



Role of paralogs in the sex-bias transcriptional and metabolic regulation of the brain-placental axis in mice

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ABSTRACT

Introduction: Duplicated genes or paralogs play important roles in the adaptive function of eukaryotic genomes. Animal studies have shown evidence for the functional role of paralogs in pregnancy, but our knowledge about the role of paralogs in the fetoplacental regulation remains limited. In particular, if fetoplacental metabolic regulation is modulated by differential expression of paralogs remains unexamined.

Methods: In this study, gene expression profiles of day-15 placenta and fetal brain were compared to identify families or groups of paralogous genes expressed in the placenta and brain of male versus female fetuses in mice. A Bayesian modeling was applied to infer directional relationship of transcriptional variation of the paralogs relative to the phylogenetic variation of the genes in each family. Gas chromatography-mass spectrometry (GC-MS) was used to perform untargeted metabolomics analysis of day-15 placenta and fetal brain of both sexes.

Results: We identified paralog groups that were expressed in a sex and/or tissue biased manner between the placenta and fetal brain. Bayesian modeling showed evidence for directional relationship between expression and phylogeny of specific paralogs. These relationships were sex specific. GC-MS analysis identified metabolites that were expressed in a sex-bias manner between the placenta and fetal brain. By performing integrative analysis of the metabolomics and gene expression data, we showed that specific groups of metabolites and paralogous genes were expressed in a coordinated manner between the placenta and fetal brain.

Discussion: The findings of this study collectively suggest that paralogs play an influential role in the regulation of the brain-placental axis in mice.

1. Introduction

When a genomic region containing a protein coding gene is duplicated, the newly created gene is referred to as duplicated gene or paralog. Paralogs contribute to evolutionary innovation of eukaryotic genomes to acquire novel adaptive functions [1,2]. They contribute to the regulation of adaptive functions including those related to reproduction and early developmental processes in animals [3–6]. Gene duplication generates novel ligand proteins to promote cellular interactions within and between tissues for novel adaptive functions [7]. In addition, paralogs also contribute to functional diversity of metabolic pathways to respond to different physiological conditions [8].

Both paralogs (i.e. homologous genes within species) and orthologs (i.e. homologous genes between species) contribute to the evolution of genome function. The theory of ortholog conjecture suggests that orthologous genes are functionally more similar than paralogous genes [9]. However, gene expression data suggest that functional similarity among genes is more reliant on the biological function of the tissue and cell types in which the genes are expressed rather than the orthology or paralogy relationship among the genes [10,11]. Moreover, selection on gene expression is also known [12,13]. Directional selection refers to the process when a trait, either molecular or phenotypic, changes in a directional manner with the phylogenetic relationships of the genes [12–15]. Neofunctionalization is a selective process that shapes

Abbreviations: Paralog groups, (PG); Gas chromatography–mass spectrometry, (GC-MS); Markov Chain Monte Carlo, (MCMC); Log Bayes Factor, (LBF); Gene Ontology, (GO).

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transcriptional diversity of newly duplicated genes [1,10,16]. On the other hand, sex-bias gene expression helps resolve conflicts in reproductive fitness of genes between males and females, also known as sexual conflict [17–19].

Animal experiments have shown that paralogs play important roles in the adaptive function to respond to stress conditions during pregnancy [20,21]. In mammalian reproduction, the placenta plays adaptive functions to support fetal survival and growth. Placental functions safeguard the fetus from nutrient deficiency [22], metabolic abnormalities [23–25], and adverse maternal conditions [26–28]. Our previous study has shown that several paralogs are coordinately regulated between the placenta and fetal brain in mice [29]. In addition, we have shown that placenta differentially expresses a set of ligand genes when the uterus lacks the Forkhead box a2 (*Foxa2*), a key gene for embryo implantation [30]. Subsequently, we also showed that mouse fetal brain regulates gene expression in a sex-bias manner relative to the placenta in response to the uterine ablation of *Foxa2* [31]. Our research further demonstrated that epigenetic and metabolic changes in the placenta differentially influence the aging program of male versus female fetal brain [32,33]. Whilst these studies have suggested functional relevance of the brain-placental axis to the developmental programming of the fetus [28], role of paralogs in the regulation of brain-placental axis remains poorly understood.

The present study has three specific aims. The first aim is to identify paralogs that are expressed in the placenta and fetal brain. The second aim is to determine if fetoplacental expression of paralogs is correlated with phylogenetic relationship of the genes. In the third aim, we investigate the relevance of paralogs in the metabolic regulation of the brain-placental axis.

2. Materials and method

2.1. Animal breeding

The C57BL/6J mice were obtained from the Jackson Laboratory. Three male and three female mice, all about 8-weeks old, were used to establish timed pregnancy as described earlier [29]. The vaginal plug was observed to mark the start time of pregnancy (day 1). On day 15, the pregnant mice were euthanized, and the fetuses and corresponding placentae were collected [31]. From each fetus, the whole brain was dissected [32]. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri, and were conducted according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA).

2.2. Inferring phylogeny of paralogous genes expressed in the placenta and fetal brain

The gene expression data of day-15 placenta and fetal brain of both sexes (accession number GSE157556) were analyzed to identify paralogs expressed in the placenta and fetal brain. The mean expression values of individual genes, as the read counts per million mapped reads, were used in the analysis. The *Ensembl* homology annotation [34] was used to identify paralog groups (PGs) expressed in the placenta and fetal brain. We defined PG as a group of paralogs that satisfied two conditions: 1) at least 25 % sequence similarity among the member genes in the group, and 2) at least 5 genes are expressed in the placenta and fetal brain in the group. We used these criteria to make sure that all the phylogenetic trees we constructed in our analysis had at least 5 genes, and all these genes were expressed in the placenta and fetal brain. Genes annotated as predicted coding genes (genes with no symbol) were excluded. Therefore, not all genes annotated as paralogs by *Ensembl* were included in our analysis. We have provided the computer codes that were used to extract the paralog groups from the *Ensembl* annotation of mouse genes (Supplementary Text 1). After identifying the PGs, the coding sequences of the paralogs were extracted from the reference genome, and subjected to

multiple sequence alignment by *Clustal Omega* [35] for each PG. The aligned sequences were used to generate neighbor-joining phylogenetic trees using *Phyx* [36]. The root of each tree was placed at the common ancestor of the PG based on *Ensembl* homology [34] using *Ape* 5.0 [37]. The timeline of common ancestor was according to the *TimeTree* annotation [38].

2.3. BayesTraits modeling for inferring directional selection of paralog expression

Gene expression changes between placenta and fetal brain, within each PG, were modeled using the *BayesTraits* tool [39]. *BayesTraits* is a Bayesian framework to perform model-based analyses of evolutionary selection of phenotypic or molecular traits [40–42]. We used *BayesTraits* directional selection models [39] to test if expression changes of paralogs were correlated with the root to tip distances (i.e. directional changes) of the phylogeny. This was achieved using a nested modeling approach implemented in *BayesTraits*. In this approach, first we analyzed the trait data (gene expression) and the rooted phylogenetic tree (in nexus format) of each PG using model A which is referred to as the random walk model. This model assumes that the relationship between phylogeny and gene expression is random and there is no correlation of gene expression changes with root to tip distances of the tree. Next, we analyzed the same data using model B which is the directional model. Model B assumes that gene expression changes are correlated with the root to tip distances of the phylogenetic tree. We used MCMC (Markov chain Monte Carlo) and Stepping stones iteration (100 stones and 1,000 iterations per stone) methods [43] implemented in the *BayesTraits* to estimate the log marginal likelihood (LML) of model A and model B for each PG. The likelihood estimates were then compared between the two models to infer significance of directionality based on the Log Bayes Factor (LBF). The LBF is calculated as the difference in the LML value of model B relative to that of model A and then multiplied by 2. The LBF value > 2 suggests evidence for directional selection [44] implying significant correlation of gene expression changes with the phylogeny. We have provided the computer codes (Supplementary Text 2) to perform the *BayesTraits* analysis.

2.4. Metabolomics analysis

The day-15 placenta and fetal brain of male and female fetuses, each in three replicates, were used for untargeted metabolomic profiling by gas chromatography–mass spectrometry (GC-MS). An Agilent 6890 GC coupled to a 5973 N MSD mass spectrometer was used for GC-MS analysis. Sample processing and GC-MS analysis were performed at the University of Missouri Metabolomics Center. Sample processing was performed as described in detail in our earlier work [33]. The spectral analysis was performed by the AMDIS (Automated Mass Spectral Deconvolution and Identification System), and metabolites were identified using a commercial NIST17 mass spectral library. The abundance of the metabolites was determined by the Metabolomics Ion-Based Data Extraction Algorithm (MET-IDEA).

2.5. Integrative metabolomics and transcriptomics analysis

The metabolites identified from the GC-MS analysis were mapped to the gene-chemical interaction database [45] which curates experimentally verified interactions between genes and metabolites. The mapping allowed us to identify the paralog-metabolite pairs expressed in the placenta and fetal brain, and to integrate the RNA-seq and metabolomics data. The combined metabolomics and transcriptomic data were then subjected to hierarchical cluster analysis to identify association between metabolites and paralogs in the placenta and fetal brain.

2.6. Computational and statistical analysis

All computational analyses including gene sequence extraction, sequence alignment, and phylogeny inference were performed using published open source tools mentioned above. The *BayesTraits* data modeling was performed using the University of Missouri high performance computing (HPC) cluster *Lewis* via batch job submission through *Slurm*. All statistical analyses and plotting were performed in *R*.

3. Results

3.1. Identification of paralogs expressed in the placenta and fetal brain

The transcriptomic data of placenta and fetal brain from day-15 pregnant C57BL/6J mice (accession # GSE157555) were analyzed, as shown in Fig. 1A, to identify expression of paralogs. The analysis identified a total of 1,725 paralogs expressed in the placenta and fetal brain of both sexes (Supplementary Table 1). Based on *Ensembl* homology annotation [34], we identified a total of 183 groups of paralogs (PGs). The groups varied in number of member genes. The majority of the groups contained less than 20 genes, but a few groups showed relatively larger size (Supplemental Fig. 1). The paralogs showed different phylogenetic relationships among each other within each group (Supplementary Table 2). By integrating data of *Ensembl* [34] and *TimeTree* [38] annotation with the inferred phylogeny of the paralogs, we observed that the majority of the paralogs expressed in the placenta and fetal brain were duplicated prior to the origin of placenta, which is estimated ca. 90 million years ago [46]. On the other hand, paralogs that originated post-placentalia were significantly over-represented (hypergeometric test, $p < 0.05$) by genes that were recently duplicated within the mouse genome (Supplemental Fig. 1) suggesting a relationship between phylogeny and expression variation of the paralogs.

3.2. Evidence for directional expression of paralogs between placenta and fetal brain

To dissect the relationship between phylogenetic variation and

expression variation of the paralogs, we modeled expression data of the paralogs relative to gene phylogeny using *BayesTraits* [39]. The overview of the modeling strategy is illustrated in Fig. 1B, and described in detail in the methods section. The results of this analysis revealed evidence for sex-specific directional selection of gene expression within specific PGs ($n = 12$) (Supplementary Table 3). Of the 12 significant PGs, 7 were specific to female fetuses and the other 5 were specific to male fetuses. The directional expression changes of paralogs of PG67 were significant in females but not in males, whereas that of PG181 were significant in males but not in females. The paralogs in both PG181 and PG67 were expressed at a higher level in the placenta compared to the fetal brain. However, the variability of gene expression within PG181 was relatively higher in males than females whereas that of PG67 showed an opposite pattern (Fig. 2). We further observed that expression of individual member genes varied differentially within groups (Fig. 3). Fig. 3 shows expression patterns of pregnancy-specific glycoprotein (Psg) genes in the group PG34. The group PG34 showed evidence of directional selection based on *BayesTraits* modeling (Supplementary Table 3). Within this group, specific paralogs (Psg21, Psg23 and Psg27) showed distinct cluster patterns in gene expression as well as genetic variation compared to other member genes (Supplementary Fig. 2).

3.3. Paralogs associated with metabolic regulation between placenta and fetal brain

Based on Gene Ontology (GO) enrichment analysis, the paralogs that showed evidence for directional selection were significantly over-represented (Fisher Exact test, $p < 0.05$) with different metabolic processes (Supplementary Table 4). To validate this prediction and further examine the role of paralogs in the brain-placental metabolism, we performed an untargeted metabolomics analysis of day-15 placenta and fetal brain of both sexes by gas chromatography-mass spectrometry (GC-MS). A total of 139 metabolites were detected in the placenta and brain of male and female fetuses (Supplementary Table 5). We identified specific metabolites ($n = 8$) that were significantly ($p < 0.05$) male-biased in the fetal brain (Fig. 4A). While no male-bias metabolite was observed in the placenta, a relatively greater number of metabolites (n

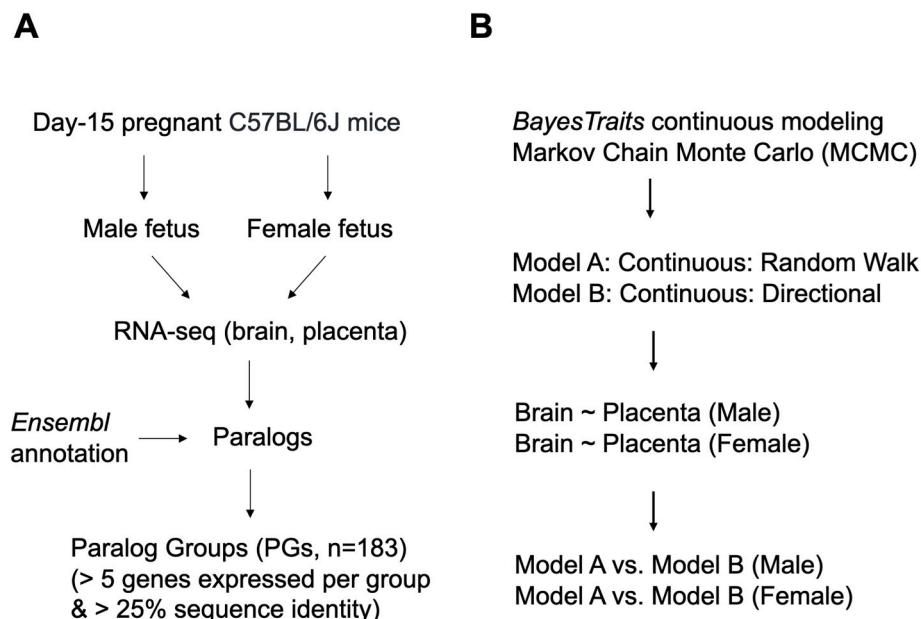


Fig. 1. Overview of the study approach. A) RNA-seq data of placenta and fetal brain of both sexes from day15-pregnant C57BL/6J mice were analyzed to identify paralogs based on *Ensembl* homology annotation of mouse genes. If five or more paralogous genes were expressed in the placenta and fetal brain, and sequence similarities among these genes were more than 25 %, the genes were assigned to a single paralog group (PG). B) Gene expression variation between placenta and fetal brain was modeled relative to the phylogenetic tree of the member genes within each PG using *BayesTraits*. Model likelihoods were estimated by MCMC in males and females separately, and directional selection of paralog expression was inferred based on Log Bayes Factor (see methods for detail).

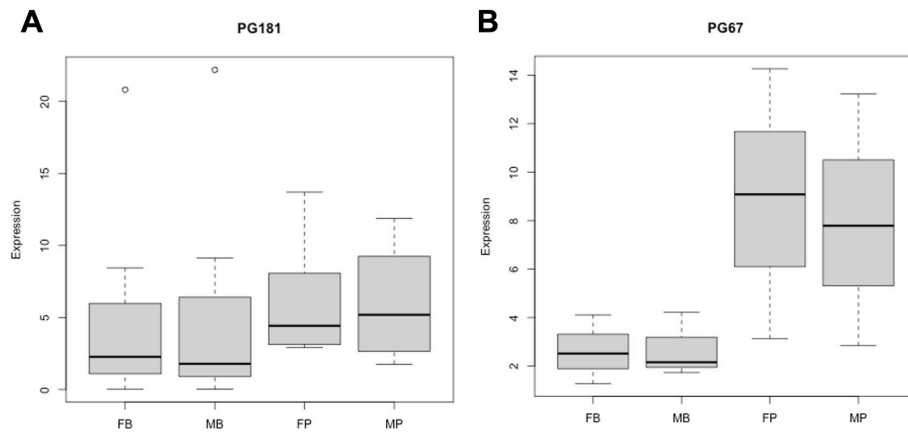


Fig. 2. Variation of paralog expression between sexes. The boxplots show expression patterns of genes within PG181 (A) and PG67 (B) in the placenta and brain of male and female fetuses.

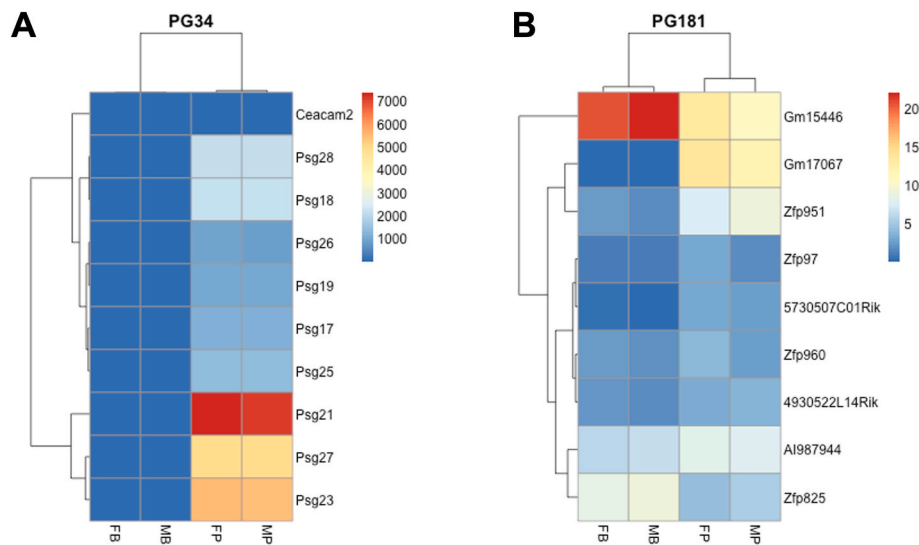


Fig. 3. Heatmap of gene expression variation within paralog groups. Specific genes are expressed at a higher level than other member genes in the placenta compared to the fetal brain (A) or vice versa (B). Gene expression level is represented by the color-coded scales.

= 39) showed female-bias expression in the placenta and/or fetal brain (Fig. 4B). Among these, 13 metabolites were commonly female-biased in both the placenta and fetal brain (Fig. 4C). They included butylmethylsilane, trimethylsilane, dimethyl oxalate, ethylbis(trimethylsilyl) amine, proline, umbelliferone, and 1-Docosanol. Cholesterol was the only metabolite that showed significant sex differences (2x2 contingency test, $p < 0.0001$) between placenta and fetal brain but in a differential manner (Fig. 4D). It was male-biased in the fetal brain but female-biased in the placenta.

Next, we mapped the metabolites to the gene-chemical interaction database [45], which curates experimentally verified interactions between genes and chemical compounds, to identify paralogs associated with the metabolites expressed in the placenta and fetal brain (Supplementary Table 6). Hierarchical cluster analysis of the combined metabolomics and transcriptomic data showed two distinct clusters A and B (Fig. 5A). The cluster A represented methionine-associated genes of PG40, PG18 and PG104. The cluster B contained two subclusters: B1 and B2. The B1 sub-cluster represented cholesterol-associated genes of PG66, PG48, PG135 and PG36. On the other hand, the B2 sub-cluster represented genes associated with multiple metabolites including succinic acid, citric acid, umbelliferone, glycine, steric acid, linoleic acid, and oleic acid. These genes were identified as paralogs of PG60, PG151 and PG169. The top 10 metabolites and paralogs associated with these

clusters are shown in Fig. 5 B and C. The distinct expression patterns of the paralogs and metabolites suggested that metabolism of the brain-placental axis was regulated by coordinated expression of paralogs in distinct clusters. Based on these results, we propose a model (Fig. 6) relating to the metabolic regulation of the brain-placental axis, which is discussed further in the following section.

4. Discussion

The current study investigated expression variation of paralogs between placenta and fetal brain in a phylogenetic context. A key finding of this study is that expression of specific paralogs varies between placenta and fetal brain in a directional manner with gene phylogeny. We show evidence for sex-specific directional selection of paralog expression in the brain-placental axis. While adaptive functions of the placenta help safeguard fetal growth during adverse pregnancy conditions such as hypoxia and malnutrition [47], in normal pregnancy the placenta is also adaptive to sex differences of the fetus [48]. Recently, we showed that the placental epigenome is linked to the sex-bias aging of the mouse brain [32]. While placenta safeguards fetuses of both sexes, male fetuses are more vulnerable to gestational risks compared to female fetuses [49]. Also, male and female fetal brains are associated with differential metabolic changes during early development [25]. The sex

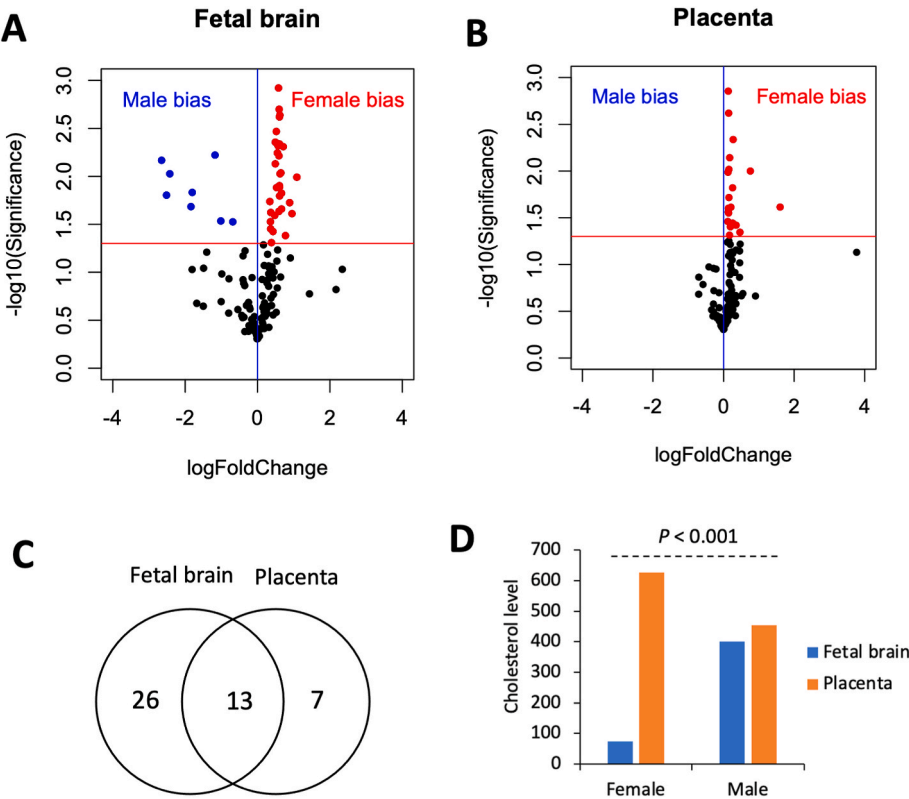


Fig. 4. Sex-bias metabolism of placenta and fetal brain. Volcano plot shows sex-bias metabolites in the fetal brain (A) and placenta (B). Each dot represents a metabolite. The color codes are as follows-blue: male bias, red: female bias, and black: no significant bias. The red horizontal line represents a significance level of 0.05 (in log scale). The vertical blue line shows fold change of metabolites between sexes (in log scale). C. Venn diagram shows female-biased metabolites observed both in the placenta and fetal brain. Off note, there was no male-bias metabolite common in fetal brain and placenta. D. Cholesterol level in the placenta and brain of the male versus female fetuses (significance level shown).

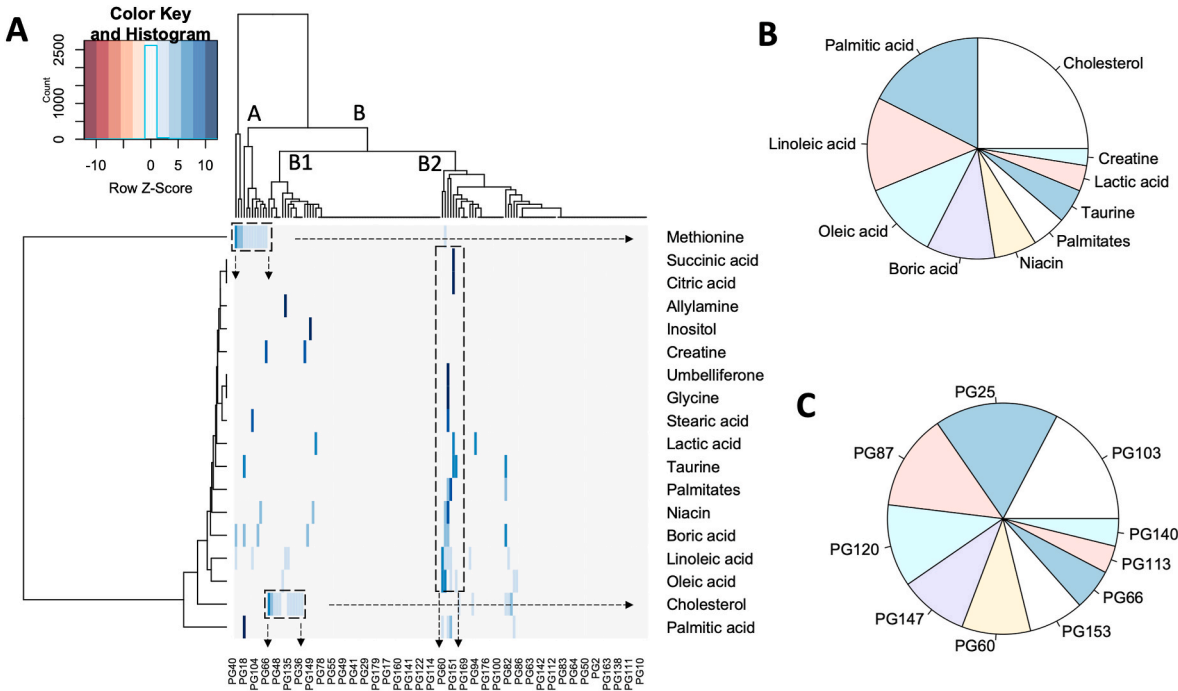


Fig. 5. Association between metabolites and gene paralogs. A. Heatmap showing cluster patterns of metabolites and gene expression of the paralog groups (dotted boxes). Arrows indicate the metabolites and PGs associated with each cluster. B. The top ten metabolites that represented the metabolite-gene association are shown. The pie chart shows the proportion of PGs associated with each metabolite. C. The top ten PGs that represented the metabolite-gene association are shown. The pie chart shows the proportion of metabolites associated with each PG.

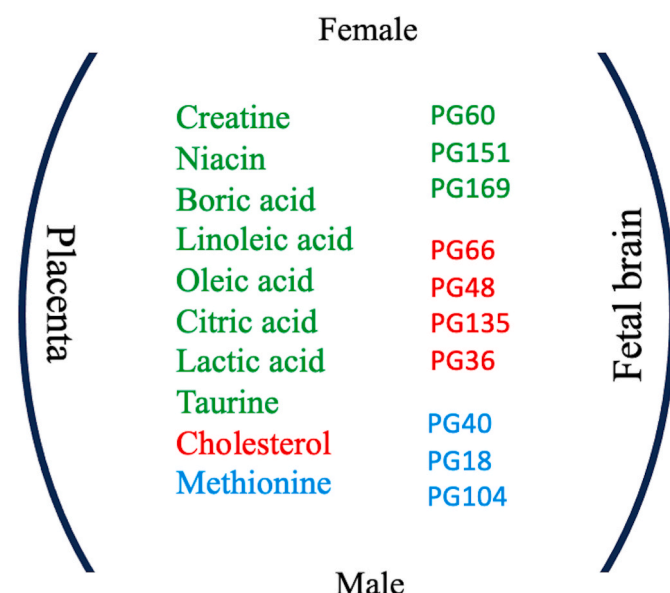


Fig. 6. A summarized finding of the study. It illustrates a simplified model relating to the crosstalk between the metabolites and paralog groups in the placenta and fetal brain. The paralog-metabolite associations are shown by matching colors. These metabolites and paralogs are expressed in a sex or tissue bias manner between the placenta and fetal brain.

differences in gene expression of the fetal brain are not specific to hemochorial placenta but also observed in epitheliochorial placenta of pigs, as shown in our earlier studies [50,51].

The results of the current study support the idea that paralogs contribute to the adaptive functions of placenta during mammalian pregnancy. While purifying selection helps maintain the duplicated gene copies in the genome [52], persistent divergence of the gene copies leads to adaptive function of the paralogs [53]. Post-duplication neofunctionalization contributes to the transcriptional diversity of paralogs within and between tissues [70]. In mice, our previous study [29] showed that expression changes of paralogs were relatively higher in the placenta compared to the fetal brain. The same study identified specific paralogs such as *Prl2c2/Prl2c3*, *Psg26/Psg28*, *Serpib9f/Serpib9g*, and *Xlr4a/Xlr4b* that were coordinately regulated in the placenta relative to the fetal brain. It is known that specific prolactin (*Prl*) paralogs play a role to respond to physiological stresses during pregnancy [8,9].

Another key finding of this investigation is that specific paralogs are associated with metabolites expressed in the placenta and fetal brain. We summarize the findings in a model illustrated in Fig. 6. It shows the relationship between metabolites and paralog groups expressed in the placenta and fetal brain. The paralogs associated with cholesterol showed a distinct expression pattern relative to paralogs associated with methionine. This was evident from hierarchical cluster analysis of the combined metabolomics and transcriptomics data (see Fig. 5). Methionine functions as a suppressor of cholesterol by downregulating cholesterol biosynthesis and increasing excretion [55]. During pregnancy, methionine metabolism shows distinct expression patterns between the placenta and fetus [56]. In humans, a higher rate of transsulfuration (interconversion between homocysteine and cysteine) is observed during early pregnancy whereas a higher rate of transmethylation (recovery of methionine from homocysteine) occurs during late stages of pregnancy [57]. Also, increase in maternal methionine shows a positive effect on fetal development suggesting an important role of methionine in early development [58]. Similar to methionine, cholesterol plays a vital role in fetal development [59–61]. Though a majority of the cholesterol is synthesized in the fetus, cholesterol from maternal circulation is also transported to the fetus which increases during late gestation to support rapid growth of the fetus [60]. While

evidence for interactions between methionine and cholesterol are well documented [62–66], the results of the current study show that changes of paralog expression are significantly associated with the changes of methionine and cholesterol level between the placenta and fetal brain.

The overall implication of this study is that paralogs play a role to influence fetoplacental metabolism. Placental function is highly adaptive to support sex-specific developmental needs of the fetus. It is well established that gene duplication contributes to evolutionary innovation of eukaryotic genomes to acquire novel adaptive functions [15]. The duplicated copies contribute to the regulation of adaptive functions including those related to pregnancy and early developmental processes in animals [4,5]. Evolutionary studies indicate that upon gene duplication, paralogous copies are maintained in the genome by purifying selection [7]. Such selection stabilizes expression of the paralogs [67] and promotes functional diversity of paralog-encoded ligand proteins that are required for cellular interactions for novel adaptive functions [7]. This is supported from a recent single-cell RNA-seq study which shows that paralogs are regulated differentially in different cell populations [54]. Recently duplicated ligand genes are selectively distinct from those duplicated during early mammalian evolution [68]. As a result, duplicated genes can produce novel ligand molecules to generate receptor-ligand interactions to adapt to different developmental and physiological niches [69]. This is further supported by the idea that most ligands are evolutionarily younger than their cognate receptors [70]. Besides receptor/ligand diversity, paralogs also contribute to the degeneracy of metabolic functions where differential regulation of a metabolic pathway allows specific cells or tissues to respond to different physiological or genetic conditions [71,72]. Metabolic degeneracy is a concept of the broader idea of biological degeneracy that is argued to shape the functional complexity of eukaryotic genomes [73].

It is however important to note that there are limitations in our current investigation. This study doesn't provide direct experimental evidence to support the role of paralogs in the metabolic regulation between placenta and fetal brain. We have not performed any experiment to manipulate the availability of specific metabolite(s), and determine its effects on the differential expression of the cognate paralogs between the placenta and fetus. Conversely, changes in fetoplacental metabolism can be investigated using mouse models that lack specific paralogs. Our future direction of this study is directed to address these limitations. Nevertheless, the findings of the current study highlights the need for further work to investigate the role of paralogs in mammalian reproduction.

Data statement

The metabolomics data is provided as a supplementary data file with this paper.

CRediT authorship contribution statement

Maliha Islam: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Susanta K. Behura:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.placenta.2023.12.019>.

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