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Quantitative Analysis of *FMO* Gene mRNA Levels in Human Tissues

JUN ZHANG and JOHN R. CASHMAN

Human BioMolecular Research Institute, 5310 Eastgate Mall, San Diego, California 92121

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Corresponding author:

John R. Cashman

Human BioMolecular Research Institute, 5310 Eastgate Mall, San Diego CA, 92121

Tel. (858) 458-9305; Fax. (858) 458-9311; Email: jcashman@hbri.org

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Abbreviations: FMO, flavin-containing monooxygenase; PCR, polymerase chain reaction; RT-PCR, reverse transcription - polymerase chain reaction; q-PCR, quantitative polymerase chain reaction; TBP, TATA-Box binding protein; HPRT, hypoxanthine phosphoribosyltransferase; PTC, premature termination codon.

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Abstract

The developmental- and tissue-specific expression of FMO enzymes has been previously characterized in a number of animal species, including humans, mice, rats, and rabbits. In this study, we used sensitive real-time RT-PCR methodology to systematically quantify the steady-state mRNA levels of *FMO1*, 2, 3, 4, and 5 in human tissues. We examined the developmental regulation of these enzymes in brain tissue. *FMO1* was found to be down-regulated in human adult brain. Amount of other *FMO* mRNAs in human brains in different age groups was not significantly different. The study also provided a systematic quantitative comparison of the steady-state mRNA levels of *FMO1* to 5 in several major human organs (i.e., liver, lung, kidney, small intestine, and brain). The nature of the quantitative analysis allowed a comprehensive comparison of each *FMO* mRNA in different tissues as well as among *FMO* isoforms in the same tissue. A comparison between fetal liver and adult liver showed that *FMO1* was the only *FMO* that was down regulated; all other *FMOs* had greater amounts of mRNA in adult liver. *FMO5* was the most prominent *FMO* form detected in fetal liver. *FMO5* mRNA level was nearly as abundant as *FMO3* in adult liver. While other *FMOs* displayed significant dominant tissue-specific mRNA profile (i.e., *FMO1* in kidney, *FMO2* in lung, *FMO3* and *FMO5* in adult liver), *FMO4* mRNA was observed more broadly at relatively comparable levels in liver, kidney, lung, and small intestine.

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Introduction

Flavin-containing monooxygenases (FMOs) (EC 1.14.13.8) are a family of NADPH- and FAD-dependent enzymes that catalyze the oxygenation of a wide variety of compounds containing nucleophilic nitrogen, sulfur, and phosphorous heteroatoms (Cashman, 1995; Ziegler, 1988). The enzyme is recognized as a broad spectrum monooxygenase and functional diversity of this family is determined by the expression of five *FMO* genes, named *FMO1* to *FMO5*, and their variants. There are also a large number of *FMO* splice variants detected in human tissue (Lattard et al., 2004). A number of other *FMO* pseudogenes have been described, but apparently no other functional proteins are expressed (Hernandez et al., 2004; Hines et al., 2002).

The developmental- and tissue-specific expression of FMOs has been characterized in a number of animal species, including humans, mice, rats, and rabbits (Hines et al., 1994). Methods that have been used to characterize FMO expression include RNase protection assays for *FMOs* in human kidney and liver (Dolphin et al., 1996) and mouse (Janmohamed et al., 2004) to quantify mRNA; western blot analysis in human liver to quantify protein level (Koukouritaki et al., 2002); microsomal activity measurements for human liver FMO (Overby et al., 1997) and rabbit FMOs (Shehin-Johnson et al., 1995). The functional characterization of FMO previously reported has mainly focused on hepatic- and kidney-mediated metabolism, where the majority of the metabolism of drugs and exogenous chemicals occur. Limited animal studies, mostly using rodents, show that FMO activity exists in brain (Blake et al., 1996; Kawaji et al., 1995; Lattard et al., 2003). To further our understanding of the contribution of FMO function to chemical metabolism in brain, a systematic quantitative analysis of expression of brain FMO is essential. A gap in our knowledge of brain FMOs may be related to the significantly lower amounts of

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FMOs in brain tissue compared with that of liver or kidney. In addition, as discussed above, the developmental- and tissue-specific expression profile of FMOs in tissues examined thus far (i.e., liver and kidney) are fairly distinct among different animal species. Studies of FMO in brain of rodents are not easily interpretable for predicting the expression and regulation of FMO in human brain.

In this study, we used highly selective real-time RT-PCR methodology to systematically categorize the mRNA of *FMO1* to *FMO5* in human brain tissues to quantify the steady state mRNA levels of *FMO1* to *5*. We examined the developmental regulation and gender-dependent mRNA level in brain for these genes. In addition, the study provided a quantitative systematic comparison of the mRNAs of *FMO1* to *FMO5* in the major human organs (i.e., liver, lung, kidney, small intestine and brain). The nature of the quantitative analysis allowed a comparison between samples as well as among *FMO* genes, and also allowed quantification of low amounts of transcripts in tissues such as brain.

Materials and Methods

Chemicals and Reagents

cDNA synthesis components were purchased from Invitrogen (Carlsbad, CA). q-PCR reagents were purchased from Bio-Rad (Emeryville, CA). All chemicals and reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) in the highest purity commercially available. Other reagents, buffers and solvents were obtained from VWR Scientific (San Diego, CA)

RNA and Tissue Samples

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Standard human tissue RNA samples were purchased from BD Biosciences Clontech (Palo Alto, CA). Adult human brain RNA was obtained from whole brain from three healthy male/female Caucasians, ages 21-45 after sudden death from a fatal accident. Human fetal brain RNA was isolated from normal fetal brain tissue pooled from 21 spontaneously aborted male/female Caucasian fetuses, ages 26-40 weeks. Adult human liver RNA was pooled from healthy liver from seven male/female Caucasians, ages 22-40 after sudden death from a fatal accident. Human fetal liver RNA was isolated from healthy fetal livers from a pool of 63 spontaneously aborted male/female Caucasian fetuses, ages 22-40 weeks. Human kidney RNA was from a pool of 6 male/female Caucasians, ages 20-52 after sudden death from a fatal accident. Human lung RNA was from a pool of healthy lung tissue from three male/female Caucasians ages 15-40 that died from sudden death. Human small intestine RNA was isolated from a pool of small intestine tissue from five male/female Caucasians, ages 20-61 that died from sudden death.

Human brain tissues were obtained from the NIH-supported Brain and Tissue Bank for Development and Disorder at the University of Miami (Miami, FL) and the Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD). The ethics committee of Independent Review Consulting, Inc. (San Anselmo, CA) approved this study. Tissue samples were collected using protocols approved by the Tissue Banks. The midbrain sections were requested based on our earlier studies that showed the substantia nigra region of human brain was selectively labeled with anti-human FMO3 antibody (Cashman and Zhang, 2002). However, due to the small size of the embryonic brain, the portion of the section that represented the whole brain could be different compared to that of the adult brain. Human brain RNA samples were prepared from about 100 mg of tissue with Trizol reagent using a protocol

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from manufacturer (Invitrogen, Carlsbad, CA). RNA samples were prepared and stored at -80°C until analysis.

Reverse Transcription

cDNA was synthesized from human RNA samples using Superscript III (Invitrogen, Carlsbad, CA) and analyzed for housekeeping gene transcription. Based on results of housekeeping gene quantification (i.e., TATA-Box binding protein (TBP) and hypoxanthine phosphoribosyltransferase (HPRT)), samples that displayed less than 10% of the median level housekeeping gene expression were considered to possess poor RNA quality and were eliminated from further analysis. 60 cDNA samples were selected for *FMO* mRNA quantification. The cDNAs were stored at -20 °C until further evaluation.

Validation of Q-PCR conditions

Gene-specific primers were designed for human *FMO1-5* and housekeeping genes *TBP* and *HPRT* (Table 1). All q-PCR reactions were run in 96-well PCR plates using iCycler Thermal Cycler (BioRad, Emeryville, CA). The PCR reactions were prepared in 20 µl volumes for each sample using the following components: Bio-Rad SYBR super mix (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM each of dNTP, 3 mM MgCl₂, 25 U/ml iTaq DNA polymerase, SYBR Green I, 10 nM fluorescein, and stabilizers), gene specific primer, MgSO₄, H₂O and cDNA. The q-PCR conditions, including annealing temperature, primer concentration, and Mg²⁺ concentration were optimized using plasmid DNA as a template (for *FMO1* to 5) or standard tissue RNA as a template (i.e., for *TBP* and *HPRT*). The concentration of the specific primers and Mg²⁺ used were as indicated in Table 1. The q-PCR was run at 95 °C for 5 minutes followed

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with 50 cycles at 95 °C for 15 seconds, 55 °C (or as indicated for each specific gene listed in Table 1) for 30 seconds, and 72 °C for 30 seconds. At the end of the PCR cycling steps, melting curve data collection and analysis was enabled. For each plate, samples were set up in duplicate. For each standard series, duplicate no-template controls (NTC) were included for each gene analyzed on the same plate.

Data normalization and gene transcription quantification by a standard curve method.

To normalize the inter-sample variation in quality inherently associated with RNA preparation from human tissues, the transcription level of two housekeeping genes, *TBP* and *HPRT*, were quantified for all samples to obtain the normalization factors. The two housekeeping genes were selected because their low amount of transcription was comparable to the low amount of transcription of *FMO* in tissues such as human brain. Fetal brain RNA (BD Bioscience, Palo Alto, CA) was used to generate a standard curve. Fetal brain RNA was diluted in the presence of 100 ng/μl yeast tRNA to obtain 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 ng fetal brain RNA/μl to create a standard series. RNA standards were reverse-transcribed to generate the cDNA samples together with other tissue RNA samples. The standards and sample cDNAs were then subjected to q-PCR analysis for both *HPRT* and *TBP* transcription. A normalization factor for each individual sample was calculated based on the standard curve generated from the fetal brain cDNA standards. The normalization factors calculated from the standard curves were used for normalization of all *FMO* transcription data obtained from q-PCR analyses.

External calibration curves of recombinant DNA were generated from human *FMO* plasmid DNA and used for quantification calculations. Human *FMO1*, 2, 3, 4, and 5 gene fragments

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were amplified through RT-PCR and directly cloned into pCR2.1 using TA cloning methods as described by the manufacturer (Invitrogen, Carlsbad, CA). The sequence of selected clones was verified by re-sequencing and analysis. Plasmid DNA containing the specific *FMO* genes were prepared with midi-prep kits (Qiagen, Valencia, CA) and quantified based on UV absorbance at 260 nm. The plasmid DNA was then serial-diluted to 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 ag/ μ l to generate standard calibration curves for q-PCR analysis. Gene copy number for each gene was then calculated based on the molecular weight of each plasmid. Transcription levels of *FMO1*, 2, 3, 4, and 5 were analyzed with q-PCR for cDNA derived from human tissues.

Data processing and statistic analysis.

Statistical analysis was done with GraphPad Prism Programs. For analyzing age-dependent changes of *FMO* transcripts in brain, the 60 brain samples were placed into 5 groups based on their age distribution. The data were first analyzed with Kolmogorov-Smirnov (KS) test for data distribution normality. The data were then analyzed with Bartlett's test for equal variance or homogeneity. The confidence interval was set to be at least 95%. For data that passed Bartlett's test, the primary data was analyzed with an ANOVA1 test followed by Tukey's multiple comparison test to compare the significant difference between the different age groups. For data that did not pass Bartlett's test, primary data were transformed into lnY to reduce sample variability. The transformed data passed Bartlett's test and was then analyzed with an ANOVA1 test followed by Tukey's multiple comparison test to compare the significant difference between different age groups. For the analysis of transcription of each *FMO* gene in different tissues, data was analyzed with an ANOVA1 test followed by Tukey's multiple comparison tests.

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Results

Validation of the q-PCR assay.

In this study, we designed, optimized, and validated real-time RT-PCR methods for the quantification of human *FMO1*, 2, 3, 4 and 5 in liver, lung, kidney, small intestine and brain. The amplification of *FMO* (reverse transcribed cDNAs and external calibration standard plasmids) was linear over a wide range of input copies, and great sensitivity was achieved. As few as 10 molecules of *FMO* could be detected with the quantification methods established in this study. The large range of linearity of the assay (i.e., 10 - 10^5 copies/reaction) permitted the quantification of *FMO* transcripts in both high transcription tissue and low transcription tissue simultaneously and allowed direct quantitative comparison between the samples examined that possessed large differences. The quantification calculations were based on standard curves that determined the absolute amount of each *FMO* transcript, and it was therefore possible to cross-compare *FMO* transcript levels in different tissues, as well as among different genes.

Amplification of genomic DNA was avoided by using primer pairs that were located on different exons, and consequently, a DNase treatment of total tissue RNA samples was omitted. A two-step q-PCR approach was used, so that a single cDNA sample was generated from each tissue RNA. Random hexamers and oligodTs were used for the analysis of all genes examined (i.e., *FMO* genes and housekeeping genes, *TBP* and *HPRT*). This approach minimized data variation generated from multiple sample handling and multiple reverse transcription. Two housekeeping genes, *TBP* and *HPRT* were quantified for normalization of the cDNA input in each q-PCR reaction. These two genes were selected because their low level of mRNA was comparable to the low level of *FMO* transcripts in human brain. However, during the age-dependent analysis

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of brain, *HPRT* mRNA level was found to be down-regulated during later stages of gestation (i.e., 22-39 weeks) and in adult, while the *TBP* mRNA level remained constant (data not shown). Based on this finding, all analyses for *FMO* mRNA level presented below were normalized with *TBP* mRNA level.

Age-dependent mRNA regulation of FMO isoforms in brain. Steady state mRNA levels of *FMO1*, 2, 3, 4 and 5 in brain were examined in 60 samples. Based on the age of the donor, the samples were separated into 5 age groups (i.e., prenatal 18-21 weeks, prenatal 22-39 weeks, 0-8 months, 15-40 years, and 41-60 years) as shown in Figure 1. While the age groups were selected as above, correlation analysis was done between each age group using a Graphpad Prism Program and showed no significant difference of *FMO* mRNA level and each age group. Similarly, regression tree analysis broke the samples into four groups and also did not show a difference between *FMO* mRNA levels and each age group. When 25 and 75 percentile values were analyzed for each group (Graphpad Prism software), variation within each group was less than 10-fold (data not shown) except for the age 15-40 years group for *FMO1* (21-fold difference between the 25% and the 75% value). The scatter plot of data from all 60 samples was presented in Figure 1. Data from all age groups were analyzed for Gaussian distribution using the Kolmogorov-Smirnov test (Graphpad Prism software) to evaluate normal distribution. All data presented in Figure 1 passed the normality test (data not shown).

FMO1. *FMO1* mRNA level in brain was much less than that in other tissues examined and represented less than 1% of *FMO1* in a tissue rich in *FMO1* such as the kidney (see below). Changes in mRNA level of brain *FMO1* as a function of age were studied. Adult brain contained

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less *FMO1* than prenatal brain (Figure 1). An ANOVA¹ test followed by Tukey's multiple comparison test showed a significant difference between prenatal 18-21 week samples and the three postnatal groups (i.e., 0-8 months ($p<0.05$), 15-40 yrs ($p<0.05$), and 41-61 yrs groups ($p<0.001$)). The results indicated that the steady state of *FMO1* mRNA in brain was down-regulated after birth.

FMO2. *FMO2* mRNA level in brain was among the lowest observed for *FMO2* in the tissues examined and represented less than 1% of *FMO2* in lung (see Figure 2). No significant difference for *FMO2* was observed between the different age groups examined through prenatal and postnatal samples ($p>0.05$) (Figure 1).

FMO3. *FMO3* mRNA level in brain was among the lowest observed for *FMO3* in the tissues examined and represented less than 1% of *FMO3* in adult liver (see Figure 2). No significant statistical difference was observed for *FMO3* between the different age groups examined through prenatal and postnatal samples ($p>0.05$) (Figure 1).

FMO4. *FMO4* mRNA level in brain was among the lowest in the tissues examined and represented less than 1% of *FMO4* in tissues rich in *FMO4* such as adult liver or kidney (see below). No significant statistical difference was observed for *FMO4* between the different age groups examined from prenatal to postnatal samples ($p>0.05$) (Figure 1).

FMO5. *FMO5* mRNA level in brain was among the lowest in the tissues examined and represented less than 1% of *FMO5* in a tissue rich in *FMO5* such as adult liver (see below). No

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significant difference was observed for *FMO5* between the different age groups examined from prenatal to postnatal samples ($p>0.05$) (Figure 1).

Gender-selective regulation of mRNA levels of FMO isoforms in brain. Q-PCR data was also analyzed based on the gender of the sample donor. Because adult female samples were somewhat limited, we analyzed two age groups (i.e., prenatal samples, $n=11$ female and 13 male), and 0-4 months postnatal samples (i.e., $n=5$ female and 5 male). No significant differences were found for all *FMO* isoforms (i.e., *FMO1-5*) analyzed between the male and female groups at the indicated age (data not shown). Therefore, based on the limited number of samples examined, gender-specific regulation did not appear to affect *FMO* isoform mRNA steady state level during prenatal and early postnatal age in human brain.

Steady state mRNA levels of FMO isoforms in different tissues. Total human RNA from human liver, lung, kidney and small intestine was also quantified with the same standards used in the studies employing brain samples described above. The data was plotted in Figure 2 for specific *FMO* isoforms and in Figure 3 for specific tissues.

FMO1 mRNA was mainly detected in kidney, that contained *FMO1* in significantly greater amounts compared to all other tissues examined ($p<0.001$). *FMO1* transcripts in fetal liver and small intestine were 10-14 fold less than that observed in kidney, but there was no significant difference between fetal liver and small intestine ($p>0.05$). Transcripts of *FMO1* in lung were at the level of about 2.8% of *FMO1* in kidney. *FMO1* transcripts in brain and adult liver

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represented less than 1% of *FMO1* quantified in kidney. Adult liver contained significantly less *FMO1* than that of fetal liver ($p<0.001$).

FMO2 mRNA was mainly detected in lung tissue and contained *FMO2* in significantly greater amounts compared to all other tissues examined ($p<0.001$). *FMO2* transcripts in kidney were about 7-fold less than that observed in lung. The mRNA of adult liver and small intestine *FMO2* was approximately 2% of the amount of *FMO2* detected in the lung. Brain and fetal liver showed less than 1% of *FMO2* mRNA in the lung. Adult liver contained significantly more *FMO2* than that observed in fetal liver ($p<0.001$).

FMO3 mRNA was mainly detected in adult liver, which contained *FMO3* in significantly larger amounts compared to all other tissues examined ($p<0.01$). Lung, kidney and fetal liver contained similar amounts of *FMO3* mRNA that was approximately 4.5%, 3.7% and 2.1% of the amount in adult liver, respectively. Small intestine and both fetal and adult brain tissues contained *FMO3* mRNA in amounts less than 1% of the amount in adult liver.

FMO4 mRNA was mainly detected in adult liver and kidney, which contained *FMO4* in significantly greater amounts compared to others tissues examined ($p<0.01$). Fetal liver, small intestine, and lung contained approximately 10.9%, 10.8% and 7.0%, respectively, of the amount of *FMO4* in adult liver. Both fetal and adult brain contained *FMO4* in amounts less than 1% that of *FMO4* observed in adult liver.

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FMO5 mRNA was mainly detected in adult liver, which contained *FMO5* in significantly greater amounts compared with other tissues examined ($p < 0.001$). Steady state mRNA levels of *FMO5* in fetal liver, small intestine and kidney were at 18.1%, 12.8% and 9.8%, respectively, of the amount in adult liver and there was no statistically significant difference between these tissues ($p > 0.05$). *FMO5* mRNA levels in lung were 4% of the amount detected in adult liver. Both fetal and adult brain contained *FMO5* in an amount less than 1% of the *FMO5* present in adult liver.

Steady state mRNA of different FMO isoforms in the same tissue.

In adult liver, the mRNA levels of *FMO3* and *FMO5* were at a similar level ($p > 0.05$), even though *FMO3* was the FMO isoform commonly reported to be prominently expressed in adult liver (Cashman, 1995). Although *FMO5* represented approximately 46% or more of the total FMO transcripts in adult human liver, the enzyme activity contribution of *FMO5* to hepatic metabolism has not been clearly established. *FMO5* does not apparently catalyze the oxygenation of common FMO substrates (i.e., methimazole, ranitidine, cimetidine) (Overby et al., 1997). The question whether *FMO5* has a selective substrate specificity different from other FMOs remains to be determined. *FMO4* mRNA levels in adult liver were found to be about 18% of *FMO3* and *FMO5*. *FMO1* and *FMO2* mRNA levels were at the level of 0.4% and 4% of that of *FMO5*, respectively.

In fetal liver, *FMO5* mRNA was the prominent *FMO* form and was present at the highest amounts measured. Other *FMOs* showed moderate mRNA amounts with *FMO1* at 23%, and *FMO3* and *FMO4* at 11% of the amount of *FMO5* mRNA levels in fetal liver. *FMO2* mRNA in fetal liver was at about 2.3% of the amount of *FMO5*.

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Comparison of the mRNA level of *FMO* between fetal and adult liver showed that *FMO1* was down-regulated about 10-fold, and *FMO3* was up-regulated about 50-fold from fetal liver to adult liver. We also observed that *FMO1* was the only *FMO* isoform that was down-regulated in adult liver, and all other *FMOs* were detected with greater amounts in adult liver compared with fetal liver.

In kidney, *FMO1* was the prominent isoform observed. *FMO2* was observed at approximately 76% of the amount of *FMO*. Transcripts of *FMO3*, *FMO4* and *FMO5* were 11.7-, 2.8- and 4.1-fold less, respectively, than that of *FMO1*

In lung, results showed that the mRNA level of *FMO2* was over 150-fold higher than that of *FMO1* and *FMO4* and about 50-fold higher than that of *FMO3* and *FMO5*. *FMO2* has been reported to be the dominant form of *FMO* transcribed in lung in rabbit (Larsen-Su et al., 1999). Because a functional allele encoding full-length functional *FMO2* is lacking in Caucasians and is at a frequency of 13-20% in African Americans (Whetstone et al., 2000), the functional significance of high amounts of *FMO2* transcripts remains to be established.

In small intestine, *FMO5* was the dominant form detected. *FMO1*, *FMO2*, and *FMO4* mRNAs were found at similar levels of 22%, 39% and 15%, respectively, to that of *FMO5*. Of all the *FMOs* examined, *FMO3* was at the lowest amount in small intestine.

Discussion

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Quantitative RT-PCR was used to determine the tissue-specific steady state mRNA levels for *FMOs* 1, 2, 3, 4 and 5, and set the stage for future investigations of tissue-specific regulation of FMO isoforms in human. Steady state mRNA levels in tissue are generally controlled by transcription regulation, alternative splicing, and mRNA degradation. Information regarding the regulation of *FMO* transcription is beginning to emerge. Characterization of the promoter region and identification of regulatory elements for *FMO* have been reported recently (Hines et al., 2003; Koukouritaki et al., 2005; Shehin-Johnson et al., 1996). Dominant mRNA levels of *FMO1* in adult kidney, *FMO2* in lung, and *FMO3* and *FMO5* in adult liver, suggest the promoter regions of these FMOs likely contain regulatory elements responsive to transcription factors specifically present in corresponding tissues. Identification of such regulator elements at the genetic level and tissue-specific transcription factors at the protein level will be essential for further understanding the regulatory mechanism for FMO isoforms. Alternative splicing of *FMOs* have been reported in human tissues including liver, kidney, and brain (Lattard et al., 2004). With the exception of *FMO4* in human brain, alternative splicing does not appear to be the major factor contributing to the tissue-specific regulation of FMOs. To minimize the complexity of the current study, the q-PCR primers were carefully designed to avoid *FMO* alternative spliced regions and allow quantification of total transcripts. There is little information available regarding the RNA stability regulation for FMO isoforms. The RNA stability regulation is especially interesting for *FMO2* because human *FMO2* mRNA is present at high levels in lung even though there is generally no functionally active enzyme expressed (i.e., whites, Asians) due to a common polymorphism resulting in a premature termination of the protein open reading frame, with the exception of a small percentage of population carrying the functional form (Krueger et al., 2004). Full length *FMO2* is expressed in rabbit, monkey and

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wild rat. Apparently, humans retain the genetic regulatory elements present in other small animals (i.e., rabbit and rat) for high level transcription of *FMO2* in lung despite the non-functional common polymorphism. Generally, mammals are protected from accumulation of transcripts containing a premature termination codon (PTC) like *FMO2* by nonsense-mediated decay, a mRNA surveillance mechanism, that leads to selective degradation of PTC-containing transcripts (Holbrook et al., 2004). However, rare mutations sometimes give rise to truncated proteins that overwhelm the proteolytic system of the cell and cause toxic precipitation of insoluble protein chains (Thein et al., 1990). Abnormal transcripts not degraded could also result in synthesis of truncated, dominant oncoproteins. To date, neither of these situations has been observed for *FMO2*.

Age-dependent regulation of mRNA levels are not statistically significant for *FMO2*, 3, 4 and 5 in human brain. *FMO1* mRNA levels in the brain are down-regulated in adult relative to the neonate. Overall, *FMO* mRNA levels in brain are much lower than that in most other tissues examined and at the level of less than 1% as compared to the most abundant tissues observed (i.e., *FMO1* in kidney, *FMO2* in lung, and *FMO3* and 5 in liver). Although FMO functional activity has been reported in animal (Bhamre et al., 1995; Bhamre et al., 1993; Kawaji et al., 1995) and human brain (Bhagwat et al., 1996; Bhamre et al., 1995), based on the data herein, it is not clear whether FMOs make a significant contribution to functional chemical metabolism in human brain. This study focuses on brain tissue from the substantial nigra where FMO has been reported (Cashman and Zhang, 2002), other regions of the brain may be selectively enriched in FMO. For example, in mouse brain, *FMO1* and 5 was localized to newborns of the cerebrum (Janmohamed et al., 2004) and *FMO1* was detected in the choroid plexus, a region rich

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in blood vessels. Age-dependent mRNA levels of FMOs were also evaluated using pooled fetal liver and adult liver. With the exception of FMO1, whose mRNA level is higher in fetal liver, all other FMOs (i.e., FMO 2, 3, 4, and 5) show substantially higher mRNA level in adult liver. Future studies will be required to more fully define the transcription regulation and functional consequences of FMO expression, and examine whether exposure during the neonate time period is different from that of adult for chemicals and drugs detoxicated by FMOs.

Based on the size of the kidney and the *FMO* transcripts copy number, we estimate the *FMO1* transcripts in adult kidney is approximately 33-fold less than *FMO3* transcripts in the adult human liver. Since FMO1 accepts larger substrates than FMO3 (Kim and Ziegler, 2000), it is interesting to investigate whether larger substrates are oxygenated by kidney FMO1 in preference to hepatic FMO3. Supporting previous work, *FMO3* transcripts are present in kidney, lung, and small intestine in low abundance, and are upregulated in the adult liver of humans. In male mice, *FMO3* transcripts were shown to be down-regulated in liver, but female mice contained 80-fold more FMO3 mRNA than male mice (Cherrington et al., 1998; Janmohamed et al., 2004). The gender specific transcription is not found in human brain, and is not determined in human liver in this study. While *FMO4* transcripts are present in adult liver and kidney to a similar extent, the functional significance of human FMO4 is not clearly understood. Contributing to the uncertainty of FMO4 functional activity is the difficulty of expression and purification of recombinant FMO4 and the lack of stability of the isolated protein (Lattard et al., 2003). The paucity of human FMO4 functional studies has hampered establishment of substrate specificity and an estimate of the contribution of FMO4 to FMO-mediated metabolism. *FMO5* transcripts are found to be equally prevalent as *FMO3* in human adult liver. *FMO5* is also the

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most abundant *FMO* transcripts present in human small intestine and may contribute to intestinal first pass metabolism. The functional significance of FMO5 is not clear because FMO5 does not oxygenate typical FMO substrates (i.e., methimazole, ranitidine or cimetidine) (Cherrington et al., 1998; Overby et al., 1995; Rettie et al., 1994). FMO5 has been reported to *S*-oxygenate thioethers with a proximal carboxylic acid, a somewhat unique FMO substrate activity (Ohmi et al., 2003). It has been difficult to establish a substrate specificity for FMO5 due to the relatively low contribution of enzyme functional activity associated with the majority of FMO-mediated hepatic metabolism. We hypothesize that, as new specific substrates are discovered, FMO5 may show selective functional activity.

The quantitative nature of this study provides information on an absolute level for *FMO* transcripts and makes it possible to compare amounts of *FMO* mRNA from different tissues, at different ages, and at different stages of development. However, this study is not focused on the amount of protein expression or the activity of the FMO isoforms. A comparison of the mRNA levels of different *FMOs* with previously reported FMO protein levels in various tissues and during development indicates that, in most situations examined, the level of mRNA detected in this study reflects the FMO protein expression profile qualitatively, indicating regulation at this level is presumably one of the major regulatory mechanisms for FMO tissue-specific expression. For example, human hepatic FMO1 expression is restricted to the fetus whereas FMO3 and FMO5 are expressed in adult liver. However, the relationship between steady state mRNA level and the FMO enzyme level does not always prove to be quantitatively translated. Overby et al., reported human adult liver FMO3 and FMO5 levels that ranged from 12.5 to 117 and 3.5 to 34 pmol/mg of microsomal protein, respectively (Overby et al, 1997). In the previous study, the

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concentration of FMO3 was greater than that of FMO5 in all samples examined, but the ratio of FMO3 to FMO5 varied from 2:1 to 10:1. The mRNA levels of FMO3 and FMO5 we observed in human liver show no significant differences. For FMO1, maximum protein levels in the fetal liver during the first trimester was reported with a median value of 7 pmol/mg of microsomal protein, although values ranged as high as nearly 20 pmol/mg of microsomal protein (Koukouritaki et al. 2002). This was in agreement with another report of fetal liver FMO1 levels of 14.4 ± 3.5 pmol/mg microsomal of protein (Yeung et al. 2000). In contrast, adult FMO1 kidney levels were reported to be 47.0 ± 9.0 pmol/mg of microsomal protein. The fold-difference in FMO1 protein levels between fetal liver and kidney previously reported was considerably less than the 10.7-fold difference in *FMO1* transcript levels identified in the study reported herein. High level of FMO2 transcripts in lung does not translate to functional FMO2 protein in most of the population as discussed earlier. Therefore, the systematic study herein provides a general picture for *FMO* transcripts in human liver, kidney, lung, small intestine, and brain. How does such information translate to the functional aspects at the protein level is controlled by various other aspects of regulation and remains to be evaluated empirically.

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Footnote:

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Legends for Figures

Figure 1. Analysis of age-dependent steady-state mRNA levels of *FMO* isoforms in human brains. Column scatter plots (GraphPad Prism Software) were used to show *FMO* isoform mRNA in different age groups (i.e., prenatal 18-21 weeks, n=12; prenatal 22-39 weeks, n=12; 0-8 month, n=15; 15-40 years, n=9; and 41-61 years, n=12). The mRNA levels displayed represented copies of respective *FMO* transcripts per μg RNA normalized with mRNA level of *TBP*. The horizontal line in each column represented the median transcription value for the specific age group. Statistic analysis was described in the text.

Figure 2. Steady-state mRNA levels of *FMO* isoforms in different tissues. Column bar graphs (GraphPad Prism Software) were used to show the mRNA levels of each *FMO* isoform in different human tissues examined. The mRNA levels displayed represented copies of respective *FMO* transcripts per μg RNA normalized with mRNA level of *TBP*. Statistical differences were determined by ANOVA1 test followed by Tukey's multiple comparison test: ** indicated $p<0.001$ with comparison to all other groups, * indicated $p<0.01$ with comparison to all other groups. For the *FMO4* graph, **a indicated $p<0.001$ with comparison to all other groups except kidney ($p>0.05$), *b indicated $p<0.01$ with comparison to all other groups except AL ($p>0.05$)).

Figure 3. Tissue-dependent mRNA levels of *FMO* in human. Column bar graphs (GraphPad Prism Software) were used to show the mRNA levels of *FMO* isoforms in the different human tissues examined. The mRNA levels displayed represented copies of respective *FMO* transcripts per μg RNA normalized with mRNA level of *TBP*.

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Table 1. Q-PCR primers and conditions. PCR primers used for the q-PCR analysis of target *FMO* genes and house keeping genes are listed with their exon location indicated. Validated q-PCR conditions including annealing temperature (T_m), Mg^{2+} concentration (Mg^{2+} , mM), primer concentration (Primer nM), and amplification efficiency (E) calculated from standard curves are listed.

Target Gene	Primers and exon	primer sequence	T_m	Mg^{2+} (mM)	Primer (nM)	E
<i>hFMO1</i>	F1F711, ex5/6	acctggcggaaaaggtgt	55°C	4	400	1.87
	F1R823, ex6	catgttctgaaagcgtgtcat			800	
<i>hFMO2</i>	F2F1187, ex7	gttccattttcccaactgct	54°C	4	400	1.65
	F2R1333, ex9	tctggcttttccaaacaggt			800	
<i>hFMO3</i>	hF3F1223, ex7	ctgccattcccacagttgac	55°C	3	800	2.0
	hF3R1420, ex9	ccccaatgaaggaggagagt			400	
<i>hFMO4</i>	hF4F627, ex4	ggactatctccaagaatttgctg	54°C	4	400	1.96
	hF4R755, ex5	ctctatttgcttgccctctg			800	
<i>hFMO5</i>	F5F1273, ex8	acattgccctcacagagtga	54°C	4	400	1.86
	F5R1411, ex9	ccaccaaatacgaagctct			200	
<i>HPRT</i>	HPRTF407 ex4	gaccagtcaacaggggacat	56.5°C	4.5	800	2.13
	HRPTR588 ex7	gtcctttcaccagcaagct			800	
<i>TBP</i>	TBPF243 ex2	acaacagcctgccaccttac	55°C	3	500	2.01
	TBPR430 ex3	ctgctgctgtgtgttgct			500	

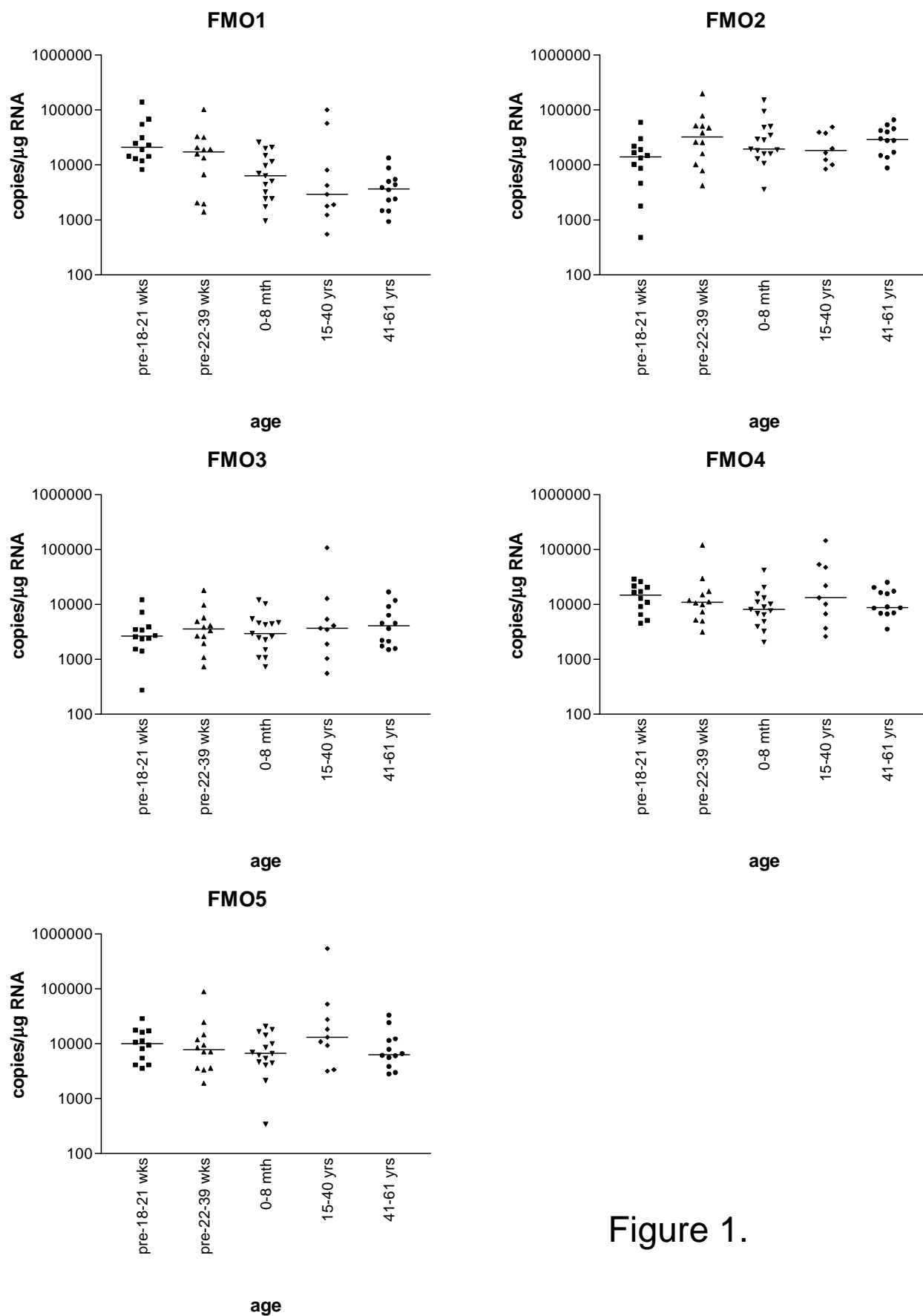


Figure 1.

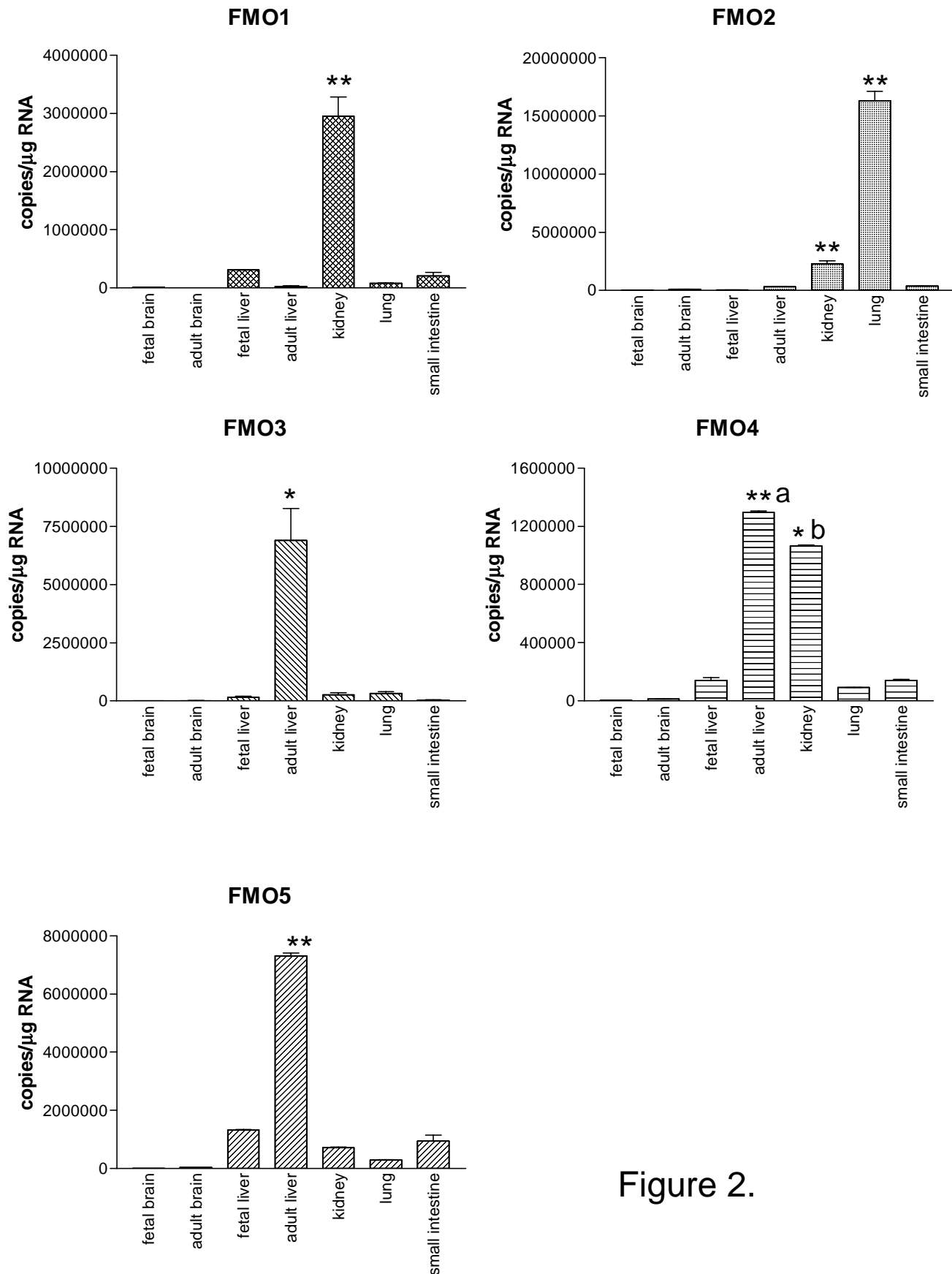


Figure 2.

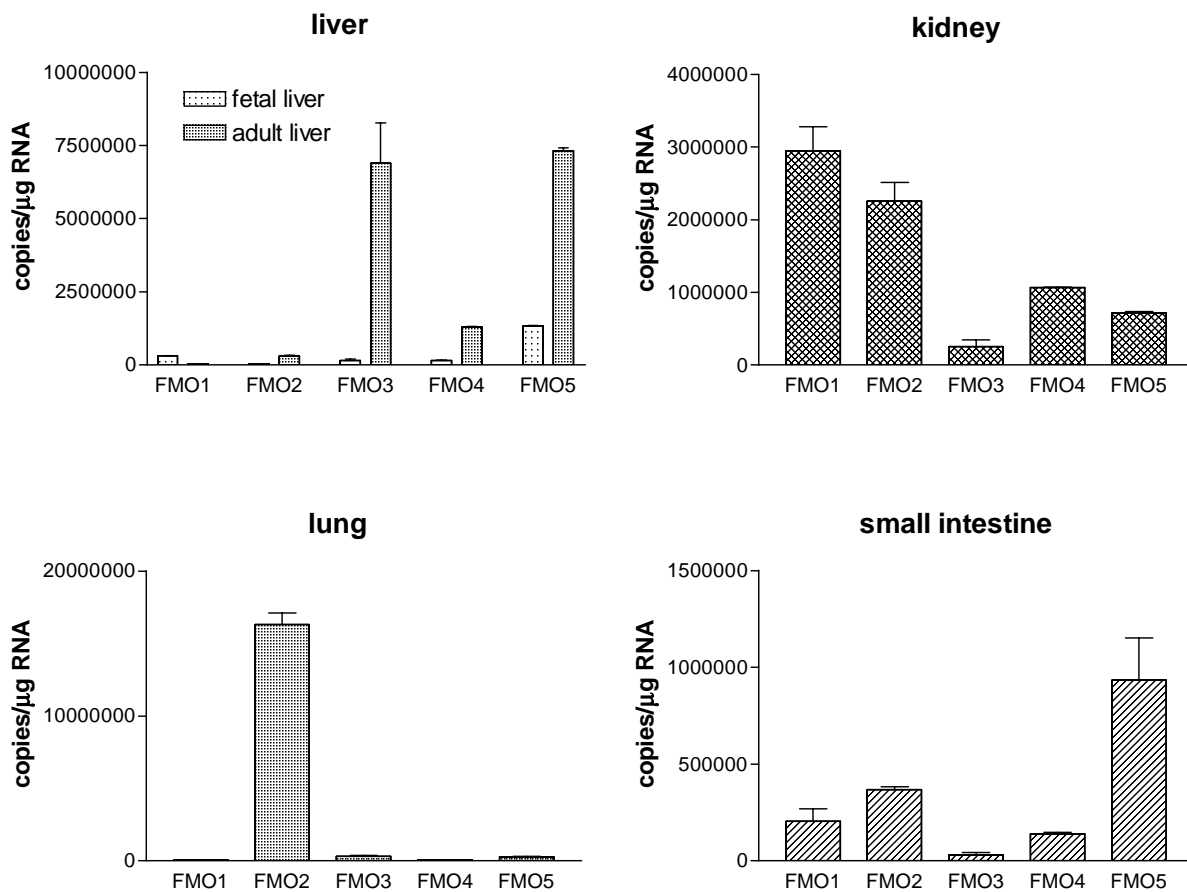


Figure 3.