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 *Hnf-6* 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.

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 xenostrogens 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 miRNAs 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 IGF1 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 IGF1 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, Drd2loxP/loxP mice (Bello et al. 2011) were crossed with B6.Cg-Tg(Nes-cre)1Kln/j to obtain cohorts of Drd2loxP/loxP (control mice) and Drd2loxP/loxP.B6.Cg-Tg(Nes-cre)1Kln/j littermates (Noain et al. 2013). Thereafter, breeding pairs of Drd2loxP/loxP and Drd2loxP/loxP.Tg(Nes-Cre)1Kln/j mice were used to generate Drd2loxP/loxP (control) and Drd2loxP/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 Drd2loxP/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 Drd2loxP/loxP sex-matched mice. NeuroDrd2KO males also had decreased Mup1 mRNA levels compared to Drd2loxP/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

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**Table 1** GH axis in neuroDrd2KO mice.

|                  | Female                      | Male                        |
|------------------|-----------------------------|-----------------------------|
|                  | Drd2loxP/loxP | NeuroDrd2KO | Drd2loxP/loxP | NeuroDrd2KO |
| **BW (g)**       | 27.0 ± 0.7 | 22.0 ± 0.5 | 33.0 ± 0.8 | 26.0 ± 0.5 |
| **n**            | 12           | 8           | 9            | 7           |
| **Pituitary GH (ng/ug prot)** | 263 ± 87 | 108 ± 38 | 398 ± 84 | 136 ± 0.6 |
| **n**            | 7           | 6           | 13          | 16          |
| **Liver Igf1**   | 116 ± 11 | 45 ± 4   | 100 ± 11 | 37 ± 7 |
| **n**            | 8           | 9           | 10          | 6           |
| **Liver Igfbp3** | 147 ± 29 | 165 ± 25 | 100 ± 12 | 134 ± 28 |
| **n**            | 8           | 9           | 10          | 6           |
| **Liver Mup1**   | 18 ± 4     | 12 ± 5     | 100 ± 18 | 30 ± 4 |
| **n**            | 8           | 9           | 7           | 8           |
| **Serum IGF1 (ng/mL)** | 792 ± 83 | 393 ± 58 | 648 ± 45 | 463 ± 24 |
| **n**            | 8           | 9           | 10          | 8           |
| **Urine MUP (AU)** | 48.2 ± 8.9 | 15.4 ± 3.5 | 100.0 ± 5.8 | 54.2 ± 3.5 |
| **n**            | 12          | 5           | 18          | 8           |
| **Prolactin (ng/mL)** | 235 ± 60 | 267 ± 57 | 182 ± 32 | 182 ± 51 |
| **n**            | 8           | 9           | 10          | 8           |

Data are presented as means ± s.e.m. Genotype: indicates significant differences between genotypes (P ≤ 0.05) in both sexes. Sex: indicates significant differences between males and females (P ≤ 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 10X Buffer (1×), 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 ½ 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 H₂O.

**Treatment with methylation sensitive restriction enzymes**

The Mix was prepared at room temperature at a final volume of 50 µL (1× 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, spun 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 |
|------------------|-------------------------|-------------------------|--------|
| **Primer sequence** | **Sense** | **Antisense** | **Source** | **Enzyme** |
| **Primers used in mRNA studies** | | | | |
| Adh1             | GCTCTGCGGTCAAGTCGCA     | ACCGACACCTCCGAGGCAA     | Designed by PrimerBlast | |
| Hnf6             | AAGCCCTGGAGCAAATCTAA    | CCAATCTCTCCGGAAGTCTC   | Designed by PrimerBlast | |
| Prlr             | CACAGTAATGCCCCAGACAAG   | GGCAACCATTTTACCCACAG   | Designed by PrimerBlast | |
| Cyp7b1           | TGAGGCTGAGCCTGCTGCT     | TCCGCACTTCTCGGATGATG   | Holloway et al. 2006  | |
| Cyp2a4           | AGCGAGCTACCTCGACTGG     | GCTGCTGAGGCTATGCA      | Wiwi et al. 2004     | |
| Cyclophilin      | CAGAACATATCCCTGCAT      | GCCAGCTCTGGGATGACCTT   | Designed by PrimerBlast | |
| **Primers used in methylation studies** | | | | |
| Adh1             | AATGTGCTAGGCCTATGGG     | ATCAGGACTCAAGGGCTCTG   | Designed by PrimerBlast | Hpall |
| Hnf6             | TGGGCGCTGCTGGCTTTTG     | AGATGCACCTGGAGCC       | Designed by PrimerBlast | Hpall |
| Prlr             | CTTGATGGAAGAGGTCCCTCT   | GAAAGCCAGCTGAGAACG     | Designed by PrimerBlast | HpaI |
| Cyp7b1           | GCCAGACCCCTAGTGCC       | CAGGCGCTACCTGGTGC      | Designed by PrimerBlast | HpaI |
| Cyp2a4           | CCAAAAGCTAAGGCCTTC     | GCCGATATCAGGGCTGTCTT   | Designed by PrimerBlast | HpaI |
| **Primers used in miRNA studies** | | | | |
| mmu-miR-155-5p   | miRNA Rv UNIVERSAL     | TGGTGCAGGGTCAGGTATT    | Designed by PrimerBlast | |
|                  | RT-miR-155-5p STEM      | GCTCCTCTGGTGACGAGGTCAGGTATTTCGCACCAGGGAGACCCCTT | Designed by PrimerBlast | |
| mmu-miR-142a-3p  | miR-155-5p Fw          | GGGCCGGGTAAATGCTAATTTG | Designed by PrimerBlast | |
|                  | RT-miR-142a-3p STEM     | GCTCCTCTGGTGACGAGGTCAGGTATTTCGCACCAGGGAGACCCCTA | Designed by PrimerBlast | |
|                  | miR-142a-3p Fw         | GCCGCGTATGTTTCTCTACTT  | Designed by PrimerBlast | |

*Primers detect all Prlr isoforms.*
Specific methylation and miRNA liver gene control

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 (Messeguer 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±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. Kruskall–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≤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 Drd2loxP/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 Drd2loxP/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 Drd2loxP/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 Drd2loxP/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 ≤ 0.05 vs females (genotype matched); ‘a’ P ≤ 0.05 vs Drd2loxP/loxP sex-matched mice. A full colour version of this figure is available at https://doi.org/10.1530/JME-19-0205.

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

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Neonatal androgenization of females decreased the expression levels of this gene compared to control females (Fig. 1D), but methylation of the Hif6 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).

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).
Specific methylation and miRNA liver gene control

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 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).
In silico prediction of transcription binding sites in the CpG islands of proximal promoters analysed for Prlr, Cyp2a4 and Hnf6

For this analysis we chose the three genes which showed an association of methylation of CpGs in gene expression. For the Hnf6 gene a large CpG island was found in the proximal promoter and exon 1. In silico prediction, using a bioinformatic tool together with the validation of Chip-seq analysis from the literature, showed that STAT5B, HNF4 and HNF6-binding sites were found in the CpG island studied. The analysed CpG sites sensitive to enzyme HpaII encompassed by tested primers were located proximal to binding sites for CCAAT/enhancer binding protein (C/EBP) C/EBP, SP1 and HNF4 transcription factors (Fig. 6A).

For the Prlr gene there was a CpG island located in exon 1. In silico prediction, together with validated published data, showed that the analysed CpG sites in the Prlr gene sensitive to the enzyme HpaII were located proximally to C/EBP-binding sites and that HNF4 and STAT5B-binding sites were found in this CpG island (Fig. 6B).

For the Cyp2a4 gene a CpG island was found within exon 1. In silico prediction and validated data in the literature showed that STAT5B binding sites were found in the CpG island studied, while in the proximal promoter HNF4, DBP and STAT5B-binding sites were present. The analysed CpG sites sensitive to enzyme Hhal encompassed by tested primers were located proximal to STAT5B-binding sites (Fig. 6C).

Mmu-miR-155-5p may account for sexual differences in hepatic Cyp7b1 expression, while mmu-miR-142a-3p is not involved in Prlr sexually dimorphic expression

We performed an in silico analysis using the bioinformatic tool miRSystem (Lu et al. 2012), a database which integrates seven well-known miRNA target gene prediction programs: DIANA, miRanda, miRBridge, PicTar, PITA, m22, and TargetScan. We selected miRNAs with hits in at least three of the previously mentioned data bases. Mmu-miR-155-5p and mmu-miR-142a-3p were detected as promising miRNAs to target the Cyp7b1 and Prlr genes, respectively.

The mmu-miR-155-5p which was selected as a possible modulator of Cyp7b1 expression revealed a sexually dimorphic pattern of expression (P=0.0019; Fig. 7A), with lower levels in male livers in accordance with high Cyp7b1 mRNA expression in males (Fig. 5A).

On the other hand the Prlr-directed mmu-miR-142a-3p was also sexually dimorphic (Fig. 7B; P=0.012), but it did not correlate inversely but positively with Prlr expression, indicating that it is probably not involved in suppressing Prlr mRNA expression in males, but may impact on other sexually dimorphic genes as well.

No significant differences were observed in mmu-miR-155-5p and mmu-miR-142-3p expression in neuroDrd2KO mice (Fig. 7C and D).

Finally, mmu-miR-155-5p was similar in females and TP females (Fig. 7E) in accordance with the lack of change of Cyp7b1 mRNA expression in these two groups (Fig. 5E), while there was a tendency to lower levels in males (as expected due to the male dominance of this gene, P=0.13).
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

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**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.

**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<sup>loxP/loxP</sup> mice, and (E and F) females, neonatally androgenized females (TP female) and males. *P ≤ 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 miRNAs 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), 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.

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