1 **Full Title**

2 Sex differences in early and term placenta are conserved in adult tissues

3

4 **Short Title**

5 Placenta sex differences

6

7 **Authors**

8 Kimberly C. Olney[1,2], Seema B. Plaisier[1,2], Tanya N. Phung[1,2], Michelle Silasi[3], Lauren Perley[4],

9 Jane O'Bryan[4], Lucia Ramirez[1,2], Harvey J. Kliman[4], and Melissa A. Wilson[1,2,5*]

10

11 **Affiliations**

12 1. School of Life Sciences, Arizona State University, Tempe AZ 85282 USA

13 2. Center for Evolution and Medicine, Arizona State University, Tempe AZ 85282 USA

14 3. Department of Obstetrics and Gynecology, Division of Maternal-Fetal Medicine, Mercy Hospital

15 St. Louis, St. Louis, MO 63141

16 4. Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of

17 Medicine, New Haven, CT 06520

18 5. The Biodesign Center for Mechanisms of Evolution, Arizona State University, Tempe AZ 85282

19 USA

20

21 *Corresponding author

22 Melissa A. Wilson

23 School of Life Sciences | Arizona State University | PO Box 874501 | Tempe, AZ 85287-4501

24 mwilsons@asu.edu

Abstract

Background: Pregnancy complications vary based on the fetus's genetic sex, which may, in part, be modulated by the placenta. Further, developmental differences early in life can have lifelong health outcomes. Yet, sex differences in gene expression within the placenta at different time points throughout pregnancy and comparisons to adult tissues remains poorly characterized.

Methods: Here, we collect and characterize sex differences in gene expression in term placentas ($\geq$ 36.6 weeks; 23 male XY and 27 female XX). These are compared with sex differences in previously collected first trimester placenta samples and 42 non-reproductive adult tissues from GTEx.

Results: We identify 268 and 53 sex differentially expressed genes in the uncomplicated late first trimester and term placentas, respectively. Of the 53 sex differentially expressed genes observed in the term placentas, 31 are also sex differentially expressed genes in the late first trimester placentas. Furthermore, sex differences in gene expression in term placentas are highly correlated with sex differences in the late first trimester placentas. We found that sex differential gene expression in the term placenta is significantly correlated with sex differences in gene expression in 42 non-reproductive adult tissues (correlation coefficient ranged from 0.892 to 0.957), with the highest correlation in brain tissues. Sex differences in gene expression were largely driven by gene expression on the sex chromosomes. We further show that some gametologous genes (genes with functional copies on X and Y) will have different inferred sex differences if the X-linked gene expression in females is compared to the sum of the X-linked and Y-linked gene expression in males.

Conclusions: We find that sex differences in gene expression are conserved in late first trimester and term placentas and that these sex differences are conserved in adult tissues. We demonstrate that there are sex differences associated with innate immune response in late first trimester placentas but there is no significant difference in gene expression of innate immune genes between sexes in healthy full term placentas. Finally, sex differences are predominantly driven by expression from sex-linked genes.

Keywords: sex differences, gene expression, RNAseq, pregnancy, life course, development, alignment, gametolog, sex chromosome

56 Abbreviations

57 Body mass index (BMI)

58 Genomic Analysis ToolKit (GATK)

59 Million (M)

60 Multidimensional scaling (MDS)

61 Spontaneous vaginal delivery (SVD)

62 Highlights

63 ● Sex differences in gene expression in late first trimester placentas are positively
64   correlated with sex differences in gene expression in full term placentas; sex differences
65   develop early and are maintained.
66 ● Sex differences in gene expression on the sex chromosomes in the placenta are
67   correlated to sex differences in adult tissues.
68 ● Sex-linked gametolog genes require additional methodological approaches for accurate
69   quantification.

70 Background

71 The placenta is a specialized organ that is part of and supports a developing fetus by facilitating
72 nutrient transfer, waste removal, and immune regulation with the maternal body (Burton et al,
73 2015). Trophoblast cells are the first cells to differentiate from the fertilized oocyte forming the
74 outer layer of the blastocyst and invade into maternal uterine tissue forming an interface that
75 grows with the developing embryo and fetus throughout pregnancy. Signals from the placenta
76 elicit proinflammatory TH1 responses in the first and third trimester to create an environment
77 free from pathogens for implantation and prepare the fetus for interaction with the outside world,
78 while an anti-inflammatory response occurs in the second trimester to prevent rejection of the
79 fetus as it undergoes rapid growth and development (Mor et al., 2011). Changes in placental
80 development can have major effects on the fetus and many studies have demonstrated that
81 altered placenta function is associated with pregnancy complications such as preeclampsia and
82 intrauterine growth restriction (Haram et al., 2020). Healthy placental tissue may provide a
83 common reference for studying the developmental origins of sex differences in adverse health

84 outcomes that arise during pregnancy that have continued effects after birth (Burton et al., 2016;
85 Goldstein et al., 2020; Keleher et al., 2021).

86

87 Sex-specific differences have been observed in the placenta, the developing fetus, infants, and
88 adults. The specific placental biomarkers s-FLt1, PAI-2, and PLGF have been measured at
89 higher concentrations in female placenta compared to male placenta (Brown et al., 2014), while
90 male placentas have been reported to have higher expression of induced TNF-a response
91 involved in inflammation relative to female placentas (Yeganegi et al., 2009). Male fetuses have
92 a larger crown-to-rump length than females in the first trimester and a slower growth rate of
93 head circumference, while male neonates are heavier than female neonates at birth (Brown et
94 al., 2014). Further, a recent study of adult tissues from the Genotype-Tissue Expression Project
95 (GTEx) demonstrated that sex influences gene expression and cellular composition throughout
96 the body with 37% of all genes showing sex differences in expression in at least one tissue
97 (Oliva et al., 2020). In mouse, chromosomal sex contributes, along with organizational gonadal
98 sex and activational hormones, to sex differences in gene expression in adult tissues (Blencowe
99 et al., 2022) in adult tissues. However, sex differences in early formed placental tissues have
100 not been compared with sex differences in adult tissues.

101

102 Therefore, we studied sex differences in the placental gene expression in early and late stage
103 pregnancy and compared with adult tissues. We generated RNA and DNA from 30 male and 30
104 female term ($\geq$ 36.6 weeks) placentas from uncomplicated births. We compared sex
105 differences in term uncomplicated placentas to late first trimester placenta samples (Gonzalez
106 et al., 2018) and to 42 non-reproductive adult tissues to better understand the consistency of
107 sex differential expression across the life span. We found that sex differences in gene
108 expression in term placentas are highly correlated with sex differences in the late first trimester
109 placentas. We found that genes related to immune function are sex differentially expressed in
110 the late first trimester but not at full term. Genes on the sex chromosomes, but not the
111 autosomes, tend to show similar sex differences between the placenta and adult tissues, with
112 the highest correlations between the placenta and brain tissues. Furthermore, we discuss
113 approaches for quantifying gametologous gene expression (expression from genes with
114 functional copies on the X and Y chromosomes due to shared ancestry). Understanding sex-
115 specific changes in placental gene expression during and after pregnancy can provide insight
116 on the mechanisms underlying human growth and development *in utero* and beyond in health
117 and disease.

Methods 118

Samples 119

120 Working with the Yale University Reproductive Sciences (YURS) Biobank, we collected 60 term

121 (≥ 36.6 weeks) placentas from uncomplicated pregnancies, 30 assigned female at birth and 30

122 assigned male at birth. Individuals were chosen for inclusion in this study based on self-reported

123 race – Black (33%), White (33%), Asian (23%), and Hispanic (7%) – to increase the likelihood of

124 a genetically diverse sample (Additional Table 1; see demographic analysis below). The

125 placenta samples here were carefully selected to represent the fetal component of the placenta.

126 Tissue samples were taken midway between the chorionic and basal plates from the periphery

127 of the lobules avoiding maternal tissue. Placentas were oriented maternal side up and the basal

128 plate was removed to remove the maternal tissue. Four tissue samples weighing roughly 50 mg

129 were placed in a cryovial, snap frozen in liquid nitrogen, and stored at -80°C until processed for

130 sequencing. Chosen sampling sites were free of visible infarction, calcification, hematoma, and

131 tears. Three samples were obtained from each placenta, one for whole exome sequencing, and

132 two tissue samples from opposing quadrants for RNA sequencing (RNAseq) for a total of 120

133 RNAseq placenta samples. The placentas were collected and sequenced at two different times,

134 with 12 male and 12 female placentas in the first batch and 18 male and 18 female placentas in

135 the second batch. All placenta samples were collected immediately following live birth via

136 cesarean section (CS) except for one male placenta, which was collected following

137 spontaneous vaginal delivery (SVD), sample ID YPOPS0007M. However, the spontaneous

138 vaginal delivery sample was removed from the study due to failed GC content (Additional Table

139 1).

RNAseq data processing 140

141 RNAseq libraries were constructed using Illumina TruSeq reverse forward stranded RiboZero

142 library prep to deplete cytoplasmic polyadenylated tails. Samples were sequenced to 50 million

143 (M) 2 x 100 bp paired-end reads. Samples were checked for quality using FastQC version

144 0.11.8 (Andrews, 2010) and aggregated using MultiQC version 0.9 (Ewels et al., 2016). For

145 preprocessing of the RNAseq data, we trimmed adapters using bbduk as part of bbmap version

146 38.22 (Bushnell, 2014) with the following parameters: `qtrim=rl trimq=30 minlen=75`

147 `maq=20`. Post trimming quality was again checked using FastQC version 0.11.8 (Andrews,

148   2010) and MultiQC version 0.9 (Ewels et al., 2016). Post trimming samples had an average of

149   35.18M and median of 35M reads (Additional Table 2).

150

151   All RNAseq samples were aligned to Gencode GRCh38.p12 human reference genome informed

152   on the sex chromosome complement of the sample (Olney et al., 2020; Webster et al., 2019)

153   using HISAT2 version 2.1.0 for alignment (Kim et al., 2015) and SubRead FeatureCounts

154   version 1.5.2 for quantification (Liao et al., 2014). Briefly, the sex chromosome complement of

155   the sample was first checked by investigating the expression of five Y-linked (*EIF1AY, KDM5D,*

156   *UTY, DDX3Y, RPS4Y1*) genes and one X-linked gene (*XIST*)*.* A sample with presence of a Y

157   chromosome will show expression for most or all the Y-linked genes while samples with at least

158   two X chromosomes will show expression for *XIST* (Additional Figure 1)*.* Samples with no

159   evidence of a Y chromosome were aligned to a reference genome with the entire Y

160   chromosome masked with Ns to avoid mis-mapping of homologous X-Y sequence reads (Olney

161   et al., 2020). Samples with evidence of a Y chromosome were aligned to a reference genome

162   with the Y chromosome pseudoautosomal regions (PARs) masked out as those regions are

163   replicated 100% on the X chromosome PARs in GRCh38.p12. We followed the XY_RNAseq

164   readme (Olney et al., 2020) to utilize a Ymasked, and YPARs masked reference genome and

165   HISAT `-x index` function to create two sex chromosome complement reference indexes used

166   for alignment. HISAT2 alignment was performed with the following parameters, `--dta` for

167   downstream transcriptome assembly, `--rna-strandness RF` to indicate the sequences are

168   reverse forward, `--phred 33` encoding, and pair-end alignment. RNAseq alignment files were

169   then sorted, read groups were added, duplicates were marked, and files were indexed using

170   bamtools 2.5.1 (Barnett et al., 2011) and Picard 2.9.2 (*Picard Tools - By Broad Institute*, n.d.).

171   FeatureCounts was employed using `--primary` to only use primary alignments and `-p 2` to

172   specify the minimum number of consensus reads from the same pair, suggested for paired-end

173   read data (Liao et al., 2014). FeatureCounts uses the gene annotation file to infer exon-exon

174   junctions from connecting each pair of neighboring exons from the same gene (Liao et al.,

175   2014). FeatureCounts was run twice for each RNAseq sample, once for the gene level using `-g`

176   `gene_name`. There are 57,133 genes in the Gencode GRCh38.p12 human reference genome

177   used in this analysis.

Exome data processing

We used FastQC version 0.11.8 (Andrews, 2010) and MultiQC version 0.9 (Ewels et al., 2016) for visualizing quality for whole-exome data. We trimmed adapters using bbduk as part of bbmap version 38.22 (Bushnell, 2014) with the following parameters: `qtrim=rl trimq=30 minlen=75 maq=20`. We used bwa-mem version 0.7.17 (Li, 2013) to align the whole exome samples. Samples were aligned to a sex chromosome complement reference (see RNAseq data processing for more details (Olney et al., 2020; Webster et al., 2019)). Post alignment, PCR duplicates were marked using Picard version 2.18.27 (*Picard Tools - By Broad Institute*, n.d.). To genotype variants, we used GATK version 4.1.0.0 (DePristo et al., 2011; McKenna et al., 2010; Van der Auwera et al., 2013). We first used GATK's HaplotypeCaller to generate GVCF files. Second, we combined GVCF from 60 individuals, 30 male XY, and 30 female XX, using GATK's CombineGVCFs.

Late first trimester placentas

Late first trimester, 10.5 - 13.5 weeks, from last menstrual period when available, human placenta RNAseq samples from Gonzalez et al. 2018 (Gonzalez et al., 2018) were downloaded from NCBI GEO Accession GSE109082 using `fastq-dump -I --split-files` (Leinonen et al., 2011). There are 17 female XX and 22 male XY placenta samples in this data set, all of which self-reported as white (Gonzalez et al., 2018). GSE109082 were processed similarly as the full-term uncomplicated placentas with one exception, trimming for quality. GSE109082 transcriptome samples before trimming had an average of 22.53M 2 x75 bp paired-end reads. GSE109082 paired-end reads were checked for quality using FastQC version 0.11.8 (Andrews, 2010) and MultiQC version 0.9 (Ewels et al., 2016). We preprocessed the samples from GSE109082 similarly to the newly generated samples, with minor changes due to the difference in the RNAseq data: trimmed adapters using bbduk as part of bbmap version 38.22 (Bushnell, 2014) with the parameters `qtrim=rl trimq=25 minlen=40 maq=10`. Post-trimming quality was checked using Fastqc and MultiQC. Post trimming samples had an average of 20.6M and a median of 18.8M reads (Additional Table 2). Reads were then aligned to a sex chromosome complement Gencode GRCh38.p12 reference genome using HISAT2 (Kim et al., 2015). Gene level counts were obtained using SubRead FeatureCounts (Liao et al., 2014). The late first trimester placentas were reverse forward sequencing, the same as the term placentas presented here. Thus, the HISAT2 and FeatureCounts parameters were the same for both the late first trimester (Gonzalez et al., 2018) and the full-term placentas.

Multidimensional scaling

Multidimensional scaling (MDS) of the expression counts following subRead FeatureCounts was generated using `plotMDS` of the limma package (Law et al., 2014). `plotMDS` is a slightly modified MDS that plots the transcript expression profiles on a two-dimensional scatterplot so that distances on the plot approximate the typical log_2 fold changes between the samples. MDS plots were generated using the `gene.selection` parameter and selecting "`common`" for all shared genes. This was repeated for the top 100 genes that show the most extensive standard deviations between samples (Additional Figure 2). The full-term placentas were sequenced at two different time points in batch 1 and batch 2. Before clustering with MDS, we accounted for batch effects using the `removeBatchEffect` part of the limma package (Law et al., 2014). This was for visualization purposes only. Batch was included as a covariant in the linear model downstream; see Differential expression.

Excluding RNAseq samples

Samples that failed quality control (QC) were removed from downstream analysis. Samples were removed that had less than 12.5M or higher than 90M sequences remaining after trimming. If more than 30% of the reads deviated from the sum of the deviations from the normal distribution of the per-sequence GC content as defined by the FASTQC report, then the sample was removed (Additional Table 3). Samples were also excluded that did not cluster with reported sex assigned at birth (Additional Figure 2 & Table 3). There were 23 male XY and 27 female XX full-term placentas that passed QC and were included in the downstream analyses. All 17 female XX and 22 male XY late first trimester placentas from Gonzalez et al. 2018 passed QC and were kept for downstream analysis.

Subject demographic analysis

We inferred population ancestry from the variants obtained from the whole-exome data using Peddy (Pedersen & Quinlan, 2017) (Additional Figure 3 & Additional Table 1). The resulting outputs of the PCA analysis in Peddy yielded principal components (PC) that were used to assign predicted ancestry. PC1 and PC2 were used later downstream as covariant in the linear model for the differential expression analysis. We did not infer population ancestry from the late first trimester GSE109082 placentas as this data set only included RNAseq data, and not DNA.

Quantify technical and biological variation in RNAseq expression data

239

240   Utilizing variancePartition (Hoffman & Schadt, 2016), a linear mixed model was employed to

241   quantify variation in each expression trait attribute. Variation within gestational age (GA),

242   sequencing lane, sex, reported race, and birth weight was examined. Variation in placenta

243   expression for maternal clinical data, including parity, gravidity, pre-pregnancy body mass index

244   (BMI), and maternal age, were also examined (Additional Figure 4). We did not run

245   variancePartition for all the first trimester placentas because we did not have access to clinical

246   data for this sample set. We additionally examined sex differences for clinical information for

247   full-term placentas for maternal age at delivery, pre-pregnancy BMI, gravidity and parity,

248   gestational age, method of conception, self-reported race, and birth weight. Sex differences for

249   continuous variables were tested using a t-test, p-value < 0.05. A Fisher's exact test was used

250   to test for sex differences for categorical variables, p-value < 0.05 (Additional Table 4 &

251   Additional Figure 5).

X and Y gametolog gene expression

252

253   A list of X and Y gametologous genes (genes with shared sequence homology and ancestry

254   due to existence as homologs on ancestral autosomes that became sex chromosomes) were

255   curated from a combination of Skaletsky et al. 2003 and Godfrey et al. 2020 (Godfrey et al.,

256   2020; Skaletsky et al., 2003) (Additional Table 5). In samples determined to have a Y

257   chromosome, the CPM value of the X-linked gametolog and the Y-linked gametolog were

258   summed and included in a single value under the X-linked gametolog label. Then we compared

259   expression between the XX female X-linked gametolog expression to XY male X-linked

260   gametolog plus Y-linked gametolog gene expression using a Wilcox rank-sum, p-value < 0.05

261   (Figure 4, Additional Table 5, Additional Figure 6).

Differential expression

262

263   Sex differential expression analysis between male XY and female XX placentas was performed

264   using the limma/voom pipeline (Law et al., 2014). Quantified read counts from each sample

265   were generated using SubRead featureCounts and combined into a count matrix, with each row

266   representing a unique gene id, and each column representing the gene counts for each unique

267   sample. Using the DGEList function in the limma package the counts matrix and a tab-delimited

268   file containing sample ID, sex, race, batch, lane, GA, parity, maternal age, gravidity, pre-

269   pregnancy BMI, birth weight, PC1 and PC2 from the whole exome data were read into R

270   (Additional Table 1). Technical replicates from within a placenta were summed together using

271   the sumTechReps function in version 3.14.0 (Robinson et al., 2010). Normalization factors were

272   calculated using the calcNormFactors function in EdgeR (Robinson et al., 2010). Lowly

273   expressed genes were filtered out using a minimum threshold of 1 Fragments Per Kilobase

274   Million (FPKM) in at least one group being compared. Then we ran Trimmed Means Method

275   (TMM) to normalize for library size variation between samples (Robinson & Oshlack, 2010).

276   Counts were then transformed to $\log_2(\text{CPM}+0.25/L)$, where CPM is counts per million, L is

277   library size, and 0.25 is a prior count to avoid taking the log of zero (Law et al., 2014). For each

278   comparison of interest, a model was created to compare between the groups where each

279   coefficient corresponds to a group mean. The model.matrix function in the 'stats' library in R

280   was used to specify the model being fitted. The model was fitted for sex for both the term and

281   first-trimester placenta analyses. Additional covariates (batch, birth weight, lane, and exome

282   sequencing principal components used to account for ancestry) were also added for the term

283   placentas. There were no covariates to add to the model matrix for the late first trimester

284   placentas because clinical and technical information was not made available for this data set.

285   For each differential expression analysis, a linear model was fitted to the DGEList-object, which

286   contained the normalization factors for each gene counts for each sample, using the limma lmfit

287   function which will fit a separate model to the expression values for each gene (Law et al.,

288   2014). Comparisons between groups were then obtained as contrasts of the fitted linear model.

289   An empirical Bayes approach was applied to smooth the standard errors. Genes are defined as

290   being differentially expressed between groups when the adjusted p-value is ≤ 0.05 using a

291   Benjamini-Hochberg false discovery rate (Law et al., 2014) (Figure 1).

Quantifying sex differences for innate immune gene expression

293   Differential expression between male XY and female XX placentas for 979 innate immune

294   genes, as defined by InnateDB (Breuer et al., 2012) (Additional Table 6). The InnateDB is a

295   publicly available database of genes, proteins, experimentally verified interactions, and signaling

296   pathways involved in the innate immune response to microbial infection. Sex differential

297   expression of the 979 innate immune genes was performed using the limma/voom pipeline (Law

298   et al., 2014). We repeated this analysis for both the term and late first trimester placentas. The

299   model matrix for term placentas was the same when looking at the differential expression for the

300   whole transcriptome. Only genes determined to be expressed in at least one sex were included

301   in the analysis (see Methods). In addition to differential expression, we generated an MDS plot

302   on the expression data for only the innate immune genes to determine immune gene expression

303   similarity among samples (Figure 2).

Gene function and enrichment network analysis

305   We used the TopFunn webtool to infer the function of genes and identify enriched biological

306   processes, molecular functions, and gene families (J. Chen et al., 2009). Additionally, we looked

307   at each sex differentially expressed gene from the late first trimester and full-term placenta

308   comparisons using genecards.org and a literature review of genome-wide association studies

309   and expression quantitative trait loci (eQTL) to investigate if that gene is involved in known

310   diseases or disorders, particularly with known pregnancy complications (Additional Table 12).

Sex differences in adult GTEx tissues

312   For each of the 42 non-reproductive adult tissues in the Genotype by Tissue Expression (GTEx)

313   consortium, we computed the $\log_2$ female-to-male expression ratios from the reported

314   Transcripts Per Kilobase Million (TPM) counts version 2017-06-06_v8 (Additional Table 8)

315   (Carithers et al., 2015; Oliva et al., 2020). To determine if sex differences in gene expression

316   within the placenta are correlated with sex differences in adult tissues, we computed the

317   coefficient of correlation, r, of the $\log_2$ female-to-male expression ratios for sex differentially

318   expressed genes found in the placenta to the $\log_2$ female-to-male expression ratios of the same

319   genes in each adult tissue (Figure 3).

320

Results

Unsupervised separation of placenta samples by sex

323   Multidimensional Scaling (MDS) analysis was performed on full-term and late first trimester

324   placentas to determine trends in the placenta transcriptome. MDS of the full-term placentas

325   show separation of placenta by genetic sex on the first dimension showing that sex is a key

326   factor in gene expression in the placenta. Each placenta sample from term placentas included

327   two replicate samples, all passed our QC except one female XX placenta replicate, so both

328   replicates for this sample was removed from the downstream analyses. The replicate failed QC

329   due to a low number of sequence reads after filtering and falling outside of the expected MDS
330   cluster (Additional Figure 2 and Additional Table 3).

Population ancestry inferred from whole exome data

332   Principal component analysis of the full-term placenta whole exome data shows the samples
333   separated by reported and inferred ancestry in many cases (Additional Table 1). The self-
334   reported race and ethnicity for the placenta samples included 14 Asian, 20 Black, 4 Hispanic, 20
335   White, and 2 unknown (Additional Table 1). The ancestry prediction estimates that the
336   population ancestry of the samples is: 14 Asian (5 South Asian, 3 East Asian, 1 European, and
337   2 unknown), 20 Black (17 African, 3 unknown), 4 Hispanic (1 European, 3 unknown), 20 white
338   (15 European, 1 American, 1 South Asian, 1 African, 2 unknown) and 2 unknown (1 American, 1
339   South Asian). To account for population ancestry differences among the samples, PC1 and PC2
340   from the whole exome data was included as covariates in the linear model used to identify sex
341   differential expression.

Modeling parameters to determine sex differences in placental gene expression

344   To determine differentially expressed genes in the placenta, specifically because of differences
345   in infant sex, we assessed non-sex variables that show differences between the sample groups
346   that could confound our results. First, we examined differences in clinical phenotypes collected
347   in this study. Nearly all the full-term placentas collected for this study were spontaneous
348   methods of conception (all but one male from an IVF pregnancy and one female placenta from
349   intrauterine insemination) (Additional Table 1) making this an unlikely confounding variable for
350   sex differences in gene expression. Maternal age at delivery ranged from age 22 to 45 years
351   old, and pre-pregnancy BMI ranged from 19.40 to 66.30. There was no significant difference in
352   maternal age or pre-pregnancy body mass index (BMI) for women who carried a male XY
353   versus women who carried a female XX (t-test p-values = 0.39, and 0.73, respectively).
354   Gravidity, the number of pregnancies the mother had achieved, ranged from 1 to 9, and parity,
355   the number of pregnancies reaching higher than 20 weeks, ranged from 0 to 4; the current
356   pregnancy at the moment the data was collected was not included in the parity counts. Gravidity
357   and parity did not show a significant difference in women that carried a male XY or female XX
358   pregnancy (t-test p-values of 0.43 and 0.61, respectively). Gestational age ranged from 36.6 to
359   41.1 weeks with no significant difference between people who carried a male XY versus a

360   female XX pregnancy (t-test p-value = 0.90). The difference in average birth weight approached

361   statistical significance between the sexes, with a male mean of 3.6 kilograms in males and

362   female mean of 3.3 kilograms (t-test p-value = 0.056; Additional Figure 5 & Additional Table 4).

363   Since a previous study demonstrated sex differences in birth weight and placental genes

364   differentially expressed by birth weight (Crawford et al., 1987; Sõber et al., 2015), we included

365   birth weight as a covariate in our model. Factoring out the birth weight by including it as a

366   covariate allowed us to focus our results on genes differentially expressed solely based on

367   genetic sex, as this is what our study was designed to assess. If birth weight was not included in

368   the model as a covariate, we identify 19 more genes as differentially expressed, only one of

369   which, ASMTL, was also differentially expressed in the late first trimester placentas (Additional

370   Table7). In addition to assessing differences in clinical variables between sexes, we used

371   variance partitioning to assess the extent to which clinical features as well as technical features

372   explained the variance in gene expression between our placenta samples. Variance partitioning

373   was performed for the full-term placenta RNAseq samples revealing that the sequencing lane

374   explained the largest amount of variance in our data (see methods; Additional Figure 4).

375   Maternal variables tested explained very little of the variance in placenta gene expression,

376   consistent with the same finding reported in a previous placenta gene expression study (Sõber

377   et al, 2015). Our term placenta samples were collected in two batches, so the batch was also

378   included as a covariate. Using results from all of these approaches, we constructed a linear

379   model to determine sex differentially expressed genes that included batch, lane, the first

380   principal components from exome sequencing which captured differences in population

381   ancestry, and birth weight as covariates.

382   ## Sex differential expression from male XY and female XX term uncomplicated

383   ## human placentas

384   We observed 14,441 genes expressed with an FPKM > 1 in at least all the male XY or all

385   female XX placenta samples (Additional Table 9). 53 genes exhibited sex differentially

386   expressed with an adjusted p-value < 0.05 (Figure 1 & Additional Table 7). Thirty genes showed

387   higher expression in the female XX placentas and 23 genes showed higher expression in the

388   male XY placentas. Of the 30 genes that were more highly expressed in the female XX

389   placentas than the male placentas, 28 are X-linked and two are autosomal (EIF2S3B, and

390   EIF1AXP1). Of the 23 genes more highly expressed in males, 18 are Y-linked, 4 are X-linked

391   (CD99, RPS6KA6, VDAC1P1, VAMP7), and one is autosomal (PRKCE) (Figure 1 & Additional

392   Table 7).

Sex differential expression within late first trimester placentas

394   There were 13,502 genes expressed with an FPKM > 1 in at least the male XY or female XX

395   late first-trimester placenta samples that were reprocessed here using the same approach as

396   the term placentas (see Methods) (Gonzalez et al., 2018). After reprocessing the sequencing

397   data to improve alignment to sex chromosomes, we identified 268 genes with a differential

398   expression between male and female placentas with an adjusted p-value < 0.05 (Figure 1). Of

399   these, 180 genes showed higher expression in the female XX placentas and 88 genes showed

400   higher expression in the male XY placentas.

401   Figure 1. Sex differential gene expression in late first trimester and term placentas are

402   highly correlated.

403   Sex differences in gene expression, $\log_2(\text{CPM} + 0.25/L)$, between 17 female and 22 male late

404   first trimester (10.5 - 13.5 weeks) placentas on the left and 27 female and 23 male term ($\geq$ 36.6

405   weeks) placentas shown on the right (A). Each point represents a gene. Genes that are sex

406   differentially expressed, adjusted p-value < 0.05, are indicated in purple for autosomal, orange

407   for Y-linked, and green for X-linked. The number of uniquely sex differentially expressed genes

408   and shared between the late first trimester and the term placentas is shown in (B). More genes

409   are sex differentially expressed in the later first trimester (10.5 - 13.5 weeks) than in the term ($\geq$

410   36.6 weeks) placentas. There are 237 genes that are uniquely called as sex differentially

411   expressed in the late first trimester placentas that are not called as sex differentially expressed

412   in the term placentas; however, the $\log_2$ female-to-male expression ratio for those genes are

413   highly correlated between the later first trimester (Y-axis) and the term (X-axis) placentas r =

414   0.879 (C). There is also a high correlation for the 31 sex differentially expressed genes that are

415   called in both the late first trimester placentas and the term placentas, r = 0.986 and for the 22

416   genes uniquely called in the term placentas r = 0.982.

417
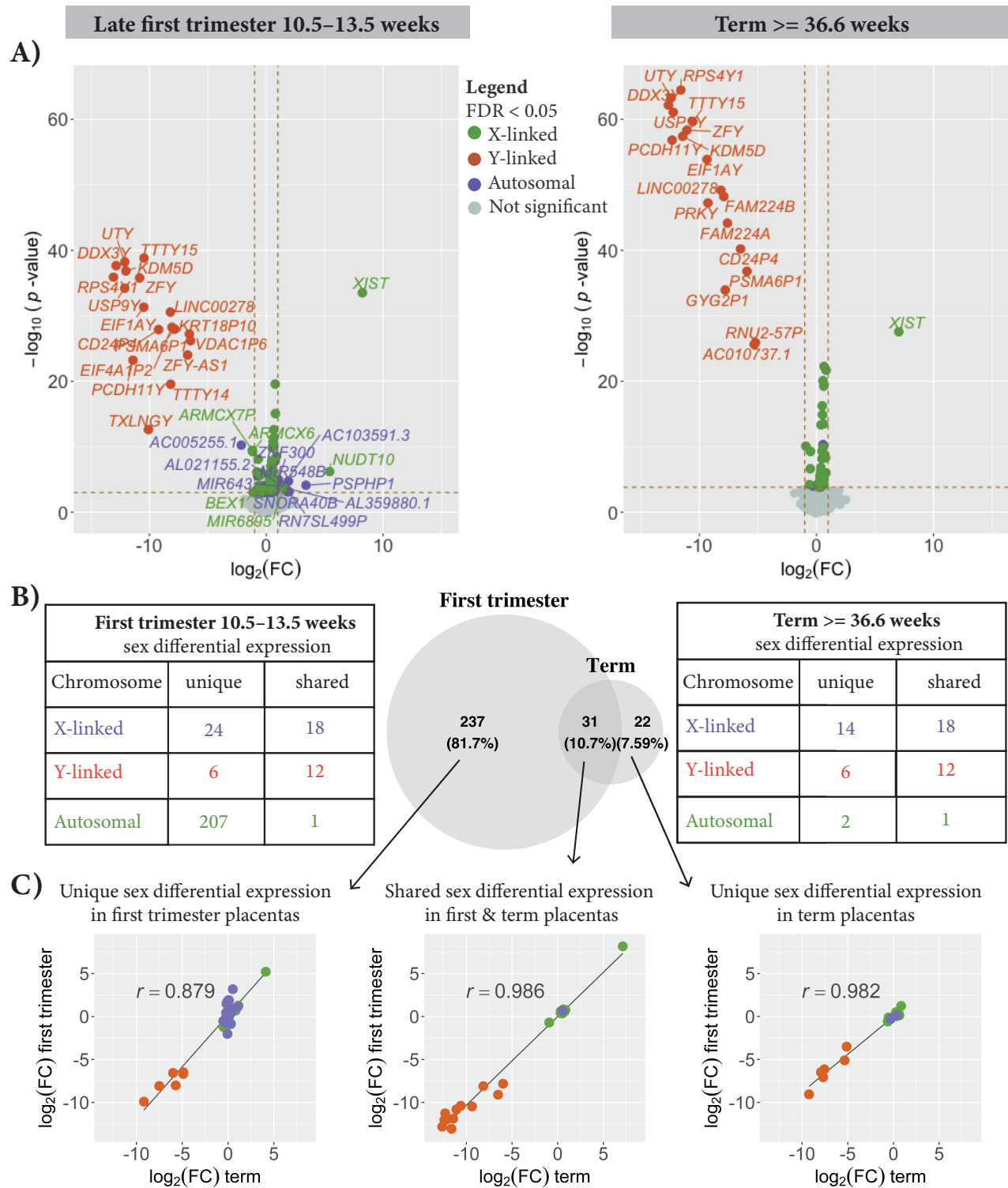
Sex differential expression shared between late first trimester and full-term placentas

We observed that sex differences in gene expression ratios between female and male placentas were highly correlated between late first trimester and term placentas. The late first trimester (Gonzalez et al., 2018) and full-term placenta RNAseq samples were processed using the same tools and only differed in the trimming parameters (bbduk minlen, see Methods) and the covariates added to the linear model for computing sex differential expression (see above). Of the 268 genes that were sex differentially expressed in the late first trimester placentas, 31 or 10.7% were shared with the genes identified as sex differentially expressed in the full-term placentas (Figure 1). Although there were more genes identified as sex differentially expressed in the late first trimester placentas given our adjusted p-value threshold < 0.05, the $\log_2$ female-to-male expression ratio for these genes was highly correlated between late first trimester and term placentas (Figure 1C). For the 237 genes that were uniquely called sex differentially expressed in the late first trimester placentas, the correlation coefficient, r, for the $\log_2$ female-to-male expression ratio between late first trimester and term placentas was 0.879. The correlation coefficient r for the $\log_2$ female-to-male expression ratio between the late first trimester and term placentas for the 31 genes differentially expressed in both placenta datasets was 0.986. Twenty-two genes were uniquely identified as sex differentially expressed in the term placentas compared to the late first trimester placentas; with the correlation coefficient of $\log_2$ female-to-male expression ratio for these genes being 0.982.

Biological processes and molecular functions enriched in placenta of a specific sex

We investigated enrichment of biological functions and processes in genes that were overexpressed in one sex versus the other sex for placentas collected from full-term uncomplicated pregnancies and late first trimester (Gonzalez et al., 2018) (adjusted p-value < 0.05) (Additional Table 7, Additional Table 11). Genes more highly expressed in male XY term placentas than female XX term placentas were found to be involved in histone lysine and protein demethylation processes and histone demethylase activity, driven by Y-linked genes, including UTY and KDM5D (previously also called SMCY or JARID1D). Serine/threonine kinases RPS6KA6 (chrX), PRKCE (chr2), and PRKY (chrY) were also more highly expressed in term male XY placentas. Five genes expressed exclusively in term male XY placentas were identified as members of minor histocompatibility antigen gene family: UTY, DDX3Y, RPS4Y1,

450   KDM5D, and USP9Y (all Y-linked genes). In contrast, genes more highly expressed in female

451   XX term placentas than in male XY term placentas were identified as involved in translational

452   initiation and regulation of sister chromatid cohesion, driven mainly by X-linked genes including

453   RPS4X, DDX3X, NAA10, EIF1AX, EIF2S3, and HDAC8. In the late first trimester placentas, as

454   in term placentas, genes more highly expressed in male XY placentas compared to female

455   placentas also included minor histocompatibility antigen genes. Genes more highly expressed in

456   the female late first trimester placentas than male late first trimester placentas were shown to be

457   involved in diphosphatase and phosphotransferase activity driven by DCP2, NUDT10, NUDT14,

458   PIGF, and PIGN and branched-chain amino acid catabolic and metabolic processes driven by

459   ACADSB, DBT, HSD17B10, ALDH6A1. In both term and late first trimester placentas, about

460   30% of the sex differentially expressed genes were associated with pregnancy complications

461   (Additional Table 12). Miscarriage and preeclampsia were the most frequently observed

462   complications, but there were others, such as gestational diabetes and fetal growth restriction.

More sex differences in expression of immune and immune modulator genes in late first trimester placentas than in term placentas

465   We tested for sex differences in a subset of genes with known connections to innate immune

466   function in this RNAseq study of late first trimester and term placentas. Of the 979 innate

467   immune genes reported from InnateDB, 628 were expressed in the term placentas. Unlike with

468   all genes where placenta samples clearly clustered by sex (Additional Figure 2),

469   multidimensional scaling with only the innate immune genes showed no distinguishable pattern

470   (Figure 2). Of the 628 innate immune genes expressed in the term placentas, only DDX3X

471   showed a statistically significant difference in expression (adjusted p-value = 2E-5; Figure 2;

472   Additional Table 6). This result was further investigated in the gametolog section as DDX3X has

473   a functional Y-linked copy, DDX3Y.

474

475   Unlike in the term placentas where only one innate immune gene showed differential expression

476   by sex, the late first trimester placentas showed 37 innate immune genes as differentially

477   expressed between the sexes (adjusted p-value < 0.05; Additional Table 6). Of the 979 innate

478   immune genes reported from InnateDB, 626 were expressed in the late first trimester placentas

479   (see Methods). Like the term placentas, DDX3X also showed a difference in expression (See

480   Gametolog section; Additional Tables 5 & 6). SERPING1 showed the highest fold change, with

481   1.84 times greater average expression in late first trimester male XY placentas than in female

482   XX placentas (Figure 2). Seven of the differentially expressed innate immune genes had

483   transcription activator activity (SREBF1, REL, RELA, CREB1, ATF2, DDIT3, and STAT6), seven

484   had kinase activity (PIK3CA, SOCS6, SOCS5, TRIM28, GSK3A, DDX3X, RBCK1), and seven

485   had ubiquitin transferase activity (BIRC2, TRIM13, TRIM28, HERC5, RBCK1, HACE1).

486   Figure 2. Innate immune gene expression between male XY and female XX placentas.

487   Of the 979 innate immune genes from InnateDB, 625 genes are expressed in the late first

488   trimester placentas and 628 are expressed in the term placentas. (A) MDS plot shows no

489   clustering by genetic sex in either the late first trimester (left) or term (right) placentas. (B)

490   volcano plot of the sex differential expression for late first trimester placentas (left) and term

491   placentas (right). Each point represents a gene. Genes that are sex differentially expressed,

492   adjusted p-value < 0.05, are indicated in purple for autosomal, orange for Y-linked, and green
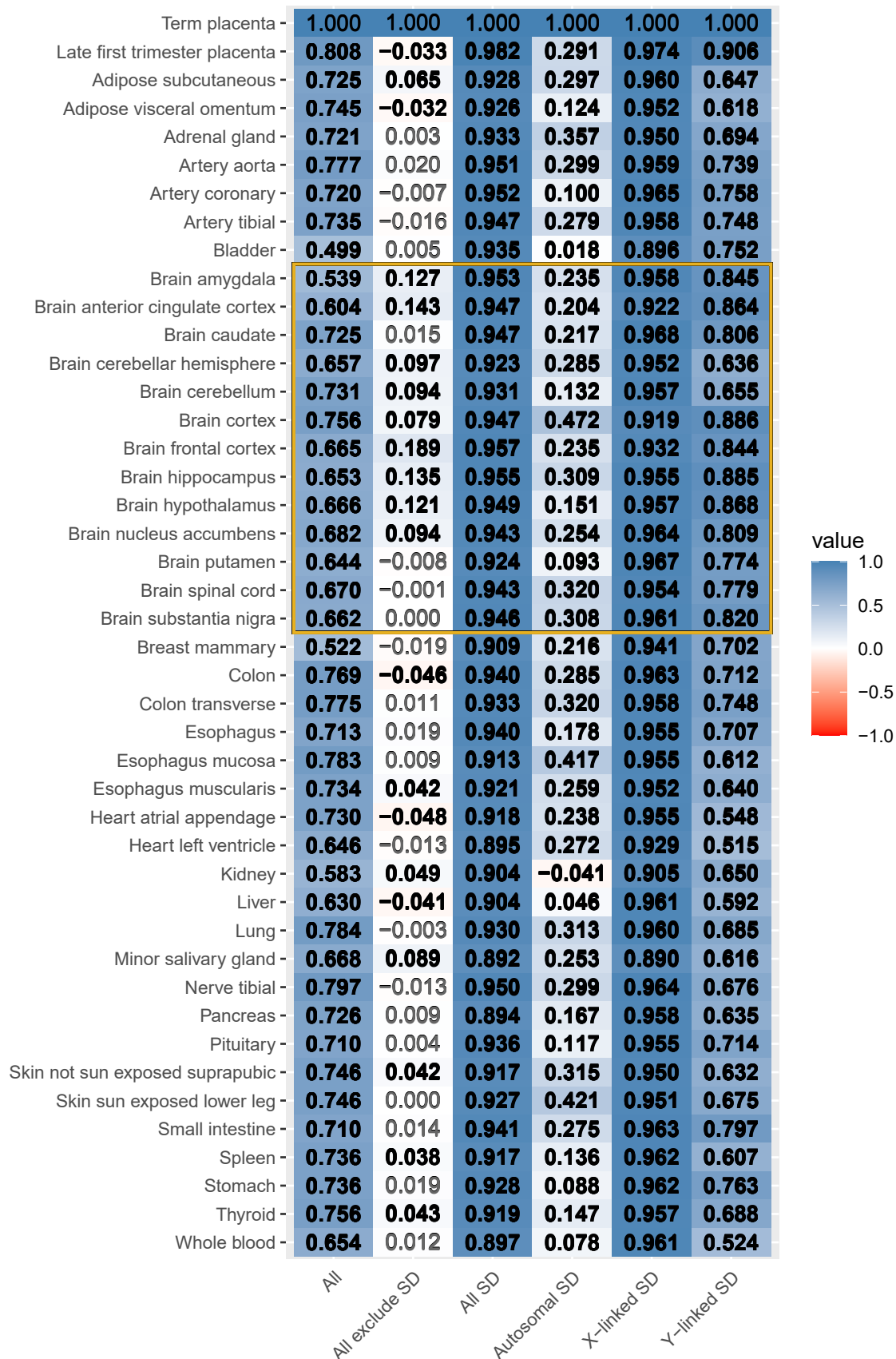
493   for X-linked.

494
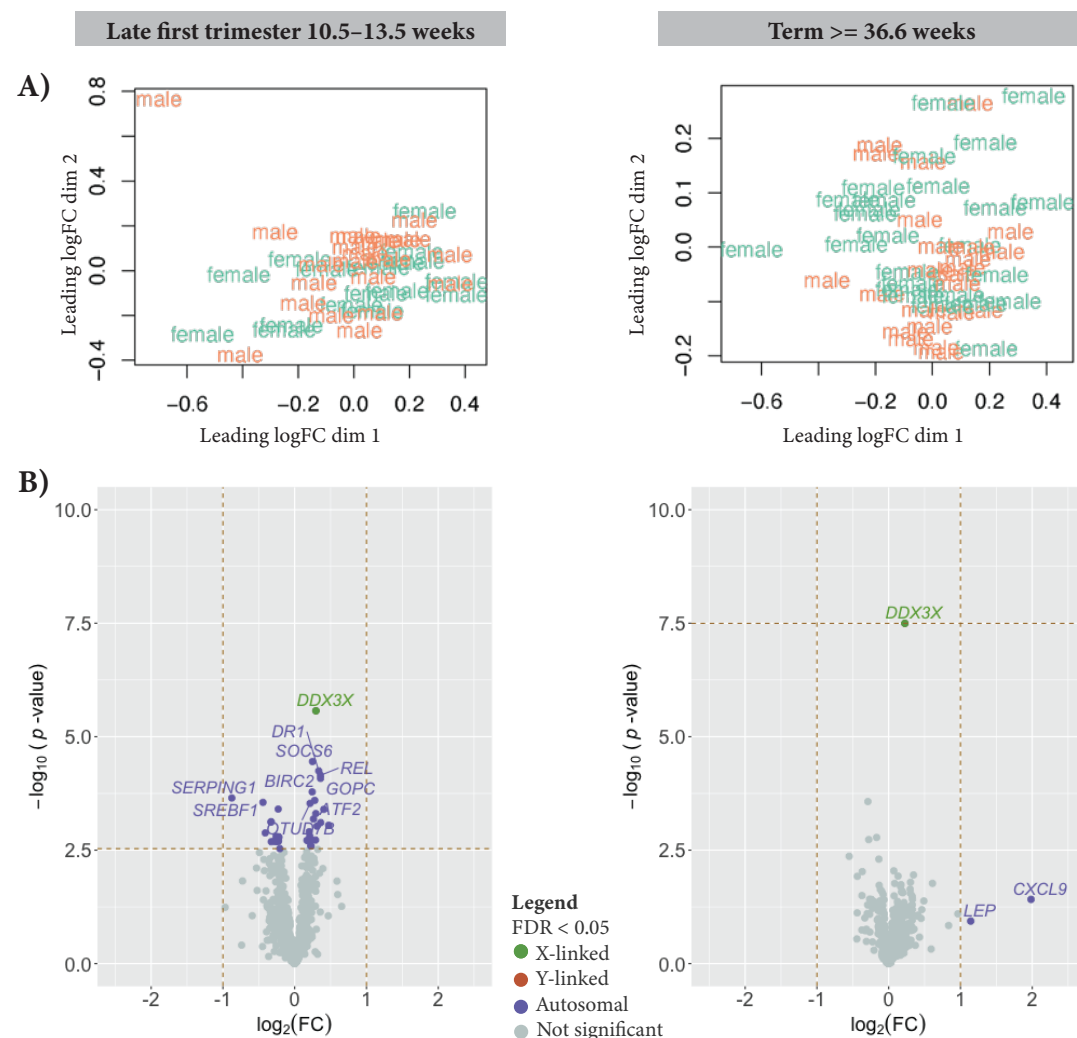
Female-to-male gene expression ratios in the placenta are correlated with adult tissues

Sex differences in gene expression in the human placenta were highly positively correlated with sex differences in adult tissues, and this was largely driven by sex-linked genes (Figure 1, 3, & Additional Table 7). We first observed that all genes expressed in the placenta showed a significant correlation between the $\log_2$ ratio of female-to-male expression in the placenta and adult tissues ("All" in Figure 3). However, when we removed sex-differentially expressed genes, this correlation was markedly decreased ("Exclude SD" in Figure 3). Conversely, we observed high correlation between the subset of sex-differentially expressed genes in the placenta and 42 adult tissues, with a pairwise r ranging from 0.892 to 0.982 (adjusted p-value < 0.05 for all comparisons; "All SD" in Figure 3). The adult tissue with the highest correlation to that of term placentas in the $\log_2$ female-to-male expression was the frontal brain cortex with an r of 0.957 (and adjusted p-value < 0.05). Notably, the $\log_2$(female-to-male expression ratio) correlation for placenta sex differentially expressed genes between term placentas and adult tissue brain regions ranged from r of 0.923 to 0.957(adjusted p-value < 0.05; "All SD" in Figure 3).

The high r of the $\log_2$female-to-male expression between term placenta to adult tissues for genes found to be sex differentially expressed in the placenta was largely driven by sex-linked genes (Figure 3). Of the 243 sex differentially expressed genes in the placenta and adult GTEx tissues, 182 were not sex-linked (autosomal or mtDNA), and the remaining 61 were on the sex chromosomes, X or Y (Additional Table 7). Despite showing sex differences in the placenta, the autosomal genes showed much lower, and largely not significant, correlations between placenta and adult tissues compared to the sex-differentially expressed genes ("Autosomal SD" in Figure 3). In contrast, we generally observed equal or higher correlations (r ranges from 0.890 to 0.967, adjusted p-value < 0.05) between the placenta and adult tissues for X-linked genes compared to any other group of sex-differentially expressed genes—again with the highest correlations between the placenta and adult brain tissues ("X-linked SD" in Figure 3). Y-linked genes showed an intermediate range of correlation values (r from 0.515 to 0.886; "Y-linked SD" in Figure 3), but all were significant after correction for multiple testing, unlike autosomal sex-differentially expressed genes.

525   Figure 3. Correlation of female-to-male expression between placenta and 42 non-

526   reproductive adult tissues.

527   From left to right: The first column is All genes which includes 11,179 expressed genes in the

528   placenta and contains count information in GTEx. Of the 11,179 genes 10,762 are autosomal or

529   MT genes, and 417 are sex-linked (X or Y-linked). The second column is All genes but

530   excluding sex differentially (SD) genes. Exclude SD genes comprises 10,936 genes, of which,

531   10,580 are autosomal or MT genes, and the remaining 356 are X-linked. All SD genes includes

532   243 sex differentially expressed genes in the placenta (late first trimester or term) adjusted p-

533   value < 0.05 and contains count information for GTEx tissues. Of the 243 sex differentially

534   expressed genes, 182 are autosomal and 61 are sex-linked (49 X-linked and 12 Y-linked). The

535   last three columns show the coefficient correlation, r, in the $\log_2$ female-to-male expression

536   ratios between term placenta, late first trimester placentas, and 42 non-reproductive adult GTEx

537   tissues when only exaiming the autosomal sex differentially expressed genes (182 genes), SD

538   and X-linked (49 genes) and SD and Y-linked (12 genes). The gold box highlights adult samples

539   from many regions of the brain which all showed high correlation of with differential expressed

540   sex-linked genes in the placenta. Black and bold indicates a significant correlation after

541   correcting for multiple testing, adjusted p-value < 0.05.

	All	All exclude SD	All SD	Autosomal SD	X-linked SD	Y-linked SD
Term placenta	1.000	1.000	1.000	1.000	1.000	1.000
Late first trimester placenta	0.808	−0.033	0.982	0.291	0.974	0.906
Adipose subcutaneous	0.725	0.065	0.928	0.297	0.960	0.647
Adipose visceral omentum	0.745	−0.032	0.926	0.124	0.952	0.618
Adrenal gland	0.721	0.003	0.933	0.357	0.950	0.694
Artery aorta	0.777	0.020	0.951	0.299	0.959	0.739
Artery coronary	0.720	−0.007	0.952	0.100	0.965	0.758
Artery tibial	0.735	−0.016	0.947	0.279	0.958	0.748
Bladder	0.499	0.005	0.935	0.018	0.896	0.752
Brain amygdala	0.539	0.127	0.953	0.235	0.958	0.845
Brain anterior cingulate cortex	0.604	0.143	0.947	0.204	0.922	0.864
Brain caudate	0.725	0.015	0.947	0.217	0.968	0.806
Brain cerebellar hemisphere	0.657	0.097	0.923	0.285	0.952	0.636
Brain cerebellum	0.731	0.094	0.931	0.132	0.957	0.655
Brain cortex	0.756	0.079	0.947	0.472	0.919	0.886
Brain frontal cortex	0.665	0.189	0.957	0.235	0.932	0.844
Brain hippocampus	0.653	0.135	0.955	0.309	0.955	0.885
Brain hypothalamus	0.666	0.121	0.949	0.151	0.957	0.868
Brain nucleus accumbens	0.682	0.094	0.943	0.254	0.964	0.809
Brain putamen	0.644	−0.008	0.924	0.093	0.967	0.774
Brain spinal cord	0.670	−0.001	0.943	0.320	0.954	0.779
Brain substantia nigra	0.662	0.000	0.946	0.308	0.961	0.820
Breast mammary	0.522	−0.019	0.909	0.216	0.941	0.702
Colon	0.769	−0.046	0.940	0.285	0.963	0.712
Colon transverse	0.775	0.011	0.933	0.320	0.958	0.748
Esophagus	0.713	0.019	0.940	0.178	0.955	0.707
Esophagus mucosa	0.783	0.009	0.913	0.417	0.955	0.612
Esophagus muscularis	0.734	0.042	0.921	0.259	0.952	0.640
Heart atrial appendage	0.730	−0.048	0.918	0.238	0.955	0.548
Heart left ventricle	0.646	−0.013	0.895	0.272	0.929	0.515
Kidney	0.583	0.049	0.904	−0.041	0.905	0.650
Liver	0.630	−0.041	0.904	0.046	0.961	0.592
Lung	0.784	−0.003	0.930	0.313	0.960	0.685
Minor salivary gland	0.668	0.089	0.892	0.253	0.890	0.616
Nerve tibial	0.797	−0.013	0.950	0.299	0.964	0.676
Pancreas	0.726	0.009	0.894	0.167	0.958	0.635
Pituitary	0.710	0.004	0.936	0.117	0.955	0.714
Skin not sun exposed suprapubic	0.746	0.042	0.917	0.315	0.950	0.632
Skin sun exposed lower leg	0.746	0.000	0.927	0.421	0.951	0.675
Small intestine	0.710	0.014	0.941	0.275	0.963	0.797
Spleen	0.736	0.038	0.917	0.136	0.962	0.607
Stomach	0.736	0.019	0.928	0.088	0.962	0.763
Thyroid	0.756	0.043	0.919	0.147	0.957	0.688
Whole blood	0.654	0.012	0.897	0.078	0.961	0.524

value
1.0
0.5
0.0
−0.5
−1.0

542

Sex differences in expression for X-linked gametolog genes

X-linked gametolog genes showed a difference in expression between female and male placentas, but the bias of expression differed whether the Y-linked gametolog was included in the analysis (Figure 4). Of the 23 gametologous gene pairs (genes with a functional copy on the X chromosome and Y chromosome due to shared ancestry on the sex chromosomes), 14 were expressed in the late first trimester and term placenta samples (FPKM > 1 in at least all the male or all the female samples; Additional Table 9). In the late first trimester placentas, 7 out of the 14 X-linked gametolog genes showed higher expression in females compared to males (DDX3X, ZFX, KDM6A, PRKX, PRS4K, EIF1AX; Additional Table 5). When we summed the expression of the X and Y-linked gametolog for male samples, we no longer saw a sex difference in expression for ZFX, PRKX, and EIF1AX, Wilcoxon test p-value > 0.05 (Additional Table 5). Three sex differentially expressed genes, DDX3X, KDM5C, and RPS4X, continued to show higher expression in female XX compared to male XY late first trimester placentas. On the other hand, when we took the sum X + Y-linked expression in males, KDM6A flipped direction and showed higher expression in male compared to female late first-trimester placentas, Wilcoxon test p-value < 0.01 (Additional Table 5). Additionally, PCDH11X changed from showing no sex difference in expression to showing higher male expression compared to female in late first trimester placentas, Wilcoxon test p-value < 0.01 (Figure 4 & Additional Table 5).

In term placentas, we observed a similar pattern when we summed the X and Y-linked expression for males. The same 7 X-linked gametolog genes that showed higher expression in the female late first-trimester placentas also showed higher expression in the female term placentas compared to male placentas (Additional Table 5, Additional Figure 6). When we took the sum expression of the X and Y-linked copy for male XY term placentas, we no longer saw a sex difference in expression for ZFX, PRKX, and RPS4X, p-value > 0.05 (Additional Table 5). This differed from the late first trimester placentas that showed sex higher expression in female placentas for RPS4X, regardless of accounting for the Y-linked gametolog (Additional Table 5). Three genes, DDX3X, KDM5C, and EIF1AX, continued to show higher expression in female compared to male XY term placentas compared to female XX placentas after accounting for Y-linked gametolog expression, Wilcoxon p-value < 0.01 (Additional Table 5). Similar to late first trimester placentas, when we accounted for Y-gametolog expression, KDM6A flipped direction and showed higher expression in male compared to female term placentas and PCDH11X changed from showing no sex difference in expression to showing higher expression in male

576   compared to female term placentas, Wilcoxon test p-value < 0.01 (Figure 4 & Additional Table

577   5).

578   Figure 4. Chromosome X+Y expression of gametologs in XY male placentas.

579   There is a significant difference in male XY to female XX expression for ZFX and KDM6A (UTX)

580   when only looking at the X chromosome CPM expression value. When we add the Y

581   chromosome-linked CPM expression count for these genes for male samples, there is no longer

582   a difference in expression between males XY and females XX for ZFX. KDM6A changes

583   directionality and shows males as having significantly higher expression than females when

584   accounting for gametolog expression. PCDH11X, when adding Y-linked CPM expression,

585   shows a significantly higher expression than females. T-test to see if there is a difference

586   between the female CPM and the male CPM for each gene, p-value < 0.05.

587
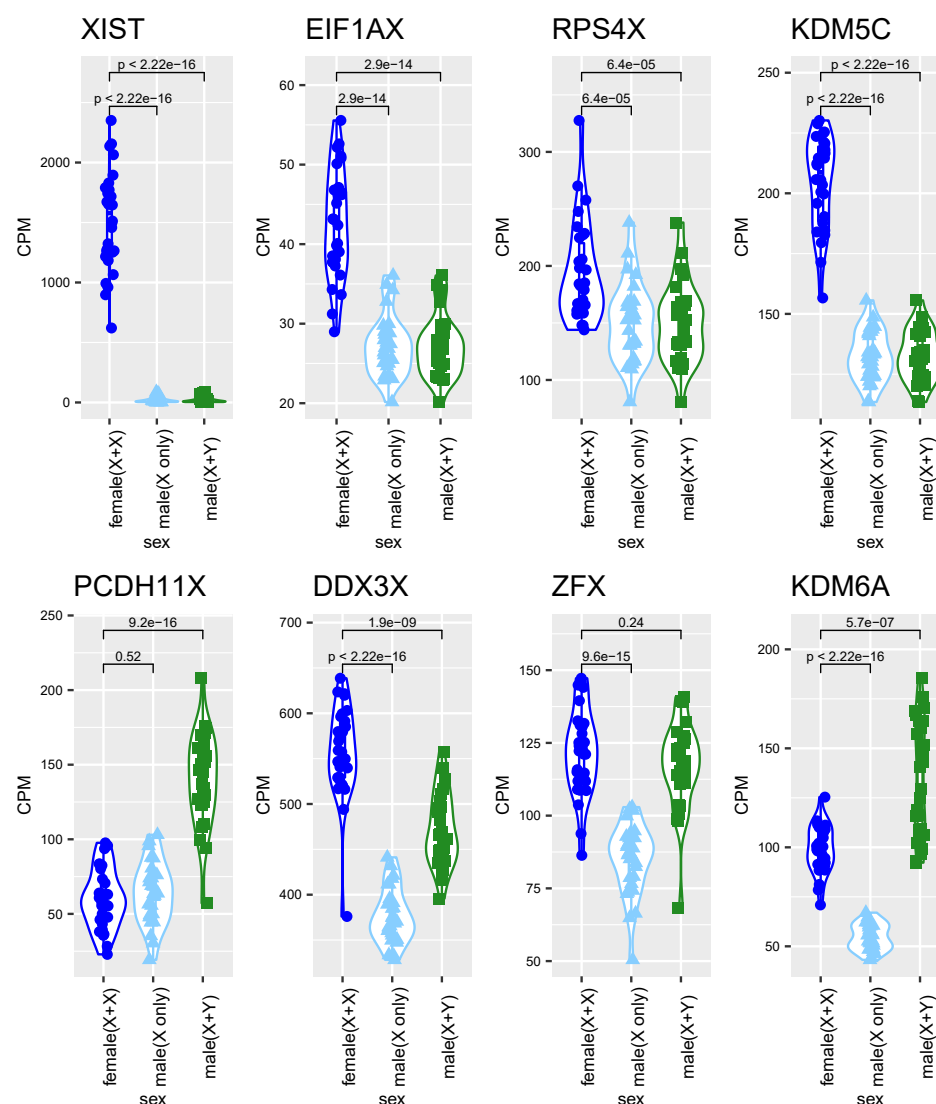
Discussion

Sex differences in gene expression begin in early pregnancy and continue through adulthood

We observed a positive correlation in the $\log_2$ female-to-male expression ratio for sex differentially expressed genes between the late first trimester (Gonzalez et al., 2018) and term placentas, and between term placentas and 42 non-reproductive adult GTEx tissues (Figure 3). A previous study using microarrays compared first trimester and term placentas and found thousands of genes to be differentially expressed, but they did not characterize sex differences (Sitras et al., 2012). A previous microarray study on placental cell types reported 11 genes that were sex differentially expressed in all trophoblast and endothelial cell types, all of which are sex differentially expressed in the late first trimester and term placenta samples analyzed here (Cvitic et al., 2013). Another study of term placentas reported only X- and Y-linked genes as differentially expressed between female and male placentas (Sõber et al, 2015), consistent with our results which revealed the majority of sex differentially expressed genes on chromosomes X and Y. When we looked at all genes located on the sex chromosomes, X & Y, the $\log_2$ female-to-male expression ratio was positively correlated between late first trimester and term placentas, as well as adult tissues (Figure 3). When this was repeated for only autosomal genes, 1-22 and mitochondrial chromosomes, we did not observe the same positive correlation between late first trimester, term placentas, and adult tissues (Figure 3). These findings suggest that sex differences in gene expression for sex-linked genes develop early in embryonic tissue and are replicated in adult tissues. Sex differential expression for autosomal genes may be more tissue-dependent, as previously suggested by Lopes-Ramos et al. 2020. Lopes-Ramos et al. 2020 found that sex differentially expressed genes common among adult tissues were enriched for sex chromosome genes, and sex differences for autosomal genes were tissue-specific (Lopes-Ramos et al., 2020). In summary, we observed a positive and significant correlation in the $\log_2$ female-to-male expression ratio for sex-linked genes between term placentas and adult tissues (Figure 3).

Our study showed that sex differences in placental gene expression is correlated to sex differences in adult tissues, particularly in the brain. In addition to hormones, the placenta produces a host of neurotransmitters to facilitate fetal brain formation (Rosenfeld, 2021). Dopamine and norepinephrine are expressed in placenta cytotrophoblasts in early pregnancy

620 and syncytiotrophoblast cells in late pregnancy (Zhu et al., 2002). This normal expression was

621 altered in pregnancy complications such as pregnancy-induced hypertension syndrome and

622 developmental exposure to endocrine-disrupting chemicals such as bisphenol A and S (Mao et

623 al., 2020; Zhu et al., 2002). Sex differences in fetal brain development have been associated

624 with sex-linked genes such as O-linked N-acetylglucosamine transferase (OGT), which is

625 expressed at a higher level in XX females because it escapes X chromosome inactivation,

626 leading to increased histone methylation and decreased gene expression thought to provide a

627 neuroprotective state in females (Bale, 2016). Taken together, sex differentially expressed

628 genes in the placenta that are correlated to sex differentially expressed genes in the adult brain

629 could provide insight into mechanisms driving brain development differently in each sex and

630 potentially how these mechanisms are altered in neurological disorders.

631

Gene enrichment of sexually dimorphic genes reveals genes that may be involved in pregnancy complications

634 Sex differentially expressed genes may be involved in biological pathways related to pregnancy

635 complications. In term placentas, genes upregulated in XY males are involved in histone lysine

636 and protein demethylation processes and histone demethylase activity, driven by Y-linked

637 genes, including UTY and KDM5D (Additional Table 11). A review of ruminant placenta gene

638 targeting found histone lysine demethylase 1A and androgen signaling involved in gene

639 networks for cell proliferation and angiogenesis (Hord et al., 2020). The authors also note

640 previous studies that have examined exposure to testosterone during pregnancy leading to

641 ovarian dysfunction and low-birth-weight for female offspring suggesting that increased

642 androgen signaling dysregulates fetal development for female offspring (Hord et al., 2020). Five

643 genes in the minor histocompatibility antigen gene family were also upregulated in term male

644 placentas. It has been shown that minor histocompatibility antigens are involved with maternal

645 immune exposure to fetal antigens needed to prevent fetus rejection (Linscheid & Petroff, 2013).

646 Genes upregulated in XX female term placentas are involved in translational initiation and

647 regulation of sister chromatid cohesion, driven mainly by X-linked genes, including NAA10.

648 NAA10 is involved in post-translational protein modifications, and mutations in NAA10 are

649 known to cause Ogden syndrome, which may lead to growth failure (Lee et al., 2017)

650 (Additional Table 11). In a Naa10 mouse knockout study, the authors reported placental

651 insufficiency that contributed to embryonic and neonatal lethality (Lee et al., 2017). Naa10

652   mouse knockouts showed low birth weight and postnatal growth failure compared to control

653   mice (Lee et al., 2017). Loss of NAA10 plays a role in the development of cardiovascular and

654   growth defects in humans and mice (Lee et al., 2017; Wu & Lyon, 2018). In our study, NAA10 is

655   upregulated in females compared to male term uncomplicated placentas (Additional Table 7).

656

657   Using novel sex-informed alignment and functional enrichment analysis, we reanalyzed the

658   Gonzalez et al. 2018 late first-trimester placenta data to compare to our newly collected full-term

659   placenta data. We found genes upregulated in male XY compared to female XX late first

660   trimester placentas are involved in positive regulation of anoikis (a form of programmed cell

661   death). We found that genes upregulated in the female late first trimester placentas are involved

662   in organonitrogen compound catabolic, branched-chain amino acid catabolic, and positive

663   regulation of protein K63-linked ubiquitination processes. NUDT10 was shown to be enriched in

664   these biological processes and is upregulated in female XX late first trimester placentas and

665   was reported in the Gonzalez et al. 2018 study as well (Gonzalez et al., 2018). The role of

666   NUD10 in placenta function remains to be further explored. Additionally, of the 58 genes

667   previously identified as sex differentially expressed in the late first-trimester placentas by

668   Gonzalez et al. 2018, we found 45 of those genes to be sex differentially expressed in the

669   samples using different tools to process the data. Overall, we replicated the findings from

670   Gonzalez et al. 2018 and identified 210 additional genes to be sex differentially expressed

671   among the late first trimester placentas, several of which have been associated with pregnancy

672   complications (Additional Table 10 and 12). We further observed a high positive correlation in

673   the $\log_2$ female-to-male expression ratio for all 268 sex differentially expressed in the late first

674   trimester placentas compared to that of the term placentas (Figure 1).

Sex differences in immune gene expression linked to pregnancy complications

677   The placenta is an immune modulator in the uterine environment interacting with the maternal

678   decidua cells to promote an immunosuppressive environment for maintaining fetal tolerance

679   (Mor et al., 2011; PrabhuDas et al., 2015; Xin et al., 2014). The placenta promotes inflammation

680   response with up-regulation of pro-inflammatory cytokines during early implantation and late in

681   pregnancy (Mor et al., 2011; PrabhuDas et al., 2015). Placentas from preeclampsia pregnancies

682   have been reported to show lower expression of immune protein CD74 and enrichment for the

683   IL-1-signaling pathway compared to uncomplicated placentas (Przybyl et al., 2016).

684   Furthermore, differences in maternal immune function are different based on the sex of the

685   fetus, such as maternal cytokine production increases throughout pregnancy and are higher

686   when carrying a female fetus (Mitchell et al., 2017). Immune gene expression within the

687   placenta plays a role in maintaining a pregnancy to term (PrabhuDas et al., 2015; Xin et al.,

688   2014); we, therefore, sought to characterize sex differences in immune gene expression in the

689   late first trimester (Gonzalez et al., 2018) and term uncomplicated placentas to expand on

690   previously reported sex differences among uncomplicated placentas (Gonzalez et al. 2018 and

691   Sood et al. 2006).

692

693   While an inflammatory response has been shown to happen in the first and third trimesters of

694   pregnancy (Mor et al., 2011), we observed sex differences in immune genes almost exclusively

695   in late first trimester placentas. In the term placentas, when looking at sex differences for only

696   innate immune genes, only DDX3X showed a difference in expression between the sexes, with

697   higher expression in female placentas compared to males (adjusted p-value = 5.55 x 10^{-8})

698   (Figure 2). DDX3X is essential in cell cycle control, and loss of Ddx3x in male mice resulted in

699   early post-implantation lethality (C.-Y. Chen et al., 2016). In female mice, inactivation of a

700   paternal Ddx3x copy resulted in placental abnormalities and embryonic lethality (C.-Y. Chen et

701   al., 2016). This suggests that the expression of DDX3X in the placenta may be critical for proper

702   placental development. DDX3X may show higher expression in female than male placentas

703   because DDX3X escapes X chromosome inactivation in female uncomplicated placentas,

704   showing expression of both the maternal and paternal gene copy (Phung et al., n.d.). Although

705   not significantly differentially expressed between the sexes, there were several immune genes

706   that showed higher mean expression in female or male placentas. CXCR4 showed 1.5 fold

707   higher expression in term male placentas (p-value = 0.004). CXCR4 is a chemokine receptor

708   shown to be expressed in both early and term placenta (Kumar et al., 2004) and shown to have

709   higher expression in mothers with preeclampsia and gestational hypertension (Zheng et al.,

710   2021). CXCR4 showed sex-dependent overexpression in lung adenocarcinomas and the signal

711   was shown to be higher in premenopausal women compared to postmenopausal women and

712   men (Rodriguez-Lara et al., 2014). AQP3 showed a 2 fold higher mean expression in term

713   female placentas. AQP3 is an aquaporin water channel cell membrane protein expressed in

714   both placenta and fetal membranes that has been suggested to be involved in regulation of

715   amniotic fluid homeostasis (Szpilbarg & Damiano, 2017). Cell line studies showed that the

716   inhibition of AQP3 reduces cell migration in trophoblast cells (Alejandra et al., 2018) and

717   expression of AQP3 was found to be significantly reduced in preeclampsia (Szpilbarg &

718   Damiano, 2017). Sex differences in expression of other members of the aquaporin family have

719   been observed in other organs in rodent models (Fan et al., 2005; Nicchia et al., 2001; Sun et

720   al., 2007), but sex differences in placenta expression has yet to be studied. Overall, except for

721   DDX3X, we observed a lack of sex differences in gene expression of innate immune genes

722   among uncomplicated term placentas (Figure 2 & Additional Table 6), suggesting expression of

723   these genes may be important for maintaining all pregnancies to term regardless of the sex of

724   the fetus.

725

726   In the late first trimester placentas (Gonzalez et al., 2018), 37 innate immune genes were

727   differentially expressed between the sexes (adjusted p-value < 0.05), including DDX3X

728   (Additional Table 6). Of the 37 innate immune genes differentially expressed between the

729   sexes, SERPING1 showed the highest expression ratio between male to female placentas

730   ($\log_2$(fold change=1.7). SERPING1 encodes for a highly glycosylated protein and is involved in

731   inhibiting C1r and C1a of the complement component. SERPING1 may be involved in the

732   placental circulatory function, the dyssregulation of which could lead to vascular leakiness and

733   subsequent edema—which often accompany preeclampsia (Vaiman et al., 2005). Intriguingly,

734   unmethylated CpG islands by the transcription start site of SERPING1 have been associated

735   with eight times higher gene expression in placenta tissue from patients with preeclampsia

736   (Blanch et al., 2003; Chelbi et al., 2007). Similarly, SREBF1, which also showed significantly

737   higher expression in males, showed lower methylation rates and higher expression in placentas

738   in pregnancies after using assisted reproductive technologies (Lou et al., 2014), suggesting

739   potential links to uterine conditions that hinder implantation. Overall, we observed many genes

740   that showed sex differences in expression for innate immune genes in the late first trimester

741   placentas, but not in the term placentas, though some of this may have been due to our ability

742   to account for covariates in the analysis of term placentas.

Quantification of X-linked gametolog genes

744   Measuring gene expression on sex chromosomes is complicated due to areas of highly

745   conserved regions between the X and Y chromosomes (Olney et al., 2020). Quantifying gene

746   expression is particularly problematic for gametolog genes which have functional copies on both

747   the X and Y chromosomes that are thought to be expressed off of both X and Y chromosomes

748   in males (Wilson & Makova, 2009). For genes present only on the X chromosome, silencing of

749   one X in XX females is thought to equalize expression of the one X in XY males. However,

750   some genes escape X inactivation in XX females and some gametologs show expression from

751   the Y chromosomes in XY males making functional quantification of these genes more difficult

752   (Godfrey et al., 2020; Wilson Sayres & Makova, 2013). In this study we assessed how summing

753   expression of gametologs with X- and Y-linked expression in XY males may alter how we

754   interpret sex differences in gene expression, in contrast with comparing only X-linked gametolog

755   expression. Gene expression differences between female and male placentas in both the full-

756   term placentas (Figure 4, Additional Table 5) and late first trimester placentas (Additional Figure

757   6, Additional Table 5) for DDX3X, ZFX, PCDH11AX, and KDM6A (UTX) would all be interpreted

758   very differently if expression from X- and Y-chromosomes would have been summed in XY

759   males. For all 4 of these genes, previous literature has demonstrated that they are transcribed

760   from both the X- and Y-chromosomes and that the gametologous genes have similar functions.

761   DDX3X and DDX3Y share over 90% sequence similarity and were observed to have redundant

762   roles in protein synthesis (Venkataramanan et al., 2021). KDM6A (also called UTX) has been

763   shown to escape X-inactivation, and mouse model studies of Y-linked gametolog UTY showed

764   that it maintains some of its function (Gažová et al., 2019). For the same reasons, ZFX and ZFY

765   and protocadherin PCDH11X and PCDH11Y expression were both previously summed in cell

766   line studies that demonstrate dosage compensation on genes from the sex chromosomes

767   (Johansson et al., 2016; Schneider-Gädicke et al., 1989). Properly quantifying gene expression

768   of gametologs from the sex chromosomes can help us to more accurately identify sex

769   differences in gene expression.

Perspectives and Significance

770

771   Overall, these results highlight the importance of considering sex-shared and sex differences in

772   gene expression. While we found some differences in the exact genes that show significant

773   differences in gene expression, we observed that overall genes that showed sex differences in

774   gene expression in term placentas also exhibited sex differences in late first trimester, and

775   across most adult tissues, including all regions of the brain. Because of the comparisons we had

776   the capacity to make here, we cannot fully distinguish the relative importance of sex

777   chromosomes, sex hormones and gonadal sex on driving sex differences in gene expression in

778   the samples studied here. Recent work in adult tissues (liver and adipose) in a mouse model

779   reports the strongest effect of activational hormones, followed by a lesser organizational gonad

780   effect, with a third strongest role of sex chromosome complement on sex differences in gene

781   expression (Blencowe et al., 2022). Thus, one can hypothesize that a similar hierarchy may be

782   in place for shaping sex differences in gene expression in humans. It is potentially provocative,

783 then, that we observed that the most conserved sex differences across tissues and

784 developmental timepoints were found on the X chromosome, suggesting a strong interaction

785 between hormones, gonads, and chromosomes in determining sex differences in gene

786 expression.

787 Declarations

788 Ethics approval and consent to participate

789 This research was approved by HIC#1309012696 to The Yale University Reproductive

790 Sciences Data and Specimen Biorepository, a component of the Department of Obstetrics,

791 Gynecology & Reproductive Sciences, Yale School of Medicine, New Haven, CT.

792 Consent for publication

793 Consent for publication was included in HIC#1309012696.

794 Availability of data and materials

795 The datasets generated during this study are available via controlled access: phs002240.v1.p1

796 NIGMS Sex Differences Placentas at https://www.ncbi.nlm.nih.gov/projects/gap/cgi-

797 bin/study.cgi?study_id=phs002240.v1.p1. The data from Gonzales et al was accessed here:

798 NCBI GEO Accession GSE109082. The GTEx data was accessed here:

799 https://storage.googleapis.com/gtex_analysis_v8/rna_seq_data/GTEx_Analysis_2017-06-

800 05_v8_RNASeQCv1.1.9_gene_tpm.gct.gz. The code generated during this study is available at

801 https://github.com/SexChrLab/Placenta_Sex_Diff.

802 Competing interests

803 The authors declare that they have no competing interests.

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Authors' contributions

KCO: Conceptualization, Supervision, Formal Analysis, Investigation, Visualization, Writing - Original Draft, Writing - Review and Editing

SBP: Formal Analysis, Investigation, Visualization, Writing - Original Draft, Writing - Review and Editing

TNP: Conceptualization, Supervision, Visualization, Writing - Original Draft Preparation, Writing - Review and Editing

MS: Assistance with collecting the placenta samples

JO: Assistance with collecting the placenta samples

LP: Assistance with collecting the placenta samples

LR: Investigation

HJK: Conceptualization, Assistance with collecting the placenta samples, Writing – Review and Editing

MAW: Conceptualization, Supervision, Visualization, Resources, Project Administration, Writing - Original Draft Preparation, Writing - Review and Editing, Funding Acquisition

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