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Expression and methylation status of female-predominant GH-dependent liver genes are modified by neonatal androgenization in female mice

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ABSTRACT

Neonatal androgenization masculinizes the GH axis and thus may impact on liver gene regulation. Neonatal testosterone administration to female mice decreased (defeminized) female predominant GH-dependent liver gene expression (*Hnf6*, *Adh1*, *Prlr*, *Cyp3a41*) and did not modify male predominant genes (*Cyp7b1*, *Cyp4a12*, *Slp*). Female predominance of *Cis* mRNA, an inhibitor of episodic GH signaling pathway, was unaltered. At birth, *Cyp7b1* promoter exhibited a higher methylation status in female livers, while the *Hnf6* promoter was equally methylated in both sexes; no differences in gene expression were detected at this age. In adulthood, consistent with sex specific predominance, lower methylation status was determined for the *Cyp7b1* promoter in males, and for the *Hnf6* promoter in females, and this last difference was prevented by neonatal androgenization. Therefore, early steroid treatment or eventually endocrine disruptor exposure may alter methylation status and sexual dimorphic expression of liver genes, and consequently modify liver physiology in females.

1. Introduction

In the liver many metabolic pathways are expressed in a sexually specific manner, and this organ is the center for controls contributing to sex differences in energy homeostasis, lipid and steroid hormone metabolism, and degradation of xenobiotics, among other liver functions (Waxman and O'Connor, 2006; Waxman and Holloway, 2009). Almost 94% of hepatic genes differentially expressed between sexes are pituitary dependent, and mostly regulated by the pattern in pituitary growth hormone (GH) secretion, which is pulsatile in males and more continuous in females (Wauthier et al., 2010). This proposes an interesting scenario in which the central nervous system, indirectly setting GH profiles through GHRH and somatostatin control, regulates sex dependent liver activity in accordance with brain sex differences, and the need for sex-specific steroid metabolism (Noain et al., 2013).

In fact, in both humans and mice, sex specific GH patterns may condition thrombosis, dislipidemia, coronary artery disease, liver lesions or hepatocellular carcinoma development (Liao et al., 1993; Wong et al., 2008; Zhang et al., 2011). Additionally, marked differences in drug pharmacokinetics between women and men have been described (Anderson, 2005).

Male predominant and female predominant liver genes represent 63% and 37%, respectively, of total sex-specific liver gene expression. Hypophysectomy (mostly loss of GH) abolishes sex specificity for 95% and 91% of male- and female-predominant genes, respectively, highlighting the importance of GH regulation of liver gene sex differences (Wauthier et al., 2010). Sexually dimorphic plasma GH profiles first emerge at puberty but are set and ultimately regulated by gonadal steroid imprinting during the neonatal period (Jansson and Frohman, 1987; Ramirez et al., 2010). During very restricted or critical periods of neural differentiation gonadal hormones exert an organizational influence on the developing central nervous system (Arnold and Gorski, 1984; Becu-Villalobos and Libertun, 1995; Becu-Villalobos et al., 1997). Exposure of the developing brain to the presence or absence of androgen or estrogen results in the differentiation of a broad spectrum of responses which are congruent with the genotype. This process, referred to as sexual differentiation, assures adequate behavioral and neuroendocrine responses in males and females.

There is an emerging interest in the epigenetic mechanisms contributing to the maintenance of permanent changes established during hormonally induced sexual differentiation of the brain (Lenz et al., 2012; Nugent and McCarthy, 2011). Early hormonal inputs may alter DNA methylation, and histone modification patterns that can result in lifelong changes in gene expression. These epigenetic marks are therefore considered a potential

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mechanism by which steroid exposure during the critical period of sexual differentiation of the brain maintains long-term sex differences in gene expression.

The impact of sexual differentiation on GH control of liver genes and, in particular, on the methylation status of liver gene promoters has not been studied in detail. We have shown that sexually differentiated expression of some GH-dependent liver genes may be permanently modified by neonatal androgen exposure in female mice (Ramirez et al., 2010), and by neonatal xenoestrogens in rats (Ramirez et al., 2012). We now explore the possibility that changes in gene expression in females could be related to the early imprinting by neonatal testosterone on GH signaling in the liver, or to epigenetic factors, such as the methylation status of gene promoter regions. To address these hypotheses, we first evaluated the effect of neonatal testosterone administered to female mice in the expression pattern of a group of female- and male- predominant liver genes in adult mice. This experimental model has been extensively used in the study of brain sexual differentiation in rodents, as well as in the evaluation of effects caused by early endocrine disruptors (Becu-Villalobos, 2007; Morris et al., 2004; Wu et al., 2009). We included genes coding for CYP450 enzymes (*Cyp3a41*, *Cyp7b1*, *Cyp4a12*), the hepatocyte nuclear transcription factor 6 (*Hnf6*), the prolactin receptor (*Prlr*), alcohol dehydrogenase (*Adh1*), the sex limited protein (*Slp*), and the inhibitory protein of GH transduction signaling pathway (*Cis*). We next selected a female and a male predominant gene, *Cyp7b1* and *Hnf6*, respectively, because their promoters have important CpG islands, studied the methylation status at birth and in adult mice, and correlated methylation status of the promoter with gene expression.

Our results propose that sexual differences in the expression of hepatic liver genes which depend on GH may be influenced by neonatal testosterone, and that a dynamic methylation process, and/or intrinsic differences in GH signaling pathway participate in setting the differences. Therefore, neonatal steroid or eventually endocrine disruptor exposure may impact on liver physiology and drug metabolism in females.

2. Materials and methods

2.1. Animals

C57BL/6J mice were housed in temperature-controlled room with lights on at 0700 h and lights-off at 1900 h, and free access to laboratory chow and tap water. A total of 18 litters were used: 20 females, 25 TP females and 23 males. On the day of birth (designated day 0) the pups were left undisturbed, and on the second day they were sexed and each pup was randomly divided into treatment groups: females were injected sc with 100 μg testosterone propionate (Sigma, St. Luis, MO) in 0.010 ml castor-oil (neonatally androgenized females: TP females). The dose was chosen as it has been described to effectively androgenize neonatal brain in mice (Ingman and Robertson, 2007; Livne et al., 1992). Females and males from the same cohort were injected with castor oil and used as controls. Animals were weighed and euthanized at 4 months of age. As previously described (Ramirez et al., 2010) TP females were 10% heavier than control females, had increased percentage of pituitary somatotropes and GH concentration, as well as higher serum IGF-I levels compared to females (Table 1). Control females in diestrus and TP females in diestrus/anestrus were used, as TP females had highly irregular cycles. A group of untreated female and male pups were euthanized at postnatal day one, for ontogenic studies. All experimental procedures were approved by the Institutional Animal Use and Care Committee.

2.2. Reagents

Unless otherwise specified, all chemicals were purchased from Sigma (St. Louis, MO).

2.3. Tissue RNA extraction and total cDNA preparation for Hnf-6, Adh1, Prlr, Cis, Cyp3a41, Cyp7b1, Cyp4a12 and Slp expression by real time PCR

After euthanasia liver samples (≅50 mg) were immediately homogenized in TRIzol reagent (Invitrogen, Buenos Aires) and stored at −70 °C until used. Total RNA was isolated from tissue homogenates by use of the TRIzol reagent method. The RNA concentration was determined on the basis of absorbance at 260 nm, its purity was evaluated by the ratio of absorbance at 260/280 nm (>1.8), and its integrity by agarose gel electrophoresis. RNAs were kept frozen at −70 °C until analyzed. First-strand cDNA was synthesized from 3 μg of total RNA in the presence of 10 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} Tris·HCl (pH 8.6), 75 mol l^{-1} KCl, 0.5 mM deoxy-NTPs, 1 mol l^{-1} DTT, 1 U/μl RnaseOUT (Invitrogen), 0.5 μg oligo$(dT)_{15}$ primer (Biodynamics, Buenos Aires, Argentina), and 20 U of MMLV reverse transcriptase (Epicentre, Madison, WI). To validate successful deoxyribonuclease I treatment, the reverse transcriptase was omitted in control reactions. The absence of PCR-amplified DNA fragments in these samples indicated the isolation of RNA free of genomic DNA.

2.4. Quantitative real-time PCR

Sense and antisense oligonucleotide primers were designed on the basis of the published cDNA or by the use of PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Oligonucleotides were obtained from Invitrogen. The sequences are described in Table 2.

Quantitative measurements of specific mRNA levels were performed by kinetic PCR using HOT FIREPol EvaGreen qPCR Mix Plus (ROX), Solis BioDyne, 0.4 μmol l^{-1} primers, and 150 ng cDNA in a final volume of 10.4 μl. After denaturation at 95 °C for 15 min, the cDNA products were amplified with 40 cycles, each cycle consisting of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, and optical reading stage at 80 °C for 33 s. The accumulating DNA products were monitored by the ABI 7500 sequence detection system (Applied Biosystems), and data were stored continuously during the reaction. The results were validated on the basis of the quality of dissociation curves generated at the end of the PCR runs by ramping the temperature of the samples from 60 to 95 °C, while continuously collecting fluorescence data. Product purity was confirmed by agarose gel electrophoresis. Each sample was analyzed in duplicate. Relative gene expression levels were calculated according to the comparative cycle threshold (CT) method. Normalised target gene expression relative to *Gapdh* or cyclophilin was obtained by calculating the difference in CT values, the relative change in target transcripts being computed as $2^{-\Delta CT}$. To validate the comparative CT method of relative quantification, the efficiencies of each target and housekeeping gene amplification (endogenous *Gapdh*) were measured and shown to be approximately equal.

2.5. Bisulfite treatment of DNA

DNA was isolated from liver biopsy specimens at the same time as RNA by standard methods. DNA bisulfite modification was based on bisulfite treatment of genomic DNA, which converts all the unmethylated cytosines to uracils while conserving the methylated cytosines. DNA (2 μg) in a volume of 50 μl was denatured with NaOH for 20 min at 37 °C. Thirty microliters of 10 mM

Table 1
Neonatal testosterone partially masculinizes the GH axis in female mice.

	Female	TP female	Male
Body weight (g)	19.0 ± 0.4 (a)	20.9 ± 0.4 (a, b)	26.0 ± 0.4 (b)
Somatotropes (%)	18.9 + 2.2 (a)	27.5 + 2.6	30.5 + 1.5 (b)
Pituitary GH (ng/ug protein)	5.9 ± 2.5 (a)	36.2 ± 13.2	71.7 ± 19.2 (b)
Serum IGF-I (ng/ml)	309.4 ± 19.1(a)	363.1 ± 19.0 (b)	364.8 ± 19.5 (b)

(a) P 0.05 vs. males; (b) P 0.05 vs. females. N = 11–13 for body weight and serum IGF-I, and 5-8 for somatotropes and pituitary GH.

Table 2
Description of primers used for real-time PCR.

Gene	Strand	Primer sequence (5′-3′)	Source
Cyp4a12	Sense	GAACAATCTCTTTTTTCTCCGTGTG	(Wiwi et al., 2004)
	Antisense	GCAGGCACTGTTGGCCAA	
Slp	Sense	GACCAAGGACCATGCTGTGC	(Holloway et al., 2006)
	Antisense	AACTGCTGGATCCGAACGTGG	
Hnf6	Sense	AAGCCCTGGAGCAAACTCAA	Designed by PrimerBlast
	Antisense	CCACATCCTCCGGAAAGTCTC	
Adh1	Sense	GCTCTGCCGTCAAAGTCGCCA	Designed by PrimerBlast
	Antisense	ACCGACACCTCCGAGGCCAA	
Prlr	Sense	CACAGTAAATGCCACGAACG	Designed by PrimerBlast
	Antisense	GGCAACCATTTTACCCACAG	
Cis	Sense	TGCATAGCCAAGACGTTCTC	Designed by PrimerBlast
	Antisense	GTGGGTGCTGTCTCGAACTA	
Cyp7b1	Sense	TGAGGTTCTGAGGCTGTGCTC	Holloway et al. (2006)
	Antisense	TCCTGCACTTCTCGGATGATG	
Cyp3a41	Sense	GTGGAGAAAGCCAAAGGGATT	Designed by PrimerBlast
	Antisense	GAAGACCAAAGGATCAAAAAAGTCA	
Gapdh	Sense	CAGAACATCATCCCTGCAT	Designed by PrimerBlast
	Antisense	GTTCAGCTCTGGGATGACCTT	

hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (Sigma) (pH5), freshly prepared, were added and mixed, and samples were incubated in mineral oil at 55 °C for 16 h (37). Modified DNA was purified using Wizard DNA purification resin according to the manufacturer (Promega) and eluted into 50 μl of heated water (60–70 °C). Modification was completed by NaOH treatment for 15 min at 37 °C, followed by ethanol precipitation. DNA was resuspended in 20 μl of water and used immediately or stored at −70 °C.

2.6. Methylation-specific polymerase chain reaction of bisulphite modified DNA

The chemically modified DNA was subsequently used as a template for a methylation specific polymerase chain reaction to determine the promoter methylation status of the selected CpG dinucleotides in the *Cyp7b1* and *Hnf6* promoters. Two pairs of primers were used: one pair was specific for bisulfite-modified methylated DNA (M primers) and the other pair was specific for bisulfite-modified unmethylated DNA (U primers). Three sets of primers (M and U) were used for *Cyp7b1*, and two sets for *Hnf6*. For each sample studied, PCRs were performed simultaneously using the M and U primer pairs. For primer design, a sequence starting 1000 bp upstream from the transcriptional start site (TSS) of *Cyp7b1* and *Hnf6* was used in the Methyl-Primer Express V1.0 program (Applied Biosystems) to search for regions with potentially methylated CpG sites. The sequence was retrieved from the Database of Transcriptional Start Sites (http://dbtss.hgc.jp/) with the following ID numbers: NM_007825, chromosome: 3: 17954811…18160476, TSS: 18143338 (ENSMUST00000035625) for *Cyp7b1*, and NM_008262, chromosome: 9: 74692135…74741723, TSS: 74709728 (ENSMUST00000056006) for *Hnf6*.

For maximal discrimination between methylated and unmethylated alleles, M and U primers were designed to contain at least one CpG site at the end, and 2–4 CpG sites in their sequences (see Figs. 5A and 6A). Primer sequences are shown in Tables 3 and 4. The level of methylated DNA was expressed as the ratio of methylated DNA to unmethylated (U/M)DNA levels, calculated for each sample using the fluorescence threshold cycle values. The assay was based on quantitative real-time PCR (RT-PCR) in an iCycler thermocycler (Bio-Rad, Hercules, CA) using EvaGreen (HOT FIREPol EvaGreen HRM mix, Solis BioDyne) as a fluorescent dye. All the samples were performed in duplicate. The CV% was observed to be less than 5%. The specificity of amplification and the absence of primer dimers were confirmed by way of melting curve analysis at the end of each run and agarose electrophoresis.

The Web-based AliBaba2 program (http://www.gene-regulation.com/pub/programs/alibaba2) was used for *in silico* prediction of transcription factor–binding sites in the studied DNA sequences *a posteriori* of the sequence and primer selection.

2.7. RIAs

GH was measured by RIA using kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK; Dr. A.F. Parlow, National Hormone and Pituitary Program (NHPP), Torrance, CA]. Results are expressed in terms of mouse GH standard AFP-10783B. Intra- and interassay coefficients of variation were 8.4% and 13.2%, respectively.

For IGF-I RIA, serum samples (15 μl) and IGF-I standards were subjected to the acid–ethanol cryoprecipitation method as previously described (Lacau-Mengido et al., 2000). IGF-I was determined using antibody (UB2-495) provided by Drs. L. Underwood and J.J. Van Wyk, and distributed by the Hormone Distribution Program of the NIDDK. Recombinant human IGF-I (Chiron Corp., Emeryville, CA) was used as radioligand and unlabeled ligand. The assay sensitivity was 6 pg per tube. Intra- and interassay coefficients of variation were 8.2% and 14.1% respectively.

Pituitaries (1–1.5 mg) were homogenized in ice-cold PBS and centrifuged at 3000 rpm for 5 min. Supernatant protein contents were measured with the QUBIT Fluorometer and the QUANT-IT protein Assay Kit (Invitrogen, Buenos Aires, Argentina). Aliquots of equal quantity of protein were used to assay pituitary GH content.

2.8. Immunohistochemistry and morphometric analysis

Pituitaries from 4-month-old animals fixed in formalin were embedded in paraffin (using a similar orientation in each sample

Table 3
Description of primers used for MSPq to study the methylation status of the *Hnf6* promoter.

Primer set	Sense	Antisense
M1	5′ TGTTGGGACGTTACGGAGC 3′	5′ ACGATTCCGTAACCGCCG 3′
U1	5′ TTGTGTTGGGATGTTATGGAGT 3′	5′ ACTACAATTCCATAACCACCAC 3′
M2	5′ CGAGTTATGGTTCGAGTTGGC 3′	5′ AAAAACGCGATACCCCTAACC 3′
U2	5′ TGAGTTATGGTTTGAGTTGGT 3′	5′ AAAACACAATACCCCTAACC 3′

M: methylated.
U: unmethylated.

Table 4
Description of primers used for MSPq to study the methylation status of the *Cyp7b1* promoter.

Primer set	Sense	Antisense
M1	5′ TCGGGATGTAGGGAGTTAC 3′	5′ AAACGCTTACCTAATACGCC 3′
U1	5′ GTTGGGATGTAGGGAGTTAT 3′	5′ CAAACACTTACCTAATACACC 3′
M2	5′ AGGGTTTTTGTTATCGGAGC 3′	5′ ATCCCGACGAACTAACGAC 3′
U2	5′ GGTAGGGTTTTTGTTATTGGAGT 3′	5′ ACATCCCAACAAACTAACAACT 3′
M4	5′ CGTCGGGATGTAGGGAGTTAC 3′	5′ TCCCTACCGAACTCGAATATCC 3′
U4	5′ TGTTGGGATGTAGGGAGTTAT 3′	5′ CCCTACCAAACTCAAATATCC 3′

M: methylated.
U: unmethylated.

for cutting the tissue), and immunohistochemistry was performed using fluorescence detection (Garcia-Tornadu et al., 2006). We used rabbit polyclonal antibody against mouse GH (dilution 1:750, NHPP, NIDDK-AFP-5672099). Secondary antibody was fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (dilution 1:100; Zymed Laboratories Inc., San Francisco, CA). Four-five animals per experimental group and 3 pituitary sections per animal were used. Morphometric analysis was performed using a Carl Zeiss transmitted light microscope at a magnification of X250, and X400. Image analysis of pituitary sections for performed by Image J, version 6.0 software. The number of GH-immunoreactive cells was scored and used to calculate cell percentage (number of positive cells per total nuclei in the sections).

2.9. Statistical analysis

Data are expressed as means ± SEM. The differences between means were analyzed by ANOVA followed by Newman-Keuls test or Tukey's honestly significant difference test for unequal N (for liver mRNA expression, GH content, serum IGF-I, body weight). Two-way ANOVA was used to analyze ontogenic mRNA levels (effects of group and age), followed by Newman–Keuls test or Tukey's honestly significant difference test for unequal *N*.

Student's *t* test (2 groups) or ANOVA (for 3 groups) was used to determine differences in M/U DNA for each primer set. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of neonatal testosterone on GH-dependent female-(Hnf6, Adh1, Prlr and Cyp3a41) and male-(Cyp7b1, Cyp4a12, Slp) predominant liver genes

Female predominant expression was confirmed for *Hnf6*, *Adh1*, *Prlr* and *Cyp3a41* mRNA levels, with female/male ratios of 3.7, 2.5, 6.3 and >100, respectively. Neonatal androgenization of female pups induced a defeminization (decrease) of the four liver genes in adulthood, leading to a loss of sex-specific expression in the case of *Hnf6* and *Adh1* ($P \leqslant 0.02$ for TP females vs. females; and NS for TP females vs. males, Fig. 1A and B). Defeminization of *Prlr* and *Cyp3a41* mRNA expression was partial, as levels in TP females were different from males and females ($P \leqslant 0.05$ TP females vs. females and males, Fig. 1C and D).

Cyp7b1, *Cyp4a12*, and *Slp* mRNA levels were expressed predominantly in livers of male compared to female mice, and male/female ratios obtained were 10.7, 69.9 and 3.5, respectively. Neonatal androgenization did not masculinize the expression of these male-predominant genes in female livers (Fig. 2A–C).

3.2. Effect of neonatal androgenization on a female-predominant liver gene induced by GH, Cis

CIS is a member of the suppressors of the cytokine signaling family that normally inhibits GH-activated JAK2/STAT5b signaling pathway (Landsman and Waxman, 2005). Female predominance was observed for this gene, and neonatal androgenization of female pups did not alter liver *Cis* mRNA expression in adulthood (Fig. 3).

3.3. Ontogenic mRNA levels of Hnf-6 and Cyp7b1

We found that mRNA levels of the female predominant gene, *Hnf-6*, and the male predominant gene, *Cyp7b1*, markedly increased from birth to 4 months of age, and sex differences in their expression found in adult livers were absent in one day old mice (Fig. 4A and B). As previously shown neonatal androgenization defeminized *Hnf-6* expression.

3.4. Role of DNA methylation status on sex-specific gene expression; effect of neonatal androgenization

To determine whether the status of liver DNA methylation was associated with the sexual dimorphism and the effect of neonatal androgenization on liver genes we studied the level of DNA methylation of putative methylation target sites (CpG) in the promoters of a masculine predominant and a female predominant gene, *Cyp7b1* and *Hnf6*, respectively. We chose these genes because *in sílico* analysis revealed that they had robust CpG islands (Figs. 5A and 6A). Analysis of a sequence spanning 1000 bp upstream from

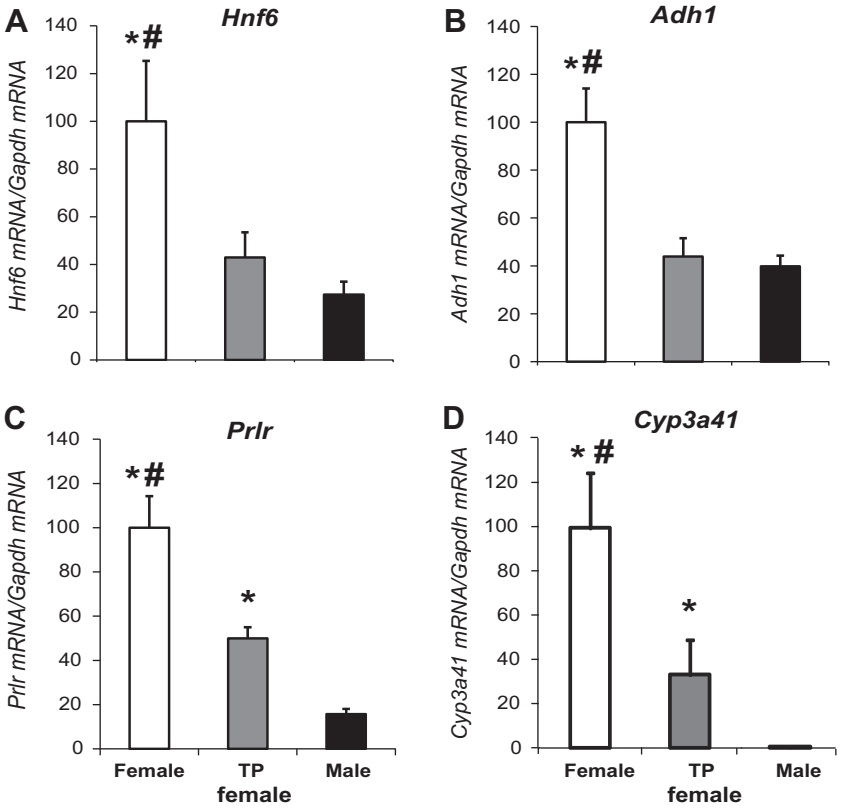

Fig. 1. Expression of female predominant liver genes normalized to *Gapdh* mRNA levels expressed in arbitrary units, in 4 month old females, neonatally androgenized females (TP females), and males (A) *Hnf6* ($N = 7$, 10 and 6, respectively), (B) *Adh1* ($N = 8$, 9 and 7, respectively), (C) *Prlr* ($N = 10$, 10 and 8, respectively) and (D) *Cyp3a41* ($N = 8$, 11 and 10). For all panels $^{*}P < 0.05$ vs. males, and $^{\#}P < 0.05$ vs. TP females.

the transcriptional start site (TSS) to 470 bp of the first exon of the mouse *Hnf6* gene using Methyl-Primer Express V1.0 software, revealed a 1.2 kb CpG island covering −715 bp upstream the TSS and 460 bp of exon I (Fig. 5A). More than 70 CpG doublets were found in the island. Two sets of primers were designed (located at positions relative to TSS = Set 1 (−49,−146, forward and reverse, respectively), and Set 2 (−9,+118, forward and reverse, respectively). *In silico* prediction showed that the CpG sites chosen were located at putative Sp1 and CCAAT/enhancer binding proteinα (C/EBPα) transcription binding sites (Fig. 5A).

In one-day old mice there were no sex differences in the ratio of methylated/unmethylated DNA of *Hnf6* promoter, while in adult animals, the ratio was higher in males using both sets of primers (Fig. 5B). Neonatal androgenization increased methylation status in adult females ($P \leqslant 0.03$ TP females vs. females for primer sets 1 and 2, respectively, Fig. 5B). This methylation status was in accordance with the higher female expression of the gene, as well as it defeminization in adult female livers (Figs. 1A and 4).

For *Cyp7b1* analysis, Methyl-Primer Express V1.0 software revealed a 0.83 kb CpG island covering 190 bp of the 5'flanking sequence, the entire exon I (251 bp) and 389 bp of intron 1 (Fig. 6A). Three sets of primers were designed (located at positions relative to TSS: Set 1 (+130,+241), Set 2 (−88,+118), and Set 4 (+130,+330). *In sílico* prediction indicated that these positions were located at putative Sp1 transcription binding sites and Chip analysis predicted GABP binding encompassed by Set 2 (Fig. 6A),. In contrast with results obtained in the feminine predominant gene, the promoter of the male predominant *Cyp7b1* gene had lower methylated/unmethylated DNA ratio (for the 3 sets chosen) in males compared to females already at birth, a situation which was maintained in livers of adult mice for sets 1 and 2 (Fig. 6B). Furthermore, neonatal androgenization did not modify methylation status in adult TP females compared to females ($P > 0.5$ TP females vs. females for primer sets 1 and 2, respectively, and $P < 0.02$ for TP females, or females vs. males, Fig. 6B). This methylation status was in accordance with the higher expression of the gene in adult male mice, as well as the lack of masculinization in TP females (Figs. 2A and 4).

Further analysis of transcription binding sites identified genome-wide by ChiP-seq indicated that other GH-regulated transcription factors bound to the proximal promoters and CpG islands studied of *Hnf-6* and *Cyp7b1* genes (Figs. 5A and 6A), even though these binding sites were mostly not encompassed by our primers. Such was the case of HNF-1, HNF-4, HNF-6, GABP (Leuenberger et al., 2009; Odom et al., 2007), and STAT (consensus site) (Lahuna et al., 2000) binding locations at the *Hnf6* promoter, and HNF-4, HNF-1, GABP (Leuenberger et al., 2009; Odom et al., 2007), and PPARα (Boergesen et al., 2012) at the *Cyp7b1* proximal promoter. Other important GH-regulated transcription factors bind at distal sites of these liver genes, for example BCL6, CUX2 and FOXA2 (Conforto et al., 2012; Li et al., 2012; Zhang et al., 2012).

4. Discussion

Sex-specific expression in the liver extends beyond the genes involved in steroid hormone metabolism or reproductive behavior, to include large numbers of nuclear factors, receptors, signaling molecules, secretory products and enzymes, many of which may contribute as mediators of a wide range of biological processes including steroid and lipid metabolism, behavior, inflammation and diseased states (Noain et al., 2013; Waxman and O'Connor, 2006; Waxman and Holloway, 2009). Sex differences in drug metabolism and pharmacokinetics have long been recognized,

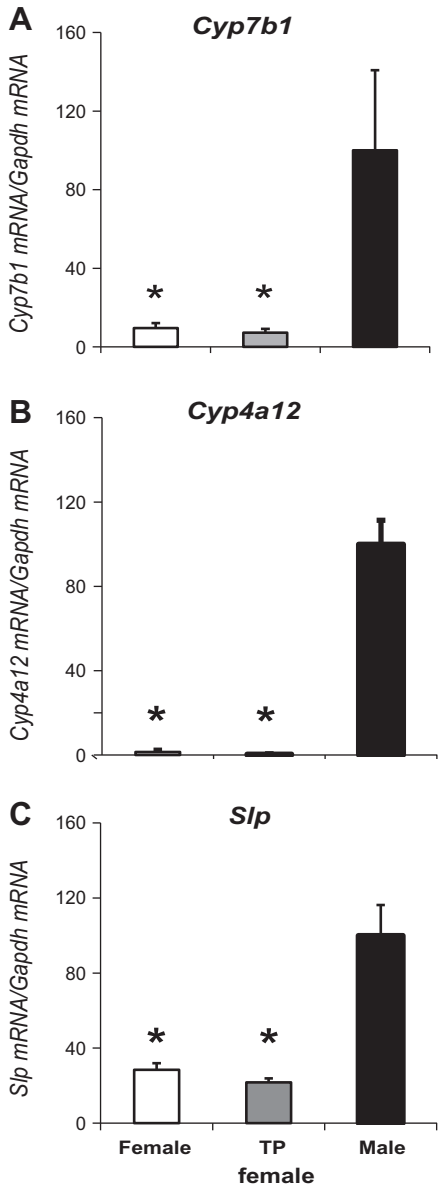

Fig. 2. Expression of male predominant liver genes normalized to *Gapdh* mRNA levels expressed in arbitrary units, in 4 month old females, neonatally androgenized females (TP females), and males (A) *Cyp7b1* ($N = 8$, 10 and 8, respectively), (B) *Cyp4a12* ($N = 10$, 13 and 11, respectively), and (C) *Slp* mRNA levels ($N = 9$, 13 and 11, respectively. For all panels $^{*}P < 0.05$ vs. males.

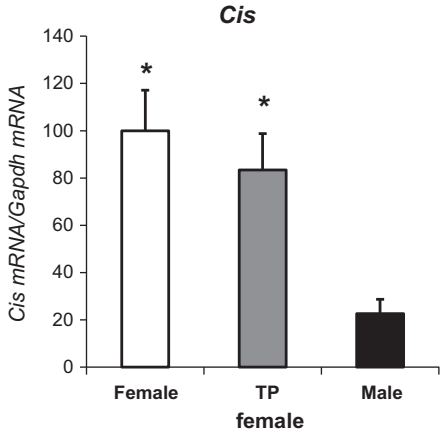

Fig. 3. Expression of a female predominant GH-induced liver gene, *Cis*, normalized to *Gapdh* mRNA levels expressed in arbitrary units, in 4 month old females, neonatally androgenized females (TP females), and males ($N = 8$, 10 and 6, respectively. $^{*}P < 0.05$ vs. males.

and are partly a consequence of the sex-biased expression of liver cytochrome P450 and other drug metabolizing enzymes, which is mostly dictated by the sexually dimorphic plasma profiles of GH (Waxman and Holloway, 2009). In humans, sex differences in cardiovascular disease risk, fatty liver disease and hepatocelullar carcinoma (Ayonrinde et al., 2011; Mueller et al., 2012; Rothwell et al., 2005; Takemoto et al., 2005; Zhang et al., 2011) have been described associated to the widespread sex differences in liver gene expression. Understanding how sex functions as a disease modifier is an increasing concern.

In rats and mice, many of the brain sex differences which set GH secretory profiles are determined during a critical period of development, when testosterone synthesized by the developing male testes impacts on the brain and is locally converted to estradiol (Dorner et al., 1987). This early steroid exposure permanently differentiates male and female specific brain regions which imprint sex-dependent patterns of GH release (Chowen et al., 1993; Ramirez et al., 2010), ultimately controlling most of the sex dependent liver gene expression.

We now show that neonatal androgenization of female mice, which partially masculinized the GH axis (i.e. lower GH and somatotrope content in the pituitary, and lower serum IGF-I levels) (Ramirez et al., 2010), has different effects on male and female predominant liver genes. In adulthood, the expression of the four female predominant genes studied was partially or totally defeminized (decreased), while none of the three male predominant genes were masculinized or increased in female livers.

Lack of masculinization of male genes in females has also been described in different experimental settings. Female hepatocytes could not be stimulated, either *in vitro* or *in vivo* (Thangavel et al., 2006; Thangavel and Shapiro, 2007) to express male-like levels of male-specific P450s, and accordingly, drug metabolism in males and females remains irrevocably different. This lack of gene induction may be dependent on the lack of induction of a fully male episodic GH secretion, the imprinting of GH signaling by neonatal hormones, GH independent mechanisms, or epigenetic factors, such as methylation status of gene promoter regions, among others.

We also found an ontogenic increment and no sexual differences in *Cyp7b1* and *Hnf-6* mRNA expression in both sexes at birth, similar to the developmental increase described for many liver genes (Cui et al., 2012),

Epigenesis is a complex process which refers to modifications made to the genome that can impact gene expression without affecting the underlying DNA sequence. This process is controlled by numerous factors such as chromatin modifications, DNA methylation, chromatin accessibility, transcription factor binding, modifications to histones, and non-coding RNA. One of the most intriguing modifications to chromatin that can impact gene expression is methylation of CpG sites as this modification can be duplicated across cellular division and maintained throughout a lifespan. DNA methylation evokes a decrease in gene expression by recruitment of methyl-binding and chromatin remodeling proteins, which cause chromatin to condense, or by blocking transcriptional machinery from accessing start sites on a gene's promoter (Garcia-Carpizo et al., 2011). In general, methylation of a promoter is inversely associated with gene expression. Support for a role of epigenetic factors in the regulation of sex-specific liver gene expression comes from the present findings which show that the methylation status of the *Cyp7b1* and *Hnf6* promoters in the

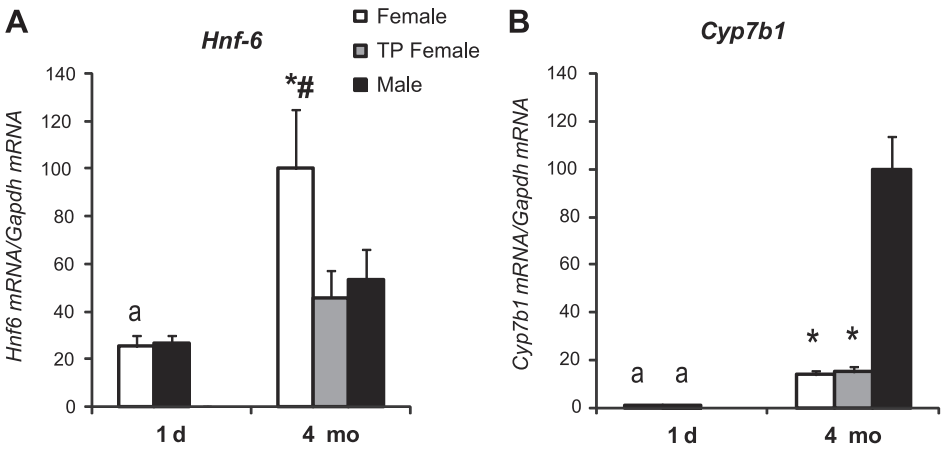

Fig. 4. Ontogenic expression of a female and a male predominant liver genes normalized to *Gapdh* mRNA levels expressed in arbitrary units, in one day-old female and male, and four month-old females, neonatally androgenized females (TP females), and males (A) *Hnf-6* (N = 10, 9, 7, 10 and 10, respectively) and (B) *Cyp7b1* (N = 10, 9, 9, 10 and 10, respectively). $^{*}P < 0.05$ vs. males, $^{\#}P < 0.05$ vs. TP females and (a) $P < 0.05$ vs. 4 month-old sex-matched group.

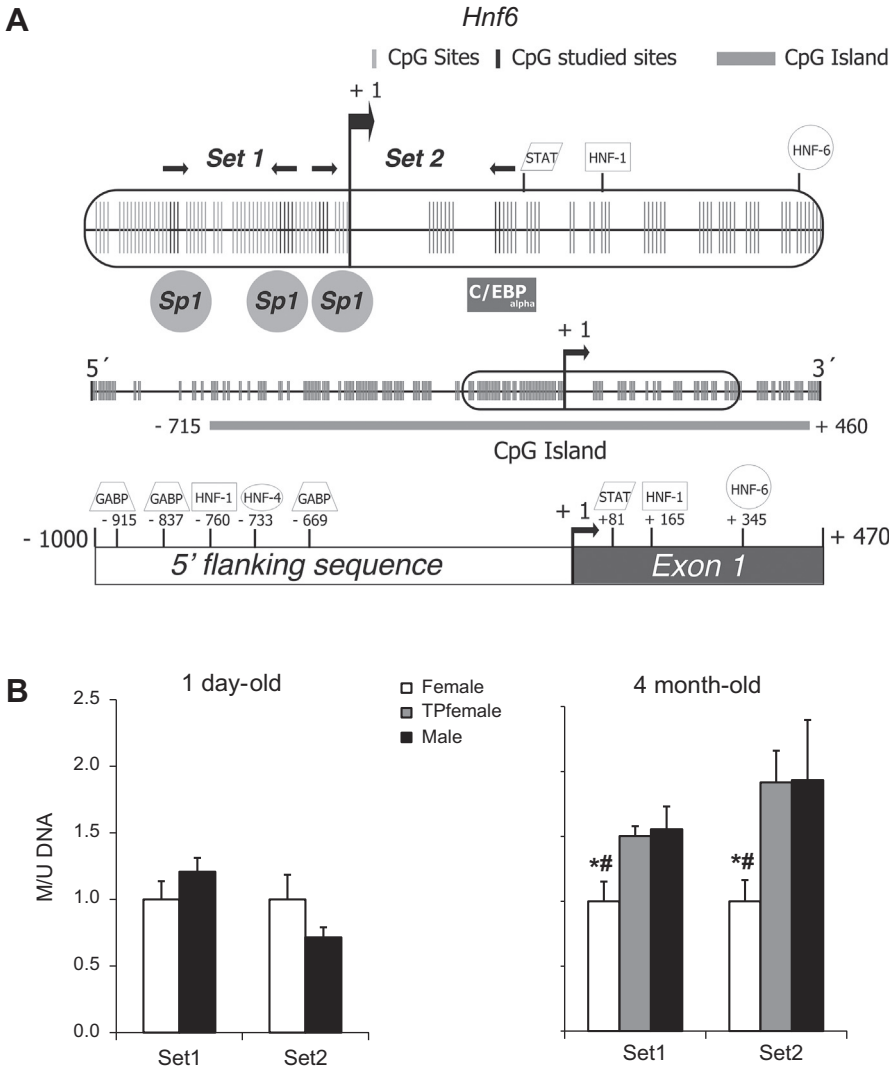

Fig. 5. Methylation status of the proximal *Hnf-6* promoter. (A) Upper: Location at the CpG island studied of the two sets of primers used, [each set consisting of 2 primers (Table 3)] is indicated by arrows. Binding sites for GH-regulated transcription factors, data obtained from genome-wide by ChIP-seq data for HNF-1 and HNF-6 (Odom et al., 2007) or a consensus site for STAT (Lahuna et al., 2000) are shown. Below the line of the CpG island, in sílico-predicted transcription factors which may bind to the CpGs analyzed are shown (SP1 and C/EBPα). Below: CpG plot of the mouse *Hnf6* promoter, a 1470 bp DNA sequence, including 1000 bp of the 5'flanking sequence, and 470 bp of exon I was analyzed. The CpG island covers 715 bp of 5'flanking sequence and 460 bp of exon I. Binding sites for GH-regulated transcription factors are depicted. (B) Methylated DNA/unmethylated DNA ratio (M/U DNA) for the liver *Hnf6* promoter, using two sets of primers (each set included the methylated and unmethylated sequence), after bisulphite treatment of DNA, in one day old (N = 5–6), and four month old mice (N = 4–6 for each group). $^{*}P < 0.05$ vs. males, and $^{\#}P < 0.05$ vs. TP females.

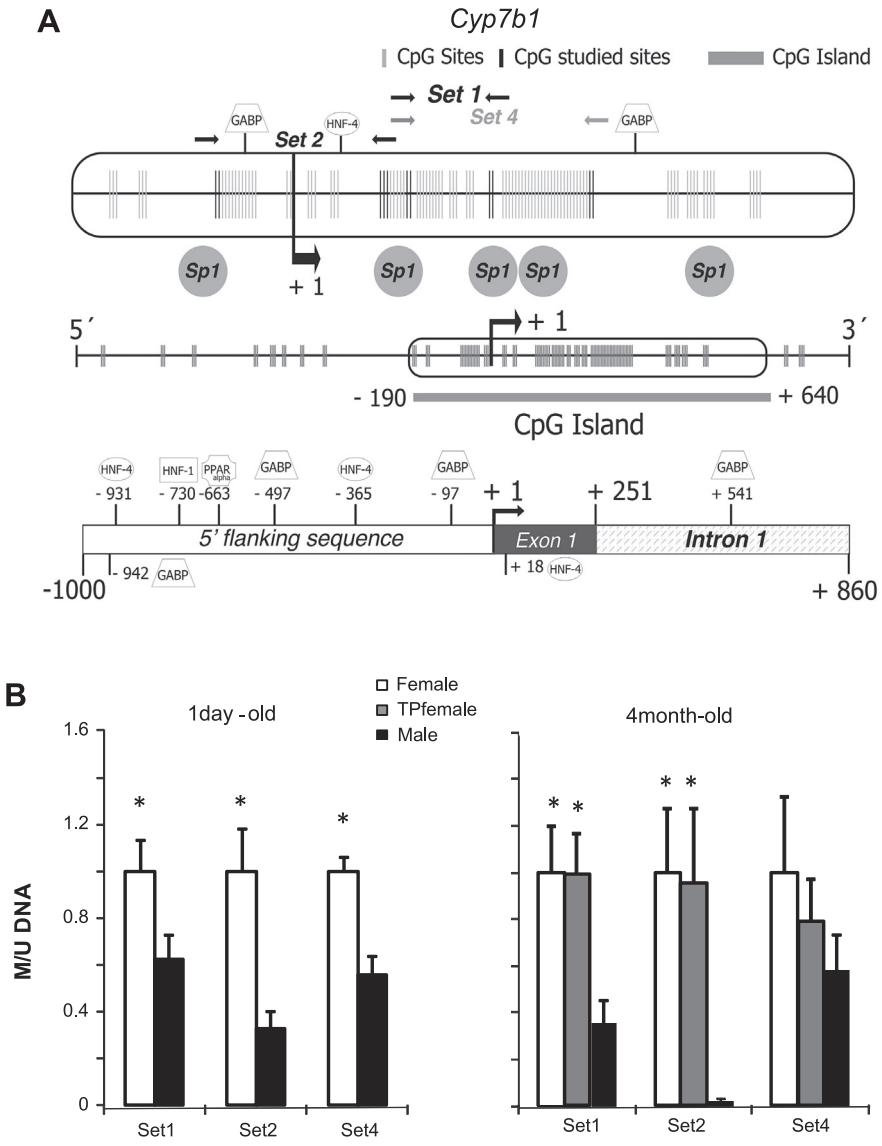

Fig. 6. Methylation status of the proximal *Cyp7b1* promoter. (A) Upper: Location of the three sets of primers used (Table 4) is indicated by arrows. Binding sites for GH-regulated transcription factors, data obtained from genome-wide by ChIP-seq data for HNF-4 (Odom et al., 2007) and from ChiP analysis for GABP (Leuenberger et al., 2009) are shown. Below the line of the CpG island, in sílico- predicted binding site (SP1) which may bind to the CpGs analyzed are shown. Below: *Cyp7b1* promoter. A 1860 bp DNA sequence, including 1000 bp of the 5'flanking sequence, exon I and 609 bp of intron I sequence was analyzed. The CpG island covers 190 bp of 5'flanking sequence, the entire exon I (251 bp) and 389 bp of intron I. Binding sites for GH-regulated transcription factors obtained from genome-wide by ChIP-seq data are depicted for HNF-1,HNF-4, HNF-6, GABP and PPARα (Boergesen et al., 2012; Leuenberger et al., 2009; Odom et al., 2007) B) Methylated DNA/unmethylated DNA ratio (M/U DNA) for the liver *Cyp7b1* promoter, using three sets of primers, after bisulphite treatment of DNA, in one day old ($N = 5–6$), and four month old mice ($N = 4–6$ for each group), $^{*}P < 0.05$ vs. males.

adult liver correlated with mRNA expression of the respective genes: higher methylated/unmethylated DNA was determined for the *Cyp7b1* promoter in females, and for the *Hnf6* promoter in males, consistent with male and female predominance of gene expression, respectively. Interestingly, *Cyp7b1* promoter exhibited a higher methylation status in females at birth and at 4 months of age, while the *Hnf6* promoter was equally methylated in livers of male and female neonates. The methylation status of *Hnf6* promoter changed in livers of adult mice, and higher methylation was observed not only in adult males, but also in neonatally androgenized females compared to females, in accordance with the defeminization of the gene induced by neonatal testosterone. On the other hand, the lower methylation status observed the *Cyp7b1* gene promoter in females at birth was maintained in adulthood and could not be modified by neonatal testosterone. Therefore CpG sites in the promoters of these genes exhibit sex-specific methylation patterns which correlate with their expression in the livers of adult and not newborn mice. We cannot conclude this is a general phenomenon which dictates sexual dimorphism and lack of masculinization of male predominant genes in females, because some of the genes studied lacked CpG islands. Nevertheless, Yokomori *et al* also described that the female *Cyp2a4* gene was equally methylated at birth in both sexes, and underwent developmental demethylation in females, while the male predominant *Cyp2d9* promoter exhibited higher methylation status in female compared to male livers already in 2 day-old mice, and CpG sites in this promoter exhibits a sex-preferential demethylation which correlates with the ontogeny and sex differential expression of its mRNAs in the livers of mice (Yokomori et al., 1995).

Because DNA methylation of cytosine residues can interfere with the binding of transcription factors, and thus prevent transcription, we examined whether the DNA sequences encompassed by the primer sets contained putative transcription factor-binding sites. *In silico* prediction showed that the analysed CpG sites in the *Cyp7b1* promoter were located at Sp1 binding sites, and Chip analysis predicted a GABP binding site (Leuenberger et al., 2009); while

CpG sites in the *Hnf6* promoter included C/EBPα and SP1 binding sites. The Sp1 proteins are ubiquitously expressed, and interact with general transcription factors, and in particular have an important role for basal *CYP7B1* transcription. Mutagenesis of Sp1 binding sequences markedly reduced promoter *CYP7B1* activity (Wu and Chiang, 2001). C/EBPα regulates expression of hepatocyte-specific genes, in particular HNF6-dependent transcription (Yoshida et al., 2006). Methylation of transcription binding sites generally prevents the transcriptional machinery from accessing start sites on a gene, therefore it is conceivable that higher methylation of Sp1 binding sites in the *Cyp7b1* promoter in females, and of Sp1 and C/EBPα binding sites in the *Hnf6* promoter in males is causally related to sex differences in their gene expression.

Furthermore, many liver transcription factors have now been mapped to the genome in mouse liver, and thousands of binding sites have been identified genome-wide by ChIP-seq, for several liver HNF's and GH-regulated transcription factors. Analysis of these data indicated that other GH-regulated transcription factors could bind to the *Hnf-6* and *Cyp7b1* CpG islands studied, even though these binding sites were not encompassed by our primers. Such was the case for HNF-4, HNF-1, HNF-6 and GABP for *Hnf6*, and HNF-4, HNF-1, GABP and PPARα for *Cyp7b1* (Boergesen et al., 2012; Leuenberger et al., 2009; Odom et al., 2007). Therefore it is conceivable that methylation may modulate the binding and interaction of multiple transcription factors which are regulated by GH secretory patterns, and may also participate in dictating sexual dimorphism in liver gene expression.

There is evidence that the degree of promoter methylation determines the expression of other female predominant genes such as *Cyp2a4* in mice (Yokomori et al., 1995) and prolactin in rats (Ngo et al., 1996). Furthermore, greater acetylation of histone-4-lysine-8 of the *Cyp3a41* chromatin has been described in female compared to male livers (Bhadhprasit et al., 2011), and female-specific expression of the *CYP2C12* gene in rats results from inaccessibility of transcription factors to target elements due to chromatin condensation in male rat livers (Endo et al., 2005).

Results obtained using genome-wide analysis point to a complex scenario in the epigenetic processes which intervene in GH-dependent liver gene sexual dimorphism. Chromatin structure can impact on sex-differential chromatin accessibility and sex-biased gene expression. Furthermore, sex differences in chromatin environment are commonly seen at male-biased and female biased DNAse hypersensitive sites (DHS), and not at sex-biased TSS, which mostly lack sex differences in local chromatin activating or repressing marks (Sugathan and Waxman, 2013). This suggests that many sex-biased genes are controlled by distal regulatory sites, which were not studied in the present experiments. Besides, sex-biased repressive or activating marks at histones participate in chromatin accessibility, and in particular, male-dependent suppressive mark, trimethlytion of histone H3 at K27, is found at the highly female-biased *Cyp2b9* gene and not in any of the male predominant genes studied. Furthermore, distinct activating marks at histone 3 are found in each sex, for example female- enriched activating K4me1 and K27ac histone marks (associated with enhancers) are found within the female predominant *Cyp3a41*, *Cyp2a4* and *Cyp2b9* genes, and male-enriched activating marks are found in the male-predominant *Cyp7b1*, *Slp* and *Cyp4a12* genes (Sugathan and Waxman, 2013). This indicates that not only CpG methylation but different epigenetic events participate in the determination sex-biased gene expression in the liver.

Lack of masculinization of male predominant genes in females may be related to a lack of induction of a fully episodic GH pattern of release in females, as suggested by the lack of masculinization of the *Mup* gene (Ramirez et al., 2010), or to a muted response of the signal transduction pathways activated by GH. GH signaling in the liver is initiated by hormone binding of GH receptors on the surface of target cells. Subsequently two molecules of Janus Kinase-2 (JAK2) are activated, and cross phosphorylate each other, as well as the GH receptor. Signal transducers and activators of transcription (STAT5B) and other transcription factors may bind to these phosphorylated receptor docking sites, are in turn phosphorylate, homodimerize, and translocate to the nucleus where they bind to promoter sites. An important negative regulatory mechanism of GH signaling are the suppressors of cytokine signaling (SOCS/CIS) family of inhibitory proteins, which inhibit GH signaling by competing with STAT5B for common binding sites of the GH receptor. In this regard, CIS appears to be the dominant factor down-regulating the GH initiated JAK2/STAT5B signaling pathway (Thangavel and Shapiro, 2007). Activation of the JAK2/STAT5B signaling pathway of episodic GH is highly suppressed in females (Dhir et al., 2007) as a likely result of an inherent overexpression of CIS (Thangavel and Shapiro, 2007). *Cis* mRNA levels are consistently higher in female hepatocytes (Thangavel and Shapiro, 2007), even in hypophysectomized rats suggesting sex-dependent, irreversible imprinting. In the present experiments, we found that neonatal androgenization did not defeminize or decrease *Cis* mRNA expression in female livers, and therefore persistent high *Cis* expression may preclude the masculinization of male genes in TP females.

The decrease or defeminization of feminine predominant liver genes (*Hnf6, Adh1, Cyp3a41*, and *Prlr* observed in the present work, and *Cyp2a4* and *Cyp2b9* in (Ramirez et al., 2010)) is consistent with the increase in the GH axis induced by neonatal androgenization. It has been described that overexpression of GH in transgenic female mice increased liver weights, but decreased hepatic cytochrome P450 content (Cheriathundam et al., 1998). Furthermore, GH administration in the GH-deficient Little (*lit/lit*) mouse reduced feminine predominant CYP2A4 activity and mRNA levels (Noshiro and Negishi, 1986).

We conclude that early sexual differentiation of the GH axis regulates sex dependent liver gene expression, a process which may involve specific methylation of promoters and GH signaling liver molecules. In females, abnormal neonatal steroid exposure evokes a loss of sex specificity in female predominant and not in male predominant liver genes, a situation which may compromise liver function.

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