Characterization of the Tissue Distribution of the Mouse *Cyp2c* Subfamily by Quantitative PCR Analysis^S

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ABSTRACT

The CYP2C subfamily of the cytochrome P450 gene superfamily encodes heme-thiolate proteins that have a myriad of biologic functions. CYP2C proteins detoxify xenobiotics and metabolize endogenous lipids such as arachidonic acid to bioactive eicosanoids. We report new methods and results for the quantitative polymerase reaction (qPCR) analysis for the 15 members of the mouse *Cyp2c* subfamily (*Cyp2c29*, *Cyp2c37*, *Cyp2c38*, *Cyp2c39*, *Cyp2c40*, *Cyp2c44*, *Cyp2c50*, *Cyp2c54*, *Cyp2c55*, *Cyp2c65*, *Cyp2c66*, *Cyp2c67*, *Cyp2c68*, *Cyp2c69*, and *Cyp2c70*). Commercially available TaqMan primer/probe assays were compared with developed SYBR Green primer sets for specificity toward the mouse *Cyp2c* cDNAs and analysis of their tissue distribution. TaqMan primer/probe assays for 10 of the mouse *Cyp2c* isoforms were shown to be specific for their intended mouse *Cyp2c*

cDNA; however, there were no TaqMan primer/probe assays specific for the mouse *Cyp2c29*, *Cyp2c40*, *Cyp2c67*, *Cyp2c68*, or *Cyp2c69* transcripts. Each of the SYBR Green primer sets was specific for its intended mouse *Cyp2c* cDNA. The two qPCR methods confirmed similar patterns of *Cyp2c* tissue expression: *Cyp2c37*, *Cyp2c38*, *Cyp2c39*, *Cyp2c44*, *Cyp2c50*, *Cyp2c54*, and *Cyp2c70* were most highly expressed in liver; *Cyp2c55* was highly expressed in large intestine; *Cyp2c65* was highly expressed in stomach, duodenum, and large intestine; and *Cyp2c66* was highly expressed in both duodenum and jejunum. For isoforms without specific TaqMan primer/probe assays, the SYBR Green primer sets detected high level expression of *Cyp2c29*, *Cyp2c40*, *Cyp2c67*, *Cyp2c68*, and *Cyp2c69* in the liver. Lower expression levels of the mouse *Cyp2cs* were also detected in other tissues.

Introduction

Members of the cytochromes P450 (P450s) gene superfamily have been intensely studied because of their ability to catalyze the metabolism of a broad array of xenobiotics and endogenous compounds (Nelson et al., 1996; Kroetz and Zeldin, 2002; Nebert and Russell, 2002). Arachidonic acid is a polyunsaturated fatty acid present in mammalian cell membranes that can undergo NADH-dependent oxidation by certain P450s to generate bioactive metabolites such as epoxyeicosatrienoic acids (EETs) (Capdevila et al., 2000; Kroetz and Zeldin, 2002; Nebert and Russell, 2002). EETs have a wide range of physiologic functions including anti-inflammatory (Node et al., 1999), cardioprotective (Seubert et al., 2004, 2006), fibrinolytic (Node et al., 2001), angiogenic (Wang et al., 2005), and vasodilatory (Campbell et al., 1996; Larsen et al., 2006) effects both in vitro and in vivo.

One of the largest subfamilies of the P450s is the CYP2C subfamily. Many species contain multiple CYP2C subfamily members; rats have nine *Cyp2c* genes, whereas humans have four CYP2C genes (Nelson et al., 1996). The mouse *Cyp2c* subfamily is made up of 19 subfamily members, 4 of which are pseudogenes with no open reading frame. Eighteen of the nineteen genes are located within a ~1.5 Mb cluster on

mouse chromosome 19. The 19th gene, *Cyp2c44*, is located ~3.8 Mb away from the *Cyp2c* cluster (Nelson et al., 1996; Wang et al., 2004). Previous nonquantitative reverse-transcription polymerase chain reaction (RT-PCR) studies determined that most of the mouse *Cyp2c* subfamily members are highly expressed in the liver, but some are also expressed in extrahepatic tissues. *Cyp2c29*, *Cyp2c37*, *Cyp2c38*, *Cyp2c39*, *Cyp2c40*, *Cyp2c50*, and *Cyp2c54* are all predominantly expressed in liver (Luo et al., 1998; Wang et al., 2004). *Cyp2c55* is highly expressed in colon (Wang et al., 2004), whereas the *Cyp2c44* epoxigenase has been shown to be expressed in liver and the renal collecting duct (DeLozier et al., 2004; Capdevila et al., 2014). The remaining mouse *Cyp2c* cDNAs (*Cyp2c65*, *Cyp2c66*, *Cyp2c67*, *Cyp2c68*, *Cyp2c69*, and *Cyp2c70*) were not previously cloned, and no analysis of their tissue distribution at the RNA level has been reported.

Although nonquantitative RT-PCR detects whether a particular transcript is present or not, it does not allow for the quantification of the transcript level. Quantitative polymerase chain reaction (qPCR) monitors the amplification of targeted sequences in real time, which allows accurate measurement of gene expression. For all qPCR reactions cDNA-specific primer pairs are used to amplify a target sequence from the transcript of interest. The SYBR Green qPCR method uses a double-stranded DNA-binding dye as the reporter (Ponchel et al., 2003). TaqMan or Quantifast qPCR methods rely on endonuclease activity cleaving a fluorophore from a transcript-specific probe containing a fluorescent quencher (Heid et al., 1996). Of the two methods, Taqman primer/probe assays have the advantage of increased selectivity due to their target-specific fluorescent probe. Unfortunately, researchers cannot

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ABBREVIATIONS: EET, epoxyeicosatrienoic acid; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction.

confirm whether the measured fluorescence of primer/probe assays is due to a single target amplicon as opposed to multiple off-target transcripts. This becomes especially important when quantifying members of a highly conserved subfamily of genes such as the mouse *Cyp2cs*, which have 53–98% cDNA sequence identity (Table 1). DNA-binding assays such as SYBR Green might also fail to distinguish between highly related transcripts; however, they allow for performance of an end-point melting curve assay that can determine whether the observed fluorescence is due to a single amplicon or multiple unintended amplicons. Although the SYBR Green melting curve assay will not differentiate multiple amplicons of the same melting temperature, the end-point assay does provide a level of confirmation that is not possible with primer/probe qPCR assays.

In this study, SYBR Green primer sets were developed for use in qPCR assays based on published and cloned cDNA sequences of members of the mouse *Cyp2c* subfamily. These primer sets were compared with the commercially available mouse *Cyp2c* TaqMan primer/probe assays. Ten TaqMan primer/probe assays were shown to be specific for their intended transcript. All 15 of the SYBR Green primer sets were found to be specific for their intended transcript. Similar tissues distribution profiles were obtained using both qPCR methods.

Materials and Methods

Reagents and Supplies. SYBR Green primer set oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). TaqMan primer/probes, MicroAmp Optical 384-Well Reaction Plates, an ABI Prism 7700 Sequence Detection System, and an ABI ViiA 7 Sequence Detection System were all purchased from Life Technologies (Carlsbad, CA).

RNA Isolation and cDNA Archives. Total RNA was prepared from three female and three male wild-type C57BL/6 mice for each tissue using RNeasy Midi or Mini Kits from Qiagen (Valencia, CA) following the manufacturer's protocols. For all the RNAs, 1 μ g of total RNA was treated with 1 unit of DNase I (Life Technologies) for 15 minutes and then inactivated by addition of 1 μ l 25 mM EDTA and heat treatment at 65°C for 10 minutes. RNase-free water was then added to a total volume of 50 μ l. The High Capacity cDNA Reverse Transcription Kit from Life Technologies was used according to the manufacturer's protocol to produce cDNA from the DNase I treated RNA. The final concentration of the cDNA archives was 10 ng/ μ l, which was diluted to a working concentration of 1 ng/ μ l with RNase-free water.

Cloning of cDNAs for *Cyp2c* Subfamily Members. The cDNAs for *Cyp2c65*, *Cyp2c67*, *Cyp2c68*, and *Cyp2c69* were identified by sequence analysis using the National Center for Biotechnology Information database's Basic Local Alignment Search Tool (BLAST) and the Lasergene DNASTar v12 package

(Madison, WI). Primer pairs were designed to amplify the coding regions of *Cyp2c67*, *Cyp2c68*, and *Cyp2c69* cDNAs (Table 2). Primers for the *Cyp2c65* cDNA included the 3' untranslated region (UTR) sequence. Briefly, 60 ng of stomach or liver cDNA archives (as described above) were amplified using Phusion HF DNA polymerase from New England BioLabs (Ipswich, MA) in a total volume of 25 μ l. The PCR conditions were as follows: 96°C for 3 minutes, 35 cycles of 96°C for 10 seconds, 58°C for 10 seconds, and 72°C for 1 minute; the final extension was at 72°C for 2 minutes. The *Cyp2c68* post-PCR reaction mix was digested with the restriction endonuclease enzymes NdeI and XmnI to eliminate any *Cyp2c40*, *Cyp2c67*, and *Cyp2c69* amplicons. The PCR products were then gel isolated using a Qiagen kit. An A-overhang was added using New England BioLabs' Taq DNA polymerase, and the cDNA was cloned into the pRC2.1-TOPO vector from Life Technologies. Positive clones were identified using blue-white selection. Sequence fidelity was verified by DNA sequencing (GeneWiz, Inc., Research Triangle Park, NC).

TaqMan qPCR. Life Technologies' TaqMan primer/probe assays for the mouse *Cyp2jc* subfamily members are listed in Table 3. For all qPCR reactions, 0.5 μ l of the 20 $\times$ primer/probe assays and 5 μ l of Maxima Probe/ROX qPCR Master Mix (2 $\times$) from Thermo Scientific (Pittsburgh, PA) were used in a 10 μ l total reaction volume, along with 1 ng of the cDNA archive. The conditions used were 50°C, 2 minutes; 95°C, 10 minutes, followed by 40 repeat cycles of 95°C, 15 seconds; 60°C, 1 minute. Six individual samples for each tissue examined (three female and three male) were each run in triplicate (18 wells per tissue) on the same qPCR plate, except for sex-specific tissues where three individual tissue samples were each run in triplicate (nine wells per tissue) on the same plate.

SYBR Green qPCR. Specific primer sets of oligonucleotides were designed, spanning exon-exon boundaries when possible, for the protein coding members of the mouse *Cyp2c* subfamily (Table 4). Due to high sequence homology with other paralogs, the primer set for *Cyp2c65* was designed in the 3' UTR. Additional mouse *Cyp2c* primer sets that were used had previously been published for use in nonquantitative RT-PCR (Luo et al., 1998; Wang et al., 2004). All SYBR Green qPCR assays were run in 10 μ l volume with final primer concentration of 0.5 μ M for both the forward and reverse primers. Five microliters of Power SYBR Green PCR Master Mix (Applied Biosystems by Life Technologies) was used in the master mix for all the SYBR Green qPCR reactions, along with 1 ng of cDNA archive. The conditions used were 50°C, 2 minutes; 95°C, 10 minutes, followed by 40 repeat cycles of 95°C, 15 seconds; 60°C, 1 minute (two-step PCR with single-stage annealing and extension). A melt curve dissociation stage was added of 95°C, 15 seconds and then 60°C to 95°C at 0.05°C/s. For *Cyp2c29*, *Cyp2c67*, and *Cyp2c68*, an annealing/extension temperature of 68°C was used. For *Cyp2c39* and *Cyp2c40*, an annealing/extension temperature of 64°C was used. For *Cyp2c54*, an annealing/extension temperature of 62°C was used. For *Cyp2c37* and *Cyp2c69* an annealing/extension temperature of 66°C was used. Six individual samples for each tissue examined (three female and three male) were each run in triplicate (18 wells per tissue) on the same qPCR plate, except

TABLE 1

Nucleic acid sequence identity of the mouse *Cyp2c* subfamily members

Full-length mouse *Cyp2c* cDNAs were compared using the Lipman-Pearson method in the DNASTar package (Madison, WI). Percent sequence identities are shown.

	<i>Cyp2c29</i>	<i>Cyp2c37</i>	<i>Cyp2c38</i>	<i>Cyp2c39</i>	<i>Cyp2c40</i>	<i>Cyp2c44</i>	<i>Cyp2c50</i>	<i>Cyp2c54</i>	<i>Cyp2c55</i>	<i>Cyp2c65</i>	<i>Cyp2c66</i>	<i>Cyp2c67</i>	<i>Cyp2c68</i>	<i>Cyp2c69</i>	<i>Cyp2c70</i>
<i>Cyp2c29</i>	100	75.8	86.9	87.9	69	55.4	77.3	71.9	64.6	70.6	69	67.4	68.3	68.9	62.6
<i>Cyp2c37</i>		100	72.8	88.3	72.6	63.9	95.8	81.9	64.8	73.1	68.2	73.3	71.6	72.8	60.7
<i>Cyp2c38</i>			100	89.5	67	56	77.1	70.5	62.6	73.8	75.4	70.2	68	65.9	58
<i>Cyp2c39</i>				100	70.7	55.3	80.1	69	65.9	72.3	75.3	70.6	67.6	67.9	62.2
<i>Cyp2c40</i>					100	59.1	74.4	70.4	58.5	69.7	66.9	98.7	97.9	97.2	57.2
<i>Cyp2c44</i>						100	58.2	53.2	56.2	65.3	62.2	54.3	52.8	56.1	60.2
<i>Cyp2c50</i>							100	84.4	61.6	73.4	70.8	76.9	73.1	75.6	62.8
<i>Cyp2c54</i>								100	66.7	72.4	68.4	70.7	75.1	70.4	62.2
<i>Cyp2c55</i>									100	74.2	70.6	65.1	64.8	65.9	63.8
<i>Cyp2c65</i>										100	98	66.4	64.3	64.7	62.7
<i>Cyp2c66</i>											100	61.2	60.3	62.8	63.1
<i>Cyp2c67</i>												100	97.7	97.7	58.4
<i>Cyp2c68</i>													100	96.1	57.5
<i>Cyp2c69</i>														100	57.4
<i>Cyp2c70</i>															100

TABLE 2

Primer sequences used for the cloning of mouse *Cyp2c65*, *Cyp2c67*, *Cyp2c68*, and *Cyp2c69* cDNAs

Corresponding positions are relative to the ATG initiating codon designated as +1.

cDNA and Direction	Primer Sequence 5' to 3'	Corresponding Position
<i>Cyp2c65</i>		
Forward	GCAAACAATGGTTCTAGGTGTGTT	-6 to +17
Reverse	CCATCAGATAAGAACAGGGCTGA	+1807 to +1829
<i>Cyp2c67</i>		
Forward	GATCCATTGTAGTCTTGGTGCTTTGTC	+4 to +31
Reverse	ATAGCAGAATCTTTCCTCATCTTCACTT	+1491 to +1518
<i>Cyp2c68</i>		
Forward	ATCATACCAAGTGTCAAGTAAGAAGGG	-29 to -5
Reverse	ACTGTTAGCCTTGGGCAATTAGATA	+1605 to +1629
<i>Cyp2c69</i>		
Forward	GAATGCCTGATCATAACAGTGTGAC	-38 to -14
Reverse	ATAACAGAGAACTTTCAGCCTTGGG	+1611 to +1635

for sex-specific tissues where three individual tissue samples were each run in triplicate (nine wells per tissue) on the same plate.

qPCR Analysis. Data generated from qPCR was analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). All graphs were generated using the GraphPad Prism 6.0C program (GraphPad Software, San Diego, CA). Mouse tissue expression values were normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) expression values. To confirm specificity against cloned cDNAs, SYBR Green qPCR assay values were normalized to β -lactamase (ampicillin resistance) from the cDNA vector. Outliers were determined using the Grubbs Outlier test (Grubbs, 1950). Significance for sex differences in various tissues was determined using the Student's *t* test. No statistical comparisons were performed between tissues. The cDNA and tissue heat maps were generated using the Microsoft Excel program (Microsoft, Redmond, WA). Relative tissue expression was normalized to *Gapdh*; ΔC_T values (*Cyp2c-Gapdh*) are reported in the heat map (Fig. 2).

Results

Specificity of the Mouse *Cyp2c* TaqMan Primer/Probes and SYBR Green Primer Sets. The mouse *Cyp2c* subfamily cDNAs, except for *Cyp2c65*, *Cyp2c67*, *Cyp2c68*, and *Cyp2c69*, were previously cloned in collaboration with Dr. Joyce Goldstein's laboratory (Luo et al., 1998; DeLozier et al., 2004; Wang et al., 2004). Each cDNA was cloned into either the pBS vector (Agilent Technologies, Santa Clara, CA) or the pCR2.1 vector from Life Technologies. We cloned the *Cyp2c65* cDNA from mouse stomach and the *Cyp2c67*, *Cyp2c68*, and *Cyp2c69* cDNAs from mouse liver. The four new cDNAs were cloned into the pCR2.1 vector (ThermoFisher Scientific, Inc., Waltham, MA).

Ten of the eleven commercially available gene-specific mouse *Cyp2c* TaqMan primer/probe assays (Table 3) preferentially detected their target cDNAs (Fig. 1A). Only the *Cyp2c29* probe failed to detect its respective cDNA and had cross-reactivity with the *Cyp2c50*, *Cyp2c54*, *Cyp2c55*, and *Cyp2c66* cDNAs. The TaqMan *Cyp2c29* probe amplifies an 89-bp amplicon completely located within terminal exon 9. It may be that this amplicon spans both coding and noncoding sequence of exon 9. If this is the case, then the assay may be unable to detect the *Cyp2c29* ORF clone, which lacks the complete 3' UTR. The *Cyp2c38* TaqMan primer/probe recognized the *Cyp2c38* cDNA but also had some (30–35%) cross-reactivity to the *Cyp2c66* isoform (Supplemental Fig. 1A). Both the *Cyp2c44* and the *Cyp2c54* TaqMan primer/probes had low but detectable cross-reactivity to the *Cyp2c39* and *Cyp2c29* transcripts, respectively (Fig. 1A). Four TaqMan primer/probe assays (Table 3) were all advertised as having cross-reactivity between the *Cyp2c40*, *Cyp2c67*, *Cyp2c68*, and *Cyp2c69* subfamily members. Results confirmed cross-reactivity of all four TaqMan primer/probe assays for these four mouse *Cyp2c* isoforms (Fig. 1A).

The 15 mouse *Cyp2c* SYBR Green primer sets were specific for their intended ORF targets (Fig. 1B). The *Cyp2c29* primer set had low but

detectable cross-reactivity to the *Cyp2c38* and *Cyp2c50* cDNAs (Fig. 1B; Supplemental Fig. 1B), whereas the *Cyp2c69* primer set had low but detectable cross-reactivity to the *Cyp2c40*, *Cyp2c67*, and *Cyp2c68* isoforms (Fig. 1B).

The mouse *Cyp2c* and *Cyp2j* gene subfamilies are related with approximately 25–66% nucleotide identity (data not shown). To test for any cross-reactivity, the *Cyp2c* TaqMan primer/probes and SYBR Green primer sets were used in qPCR with the seven mouse *Cyp2j* cloned ORFs. None of the *Cyp2c* TaqMan primer/probes or SYBR Green primers sets detected any of the *Cyp2j* members (data not shown).

Tissue Distribution of the Mouse *Cyp2c* mRNAs by qPCR. By using the SYBR Green primer set, *Cyp2c29* expression was most abundant in male liver with significantly lower expression in female liver; much lower expression was detected in duodenum and testis (Fig. 2B; Table 5; Supplemental Fig. 1D). Detection of *Cyp2c29* expression in liver is consistent with published nonquantitative RT-PCR data, although no difference in expression between the sexes was previously reported (Luo et al., 1998). *Cyp2c29* was also reported to be expressed in the intestines by nonquantitative RT-PCR (Luo et al., 1998), which is consistent with the expression of *Cyp2c29* in duodenum by the SYBR Green primer set in the current work. *Cyp2c29* was also detected in jejunum by the SYBR Green primers at low levels; however, the expression of *Cyp2c29* in brain, kidney, lung, and heart by the nonquantitative RT-PCR method (Luo et al., 1998) was not confirmed using the SYBR Green qPCR method. There is an approximate melting temperature of 82.5°C for the dissociation curve of liver with the

TABLE 3.

TaqMan primer/probes for the mouse *Cyp2c* subfamily

Subfamily Member	TaqMan Primer/Probe
<i>Cyp2c29</i>	Mm00725580_s1
<i>Cyp2c37</i>	Mm00833845_m1
<i>Cyp2c38</i>	Mm00658527_m1
<i>Cyp2c39</i>	Mm04207909_g1
<i>Cyp2c(40/67/69)</i>	Mm04204172_mH
<i>Cyp2c44</i>	Mm01197188_m1
<i>Cyp2c50</i>	Mm00663066_gH
<i>Cyp2c54</i>	Mm02602271_mH
<i>Cyp2c55</i>	Mm00472168_m1
<i>Cyp2c65</i>	Mm02344065_g1
<i>Cyp2c66</i>	Mm01212038_g1
<i>Cyp2c(67/69)</i>	Mm03411955_mH
<i>Cyp2c(68/67/69)</i>	Mm04204171_mH
<i>Cyp2c(69/67/68)</i>	Mm04204173_gH
<i>Cyp2c70</i>	Mm00521058_m1
<i>Gapdh</i>	Mm99999915_g1

TABLE 4
SYBR Green primer set sequences for the mouse *Cyp2c* subfamily

Primer	(Direction)	Sequence (5'–3')	Reference	Annealing/Extension Temperature
<i>Cyp2c29</i>	Forward	GCTCTCCTACTCCTGCTGAAGT		68°C
	Reverse	ATGTGGCTCCTGTCTTGCATGC		
<i>Cyp2c37</i>	Forward	CACGAGGCGTTTCTCACTCA		66°C
	Reverse	AGGGCTGCTCAGAATCTTTGT		
<i>Cyp2c38</i>	Forward	CACTATGGAGACAGAGGTCTA	Luo et al., 1998	60°C
	Reverse	CCAAATACAGAGTGAAACCG		
<i>Cyp2c39</i>	Forward	GGAGACAGAGCTGTGGC	Luo et al., 1998	64°C
	Reverse	TAAAAACAATGCCAAGGCCG		
<i>Cyp2c40</i>	Forward	GGATTCCGGTTTTTGACAAGTTTCTAC		64°C
	Reverse	TGATTCTTCATGTCTTTTATTTC		
<i>Cyp2c44</i>	Forward	GGCTATGATGTGGTGAAGGAAGC		60°C
	Reverse	TCGCAGGAGTTCCACCTCTCT		
<i>Cyp2c50</i>	Forward	GAAAACGGCAACTATCCAT	Wang et al., 2004	60°C
	Reverse	CTTATGAAATTATGACAGGC		
<i>Cyp2c54</i>	Forward	CATGCCCTATACAAATGC	Wang et al., 2004	62°C
	Reverse	GGCAGTGTGTTCAGTATGG		
<i>Cyp2c55</i>	Forward	AGGAAGCTCTGGATGACCTGG		60°C
	Reverse	TTGAGAAGCGCCGAAGCTCCTT		
<i>Cyp2c65</i>	Forward	TGTCCCAAGATGTTTCTTACCA		60°C
	Reverse	TTGCAGACTTGCTTTGCAGT		
<i>Cyp2c66</i>	Forward	GGAGTTTGCTGGAAGGAACC		60°C
	Reverse	GCGTCTCATCTCTTCCAGGTC		
<i>Cyp2c67</i>	Forward	GGAAGGTTTCCGTTTTTGACAAGGTTA		68°C
	Reverse	CCATGCCCAAGTTCCTCAAGGTATTGAT		
<i>Cyp2c68</i>	Forward	CTGTCCTTCTGCTTCTCTGTAC		68°C
	Reverse	AATACAGAGTGAACACAGGCCATATA		
<i>Cyp2c69</i>	Forward	GGAGAAGTGTCTCTGGAAGAGG		68°C
	Reverse	GTGAAGACCTTGTGGCTTTCC		
<i>Cyp2c70</i>	Forward	TGGGCTTTTGCTCCTGCTGAAG		60°C
	Reverse	TCAGTGTACGGCATGTGGTTCC		
<i>Gapdh</i>	Forward	TTGATGGCAACAATCTCCAC		60°C
	Reverse	CGTCCCGTAGACAAAATGGT		
β -Lactamase	Forward	CGCAGAAGTGGTCCTGCAAC		60°C
	Reverse	TCTGCTATGTGGCGCGGTAT		

Cyp2c29 SYBR Green primer set (Supplemental Fig. 2A). The *Cyp2c29* TaqMan primer/probe assay gave a similar tissue distribution pattern to that seen by the SYBR Green primer set (Fig. 2A; Supplemental Fig. 1C). It is interesting to note that *Cyp2c29* is the only isoform with higher expression in male liver than in female liver.

Previous nonquantitative RT-PCR experiments, which used only female mouse tissues, demonstrated liver-specific expression of *Cyp2c37* (Luo et al., 1998). Both qPCR methods also revealed *Cyp2c37* expression was highest in liver, but with significantly greater expression in female liver than in male liver (Fig. 2; Table 5; Supplemental Fig. 3). With liver, a single dominant peak for the dissociation curve with the *Cyp2c37* SYBR Green primers had a melting temperature of approximately 82°C (Supplemental Fig. 2B).

Prominent *Cyp2c38* transcripts were found in both male and female livers with a significantly higher expression in female versus male liver using both qPCR methods (Fig. 2; Table 5; Supplemental Fig. 4). Much lower transcript levels were detected with the *Cyp2c38* TaqMan qPCR assay in duodenum, jejunum, kidney, lymph node, skin, testis, and uterus, whereas the SYBR Green primer set detected lower transcript levels only in duodenum and ovary. For the SYBR Green *Cyp2c38* primer set, the dissociation curve for liver was found to have a single dominant peak with a melting temperature of approximately 78.5°C (Supplemental Fig. 2C). The expression detected in duodenum, jejunum, kidney, lymph node, skin, testis, and uterus by the *Cyp2c38* TaqMan primer/probe may be explained by its cross-reactivity to *Cyp2c66*, which is highly expressed in the gastrointestinal tract and testis (Fig. 2; Supplemental Fig. 12). Nonquantitative RT-PCR, using only female mouse tissues, previously demonstrated the presence of the *Cyp2c38* transcript in liver, brain, kidney, and

intestine with much lower levels of expression in lung and heart (Luo et al., 1998).

Published nonquantitative RT-PCR experiments using only female mouse tissues demonstrated *Cyp2c39* expression specifically in liver (Luo et al., 1998). qPCR results from the *Cyp2c39* TaqMan primer/probe and SYBR Green primer set confirmed highest expression in the liver, but also revealed higher *Cyp2c39* expression in female liver than in male liver (Fig. 2; Table 5; Supplemental Fig. 5). *Cyp2c39* was also found at much lower but detectable levels in other tissues examined, including aorta, kidney, large intestine, and lung, with both qPCR methods. The dissociation curve for liver with the SYBR Green *Cyp2c39* primer set revealed a single prominent peak with a melting temperature of 80.3°C (Supplemental Fig. 2D).

For *Cyp2c40*, the highest expression using the SYBR Green primer set was in the liver. *Cyp2c40* is more highly expressed in female liver compared with male liver (Fig. 2; Table 5; Supplemental Fig. 6). A single dominant peak was obtained only for liver with an approximate melting temperature of 80.5°C (Supplemental Fig. 2E). In contrast, previous nonquantitative RT-PCR experiments detected *Cyp2c40* in liver, intestine, brain, and kidney with lower expression also present in lung and heart (Luo et al., 1998). There is no *Cyp2c40*-specific TaqMan primer/probe available. All four TaqMan primer/probes tested (Table 3) that recognized the *Cyp2c40* cDNA also recognized *Cyp2c67*, *Cyp2c68*, and *Cyp2c69* (Fig. 1; Supplemental Figs. 6A, 13A, 14A, and 15A). Therefore, although a similar expression pattern to the *Cyp2c40* SYBR Green primer assays was obtained with these four TaqMan primer/probes, none can be used as a *Cyp2c40*-specific primer/probe assay.

By nonquantitative RT-PCR methods, the *Cyp2c44* transcript was previously reported to be expressed in liver, kidney, and adrenal gland;

Fig. 1. Heat maps showing specificity of TaqMan primer/probes and SYBR Green primer sets for mouse *Cyp2c* cDNAs. (A) Both the *Cyp2c29* and *Cyp2c38* TaqMan primer/probes are cross-reactive with other *Cyp2c* genes. The probes used for *Cyp2c40*, *Cyp2c67*, *Cyp2c68*, and *Cyp2c69* comparisons are nonspecific. (B) All the mouse *Cyp2c* SYBR Green primer sets are specific to their intended *Cyp2c* gene target.

Cyp2c44 was found to be similarly expressed in male and female livers, but more highly expressed in female kidney and adrenal gland compared with male (DeLozier et al., 2004). By both qPCR methods, *Cyp2c44* transcripts were detected at approximately equal levels in male and female livers (Fig. 2; Table 5; Supplemental Fig. 7). *Cyp2c44* was also detected in kidney, with significantly higher expression in female kidney compared with male kidney. Both methods confirm the previous nonquantitative RT-PCR results. Adrenal glands were not included in the panel of tissues for the current study. Low levels of *Cyp2c44* expression were observed in several other tissues examined. Both qPCR methods detected *Cyp2c44* in liver, duodenum, kidney, testis, stomach, lung, ileum, jejunum, large intestine, esophagus, and visceral fat. The SYBR Green dissociation curves yielded a single peak for all these tissues. The dissociation curve for liver with the *Cyp2c44* SYBR Green primer set gives a melting temperature of 79.5°C, as shown in Supplemental Fig. 2F. This finding stands in contrast to the earlier nonquantitative results, which found no detectable expression level in other tissues including small intestine, colon, testis, and lung.

A prominent *Cyp2c50* transcript was detected in liver samples using both TaqMan and SYBR Green qPCR methods (Fig. 2; Table 5; Supplemental Fig. 8). Expression levels for *Cyp2c50* were approximately the same for both male and female liver using both qPCR methods. For the SYBR Green primer set, very low levels of *Cyp2c50* transcript (CT values of ~36) were detected in the remaining tissues

tested. The SYBR Green *Cyp2c50* dissociation curves were examined and only liver gave a single dominant peak with a melting temperature of approximately 81.5°C (Supplemental Fig. 2G), which suggests that the low level of detection in other tissues represents cross-reactivity with nontarget transcripts. Previous nonquantitative RT-PCR methods detected the expression of *Cyp2c50* in the liver and heart (Wang et al., 2004). Heart expression of *Cyp2c50* was not confirmed by either qPCR method used in this study.

Prominent *Cyp2c54* expression was detected in male and female liver using both qPCR methods. Significantly higher expression was detected in female liver compared with male liver (Fig. 2; Table 5; Supplemental Fig. 9). There was low level detection of *Cyp2c54* in all other tissues examined with the SYBR Green primer set. For the *Cyp2c54* SYBR Green dissociation curves, only liver produced a single prominent peak with a melting temperature of approximately 81.5°C (Supplemental Fig. 2H). A single peak was observed only for liver. The low level detection in all other tissues is most likely due to cross-reactivity with nontarget transcripts. Nonquantitative RT-PCR from previous studies reported *Cyp2c54* transcripts in liver, stomach, and kidney (Wang et al., 2004). Our methods can only confirm significant expression of *Cyp2c54* in liver.

Previous findings with nonquantitative RT-PCR (Wang et al., 2004) demonstrated that *Cyp2c55* transcripts were present in all the tissues examined (liver, kidney, lung, heart, brain, spleen, colon, small intestine, stomach, testis, skeletal muscle, aorta, adrenal gland, prostate, and ovary). By using both the TaqMan primer/probe and SYBR Green primer sets, *Cyp2c55* was found to be most abundant in the large intestine with significantly higher expression in male versus female large intestine (Fig. 2; Table 5; Supplemental Fig. 10). Lower *Cyp2c55* expression was found in the jejunum with significantly higher expression in male jejunum versus female jejunum. Low levels of *Cyp2c55* expression were also found in duodenum, ileum, stomach, and liver. The other tissues failed to give a single peak in the *Cyp2c55* SYBR Green dissociation curves. The single dominant peak for large intestine has an approximate melting temperature of 81.5°C (Supplemental Fig. 2I). This suggests off-target cross-reactivity may account for previous nonquantitative RT-PCR results.

The *Cyp2c65* SYBR Green primer set amplifies a region of the 3' UTR. The *Cyp2c65* SYBR Green dissociation curves gave a single prominent peak with an approximate melting temperature of 76.5°C for stomach, duodenum, ileum, jejunum, large intestine, testis, and esophagus (Fig. 3; Supplemental Fig. 2J). To confirm specificity for *Cyp2c65*, the SYBR Green qPCR reactions for stomach, duodenum, and large intestine were run on agarose gels. A single band was obtained for each, which was isolated using a Qiagen Gel Isolation Kit. The isolated bands were sequenced using the *Cyp2c65* SYBR Green primers. For all three tissues tested, the sequence matched the predicted *Cyp2c65* sequence (Fig. 3B). This strongly suggests that the SYBR Green *Cyp2c65* primer set specifically detect only the *Cyp2c65* transcript in these tissues. *Cyp2c65* transcripts were detected in stomach, duodenum, and large intestine with much lower expression in jejunum, testis, and esophagus by both qPCR methods (Fig. 2; Table 5; Supplemental Fig. 11). There was significantly higher expression in female large intestine than male large intestine by both qPCR methods. There was also significantly higher expression in male jejunum compared with female jejunum with the *Cyp2c65* TaqMan primer/probe. This sex difference in the SYBR Green primer assay was not statistically significant ($P = 0.07$). No previous RT-PCR studies have been performed for *Cyp2c65*.

Prominent *Cyp2c66* transcripts were detected in duodenum and jejunum, with significantly lower expression in large intestine and testis using both qPCR methods (Fig. 2; Table 5; Supplemental Fig. 12). There

Fig. 2. Heat maps showing tissue distribution of mouse *Cyp2c* transcripts using TaqMan primer/probes and SYBR Green primer sets. Mouse *Cyp2c* TaqMan primer/probes (A) and SYBR Green primer sets (B) show similar expression patterns. Relative tissue expression was normalized to *Gapdh*; Δ CT values (*Cyp2c*-*Gapdh*) are reported in the heat maps.

was significantly higher expression in male jejunum than female jejunum. Conversely, there was significantly higher expression in female large intestine compared with male large intestine using both qPCR methods. A single dominant peak for duodenum and jejunum gave an approximate melting temperature of 79.0°C for the *Cyp2c66* SYBR Green primer set (Supplemental Fig. 2K). *Cyp2c66* expression has not previously been reported.

There were no previous nonquantitative RT-PCR studies found with mouse *Cyp2c67*. We detected a prominent *Cyp2c67* transcript in liver using the SYBR Green primer set with similar expression in both sexes (Fig. 2; Table 5; Supplemental Fig. 13D). There is no *Cyp2c67*-specific TaqMan primer/probe assay available (Fig. 1). The TaqMan primer/probe (Table 3) used for comparison with the *Cyp2c67* SYBR Green primers also strongly recognizes the *Cyp2c40*, *Cyp2c68*, and *Cyp2c69* transcripts (Supplemental Fig. 13A). This cross-reactivity likely accounts for the higher expression observed in female liver in comparison with male liver, because this sex difference is also seen using the *Cyp2c40*-, *Cyp2c68*-, and *Cyp2c69*-specific SYBR Green primer sets (Supplemental Figs. 6D, 14D, and 15D). A single prominent peak was observed using the SYBR Green primers with liver that had an approximate melting temperature of 77.5°C (Supplemental Fig. 2L).

The *Cyp2c68* SYBR Green primer set detects the highest expression in liver with significantly greater expression in female liver versus male liver (Fig. 2; Table 5; Supplemental Fig. 14). *Cyp2c68* transcripts were also detected in duodenum, ileum, jejunum, large intestine, stomach, and kidney, which is confirmed by the dissociation curves. A single

dominant peak for liver has an approximate melting temperature of 80.0°C (Supplemental Fig. 2M). There was significantly higher expression of *Cyp2c68* in female large intestine compared with male large intestine. There is no specific *Cyp2c68* TaqMan primer/probe available (Fig. 1; Supplemental Fig. 14A). The expression pattern seen with the TaqMan primer/probe used for comparison (Table 3) could be due to detection of *Cyp2c40*, *Cyp2c67*, and *Cyp2c69*, as well as *Cyp2c68*, because all four isoforms are recognized by this primer/probe assay. No nonquantitative RT-PCR experiments for *Cyp2c68* expression were previously reported.

A prominent transcript is detected in liver using the *Cyp2c69*-specific SYBR Green primers (Fig. 2; Table 5; Supplemental Fig. 15). There is significantly higher *Cyp2c69* expression in female liver compared with male liver. The dissociation curves for the *Cyp2c69* SYBR Green primer set reveals a single peak with a melting temperature of approximately 77.0°C (Supplemental Fig. 2N). There is no specific TaqMan primer/probe assay for mouse *Cyp2c69*. The *Cyp2c69* TaqMan primer/probe assay (Table 3) used also recognizes *Cyp2c40*, *Cyp2c67*, and *Cyp2c68* (Supplemental Fig. 15A). The expression pattern observed with this primer/probe could be due to reactivity with any of the other four isoforms (Fig. 1). No non-quantitative RT-PCR experiments for mouse *Cyp2c69* were available as a comparison.

Finally, both qPCR methods detected *Cyp2c70* only in liver (Fig. 2; Table 5; Supplemental Fig. 16). There were no expression differences between the sexes. The dissociation curves for liver gave a single dominant peak with an approximate melting temperature of 79.5°C

TABLE 5
Mouse *Cyp2c* subfamily expression summary

Isoform	Highest Expression	Average Ct SYBR Green	Average Ct TaqMan	Other Tissues, Average SYBR Green	Other Tissues, Average Ct TaqMan
<i>Cyp2c29</i>	Liver, M > F	F, 21.0 M, 20.6	F, 19.6 M, 19.2	Duodenum, 24.7	Duodenum, 28.8
<i>Cyp2c37</i>	Liver, F > M	F, 18.0 M, 19.6	F, 26.0 M, 27.3	Testis, 28.9	Testis, 28.8
<i>Cyp2c38</i>	Liver, F > M	F, 27.3 M, 29.4	F, 28.8 M, 30.5		
<i>Cyp2c39</i>	Liver, F > M	F, 28.8 M, 31.8	F, 30.9 M, 33.8		
<i>Cyp2c40</i>	Liver, F > M	F, 28.3 M, 35.1	NA		
	Liver	22.9	26.9	Kidney, F, 26.3 Kidney, M, 27.9 Duodenum, 27.5 Testis, 29.7	Kidney, F, 29.3 Kidney, M, 30.7 Duodenum, 32.2 Testis, 29.7
<i>Cyp2c44</i>					
<i>Cyp2c50</i>	Liver	31.1	24.6		
<i>Cyp2c54</i>	Liver, F > M	F, 29.5 M, 30.5	F, 26.0 M, 26.5		
	Large Intestine, M > F	F, 24.6 M, 20.4	F, 29.1 M, 24.7	Duodenum, 26.0 Ileum, 26.4 Jejunum, 24.9 Jejunum, 25.2	Duodenum, 27.0 Ileum, 29.9 Jejunum, 28.4 Jejunum, 28.8
<i>Cyp2c55</i>					
<i>Cyp2c65</i>	Stomach Duodenum Large intestine Duodenum	Stomach, 22.2 Duodenum, 22.0 Large intestine, 24.0 Duodenum, 23.1	Stomach, 28.4 Duodenum, 27.7 Large intestine, 28.6 Duodenum, 24.5		
<i>Cyp2c66</i>	Jejunum, M>F	Jejunum, M, 23.1 Jejunum, F, 26.1	Jejunum, M, 24.8 Jejunum, F, 24.8		
<i>Cyp2c67</i>	Liver	22.1	NA		
	Liver, F > M	F, 22.3 M, 25.1	NA	Large intestine, F, 26.0 Large intestine, M, 27.4 Duodenum, 25.6 Ileum, 27.1 Jejunum, 25.6	
<i>Cyp2c68</i>					
<i>Cyp2c69</i>	Liver, F > M	F, 20.2 M, 25.2	NA		
<i>Cyp2c70</i>	Liver	21.7	22.3		

NA, Specific probes not available.

(Supplemental Fig. 2O). No previous *Cyp2c70* nonquantitative RT-PCR experiments have been reported.

Discussion

The four human *CYP2C* isoforms (*CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19*) account for 20% of hepatic P450s and are active in the metabolism of many widely used drugs (Goldstein and de Morais, 1994) and endogenous compounds (Daikh et al., 1994; Zeldin et al., 1995). In comparison, the mouse *Cyp2c* subfamily is larger and more complex, with 15 subfamily members (Nelson et al., 1996; Wang et al., 2004). Mouse *Cyp2c* enzymes also metabolize both endogenous and exogenous compounds (Luo et al., 1998; DeLozier et al., 2004; Wang et al., 2004). They are predominantly expressed in liver but are also present in extrahepatic tissues (Luo et al., 1998; DeLozier et al., 2004; Wang et al., 2004). Accurate identification of the tissue expression pattern of the mouse *Cyp2c* isoforms is a critical step in identifying their physiologic roles. The tissue distribution of nine of the fifteen members of the mouse *Cyp2c* subfamily had been previously reported using both nonquantitative RT-PCR and immunoblotting (Luo et al., 1998; DeLozier et al., 2004; Wang et al., 2004). Analysis of the mouse *Cyp2c* subfamily members in a panel of tissues by qPCR provides a more reliable tissue expression profile. Herein, we describe 1) development of new SYBR Green primer sets, 2) specificity of both TaqMan and SYBR Green qPCR methods for each of the 15 mouse *Cyp2c* isoforms, 3) tissue expression patterns for all 15 mouse *Cyp2c* subfamily members using our developed SYBR Green primer sets, and 4) tissue expression patterns of 11 of the mouse *Cyp2c* isoforms using specific TaqMan

primer/probes. In short, our work provides a valuable reference for mouse *Cyp2c* qPCR methods and catalogs their tissue distribution.

Nine of the fifteen mouse TaqMan primer/probes tested (*Cyp2c37*, *Cyp2c39*, *Cyp2c44*, *Cyp2c50*, *Cyp2c54*, *Cyp2c55*, *Cyp2c65*, *Cyp2c66*, and *Cyp2c70*) are highly specific for their respective *Cyp2c* isoforms. The *Cyp2c38* TaqMan primer/probe recognizes its intended cDNA, but has ~30–35% cross-reactivity to the *Cyp2c66* cDNA. This cross-reactivity is not surprising due to the ~75% identity between the *Cyp2c38* and *Cyp2c66* cDNAs. The *Cyp2c29* TaqMan primer/probe does not recognize its intended cDNA, but this is most likely due to the probe partially targeting the 3' UTR that is absent from our cDNA. The remaining TaqMan primer/probes used for this study are not advertised as being specific for the *Cyp2c40*, *Cyp2c67*, *Cyp2c68*, and *Cyp2c69* isoforms. These assays were included for comparison with the SYBR Green primer sets developed for these *Cyp2c* subfamily members.

The 15 mouse *Cyp2c* SYBR Green primer sets are highly specific for their intended *Cyp2c* cDNA. These include the four isoforms (*Cyp2c40*, *Cyp2c67*, *Cyp2c68*, and *Cyp2c69*) for which there are no specific TaqMan primer/probes.

Tissue expression profiles of the mouse *Cyp2c* isoforms were generally similar using both qPCR methods. Of the 15 mouse *Cyp2c* subfamily members, 12 were most highly expressed in liver. Mammalian P450s highly expressed in liver have important roles in the metabolism of certain drugs (Guengerich, 1992). Human *Cyp2cs* are known to metabolize clinically relevant drugs such as tolbutamide, which is used for the management of type 2 diabetes (Veronese et al., 1991), as well as anti-inflammatory drugs such as ibuprofen and acetylsalicylic acid (aspirin) (Leemann et al., 1993). The mouse

Fig. 3. *Cyp2c65* SYBR Green primer set dissociation curves and sequence. (A) A single peak in the *Cyp2c65* melting curve plot was detected for duodenum, ileum, jejunum, large intestine, stomach, testis, and esophagus. (B) Sequencing of the single band gel-isolated from the stomach, large intestine, and duodenum SYBR Green qPCR reactions gave the expected *Cyp2c65* cDNA sequence. The primer sequences are indicated by capital letters.

Cyp2c29, *Cyp2c38*, and *Cyp2c39* isoforms have been shown to metabolize tolbutamide (DeLozier et al., 2004). Mouse *Cyp2c29* and *Cyp2c37* are inducible by phenytoin and the *Cyp2c29*, *Cyp2c37*, and *Cyp2c55* isoforms are inducible by phenobarbital (Jackson et al., 2004, 2006; Konno et al., 2010). It will be important to test the remaining mouse *Cyp2cs* for their ability to metabolize drugs to better understand their physiologic roles in the liver.

Six of the twelve mouse *Cyp2c* enzymes that are most highly expressed in the liver (*Cyp2c37*, *Cyp2c38*, *Cyp2c39*, *Cyp2c40*, *Cyp2c54*, and *Cyp2c69*) are found to have significantly higher expression in female liver compared with male liver. There is only one isoform (*Cyp2c29*) that has significantly higher expression in male liver compared with female liver. It is well known that sex differences found in the metabolism of many pharmaceuticals are due, at least in part, to sex differences in expression of hepatic enzymes, including P450s (Waxman and Azaroff, 1992; Waxman and Holloway, 2009). Identifying these sexually dimorphic mouse *Cyp2c* enzymes in liver may aid in understanding differences in drug potency observed in male versus female mice. However, sexual dimorphism is much less

pronounced in human models in comparison with rodent models (Lofgren et al., 2008; Tanaka, 1999), which must be taken into account when using the mouse as a model for human disease and drug metabolism.

Cytochrome P450 enzymes primarily expressed in the gastrointestinal tract may also play an important role in drug elimination as well as biotransformation of other exogenous and endogenous compounds (Kaminsky and Fasco, 1992; Wang et al., 2004; Paine et al., 2006). Both qPCR methods show that *Cyp2c55*, *Cyp2c65*, and *Cyp2c66* are highly expressed in the gastrointestinal tract with similar tissue expression profiles for each isoform. Previously *Cyp2c40* was identified as the major *Cyp2c* isoform present in the intestine (Tsao et al., 2000); however, with more sensitive and paralog-specific qPCR methods, we identified other highly expressed *Cyp2c* isoforms in the intestinal tract and that *Cyp2c40* is predominantly expressed in female liver.

Mouse models exist that have disruption of *Cyp2c* isoforms. *Cyp2c44*^{-/-} mice have hyperactive epithelial sodium channel (ENaC) activity in the kidney and are more susceptible to salt-induced hypertension than wild-type mice. The authors concluded that

CYP2C44 normally generates EETs to suppress ENaC activity and limit sensitivity to high-salt diet (Capdevila et al., 2014). Our data suggest that *Cyp2c44* is the predominant *Cyp2c* expressed in the kidney. Another mouse model has the 14 *Cyp2c* gene cluster genetically disrupted with only the downstream *Cyp2c44* locus intact. These mice are viable and healthy, although some animals exhibited liver inflammation. They also have significantly reduced metabolism of known CYP2C substrates such as tolbutamide (Scheer et al., 2012). Together, these models will be important for elucidating the roles of mouse CYP2C enzymes in cardiovascular physiology and xenobiotic metabolism. The human ortholog of mouse *Cyp2c44* is not known. Human CYP2C8 and CYP2C9, thought to be the primary CYP2C epoxigenases, are both abundantly expressed in kidney and have a similar function (Enayetallah et al., 2004). Studies with the human single nucleotide polymorphism CYP2C8*3 have shown either a small but significant association with hypertension (Tzveova et al., 2015) or no association with hypertension (Dreisbach et al., 2005; King et al., 2005). Further studies will be needed to clarify the association between polymorphisms such as CYP2C8*3 and hypertension in humans.

Although the data in this manuscript provide a comprehensive profile of *Cyp2c* gene expression in mice, they do little to clarify the identity of the murine orthologs for the known human CYP2C genes. Humans have four CYP2C genes, *CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19* (Nelson et al., 2004). Collectively, we find murine *Cyp2c* expression is restricted largely to the liver, gastrointestinal tract, and kidney. In humans, *CYP2C8* and *CYP2C9* are expressed in all three of these tissues, but can also be found in other tissues, including heart, lung, lymph nodes, spleen, and skin (Enayetallah et al., 2004). Metabolic studies with individual CYP2C proteins will be needed to clarify whether any of the murine CYP2Cs perform similar functions as the corresponding human CYP2C enzymes. Due to the complexity and high level of homology between the mouse *Cyp2c* subfamily members, it is exceedingly difficult to develop isoform-specific antibodies. Future Western blot analysis and immunohistochemistry studies will be essential to confirm the tissue distribution and sexual dimorphism for the mouse *Cyp2c* proteins. In addition, although human CYP2Cs also display sexually dimorphic expression, this occurs to a lesser degree than in mice. It is important to note these differences to fully understand and interpret drug metabolism data by CYP2Cs in humans compared with mice.

In summary, we developed SYBR Green primers, validated their specificity, and compared them to commercially available Taqman primer/probe assays for determination of the tissue expression profiles of the 15 mouse *Cyp2c* subfamily members. Similar tissue expression profiles were observed for 11 of the mouse *Cyp2c* isoforms using both qPCR methods. Tissue expression profiles were also developed using our SYBR Green primer sets for the remaining four mouse *Cyp2c* isoforms that currently do not have paralog-specific TaqMan primer/probe assays. Although primer/probe assays may offer increased specificity of target detection, the SYBR Green end-point melting curve dissociation analysis allows for verification that observed fluorescence is due to a single amplicon (or at least amplicons of similar melting temperature). Our developed mouse *Cyp2c* SYBR Green primer sets may be used as accurate and less expensive alternatives to the commercially available TaqMan primer/probe assays for the quantitative expression of the mouse *Cyp2cs* transcripts in tissues. In addition, our *Cyp2c*-specific SYBR Green primer sets may be used for the quantitative analysis in tissues where there is no specific TaqMan assay available.

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Authorship Contributions

Participated in research design: Graves, Gruzdev, Edin, and Zeldin.

Conducted experiments: Graves.

Contributed new reagents or analytical tools: Bradbury and DeGraff.

Performed data analysis: Graves, Gruzdev, and Zeldin.

Wrote or contributed to the writing of the manuscript: Graves, Gruzdev, Edin, and Zeldin.

References

- Campbell WB, Gebremedhin D, Pratt PF, and Harder DR (1996) Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* **78**:415–423.
- Capdevila JH, Falck JR, and Harris RC (2000) Cytochrome P450 and arachidonic acid bioactivation. Molecular and functional properties of the arachidonate monooxygenase. *J Lipid Res* **41**:163–181.
- Capdevila JH, Pidkova N, Mei S, Gong Y, Falck JR, Imig JD, Harris RC, and Wang W (2014) The Cyp2c44 epoxigenase regulates epithelial sodium channel activity and the blood pressure responses to increased dietary salt. *J Biol Chem* **289**:4377–4386.
- Dai KH, Lasker JM, Raucy JL, and Koop DR (1994) Regio- and stereoselective epoxidation of arachidonic acid by human cytochromes P450 2C8 and 2C9. *J Pharmacol Exp Ther* **271**:1427–1433.
- DeLozier TC, Tsao CC, Coulter SJ, Foley J, Bradbury JA, Zeldin DC, and Goldstein JA (2004) CYP2C44, a new murine CYP2C that metabolizes arachidonic acid to unique stereospecific products. *J Pharmacol Exp Ther* **310**:845–854.
- Dreisbach AW, Japa S, Sigel A, Parenti MB, Hess AE, Srinouanprachanh SL, Rettie AE, Kim H, Farin FM, Hamm LL, et al. (2005) The Prevalence of CYP2C8, 2C9, 2J2, and soluble epoxide hydrolase polymorphisms in African Americans with hypertension. *Am J Hypertens* **18**:1276–1281.
- Enayetallah AE, French RA, Thibodeau MS, and Grant DF (2004) Distribution of soluble epoxide hydrolase and of cytochrome P450 2C8, 2C9, and 2J2 in human tissues. *J Histochem Cytochem* **52**:447–454.
- Goldstein JA and de Morais SM (1994) Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* **4**:285–299.
- Grubbs FE (1950) Sample criteria for testing outlying observations. *Ann Math Stat* **21**:27–58.
- Guengerich FP (1992) Human cytochrome P-450 enzymes. *Life Sci* **50**:1471–1478.
- Heid CA, Stevens J, Livak KJ, and Williams PM (1996) Real time quantitative PCR. *Genome Res* **6**:986–994.
- Jackson JP, Ferguson SS, Moore R, Negishi M, and Goldstein JA (2004) The constitutive active/androstane receptor regulates phenytoin induction of Cyp2c29. *Mol Pharmacol* **65**:1397–1404.
- Jackson JP, Ferguson SS, Negishi M, and Goldstein JA (2006) Phenytoin induction of the cyp2c37 gene is mediated by the constitutive androstane receptor. *Drug Metab Dispos* **34**:2003–2010.
- Kaminsky LS and Fasco MJ (1992) Small intestinal cytochromes P450. *Crit Rev Toxicol* **21**:407–422.
- King LM, Gainer JV, David GL, Dai D, Goldstein JA, Brown NJ, and Zeldin DC (2005) Single nucleotide polymorphisms in the CYP2J2 and CYP2C8 genes and the risk of hypertension. *Pharmacogenet Genomics* **15**:7–13.
- Konno Y, Kamino H, Moore R, Lih F, Tomer KB, Zeldin DC, Goldstein JA, and Negishi M (2010) The nuclear receptors constitutive active/androstane receptor and pregnane x receptor activate the Cyp2c55 gene in mouse liver. *Drug Metab Dispos* **38**:1177–1182.
- Kroetz DL and Zeldin DC (2002) Cytochrome P450 pathways of arachidonic acid metabolism. *Curr Opin Lipidol* **13**:273–283.
- Larsen BT, Miura H, Hatoum OA, Campbell WB, Hammock BD, Zeldin DC, Falck JR, and Gutterman DD (2006) Epoxyeicosatrienoic and dihydroxyeicosatrienoic acids dilate human coronary arterioles via BK(Ca) channels: implications for soluble epoxide hydrolase inhibition. *Am J Physiol Heart Circ Physiol* **290**:H491–H499.
- Leemann TD, Transon C, Bonnabry P, and Dayer P (1993) A major role for cytochrome P450TB (CYP2C subfamily) in the actions of non-steroidal antiinflammatory drugs. *Drugs Exp Clin Res* **19**:189–195.
- Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**:402–408.
- Löfgren S, Baldwin RM, Hiratsuka M, Lindqvist A, Carlberg A, Sim SC, Schülke M, Snait M, Edero A, Fransson-Steen R, et al. (2008) Generation of mice transgenic for human CYP2C18 and CYP2C19: characterization of the sexually dimorphic gene and enzyme expression. *Drug Metab Dispos* **36**:955–962.
- Luo G, Zeldin DC, Blaisdell JA, Hodgson E, and Goldstein JA (1998) Cloning and expression of murine CYP2Cs and their ability to metabolize arachidonic acid. *Arch Biochem Biophys* **357**:45–57.
- Nebert DW and Russell DW (2002) Clinical importance of the cytochromes P450. *Lancet* **360**:1155–1162.
- Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, et al. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**:1–42.
- Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, and Nebert DW (2004) Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics* **14**:1–18.
- Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, and Liao JK (1999) Anti-inflammatory properties of cytochrome P450 epoxigenase-derived eicosanoids. *Science* **285**:1276–1279.
- Node K, Ruan XL, Dai J, Yang SX, Graham L, Zeldin DC, and Liao JK (2001) Activation of Galphas mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids. *J Biol Chem* **276**:15983–15989.
- Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, and Zeldin DC (2006) The human intestinal cytochrome P450 “pie”. *Drug Metab Dispos* **34**:880–886.
- Ponchel F, Toomes C, Bransfield K, Leong FT, Douglas SH, Field SL, Bell SM, Combaret V, Puisieux A, Mighell AJ, et al. (2003) Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. *BMC Biotechnol* **3**:18.

- Scheer N, Kapelyukh Y, Chatham L, Rode A, Buechel S, and Wolf CR (2012) Generation and characterization of novel cytochrome P450 Cyp2c gene cluster knockout and CYP2C9 humanized mouse lines. *Mol Pharmacol* **82**:1022–1029.
- Seubert J, Yang B, Bradbury JA, Graves J, Degraff LM, Gabel S, Gooch R, Foley J, Newman J, Mao L, et al. (2004) Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K⁺ channels and p42/p44 MAPK pathway. *Circ Res* **95**: 506–514.
- Seubert JM, Sinal CJ, Graves J, DeGraff LM, Bradbury JA, Lee CR, Goralski K, Carey MA, Luria A, Newman JW, et al. (2006) Role of soluble epoxide hydrolase in postischemic recovery of heart contractile function. *Circ Res* **99**:442–450.
- Tanaka E (1999) Gender-related differences in pharmacokinetics and their clinical significance. *J Clin Pharm Ther* **24**:339–346.
- Tsao C-C, Foley J, Coulter SJ, Maronpot R, Zeldin DC, and Goldstein JA (2000) CYP2C40, a unique arachidonic acid 16-hydroxylase, is the major CYP2C in murine intestinal tract. *Mol Pharmacol* **58**:279–287.
- Tzveova R, Naydenova G, Yaneva T, Dimitrov G, Vandeva S, Matrozova Y, Pendicheva-Duhlenska D, Popov I, Beltheva O, Naydenov C, et al. (2015) Gender-specific effect of CYP2C8*3 on the risk of essential hypertension in Bulgarian Patients. *Biochem Genet* **53**:319–333.
- Veronese ME, Mackenzie PI, Doecke CJ, McManus ME, Miners JO, and Birkett DJ (1991) Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome P450C9. *Biochem Biophys Res Commun* **175**:1112–1118.
- Wang H, Zhao Y, Bradbury JA, Graves JP, Foley J, Blaisdell JA, Goldstein JA, and Zeldin DC (2004) Cloning, expression, and characterization of three new mouse cytochrome p450 enzymes and partial characterization of their fatty acid oxidation activities. *Mol Pharmacol* **65**:1148–1158.
- Wang Y, Wei X, Xiao X, Hui R, Card JW, Carey MA, Wang DW, and Zeldin DC (2005) Arachidonic acid epoxigenase metabolites stimulate endothelial cell growth and angiogenesis via mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways. *J Pharmacol Exp Ther* **314**:522–532.
- Waxman DJ and Azaroff L (1992) Phenobarbital induction of cytochrome P-450 gene expression. *Biochem J* **281**:577–592.
- Waxman DJ and Holloway MG (2009) Sex differences in the expression of hepatic drug metabolizing enzymes. *Mol Pharmacol* **76**:215–228.
- Zeldin DC, DuBois RN, Falck JR, and Capdevila JH (1995) Molecular cloning, expression and characterization of an endogenous human cytochrome P450 arachidonic acid epoxigenase isoform. *Arch Biochem Biophys* **322**:76–86.

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