Role of multiple microRNAs in the sexually dimorphic expression of Cyp2b9 in mouse liver

Xiaofeng Xie, Lingling Miao, Jun Yao, Chenchen Feng, Chenggang Li, Man Gao, Mingxia Liu, Likun Gong, Yizheng Wang, Xinning Qi, Jin Ren

Center for Drug Safety Evaluation and Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (XF X, LL M, J Yao, CC F, CG L, M G, MX L, UK G, XM Q, JR);

Laboratory of Neural Signal Transduction, Institute of Neuroscience, Chinese Academy of Sciences (YZ W).
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Address correspondence to: Dr XinMing Qi or Jin Ren, Center for Drug Safety Evaluation and Research, Shanghai Institute of Materia Medica, 501 Haike Road, Shanghai, 201203, Tel &Fax: 862120231000#1303, Email: xmqi@cdser.simm.ac.cn, jren@cdser.simm.ac.cn.

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Abbreviations:
3′-UTR, 3′-untranslated region
CAR, Constitute androgen receptor
CYP, Cytochrome P450
FoxA2, Forkhead box A2
GH, Growth hormone
Hnf4α, Hepatocyte nuclear receptor 4α
KO, Knockout
qRT-PCR, Quantitative real-time reverse transcription-PCR
WT, Wild type
Abstract

Mouse cytochrome P450 2b9 (Cyp2b9) is a testosterone 16 alpha-hydroxylase enzyme showing female-specific expression in many inbred mouse strains, including C57BL/6J. Previous studies have recognized that some sex-dependently secreted endogenous modulating factors were involved in the sexually dimorphic expression of Cyp2b9 through transcriptional regulation. In this study, we found evidence that some microRNAs contributed to the sexually biased expression of Cyp2b9 via post-transcriptional regulation. Cyp2b9 was up-regulated in livers of hepatocyte-specific Dicer1 knockout mice at 3 weeks. The age-dependent down-regulation of Cyp2b9 in the livers of male mice was diminished when Dicer1 was specifically knocked out in hepatocytes. When these data were combined with bioinformatics analysis and microRNA profiles of male and female mice, we found that 18 microRNAs were associated with the sexually dimorphic expression of Cyp2b9, which showed higher expression levels in male C57BL/6J mice when compared to females. Luciferase assays revealed that approximate half of these microRNAs repressed luciferase activity in a reporter system containing the 3′-untranslated region (3′-UTR) of Cyp2b9, and also inhibited Cyp2b9 protein expression. MicroRNA seed region mutation or mutations in putative binding sites of the microRNAs in Cyp2b9 3′-UTR led to the loss of the suppression of luciferase activity. There was also a negative correlation between the levels of these microRNAs and Cyp2b9. Our results suggested that multiple microRNAs participated in the regulation of Cyp2b9 expression, and that the lower expression levels of these
microRNAs potentially contributed to the female-specific expression of Cyp2b9 in the livers of C57BL/6J mice.
Introduction

The cytochrome P450 superfamily (CYP) is a large and diverse group of enzymes that catalyze the metabolism of drugs and carcinogens. Many studies have demonstrated that gender plays an important role in the pharmacological and toxicological responses to drugs. In humans, the expression of CYP1A2, CYP2E1, CYP3A4, CYP2A6, and CYP2B6 shows gender bias, which are risk factors for clinically relevant adverse drug reactions (Anderson, 2008). In animal models such as mice, there are gender differences in the expression of Cyp2a5, Cyp2b9/10/13, Cyp2d9, Cyp3a41/44, etc. (Hrycay and Bandiera, 2009). Cyp2b9 is a testosterone 16 alpha-hydroxylase enzyme showing female-specific expression in many inbred mouse strains, including C57BL/6J (Noshiro et al., 1988). Previous studies have identified some mechanisms involved in the sexually dimorphic expression of Cyp2b9 at the transcriptional level. Sex-dependent secretion of endogenous modulating factors, especially growth hormone, glucocorticoid hormone and sex hormones, are involved in this regulatory pathway (Jarukamjorn et al., 1999; Jarukamjorn et al., 2001; Jarukamjorn et al., 2002).

MicroRNAs are a large family of endogenous non-coding RNAs that regulate gene expression primarily by binding to the 3’-untranslated region (3’-UTR) of target genes, resulting in suppressed translation or decreased mRNA stability. MicroRNAs may regulate about 60% of all genes in humans (Bartel, 2009). Dicer1 is an RNase III endonuclease that is essential for the biogenesis of microRNAs, and multiple Dicer1 knockout (KO) animal models show significant down-regulation of microRNAs and...
up-regulation of microRNA targeting genes in these models (Hand et al., 2009; Sekine et al., 2009).

Recently, it has been demonstrated that microRNAs regulate cell processes necessary for sexual differentiation (Morgan and Bale, 2012). MiR-23a contributes to sex differences in the response to cerebral ischemia by regulating the expression of X-linked inhibitor of apoptosis (XIAP) (Siegel et al., 2011). Sex-based differences in miR-1 may underlie the sex-based differences in Cx43 expression in cardiomyocytes in pathologic conditions (Stauffer et al., 2011).

In this study, we used a hepatocyte-specific Dicer1 KO mouse model, bioinformatics analysis and in vitro studies to investigate the role of microRNAs in the regulation of sexually dimorphic Cyp2b9.

Materials and methods

Animals. All animal treatments were approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Materia Medica (Shanghai, China). The hepatocyte-specific Dicer1 KO C57BL/6 mouse strain was a kind gift from Professor Yizheng Wang (Institute of Neuroscience, CAS, China). Three- and six-week-old male hepatocyte-specific Dicer1 KO C57BL/6 mice were used in this study (Hand et al., 2009; Sekine et al., 2009).

Quantitative real-time reverse transcription-PCR (qRT-PCR). Total RNA was isolated from the liver by UNIQ-10/Trizol total RNA extraction kit (Sangon, Shanghai, China) and reverse-transcribed to cDNA with Primescript RT Reagent Kit (Takara,
Otus, Shiga, Japan). The primers for Cyp2b9 mRNA were 5’-CCTCCACTATGGAGTCCTGC-3’ (forward) and 5’-ACTTGGACTGTTGGAGGAAGA-3’ (reverse), and qRT-PCR analysis was performed using SYBR® Premix Ex Taq™ (Takara, Otus, Shiga, Japan). Cyp2b9 mRNA levels were normalized to mouse Gapdh mRNA detected by primers 5’-GGCTACACTGAGGACCAGGATT-3’ (forward) and 5’-TGCTGACTACGAGGAAGA-3’ (reverse).

MicroRNAs were isolated with the mirVana™ miRNA Isolation Kit (Ambion, Austin, USA) according to the manufacturer’s instructions. Reverse transcription and detection of microRNAs were carried out using NCode™ VILO™ miRNA cDNA Synthesis Kit and EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kit respectively (Invitrogen, Carlsbad, CA). MicroRNA-specific forward primers were designed according to the manufacturer’s instructions. The microRNA expression levels detected were normalized to mRnu6 levels.

**Western Blot.** Liver tissues were lysed in RIPA lysis buffer (Sangon, Shanghai, China) with phenylmethanesulfonyl fluoride. Cell samples were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 100 mM DTT. Proteins (20μg) were separated on 8% SDS-polyacrylamide gels (SDS-PAGE). Anti-mouse Cyp2b9 polyclonal antibody was generated by immunizing rabbits with a peptide of amino acids 462-473 from Cyp2b9 by Abmart (Shanghai, China). Verification for the specificity of this antibody was performed (Supplementary Figure 1). Anti-Gapdh (Sigma-Aldrich, St. Louis, MO) (1:5000) served as a loading control.
Construction of luciferase reporter plasmids. The 3’-untranslated region (3’-UTR, 376bp) of mouse Cyp2b9 (NM_010000, GeneBank) was amplified by PCR using cDNA from the liver of a female C57BL/6J mouse. The primers are 5’-TAGGCGATCGCTCGAGTTGGGGTGAGGGAGCCAGGTGTC-3’ (forward) and 5’-TTGCGGCCAGCGGCCGCCACACACTATGTGATTCTGT-3’ (reverse). This PCR fragment was cloned into the psiCHECK-2 vector (Promega, Madison, WI, USA) with the In-fusion™ Advantage PCR Cloning Kit (Clontech, Mountain View, CA).

Site-directed mutation of luciferase reporter plasmids. The mutated plasmids were cloned using the KOD-Plus-Mutagenesis Kit (TOYOBO, Japan) with standard primers (Supplementary Table 1). DNA sequencing confirmed the nucleotide sequence of these plasmids.

Luciferase assays. For luciferase reporter assays, various luciferase reporter plasmids were co-transfected with 100nM microRNA mimics and their seed region mutants (GenePharma, Shanghai, China) into Huh7 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). All the sequence of microRNA mimics was summarized in Supplementary Table 2. Luciferase activity was analyzed after 72 hours by the Dual-Luciferase Reporter Assay System according to the manufacturer’s protocol (Promega, Madison, USA).

Establishment of Huh7 cell line stably expressing. Full-length of the mouse Cyp2b9 (NM_010000) cDNA (1877bp, including 3’-UTR) was amplified by PCR using the primers of 5’-GGACTCAGATCTCGAGAAGCAGACTCTTGATTAG...
ACC-3’ (forward) and 5’-GTCGACTGCAGAATTCCACACACTATGTGATTCTGT-3’ (reverse). The fragment was cloned into pLVX-AcGFP-N1 lentiviral expression vector with In-fusion™ Advantage PCR Cloning Kit (Clontech). The nucleotide sequence of the plasmids was confirmed by DNA sequencing. The plasmid was transfected into the HEK 293T packaging cell line using the Lenti-X HT Packaging System (Clontech), and 48 h after transfection, viral supernatant was collected. Huh7 cells were transfected with pLVX-AcGFP-N1-Cypb9 virus-containing media and were selected in 4 μg/ml puromycin (Sigma) for 7 days, and the pooled cell population was used for subsequent experiments. The expression of Cyp2b9 by was confirmed by western blot (Data not shown).

**Statistical analyses.** Statistical significance was determined by unpaired, two-tailed student’s t-test. Correlation coefficients (R) were determined by Pearson’s correlation test.

**Results**

**Effects of Dicer1 disruption on the expression of Cyp2b9 in the livers of male and female mice.** We obtained the expression profile of microRNAs and CYPs in hepatocyte-specific Dicer1 KO male mice (male-KO mice). Dicer1, miR-122 and miR-192 were significantly down-regulated in male-KO mice (supplementary Figure. 2) indicating efficient KO of Dicer1. It has been previously shown that the expression of Cyp2b9 decreased in male mice during development after birth (Hashita et al., 2008). In our study, mRNA (Fig. 1A, B) and protein (Fig. 1C, D) levels of Cyp2b9 at
3 weeks were higher in the livers of male-KO mice compared to wild-type (WT) male mice. The expression of Cyp2b9 in WT male mice at 6 weeks was extremely low, while that of KO male mice was still high (Fig. 1A-D). However, these phenomena were not observed in female mice at 3 or 6 weeks (supplementary Figure. 3).

**Screening of microRNAs that may be involved in the sexually dimorphic expression of Cyp2b9.** Using bioinformatics tools (Targetscan, Microcosm and mirWalk) (Lewis et al., 2005; Griffiths-Jones et al., 2008; Dweep et al., 2011), we predicted microRNAs that potentially regulate the expression of Cyp2b9 and found that over 60 microRNAs might bind to the 3’-UTR of Cyp2b9. We combined this result with the microRNA profiles from male and female mice at 6 weeks and excluded the microRNAs with potential binding sites too close to the open reading frame of Cyp2b9, which were reported less likely to have regulatory function (Lewis et al., 2005), and finally found 22 microRNAs expressed in liver, thus we focused on studying the roles of these 22 microRNAs in the regulation of Cyp2b9 (Table 1). qRT-PCR results further revealed that 18 of 22 microRNAs had higher expression levels in male mice (Fig. 2). The screening strategy was summarized in supplementary Figure 4.

**Multiple microRNAs negatively regulated the expression of Cyp2b9.** We constructed a Cyp2b9 3’-UTR luciferase reporter plasmid to study the post-transcriptional regulation of microRNAs on Cyp2b9 expression. Luciferase reporter assays showed that 8 of 22 microRNAs decreased luciferase activity at the final concentration of 100nM (Fig. 3A, B). These microRNAs also down-regulated
the protein level of Cyp2b9 (Fig. 3C). When mutations were introduced into the putative binding sites of microRNAs (mmu-miR-139-3p, 1b-5p, 21*, 291a-5p, -297a*, 297b-3p, 467g, -667) in Cyp2b9 3’-UTR (Fig. 3A), the inhibition of luciferase activity was partially or totally abolished (Fig. 3A, B). MicroRNAs with mutations in their seed regions also lost the inhibition of the luciferase activity (Fig. 3D). These results suggested that 8 microRNAs (mmu-miR-139-3p, -1b-5p, -21*, -291a-5p, -297a*, -297b-3p, -467g, and -667) inhibited Cyp2b9 expression post-transcriptionally through binding to the 3’-UTR region.

**Correlation analysis of the expression of microRNAs and Cyp2b9 in male and female mice.** All 8 microRNAs were found to regulate Cyp2b9 had higher expression levels in male C57BL/6J mice compared to female mice (Table 1). We performed Pearson’s correlation tests to identify potential correlations between the protein levels of Cyp2b9 protein and the levels of microRNAs in male and female WT mice at 6 weeks. Expression of mmu-miR-139-3p, 21*, 297b-3p, 467g and 667 showed weak negative correlation with Cyp2b9 expression (Fig. 4A, C, F-H), while the expression of mmu-miR-1b-5p, 291a-5p and 297a* demonstrated strong negative correlation with Cyp2b9 expression (Fig. 4B, D and E). Finally, the expression of mmu-miR-490-5p, which showed no regulation of Cyp2b9 by luciferase activity assay, did not correlate with Cyp2b9 expression (Fig. 4I). The correlation coefficients between the levels of 22 microRNAs and Cyp2b9 expression were listed in Table 2.
Discussion

It has been previously reported that the expression of Cyp2b9 developmentally decreased after birth in male mice (Hashita et al., 2008). Previous studies have shown that the growth hormone (GH)-Stat5/CAR (constitute androgen receptor)/forkhead box A2 (FoxA2)/hepatocyte nuclear receptor α (Hnf4α) pathways influence the expression of female-specific genes, including Cyp2b9 (Lahuna et al., 2000; Wiwi et al., 2004; Mota et al., 2010; Baik et al., 2011). In our study, the expression levels of Stat5, FoxA2 and Hnf4α in 6-week-old male KO mice were similar to levels in WT mice, while CAR, a positive regulator of Cyp2b9 in male mice (Mota et al., 2010), was down-regulated (data not shown). These results suggest that other factors are involved in the up-regulation of Cyp2b9 in male KO mice.

We investigated the possibility that microRNAs may be involved in the regulation of Cyp2b9 and identified multiple microRNAs involved in the sexually dimorphic expression of Cyp2b9. Several lines of evidence supported our conclusion. First, Cyp2b9 up-regulation in the liver of hepatocyte-specific Dicer1 KO male mice indicated the potential role of microRNAs in gender differences of Cyp2b9 expression; this was further supported by the differential expression of microRNAs with binding sites in the 3’-UTR of Cyp2b9 between male and female mice. Second, Multiple microRNA exerted inhibitory effects on Cyp2b9 protein expression and luciferase activity of reporters containing Cyp2b9 3’-UTR, however, when mutations were introduced into Cyp2b9 3’-UTR or seed region of the microRNAs, the inhibition was reversed, which suggested multiple microRNAs regulated Cyp2b9 at
post-transcriptional levels via their seed region complementary to Cyp2b9 3’-UTR. Finally, correlation analyses showed negative correlation between the expression of microRNAs and Cyp2b9. Altogether, these data strongly suggested that microRNAs post-transcriptionally regulate the expression of Cyp2b9 and participate in the gender differences of Cyp2b9 expression.

Our work suggested that some male-predominant microRNAs may participate in the negative regulation of Cyp2b9 in male mice. A previous study showed that CAR knockout caused up-regulation of Cyp2b9 expression in female mice, suggesting a negative correlation between CAR and Cyp2b9 in female mice (Mota et al., 2010). In contrast, Cyp2b9 was down-regulated in 6-week-old Dicer1 KO female mice (Supplementary Fig. 3), which also showed a great increase of CAR (data not shown). Here, we suggested that the down-regulation of Cyp2b9 in female mice may be due to the combination of up-regulation of CAR and the low levels of predicted microRNAs (Fig. 2 & 4) in female mice. Future studies are needed to clarify the mechanisms involved in the down-regulation of Cyp2b9 in Dicer1 KO female mice.

Human CYP2B6 shows gender bias and in mouse, Cyp2b9 and Cyp2b10 also have gender difference. In our study, knockdown of DICER1 increased the mRNA levels of CYP2B6 (Supplementary Figure 5A, B). Dicer1 knockout also increased the protein levels of Cyp2b10 in the liver of male-KO mice (Supplementary Figure 5C). The luciferase activity of the reporter containing the 3’UTR of Cyp2b10 or CYP2B6 was also repressed by some microRNAs (Supplementary Figure 5D-E). Altogether, these results suggested that microRNAs may also play a role in the sexual dimorphic
expression of CYP2B6.

Gender and individual differences in the expression of CYPs are universal phenomena that are driven by multiple mechanisms including genetic variation, transcriptional factors and post-transcriptional regulation (Hrycay and Bandiera, 2009). Sex differences in the expression of CYPs lead to sex differences in the pharmacokinetics and pharmacodynamics of many drugs; this is a primary cause of sex-related pharmacokinetics and side effects (Tanaka, 1999). Between 6 to 7% of new drug applications with sex analyses show at least a 40% differential in pharmacokinetics between men and women (Anderson, 2005). In this study, we demonstrated a microRNA-directed post-transcriptional regulatory mechanism that may participate in sex-biased expression of CYPs. Our results suggest a novel potential mechanism for sex-based differences in the pharmacokinetics and pharmacodynamics of drugs, and indicate that microRNAs may be important factors in sex-biased drug administration.
Acknowledgement

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Authorship contribution

**Participated in research design:** Jin Ren, Yizheng Wang, Xinming Qi,

**Conducted experiments:** Xiaofeng Xie, Lingling Miao, Jun Yao, Chenggang Li,
Chenchen Feng, Man Gao, Mingxia Liu,

**Performed data analysis:** Xiaofeng Xie, Lingling Miao, Xinming Qi, Likun Gong

**Wrote or contributed to the writing of the manuscript:** Xiaofeng Xie, Lingling Miao,
Xinming Qi.
References


Hrycay EG and Bandiera SM (2009) Expression, function and regulation of mouse


Mota LC, Hernandez JP, and Baldwin WS (2010) Constitutive androgen receptor-null mice are sensitive to the toxic effects of parathion: association with reduced


Footnotes

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Figure legends

**Fig. 1.** Up-regulation of Cyp2b9 in the livers of hepatocyte-specific Dicer1 KO male mice. mRNA levels of Cyp2b9 in the livers of three- and six-week-old male (3w-Male and 6w-Male) WT and KO mice were detected by qRT-PCR (A) and RT-PCR (B). (C-D) Protein levels of Cyp2b9 were detected by western blot and quantified with ImageQuant Software. mRNA and protein levels of Cyp2b9 were normalized to those of Gapdh. *, P<0.05 vs. WT group.

**Fig. 2.** Sex-biased expression of microRNAs potentially targeting the 3’-UTR of Cyp2b9 in the livers of mice. Expression of 22 microRNAs was detected by qRT-PCR. Each empty cycle was marked with microRNA name from miRbase database. The difference in expression of various microRNAs between female and male mice was defined as $\Delta\Delta C_t = \Delta C_{t_{\text{female}}} - \Delta C_{t_{\text{male}}}$ (female vs. male). Significant differences in microRNA levels between female and male mice were defined by p-value. $\Delta\Delta C_t (< 0$ indicates expression of the microRNA is higher in males) and p-values ($< 0.05$) were indicated by the dotted lines.

**Fig. 3.** Post-transcriptional regulation of Cyp2b9 by multiple microRNAs. (A) Schematic of the Cyp2b9 3’UTR with putative microRNA binding sites. Underlined, the sequence of Cyp2b9 3’UTR; Red, seed region of microRNA; Short line, predicted binding site of microRNA at Cyp2b9 3’UTR; Blue and italic, mutant Cyp2b9 3’UTR. (B) 8 microRNAs individually reduced the luciferase activity of a luciferase reporter
containing the 3'-UTR of Cyp2b9. Their inhibitory effects were abolished by the mutation of microRNA binding site in Cyp2b9 3'UTR. The inhibitory effects of 22 microRNAs (Table 1) were screened in a luciferase reporter system containing the Cyp2b9 3'-UTR (wild type, WT). 6 luciferase reporters containing mutant Cyp2b9 3'-UTR (mt1-mt6) were used to examine the effects of 8 microRNAs. (C) 8 microRNAs down-regulated the protein level of Cyp2b9. Cyp2b9 was stably transfected into Huh7 cells. Protein levels of Cyp2b9 were detected by western blot and quantified with ImageQuant Software. Protein levels of Cyp2b9 were normalized to those of Gapdh. (D) Mutations in the seed regions of microRNAs reversed their inhibitory effects on luciferase activity. wt, microRNA mimics with wild type seed regions; ms, microRNA mimics with mutant seed regions. *, p<0.05; **, p<0.01 vs. negative control (NC) group; #, p<0.05; ##, p<0.01.

Fig. 4. Inverse correlation between the expression of Cyp2b9 and multiple microRNAs. Expression levels of miR-139-3p (A), 1b-5p (B), 21* (C), 291a-5p (D), 297a* (E), 297b-3p (F), 467g (G), and miR-667 (H) negatively correlated with Cyp2b9 expression in the livers of male and female mice. miR-490-5p (I) expression showed no correlation with Cyp2b9 expression. All the correlation coefficient data were shown in Table 2.
## TABLE 1 Differential expression of 22 microRNAs with binding sites in the 3'-UTR of Cyp2b9

<table>
<thead>
<tr>
<th>microRNAs</th>
<th>ΔΔCt (ΔCt(<em>{\text{female}})-ΔCt(</em>{\text{male}}))</th>
<th>p-value (student’s t-test)</th>
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</tr>
<tr>
<td>miR-139-3p</td>
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<tr>
<td>miR-490-5p</td>
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Expression of microRNAs was detected by qRT-PCR. The difference in expression of various microRNAs between female and male mice was defined as $\Delta \Delta CT = \Delta CT_{\text{female}} - \Delta CT_{\text{male}}$ (female vs. male). Significant differences in microRNA levels between female and male mice were defined by p-value.

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<th>microRNA</th>
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<td></td>
<td>(Pearson’s test)</td>
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The correlation coefficients (R²) between the levels of 22 microRNAs and Cyp2b9 expression in mice (both male and female mice included). Significant differences in microRNA levels between female and male mice were defined by p-value.
Figure 1

(A) Bar graphs showing normalized Cyp2b9 mRNA level in WT and KO mice at 3W and 6W.

(B) Western blots showing Cyp2b9 and Gapdh expression in WT and KO mice at 3W and 6W.

(C) Bar graphs showing normalized Cyp2b9 protein level in WT and KO mice at 3W and 6W.

(D) Western blots showing Cyp2b9 and Gapdh expression in WT and KO mice at 3W and 6W.
Figure 2

The scatter plot illustrates the comparison between female and male samples, with each point representing a different sample. The y-axis represents the p-value, ranging from 0.1 to 0.5, while the x-axis shows the ΔΔCt values, ranging from -4 to 1.

The plot highlights that the p-value bands for the female and male samples are distinct, with the female samples generally having a lower p-value compared to the male samples, indicating a significant difference in ΔΔCt values between the two groups.

Key:
- Female vs Male
- ΔΔCt
- P Value
- 297a*
- 297b-3p
- 297b-5p
- 28b
- 466a-3p
- 466f-3p
- 466g
- 466k
- 490-5p
- 509-5p
- 582-5p
- 669-5p
- 669o-5p
- 1188
- 139-3p
- 19a*
- 1b-3p
- 21*
- 297a
- 297b
- 297a*
- 297b-3p
- 297b-5p
- 297a*
- 466a-3p
Figure 3

(A) Schematic representation of the miR-667 and miR-291a-5p binding sites on the 3' UTR of Cyp2b9. Gene names are followed by their respective seed sequences.

(B) Normalized luciferase activity (fold of NC) for different miRNAs. The graph shows a significant decrease in luciferase activity with the mutation at position 161-240 bp.

(C) Normalized Cyp2b9 protein level for different miRNAs. The graph shows a significant increase in protein level with the mutation at position 21.*

(D) Normalized luciferase activity (fold of NC) for different miRNAs. The graph shows a significant decrease in luciferase activity with the mutation at position 297a.*