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Maternal bias and escape from X chromosome imprinting in the midgestation mouse placenta ☆

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ABSTRACT

To investigate the epigenetic landscape at the interface between mother and fetus, we provide a comprehensive analysis of parent-of-origin bias in the mouse placenta. Using F1 interspecies hybrids between *mus musculus* (C57BL/6J) and *mus musculus castaneus*, we sequenced RNA from 23 individual midgestation placentas, five late stage placentas, and two yolk sac samples and then used SNPs to determine whether transcripts were preferentially generated from the maternal or paternal allele. In the placenta, we find 103 genes that show significant and reproducible parent-of-origin bias, of which 78 are novel candidates. Most (96%) show a strong maternal bias which we demonstrate, via multiple mathematical models, pyrosequencing, and FISH, is not due to maternal decidual contamination. Analysis of the X chromosome also reveals paternal expression of *Xist* and several genes that escape inactivation, most significantly *Alas2*, *Fhl1*, and *Slc38a5*. Finally, sequencing individual placentas allowed us to reveal notable expression similarity between littermates. In all, we observe a striking preference for maternal transcription in the midgestation mouse placenta and a dynamic imprinting landscape in extraembryonic tissues, reflecting the complex nature of epigenetic pathways in the placenta.

Introduction

In mammals, while most genes are transcribed equally from the maternal and paternal allele, over 100 genes have been identified which have a strong parent-of-origin bias. This bias, called genomic imprinting, was initially discovered through genetic complementation studies (Searle and Beechey, 1978), in which crossing a male with a deletion and a female with a duplication in the same locus failed to rescue the deficiency. This suggested that healthy development requires specific contributions from both the maternal and paternal genomes. Imprinting was further confirmed by the analysis of parthenogenetic and androgenetic embryos. Parthenogenones, with two maternal genomes, show substantial undergrowth of extraembryonic tissues (Surani and Barton, 1983; Surani et al., 1984). Conversely, androgenones, with two paternal genomes, have mostly normal placental tissue (Barton et al., 1984). These data lead to a hypothesis that the paternal genome is responsible for the development of extraembryonic tissues, while the maternal genome limits their growth.

The distinctive patterns of imprinting in the murine placenta are epitomized by the placenta-specific silencing of the entire paternal X chromosome. Mammals silence one copy of the X chromosome in females in order to ensure equivalent gene expression between XX females and XY males (Lyon, 1961). This silencing is controlled by *Xist* – an RNA transcribed exclusively from the inactive chromosome, which leads to its repression (Brockdorff et al., 1991; Brown et al., 1991; Johnston et al., 1998). *Xist* is expressed from the paternal allele in cleavage-stage embryos, silencing the paternal X chromosome in early embryos and in the placenta (Brockdorff et al., 1991; Brown et al., 1991; Harper et al., 1982; Monk and Harper, 1979; Takagi and Sasaki, 1975; West et al., 1977). Maternal *Xist* is reactivated in the inner cell mass, and during tissue development, stochastic variation causes one allele to take over and one chromosome to be silenced. In contrast, this reactivation is absent in the placenta and the maternal X chromosome continues to be expressed. The story of the X chromosome highlights that imprinting in the placenta occurs at different regions, and perhaps through different mechanisms, than imprinting in other cell lineages.

The exact mechanisms underlying the placenta's unique epigenetic state, including variation in imprinting and X inactivation, are unknown. However, it is likely that chromatin modifications play a role. Overall, the placenta appears to be depleted for many

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of the repressive marks seen in embryonic tissues. Placental DNA is distinctly hypomethylated (Razin et al., 1984) and trophoblast cells can survive without any DNA methylation (Sakaue et al., 2010). It has been shown at two regions on chromosome seven that histone marks, rather than DNA methylation, control allele-specific silencing at imprinted regions in the placenta (Lewis et al., 2004; Umlauf et al., 2004). However, even these histone marks are relatively low in the placenta: in particular, H3K27me3 and arginine methylation (Chuong et al., 2013; Rugg-Gunn et al., 2010; Torres-Padilla et al., 2007). Further, the placenta is tolerant of polyploidy, as is demonstrable by tetraploid complementation, in which tetraploid cells injected into a blastocyst are excluded from the embryo but contribute to the placenta (Nagy et al., 1990). Wild-type placental cells are frequently polyploid or polynucleate, such as trophoblast giant cells, syncytiotrophoblasts, and extravillous cytotrophoblasts (Hu and Cross, 2010). These observations suggest that the placenta is the site of marked flexibility in the biochemical pathways generally controlling gene expression.

The plasticity of the placental environment, illustrated both in epigenetic marks and expression of imprinted genes, raises the question of the extent of placental imprinting. Marked improvements in sequencing technology allow for the discrimination between, not only how many imprinted genes exist, but also to what extent these genes display bias. High throughput RNA sequencing of F1 hybrids has provided an unprecedented ability to identify allele-specific expression due to imprinting (Goncalves et al., 2012; Gregg et al., 2010a, 2010b; Okae et al., 2012; Wang et al., 2011), although this technology and the associated statistical and bioinformatic approaches are still in development. While initial studies identified a small number of novel imprinted transcripts (Babak et al., 2008; Wang et al., 2008), recent studies have suggested imprinting at more than 1300 transcripts in the mouse brain (Gregg et al., 2010a, 2010b). Additionally, several recent reports have used RNA sequencing of F1 hybrids to identify an additional 200–1000 imprinted genes specific to the placenta (Okae et al., 2012; Wang et al., 2011). There is still controversy in the field as to whether this marked increase in the number of imprinted transcripts is a result of increased sequencing depth and sensitivity or noise and bias in the sequencing data (DeVeale et al., 2012). It is essential to refine methodologies for sample preparation and data analysis in order for these technologies to clearly provide new insights into genomic imprinting.

Here, we provide a new means to detect parent-of-origin bias in the placenta. We show that our methods improve identification of imprinted loci; we identify regions on the paternal X chromosome that escape imprinting; and we demonstrate a clear preference for placental expression of the maternal allele of hundreds of transcripts. Moreover, throughout this analysis, we use individual placental samples rather than pooled samples, allowing us to analyze inter-individual variation. In all, this study defines an improved approach for studying imprinting in the placenta, and sheds light on several features of the placental epigenetic environment.

Materials and methods

An overview of the general experimental procedure (covered in the following four sections) is provided in Fig. S1.

Mouse strains and crosses

Strains used were C57BL/6J and CAST/EiJ. C57BL/6J females were crossed with CAST/EiJ males to generate B6 X CAST F1 hybrids, and CAST/EiJ females were crossed with C57BL/6J males to generate CAST X B6 F1 hybrids. Placentas and yolk sacs of F1 hybrids were dissected at stage e11.5 (12 placentas from 3 litters B6 X CAST, 11 placentas from 2 litters CAST X B6) and e17.5 (6 animals from 1 litter, B6 X CAST only). To minimize maternal contamination, we removed the decidual layer of the placenta during dissection.

Illumina sequencing of 3′ ends of mRNA

RNA sequencing libraries were built for each individual placenta using an approach which targets the 3' end (3SEQ) as described previously (Beck et al., 2010). Samples were prepped in cohorts containing individuals from multiple litters dissected on different days. Total RNA was isolated from placenta using a phenol–chloroform purification with Trizol (Invitrogen). Additionally, DNA was precipitated with ethanol and purified for genotyping to verify gender. Briefly, after purification, mRNA was heat sheared before reverse transcription with the P7_oligodT primer. The P5 linker was then ligated to the free end, sequences are amplified using primers to P5 and P7, and the resulting library was sequenced from P5 using the Illumina Genome Analyzer II. In total, we sequenced and mapped 225 million reads for the B6 X CAST cross and 226 million reads for the CAST X B6 cross, with an average of 20 million reads per library.

Alignment and quantification

A *Mus musculus castaneus* genome was created in silico by applying the high-quality Castaneus SNPs (as defined by the Sanger Mouse Genomes Project) (Yalcin et al., 2012) to the reference C57-Black6/J genome. For each reference genome, a transcriptome was constructed using RefSeq annotations, including spliced transcripts of all genes separated by 200″N″ nucleotides. This spliced transcriptome sequence was added to the reference genomic sequence to create a composite genome.

BWA (Li and Durbin, 2009) was used to align all reads to both composite genomes separately, thus mapping each individual read to a genome. For each read, we retained its best alignments, but required that they all correspond to the same genomic location. This effectively disregarded reads mapping to multiple genomic locations but allowed reads to map to multiple transcriptomic positions that corresponded to the same exon or splice junction. The alignments for each read were then consolidated into a single genomic alignment, including intronic splice gaps. Since each read was aligned to both the B6 and Cast references, we chose the alignment from the reference with the fewest number of differences (selecting randomly in the case of a tie), and used this alignment for the remaining analyses.

For measuring allelic bias, only SNPs that were at least 50 bp away from Sanger-annotated short Cast indels were used for counting allelic expression; this was done to reduce potential bias due to lower mapping sensitivity of reads containing indels.

Global expression patterns were exported from DNANexus using the 3'-seq/transcriptome-based quantification analysis. These data were subsequently normalized using DESeq (Anders and Huber, 2010).

Detection of significant effects and identification of candidates

For gene specific analyses, we compared the total number of reads mapped to the Castaneus allele to the total number of reads mapped to the B6 allele. For SNP specific analyses, we split each read “vote” evenly between the SNPs it contained, and used these numbers. We used a two-sample paired *t*-test and a Wilcoxon Mann Whitney test to determine when the maternal read counts were significantly different from the paternal read counts in our sampled biological replicates. A transcript was called as