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Prolactin up-regulates female-predominant *Cyp* gene expressions and down-regulates male-predominant gene expressions in mice liver

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Running Title: PROLACTIN AND SEX-PREDOMINANT GENE EXPRESSIONS IN THE LIVER

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Abbreviations: Cyp, cytochrome P450; D2R, dopamine2 receptors; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; GHR, growth hormone receptor; Igf; insulin-like growth factor; Jak, Janus kinase; Mup, major urinary protein; PCR, polymerase chain reaction; PGH, placental growth hormone; PrlR, prolactin receptor; qPCR, quantitative real-time polymerase chain reaction; Stat, signal transducer and activator of transcription; TT, total testosterone.

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Abstract

Prolactin is a polypeptide hormone with over 300 separate biological activities and its serum level is increased during pregnancy and lactation. It has been described that pregnancy and lactation affect drug and steroid metabolism in mice and humans. Several studies reported that pregnancy or lactation influences liver cytochrome P450 (Cyp) expression and its activity, affecting the biosynthesis of steroids and xenobiotics through growth hormone or sex hormones; however, the role of prolactin as the regulator of liver *Cyp* expression has not been elucidated so far. In the present study, we focused on prolactin as the regulator of liver sex-predominant gene expressions including *Cyps*. To investigate the role of prolactin in the hepatic gene expressions, pCAGGS expression vector containing mouse prolactin cDNA was transfected by hydrodynamic injection in both male and female mice. Hyperprolactinemia phosphorylated signal transducer and activator of transcription 5 in the liver and augmented female mice liver mRNA expressions of *Cyp3a16*, *Cyp3a41*, *Cyp3a44*, *Cyp2b9* and prolactin receptor genes, whose expressions were female-predominant in hepatocytes. Moreover, liver expressions of male-predominant genes such as *Cyp2d9*, *Cyp7b1*, *Mup1* and *Alas2* were reduced in male mice with hyperprolactinemia. The serum levels of conventional regulators in hepatic gene expressions, growth hormone and testosterone, were not affected by hyperprolactinemia. We demonstrated that prolactin up-regulated female-predominant genes in female mice and down-regulated male-predominant genes in male mice. We conjecture that higher concentration of

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prolactin would alter the steroids and xenobiotic metabolisms by modulating hepatic *Cyp* gene expressions during pregnancy and lactation.

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Introduction

Prolactin is a polypeptide hormone that is mainly synthesized in and secreted from the lactotrop cells of the anterior pituitary gland, and it regulates lactation, luteotrophic actions, and reproductive and parental behavior. During pregnancy prolactin has important roles in the development of mammary epithelium, and the maintenance and secretory activity of the corpus luteum in some mammals, particularly rodents. Moreover, prolactin has over 300 separate biological activities and is synthesized and secreted from various organs and tissues such as hypothalamus, mammary gland and immune cells (Freeman et al., 2000; Bachelot and Binart, 2007). Prolactin is secreted on proestrus evening in response to ovarian estradiol and returns to basal levels the next morning during the 4-day estrous cycle of the female rat. Prolactin secretion is also transiently elevated by mating stimulus on the uterine cervix as a nocturnal and a diurnal surge in female rat (Freeman et al., 2000, Kennett and McKee, 2011). Then the diurnal or nocturnal surges of prolactin recur for 10 days if the mating is fertile and results in pregnancy, and the surges of prolactin cease after day 10 of pregnancy due to the negative-feedback action of placental lactogen, which is mainly secreted from placenta and has structural and functional similarity to pituitary prolactin (Voogt et al., 1982; Tonkowicz et al., 1983, Corbacho et al., 2002). After giving birth, suckling stimulus induces prolactin secretion again. In the rats blood prolactin concentrations begin to rise within 1-3 min of initiation of nursing, reach at 200 to

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300 ng/mL within 10 to 15 min, and are sustained at a constant level as long as nursing continues (Grosvenor and Whitworth, 1974, Moore, 1987).

It has been described that hepatic drug metabolism is influenced by sex differences, and pregnancy and lactation also affect drug and steroid metabolism in mice and humans. Several studies reported that liver cytochrome P450 (Cyp) expression and activity were different between female and male, and were altered during pregnancy, delivery and lactation (Waxman and Holloway, 2009, Zhang et al., 2008, Tracy et al., 2005, He et al., 2005, Koh et al., 2011). All known mammalian Cyp enzymes are membrane-associated proteins, located either in the endoplasmic reticulum or in the inner membrane of mitochondria (Sugimoto and Shiro, 2012). They play central roles in the biosynthesis of bioactive compounds, such as sterols, steroid hormones, eicosanoids, and vitamins, and in the oxidative metabolism of a wide range of medicines and detoxification of xenobiotics (Sugimoto and Shiro, 2012; Waxman et al, 1988; Shimada et al., 1994). The expressions of many hepatic genes including *CYP* superfamily have been reported to be influenced by sex-specific patterns of growth hormone (GH) secretion, namely pulsatile secretion in males and continuous secretion in females, resulting in sexual dimorphism of hepatic gene transcription (Norstedt and Palmiter, 1984, Waxman and O'Connor, 2006). These sex-dependent gene expression differences are most prominent in rodents, but they were also observed in humans (Waxman and Holloway, 2009). These sex-predominant hepatic genes are classified into four categories: male class I genes require pituitary hormones, principally GH

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pulse, for full expression and are therefore decreased in expression after hypophysectomy or continuous GH treatment; male class II genes are primarily regulated by the repressive actions of the female GH pattern in both male and female rodents and do not require GH; female class I genes require continuous GH exposure and are decreased in expression after hypophysectomy or GH pulse; and female class II genes are also increased in expression after hypophysectomy, and are suppressed by GH pulse replacement (Wauthier and Waxman, 2008, Waxman and Holloway, 2009, Wauthier et al., 2010). Pulsatile GH binding to GH receptor (GHR) activates the GHR-associated tyrosine kinase Janus kinase (Jak) 2, which in turn phosphorylates the cytoplasmic domain of GHR at multiple sites, generating docking sites for signal transducer and activator of transcription (Stat) 5b, resulting in the nuclear translocation and induction of target gene transcription (Waxman and O'Connor, 2006). In addition to GH, several other physiological hormones such as placental GH (PGH) or estrogens regulate liver *Cyp* expression (Waxman and O'Connor, 2006; Waxman and Holloway, 2009; Zhang et al., 2008). However, the role of prolactin as the regulator of liver *Cyp* expression and other sex-different hepatic genes has not been elucidated so far. In the present study, we focused on prolactin as the regulator of liver sex-predominant genes by using hyperprolactinemic male and female mice induced by gene transfer of murine prolactin cDNA into hepatocytes, and revealed the influence of hyperprolactinemia on hepatic gene expressions.

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Materials and Methods

Animals.

Male and female C57BL/6J mice at 10-12 weeks of age were purchased from Charles River Japan (Yokohama, Japan) and maintained in our animal facility until the implementation of the experiments. They were individually housed in cages and kept under 12-hour light and dark cycle with free access to standard chow and water. All mice were sacrificed to collect serum and liver samples 2 days after the hydrodynamic injection of expression plasmid vector. Twenty milligram of pentobarbital was injected intraperitoneally for anesthesia before sacrifice. All the animal experiments were conducted in compliance with the protocol which was reviewed by the Institutional Animal Care and Use Committee and approved by the President of Niigata University (Permit Number: #28 Niigata Univ. Res. 289-2).

Construction of expression vectors and hydrodynamic injection.

The pCAGGS vector was kindly provided by Dr. Miyazaki (Niwa et al., 1991). The coding sequence of mouse prolactin was inserted into the pCAGGS vector at *Xho*I site. Insert fragment was generated by DNA synthesis, and mock vector was used as a control. Plasmids were purified in an endotoxin-free environment using an Endofree Plasmid Maxi Kit (Qiagen, Hilden, Germany) and then dissolved in sterile PBS (5 μg of plasmid DNA per 1 mL of PBS). Each expression vector was transfected into mouse liver by the hydrodynamic method (Liu et al., 1999). A mouse was placed in a

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holding device and 2 mL of plasmid DNA solution (10 μg plasmid DNA per mouse) was injected rapidly via the tail vein using a 30-gauge needle. Total DNA was extracted from the liver of each mice treated with pCAGGS vector using a DNeasy blood and tissue kit (Qiagen), and the pCAGGS common sequence and inserted mouse prolactin cDNA were confirmed with polymerase chain reaction (PCR). Total RNA was also extracted from the liver and cDNA was synthesized by reverse transcriptase and amplified by PCR to detect glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and mouse prolactin. The primer sequences are shown in Table 1.

Measurement of specific hormones.

Prolactin and GH levels in the serum of mice were measured using ELISA kits for mouse prolactin and GH, respectively (Cloud-Clone, Houston, TX). Serum total testosterone (TT) concentration was measured by the electro-chemiluminescence immunoassay method (SRL Inc, Tokyo, Japan).

Western blot analysis.

Liver lysate or serum samples were separated using SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membrane was blocked with a Western Blocking Reagent (Roche, Manheim, Germany) for 30 min at room temperature. The blocked membrane was incubated overnight at 4^0C with goat polyclonal anti-prolactin receptor (PrlR) antibody (AF1167, 0.1 μg/mL, R&D Systems, Minneapolis, MN), rabbit polyclonal anti-Stat5 antibody (9363S, 0.1 μg/mL, Cell Signaling Technology, Danvers, MA), rabbit monoclonal anti-phospho-Stat5 (Tyr694) antibody (D47E7, 0.1

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μg/mL, Cell Signaling Technology), rabbit polyclonal anti-major urinary proteins (Mups) antibody (sc-66976, 1.0 μg/mL, Santa Cruz, Dallas, TX) or horseradish-conjugated goat polyclonal anti-albumin antibody (ab19195, 0.05 μg/mL, Abcam, Cambridge, MA). Normal goat polyclonal IgG was used as a control IgG against anti-PrlR. Horseradish peroxidase-conjugated specific secondary antibody was then reacted with anti-PrlR, anti-Stat5, anti-phospho-Stat5, anti-MUPs antibody, or control IgG, respectively. Signals were detected using Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific, Waltham, MA).

Quantitative real-time polymerase chain reaction (qPCR) analysis.

Total RNA was extracted from each liver using the RNeasy Mini Kit (Qiagen, Valencia, CA). qPCR analysis was performed using the Thermal Cycler Dice Real Time System II with One Step SYBR PrimeScript Plus RT-PCR Kit (Takara Bio, Shiga, Japan) following the manufacturer's protocol. For relative quantification analysis, target amplicons and *Gapdh* were reverse transcribed and quantified using the same template RNA. The primer sequences are shown in Table 1.

Statistical analysis.

Values are expressed as means ± S.D. Statistical differences were calculated with Student's *t* test for comparison between two groups. For comparison among four groups one-way analysis of variance was used, followed by Scheffe's F test as a post-hoc test. $P < 0.05$ was considered significant.

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Results

Induction of hyperprolactinemia by gene transfer

In order to reproduce continuously high levels of serum prolactin, we constructed an expression plasmid vector by inserting mouse prolactin cDNA into pCAGGS (pCAGGS-*mPrl*) and transfected it into mice hepatocytes by hydrodynamic injection. After the hydrodynamic injection of the vector into the male or female mice, we extracted liver DNA from the mice and confirmed the transfection of the vector into the hepatocytes by PCR using a primer pair specific for the pCAGGS vector sequence and mouse prolactin cDNA (Fig. 1A). We also extracted the total RNA from the liver of these mice and, by reverse transcription PCR with primers specific for mouse prolactin, confirmed the mouse prolactin mRNA expression in the liver of the mice treated with pCAGGS-*mPrl* (Fig. 1B). The serum level of prolactin was significantly elevated in both male and female mice treated with pCAGGS-*mPrl* as compared to that in mice treated with mock vector (Fig. 1C).

Induction of Stat5 phosphorylation by hyperprolactinemia

We examined the effect of prolactin on several sex-predominant gene expressions in the liver. The biological effects of prolactin are mediated by its interaction with PrlR, which is a member of the cytokine receptor superfamily, and is present in nearly all organs and tissues (Bole-Feysot et al., 1998; Binart et al., 2010, Tsuchida et al., 2014). Although the PrlR gene is unique in each species, alternative splicing generates different isoforms, which are identical in their extracellular domains but differ in the lengths and sequences of their intracellular domains. PrlR exists as 7 recognized isoforms in humans,

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and in mice, 1 long and 2 short isoforms have been identified as proteins, and the short forms only differ by a few amino acids in the C-terminal part of the cytoplasmic tail. Acting through long-form PrlR, prolactin activates many kinases including Jak2/Stat5, whereas short-form PrlRs are reported to have independent biological activity and to have a signaling pathway distinct from Jak/Stat signaling cascades (Bole-Feysot et al., 1998; Binart et al., 2010). Long-form PrlR was confirmed to be expressed in both male and female liver (Fig. 1D) and phosphorylation of Stat5 was detected in both male and female mice treated with pCAGGS-*mPrl* (Fig. 1E).

Sex-predominant hepatic gene expressions were affected by hyperprolactinemia

We investigated the effect of hyperprolactinemia in hepatic gene expressions representative of each of the four major classes of mouse sex-specific genes, namely class I and class II male-specific and female-specific genes (Wauthier et al, 2010, Ramirez et al., 2015). Fig. 2A demonstrates that mRNA expression of female class I genes such as *Cyp3a16*, *Cyp3a41* and *Cyp3a44* were dramatically augmented by hyperprolactinemia, especially in female mice. Female class II gene *Cyp2a4* expression was increased in male mice alone, whereas other female class II genes, *Cyp2b9* and *Prlr* were increased in expression exclusively in female mice (Fig. 2B). Male class I genes such as *Cyp2d9*, *Cyp7b1* and *Mup1* were male-predominantly expressed at basal level and were down-regulated by hyperprolactinemia, especially in male mice (Fig. 2C), whereas male class II genes such as *Alas2*, *Mcm10* and *Nox4* were also male-predominantly expressed at basal level, but only *Alas2* was

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down-regulated by hyperprolactinemia in male mice (Fig. 2D). *Cyp1a2*, male-predominant but not classified as class I or class II, was up-regulated in male mice at both basal and hyperprolactimemic condition, but was not affected by hyperprolactimenia. Other unclassified genes such as *Cyp3a11*, *Cyp3a13*, *Cyp3a25*, *Cyp19a1* and *Cyp27a1* did not differ in expressions between male and female mice, and was not affected by prolactin (Fig. 2E). The serum Mup levels were decreased in hyperprolactinemic male mice (Fig. 2F).

Hyperprolactinemia did not influence the serum levels of GH or TT

Since these sex-specific hepatic gene expressions are regulated by GH, and some of them are also affected by testosterone (Delić et al., 2010, Knopf et al., 1983), we verified the influence of hyperprolactinemia on serum levels of GH and TT. In both male and female mice treated with pCAGGS-*mPrl*, the serum levels of GH were not significantly different from those in mice treated with pCAGGS-mock. In female mice TT concentration was below the detectable level, and serum levels of TT in male mice were not affected by hyperprolactinemia (Fig 3A). In order to examine the effect of GH in the liver, liver mRNA expression of insulin-like growth factor (Igf)-1, which is mainly induced by GH (Chia, 2014), was evaluated in both male and female mice treated with pCAGGS-*mPrl* or its mock vector. The results showed that the *Igf-1* expression was not affected by hyperprolactinemia in both male and female mice. *Ghr* mRNA expressions were not affected by hyperprolactinemia, neither (Fig 3B), indicating that GH-GHR interaction in the liver was not affected

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by elevated serum prolactin level.

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Discussion

In this study, several lines of evidence showed that prolactin up-regulated mRNA expressions of some female-predominant class I and class II genes such as *Cyp3a16*, *Cyp3a41*, *Cyp3a44*, *Cyp2b9* and *Prlr* in female mice liver and induced down-regulation of male-predominant class I and part of class II genes such as *Cyp2d9*, *Cyp7b1*, *Mup1* and *Alas2* in expression in male mice liver. Especially female class I gene expressions were dramatically elevated by prolactin treatment in female mice liver.

Female class I genes, such as *Cyp3a16*, *Cyp3a41* and *Cyp3a44* are female-predominant murine *Cyp3a* isoforms expressed in the liver that belong to class IA liver genes defined as positively regulated by the female but not male GH secretory profile (Ramirez et al., 2015). *CYP*s are conveniently arranged into families and subfamilies based on the percentage of amino acid sequence identity, with *CYP2*, *CYP3*, and *CYP4* families containing far more genes than the other families in humans and mice (Nerbert et al., 2013). Murine *Cyp3a* is a subfamily of six isoforms, *Cyp3a11* (Yanagimoto et al., 1992), *Cyp3a13* (Yanagimoto et al., 1994), *Cyp3a16* (Itoh et al., 1994), *Cyp3a25* (Dai et al., 2001), *Cyp3a41* (Sakuma et al., 2000), and *Cyp3a44* (Sakuma et al., 2002). *Cyp3a16* expression is detected in mice fetal liver and starts to decrease after 2 weeks of age, and only a small amount is detectable at 5 weeks of age (Ito et al., 1994). In contrast, *Cyp3a41* is constantly expressed after birth in female mice, whereas *Cyp3a44* expression increases with age in female mice after 3 weeks of age. In male mice, the expressions of both *Cyp3a41* and *Cyp3a44* gradually decline after 5 weeks of age (Sakuma

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et al., 2002). GH was determined as the inducer of hepatic *Cyp3a16*, *Cyp3a41* and *Cyp3a44* mRNA expressions (Sakuma et al., 2002, Sakuma et al., 2008, Ramirez et al., 2015). During pregnancy, PGH levels increase continuously and replace the pituitary GH. PGH differs from GH by only 13 amino acid residues and they both bind to the same GHR with similar affinity. GH-GHR signaling induces hepatocyte nuclear factor 6 and up-regulates *Cyp3a41* expression in mice and *CYP3A4* in humans. Mouse plasma estradiol concentration is also elevated during pregnancy and has been reported to induce the liver *Cyp3a16*, *Cyp3a41* and *Cyp3a44* expression, and this up-regulation is correlated with estrogen receptor-α transcripts. Increased PGH and estrogen were considered to activate the transcription of *Cyp3a41* and *Cyp3a44* in a synergic manner during pregnancy (Zhang et al., 2008).

Recently, Ramirez et al. demonstrated high prolactin levels and increase of the *Cyp3a16*, *Cyp3a44*, and *Cyp3a41* mRNA expressions without changing pituitary GH and serum IGF-1 levels in female mice which genetically lack dopamine 2 receptors (D2R) in lactotropes. In contrast, *Mup* mRNA levels were decreased in the liver of male and female mice which genetically lack D2R in neurons, resulting in reduction of pituitary GH and serum IGF-1, but not serum prolactin (Ramirez et al., 2015). In the present study, prolactin was revealed to be the independent inducer of hepatic *Cyp3a16*, *Cyp3a41* and *Cyp3a44* mRNA expression in female mice. Considering that prolactin is secreted mainly from the pituitary gland and shares similar amino acid sequence and structural and biological features with GH (Freeman et al., 2000), it is not contradictory that prolactin exhibits similar effect as

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that of the continuous GH secretion on *Cyp3a16*, *Cyp3a41* and *Cyp3a44* induction. However, differently from the continuous GH, hyperprolactinemia induced hepatic Stat5 phosphorylation, which was in common with the pulsatile GH, resulting in alteration of hepatic gene transcriptions. It is still to be elucidated how the different gene expressions between prolactin and GH could be induced by the same Stat5 phosphorylation and activation.

We could not completely exclude the possibility that hyperprolactinemia altered the episodic GH secretion pattern in male mice, but serum GH level and liver *Igf-1* mRNA level were not significantly altered. In addition, *Cyp3a16*, *Cyp3a41* and *Cyp3a44* induction was observed in female mice whose GH secretion pattern was continuous, suggesting that the effect of prolactin is independent of the influence of the GH secretion pattern. GH receptor mRNA expression in liver of both male and female mice was not affected by hyperprolactinemia, suggesting that the influence on GH–GHR interaction have been nominal in this hyperprolactinemia model. However, prolactin alone could not induce female-predominant gene expressions in the female human hepatocytes *in vitro* (Lee et al., 2014). We conjecture that induction of female-predominant genes by prolactin requires several other factors coexist in the serum *in vivo*.

The prior studies have reported that proestrous surge of prolactin did not change the hepatic *Cyp* expression and Stat5 activity, and that neither ablation of prolactin by bromocriptine nor direct injection of prolactin affected the hepatic Stat5 activity (Choi and Waxman, 1999, Lee et al., 2012).

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Considering the short duration of proestrous prolactin surge, which consists of 2 hours of an early peak (350 to 550 ng/mL) and following 4 hours of a lower plateau (250 to 300 ng/mL) (Murai et al., 1989), and the rapid metabolic clearance of prolactin in the rat, in which half-life time of prolactin was 3.9 to 5.2 min (Koch et al., 1971), or in the human (Sievertsen et al., 1980), it is not contradictory that proestrous surge of prolactin or direct injection of prolactin, as well as continuous basal level of prolactin which could be ablated by bromocriptine, did not have any influence on the up-regulation of female-predominant *Cyp* gene expressions or Stat5 activation in the liver, which might require continuous exposure of high level prolactin, based on the fact that hydrodynamics-based cDNA transfection into the liver leads to the targeted protein synthesis 8 hours after the injection and continuously produces the protein for at least 6 days (Liu et al., 1999).

We also revealed that prolactin acted as an inhibitor of male-predominant gene expressions including *Mup1* in male mice. Mup is a pheromone carrier protein of the lipocalin family secreted in rodent urine and also acts as a pheromone itself (Finlayson et al., 1965, Beynon and Hurst, 2004, Chamero et al., 2007), and is positively regulated by testosterone or pulsatile GH secretion. (Finlayson et al., 1965, Szoka and Paigen, 1978). In contrast, continuous GH secretion and chronic occupancy of GHR lead to inhibitory production of Mups (Norstedt et al., 1984). Prolactin has been reported to influence testosterone biosynthesis by altering the sensitivity of Leydig cells to the luteinizing hormone and decrease serum testosterone level in rams (Sanford and Baker, 2010). In the present study, however,

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the level of serum GH and TT was not significantly decreased in male mice. Therefore, we conjectured that the effect of prolactin on male-predominant gene expressions including *Mup* in the liver is independent of both testosterone and GH.

In the humans prolactin secretion is increased in several physiologic and pathological conditions. During pregnancy, placental estrogen production stimulates lactotroph mitosis and prolactin synthesis, leading to a stepwise increase in serum prolactin levels, achieving mean levels of 200 ng/mL at the end of pregnancy and up to 450 ng/mL in some cases. Serum prolactin levels decline quickly after delivery but are maintained slightly increased in nursing woman, especially after breastfeeding (Bronstein, 2016). Hyperprolactinemia is the most prevalent hypothalamic-pituitary dysfunction, with prolactinomas being the main cause. Microprolactinomas (diameter <10 mm) represent about 60% of prolactin-secreting adenomas and are more common in women than in men (20:1), whereas macroadenomas have roughly the same prevalence in both genders, and there are no sex-related differences in autopsy series. Serum prolactin usually ranges from 50 to 300 ng/mL in the presence of microprolactinoma and from 200 to 5000 ng/mL in the presence of macroprolactinoma (normal values range from 2 to 15 ng/mL). Dopamine receptor blockers such as sulpiride, haloperidol, chlorpromazine, metoclopramide or domperidone are the most frequent cause of the pharmacologic hyperprolactinemia and the serum prolactin increases at the levels that usually are detected with

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prolactinomas (Bronstein, 2016). Even though physiological increase of prolactin level is specific for female, in some pathological conditions male could also be susceptible to hyperprolactinemia.

To conclude, we demonstrated the regulatory roles of prolactin in sex-predominant expression of hepatic genes. Differently from rodents, high levels of circulating prolactin during human pregnancy are well established (Egli et al., 2010). Prolactin has shown to regulate expression of various hepatic drug-metabolizing enzymes such as uridine 5'-diphospho-glucuronosyltransferase 1-6 and glutathione S-transeferase, resulting in increase of glutathione conjugation and glucuronidation of *p*-nitrophenol (Jeong H, 2013). Alteration of hepatic *CYP* expressions induced by hyperprolactinemia would be one of the causes of modifying hepatic drug metabolisms in human during pregnancy and lactation.

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Authorship Contributions

Participated in research design: Sato, Kaneko, Cho, K Goto, Otsuka, and Narita

Conducted experiments: Sato, Kaneko, and Cho

Contributed new reagents or analytic tools: Sato, Kaneko, Cho, and Maruyama

Performed data analysis: Sato, Kaneko, and Cho

Wrote or contributed to the writing of the manuscript: Sato, Kaneko, Cho, Yamamoto, S Goto, Maruyama, and Narita

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Footnotes

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Figure legends

Fig. 1. Prolactin gene transfection by hydrodynamic injection into mouse liver. (A) Expression vector pCAGGS with mouse prolactin cDNA insert (pCAGGS-*mPrl*, Prl) or mock vector was transfected into hepatocytes by hydrodynamic method in male or female mice at 10–12 weeks of age, and the transfection of each pCAGGS plasmid was examined two days after the injection by amplifying pCAGGS common sequence or mouse *Prl*-specific sequence using DNA extracted from the liver. (B) Murine *Prl* and *Gapdh* mRNA expression in the liver was examined two days after the injection by reverse transcription PCR after hydrodynamic injection of the pCAGGS-*mPrl* or mock vector; NT: mRNA from the liver of the mouse with no treatment. (C) Serum concentration of prolactin in male and female mice two days after the injection of pCAGGS-*mPrl* (Prl) or mock vector (n = 4). (D) Expression of PrlR in hepatocyte in female and male mice. PrlR in hepatocytes was detected in both male and female mice by western blot. Albumin was reacted as a loading control. Normal goat IgG was used as a control IgG. (E) Phosphorylation of Stat5 in hepatocyte in male and female mice two days after injection of pCAGGS-*mPrl* (Prl) or mock vector. $^{**}P < 0.01$. Error bars indicate S.D.

Fig. 2. Gene expressions in the liver affected by prolactin. mRNA was extracted from the liver of male or female mice treated with pCAGGS-*mPrl* (Prl) or mock vector two days after the hydrodynamic injection. Liver expressions of each gene were examined in male or female mice at 10–12 weeks of age. (A) female class I gene (n = 4), (B) female class II gene (n = 4), (C) male class I gene (n = 4), (D)

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male class II gene (n = 4), (E) unclassified gene (n = 4). Expression of each indicated mRNA was quantified and standardized with that of *Gapdh* in each individual mouse. All of the values were set to 1.0 in the female-mock group (A, B), or in the male-mock group (C, D, E), and the results were shown as fold change. (F) Serum albumin and Mup in male mice two days after the treatment with pCAGGS-m*Prl* (Prl) or pCAGGS mock vector. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Error bars indicate S.D.

Fig. 3. Effect of prolactin on serum GH and TT. (A) Serum concentrations of GH and TT of male and female mice were examined two days after the treatment with pCAGGS-m*Prl* (Prl) or pCAGGS mock vector at 10–12 weeks of age (n = 4). (B) mRNA was extracted from the liver of male or female mice two days after the treatment with pCAGGS-mPrl (Prl) or pCAGGS mock vector. *Igf-1* and *Ghr* mRNA expressions were quantified and standardized with that of *Gapdh* in each mouse. The values were set to 1.0 in the male-mock group and the results were shown as fold change. (n = 4) $**P < 0.01$, Error bars indicate S.D. N.D.; not detected.

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Table 1. Sequences of primers used in the study

Gene	Sense (5′→3′)	Antisense (5′→3′)
pCAGGS common sequence	acataacttacggtaaatggc	gatgactaatacgtagatgta
mouse prolactin cDNA insert	catcattttggcaaagaattc	tttatcgaattcaatgaacat
Gapdh	tgtgtccgtcgtggatctga	ttgctgttgaagtcgcaggag
Prl	atgaccatgaacagccaggggtcagc	ttagcagttgttttgatgggcaattt
Cyp3a16	agcaccgcgtggactttatt	gggctgtgatctcgatttcag
Cyp3a41	gccaaagggattttaagagttgact	ggtgtcaggaatggaaaaagtaca
Cyp3a44	atccccaaagggtcaatggtg	agggcagatatacgtaaggatca
Cyp2a4	agcaggctaccttcgactgg	gctgctgaaggctatgccat
Cyp2b9	caccaggaccccatcctcta	tttcttgaagctgaatgaaacact
Prlr	cacagtaaatgccacgaacg	ggcaaccattttacccacag
Cyp2d9	agtctctggcttaattcctgat	cgcaagagtatcgggaatgc
Cyp7b1	tgaggttctgaggctgtgctc	tcctgcacttctcggatgatg
Mup1	caaaacagaaaaggctggtga	ttgtgcaaacctttccttga
Alas2	gccattgtcctttcatgctgt	atgaatggcttcgggtggtt
Mcm10	gagttccccaggttggaagg	gcgctcagttttggtcttgg
Nox4	ggggttaaacacctctgcct	caggactgtccggcacatag

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Cyp1a2	ggaggctaaccatctcgtca	ggcaccaatgacgttagcca
Cyp3a11	tcacacacacagttgtagggagaa	gtccatccctgcttgtttgtc
Cyp3a13	accggcggcgcttt	attctcagagatagagatggcctttt
Cyp3a25	cttcactgtccagccttgtgaa	aattggttccctgctgatcttc
Cyp19a1	tcatggtcccggaaactgtg	atcaggagaaggaggcccat
Cyp27a1	tccaatgtggacaacctcct	atcacttgctccaagagtggg
Igf-1	tgtaaacgacccggacctac	cacgaactgaagagcatcca
Ghr	ccaactcgcctctacaccg	gggaaaggactacaccacctg

Fig. 1

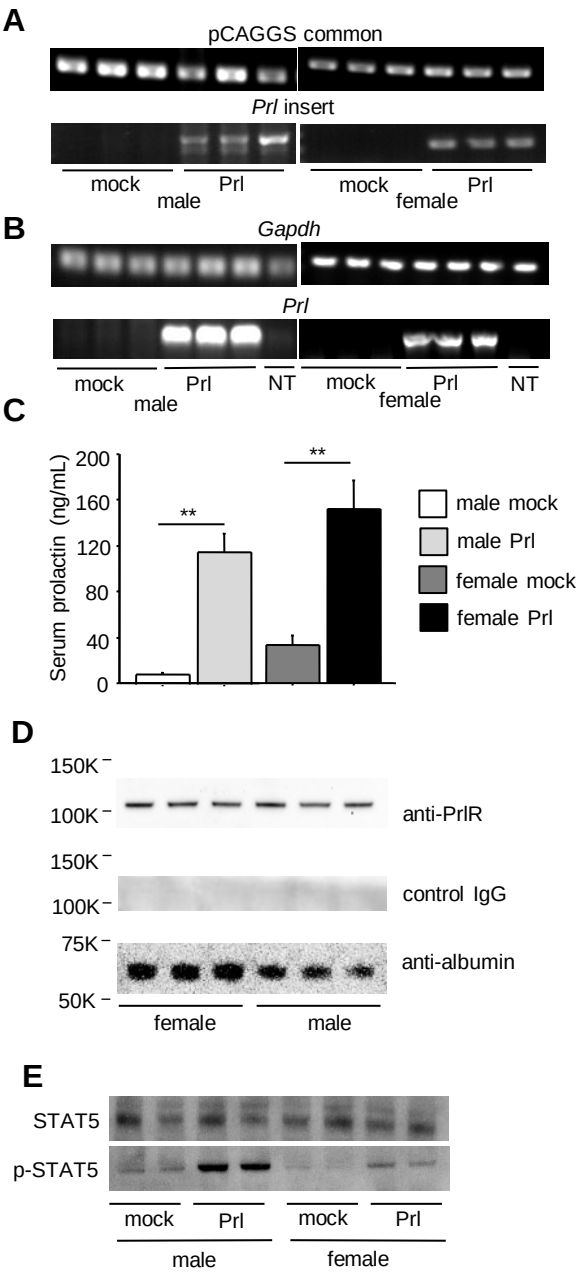

A
pCAGGS common
Prl insert
mock
Prl
mock
Prl
male
female
B
Gapdh
Prl
mock
Prl
NT
mock
Prl
NT
male
female
C
Serum prolactin (ng/mL)
200
160
120
80
40
0
**
**
male mock
male Prl
female mock
female Prl
D
150K
100K
anti-PrlR
150K
100K
control IgG
75K
50K
anti-albumin
female
male
E
STAT5
p-STAT5
mock
Prl
mock
Prl
male
female

Fig. 2

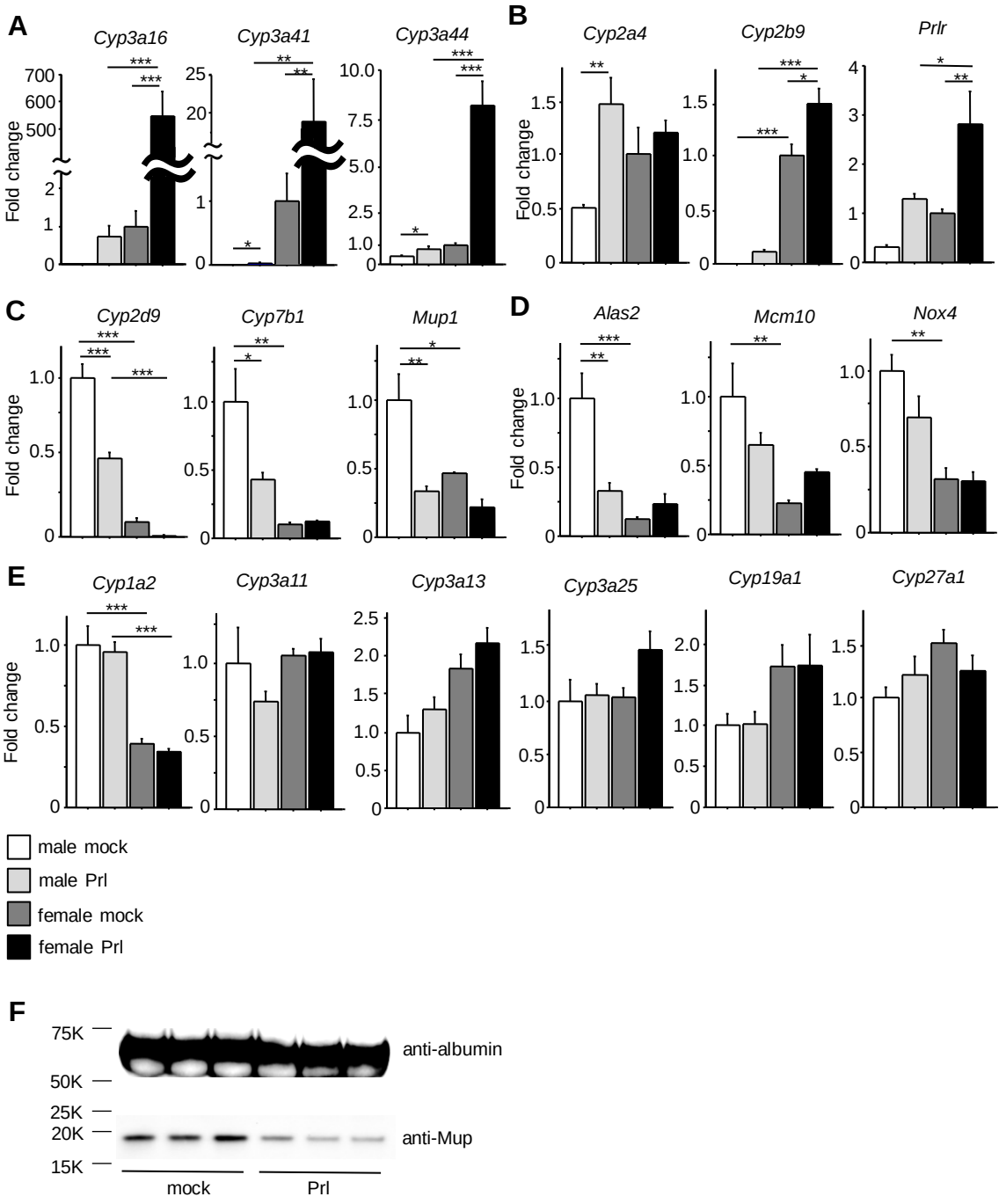
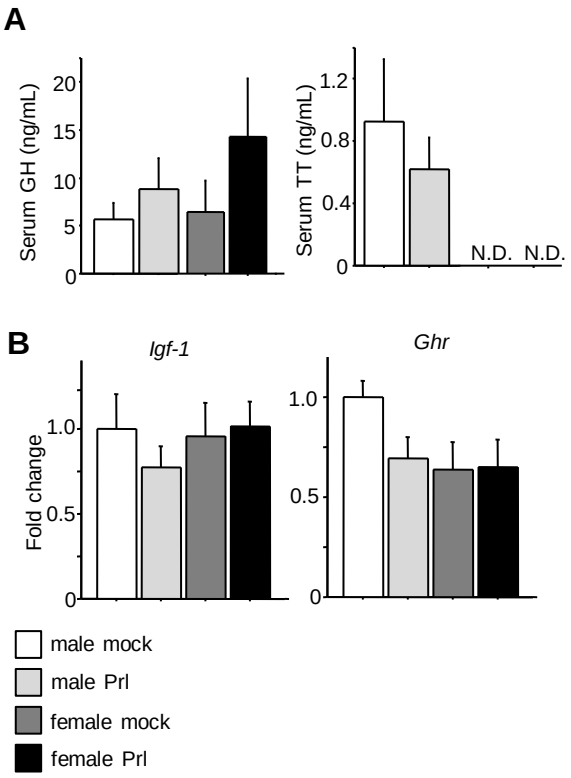

A
Cyp3a16
Cyp3a41
Cyp3a44
B
Cyp2a4
Cyp2b9
Prlr
C
Cyp2d9
Cyp7b1
Mup1
D
Alas2
Mcm10
Nox4
E
Cyp1a2
Cyp3a11
Cyp3a13
Cyp3a25
Cyp19a1
Cyp27a1
Fold change
male mock
male Prl
female mock
female Prl
F
75K
50K
25K
20K
15K
anti-albumin
anti-Mup
mock
Prl

Fig. 3


A
Serum GH (ng/mL)
20
15
10
5
0
Serum TT (ng/mL)
1.2
0.8
0.4
0
N.D.
N.D.
B
Igf-1
Ghr
Fold change
1.0
0.5
0
1.0
0.5
0
male mock
male Prl
female mock
female Prl