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A new *in vivo* analysis model to detect sexually dimorphic rat liver cytochrome P450 gene expression dependent on growth hormone secretory patterns

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Abstract: Several drug-metabolizing cytochrome P450 (CYP) enzymes exhibit sexual dimorphism depending on the pituitary growth hormone (GH) secretory patterns. However, the mechanism underlying CYP sexual dimorphism remains unclear. We previously established a transgenic (Alb-DsRed2 Tg) rat that expressed red fluorescent DsRed2 protein, particularly in hepatocytes, to visualize cell differentiation and multiplication and found that hepatic DsRed2 expression exhibited sexual dimorphism that was limited to adult males. In this study, we compared the expression patterns between sexual dimorphic *Cyps* and DsRed2 in Tg rats after experimentally reversing the GH secretory patterns in males and females. Postnatal day 1 male and female Tg rats were gonadectomized and then testosterone propionate (0.25 mg/rat) was subcutaneously administered to ovariectomized females immediately after surgery. *Cyp* mRNA and DsRed2 expression levels were quantified using RT-PCR and an *in vivo* imaging system, respectively. GH-dependent *Cyps* and hepatic DsRed2 expression patterns were reversed in males and females at 9 weeks after birth and were significantly correlated ($P<0.05$). This suggested that DsRed2 expression in these Tg rats depended on GH secretory patterns. Based on DsRed2 fluorescence, this Tg rat model could become a tool to readily and effectively evaluate changes in GH-dependent *Cyp* expression.

Key words: cytochrome P450, *in vivo* imaging, sexual dimorphism, transgenic rat

Introduction

The drug-metabolizing cytochrome P450 enzymes (CYPs) facilitate steroid and xenobiotic metabolism in the liver and are important for maintaining the body's homeostasis. CYPs are classified by specific amino acids, and more than 500 subspecies have been found in humans, with differences between males and females as well as between different animal species. Several CYP classes that reflect sexual dimorphism have been identi-

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fied [2, 4, 11, 12, 14, 15, 23]. In rats, *Cyp2c11*, a male-specific isoform that comprises 50% of total P450 in the male liver, and *Cyp2c12*, a female-specific isoform that comprises 40% of total P450 in the female liver, are typical sexually dimorphic CYPs. In humans, women exhibit higher CYP3A4 expression than do men [2], and CYP3A4 is involved in metabolizing about 50% of commercial drugs [4].

These sex differences in CYPs result in clinically important problems because of the differences between men and women in the effectiveness and side effects of drugs. However, the molecular mechanisms underlying these sex differences in CYPs remain unclear. In studies with rodents, establishing CYP sexual dimorphism depended on growth hormone (GH) secretory patterns via the hypothalamus–pituitary–liver axis, which was determined by the sex steroid hormone environment during the perinatal period [18, 21]. CYP sexual dimorphisms were disrupted by altering serum GH levels [10].

Transgenic (Tg) animals are useful models for analyzing various gene functions in a living body. In particular, Tg rats provide considerable genomic information similar to that obtained with mice, although the larger body size of the rat makes it preferable for transplant and stem cell research. Fluorescent or luminescent proteins, such as green fluorescent protein (GFP), red fluorescent protein2 (DsRed2), or firefly luciferase, can be used to identify particular cells in the body. Thus, a number of Tg animals that harbor these marker proteins have provided tools for biomedical research [6, 13].

We have generated many useful Tg rat models for use in regenerative medicine research [5, 7], including one that specifically expressed DsRed2 in the liver [17]. Alb-DsRed2 Tg rats express DsRed2, a red fluorescent protein derived from the genus *Discosoma*, particularly in hepatocytes under the control of a mouse albumin enhancer/promoter. Because DsRed2 fluorescence can be visualized only when using excitation light of a specific wavelength, it is a useful analytical tool for simply and noninvasively tracking the migration and differentiation of cells. Hepatic DsRed2 expression was detected only in adult male rats, which reflected sexual dimorphism [1]. In addition, the DsRed2 expression pattern did not change after performing gonadectomy in both male and female adult rats, although DsRed2 expression did appear in hypophysectomized females or in cultured female hepatocytes in the absence of pituitary hormones. This strongly suggested that the sexual dimorphism of DeRed2 expression in these Tg rats was regulated by hypothalamic-pituitary hormones, including GH.

In this study, to determine the relevance of GH-dependent endogenous *Cyp* expression and sexual dimorphic DsRed2 expression by Alb-DsRed2 Tg rats, we experimentally reversed the GH secretory patterns of male and female Alb-DsRed2 Tg rats. Using this model, we investigated the influence of GH secretory patterns on GH-dependent *Cyp* expression and hepatic DsRed2 expression in adult rats. We propose that liver-specific DsRed2 fluorescence in this Tg rat can be used to monitor any changes in GH-dependent *Cyp* expression that indicates a sexual dimorphism and can also become a tool to investigate the mechanisms underlying the establishment of a sexual dimorphism.

Materials and Methods

Experimental animals

For all experiments, we used Alb-DsRed2 Tg heterozygous male and female Wistar-Tg (Alb-DsRed2) 34Jmsk rats, which were established as reported previously [17]. These rats were maintained under controlled conditions, with temperature at 20–25°C, humidity of 40%–70%, and a 12 h light/12 h dark cycle (lights on at 0700). Food and water were provided *ad libitum*. All experiments in this study were conducted in accordance with the Nippon Veterinary and Life Science University Guide for Laboratory Animals.

Surgical procedures and experimental schedule

On postnatal day 1, Alb-DsRed2 Tg heterozygous male and female rats underwent castration (Cast) or ovariectomy (OVX) while under isoflurane anesthesia (Intervet, Tokyo, Japan), and were designated the Cast male and OVX female groups, respectively. To reverse the GH secretory patterns in male and female Tg rats in adulthood, according to previous reports [9], testosterone propionate (TP) (0.25 mg/head in 0.05 ml olive oil) was administered subcutaneously into selected ovariectomized females immediately after surgery, and these rats were designated the OVX+TP female group. Some female rats did not undergo ovariectomy but were injected with TP (TP female group) and used as controls. Some male and female rats did not undergo castration or ovariectomy (sham male and sham female groups).

Pups were housed with their mothers until postnatal day 20 and bred 9 weeks after birth. These rats were

euthanized by intraperitoneal administration of pentobarbital (65 mg/kg) and left liver lobes were partially removed for analysis. These rats were subsequently perfused through the heart with heparinized saline (5 IU/ml), followed by a 10% formalin buffer solution. Brains were removed and post-fixed in the same solution and then transferred to 30% sucrose in 0.1 M phosphate buffer until they settled.

Quantifying hepatic DsRed2 expression

At 9 weeks after birth, hepatic DsRed2 expression levels in each rat were quantified using an IVIS bioimaging system (Xenogen, Alameda, CA). DsRed2 fluorescence in liver tissue was excited with a light at 560 nm, after which light emission (600 nm) and images were acquired using a cooled charge-coupled device camera. Living Image software (Xenogen, Alameda, CA) was used to quantify fluorescent signals, which were expressed as digitized units (photon/sec/cm^2/steradian). DsRed2 expression was also observed using a fluorescent microscope (Leica Microsystems, Tokyo, Japan) under a 560-nm excitation light.

Semi-quantitative RT-PCR

After removal, liver samples were immediately frozen in liquid nitrogen and stored at −80°C until analyzed. Total RNA was isolated from liver tissue with TRIzol LS Reagent (Invitrogen, CA, USA). To eliminate genomic DNA, extracted RNA was incubated with 1 U/μl of DNaseI at room temperature for 15 min, 25 mM ethylenediaminetetraacetic acid was added to stop the reaction, and RNA was then incubated at 65°C for 10 min. Hepatic RNA was incubated with oligo d (T) primers (Invitrogen, CA, USA) and dNTP mix at 70°C for 10 min, and then reverse transcribed with 200 U/μl of Super Script (Invitrogen, CA, USA) at 50°C for 90 min and 70°C for 90 min. PCR was conducted using specific primer sets. For rat *Cyp2c11*: upstream 5′-CTG GTC CAA CAC CTC TCC CA-3′ and downstream 5′-GTC CGA AAA GTC GAG GGG TA −3′; for rat *Cyp2c12*: upstream 5′-CTT GCC CCA AAT GGT TTG TTG-3′ and downstream 5′-GAG TCT TGC ATA ACT CAA AT-3′; and for Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*): upstream 5′-TTC AAC GGC ACA GTC AAG-3′ and downstream 5′-TAC AAA CAC TAC CCA CAA-3′.

PCR for rat *Cyp2c11* was run for 30 cycles (95°C for 30 s, 58°C for 50 s, and 72°C for 30 s), and PCR for *Cyp2c12* was run for 32 cycles (95°C for 30 s, 54°C for 30 s, and 72°C for 30 s) using Takara rTaq (Takara, Shiga, Japan). PCR products were analyzed on a 1% agarose gel containing ethidium bromide with 1× TAE buffer. mRNA signals were visualized and signal intensity was quantified using Image J software (NIH, USA). mRNA signals were then normalized to *Gapdh* mRNA, which was used as an internal control.

Histology

Serial sections (40-μm thick) that encompassed the preoptic areas were prepared using a freezing microtome. Sections were mounted on gelatin-coated glass slides (Wako), stained with cresyl violet (MERCK, Darmstadt, German), dehydrated, and cover slipped with ENTELLAN New (MERCK, Darmstadt, German). Images were acquired using a fluorescent microscope (BZ-9000, KEYENCE).

Statistical analysis

Group results are given as means ± standard deviations. The Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used the statistical analysis. Statistical comparisons between groups were made by one-way ANOVA, followed by a Bonferroni–Dunn test. A P-value of <0.05 was considered significant.

Results

Hepatic Cyp mRNA expression profiles

Relative male-specific *Cyp2c11* mRNA expression levels in the liver in OVX+TP females were significantly increased as compared with those in sham female rats and were comparable to those in sham male rats (Fig. 1 (a)). Relative female-specific *Cyp2c12* mRNA expression levels in the liver were lower in OVX+TP females than in sham females, although this difference was not significant (Fig. 1 (b)). There were no significant differences in the expressions of these two mRNAs between OVX+TP females and sham males. There were also no significant differences in the expressions of these two mRNAs between OVX females, TP females, and sham female rats.

Cast male rats had significantly lower *Cyp2c11* mRNA expression levels and significantly higher *Cyp2c12* mRNA levels than those in sham male rats. Cast male rats had *Cyp* mRNA expression patterns similar to those of females.

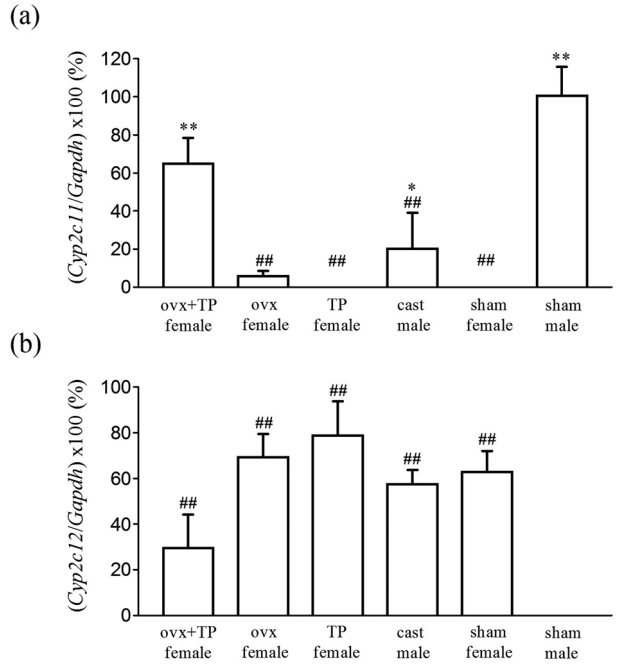

Fig. 1. Quantifying hepatic *Cyp* mRNA expression using semi-quantitative PCR. Hepatic *Cyp* mRNA expression was determined when rats were 9-weeks old. (a): Male-specific *Cyp2c11* mRNA expression levels normalized to *Gapdh* mRNA used as an internal control. (b): Female-specific *Cyp2c12* expression levels normalized to *Gapdh* mRNA. Results are means ± standard deviations (n=4) ($^{\#\#}$; vs. sham male, $P<0.01$, **; vs. sham female, $P<0.01$, *; vs. sham female, $P<0.05$).

Hepatic DsRed2 expression profiles

Neonatal OVX+TP females exhibited a high hepatic DsRed2 expression level, which was not detected among sham females (Fig. 2 (a)). Cast males did not express hepatic DsRed2 at levels that were close to the levels of sham males. Hepatic DsRed2 expression levels in OVX females and TP females were not significantly different from those in sham females.

The mean hepatic DsRed2 fluorescence expression level in OVX+TP females was $1.03 \times 10^{12} \pm 1.18 \times 10^{12}$ (Fig. 2 (b)), which was higher than the mean level in sham females ($1.43 \times 10^{10} \pm 1.78 \times 10^{9}$); however, this difference was not significant. Cast male rats had reduced DsRed2 expression levels ($5.13 \times 10^{10} \pm 9.36 \times 10^{9}$), but this level was not significantly different from that in sham females. OVX females and TP females had mean expression levels of $3.15 \times 10^{10} \pm 2.81 \times 10^{10}$ and $4.60 \times 10^{10} \pm 3.30 \times 10^{10}$, respectively. These levels were comparable with that in sham females.

Correlations between Cyp mRNA and hepatic DsRed2 expressions

We next assessed for a possible correlation between hepatic DsRed2 expression and *Cyp* mRNA expression. There was a positive correlation between DsRed2 expression and male-specific *Cyp2c11* mRNA expression, and there was a negative correlation between DsRed2 expression and female-specific *Cyp2c12* mRNA expression (Fig. 3). The R^2 value for the DsRed2 and *Cyp2c11* expression correlation result was 0.976, and that for the DsRed2 and *Cyp2c12* correlation result was 0.883. DsRed2 expression was particularly strongly correlated with *Cyp2c11* expression.

SDN-POA volumes

Sexually dimorphic nuclei of the preoptic area (SDN-POA) volumes for all rat groups are shown in Fig. 4. SDN-POA volumes were greater in male rats than in female rats. SDN-POA volumes were similar for TP females and sham males. Cast males had SDN-POA volumes that were comparable to those of sham females. OVX females had SDN-POA volumes similar to those of sham females.

Discussion

CYPs facilitate steroid and xenobiotic metabolism in the liver and are important for maintaining the body's homeostasis. These CYPs differ based on sex and animal species, and many types of CYPs that show sexually dimorphic expression have been identified [2, 4, 11, 12, 14, 15, 23]. These sex differences strongly depend on differences in GH-secretory patterns from the pituitary anterior lobe [18, 21]. We previously established a transgenic (Alb-DsRed2 Tg) rat that expressed red fluorescent DsRed2 protein, particularly in hepatocytes, to visualize cell differentiation and multiplication and found that hepatic DsRed2 expression exhibited sexual dimorphism that was limited to adult males. The mechanism of the sexual dimorphism in DsRed2 expression is unclear. In this study, we examined the relevance of the sexually dimorphic expression patterns of GH-dependent *Cyps* and that of DsRed2 in Alb-DsRed2 Tg rats. Several sexually dimorphic CYPs are dependent on pituitary GH-secretory patterns, which are determined by sex steroid hormones during the postnatal period [18, 21]. To reverse the GH secretory patterns in male and female Tg rats in adulthood, we experimentally reversed their

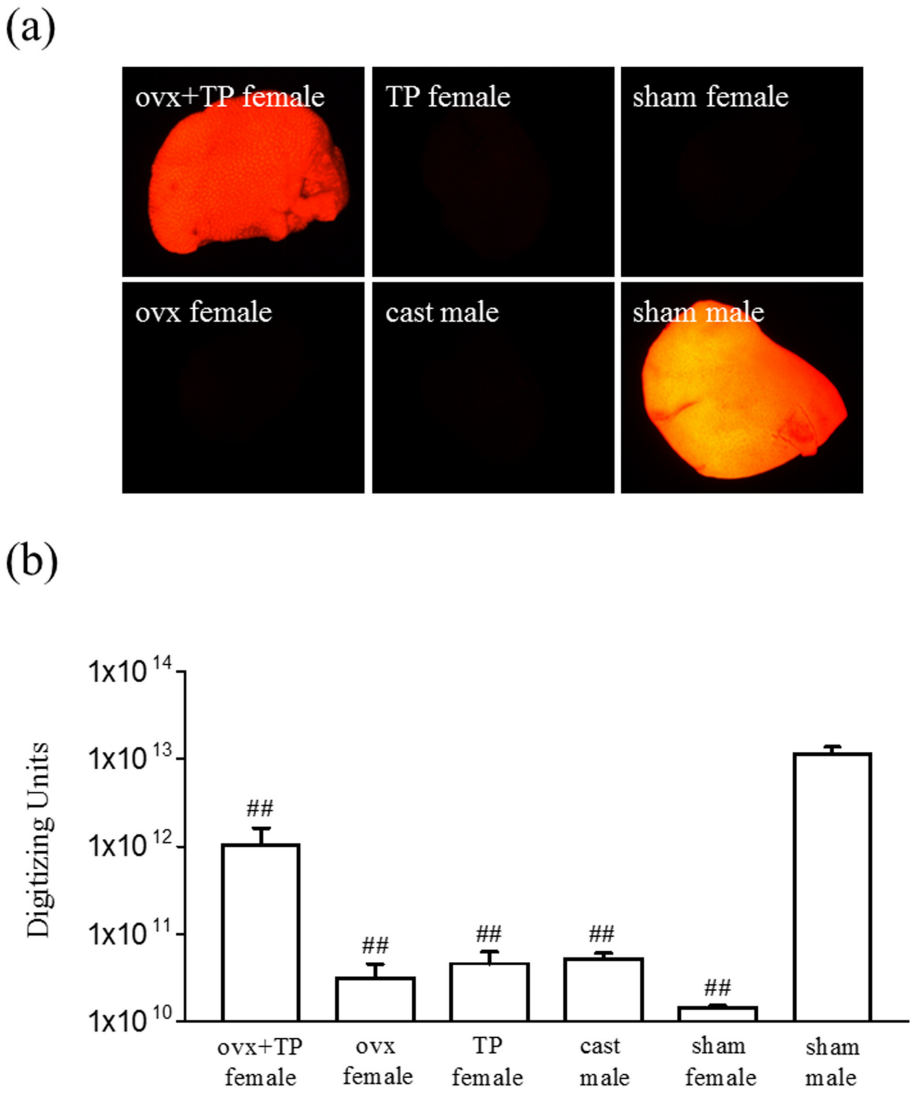

Fig. 2. Hepatic DsRed2 expression in adult Tg rats. (a): Females that underwent neonatal ovariectomy and neonatal TP treatment (OVX+TP female), females that underwent neonatal ovariectomy only (OVX female), females that underwent neonatal TP treatment only (TP female), males that underwent neonatal castration (Cast male), sham-treated females (sham female), and sham-treated males (sham male). Each sample was observed by fluorescent microscopy when rats were 9-weeks old. (b): IVIS evaluation of DsRed2 expression levels in each group and quantified using analysis software. Results are means ± standard deviations (n=4) ($^{\#\#}$; vs. sham male, $P<0.01$).

sex hormone environments at the postnatal period using previously described methods [9].

We confirmed that OVX+TP females showed male-specific patterns of hepatic *Cyp2c11* and DsRed2 expression; however, these levels were not comparable with the levels found in sham male rats. We considered that the GH-secretory patterns of the OVX+TP female rats did not completely mimic the male pattern because adult males secrete high concentrations of testosterone from their testes, whereas females obviously could not continuously produce testosterone.

The female-specific *Cyp2c12* and DsRed2 expression levels in OVX females and TP females showed that ovariectomy did not affect *Cyps* and hepatic DeRed2 expression both in newborns and adults [1], and that TP treatment only could not induce male-specific gene expression in the liver. In a previous study, we found that castrating adult Tg males did not change either *Cyp* or

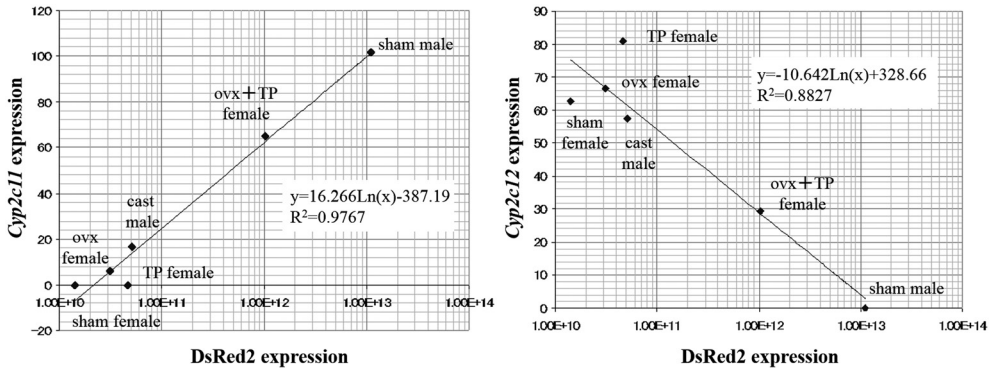

Fig. 3. Correlations between hepatic sexual dimorphic DsRed2 and *Cyp* expressions for all six groups of rats (n=4).

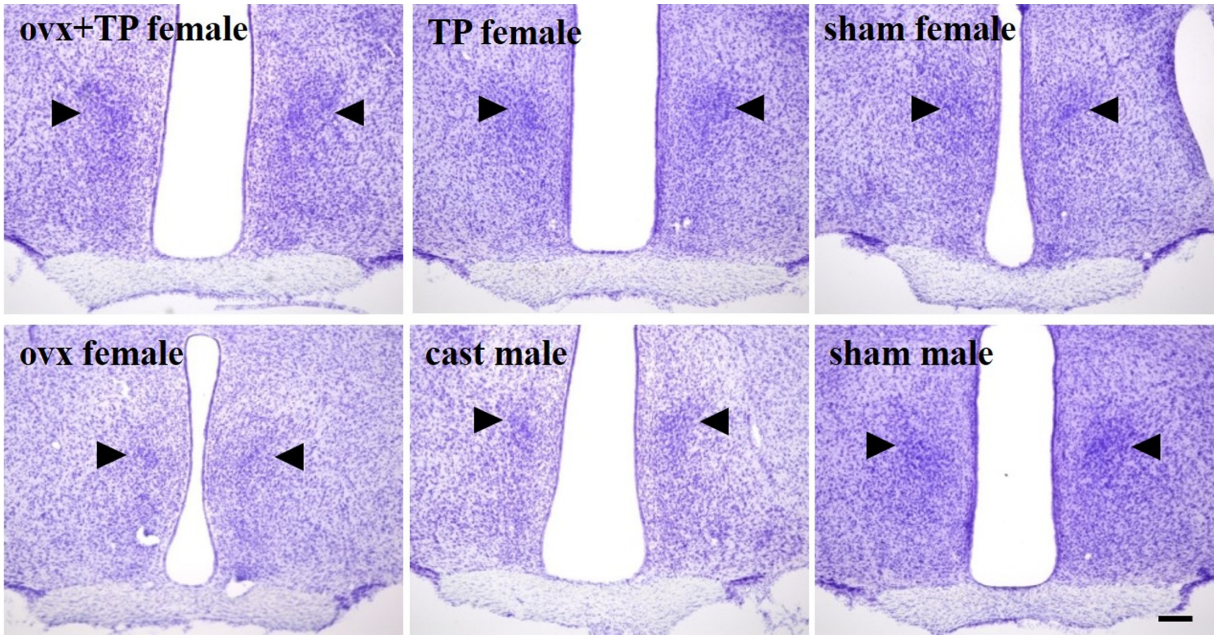

Fig. 4. Sexually dimorphic nuclei of the preoptic area (SDN-POA) volumes in 9-week-old rats. SDN-POA volumes in OVX+TP female rats, OVX female rats, TP female rats, Cast male rats, sham female rats, and sham male rats (n=4). Arrows represent SDN-POA region. Scale bar=200 μm.

DsRed2 expression [1]. These results were supported by a report that GH secretion in adult rats was altered after neonatal gonadectomy but not after prepubertal gonadectomy [8], which suggested that only neonatal castration could change the GH secretory pattern, and thus, GH-dependent gene expression was also altered in Cast males.

We found that DsRed2 expression was positively correlated with male-specific *Cyp2c11* expression and negatively correlated with female-specific *Cyp2c12* expression, which revealed a strong correlation between the expression of GH-dependent *Cyps* and DsRed2. This strongly suggested that sexual dimorphic DsRed2 expression in these Tg rats was regulated by the GH secretory pattern, which is determined during the postnatal period. However, the mechanisms for why a mouse albumin promoter with a Tg rat induces GH-dependent sexual dimorphism in rat hepatocytes remain unclear. Sexual dimorphism of hepatic DsRed2 expression in this Tg rats was also observed in another sub-line. Furthermore, transgenic rats produced by connecting another target gene to the same mouse albumin promoter showed same differences which the introduced gene was strongly expressed in adult males but not in females [20].

Therefore, we predict that the cause of the sexual dimorphism of this DsRed2 expression depends on the regulation of the promoter but not gene insertion mutation. Interestingly, transgenic mice with this mouse albumin promotor did not show any sex differences [16], and it remains to be unexplained the cause of GH-dependent sexual dimorphism in rats. It is necessary to examine the detailed property of this promoter.

In this study, we did not directly assess plasma GH secretory patterns. However, we confirmed SDN-POA, a classic marker of sexual differentiation of the rat brain, which is involved in regulating rat sexual behavior [3]. SDN-POA volumes are considerably greater in males than in females and can be clearly identified by Nissl staining. Consistent with some classic experiments, we showed that cerebral sexual differentiation could be reversed in male and female rats by reversing the sex hormone environment during the perinatal period, which also established the effect of this neonatal treatment. Together with the results of hepatic *Cyps* and DsRed2 expression profiles, we considered that this treatment during the postnatal period could reverse the GH secretory patterns in male and female rats.

It is known that exposure to sex steroid hormones during pregnancy and the perinatal period has a significant effect on the growth and physiology of the fetus. As a cause for sex differences in the efficacy and adverse effects of drugs in men and women, sexual dimorphism of CYPs has become an important clinical problem, and numerous studies on this problem have been performed. However, the molecular mechanisms that contribute to these sex differences in CYPs are less well established because of the difficulty with continuously monitoring GH secretion patterns. Monitoring GH levels requires repetitive atrial blood sampling using a catheter [22] or mimicking plasma GH patterns *in vitro* using cultured hepatocytes from hypophysectomized rats without endogenous GH [19]. This *in vivo* bio-imaging system using DsRed2 expression as an indicator may be quite beneficial for monitoring changes in GH secretion. We would like to emphasize that this model is a completely new approach for evaluating GH secretion patterns and GH-dependent gene expression.

Our results demonstrated that GH-dependent endogenous *Cyps* expression and DsRed2 expression in this Tg rat model were strongly correlated. Thus, this Tg rat model can be a beneficial analytical tool for noninvasively and simply monitoring changes in *Cyps* expression using a fluorescent protein and also for research on the mechanisms for establishing sexual dimorphism.

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