RESEARCH

Epigenetic modifications in the GH-dependent *Prlr*, *Hnf6*, *Cyp7b1*, *Adh1* and *Cyp2a4* genes

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Abstract

Many sex differences in liver gene expression originate in the brain, depend on GH secretion and may underlie sex disparities in hepatic disease. Because epigenetic mechanisms may contribute, we studied promoter methylation and microRNA abundance in the liver, associated with expression of sexual dimorphic genes in mice with selective disruption of the dopamine D2 receptor in neurons (neuroDrd2KO), which decreases hypothalamic *Ghrh*, pituitary GH, and serum IGFI and in neonatally androgenized female mice which have increased pituitary GH content and serum IGFI. We evaluated mRNA levels of the female predominant genes prolactin receptor (*Prlr*), alcohol dehydrogenase 1 (*Adh1*), *Cyp2a4*, and hepatocyte nuclear transcription factor 6 (*Hnf6*) and the male predominant gene, *Cyp7b1*. Female predominant genes had higher mRNA levels compared to males, but lower methylation was only detected in the *Prlr* and *Cyp2a4* female promoters. In neuroDrd2KO mice, sexual dimorphism was lost for all genes; the upregulation (feminization) of *Prlr* and *Cyp2a4* in males correlated with decreased methylation of their promoters, and the downregulation (masculinization) of *Hnf6* mRNA in females correlated inversely with its promoter methylation. Neonatal androgenization of females evoked a loss of sexual dimorphism only for the female predominant *Hnf6* and *Adh1* genes, but no differences in promoter methylation were found. Finally, mmu-miR-155-5p, predicted to target *Cyp7b1* expression, was lower in males in association with higher *Cyp7b1* mRNA levels compared to females and was not modified in neuroDrd2KO or TP mice. Our results suggest specific regulation of gene sexually dimorphic expression in the liver by methylation or miRNAs.

Key Words

- ▶ PRLR
- ▶ liver
- ▶ GH
- ▶ methylation
- ▶ miRNA

*Journal of Molecular
Endocrinology*
(2020) **64**, 165–179

Introduction

Many sex differences in liver gene expression originate in the brain, depend on GH secretory patterns, and are imprinted by neonatal gonadal steroids (Jansson & Frohman 1987). 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

(Nugent & McCarthy 2011, Lenz *et al.* 2012), which program sexual dimorphism and metabolic function of the liver. DNA methylation, histone modification patterns, or miRNAs can be potential mechanisms by which lifelong changes in gene expression are maintained.

Sexual dimorphism of liver genes may underlie sex differences in hepatic disease, and furthermore,

loss of dimorphism may condition disease occurrence in humans or rodents. For example, sex-specific GH patterns, by modifying gene expression in the liver, may shape the sensitivity to thrombosis, dislipidemia, coronary artery disease, liver lesions or hepatocellular carcinoma (HCC) development (Liao *et al.* 1993, Wong *et al.* 2008, Zhang *et al.* 2011). Furthermore, differential susceptibility to some liver diseases between sexes has been described. For example, chronic hepatitis, primary sclerosing cholangitis, HCC, nonalcoholic fatty liver disease (NAFLD), steatohepatitis or liver fibrosis progression predominate in males, while primary biliary cirrhosis, autoimmune hepatitis or alcoholic liver disease do so in females (Poynard *et al.* 1997, Rogers *et al.* 2007, Guy & Peters 2013, Durazzo *et al.* 2014, Buzzetti *et al.* 2017). The pituitary gland, mostly dictating GH secretory patterns is involved in this sexual dimorphism, while prolactin secretion, which regulates liver *Prlr* expression in females, may also participate.

We have previously 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 female rats (Ramirez *et al.* 2012). Furthermore, we showed that impaired GHRH-GH-IGF1 axis disrupts sexual dimorphism of many genes in mice (Ramirez *et al.* 2015), and that high levels of prolactin modify liver *Prlr* mRNA levels (Ramirez *et al.* 2015). We now explore a possible relationship between the alterations in liver gene dimorphic expression in these models and epigenetic regulation, such as the methylation status of gene promoter regions, or abundance of specific miRNAs which regulate transcripts.

DNA methylation of CpGs decreases gene expression by preventing the binding of the transcriptional machinery on a gene's promoter, or by recruiting methyl-binding and chromatin remodeling proteins which cause chromatin to condense (Garcia-Carpizo *et al.* 2011). In general, methylation of a promoter is inversely associated with gene expression. This modification can be duplicated across cellular division and maintained throughout a lifespan.

MiRNAs are short non-coding RNAs that play an important role in post-transcriptional regulation of gene expression. miRNAs repress translation, partly by targeting mRNAs for degradation based on partial complementary pairing of the miRNA seed region to the seed match site region of the target mRNA. MiRNAs have the capacity of targeting multiple genes, thus regulating the expression of several proteins. Some liver miRNAs are sexually

dimorphic (Hao & Waxman 2018), but not many have been validated in determining sex differences in liver gene expression.

We studied promoter methylation and miRNA abundance in the liver in association with sexual dimorphic expression of female predominant genes. We chose genes from a group of more than a thousand GH-dependent genes that are differentially expressed in the liver in females and males (Clodfelter *et al.* 2007), and which are important in liver function and disease: the prolactin receptor (*Prlr*), alcohol dehydrogenase 1 (*Adh1*), the steroid 15 α -Hydroxylase (*Cyp2a4*), and hepatocyte nuclear transcription factor 6 (*Hnf6*), and included a male predominant gene *Cyp7b1*. Regulation of these genes by GH has been demonstrated by us and others (Waxman & Holloway 2009, Wauthier *et al.* 2010, Ramirez *et al.* 2015).

The prolactin receptor has multiple homeostatic roles (Goffin *et al.* 2002), and, in particular, it may protect the liver from NAFLD (Shao *et al.* 2018, Zhang *et al.* 2018), prevent HCC and inflammation (Hartwell *et al.* 2014), and modify hepatic drug metabolism (Tracy *et al.* 2005). HNF6 is a transcription factor involved in glucose metabolism, bile homeostasis, inflammation, and a possible target in liver fibrosis, tumorigenesis, and cholestasis (Wang & Holterman 2012). CYP2A4 is a steroid 15 α -hydroxylase that catalyzes one of the hydroxylation reactions leading to further metabolism of the sex hormones, testosterone and estradiol, in the liver. Alcohol dehydrogenase 1 metabolizes the majority of ethanol through oxidation (Simon *et al.* 2002) and is relevant when considering the predisposition of toxic effects exerted by alcohol. And finally, CYP7B1 is cytochrome P450 enzyme that hydroxylates oxysterols and steroids and is paramount in hepatic bile salt synthesis, and in the inactivation of otherwise hepatotoxic oxysterols.

We studied female and male mice with selective disruption of the dopamine D2 receptor in neurons (neuroDrd2KO), in which hypothalamic expression of *Ghrh* is decreased, pituitary content of GH and serum IGFI are lower, and GH pulsatility is greatly impaired (Noain *et al.* 2013, Ramirez *et al.* 2015); and neonatally androgenized female mice (TP) which have increased GH pituitary content, increased serum IGFI compared to control females, but full male GH pulsatility is not achieved (Ramirez *et al.* 2010). We aimed at establishing the specificity of epigenetic modulation by promoter methylation or miRNA abundance on the regulation of sexual dimorphism, and their relation to the GH axis in each model.

Materials and methods

Animals

Neonatally androgenized females

C57BL/6J mice were housed in temperature-controlled room with lights on at 07:00 h and lights-off at 19:00 h, and free access to laboratory chow and tap water. On the day of birth (day 0) the pups were left undisturbed, and on the second day they were sexed and each pup was randomly divided into treatment groups: males, females, and females injected subcutaneously with 100 g testosterone propionate (Sigma) in 0.010 mL castor oil (neonatally androgenized females: TP females). The dose chosen has been described to effectively androgenize neonatal brain in mice (Livne *et al.* 1992, Ramirez *et al.* 2010). Females and males from the same cohort were injected with castor oil and used as controls. Animals were weighed and killed at 4 months of age. As previously described TP females had an intermediate body weight, percentage of somatotropes and pituitary GH content compared to females, and males (Ramirez *et al.* 2010). Serum IGF I levels were similar in males and TP females, and higher than females. On the other hand, liver *Mup1/2/6/8* mRNA levels, a marker of male GH pulsatility, were similar in females and TP females, and lower than in males. This indicates that even though pituitary GH content was increased in TP females, complete masculinization of male pulsatility was not achieved, suggesting that not only GH levels but also pulsatility is important for full expression of many male genes. Control females in diestrus and TP females

in diestrus/anestrus were used, as TP females had highly irregular cycles.

Mice lacking D2Rs in neurons (neuroDrd2KO)

To ablate D2Rs from cells of neural origin, *Drd2^{loxP/loxP}* mice (Bello *et al.* 2011) were crossed with B6.Cg-*Tg(Nes-cre)*^{1Kln/J} to obtain cohorts of *Drd2^{loxP/loxP}* (control mice) and *Drd2^{loxP/loxP}.B6.Cg-Tg(Nes-cre)*^{1Kln/J} littermates (Noain *et al.* 2013). Thereafter, breeding pairs of *Drd2^{loxP/loxP}* and *Drd2^{loxP/loxP}.Tg(Nes-Cre)*^{1Kln/J} mice were used to generate *Drd2^{loxP/loxP}* (control) and *Drd2^{loxP/loxP}.Tg(Nes-Cre)* (neuroDrd2KO) littermates (Noain *et al.* 2013). Body weight was lower in male and female neuroDrd2KO mice compared to their control littermates *Drd2^{loxP/loxP}* mice (Table 1). Furthermore, the GH axis was decreased in neuroDrd2KO of both sexes: they had lower pituitary GH content, liver *Igf1* mRNA levels, serum IGF1 and urine MUPs compared to *Drd2^{loxP/loxP}* sex-matched mice. NeuroDrd2KO males also had decreased *Mup* mRNA levels compared to *Drd2^{loxP/loxP}* males. Pituitary GH content, liver *Igf1* and *Mup1* mRNA levels, and urine MUPS were higher in males compared to females. Prolactin levels were higher in females, and LH and FSH were not altered by genotype. Liver *Igfbp3* was similar in all groups (Table 1) (Noain *et al.* 2013, Ramirez *et al.* 2015).

All experimental procedures were performed in 4- to 6-month-old mice. The institutional animal care and use committee of the Instituto de Biología y Medicina Experimental, Buenos Aires granted approval, protocol

Table 1 GH axis in neuroDrd2KO mice.

	Female		Male		Statistics
	<i>Drd2^{loxP/loxP}</i>	NeuroDrd2KO	<i>Drd2^{loxP/loxP}</i>	NeuroDrd2KO	
BW (g)	27.0 ± 0.7	22.0 ± 0.5	33.0 ± 0.8	26.0 ± 0.5	Genotype, sex
<i>n</i>	12	8	9	7	
Pituitary GH (ng/ug prot)	263 ± 87	108 ± 38	398 ± 84	136 ± 0.6	Genotype, sex
<i>n</i>	7	6	13	16	
Liver <i>Igf1</i>	116 ± 11	45 ± 4	100 ± 11	37 ± 7	Genotype
<i>n</i>	8	9	10	6	
Liver <i>Igfbp3</i>	147 ± 29	165 ± 25	100 ± 12	134 ± 28	NS
<i>n</i>	8	9	10	6	
Liver <i>Mup1</i>	18 ± 4	12 ± 5	100 ± 18	30 ± 4	Sex, genotype only males
<i>n</i>	8	9	7	8	
Serum IGF1 (ng/mL)	792 ± 83	393 ± 58	648 ± 45	463 ± 24	Genotype
<i>n</i>	8	9	10	8	
Urine MUP (AU)	48.2 ± 8.9	15.4 ± 3.5	100.0 ± 5.8	54.2 ± 3.5	Genotype, sex
<i>n</i>	12	5	18	8	
Prolactin (ng/mL)	235 ± 60	267 ± 57	182 ± 32	182 ± 51	Sex
<i>n</i>	8	9	10	8	

Data are presented as means ± s.e.m. Genotype: indicates significant differences between genotypes ($P \leq 0.05$) in both sexes. Sex: indicates significant differences between males and females ($P \leq 0.05$) in both genotypes. NS, non significant.

#07/2016 (in accordance with the Division of Animal Welfare, Office for Protection from Research Risks, NIH, Animal Welfare Assurance for the Institute of Biology and Experimental Medicine A#5072-01).

Tissue RNA extraction and total cDNA preparation for *Hnf-6*, *Adh1*, *Prlr*, *Cyp2a4*, and *Cyp7b1* expression by real-time PCR

After euthanasia, liver samples (50 mg) were immediately homogenized in TRI Reagent (Molecular Research Center, Inc) and stored at -70°C until total RNA was isolated as previously described (Ramirez *et al.* 2015). First-strand cDNA was synthesized from 1 μg of total RNA as previously described (Ramirez *et al.* 2015).

Quantitative real-time PCR

Sense and antisense oligonucleotide primers were designed based on previously published results 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 as previously described (Ramirez *et al.* 2015). After denaturation at 95°C for 15 min, the cDNA products were amplified for 40 cycles, each cycle consisting of denaturation at 95°C for 20 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 Biorad CFX96 Touch™ (Biorad), and data were stored continuously during the reaction. The results were validated based on 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 analysed in duplicate. Relative gene expression levels were calculated according to the comparative cycle threshold (CT) method. Cyclophilin was used as housekeeping gene, and CTs did not show sex differences and were not altered in the different experimental groups.

Stemloop RT-qPCR for microRNA expression

A specific Stemloop primer was designed for each microRNA to be analyzed (sequences in Table 2), and used for the RT reaction (instead oligoDT, from the

conventional protocol for mRNA), at a final concentration of 1 μM . It was incubated with 500 μg of RNA for two min at 65°C and then left on ice for 1 min. A master mix with 10 $\times$ Buffer (1 $\times$), DNTPS (0.4 mM), DTT (0.004 mM) and MML-V (200u; Epicentre, Madison, WI) was added to each tube. They were incubated at 16°C for 30 min, 37°C for 60 min and 85°C for 5 min. The cDNA obtained was relatively quantified through qPCR.

DNA extraction for methylation studies

The tissue was placed in liquid nitrogen and crushed with mortar (and on dry ice). The powder was suspended in digestion buffer with proteinase K (stock 20 mg/mL, 35 μL in 1.2 mL). Samples were then incubated at 50°C for 12–18 hours. They were centrifuged for 15 min at 12,000 rpm and the supernatant was preserved. One volume of phenol-chloroform-isoamyl (25:24:1, Sigma-Aldrich) was added, then mixed by inversion and left for 2 min at room temperature. The samples were centrifuged for 10 min at 12,000 g, the aqueous phase transferred to a new tube and $\frac{1}{2}$ volume of 7.5 M Ammonium Acetate and 1 volume of isopropanol were added. After incubating at -20°C for 30 min, they were centrifuged for 15 min at 12,000 rpm and the supernatant removed. The pellet was washed with 1 mL of 70% ETOH and left to dry at room temperature. Finally, it was resuspended in 80 μL of molecular H_2O .

Treatment with methylation sensitive restriction enzymes

The Mix was prepared at room temperature at a final volume of 50 μL (1 $\times$ FastDigest® Buffer, 5 μg of genomic DNA (in 10 μL of water), 5 units of FastDigest® Enzyme (treated DNA) or water for untreated samples, that served as internal controls. It was mixed gently, spinned down and incubated at 37°C for 5 min and then at 65°C for 5 min to inactivate the enzyme. A qPCR was then performed, using primers listed on Table 2, to the enzyme treated and untreated DNA. Amplification of treated DNA was relativized to that of untreated DNA of the same sample. We used the FastDigest® (Thermo Scientific) enzymes HpaII and HhaI for this test.

In silico search for CpG islands in the promoters of *Prlr*, *Hnf6*, *Cyp2a4*, *Adh1* and *Cyp7b1*

The search for CpG islands was performed using the Methyl Primer Express 1.0 software (Applied Biosystems). For each gene, the promoter region sequences were

Table 2 Description of primers used in mRNA, methylation and miRNA studies.

Gene	Primer sequence (5'–3')		Source	Enzyme
	Sense	Antisense		
Primers used in mRNA studies				
<i>Adh1</i>	GCTCTGCCGTCAAAGTCGCCA	ACCGACACCTCCGAGGCCAA	Designed by PrimerBlast	
<i>Hnf6</i>	AAGCCCTGGAGCAAACTCAA	CCACATCTCTCGGAAAGTCTC	Designed by PrimerBlast	
<i>Prlr</i>	CACAGTAAATGCCACGAACG	GGCAACCATTTTACCCACAG	Designed by PrimerBlast ^a	
<i>Cyp7b1</i>	TGAGGTTCTGAGGCTGTGCTC	TCCTGCACCTTCTCGGATGATG	Holloway <i>et al.</i> 2006	
<i>Cyp2a4</i>	AGCAGGCTACCT TCGACTGG	GCTGCTGAAGGCTATGCCAT	Wiwi <i>et al.</i> 2004	
<i>Cyclophilin</i>	CAGAACAT ATCCCTGCAT	GTTCAGCTCTGGGATGACCTT	Designed by PrimerBlast	
Primers used in methylation studies				
<i>Adh1</i>	AATGTGCTAGGCCTTATGGG	ATCAGGACTCAAGGGCTCTG	Designed by PrimerBlast	<i>HpaII</i>
<i>Hnf6</i>	TGGCGAGTGGGCTTTG	AGAGTGCAGCTGGAGCC	Designed by PrimerBlast	<i>HpaII</i>
<i>Prlr</i>	CTTGATGGAAGAAGGTCCCT	GAAGGCCAGCTCAGAAGAC	Designed by PrimerBlast	<i>HpaII</i>
<i>Cyp7b1</i>	GCCACGACCCTAGATGCC	CAGGCGCTTACCTGGTGC	Designed by PrimerBlast	<i>HhaI</i>
<i>Cyp2a4</i>	CCAAACAGCTAAGGCGCTTC	GCGGATATCAGCGTGTGCTT	Designed by PrimerBlast	<i>HhaI</i>
Primers used in miRNA studies				
<i>mmu-miR-155-5p</i>	miRNA Rv UNIVERSAL	TGGTGCAGGGTCCGAGGTATT	Designed by PrimerBlast	
	RT-miR-155-5p STEM	GTCTCCTCTGGTGAGGGTCCGAGGTATTGCGACCAGAGGAGACCCCTT	Designed by PrimerBlast	
	miR-155-5p Fw	GGGCCGGGTTAATGCTAATTGTG		
<i>mmu-miR-142a-3p</i>	RT-miR-142a-3p STEM	GTCTCCTCTGGTGAGGGTCCGAGGTATTGCGACCAGAGGAGACTCCATA	Designed by PrimerBlast	
	miR-142a-3p Fw	GGCCGGGTAGTGTTTCTACTT		

^aPrimers detect all *Prlr* isoforms.

obtained from the Ensembl program http://www.ensembl.org/Mus_musculus/Info/Index.

In silico prediction of binding sites for transcription factors in the proximal promoters of *Prlr*, *Hnf6* and *Cyp2a4*

For the identification of putative transcription factor binding sites in the proximal promoter regions of *Prlr*, *Hnf6* and *Cyp2a4*, we first used the program PROMO from the Algorithmics and Genetics Group (ALGGEN) (Messegueur *et al.* 2002). The resulting list of putative binding sites extracted using this bioinformatic tool was then narrowed down by the analysis of published data which validated, using Chip-seq, those transcription factors that bound to sequences proximal to the promoter region of the genes of interest (Mueller *et al.* 1990, Miura & Tanaka 1993, Lahuna *et al.* 2000, Yoshida *et al.* 2006, Negri-Cesi *et al.* 2008, Bolotin *et al.* 2011, Zhang *et al.* 2012).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. The differences between means were analysed by Student's *t* test (two groups); and ANOVA followed by Newman–Keuls test or Tukey's HSD test for unequal N for females, TP females and males. For the analysis of gene and miRNA expression, and promoter methylation in neuroDrd2KO mice two-way ANOVA for the effects of sex and genotype for independent measures was used. If *P* of the interaction was significant, individual means were compared by Tukey's honest significant difference test; if it was not significant, groups of means were analysed by the same test. Kruskal–Wallis test was performed when a non-parametric test was necessary. We evaluated correlation by Spearman's rank correlation coefficient, *P* < 0.05 was considered significant.

Results

Sexual dimorphism of liver *Hnf6* mRNA and promoter methylation levels in neuroDrd2KO mice and neonatally androgenized females

Expression levels of *Hnf6* was 2.80-fold higher in females compared to males confirming female predominance of this gene (*P* $\leq$ 0.05; Fig. 1A), while methylation levels of the *Hnf6* promoter were similar in both sexes (Fig. 1B).

In female neuroDrd2KO mice there was a significant downregulation of *Hnf6* mRNA expression levels compared

to female *Drd2^{loxP/loxP}* mice, and sexual dimorphism for this gene was lost (Fig. 1C). Furthermore, there was an increase in the methylation status of the *Hnf6* promoter in neuroDrd2KO females compared to controls (Fig. 1D), yielding a significant inverse correlation of mRNA levels and promoter methylation for this gene in females (Fig. 1D inset, *P* correlation = 0.016). On the other hand, expression and methylation levels of the *Hnf6* gene in males were not modified in neuroDrd2KO compared to *Drd2^{loxP/loxP}* mice (Fig. 1B and C).

Figure 1

Hnf6 mRNA levels and methylation of its promoter in (A and B) female and male mice; (C and D) female and male neuroDrd2KO and *Drd2^{loxP/loxP}* mice, and (E and F) females, neonatally androgenized females (TP female) and males. Inset (D) correlation of *Hnf6* expression and methylation of its promoter in female *Drd2^{loxP/loxP}* and neuroDrd2KO mice. *n* (for this and following figures the number of samples is defined from left to right within each panel) = 19,23 (A), 14–21 (B); 11,10,9,11 (C); 6,6,5,6 (D); 13,16,13 (E); 6,6,9 (F). **P* $\leq$ 0.05 vs females (genotype matched); 'a' *P* $\leq$ 0.05 vs *Drd2^{loxP/loxP}* sex-matched mice. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0205>.

Neonatal androgenization of females decreased the expression levels of this gene compared to control females (Fig. 1D), but methylation of the *Hnf6* promoter was similar in the three groups (Fig. 1E).

Sexual dimorphism of liver *Prlr* mRNA and promoter methylation levels in *neuroDrd2KO* mice and neonatally androgenized females

Expression levels of *Prlr* confirmed female predominance for this gene in mice (Fig. 2A; $P \leq 0.0001$; female/male ratio=3.86). Methylation of the *Prlr* promoter was significantly higher in males ($P=0.041$, Fig. 2B), and a significant inverse correlation for *Prlr* gene expression and methylation status of the promoter was verified for *Prlr* in livers from male and female mice ($P=0.018$; Fig. 2C).

In *neuroDrd2KO* mice sexual dimorphism of the *Prlr* gene was lost (Fig. 2D), indicating that the action of the central D2R is paramount in dictating sexual dimorphism of GH-dependent genes, and that the liver *Prlr* is not only a prolactin-inducible gene but also under GH control. Furthermore, in *neuroDrd2KO* males, *Prlr* expression was significantly upregulated (feminized) ($P=0.006$), and the methylation of the *Prlr* promoter decreased compared to *neuroDrd2^{Loxp/LoxP}* male mice, yielding a significant inverse correlation between mRNA levels and methylation of the promoter (P correlation=0.041, Fig. 2F).

Neonatal androgenization of females (TP females) did not alter the sexual dimorphism of this female predominant gene (Fig. 2G), or the methylation status of the *Prlr* promoter, which was higher in livers from male mice compared to females and TP females (Fig. 2H). There was also a significant inverse correlation of mRNA levels and methylation of the promoter for the *Prlr* in this model (Fig. 2I, P correlation=0.018).

Sexual dimorphism of liver *Adh1* mRNA and promoter methylation levels in *neuroDrd2KO* mice and neonatally androgenized females

Expression levels of *Adh1* confirmed female predominance for this gene (Fig. 3A; $P \leq 0.016$; female/male ratio=1.41). Methylation of the *Adh1* promoter was similar between sexes (Fig. 3B).

Adh1 mRNA levels were not modified in *neuroDrd2KO* mice compared to *Drd2^{LoxP/LoxP}* mice, though sexual dimorphism was also lost for this gene (Fig. 3C). On the other hand, neonatal androgenization decreased its expression, and sexual dimorphism was also lost (Fig. 3E).

Figure 2

Prlr mRNA levels, methylation of its promoter and correlation of expression and methylation in (A, B and C) female and male mice; (D, E and F) female and male *neuroDrd2KO* and *Drd2^{LoxP/LoxP}* mice (F: correlation of data from male *neuroDrd2KO* and *Drd2^{LoxP/LoxP}* mice), and (G, H and I) females, neonatally androgenized females (TP female) and males. $n = 16, 20$ (A), 14, 18 (B); 6, 6, 7, 8 (C); 6, 6, 7, 8 (D); 5, 6, 9 (E); 6, 7, 8 (F). * $P \leq 0.05$ vs females (genotype matched); 'a' $P \leq 0.05$ vs *Drd2^{LoxP/LoxP}* sex-matched mice, 'b' $P \leq 0.05$ vs TP females. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0205>.

Methylation pattern of the promoter did not correlate with any of the changes observed (Fig. 3D and F).

Sexual dimorphism of liver *Cyp2a4* mRNA and promoter methylation levels in *neuroDrd2KO* mice and neonatally androgenized females

Liver expression levels of *Cyp2a4* confirmed female predominance for this gene (Fig. 4A; $P=0.018$; female/male ratio=2.60). Methylation of the *Cyp2a4* promoter was higher in male mice ($P=0.0014$; Fig. 4B).

In *neuroDrd2KO* males, *Cyp2a4* was significantly upregulated (feminized) ($P=0.05$, Fig. 4C), similar to results found for the *Prlr* gene, and mRNA expression and methylation status of the promoter (Fig. 4D) correlated inversely in male mice (Fig. 4D inset, P correlation=0.041).

On the other hand, neonatal androgenization did not modify *Cyp2a4* expression or methylation status of the promoter (Fig. 4F and G).

Figure 3

Adh1 mRNA levels and methylation of its promoter in (A and B) female and male mice; (C and D) female and male neuroDrd2KO and Drd2^{loxP/loxP} mice, and (E and F) females, neonatally androgenized females (TP female) and males. $n = 16,21$ (A), $17-21$ (B); $11,12,12,11$ (C); $9,8,9,9$ (D); $14,16,16$ (E); $6,7,9$ (F). * $P \leq 0.05$ vs females (genotype matched). A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0205>.

Sexual dimorphism of liver *Cyp7b1* mRNA and promoter methylation levels in neuroDrd2KO mice and neonatally androgenized females

Expression levels of *Cyp7b1* confirmed male predominance for this gene (Fig. 5A; $P = 0.0002$; female/male ratio = 0.18), though methylation of the *Cyp7b1* promoter was similar in both sexes (Fig. 5B).

In livers from male neuroDrd2KO mice the expression of *Cyp7b1* was significantly decreased compared to livers from Drd2^{loxP/loxP} mice (Fig. 5D; $P = 0.0009$), with no

Figure 4

Cyp2a4 mRNA levels and methylation of its promoter in (A and B) female and male mice; (C and D) female and male neuroDrd2KO and Drd2^{loxP/loxP} mice (panel C is reproduced from Ramirez *et al.* (2015) by permission of Oxford University Press); and (E and F) females, neonatally androgenized females (TP female) and males. Inset D) correlation of *Adh1* expression and methylation of its promoter in female Drd2^{loxP/loxP} and neuroDrd2KO mice. $n = 12,11$ (A), $15,19$ (B); $7,9,9,8$ (C); $5,6,6,5$ (D); $13,16,13$ (E); $6,6,9$ (F). * $P \leq 0.05$ vs females (genotype matched); 'a' $P \leq 0.05$ vs Drd2^{loxP/loxP} sex-matched mice. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0205>.

modification in the methylation pattern of the promoter in either sex (Fig. 5E).

Neonatal androgenization of females did not modify the low expression of this male gene compared to untreated females (Fig. 5G), and no differences in the methylation pattern was observed for this gene (Fig. 5H).

Figure 6

Schematic representation of CpG islands, with methylation prone CpG sites, predicted binding sites for transcription factors. (A) *Hnf6*, (B) *Prlr*, and (C) *Cyp2a4*. Location of binding sites for forward and reverse primers are shown by light blue arrows, CpGs are indicated by magenta vertical lines, and CpGs sensitive to restriction enzymes tested in our studies by magenta lines with a filled circle on top. TSS is indicated in a blue box (+1). The schematic representations were designed using the Illustrator for Biological Sequences (Liu *et al.* 2015). A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0205>.

Mmu-miR-142a-3p once again showed a similar pattern to *Prlr* gene expression in female, female TP and male livers, suggesting a lack of participation in the regulation of this gene (Fig. 7F).

Discussion

Male and female predominant liver genes represent 63 and 37%, respectively, of total sex-specific liver gene expression. These sex differences depend on a highly regulated GH axis, as hypophysectomy (mostly through the loss of GH) abolishes sex specificity for the majority of male- and female-predominant genes (Wauthier *et al.* 2010). In this context, alterations in GH secretory patterns affect liver function, and have been proposed as determinants in liver disease (Brie *et al.* 2019). The magnitude, as well as

Figure 7

mmu-miR-155-5p and mmu-miR-142a-3p expression levels in (A and B) female and male mice; (C and D) female and male neuroDrd2KO and Drd2^{loxP/LoxP} mice, and (E and F) females, neonatally androgenized females (TP female) and males. $n = 12, 12$ (A), $13, 15$ (B); $3, 2, 3, 3$ (C); $3, 3, 3, 3$ (D); $6, 6, 7$ (E); $6, 6, 8$ (F). * $P \leq 0.05$ vs females. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0205>.

the pulsatility of GH secretion are important in setting sexual differences, and furthermore, complex epigenetic processes may intervene in establishing hormone-dependent liver gene sexual dimorphism (Ling *et al.* 2010, Sugathan & Waxman 2013). Chromatin structure can impact on sex-differential chromatin accessibility, sex-biased gene expression, and DNase hypersensitive sites (DHS) (Sugathan & Waxman 2013). Furthermore, methylation of gene promoters, or miRNA regulation of gene transcription may act differentially on specific GH-dependent liver genes, which indicates the need to unravel the complex genetic and epigenetic regulation of sexual dimorphism of GH-dependent liver genes.

In the present work we analysed the liver expression levels in correlation with the methylation status of the promoters of four female predominant genes, and one male predominant gene. Furthermore, we studied miRNAs which may target two of these genes. We first evaluated sexual dimorphism in gene expression and promoter methylation, and then studied the alterations evoked by disruption of the D2R in neurons or neonatal androgenization of females. The first strategy decreases GH secretion and pulsatility in males (Noain *et al.* 2013), while in TP females there is a partial masculinization of the GH axis, as pituitary GH content is increased, but male pulsatility is not fully recovered (Ramirez *et al.* 2010).

We chose sexual dimorphic GH-dependent genes that are involved in liver metabolism and health. The prolactin receptor is a type I cytokine transmembrane receptor, which can be found in cells of mammary glands, supporting its role in lactation. But multiple homeostatic roles have been attributed to this hormone, and, in accordance, PRLRs are found in the pituitary gland, brain, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal gland, uterus, skeletal muscle, and skin (Goffin *et al.* 2002). Emerging roles include its participation in immune response, food intake, or islet cell proliferation (Lopez-Vicchi & Becu-Villalobos 2017). In the liver, protective actions have been described for the PRLR in NAFLD, HCC or inflammation (Hartwell *et al.* 2014, Shao *et al.* 2018, Zhang *et al.* 2018). Notably, there is a sexually dimorphic risk for HCC and NAFLD (Rogers *et al.* 2007) (Cheung & Cheng 2016, Marin *et al.* 2016), males being more prone than females, in correlation with lower expression of liver *Prlr* in males (Ramirez *et al.* 2015). The liver PRLR has a potential clinical utility for enhancing survival of liver mass in disease, injury or surgery (Moreno-Carranza *et al.* 2013), or preventing liver cancer in high-risk patients (Hartwell *et al.* 2014). The pituitary gland, mostly dictating GH secretory patterns and prolactin secretion is involved

in its sexual dimorphic expression in the liver (Moreno-Carranza *et al.* 2013, Hartwell *et al.* 2014, Shao *et al.* 2018, Zhang *et al.* 2018).

CYP2A4 is a steroid 15 α -hydroxylase that catalyzes the metabolism of testosterone and estradiol in the liver. While, CYP7B1 a cytochrome P450 enzyme that hydroxylates oxysterols and steroids is paramount in hepatic bile salt synthesis, and in the inactivation of otherwise hepatotoxic oxysterols. Loss of CYP7B1 activity is associated with liver failure in children (Stiles *et al.* 2009).

The liver enzyme alcohol dehydrogenase metabolizes the majority of ethanol through oxidation (Simon *et al.* 2002). It is a female predominant GH-dependent enzyme in rodents (Potter & Mezey 2001, Simon *et al.* 2002, Quintanilla *et al.* 2007). The defeminization of this enzyme exacerbates alcohol-induced liver damage (Ellefson *et al.* 2011); and, studies have determined that animals with lower ADH1 activity, or lower alcohol elimination rate, have a lower voluntary ethanol intake (Forsander & Sinclair 1992). It is therefore relevant when considering the predisposition of toxic effects exerted by alcohol.

HNF6 is a transcription factor that binds to specific DNA sequences of numerous target gene promoters in the liver. It is involved in numerous functions such as cell proliferation or differentiation, migration, and in the liver, specifically in glucose metabolism, bile homeostasis, inflammation, being positioned as a possible target in liver fibrosis, tumorigenesis, and cholestasis (Wang & Holterman 2012).

Our results suggest specific regulation of mRNA expression by methylation or miRNAs, indicating a complex scenario in which epigenetic regulation participates in the expression of some but not all GH-dependent sexual dimorphic liver genes.

In *neuroDrd2KO* mice, sexual dimorphism for all hepatic genes studied was lost, indicating that the action of the central D2R is paramount in dictating sexual dimorphism of GH-dependent genes. Furthermore, in male *neuroDrd2KO* mice the *Prlr* and *Cyp2a4* genes were upregulated, or feminized, while in *neuroDrd2KO* females the *Hnf6* gene was decreased or masculinized.

Neonatal androgenization of females evoked a loss of sexual dimorphism only for the female predominant genes *Hnf6* and *Adh1*. The masculinization of these two genes may be related to the increase in GH pituitary content induced by neonatal androgenization (Ramirez *et al.* 2010).

When correlating gene expression levels and methylation of the respective promoter, we found higher

mRNA levels in females of the four female predominant genes *Hnf6*, *Prlr*, *Adh1*, and *Cyp2a4*, but lower methylation of the promoters was only detected for the *Prlr* and *Cyp2a4* genes. Furthermore, the upregulation of these two genes in male neuroDrd2KO mice correlated with a decrease in the methylation of their promoters. Therefore, our results suggest the participation of this epigenetic process in regulating the sexual dimorphism described for *Prlr* and *Cyp2a4*.

An inverse correlation between methylation of the promoter and gene expression was also found in the downregulation of *Hnf-6* in female neuroDrd2KO mice. However, in neonatally androgenized females the masculinization of *Adh1* and *Hnf6* mRNA levels was not related to the methylation of their promoters. Furthermore, no differences in the methylation patterns for *Cyp7b1* or *Adh1* were found between sexes or in the neuroDrd2KO and TP models.

There are several reports which document regulation of liver genes by promoter methylation. It has been described that a postnatal gender-specific demethylation occurs exclusively in the male liver associated to the secretion of testosterone at the time of sexual maturity. The resulting methylation profile is stable and therefore can serve as an epigenetic memory (Reizel *et al.* 2015). For example, the male predominant *Cyp2d9* promoter exhibited higher methylation status in females compared to male livers already in 2-day-old mice, and a male-preferential demethylation of this gene which correlated with the ontogeny and sex differential expression of its mRNAs in the livers of mice (Yokomori *et al.* 1995).

Furthermore, methylation patterns of specific liver genes have been associated to liver disease. For example, it has been documented that unique gene-expression alterations mediated by aberrant DNA methylation of selective genes may contribute to the development of HCC, and may have diagnostic value. Furthermore, using a NASH mouse model it was described that, even though 855 aberrantly methylated genes were found during the progression of the carcinogenic process, only 4% correlated inversely with gene expression (Dreval *et al.* 2019).

This specificity in methylation changes was also documented in human livers of an obese population. The impact of sex on DNA methylation and hepatic gene expression was studied by genome-wide DNA methylation profiling, and the methylation profile in the liver was associated with differential changes in hepatic gene expression between males and females specifically for a small proportion of genes (García-Calzón *et al.* 2018).

Nevertheless, other epigenetic changes may also participate, for example, distinct sex-specific activating

marks at histone 3 have been described, and the female-enriched activating K4me1 and K27ac histone marks (associated with enhancers) are found within the female predominant *Cyp2a4* (Sugathan & Waxman 2013). Furthermore, it has been described that in male livers, female-biased genes in an active chromatin state showed earlier responses to continuous GH infusion compared to those in an inactive chromatin state (Lau-Corona *et al.* 2017), while GH pulses increased chromatin accessibility in some but not all male genes (Connerney *et al.* 2017). Therefore, not only CpG methylation but other complex epigenetic events may participate in the determination sex-biased gene expression of the studied genes.

Because DNA methylation of cytosine residues can interfere with the binding of transcription factors preventing transcription we examined whether the DNA sequences in the CpG islands studied, and proximal to regions encompassed by selected primers, contained putative transcription factor-binding sites. *In silico* prediction, further narrowed down and validated by published data, showed that the analysed CpG sites in the *Prlr* gene contain C/EBP binding sites, and that HNF4 binding sites are found in this CpG island within exon1. C/EBP confers progesterone responsiveness leading to increased *Prlr* transcription in the mammary gland (Goldhar *et al.* 2011), and HNF4 binds to the PRLR promoter as validated by Chip analysis (Bolotin *et al.* 2011).

A large CpG island was found in the proximal promoter and exon 1 of the *Hnf6* gene. Analysed CpG sites of *Hnf6* encompassed by tested primers revealed binding sites for C/EBP, SP1 and HNF4. Furthermore, binding sites for HNF6 and STAT5B were predicted in the proximal promoter, and the beginning of exon1, within the CpG island. The SP1 proteins are ubiquitously expressed, and interact with general transcription factors, while C/EBP regulates the expression of some liver genes, in particular HNF6-dependent transcription (Yoshida *et al.* 2006). Furthermore, it has been demonstrated that GH stimulates transcription of the *Hnf6* gene by a mechanism involving binding of STAT5 and HNF4 to its promoter, and that HNF6 is not only a transcription factor but a target to the regulatory network of liver transcription factors many of which are regulated by GH (Lahuna *et al.* 2000).

Finally many binding sites for transcription factors (HNF4, albumin D-binding protein (DBP) and STAT5B) have been described in the proximal promoter of the *Cyp2a4* gene (Mueller *et al.* 1990, Miura & Tanaka 1993, Zhang *et al.* 2012). Within the CpG island located in exon 1 a STAT5B-binding sites were described (Zhang *et al.* 2012). It was shown that STAT5B nuclear localization

correlates with the repression of *Cyp2a4* in males (Sueyoshi *et al.* 1999) and therefore could contribute to the sex specificity of this gene. Similarly HNF4 regulates *Cyp2a4* in male livers (Wiwi *et al.* 2004). On the other hand, DBP is highly concentrated in liver compared to other tissues, and during chemically induced liver regeneration, its expression is rapidly downregulated, suggesting that DBP may be involved in the proliferation control of hepatocytes. Furthermore DBP can regulate *Cyp2a4* expression levels in the liver (Lavery *et al.* 1999).

Methylation of transcription binding sites generally prevents the transcriptional machinery from accessing start sites on a gene, therefore it is conceivable that higher methylation in males of HNF4 and STAT5B-binding sites in the *Cyp2a4* promoter, or HNF4 in the *Prlr* promoter may maintain a low transcription rate, while the decrease in methylation in neuroDrd2KO males may favor transcription of these two genes.

Mmu-miR-155-5p, a miRNA predicted to target *Cyp7b1* expression, was lower in males and could account for higher *Cyp7b1* mRNA levels in this sex. It was not modified by neuron deletion of D2Rs or neonatal androgenization of females. On the other hand, even when mmu-miR-142a-3p was predicted to target the *Prlr* gene, it did not correlate inversely with its expression, indicating that it is not involved in establishing the sexual differences for this gene in the liver, and that bioinformatic tools are not sufficient to determine miRNA regulation of genes. The analysis of two miRNA is indicative of gene control through this mechanism, and other sex-biased GH-dependent miRNAs in the liver may also modify hepatic sexual dimorphism (Hao & Waxman 2018). For example, the male-biased miR-1948 and the female-biased miR-802 are both regulated by sex-specific pituitary GH secretory patterns, acquire sex specificity at puberty, and are dependent on the GH-activated transcription factor STAT5 for their sex-specific expression (Hao & Waxman 2018).

Understanding the specificity of epigenetic regulation of gene expression is an increasing concern. Our results show that liver *Prlr* expression may be regulated by methylation of its promoter. This receptor stimulates normal liver growth and participates in liver regeneration (Moreno-Carranza *et al.* 2013). Downregulation of liver *Prlr* may be involved in hepatic steatosis and NAFLD occurrence in experimental models and obese patients (Shao *et al.* 2018, Zhang *et al.* 2018), and the PRLR may protect from HCC by promoting innate immune signaling, and preventing the activation of the HCC-associated gene c-Myc (Hartwell *et al.* 2014). Targeting DNA methylation for cancer therapy has long been proposed (Issa 2007) and

inhibitors of DNA methyltransferases have been approved for clinical use for what is termed epigenetic therapy. It is therefore paramount to elucidate genes whose expression may specifically be modified by hypomethylation therapy.

Furthermore, we show that, in our experimental models mmu-miR-155-5p which targets *Cyp7b1* expression may participate in the GH-dependent sexual differences observed for this gene, an important modifier of oxysterol metabolism, associated to pediatric liver failure (Stiles *et al.* 2009). To this regard, miRNA-based therapies in cancer (Garzon *et al.* 2010), neurological pathologies (Ruberti *et al.* 2012), and pathological heart hypertrophy and cardiac failure (Lv *et al.* 2015) are being developed. In the liver, miRNAs or miRNA inhibitors have been used as liver cancer therapeutics. New technologies are being developed in order to optimize the delivery of miRNAs to the liver without having any toxicity or side effects in other major organs (Drakaki *et al.* 2013). In this context, the validation of miRNA action on different tissues and biological circuits is a growing area of research.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica, Argentina; PICT 1343-2015; PICT 526-2016, Fundación Rene Barón 2018, Fundación Williams 2018, Consejo Nacional de Investigaciones Científicas y Técnicas PIP 561-2014.

References

- Bello EP, Mateo Y, Gelman DM, Noain D, Shin JH, Low MJ, Alvarez VA, Lovering DM & Rubinstein M 2011 Cocaine supersensitivity and enhanced motivation for reward in mice lacking dopamine D2 autoreceptors. *Nature Neuroscience* **14** 1033–1038. (<https://doi.org/10.1038/nn.2862>)
- Bolotin E, Chellappa K, Hwang-Verslues W, Schnabl JM, Yang C & Sladek FM 2011 Nuclear receptor HNF4alpha binding sequences are widespread in Alu repeats. *BMC Genomics* **12** 560. (<https://doi.org/10.1186/1471-2164-12-560>)
- Brie B, Ramirez MC, De Winne C, Lopez Vicchi F, Villarruel L, Soriano E, Catalano P, Ornstein AM & Becu-Villalobos D 2019 Brain control of sexually dimorphic liver function and disease: the endocrine connection. *Cellular and Molecular Neurobiology* **39** 169–180. (<https://doi.org/10.1007/s10571-019-00652-0>)
- Buzzetti E, Parikh PM, Gerussi A & Tsochatzis E 2017 Gender differences in liver disease and the drug-dose gender gap. *Pharmacological Research* **120** 97–108. (<https://doi.org/10.1016/j.phrs.2017.03.014>)
- Cheung OK & Cheng AS 2016 Gender differences in adipocyte metabolism and liver cancer progression. *Frontiers in Genetics* **7** 168. (<https://doi.org/10.3389/fgene.2016.00168>)
- Clodfelter KH, Miles GD, Wauthier V, Holloway MG, Zhang X, Hodor P, Ray WJ & Waxman DJ 2007 Role of STAT5a in regulation of sex-specific gene expression in female but not male mouse liver revealed

- by microarray analysis. *Physiological Genomics* **31** 63–74. (<https://doi.org/10.1152/physiolgenomics.00055.2007>)
- Connerney J, Lau-Corona D, Rampersaud A & Waxman DJ 2017 Activation of male liver chromatin accessibility and STAT5-dependent gene transcription by plasma growth hormone pulses. *Endocrinology* **158** 1386–1405. (<https://doi.org/10.1210/en.2017-00060>)
- Drakaki A, Hatziaepostolou M & Iliopoulos D 2013 Therapeutically targeting microRNAs in liver cancer. *Current Pharmaceutical Design* **19** 1180–1191. (<https://doi.org/10.2174/138161213804805658>)
- Dreval K, Tryndyak V, de Conti A, Beland FA & Pogribny IP 2019 Gene expression and DNA methylation alterations during non-alcoholic steatohepatitis-associated liver carcinogenesis. *Frontiers in Genetics* **10** 486. (<https://doi.org/10.3389/fgene.2019.00486>)
- Durazzo M, Belci P, Collo A, Prandi V, Pistone E, Martorana M, Gambino R & Bo S 2014 Gender specific medicine in liver diseases: a point of view. *World Journal of Gastroenterology* **20** 2127–2135. (<https://doi.org/10.3748/wjg.v20.i9.2127>)
- Ellefson WM, Lakner AM, Hamilton A, McKillop IH, Bonkovsky HL, Steuerwald NM, Huet YM & Schrum LW 2011 Neonatal androgenization exacerbates alcohol-induced liver injury in adult rats, an effect abrogated by estrogen. *PLoS ONE* **6** e29463. (<https://doi.org/10.1371/journal.pone.0029463>)
- Forsander OA & Sinclair JD 1992 Alcohol elimination and the regulation of alcohol consumption in AA and ANA rats. *Alcohol and Alcoholism* **27** 411–416. (<https://doi.org/10.1093/oxfordjournals.alcalc.a045266>)
- García-Calzón S, Perfiliev A, de Mello VD, Pihlajamäki J & Ling C 2018 Sex differences in the methylome and transcriptome of the human liver and circulating HDL-cholesterol levels. *Journal of Clinical Endocrinology and Metabolism* **103** 4395–4408. (<https://doi.org/10.1210/jc.2018-00423>)
- García-Carpizo V, Ruiz-Llorente L, Fraga M & Aranda A 2011 The growing role of gene methylation on endocrine function. *Journal of Molecular Endocrinology* **47** R75–R89. (<https://doi.org/10.1530/JME-11-0059>)
- Garzon R, Marcucci G & Croce CM 2010 Targeting microRNAs in cancer: rationale, strategies and challenges. *Nature Reviews: Drug Discovery* **9** 775–789. (<https://doi.org/10.1038/nrd3179>)
- Goffin V, Binart N, Touraine P & Kelly PA 2002 Prolactin: the new biology of an old hormone. *Annual Review of Physiology* **64** 47–67. (<https://doi.org/10.1146/annurev.physiol.64.081501.131049>)
- Goldhar AS, Duan R, Ginsburg E & Vonderhaar BK 2011 Progesterone induces expression of the prolactin receptor gene through cooperative action of Sp1 and C/EBP. *Molecular and Cellular Endocrinology* **335** 148–157. (<https://doi.org/10.1016/j.mce.2011.01.004>)
- Guy J & Peters MG 2013 Liver disease in women: the influence of gender on epidemiology, natural history, and patient outcomes. *Gastroenterology and Hepatology* **9** 633–639.
- Hao P & Waxman DJ 2018 Functional roles of sex-biased, growth hormone-regulated microRNAs miR-1948 and miR-802 in young adult mouse liver. *Endocrinology* **159** 1377–1392. (<https://doi.org/10.1210/en.2017-03109>)
- Hartwell HJ, Petrosky KY, Fox JG, Horseman ND & Rogers AB 2014 Prolactin prevents hepatocellular carcinoma by restricting innate immune activation of c-Myc in mice. *PNAS* **111** 11455–11460. (<https://doi.org/10.1073/pnas.1404267111>)
- Holloway MG, Laz EV & Waxman DJ 2006 Codependence of Growth Hormone-Responsive, Sexually Dimorphic Hepatic Gene Expression on Signal Transducer and Activator of Transcription 5b and Hepatic Nuclear Factor 4α. *Molecular Endocrinology* **20** 647–660. (<https://doi.org/10.1210/me.2005-0328>)
- Issa JP 2007 DNA methylation as a therapeutic target in cancer. *Clinical Cancer Research* **13** 1634–1637. (<https://doi.org/10.1158/1078-0432.CCR-06-2076>)
- Jansson JO & Frohman LA 1987 Differential effects of neonatal and adult androgen exposure on the growth hormone secretory pattern in male rats. *Endocrinology* **120** 1551–1557. (<https://doi.org/10.1210/endo-120-4-1551>)
- Lahuna O, Rastegar M, Maiter D, Thissen JP, Lemaigre FP & Rousseau GG 2000 Involvement of STAT5 (signal transducer and activator of transcription 5) and HNF-4 (hepatocyte nuclear factor 4) in the transcriptional control of the hnf6 gene by growth hormone. *Molecular Endocrinology* **14** 285–294. (<https://doi.org/10.1210/mend.14.2.0423>)
- Lau-Corona D, Suvorov A & Waxman DJ 2017 Feminization of male mouse liver by persistent growth hormone stimulation: activation of sex-biased transcriptional networks and dynamic changes in chromatin states. *Molecular and Cellular Biology* **37** 00301–00317. (<https://doi.org/10.1128/MCB.00301-17>)
- Lavery DJ, Lopez-Molina L, Margueron R, Fleury-Olela F, Conquet F, Schibler U & Bonfils C 1999 Circadian expression of the steroid 15 alpha-hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. *Molecular and Cellular Biology* **19** 6488–6499. (<https://doi.org/10.1128/mcb.19.10.6488>)
- Lenz KM, Nugent BM & McCarthy MM 2012 Sexual differentiation of the rodent brain: dogma and beyond. *Frontiers in Neuroscience* **6** 26. (<https://doi.org/10.3389/fnins.2012.00026>)
- Liao D, Porsch-Hallstrom I, Gustafsson JA & Blanck A 1993 Sex differences at the initiation stage of rat liver carcinogenesis—influence of growth hormone. *Carcinogenesis* **14** 2045–2049. (<https://doi.org/10.1093/carcin/14.10.2045>)
- Ling G, Sugathan A, Mazor T, Fraenkel E & Waxman DJ 2010 Unbiased, genome-wide in vivo mapping of transcriptional regulatory elements reveals sex differences in chromatin structure associated with sex-specific liver gene expression. *Molecular and Cellular Biology* **30** 5531–5544. (<https://doi.org/10.1128/MCB.00601-10>)
- Liu W, Xie Y, Ma J, Luo X, Nie P, Zuo Z, Lahrmann U, Zhao Q, Zheng Y, Zhao Y, *et al.* 2015 IBS: an illustrator for the presentation and visualization of biological sequences. *Bioinformatics* **31** 3359–3361. (<https://doi.org/10.1093/bioinformatics/btv362>)
- Livne I, Silverman AJ & Gibson MJ 1992 Reversal of reproductive deficiency in the hpg male mouse by neonatal androgenization. *Biology of Reproduction* **47** 561–567. (<https://doi.org/10.1095/biolreprod47.4.561>)
- Lopez-Vicchi F & Becu-Villalobos D 2017 Prolactin: the bright and the dark side. *Endocrinology* **158** 1556–1559. (<https://doi.org/10.1210/en.2017-00184>)
- Lu TP, Lee CY, Tsai MH, Chiu YC, Hsiao CK, Lai LC & Chuang EY 2012 miRSystem: an integrated system for characterizing enriched functions and pathways of microRNA targets. *PLoS ONE* **7** e42390. (<https://doi.org/10.1371/journal.pone.0042390>)
- Lv D, Liu J, Zhao C, Sun Q, Zhou Q, Xu J & Xiao J 2015 Targeting microRNAs in pathological hypertrophy and cardiac failure. *Mini Reviews in Medicinal Chemistry* **15** 475–478. (<https://doi.org/10.2174/1389557515666150324124751>)
- Marin V, Rosso N, Dal Ben M, Raseni A, Boschelle M, Degraffi C, Nemeckova I, Nachtigal P, Avellini C, Tiribelli C, *et al.* 2016 An animal model for the juvenile non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. *PLoS ONE* **11** e0158817. (<https://doi.org/10.1371/journal.pone.0158817>)
- Messeguer X, Escudero R, Farre D, Nunez O, Martinez J & Alba MM 2002 PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* **18** 333–334. (<https://doi.org/10.1093/bioinformatics/18.2.333>)
- Miura N & Tanaka K 1993 Analysis of the rat hepatocyte nuclear factor (HNF) 1 gene promoter: synergistic activation by HNF4 and HNF1 proteins. *Nucleic Acids Research* **21** 3731–3736. (<https://doi.org/10.1093/nar/21.16.3731>)
- Moreno-Carranza B, Goya-Arce M, Vega C, Adan N, Triebel J, Lopez-Barrera F, Quintanar-Stephano A, Binart N, Martínez de la Escalera G & Clapp C 2013 Prolactin promotes normal liver growth, survival, and regeneration in rodents: effects on hepatic IL-6, suppressor of cytokine signaling-3, and angiogenesis. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **305** R720–R726. (<https://doi.org/10.1152/ajpregu.00282.2013>)

- Mueller CR, Maire P & Schibler U 1990 DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. *Cell* **61** 279–291. ([https://doi.org/10.1016/0092-8674\(90\)90808-r](https://doi.org/10.1016/0092-8674(90)90808-r))
- Negri-Cesi P, Colciago A, Pravettoni A, Casati L, Conti L & Celotti F 2008 Sexual differentiation of the rodent hypothalamus: hormonal and environmental influences. *Journal of Steroid Biochemistry and Molecular Biology* **109** 294–299. (<https://doi.org/10.1016/j.jsmb.2008.03.003>)
- Noain D, Perez-Millan MI, Bello EP, Luque GM, Casas Cordero R, Gelman DM, Peper M, Tornadu IG, Low MJ, Becu-Villalobos D, *et al.* 2013 Central dopamine D2 receptors regulate growth-hormone-dependent body growth and pheromone signaling to conspecific males. *Journal of Neuroscience* **33** 5834–5842. (<https://doi.org/10.1523/JNEUROSCI.5673-12.2013>)
- Nugent BM & McCarthy MM 2011 Epigenetic underpinnings of developmental sex differences in the brain. *Neuroendocrinology* **93** 150–158. (<https://doi.org/10.1159/000325264>)
- Potter JJ & Mezey E 2001 Effect of STAT5b on rat liver alcohol dehydrogenase. *Archives of Biochemistry and Biophysics* **391** 41–48. (<https://doi.org/10.1006/abbi.2001.2393>)
- Poynard T, Bedossa P & Opolon P 1997 Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* **349** 825–832. ([https://doi.org/10.1016/s0140-6736\(96\)07642-8](https://doi.org/10.1016/s0140-6736(96)07642-8))
- Quintanilla ME, Tampier L, Sapag A, Gerdtsen Z & Israel Y 2007 Sex differences, alcohol dehydrogenase, acetaldehyde burst, and aversion to ethanol in the rat: a systems perspective. *American Journal of Physiology: Endocrinology and Metabolism* **293** E531–E537. (<https://doi.org/10.1152/ajpendo.00187.2007>)
- Ramirez MC, Luque GM, Ornstein AM & Becu-Villalobos D 2010 Differential neonatal testosterone imprinting of GH-dependent liver proteins and genes in female mice. *Journal of Endocrinology* **207** 301–308. (<https://doi.org/10.1677/JOE-10-0276>)
- Ramirez MC, Bourguignon NS, Bonaventura MM, Lux-Lantos V, Libertun C & Becu-Villalobos D 2012 Neonatal xenoestrogen exposure alters growth hormone-dependent liver proteins and genes in adult female rats. *Toxicology Letters* **213** 325–331. (<https://doi.org/10.1016/j.toxlet.2012.07.015>)
- Ramirez MC, Ornstein AM, Luque GM, Perez-Millan MI, Garcia-Tornadu I, Rubinstein M & Becu-Villalobos D 2015 Pituitary and brain dopamine D2 receptors regulate liver gene sexual dimorphism. *Endocrinology* **156** 1040–1051. (<https://doi.org/10.1210/en.2014-1714>)
- Reizel Y, Spiro A, Sabag O, Skversky Y, Hecht M, Keshet I, Berman BP & Cedar H 2015 Gender-specific postnatal demethylation and establishment of epigenetic memory. *Genes and Development* **29** 923–933. (<https://doi.org/10.1101/gad.259309.115>)
- Rogers AB, Theve EJ, Feng Y, Fry RC, Taghizadeh K, Clapp KM, Boussahmain C, Cormier KS & Fox JG 2007 Hepatocellular carcinoma associated with liver-gender disruption in male mice. *Cancer Research* **67** 11536–11546. (<https://doi.org/10.1158/0008-5472.CAN-07-1479>)
- Ruberti F, Barbato C & Cogoni C 2012 Targeting microRNAs in neurons: tools and perspectives. *Experimental Neurology* **235** 419–426. (<https://doi.org/10.1016/j.expneurol.2011.10.031>)
- Shao S, Yao Z, Lu J, Song Y, He Z, Yu C, Zhou X, Zhao L, Zhao J & Gao L 2018 Ablation of prolactin receptor increases hepatic triglyceride accumulation. *Biochemical and Biophysical Research Communications* **498** 693–699. (<https://doi.org/10.1016/j.bbrc.2018.03.048>)
- Simon FR, Fortune J, Iwahashi M & Sutherland E 2002 Sexual dimorphic expression of ADH in rat liver: importance of the hypothalamic-pituitary-liver axis. *American Journal of Physiology: Gastrointestinal and Liver Physiology* **283** G646–G655. (<https://doi.org/10.1152/ajpgi.00438.2001>)
- Stiles AR, McDonald JG, Bauman DR & Russell DW 2009 CYP7B1: one cytochrome P450, two human genetic diseases, and multiple physiological functions. *Journal of Biological Chemistry* **284** 28485–28489. (<https://doi.org/10.1074/jbc.R109.042168>)
- Sueyoshi T, Yokomori N, Korach KS & Negishi M 1999 Developmental action of estrogen receptor- α feminizes the growth hormone-Stat5b pathway and expression of Cyp2a4 and Cyp2d9 genes in mouse liver. *Molecular Pharmacology* **56** 473–477. (<https://doi.org/10.1124/mol.56.3.473>)
- Sugathan A & Waxman DJ 2013 Genome-wide analysis of chromatin states reveals distinct mechanisms of sex-dependent gene regulation in male and female mouse liver. *Molecular and Cellular Biology* **33** 3594–3610. (<https://doi.org/10.1128/MCB.00280-13>)
- Tracy TS, Venkataramanan R, Glover DD, Caritis SN & National Institute for Child Health and Human Development Network of Maternal-Fetal-Medicine Units 2005 Temporal changes in drug metabolism (CYP1A2, CYP2D6 and CYP3A Activity) during pregnancy. *American Journal of Obstetrics and Gynecology* **192** 633–639. (<https://doi.org/10.1016/j.ajog.2004.08.030>)
- Wang K & Holterman AX 2012 Pathophysiologic role of hepatocyte nuclear factor 6. *Cellular Signalling* **24** 9–16. (<https://doi.org/10.1016/j.cellsig.2011.08.009>)
- Wauthier V, Sugathan A, Meyer RD, Dombkowski AA & Waxman DJ 2010 Intrinsic sex differences in the early growth hormone responsiveness of sex-specific genes in mouse liver. *Molecular Endocrinology* **24** 667–678. (<https://doi.org/10.1210/me.2009-0454>)
- Waxman DJ & Holloway MG 2009 Sex differences in the expression of hepatic drug metabolizing enzymes. *Molecular Pharmacology* **76** 215–228. (<https://doi.org/10.1124/mol.109.056705>)
- Wiwi CA, Gupte M & Waxman DJ 2004 Sexually dimorphic P450 gene expression in liver-specific hepatocyte nuclear factor 4 α -deficient mice. *Molecular Endocrinology* **18** 1975–1987. (<https://doi.org/10.1210/me.2004-0129>)
- Wong JH, Dukes J, Levy RE, Sos B, Mason SE, Fong TS & Weiss EJ 2008 Sex differences in thrombosis in mice are mediated by sex-specific growth hormone secretion patterns. *Journal of Clinical Investigation* **118** 2969–2978. (<https://doi.org/10.1172/JCI34957>)
- Yokomori N, Kobayashi R, Moore R, Sueyoshi T & Negishi M 1995 A DNA methylation site in the male-specific P450 (Cyp 2d-9) promoter and binding of the heteromeric transcription factor GABP. *Molecular and Cellular Biology* **15** 5355–5362. (<https://doi.org/10.1128/mcb.15.10.5355>)
- Yoshida Y, Hughes DE, Rausa III FM, Kim IM, Tan Y, Darlington GJ & Costa RH 2006 C/EBP α and HNF6 protein complex formation stimulates HNF6-dependent transcription by CBP coactivator recruitment in HepG2 cells. *Hepatology* **43** 276–286. (<https://doi.org/10.1002/hep.21044>)
- Zhang Y, Klein K, Sugathan A, Nassery N, Dombkowski A, Zanger UM & Waxman DJ 2011 Transcriptional profiling of human liver identifies sex-biased genes associated with polygenic dyslipidemia and coronary artery disease. *PLoS ONE* **6** e23506. (<https://doi.org/10.1371/journal.pone.0023506>)
- Zhang Y, Laz EV & Waxman DJ 2012 Dynamic, sex-differential STAT5 and BCL6 binding to sex-biased, growth hormone-regulated genes in adult mouse liver. *Molecular and Cellular Biology* **32** 880–896. (<https://doi.org/10.1128/MCB.06312-11>)
- Zhang P, Ge Z, Wang H, Feng W, Sun X, Chu X, Jiang C, Wang Y, Zhu D & Bi Y 2018 Prolactin improves hepatic steatosis via CD36 pathway. *Journal of Hepatology* **68** 1247–1255. (<https://doi.org/10.1016/j.jhep.2018.01.035>)

Received in final form 2 January 2020

Accepted 21 January 2020

Accepted Manuscript published online 21 January 2020