

JPET #94367

Growth Hormone Determines Sexual Dimorphism of Hepatic Cytochrome P450 3A4 Expression in Transgenic Mice

Connie Cheung¹, Ai-Ming Yu¹, Chong-Shen Chen, Kristopher W. Krausz, Linda G. Byrd, Lionel Feigenbaum, Robert J. Edwards, David J. Waxman and Frank J. Gonzalez

Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland (C.C., A-M.Y., K.W.K., L.G.B., F.J.G); Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, The State University of New York, Buffalo, New York (A-M.Y.); Department of Biology, Division of Cell and Molecular Biology, Boston University, Boston, Massachusetts (C-S.C., D.J.W.); Laboratory Animal Science Program, SAIC, National Cancer Institute, Frederick, Maryland (L.F.); Section of Experimental Medicine and Toxicology, Division of Medicine, Imperial College London, London, United Kingdom (R.J.E.)

JPET #94367

Running Title: Sexual dimorphism of CYP3A4 developmental expression

Corresponding Author: Frank J. Gonzalez, Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, Building. 37, Room 3106, Bethesda, MD 20892, USA;
Phone: 301-496-9067; Fax: 301-496-8419; E-mail: fjgonz@helix.nih.gov

Text Pages: 30

Table: 1

Figures: 6

References: 49

Words in: Abstract: 249
Introduction: 685
Discussion: 1145

Abbreviations: CYP, cytochrome P450; GH, growth hormone; TG, transgenic; WT, wild-type.

Recommended section: Adsorption, Distribution, Metabolism and Excretion.

JPET #94367

Abstract

The impact of age and sex on the expression of hepatic cytochrome P450 3A4 (CYP3A4) was recently determined in a transgenic mouse line carrying the human *CYP3A4* gene. To further investigate the physiological regulation of human *CYP3A* genes, a novel transgenic mouse line was generated using a bacterial artificial chromosome clone containing both *CYP3A4* and *CYP3A7* genes. *CYP3A7* expression was observed in transgenic mouse fetal livers, while *CYP3A4* exhibited developmental expression characterized by sexual dimorphism in postpubertal livers. Hepatic *CYP3A4* protein and RNA were expressed in immature transgenic male mice and became undetectable after six weeks of age, whereas *CYP3A4* was expressed in both immature and adult females. *CYP3A4* was markedly elevated by the xenobiotic receptor activator phenobarbital in both male and female livers, demonstrating drug induction of *CYP3A4* transgene in this mouse model. Furthermore, continuous infusion of recombinant growth hormone (GH) in transgenic male mice, overriding the pulsatile male plasma GH profile, stimulated hepatic *CYP3A4* mRNA and protein to normal female levels. Continuous GH treatment also feminized the expression of endogenous murine *Cyp2b* and *Cyp3a44* genes. Thus, human *CYP3A4* contains all of the gene regulatory sequences required for it to respond to endogenous hormonal regulators of developmental expression and sexual dimorphism, in particular GH. These findings may help elucidate the role of GH in determining the sex-dependent expression of *CYP3A4* in human liver, and suggest that GH therapy may alter the pharmacokinetic and pharmacodynamic properties of *CYP3A4* substrates leading to enhanced metabolism and disposition of drugs in men.

JPET #94367

Introduction

The human cytochrome P450 3A (*CYP3A*) gene subfamily consist of four members, *CYP3A4*, *CYP3A5*, *CYP3A7* and *CYP3A43*, which are all located on chromosome 7q22.1 (Gellner et al., 2001). The encoded *CYP3A7* protein is a fetal-specific form (Komori et al., 1990) that accounts for about 50% of the total CYP protein present in fetal liver, whereas *CYP3A4* is the most abundant CYP enzyme in mature hepatocytes and small intestinal epithelial cells. *CYP3A4* is responsible for the metabolism of more than 50% of marketed drugs including cholesterol-lowering statins, benzodiazepines, calcium channel blockers, HIV antivirals, macrolide antibiotics and physiologically important substrates such as steroids and bile acids (Guengerich, 1999).

CYP3A4 can be regulated by many endogenous and exogenous factors, leading to considerable inter-individual variations in the metabolism of *CYP3A4* drug substrates. Nuclear receptor regulation of the *CYP3A4* gene accounts in part for some of this diversity (Willson and Kliewer, 2002). Xenobiotic drugs that activate pregnane X-receptor (PXR), constitutive androstane receptor (CAR) or vitamin D receptor are known to induce *CYP3A4* expression, potentially leading to altered pharmacokinetic profiles of drugs metabolized by this CYP enzyme. In addition, hepatocyte nuclear factor-4 α plays an important role in *CYP3A4* transcriptional regulation (Tirona et al., 2003).

CYP3A4 expression increases during the development from childhood to adulthood as suggested by a recent comprehensive study (Stevens et al., 2003). Although *Cyp3a* genes are expressed in a sexually dimorphic manner in mice and rats (Waxman et al., 1988; Kawai et al., 2000; Sakuma et

JPET #94367

al., 2002), it has not been firmly established whether CYP3A4 expression is sex-dependent in humans (Hines and McCarver, 2002; Gandhi et al., 2004; Parkinson et al., 2004), which has been studied in vitro using human liver preparations, and in vivo using CYP3A4 probe drugs. Several lines of evidence indicate that the metabolism of several clinical drugs, mainly oxidized by CYP3A4, is more efficient in women than in men (Greenblatt et al., 1980; Watkins et al., 1989; Gorski et al., 1998). Recent studies show that women have higher hepatic CYP3A4 content and drug-metabolizing capacity than men (Wolbold et al., 2003), however, this conclusion is opposed by other observations (Schmucker et al., 1990; George et al., 1995).

Investigation of the sex-specificity of CYP3A4 in humans is complicated by interindividual differences in exposure to dietary supplements, drinking, smoking and medication history, all of which are known to affect CYP3A4 expression or enzymatic activity. These factors can be controlled in the case of animals maintained under defined dietary and environmental conditions, suggesting that animal models, including transgenic mice, may be useful for investigation of the sexual dimorphism and developmental expression of human CYP enzymes. Extensive investigations carried out in rats and mice, noted above, have demonstrated that Cyp3a expression is sex-dependent in these species, although the sexual dimorphism of certain CYP enzymes in rats may be opposite to that in humans (Rich and Boobis, 1997). In addition, a recent study with a *CYP3A4*-transgenic mouse line (Granvil et al., 2003) revealed a sexual dimorphism in the developmental expression of the *CYP3A4* transgene (Yu et al., 2005).

To further investigate the sexual dimorphism and developmental regulation of human *CYP3A* genes, a novel transgenic mouse line carrying human *CYP3A4* and *CYP3A7* was produced with a

JPET #94367

bacterial artificial chromosome (BAC) clone. Sexual dimorphism of CYP3A4 expression was observed in transgenic mouse livers, while CYP3A7 was expressed in fetal livers. Phenobarbital, an activator of the xenobiotic receptors CAR and PXR, markedly induced the expression of the *CYP3A4* transgene and of the endogenous murine *Cyp3a* genes. Because sexually dimorphic growth hormone (GH) secretion is an important determinant of gender-specific patterns of growth and metabolism (Jaffe et al., 1998), and GH treatment markedly alters *Cyp3a* expression as demonstrated by extensive studies with rats and mice (Waxman et al., 1988; Robertson et al., 1998; Kawai et al., 2000; Sakuma et al., 2002), transgenic adult mice were treated with GH to determine the hormone responsiveness of the CYP3A4 transgene. Our findings show that continuous GH treatment stimulated hepatic CYP3A4 expression in transgenic male mice. These results may provide insight into the physiological regulation of CYP3A4 expression in humans and its potential significance in drug therapy.

JPET #94367

Materials and Methods

Chemicals, Enzymes and Other Reagents. Phenobarbital, 4-amidinophenylmethanesulfonyl fluoride (APMSF) and disodium ethylenediaminetetraacetate were purchased from Sigma (St. Louis, MO). Recombinant human P450 Supersomes™ and pooled human liver microsomes (coded H161) were purchased from BD Discovery Labware, Inc. (Woburn, MA). Restriction enzymes were purchased from Roche Applied Science, Life Technologies (Gaithersburg, MD), or New England Biolabs (Ipswich, MA) and were used in buffer systems provided by the manufacturers. Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was purchased from Chemicon International Inc. (Temecular, CA). P450 immunoblot analysis was carried out using antibodies to human CYP3A7 (Sim et al., 2005), CYP3A4 (MAb 275-1-2), and rat CYP3A1 (MAb 2-13-1) (Gelboin et al., 1995), CYP1A2 (MAb 22-341) (Park et al., 1982), CYP2A3 (Nagata et al., 1987) and CYP2B1 (Yamano et al., 1989). Highly purified recombinant mouse growth hormone (GH) was purchased from the National Hormone and Peptide Program-UCLA (Torrance, CA).

Animals and Treatments. Mice were maintained under controlled temperature ($23 \pm 1^{\circ}\text{C}$) and lighting (lights on 6:00 A.M. - 6:00 P.M.) with chow and water provided ad libitum. Animal handling was in accordance with the NIH guidelines for the care and use of laboratory animals and study protocols approved by the Institutional Animal Care and Use Committee at National Cancer Institute. Phenobarbital sodium salt was dissolved in saline at a concentration of 8 mg/ml. Mice (male or female, 6- to 7-week-old, 2-3 in each group) were administered i.p. with phenobarbital (80 mg/kg) or saline (as control) everyday for four days, following which the mice were killed, livers were collected and stored at -80°C . For the GH treatment study, 7-week-old

JPET #94367

male and female mice were implanted subcutaneously with an Alzet mini-osmotic pump (Durect Corporation, Cupertino, CA) filled with 0.8 µg/µl recombinant mouse GH that was released at a rate of 0.5 µl/hr. Mice with surgery but no pump implantation (sham-treatment) were used as a control. At least four mice were included in each treatment group. All mice were killed one week later, at which point the livers were collected and snap frozen in liquid nitrogen and then stored at -80°C.

Generation of *CYP3A4-CYP3A7* Transgenic Mice. The BAC clone RP11-757A13 (Resgen/Invitrogen Corporation, Huntsville, AL) containing the complete *CYP3A4* and *CYP3A7* gene sequences, including 5' and 3' flanking sequence (Fig. 1A) was identified. The BAC clone was purified by using a maxi prep kit (Qiagen, Valencia, CA) and verified by Southern blot analysis with ³²P-end labeled *CYP3A4* cDNA and DNA oligonucleotide probes recognizing specific regions (Exon 1 and 13, and -10 kb upstream) of the human *CYP3A4* gene (Fig. 1B). The BAC clone was linearized by restriction enzyme digestion (P1-Sce) and purified before microinjection into fertilized FVB/N mouse eggs. Several transgenic founders were identified by Southern blot analysis and bred with C57BL/6 mice. Mice resulting from this breeding step that were positive for the human *CYP3A4* transgene by Southern blot analysis were tested for *CYP3A4* protein expression by Western blotting. Mice derived from the C1 and C2 founder lines expressed *CYP3A4* protein (Fig. 2A). The C2 founder line was selected and further mated to C57BL/6 mice for at least five generations. Homozygous mice were then generated by breeding mice positive for the human *CYP3A4* transgene and were designated as *CYP3A4*- and *CYP3A7*-transgenic mice (TG). The *CYP3A4*-negative littermates obtained from the >5 generations

JPET #94367

backcross into the C57BL/6 strain were bred with each other and were used as wild-type (WT) controls for subsequent studies.

Southern Blot Analysis. DNA was digested with Pst I, EcoR I, Bgl II, BamH I or Apa I for the analysis of the BAC clone. Genomic DNA was isolated from mouse tail and digested with BamH I for the determination of individual mouse genotype. Electrophoresis and southern hybridization conditions described previously were followed (Granvil et al., 2003). Random-primer ³²P-labelled DNA oligonucleotide probes recognizing specific regions of the human *CYP3A4* gene were used for analysis of the BAC clone. A random-primer ³²P-labelled *CYP3A4* cDNA (Gonzalez et al., 1988) probe was used for screening mice containing human *CYP3A4* and *CYP3A7* transgenes.

Preparation of Microsomes. Tissues were homogenized in ice-cold buffer [50 mM Tris-HCl, 150 mM KCl, 20% (v/v) glycerol, 1 mM disodium ethylenediaminetetraacetate and 0.5 mM APMSF, pH 7.4], and microsomes were isolated by differential centrifugation as described previously (Granvil et al., 2003; Cheung et al., 2005). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce Chemical Co. Rockford, IL), following the manufacturer's instructions.

Western Blot Analysis. Microsomal proteins (5-30 µg) from each sample were separated by SDS-PAGE through 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). After probing with antibodies against human *CYP3A4* and *CYP3A7* and rat *CYP3A1*, *CYP1A2*, *CYP2A3*, *CYP2B1*, *CYP2E1* or mouse glyceraldehydes 3-

JPET #94367

phosphate dehydrogenase (GAPDH), the membranes were incubated with the appropriate secondary antibodies (anti-rabbit or anti-mouse IgG horseradish peroxidase purchased from Sigma) followed by ECL detection (Pierce Chemical Co., Rockford, IL) as described previously (Granvil et al., 2003; Cheung et al., 2005; Yu et al., 2005). Blots were scanned and the relative intensity of each band was analyzed using Kodak 1D (V.3.6.3) Scientific Imaging Systems software (New Haven, CT).

RNA Analysis. Total RNA was isolated from liver tissues with Trizol reagent (Invitrogen, Carlsbad, CA) by following the manufacturer's instructions, and the concentrations were determined by optical densitometry at 260 nm. SYBR green-based quantitative real-time PCR (QPCR) analysis of the specific CYP and 18S mRNAs in liver samples was carried out using an Applied Biosystems 7900HT instrument and gene-specific primers under conditions reported previously (Wiwi et al., 2004). Primers used for analysis of CYP3A4 were 5'-ATG AAA GAA AGT CGC CTC GAA-3' and 5'-AAG GAA ATC CAC TCG GTG CTT-3'.

Statistical Analysis. All values are expressed as the means \pm Standard Deviation (S.D.) unless indicated otherwise. All data were analyzed by paired or unpaired Student's *t* test (GraphPad Prism 3.02, San Diego, CA), and the difference was considered significant if the probability (*P* value) was less than 5%.

JPET #94367

Results

Generation of CYP3A4-CYP3A7 Transgenic Mice. A transgenic mouse line was created using the BAC clone RP11-757A13, containing the complete *CYP3A4* and *CYP3A7* genes together with over 35 kb of 5' flanking DNA and 9 kb of 3' flanking DNA (Fig. 1A). Hybridization analyses were performed with ³²P-labeled probes including *CYP3A4* cDNA, and oligonucleotides recognizing specific regions of Exon 1, Exon 13, and sequences -10 kb upstream of the *CYP3A4* gene were used to verify the BAC clone. The size of each hybridized band corresponded well with the size predicted from the map of *CYP3A4* gene sequence (Fig. 1B). Transgenic mice containing the human *CYP3A4* and *CYP3A7* genes were generated (see Methods), and the presence of the *CYP3A4* transgene was confirmed by Southern blotting at each breeding step. Two positive founders, designated C1 and C2, were shown to carry the *CYP3A4* transgene. Western blot analysis with a monoclonal antibody that recognizes *CYP3A4* but not any endogenous murine Cyp3a proteins, revealed that *CYP3A4* was expressed in the livers of 3-week-old *CYP3A4*-positive mice from both founders (Fig. 2A). The C2 founder was selected without any bias to generate a homozygous transgenic mouse line for the following studies.

Tissue Distribution of CYP3A4 and CYP3A7 Proteins in Transgenic Mice. Western blots were performed to assess the expression of *CYP3A4* and *CYP3A7* proteins in transgenic mouse tissues. *CYP3A4* protein was detected in the liver, but was not detectable in brain, kidney, heart, lung, stomach, spleen or colon of 3-week-old mice (Fig. 2B). To determine the expression of *CYP3A4* in the gut, small intestine was segmented into duodenum, jejunum and ileum. Western blots of microsomal proteins indicated that *CYP3A4* was expressed in the duodenum and

JPET #94367

jejunum but not in the ileum. Interestingly, CYP3A4 levels are comparable between duodenum and jejunum for females whereas it is lower in jejunum for males (Fig. 2B). Analysis of GAPDH protein as a loading control verifies that the intestinal protein was of similar quality and quantity between male and female jejunum samples thus indicating that low expression in males was not due to degradation. Furthermore, CYP3A7 protein was expressed in transgenic fetal liver (Fig. 2C) as shown using a specific anti-peptide antibody. While the CYP3A4 was not detected in kidney from 3-week-old transgenic mice by Western blotting, we cannot conclude that the protein is absent since this tissue, unlike liver and gut, is complex with many different cell types. Further studies using immunohistochemistry and tissues from mice at different age will be required to address whether CYP3A4 is expressed in kidney and other complex tissues like the brain.

Sexual Dimorphism of CYP3A4 Expression in Transgenic Mice. The impact of sex and age on the expression of CYP3A4 in the transgenic mouse livers was investigated, since both factors can affect hepatic CYP3A4 expression (Yu et al., 2005). CYP3A4 protein was expressed in the livers of male mice from 1- to 4-week of age, decreased in 5-week-old mice, and was undetectable from age 6- to 24-weeks of age. By contrast, hepatic CYP3A4 expression remained at similar levels in the 5- to 24-week-old female mice of this transgenic line (Fig. 3). In contrast to the differential expression of CYP3A4 between adult males and females, the developmental expression patterns for murine Cyp1a2 and GAPDH protein showed no difference between the sexes from age 3 to 24 weeks (Fig. 3). Further studies revealed similar age- and sex-dependent profiles for hepatic CYP3A4 mRNA, namely, suppression in males but not females after the age of 5 weeks. Moreover, similar profiles were apparent for the endogenous mouse liver gene

JPET #94367

Cyp3a44, whose expression was also female-specific in adults (Table 1). By contrast, expression of a related mouse gene, *Cyp3a25*, was not age or sex-dependent.

Induction of CYP3A4 and Murine Cyp3a by Phenobarbital. Treatment of the transgenic mice with phenobarbital induced the *CYP3A4* transgene (Fig. 4). CYP3A4 was not detectable in the livers of 6- to 7-week-old transgenic male mice, in agreement with the results shown in Figure 3, but was detected in females treated with saline. Phenobarbital, a known activator of the nuclear receptors PXR and CAR, induced CYP3A4 expression ~11-fold in the transgenic female livers. Moreover, phenobarbital induced CYP3A4 in the male transgenic livers to a level comparable to that induced in the females. Murine Cyp3a protein levels were also elevated, about 3 to 4-fold, compared to saline-treated controls (Fig 4). Induction of CYP3A4 and murine Cyp3a was associated with significantly elevated mRNA levels and resulted in sharply increased Cyp3a enzymatic activity (data not shown), indicating the potential to alter the metabolism and pharmacokinetics of Cyp3a drugs.

Growth Hormone Stimulates Expression of Human CYP3A4 and Murine Cyp3a44 in Transgenic Male Mice. Because plasma GH profiles are an important physiological regulator of sex-dependent CYP3A gene expression, we evaluated the effect of GH treatment on CYP3A4 expression in transgenic mouse livers. Recombinant mouse GH was administered as a continuous infusion over 7 days, which overrides the male, pulsatile plasma GH pattern, and in the case of the rat feminizes liver gene expression (Mode et al., 1981; Ahluwalia et al., 2004; Holloway et al., in press). Western blot analysis revealed that liver CYP3A4 expression was strongly induced in the transgenic males treated with continuous GH. By contrast, GH treatment

JPET #94367

did not alter hepatic CYP3A4 expression in the females (Fig. 5). Continuous GH infusion also stimulated the expression of a female-specific murine Cyp2b form in the male mice. Murine Cyp1a2, Cyp2a and Cyp2e1 levels were lower in both male and female mice treated with GH, compared to sham operated controls (Fig. 5). As a control, the content of GAPDH protein was shown to be unchanged in both males and females following GH treatment. In addition, CYP3A7 protein was undetectable in both the control and GH-treated male and female mouse livers (data not shown).

To further investigate whether the effects of GH are due to transcriptional regulation, and to provide more quantitative analysis, mRNA levels were determined by QPCR analysis. CYP3A4 mRNA was sharply increased in the GH-treated male mice (about 30-fold higher than in sham-treated control males), whereas there was no change in the GH-treated females (Fig. 6A), a result that is consistent with Western blot analysis (Fig. 5). Continuous GH treatment also induced the endogenous murine Cyp3a44 gene in the transgenic male livers, to a level ~130-fold higher than sham control males and comparable to that of correspondingly treated females (Fig. 6B). By contrast, GH had no significant effect on Cyp3a11 and Cyp3a25 mRNA levels in males or females (Fig. 6C, 6D), which serve as examples of sex-independent, and GH-unresponsive, hepatic Cyp3a genes. Together, these findings demonstrate that the plasma GH profile is an important factor in modulating sex-dependent expression of the *CYP3A4* transgene at the transcriptional level, as it is in modulating the sex-dependent expression of the endogenous mouse *Cyp3a44* gene.

JPET #94367

Discussion

This study characterizes a new transgenic mouse line carrying human *CYP3A4* and *CYP3A7* transgenes and its use for investigation of CYP3A gene regulation. The results reveal that hepatic CYP3A4 is subject to developmental expression that is sexually dimorphic, as manifest by the low to undetectable level of CYP3A4 protein and mRNA in the postpubertal male transgenic mice. Moreover, CYP3A4 expression in adult male livers is shown to be induced by GH administered as a 7-day continuous infusion.

Sexual dimorphism in the expression of several hepatic CYP drug-metabolizing enzymes has been demonstrated and studied extensively in rats (Waxman et al., 1991; Shimada et al., 1995; Robertson et al., 1998; Kawai et al., 2000). In particular, rat *CYP3A2*, *CYP3A9* and *CYP3A18* genes are expressed in a male-specific manner, whereas *CYP3A9* is female-specific. Murine *Cyp3a41* and *Cyp3a44* correspond to female-specific forms (Sakuma et al., 2002). Human CYP3A4 appears to be expressed at higher levels in women than men (Greenblatt et al., 1980; Watkins et al., 1989; Gorski et al., 1998; Wolbold et al., 2003). However, other studies show no sex dependence for CYP3A4 (Schmucker et al., 1990; George et al., 1995). A recent study using a large collection (n = 94) of well-characterized surgical liver samples revealed, however, a 2-fold higher level of CYP3A4 protein and RNA in women compared to men (Wolbold et al., 2003). Previous investigations with a *CYP3A4*-transgenic mouse line (Yu et al., 2005) and in the present study using a mouse line made with a larger transgene containing both the *CYP3A4* and *CYP3A7* genes and their 5' and 3' flanking DNA, revealed that CYP3A4 expression is sexually dimorphic in transgenic adult mice, but not in prepubertal mice. Given the dominant role of CYP3A4 in human hepatic drug metabolism, and given the finding that the drug efflux pump P-

JPET #94367

glycoprotein does not show significant sexual dimorphism in human liver (Wolbold et al., 2003), CYP3A4 and its sexually dimorphic expression could be a major factor contributing to sex-dependent drug clearance in humans. Nevertheless, the question is still open whether CYP3A5 can compensate for lower CYP3A4 levels since CYP3A4 expression is known to be highly variable in humans (Stevens et al., 2003).

The polypeptide hormone GH, which is secreted by the pituitary gland in a sexually dimorphic manner, regulates expression sex-specific or sex-dominant *CYP* genes. As an essential hormone for normal linear growth, GH is secreted in pulses, but with differentiated levels and frequencies in men and women (van den Berg et al., 1996; Jaffe et al., 1998). Treatment with exogenous GH has been shown to alter CYP3A expression in rats (Robertson et al., 1998; Kawai et al., 2000), elevate *Cyp3a41* and *Cyp3a44* expression in mice (Sakuma et al., 2002) and increase CYP3A4 activity in humans (Jaffe et al., 2002). In agreement with these findings, the present study demonstrates that GH plays a key role in the sexually dimorphic expression of the *CYP3A4* transgene in mice, through a pretranslational mechanism. Signal transducer and activator of transcription (STAT) 5b has been identified as an essential determinant of GH-mediated sexual expression of CYP enzymes (Park et al., 1999; Holloway et al., in press), however, the precise intracellular regulatory cascade that STAT5b activates leading to the regulation of hepatic CYP expression is only partially understood and awaits more extensive investigation. Furthermore, the absence of CYP3A4 protein in transgenic adult male mice, is in stark contrast to the stable, albeit variable expression of CYP3A4 in humans, suggesting potential influences of the hormonal environment, inducers or other regulatory mechanisms in human subjects that are absent in the mouse model. Rather, the present finding establishes that the human *CYP3A4* gene contains all

JPET #94367

of the DNA sequence elements required to respond to the endogenous mouse hormonal environment, leading to a pattern of postnatal developmental regulation, adult sexual dimorphism and plasma GH-responsiveness that is very similar to that of the endogenous mouse *Cyp3a44* gene. This is an important observation that may facilitate further studies on these regulatory events and the extent to which they are conserved between mice and humans.

In clinic pharmacotherapy, GH has been used to treat GH-deficient growth-retarded children. GH treatment stimulates normal linear growth and can be used to improve bone density in prepubertal patients (Lanes et al., 1996; Powell et al., 1997). The elderly and GH-deficient adults can also benefit from GH replacement therapy, as indicated by increased lean mass, declined adiposity and obviously restored skin thickness (Rudman et al., 1990; Hana et al., 2004), although the balance of benefits and risks and GH dosage have not been defined (Marcus and Hoffman, 1998; Cummings and Merriam, 2003). In one placebo-controlled clinical study, where the effects of GH treatment on CYP activity were investigated in elderly men, a reduction in CYP1A2 activity was observed with no significant change in CYP3A4 activity (Jurgens et al., 2002). The lack of effect on CYP3A4 activity could be due to the utilization of endogenous cortisol metabolism as the marker of CYP3A4 activity, which may not be a reliable indicator of CYP3A4-dependent metabolism in vivo (Galteau and Shamsa, 2003). The present *CYP3A4* transgenic model, in agreement with earlier studies using human hepatocyte cultures (Liddle et al., 1998) and human subjects (Jaffe et al., 2002), suggests that GH treatment not only suppresses CYP1A2 expression but also increases CYP3A4 expression and activity. These GH-dependent changes in hepatic CYP expression may significantly alter the pharmacokinetics and pharmacodynamics of drugs metabolized by CYP1A2 or CYP3A4. Changes in drug efficacy

JPET #94367

may result, perhaps leading to adverse effects as a consequence of the interactions between GH and CYP-metabolized drugs.

The development of *CYP*-humanized mouse models (Gonzalez, 2003) provides a means to overcome species differences in drug metabolism, which are, in large part, associated with differences in the genes encoding CYP drug-metabolizing enzymes. These humanized mouse models have provided valuable tools for studying the function and regulation of CYPs in a whole animal system, where the functional significance of human CYP expression can be evaluated under controlled conditions, and the regulatory networks and mechanistic basis for responses to xenobiotics can be delineated in preclinical studies. These models could also have implications in the understanding of hormone homeostasis and human disease. However, while the physiological environments are obviously different between mice and humans, particularly in body weight, blood flow rate and hormone profiles, this gap may be filled by using an allometric scaling approach to provide quantitative assessments.

In conclusion, the human *CYP3A* transgenic mouse line described here provides a means for studying the regulation and function of human *CYP3A4* and *CYP3A7* genes in a whole animal model. Sexually dimorphic expression of *CYP3A4* was observed and could lead to significant differences in drug metabolism and disposition, pharmacokinetics and pharmacodynamics between men and women. These findings, taken in the context of earlier reports, suggest that caution should be advised regarding the potential for altered drug metabolism when prescribing *CYP3A4* drugs to patients receiving GH replacement therapy.

Acknowledgements: We thank John R. Buckley for technical assistance.

JPET #94367

References

- Ahluwalia A, Clodfelter KH and Waxman DJ (2004) Sexual dimorphism of rat liver gene expression: regulatory role of growth hormone revealed by deoxyribonucleic Acid microarray analysis. *Mol Endocrinol* **18**:747-760.
- Cheung C, Yu AM, Ward JM, Krausz KW, Akiyama TE, Feigenbaum L and Gonzalez FJ (2005) The cyp2e1-humanized transgenic mouse: role of cyp2e1 in acetaminophen hepatotoxicity. *Drug Metab Dispos* **33**:449-457.
- Cummings DE and Merriam GR (2003) Growth hormone therapy in adults. *Annu Rev Med* **54**:513-533.
- Galteau MM and Shamsa F (2003) Urinary 6beta-hydroxycortisol: a validated test for evaluating drug induction or drug inhibition mediated through CYP3A in humans and in animals. *Eur J Clin Pharmacol* **59**:713-733.
- Gandhi M, Aweeka F, Greenblatt RM and Blaschke TF (2004) Sex differences in pharmacokinetics and pharmacodynamics. *Annu Rev Pharmacol Toxicol* **44**:499-523.
- Gelboin HV, Krausz KW, Goldfarb I, Buters JT, Yang SK, Gonzalez FJ, Korzekwa KR and Shou M (1995) Inhibitory and non-inhibitory monoclonal antibodies to human cytochrome P450 3A3/4. *Biochem Pharmacol* **50**:1841-1850.
- Gellner K, Eiselt R, Hustert E, Arnold H, Koch I, Haberl M, Deglmann CJ, Burk O, Buntfuss D, Escher S, Bishop C, Koebe HG, Brinkmann U, Klenk HP, Kleine K, Meyer UA and Wojnowski L (2001) Genomic organization of the human CYP3A locus: identification of a new, inducible CYP3A gene. *Pharmacogenetics* **11**:111-121.
- George J, Byth K and Farrell GC (1995) Age but not gender selectively affects expression of individual cytochrome P450 proteins in human liver. *Biochem Pharmacol* **50**:727-730.

JPET #94367

Gonzalez FJ (2003) Role of gene knockout and transgenic mice in the study of xenobiotic metabolism. *Drug Metab Rev* **35**:319-335.

Gonzalez FJ, Schmid BJ, Umeno M, McBride OW, Hardwick JP, Meyer UA, Gelboin HV and Idle JR (1988) Human P450PCN1: sequence, chromosome localization, and direct evidence through cDNA expression that P450PCN1 is nifedipine oxidase. *DNA* **7**:79-86.

Gorski JC, Jones DR, Haehner-Daniels BD, Hamman MA, O'Mara EM, Jr. and Hall SD (1998) The contribution of intestinal and hepatic CYP3A to the interaction between midazolam and clarithromycin. *Clin Pharmacol Ther* **64**:133-143.

Granvil CP, Yu AM, Elizondo G, Akiyama TE, Cheung C, Feigenbaum L, Krausz KW and Gonzalez FJ (2003) Expression of the human CYP3A4 gene in the small intestine of transgenic mice: in vitro metabolism and pharmacokinetics of midazolam. *Drug Metab Dispos* **31**:548-558.

Greenblatt DJ, Allen MD, Harmatz JS and Shader RI (1980) Diazepam disposition determinants. *Clin Pharmacol Ther* **27**:301-312.

Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39**:1-17.

Hana V, Silha JV, Justova V, Lacinova Z, Stepan JJ and Murphy LJ (2004) The effects of GH replacement in adult GH-deficient patients: changes in body composition without concomitant changes in the adipokines and insulin resistance. *Clin Endocrinol (Oxf)* **60**:442-450.

Hines RN and McCarver DG (2002) The ontogeny of human drug-metabolizing enzymes: phase I oxidative enzymes. *J Pharmacol Exp Ther* **300**:355-360.

JPET #94367

Holloway MG, Laz EV and Waxman DJ (in press) Co-regulation of GH-responsive, sexually dimorphic hepatic gene expression by STAT5b and HNF4 α *. *Mol Endocrinol*.

Jaffe CA, Ocampo-Lim B, Guo W, Krueger K, Sugahara I, DeMott-Friberg R, Bermann M and Barkan AL (1998) Regulatory mechanisms of growth hormone secretion are sexually dimorphic. *J Clin Invest* **102**:153-164.

Jaffe CA, Turgeon DK, Lown K, Demott-Friberg R and Watkins PB (2002) Growth hormone secretion pattern is an independent regulator of growth hormone actions in humans. *Am J Physiol Endocrinol Metab* **283**:E1008-1015.

Jurgens G, Lange KH, Reuther LO, Rasmussen BB, Brosen K and Christensen HR (2002) Effect of growth hormone on hepatic cytochrome P450 activity in healthy elderly men. *Clin Pharmacol Ther* **71**:162-168.

Kawai M, Bandiera SM, Chang TK and Bellward GD (2000) Growth hormone regulation and developmental expression of rat hepatic CYP3A18, CYP3A9, and CYP3A2. *Biochem Pharmacol* **59**:1277-1287.

Komori M, Nishio K, Kitada M, Shiramatsu K, Muroya K, Soma M, Nagashima K and Kamataki T (1990) Fetus-specific expression of a form of cytochrome P-450 in human livers. *Biochemistry* **29**:4430-4433.

Lanes R, Gunczler P, Orta N, Bosquez M, Scovino R, Dominguez L, Esaa S and Weisinger JR (1996) Changes in bone mineral density, growth velocity and renal function of prepubertal uremic children during growth hormone treatment. *Horm Res* **46**:263-268.

Liddle C, Goodwin BJ, George J, Tapner M and Farrell GC (1998) Separate and interactive regulation of cytochrome P450 3A4 by triiodothyronine, dexamethasone, and growth hormone in cultured hepatocytes. *J Clin Endocrinol Metab* **83**:2411-2416.

JPET #94367

Marcus R and Hoffman AR (1998) Growth hormone as therapy for older men and women. *Annu Rev Pharmacol Toxicol* **38**:45-61.

Mode A, Norstedt G, Simic B, Eneroth P and Gustafsson JA (1981) Continuous infusion of growth hormone feminizes hepatic steroid metabolism in the rat. *Endocrinology* **108**:2103-2108.

Nagata K, Matsunaga T, Gillette J, Gelboin HV and Gonzalez FJ (1987) Rat testosterone 7 alpha-hydroxylase. Isolation, sequence, and expression of cDNA and its developmental regulation and induction by 3-methylcholanthrene. *J Biol Chem* **262**:2787-2793.

Park SH, Liu X, Hennighausen L, Davey HW and Waxman DJ (1999) Distinctive roles of STAT5a and STAT5b in sexual dimorphism of hepatic P450 gene expression. Impact of STAT5a gene disruption. *J Biol Chem* **274**:7421-7430.

Park SS, Cha SJ, Miller H, Persson AV, Coon MJ and Gelboin HV (1982) Monoclonal antibodies to rabbit liver cytochrome P-450LM2 and cytochrome P-450LM4. *Mol Pharmacol* **21**:248-258.

Parkinson A, Mudra DR, Johnson C, Dwyer A and Carroll KM (2004) The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. *Toxicol Appl Pharmacol* **199**:193-209.

Powell DR, Liu F, Baker BK, Hintz RL, Lee PD, Durham SK, Brewer ED, Frane JW, Watkins SL and Hogg RJ (1997) Modulation of growth factors by growth hormone in children with chronic renal failure. The Southwest Pediatric Nephrology Study Group. *Kidney Int* **51**:1970-1979.

JPET #94367

Rich KJ and Boobis AR (1997) Expression and inducibility of P450 enzymes during liver ontogeny. *Microsc Res Tech* **39**:424-435.

Robertson GR, Farrell GC and Liddle C (1998) Sexually dimorphic expression of rat CYP3A9 and CYP3A18 genes is regulated by growth hormone. *Biochem Biophys Res Commun* **242**:57-60.

Rudman D, Feller AG, Nagraj HS, Gergans GA, Lalitha PY, Goldberg AF, Schlenker RA, Cohn L, Rudman IW and Mattson DE (1990) Effects of human growth hormone in men over 60 years old. *N Engl J Med* **323**:1-6.

Sakuma T, Endo Y, Mashino M, Kuroiwa M, Ohara A, Jarukamjorn K and Nemoto N (2002) Regulation of the expression of two female-predominant CYP3A mRNAs (CYP3A41 and CYP3A44) in mouse liver by sex and growth hormones. *Arch Biochem Biophys* **404**:234-242.

Schmucker DL, Woodhouse KW, Wang RK, Wynne H, James OF, McManus M and Kremers P (1990) Effects of age and gender on in vitro properties of human liver microsomal monooxygenases. *Clin Pharmacol Ther* **48**:365-374.

Shimada M, Murayama N, Yamazoe Y, Hashimoto H, Ishikawa H and Kato R (1995) Age- and sex-related alterations of microsomal drug- and testosterone-oxidizing cytochrome P450 in Sprague-Dawley strain-derived dwarf rats. *J Pharmacol Exp Ther* **275**:972-977.

Sim SC, Edwards RJ, Boobis AR and Ingelman-Sundberg M (2005) CYP3A7 protein expression is high in a fraction of adult human livers and partially associated with the CYP3A7*1C allele. *Pharmacogenet Genomics* **15**:625-631.

JPET #94367

Stevens JC, Hines RN, Gu C, Koukouritaki SB, Manro JR, Tandler PJ and Zaya MJ (2003)

Developmental expression of the major human hepatic CYP3A enzymes. *J Pharmacol Exp Ther* **307**:573-582.

Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y,

Gonzalez FJ, Schuetz EG and Kim RB (2003) The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* **9**:220-224.

van den Berg G, Veldhuis JD, Frolich M and Roelfsema F (1996) An amplitude-specific

divergence in the pulsatile mode of growth hormone (GH) secretion underlies the gender difference in mean GH concentrations in men and premenopausal women. *J Clin Endocrinol Metab* **81**:2460-2467.

Watkins PB, Murray SA, Winkelman LG, Heuman DM, Wrighton SA and Guzelian PS (1989)

Erythromycin breath test as an assay of glucocorticoid-inducible liver cytochromes P-450. Studies in rats and patients. *J Clin Invest* **83**:688-697.

Waxman DJ, LeBlanc GA, Morrissey JJ, Staunton J and Lapenson DP (1988) Adult male-

specific and neonatally programmed rat hepatic P-450 forms RLM2 and 2a are not dependent on pulsatile plasma growth hormone for expression. *J Biol Chem* **263**:11396-11406.

Waxman DJ, Pampori NA, Ram PA, Agrawal AK and Shapiro BH (1991) Interpulse interval in

circulating growth hormone patterns regulates sexually dimorphic expression of hepatic cytochrome P450. *Proc Natl Acad Sci U S A* **88**:6868-6872.

Willson TM and Kliewer SA (2002) PXR, CAR and drug metabolism. *Nat Rev Drug Discov*

1:259-266.

JPET #94367

- Wiwi CA, Gupte M and Waxman DJ (2004) Sexually dimorphic P450 gene expression in liver-specific hepatocyte nuclear factor 4alpha-deficient mice. *Mol Endocrinol* **18**:1975-1987.
- Wolbold R, Klein K, Burk O, Nussler AK, Neuhaus P, Eichelbaum M, Schwab M and Zanger UM (2003) Sex is a major determinant of CYP3A4 expression in human liver. *Hepatology* **38**:978-988.
- Yamano S, Nhamburo PT, Aoyama T, Meyer UA, Inaba T, Kalow W, Gelboin HV, McBride OW and Gonzalez FJ (1989) cDNA cloning and sequence and cDNA-directed expression of human P450 IIB1: identification of a normal and two variant cDNAs derived from the CYP2B locus on chromosome 19 and differential expression of the IIB mRNAs in human liver. *Biochemistry* **28**:7340-7348.
- Yu AM, Fukamachi K, Krausz KW, Cheung C and Gonzalez FJ (2005) Potential role for human cytochrome P450 3A4 in estradiol homeostasis. *Endocrinology* **146**:2911-2919.

JPET #94367

Footnotes

Supported by the Intramural Research Program of the National Cancer Institute (F.J.G.), NIH grant DK33765 (D.J.W.), and a Wellcome Trust postdoctoral research fellowship 064866 (C.C.).

¹These authors contributed equally to this study.

JPET #94367

Legends for Figures

Fig. 1. Verification of the BAC clone used for the production of *CYP3A4* and *CYP3A7* transgenic mouse line. (A) Diagram of the wild-type *CYP3A4* and *CYP3A7* genes in BAC clone (123778 bp in length) used for microinjection. Vertical black bars represent *CYP3A4* and *CYP3A7* exons. (B) Southern blot analyses of the BAC clone after purification and digestion with Pst I, EcoR I, Bgl II, BamH I, or Apa I. Hybridizations were performed by using ³²P-end labeled *CYP3A4* cDNA and DNA oligonucleotide probes that recognize Exon 1, Exon 13, and -10 kb upstream sequence of human *CYP3A4* gene, respectively. A BAC clone that does not contain the *CYP3A4* gene was digested with EcoR I and serves as a negative control.

Fig. 2. Expression of *CYP3A4* and *CYP3A7* in transgenic mice. Western blot analyses show that (A) *CYP3A4* is expressed in the liver of *CYP3A4* genotype-positive 3-week-old mice from founder lines C1 (male liver sample) and C2 (female liver sample), (B) expression of *CYP3A4* is detected in the liver, duodenum and jejunum of 3-week-old male and female mice (3 in each group, pooled samples were used in the study) of the C2 founder line, and (C) the human fetal form *CYP3A7* is expressed in the transgenic fetal liver between embryonic day 16 and 17 (pooled sample from >10 individuals). Microsomal proteins (20 µg each well) were separated by SDS-PAGE, transferred onto a membrane, and *CYP3A4*, GAPDH and *CYP3A7* were detected using the Enhanced Chemiluminescence Kit (Pierce Chemical Co., Rockford, IL). The monoclonal antibody against *CYP3A4* (MAb 275-1-2) (Gelboin et al., 1995) used in these experiments reacts with *CYP3A4* but does not recognize murine Cyp3a or other liver proteins. This antibody was used to screen positive founder lines and to assay the tissue distribution of *CYP3A4*. A specific polyclonal antibody that only recognizes *CYP3A7* (Sim et al., 2005) was

JPET #94367

used for the evaluation of CYP3A7 expression in fetal livers. Recombinant CYP3A4 and CYP3A7 protein and pooled human liver microsomes (HLM) were used as positive controls.

Fig. 3. CYP3A4 developmental regulation in transgenic male and female mouse livers. Liver tissues were collected from transgenic male and female mice of different ages and microsomes were prepared by differential centrifugation. Pooled (4-5 livers in each group) samples were used for Western blot analysis of CYP3A4, murine Cyp1a2 and GAPDH protein levels. CYP3A4 protein is essentially absent in the male livers beginning at week 6, whereas it is consistently expressed in female liver at a level that is similar at all ages after 2 weeks.

Fig. 4. Induction of hepatic CYP3A4 and murine Cyp3a proteins by phenobarbital (PB). Transgenic mice (6-7 week old) were treated daily for 4 days with saline or phenobarbital as detailed under Methods. Liver microsomes from individual mouse were prepared and analyzed by SDS-PAGE. CYP3A4 and murine Cyp3a proteins were detected with MAb 275-1-2 and MAb 2-13-1 (Gelboin et al., 1995), respectively, by using the Enhanced Chemiluminescence Kit. MAb 2-13-1 reacts strongly with murine Cyp3a and very weakly with human CYP3A4 (Yu et al., 2005). Although CYP3A4 is not detectable in control male livers, phenobarbital induces CYP3A4 expression in the males, resulting in a level comparable to that of phenobarbital-treated females.

Fig. 5. Stimulation of CYP3A4 protein expression in transgenic male mouse livers by continuous GH treatment. Mice (7-week-old, 4-5 in each group) were treated with recombinant mouse GH by continuous infusion for 7 days using Alzet osmotic mini-pumps. Sham-treated mice were

JPET #94367

used as controls. Tissue microsomes, prepared from individual mouse liver by differential centrifugation, were separated by SDS-PAGE, and subjected to Western blot analysis with enhanced chemiluminescence detection of CYP3A4 and murine Cyp2b, Cyp1a2, Cyp2a, Cyp2e1 and GAPDH. GH treatment induced CYP3A4 protein expression in transgenic male mice. Induction of a female-specific Cyp2b protein band was also seen in the GH-treated males. Partial decreases in several of the other CYP proteins, most notably Cyp1a2, were seen in the GH-treated males and females.

Fig. 6. QPCR analysis of hepatic CYP3A4 and murine Cyp3a mRNA levels in mice treated with GH. Total mRNAs were prepared from mouse livers by using Trizol reagent and cDNA was synthesized by reverse transcription. QPCR analysis was carried out with SYBR Green I PCR master mix (Applied Biosystems). Data shown are relative expression levels after normalized to the level of 18S RNA, and represent mean \pm S.E. values for 4-5 individual livers per group, with the sham-treated male RNA level set to 1. Hepatic CYP3A4 and Cyp3a41 mRNA levels were significantly higher ($p < 0.05$) in the GH-treated males (M) than in sham-treated males. GH treatment of female (F) mice had no significant impact on CYP mRNA levels.

Table 1. Sex-dependent developmental regulation of human CYP3A4 and mouse Cyp3a genes in transgenic mice.

Hepatic RNA levels were determined for each CYP3A gene by QPCR using transgenic liver tissue isolated from mice at each age, as indicated. Data shown are mean \pm S.E. values for n = 4-5 livers/group, normalized to 18S RNA levels, with the 8 week male liver level of each RNA set to 1. ^aValues are significantly (p<0.05) different from 2 week values for the same sex; ^bvalues are significantly (p<0.05) different from female values for the same age.

Age (weeks)	<i>CYP3A4</i> Transgene		Cyp3a44		Cyp3a25	
	Male	Female	Male	Female	Male	Female
2	57 \pm 8	67 \pm 17	20 \pm 3	14 \pm 4	1 \pm 0.1	0.7 \pm 0.1
5	13 \pm 4 ^{a, b}	109 \pm 4 ^a	1.2 \pm 0.1 ^{a, b}	32 \pm 5 ^a	0.7 \pm 0.2	0.5 \pm 0.1 ^a
6	0.1 \pm 0.05 ^{a, b}	66 \pm 21	0.4 \pm 0.1 ^{a, b}	22 \pm 5	0.6 \pm 0.1 ^a	0.4 \pm 0.1 ^a
8	1 \pm 0.4 ^{a, b}	196 \pm 54 ^a	1 \pm 0.3 ^{a, b}	9 \pm 4	1 \pm 0.1	0.8 \pm 0.3

Figure 1

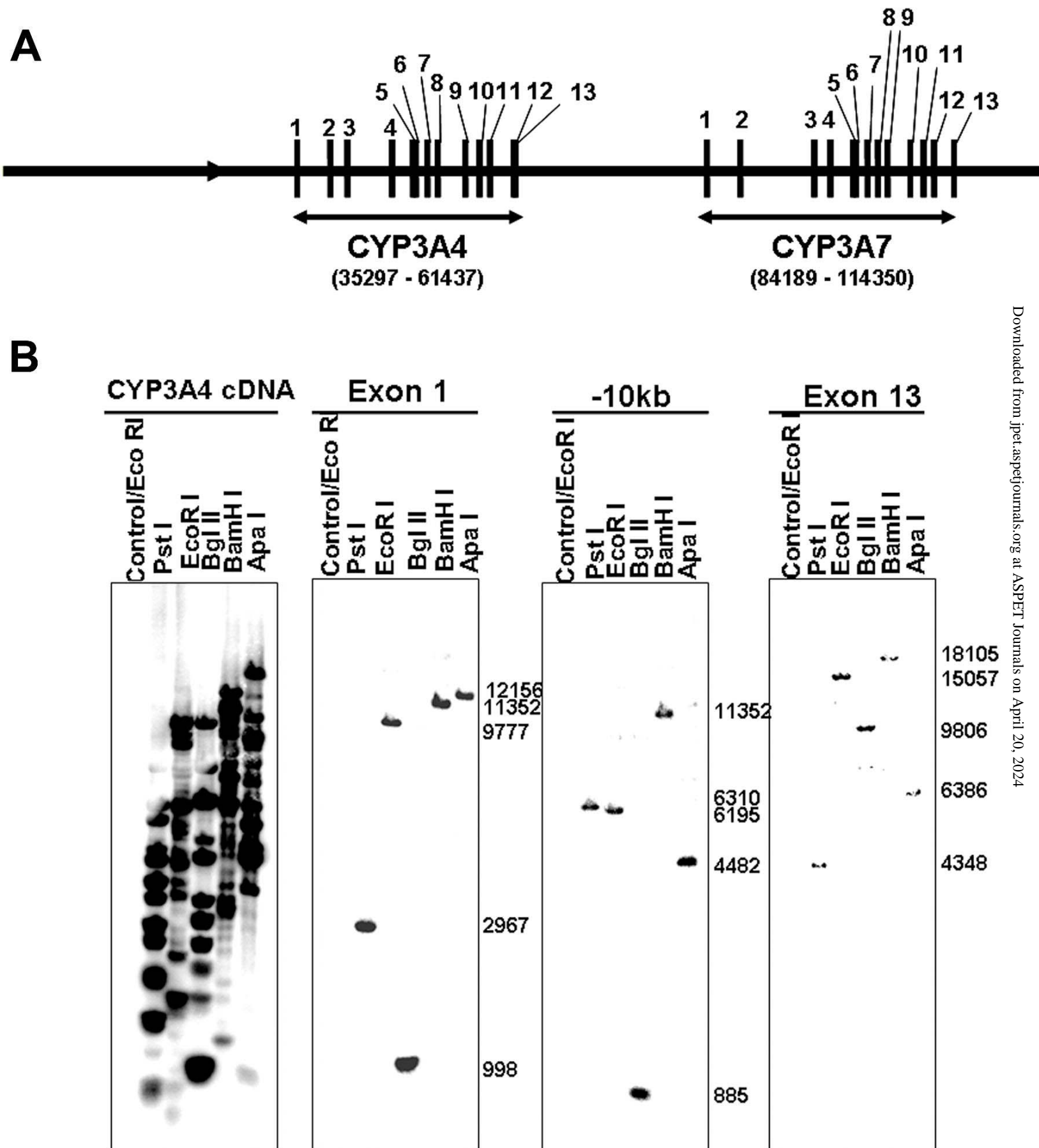


Figure 2

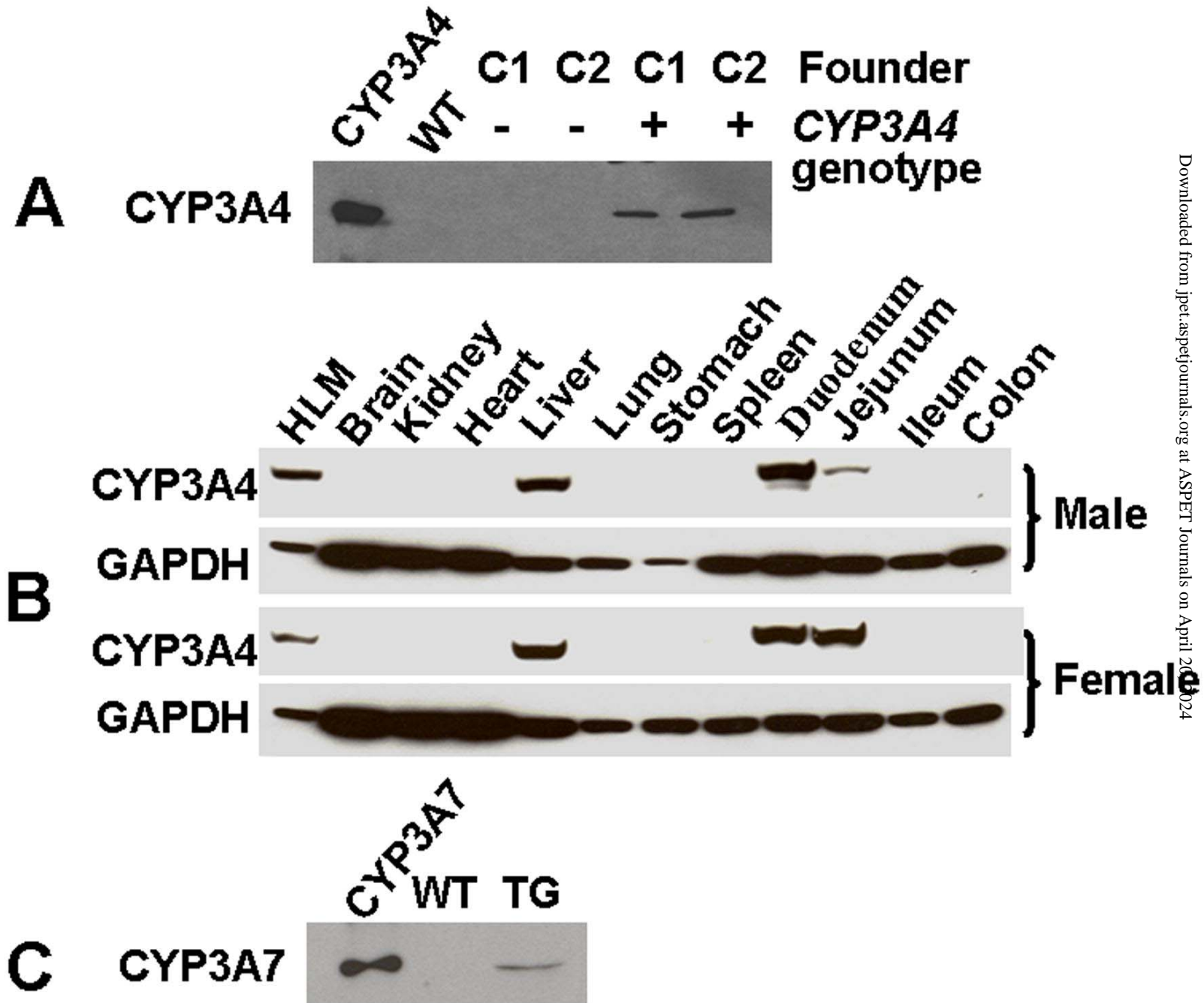


Figure 3

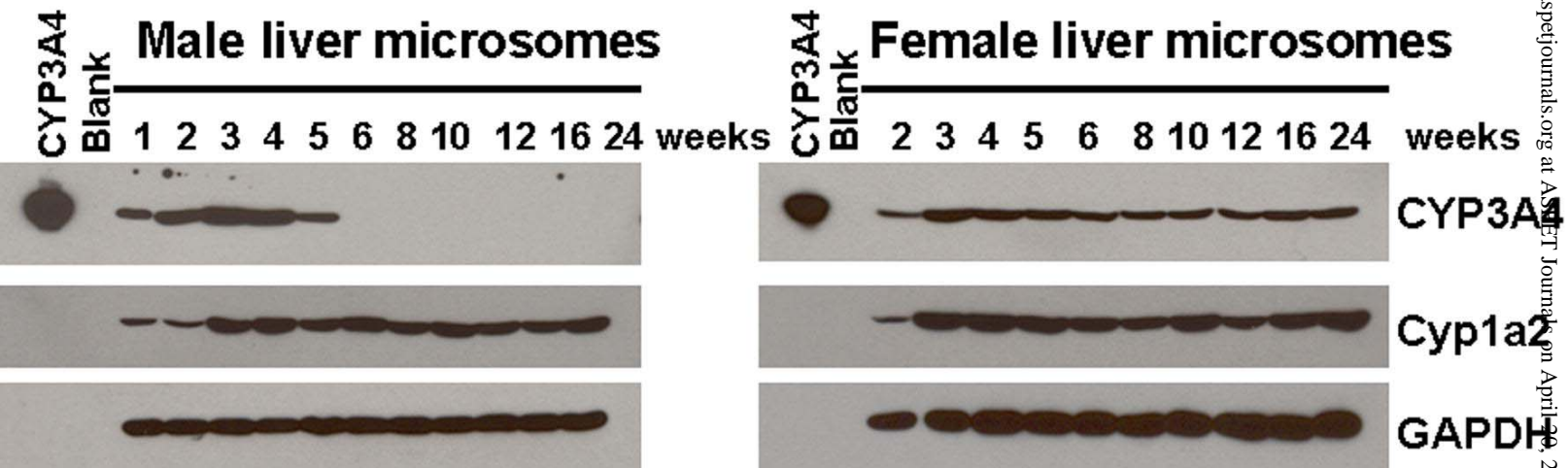


Figure 4

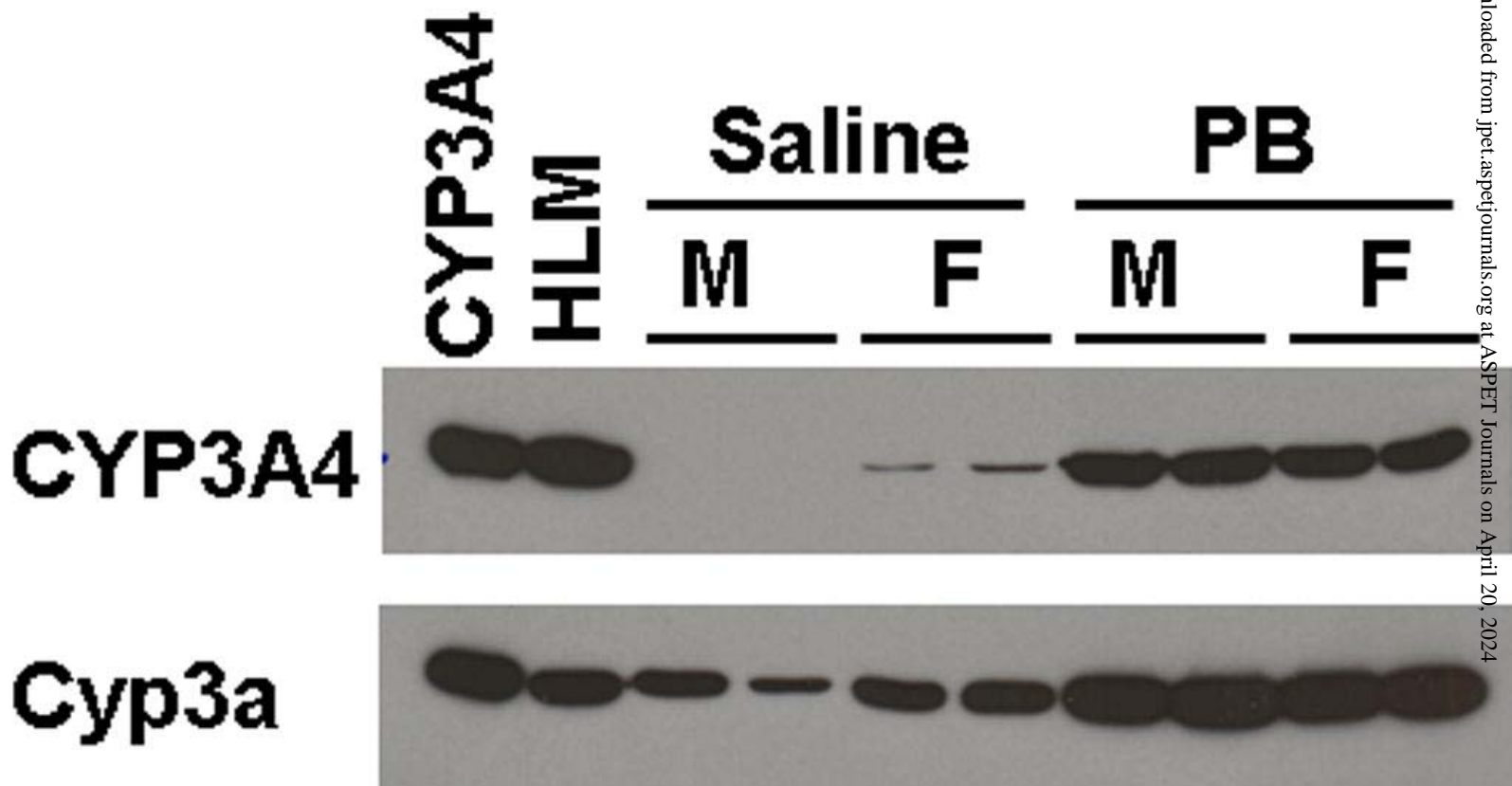


Figure 5

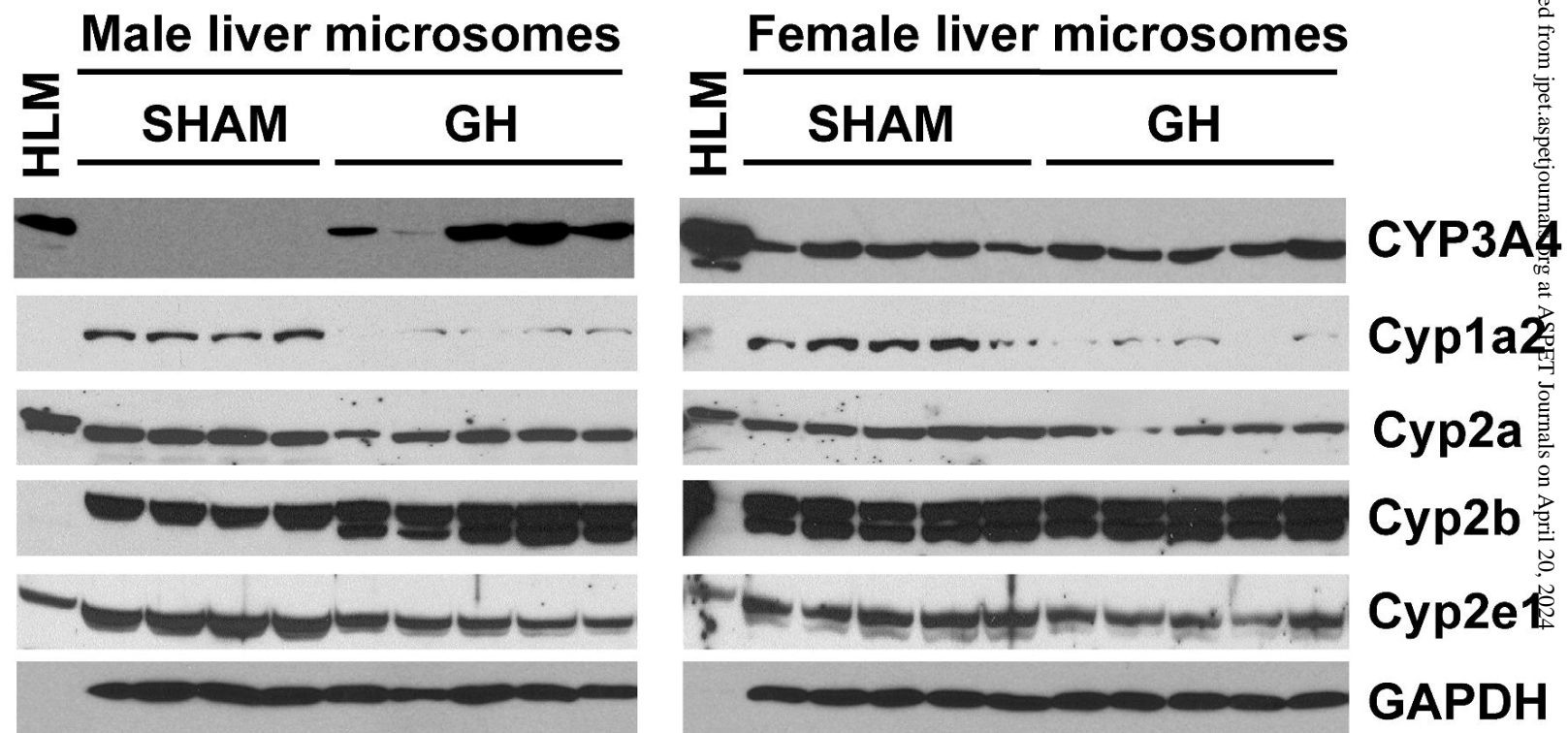


Figure 6

