocyte culture medium (Biocoat Cell Environments, BD Biosciences, Bedford, USA) with epidermal growth factor (50 ng/ml) and then cultured. DsRed2 expression was observed using fluorescent microscopy under 560-nm excitation light.

Results

Evaluation of hepatic DsRed2 expression levels in vivo using an in vivo bio-imaging system

To investigate the regulation of hepatic DsRed2 expression, we first confirmed measurements of *in vivo* DsRed2 expression. We compared results from the *in vivo* bio-imaging system (IVIS) with those obtained from fluorescent microscopy using excitation light at 560 nm. As shown in Fig. 1A, there was a good correlation between the results obtained from each technique, and we concluded that the IVIS could accurately quantify hepatic DsRed2 expression levels *in vivo*.

Differential expression of DsRed2 in adult male and female liver

As reported previously, liver-specific DsRed2 expression observed from embryonic day 14.5 during development was maintained in newborn Tg rats [11]. Indeed, DsRed2 fluorescence was strong in newborns of both sexes, being $2.07 \times 10^8 \pm 0.65 \times 10^8$ and $3.74 \times 10^8 \pm 0.65 \times 10^8$ units (mean ± SD; $n = 4$) in male and female rats, respectively. There was no statistical difference between DsRed2 levels of male and female newborn rats. However, DsRed2 expression differed between male and female rats by the age of 5 weeks, with fluorescence levels being $2.09 \times 10^9 \pm 1.55 \times 10^9$ units (mean ± SD; $n = 4$) in male rats and $2.52 \times 10^7 \pm 0.84 \times 10^7$ units (mean ± SD; $n = 4$) in female rats. To determine when the sexual dimorphic expression of hepatic DsRed2 occurred during prepubertal development, the fluorescence level of DsRed2 in individual male and female rats was determined every 7 days after birth (Fig. 1B). The hepatic DsRed2 level increased slightly 7 days after birth in males (2.20×10^9 units) and females (1.41×10^9 units), and progressively decreased 21 days after birth in both sexes. The DsRed2 level remained undetectable in the female rat liver beyond the 28th day. In contrast, the hepatic DsRed2 level in males increased 35 days after birth (3.96×10^9 unit). Similar sex-specific profiles were observed for an additional 3 males and 1 female (data not shown).

To compare these results with the endogenous sexual dimorphic gene expression, we analyzed the hepatic mRNA levels of *CYP2C11* (male-specific) and *CYP2C12* (female-specific) in male and female Tg rats 7, 21 and 42 days after birth. As shown in Fig. 1C, expression of *CYP2C11* was not detected in female liver until day 42. In contrast, male hepatic *CYP2C11* mRNA was not detected until day 21, and increased drastically by day 42. The expression of hepatic *CYP2C12* increased in both sexes by day 21, and male hepatic *CYP2C12* expression disappeared after 42 days. These results suggested that the DsRed2 expression profile is similar to the *CYP2C11* expression profile except during the early stage of pre-pubertal development.

Female hepatic DsRed2 expression is repressed by pituitary hormone

To determine the effect of sex hormones on the sexual dimorphic expression of DsRed2 in the liver, 3 male and 3 female Tg rats were gonadectomized at 5 weeks of age (Fig. 2). The fluorescence level of hepatic DsRed2 in pre-castrated male rats was $1.13 \times 10^9 \pm 0.47 \times 10^9$ units (mean ± SD); this level was maintained ($1.21 \times 10^9 \pm 0.30 \times 10^9$ units; mean ± SD) 3 weeks after castration. On the other hand, the fluorescence level of DsRed2 in female liver was undetectable in both the pre- and post-ovariectomy (3 weeks after operation) stages. These results suggested that sex hormones do not maintain the sexual dimorphic expression of DsRed2 in adult Tg rats. We then investigated the effect of pituitary hormone on the DsRed2 expression. Three male and 3 female rats were hypophysectomized (Hypox) at 8 weeks of age, and the expression level of DsRed2 was determined before (pre-Hypox) and 72 h after (post-Hypox) the hypophysectomy. As shown in Fig. 3A, the fluorescence level of hepatic DsRed2 in pre-Hypox male rats was $8.18 \times 10^9 \pm 5.13 \times 10^9$ units (mean ± SD); this level increased by a factor of 1.3 ($1.08 \times 10^{10} \pm 0.46 \times 10^{10}$ units; mean ± SD) in post-Hypox male rat liver. The fluorescence level of hepatic DsRed2 in pre-Hypox female rats was $1.14 \times 10^8 \pm 0.87 \times 10^8$ units (mean ± SD), and increased by a factor of 36 ($4.10 \times 10^9 \pm 2.99 \times 10^9$ units; mean ± SD) in post-Hypox female

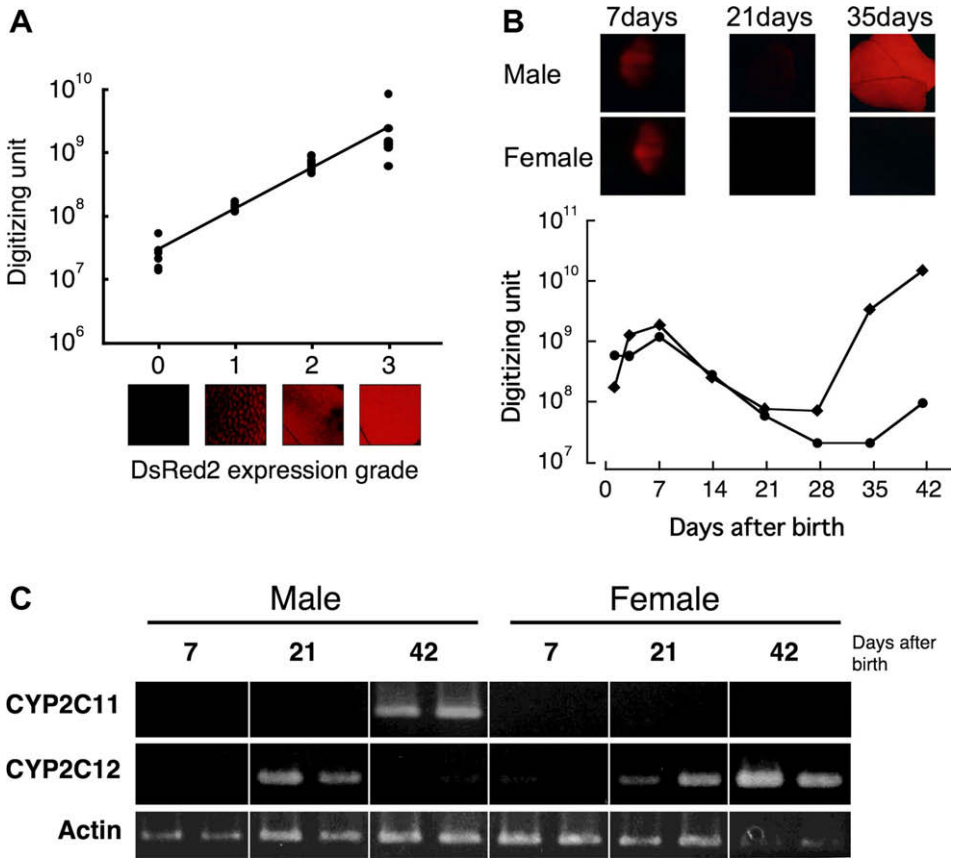

Fig. 1. DsRed2 expression in the transgenic rat liver is regulated in a sexually dimorphic manner. (A) The visible DsRed2 fluorescent signals correlate with the calculated digitizing units from the IVIS. Expressed DsRed2 was visualized by fluorescent microscopy under 560-nm excitation light. The DsRed2 expression grade was determined as indicated in the lower panel (grades 0–3; typical results are shown). Hepatic DsRed2 expression levels in the liver were detected simultaneously using the IVIS as described in Materials and methods. (B) Hepatic DsRed2 expression at 7, 21 and 35 days after birth was visualized using fluorescent microscopy (upper panels). DsRed2 expression levels in individual male (closed diamond) and female (closed circle) rat liver were detected every 7 days using the IVIS (lower panel). (C) *CYP2C11* and *CYP2C12* mRNA levels in the transgenic rat liver. Total hepatic RNA was extracted from 7-, 21- and 42-day-old transgenic animals. Reverse transcripts were amplified with a PCR technique using CYP2C11-, CYP2C12- and actin-specific primer sets as described in Materials and methods. The results obtained from two different animals are shown.

rat liver. Furthermore, we checked the expression level of hepatic *CYP2C11* and *CYP2C12* mRNAs in Hypox Tg rats (Fig. 3B). The expression level of *CYP2C12* mRNA did not change in pre- and post-Hypox female Tg rat liver. In contrast, hepatic *CYP2C11* mRNA significantly increased in post-Hypox female Tg rats that coincided with the DsRed2 expression.

DsRed2 expression in cultured Tg rat hepatocytes

Cultured hepatocytes isolated from Tg rat liver were used to evaluate the effect of circulating hormone(s) on DsRed2 repression.

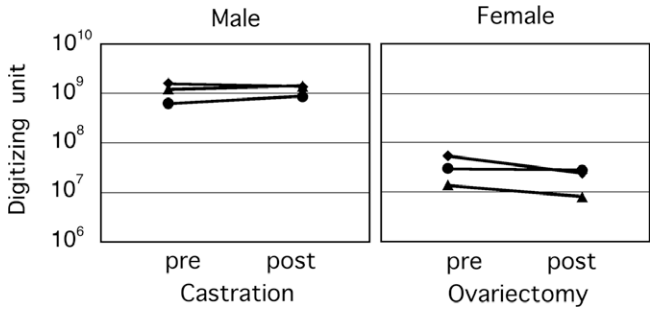

Fig. 2. Gonadal hormones have no effect on maintenance of sexual dimorphic DsRed2 expression in transgenic rat liver. Hepatic DsRed2 levels in male and female transgenic rats were detected before (pre) and 3 weeks after (post) gonadectomy. Hepatic DsRed2 levels from individual animals were detected using IVIS and the results are shown for respective animals.

As shown in Fig. 4, the expression of DsRed2 was maintained until 96 h after culture in male Tg rat hepatocytes. On the other hand, the expression of DsRed2 in female Tg rat hepatocytes was detected at 24 h following culture and increased gradually to reach a maximum at 96 h. These results support the notion that the pituitary hormone(s) in circulation represses DsRed2 expression in the female liver.

Discussion

The secretion of pituitary GH is regulated in a sexually dimorphic manner [5,8]. Adult male rats secrete GH in a highly pulsatile manner followed by GH-free intervals. In contrast, adult female rats are characterized by a more frequent pituitary GH release and the near-continuous presence of GH in plasma. The sexual differences regarding pulsatile plasma GH profile associated with adult male rats transiently induce repeated cycles of liver STAT5b activation, followed by STAT5b deactivation [17]. The continuous presence of GH in adult female rats leads to the activation of STAT5b at a low level [18]. It is thought that the status of STAT5b controls male-dominant gene expression. In this study, we demonstrated that hepatic DsRed2 levels in hypophysectomized Tg rats increased drastically in females and slightly in males (Fig. 3A). Furthermore, quiescent DsRed2 expression in female hepatocytes was activated during culture without GH (Fig. 4). These results suggest that DsRed2 expression is not activated by a GH-STAT5b pathway. The expression of DsRed2 is controlled by a mouse albumin

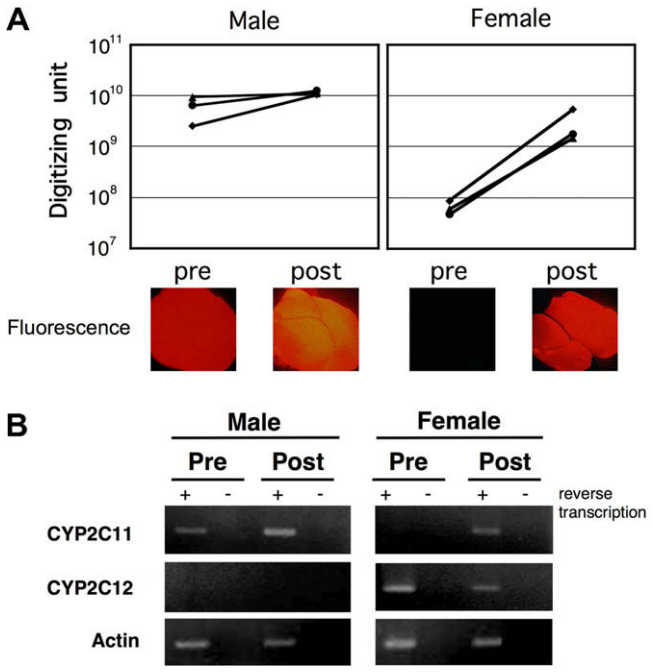

Fig. 3. Pituitary hormone represses DsRed2 expression in female transgenic rat liver. (A) Hepatic DsRed2 levels in male and female transgenic rats were detected before (pre) and 72 h after (post) hypophysectomy. Hepatic DsRed2 levels from individual animals were detected using IVIS and the results are shown for respective animals (upper panel). Hepatic DsRed2 expression was visualized using fluorescent microscopy as described Fig. 1A. A representative result is shown in the lower panel. (B) *CYP2C11* and *CYP2C12* mRNA levels in transgenic rat liver. Total RNA from hypophysectomized transgenic rat liver was reverse transcribed (RT) with (+) or without (−) reverse transcriptase. RT products were amplified with a PCR technique using CYP2C11-, CYP2C12- and actin-specific primer sets as described in Materials and methods. Representative results are shown.

promoter, which contains a −10.5 to −8.5 kb region of a 5′-flanking sequence of a mouse albumin gene and works specific in the liver [11]. We, therefore, examined the effect of a STAT5-mediated GH signal on this promoter activity using g2A-rbGHR-Jak2 cells (provided by Dr. Stuart J. Frank), and subsequently confirmed that this promoter activity was not affected by GH (data not shown). These results suggest that the repression of DsRed2 expression in the female liver is a critical factor in the sexually dimorphic expression of DsRed2.

We demonstrated that the expression of the *CYP2C11* gene, endogenous male liver-specific gene, was activated in the Hypox female rat liver similar to hepatic DsRed2 expression (Fig. 3B). The results suggest that the pituitary hormone-mediated repression of male-specific genes in the female liver may represent a general regulation of sexual dimorphic gene expression in the liver. The Alb-DsRed2 Tg rat system provides a good tool to analyze the physiological regulation of quiescent male-specific genes in female liver.

By utilizing recent advances in *in vivo* bio-imaging systems, we were able to quantify hepatic DsRed2 expression without killing experimental animals. This is an advantage if analysis is required during pre-pubertal development. For example, our data successfully suggest that 21–28 days after birth is the critical point concerning the sexual dimorphic expression of hepatic DsRed2. It is well known that neonatal exposure to testosterone produces hepatic sexual dimorphic gene expression [5]. The Alb-DsRed2 Tg animal and DsRed2 fluorescence analysis may be useful in revealing important time points regarding testosterone exposure and the effect of environmental hormones on hepatic sexual dimorphism. Furthermore, our results suggest that hepatic DsRed2 expression in Alb-DsRed2 Tg rats should be a good indicator for the physiological regulation of the hypothalamo-pituitary-liver axis in regard to adult sexual dimorphic gene expression. In fact, Liao et al. [19] demonstrated that the diethylnitrosamine (DEN)-mediated reduction of sexual dimorphism in the rat liver is partly caused by pituitary damage. It is conceivable that Alb-DsRed2 Tg animals may be useful in screening DEN-like chemicals. Thus, the Alb-DsRed2 Tg rat in couple with a novel bio-imaging system becomes a powerful tool for the study of chemicals that affect the hypothalamo-pitui-

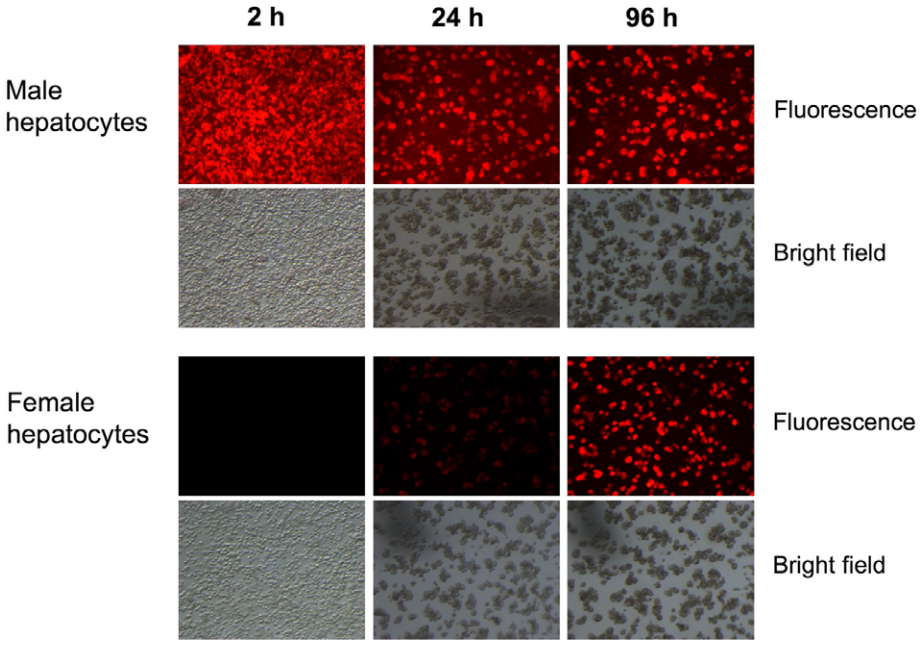

Fig. 4. DsRed2 expression in female Tg rat hepatocytes is induced during culture. Male and female hepatocytes were cultured for 96 h as described in Materials and methods. DsRed2 was detected using a fluorescent microscope under 560-nm excitation light 2, 24 and 96 h after spreading the cells (DsRed2 expressing cells are shown as Fluorescence).

tary-liver axis in the regulation of sexual dimorphic gene expression in the neonate and adult.

Acknowledgments

We thank all personnel in the Division of Organ Replacement Research, Center for Molecular Medicine, Jichi Medical University. This study was supported by a grant from the "Strategic Research Platform" for Private Universities: matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The Alb-DsRed2 Tg rat embryo is available from the Health Science Research Resources Bank (hsrrb@osa.jhsf.or.jp), National Bio-Resource Project for the Rat (nbrprat@anim.med.kyoto-u.ac.jp), or Comparative Medicine Center and Research Animal Diagnostic Laboratory, College of Veterinary Medicine, University of Missouri, MO 65211, USA (Dr. John K. Critser: critserj@missouri.edu).

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