Placental effects on maternal brain revealed by disrupted placental gene expression in mouse hybrids

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Abstract

The mammalian placenta is both the physical interface between mother and fetus, and the source of endocrine signals that target the maternal hypothalamus, priming females for parturition, lactation and motherhood. Despite the importance of this connection, the effects of altered placental signaling on the maternal brain are understudied. Here, we show that placental dysfunction alters gene expression in the maternal brain, with the potential to affect maternal behavior. Using a cross between the house mouse and the Algerian mouse in which hybrid placental development is abnormal, we sequenced late gestation placental and maternal medial preoptic area transcriptomes and quantified differential expression and placenta-maternal brain co-expression between normal and hybrid pregnancies. The expression of *Fmn1, Drd3, Caln1* and *Ctsr* was significantly altered in the brains of females exposed to hybrid placentas. Most strikingly, expression patterns of placenta-specific gene families and *Drd3* in the brains of house mouse females carrying hybrid litters matched those of female Algerian mice, the paternal species in the cross. Our results indicate that the paternally-derived placental genome can influence the expression of maternal-fetal communication genes, including placental hormones, suggesting an effect of the offspring's father on the mother’s brain.

Introduction

The placenta is a unique, transient organ shared by two organisms. Placental morphology is surprisingly diverse across vertebrates and is subject to rapid evolutionary change and convergent evolution (Blackburn 1993; Reznick et al. 2002; Roberts et al. 2016; Armstrong et al. 2017). In most eutherian mammals, including mice and humans, successful blastocyst implantation relies on endometrial invasion by the embryonic trophoblast cells that give rise to the mature placenta (Cross et al. 1994). As such, the placenta provides the closest physical and molecular link between mother and offspring seen in any animal (Wagner et al. 2014). This intimate connection promotes an array of maternal-fetal interactions, including bidirectional hormonal regulation and even the exchange of entire cells. These interactions are not spatially limited, but extend to both the fetal and the maternal brain (Bridges et al. 1996; Ladyman et al. 2010; Boddy et al.2015).

Throughout pregnancy the placenta mediates the regulation of resource allocation, immune tolerance, fetal development and, importantly, hormonal priming of the maternal brain. A key subset of placenta-secreted molecules are involved in priming maternal physiology for parturition and lactation, and promoting the onset of maternal behaviors in late gestation (reviewed in Bridges 2015; Creeth et al. 2018). In rodents, the medial preoptic area (MPoA) in the hypothalamus is thought to be the primary neural target of these placental molecules (Bridges et al. 1996; Mann and Bridges 2001; Larsen and Grattan 2012). The MPoA has been characterized as the central hub of parenting behavior (Kohl and Dulac 2018): receptors for key pregnancy hormones and neurotransmitters, including estrogen, prolactin and dopamine, are highly expressed in this nucleus and interact with ligands of both maternal and placental origin (Numan and Insel 2003).

62 Two classes of placental genes are of particular importance to the interaction between
63 placenta and maternal brain: imprinted genes (IGs) and placenta-specific gene families (PSFs).
64 Several IGs and PSF genes are expressed in the same placental compartment, especially in the
65 placental endocrine compartment (Simmons et al. 2007, 2008; Tunster et al. 2013). IGs are
66 exclusively or predominantly expressed from one allele, and are highly expressed in placenta and
67 brain. The allelic expression is initiated by heritable epigenetic marks ("imprints") in maternal
68 and paternal germ cells, such that some IGs are maternally silenced and paternally expressed,
69 whereas others are paternally silenced and maternally expressed (Reik and Walter 2001;
70 Ferguson-Smith 2011). During pregnancy, IGs are critical to placental development and
71 function, maintaining the balance between maternal supply and embryonic demand, and
72 regulating maternal-fetal exchange (Constancia et al. 2005; Lefebvre 2012; Tunster et al. 2013).
73 PSFs arose through lineage-specific gene duplication events during placental evolution
74 (Rawn and Cross 2008). In rodents, these are the prolactin gene family (placental lactogens
75 (Prls)), placental cathepsin proteases and their inhibitors (PECs) and pregnancy specific
76 glycoproteins (PSGs) (Zebhauser et al. 2005; Soares et al. 2007; Mason 2008). PSF gene
77 products are mainly expressed from the placental endocrine compartment and many are secreted
78 into the maternal bloodstream; key functions include placental development, immunoregulation,
79 and physiological and neurological priming of the maternal organism (Rawn and Cross 2008;
80 Bridges et al. 1996; Mann and Bridges 2001). Most notably, a subset of PRLs are able to bind
81 the prolactin receptor that is expressed in the maternal MPoA leading to the proposal that
82 placental hormones directly affect maternal endocrine state and behavior (Bridges et al. 1996;
83 Larsen and Grattan 2012). IGs are implicated in regulating PSF secretion via their effects on the
84 structure and function of the placental endocrine compartment (John 2017). However, our

85 current understanding of the role of IGs in PSF signaling is rudimentary, and the relationship
86 between gene expression in placenta and the maternal MPoA is uncharted.

87 The majority of the placenta, including the endocrine compartment, is fetally-derived.
88 Placental representation of both parental genomes sets the stage for conflict (maternal-paternal
89 and parent-offspring), and for coadaptation (mother-offspring), with imprinted genes uniquely
90 positioned to mediate both types of interactions (Moore and Haig 1991; Wolf and Hager 2006,
91 Keverne and Curly 2008; Haig 2014). However, while evolutionary models for imprinted gene
92 expression abound (reviewed in Patten et al. 2014), few have considered the interaction between
93 paternally-derived placental signals and signal reception in the maternal brain (Janssen et al.
94 2016a; McNamara et al. 2018; Creeth et al. 2018).

95 Here, we use a natural hybrid system to explore the effects of placental dysregulation on
96 gene expression in the maternal brain. Over- or under-growth that depends on the direction of the
97 cross is a signature of disrupted imprinting in mammalian hybrids (Vrana 2007). This pattern is
98 documented in several orders (Gray 1972), with the best-studied examples in rodents (multiple
99 species in the genera *Peromyscus, Mus* and *Phodopus* (Zechner et al. 1996; Vrana et al. 1998;
100 Brekke and Good 2014)). Parent-of-origin growth effects in the cross between the house mouse,
101 *Mus m. domesticus* (*Dom*) and the Algerian mouse, *M. spretus* (*Spret*), were first described over
102 20 years ago: placentas are undersized when the mother is *Dom* and the father is *Spret*, and
103 severely oversized in the reciprocal cross, with more extreme size effects in both directions of
104 the backcross (Zechner et al. 1996). Subsequent studies confirmed altered expression and
105 methylation of candidate IGs, and disrupted placental organization (Hemberger et al. 1999;
106 Zechner et al. 2002, 2004; Shi et al. 2005). Specifically, the placental endocrine compartment (or
107 junctional zone) was shown to be reduced and disorganized (Zechner et al. 1996; Kurz et al.

108 1999). However, the extent of placental misexpression has not been measured on a genome
109 scale, and this system's potential to uncover the maternal consequences of altered placental
110 signaling has not been considered.

111 The characteristics of this classic system, together with the availability of high quality
112 genomes for both parental species (Keane et al. 2011), make the cross between *Dom* and *Spret* an
113 excellent model in which to explore the effects of placental disruptions on the maternal brain. By
114 comparing MPoA expression between females of the same species that differ only in the type of
115 pregnancy/placenta they experience (hybrid vs. conspecific), we specifically isolate the effect of
116 placental gene expression differences on the maternal brain (Fig. 1). Characterization of altered
117 gene expression at the maternal-fetal interface provides insight into the mechanisms of maternal-
118 fetal communication, the contribution of the paternal genome to this interaction, and identifies
119 promising candidate genes for future evolutionary and biomedical work.

120

121 **Results**

122 To study the relationship between placenta and maternal MPoA on a molecular level we
123 produced three crosses resulting in three types of pregnancy: *Dom* x *Dom* (*Dom* pregnancy, n=5,
124 litter size range = 4-6, sex ratio range [proportion males] = 0.3-0.6), *Dom* x *Spret* (hybrid
125 pregnancy, n=5, litter size range = 3-6, sex ratio range = 0.3-1.0) and *Spret* x *Spret* (*Spret*
126 pregnancy, n=5, litter size range = 5-6, sex ratio range = 0.2-0.8) (in all crosses, female is first).
127 For each type of cross we produced 5 biological replicates and extracted the maternal MPoA and
128 placentas from each pregnant female in late gestation at embryonic day (e) 17.5. During late
129 gestation the effect of placental signaling on the maternal MPoA is specifically important for the
130 onset of maternal care at parturition (Bridges et al. 1996; Mann and Bridges 2001; Larsen and

131 Grattan 2012). Placenta weights were compared to confirm that hybrid placentas were
132 hypoplastic as expected (Zechner et al. 1996). Since litter size can have an effect on placental
133 and embryo size we corrected weights for litter size. Hybrid placentas weighed significantly less
134 than placentas of both parental species with no significant differences between parental species
135 (One-way ANOVA (Females): $F(2)=56.95$, $p<0.001$, Tukey HSD: Hybrid-Dom: diff=-0.1,
136 $p<0.001$, Hybrid-Spret: diff=0.1 , $p<0.001$, Dom-Spret: diff=0.002, $p=0.97$). We sequenced the
137 maternal MPoA transcriptome and the placental transcriptomes of 1 male and 1 female per
138 mother (n=9-10/type of pregnancy, in hybrid pregnancies one litter consisted of only males, thus
139 n=4 for females), and evaluated differential expression between all pregnancy types (Fig. 1).
140 Because the maternal brain is exposed to the placental signals of both sexes simultaneously, male
141 and female placental expression was analyzed jointly. Additionally, we assessed co-expression
142 between the two tissues for each type of pregnancy and determined the differences in co-
143 expression between pregnancy types (Fig. 1).
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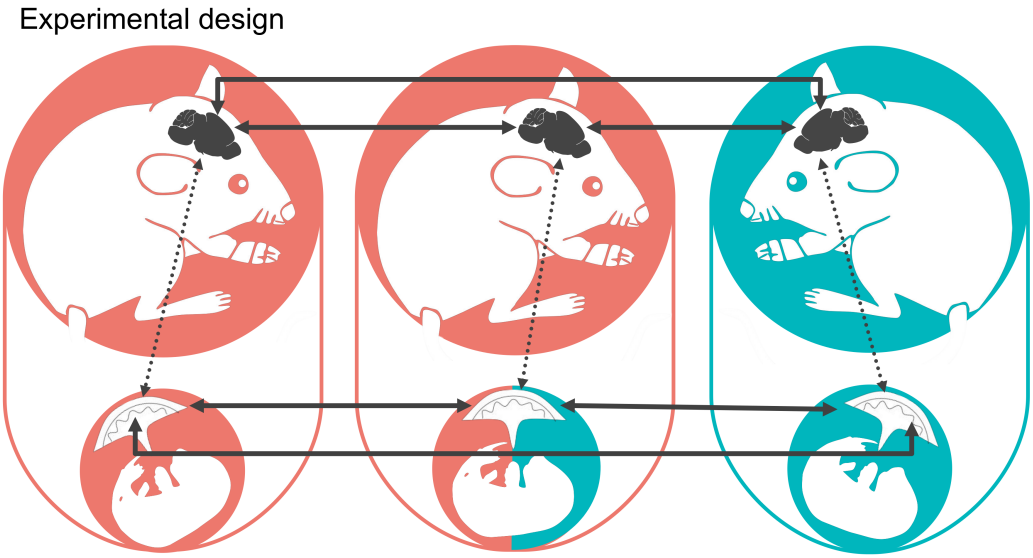

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147 **Figure 1**. Experimental design. Schematic representation of the comparisons performed to test for differential gene
148 expression in the medial preoptic area of the maternal brain and the placenta during late gestation. *Mus m.*
149 *domesticus* is depicted in red and *Mus spretus* in blue. Hybrid tissue is indicated by a combination of red and blue.
150 Solid arrows indicate differential gene expression analysis. Dashed arrows indicate co-expression analysis.
151

152 **Differential expression in the placenta**

153 We tested for differential expression in three pairwise comparisons: hybrid vs. *Dom*, hybrid vs.
154 *Spret,* and *Dom* vs. *Spret* placentas. For placental comparisons, only genes with log2 fold change
155 (LFC) in expression ≥ 0.5 (1.5 times higher or lower expression), and Benjamini-Hochberg-
156 corrected p≤0.05, were considered significantly differentially expressed (DE). We define
157 transgressive expression in hybrids as expression that is significantly higher or lower compared
158 to both parental species. Hybrid genes that were DE compared to *Dom* but not to *Spret* are
159 defined as having *Spret*-like expression patterns, and vice versa.

160 In hybrid placentas 9.73% of all tested genes were expressed higher and 7.79% lower
161 compared to *Dom* placentas (up: 1,781/18,298, including 11 IGs; down: 1,426/18,298, including
162 3 IGs) (Fig. 2*,* Supplemental Fig. S1 and Supplemental Dataset S1). Compared to *Spret*
163 placentas, 16.32% of genes were expressed higher and 9.6% lower in hybrids (up: 3,036/18,529,

164 including 16 IGs; down: 1,801/18,529, including 7 IGs) (Fig. 2*,* Supplemental Fig. S2,
165 Supplemental Dataset S1). Thirty-two percent of all tested genes were DE between *Dom* and
166 *Spret* (up: 4,014/19,079, including 19 IGs; down: 3,278/19,079, including 13 IGs) (Supplemental
167 Fig. S3, Supplemental Dataset S1). Hybrids clustered slightly closer to Dom samples in the
168 diagnostic PCA plot (Supplementary Fig. 4).

169 To explain hybrid placental phenotypes that are not intermediate to both parents*,* genes
170 with transgressive expression are of specific interest. We found 275 genes that were expressed at
171 higher, and 167 at lower, levels in hybrids compared to both parental species (Fig. 2*A*).
172 Transgressively higher expressed genes were significantly enriched for B-cell receptor activation
173 and integrin cell surface interaction pathways (Fig. 2*S,* Supplemental Dataset S1). Interestingly,
174 transgressively lower expressed genes were enriched for prolactin and growth hormone receptor
175 signaling, ERBB signaling, and cytokine signaling in immune system, among others (Fig. 2*A,*
176 Supplemental Dataset S1). Many Prls are involved in these pathways, along with other genes.
177 PSF genes were highly overrepresented among DE genes in hybrids compared to both *Dom*
178 (Fisher's exact test: $p<0.001$, odds ratio = 5.65) and *Spret* ($p<0.001$, odds ratio = 3.41). Multiple
179 members of these gene families were misexpressed in the hybrid placenta, with the majority
180 being expressed lower compared to both parental species (14 transgressively lower, 7 DE
181 intermediate) (Table 1).

182 Notably, approximately twice as many genes in hybrid placentas were uniquely DE
183 relative to *Spret* (3,271) as opposed to *Dom* (1,659) (Fig. 2*A*). Thus, the general expression
184 pattern in hybrid placentas was more similar to the maternal species. *Dom*-like expressed genes
185 were enriched for multiple immune related pathways, together with angiogenesis, vascular
186 development and hemostasis related terms, among others (Supplemental Dataset S1).

187 Genes with a *Spret*-like expression pattern in hybrid placentas are of particular interest,
188 since these have the potential to alter communication with the *Dom* maternal brain. Of the 1,659
189 genes with *Spret*-like expression (Fig. 2*A*), 12 were PSF genes and 6 were IGs (Tables 1 and 2).
190 *Spret*-like expressed genes in the hybrid were enriched for WNT signaling and extracellular
191 matrix organization pathways, among others (Fig. 2*A,* Supplemental Dataset S1).
192 IGs were significantly overrepresented among hybrid DE genes compared to both *Spret*
193 (Fisher's exact test: p=0.02, odds ratio=0.59) and *Dom* (p=0.03, odds ratio=0.55). Three IGs
194 (*Tnfrsf23, Phlda2* and *Klf14*) were transgressively higher expressed and two, (*Ascl2* and *Sfmbt2*)
195 were transgressively down-regulated. Two additional IGs, *Tspan32* and *Th*, were significantly
196 DE compared to both parental species but intermediate between the two. Four of these
197 misexpressed IGs belong to the same imprinting cluster (IC2) on the distal part of mouse
198 chromosome 7 (dist7) (MouseBook (https://www.mousebook.org (03.22.18)), and are normally
199 maternally expressed (Table 2*,* Supplemental Dataset S1). We note that *Tnfrsf23* is expressed in
200 decidual cells at the junction between the maternally derived decidua and the extraembryonic
201 placenta (Clark et al. 2002).
202

203

Table 1. Differential expression of PSF genes in hybrid placenta compared to both parental species

	Chr.	Hybrid vs. *Dom* placenta			Hybrid vs. *Spret* placenta			Hybrid vs. both parental species
		LFC	Stat	padj	LFC	Stat	padj	
Prl3c1	13	-1.60	-7.81	<0.001	-2.31	-8.78	<0.001	Transgressive lower
Prl7d1	13	-0.95	-7.80	<0.001	-0.93	-4.36	<0.001	Transgressive lower
Prl2c5	13	-0.89	-7.04	<0.001	-1.26	-5.75	<0.001	Transgressive lower
Ceacam12	7	-0.87	-4.72	<0.001	-0.88	-3.73	<0.001	Transgressive lower
Ctsm	13	-0.82	-3.19	0.01	-1.22	-4.24	<0.001	Transgressive lower
Prl7a1	13	-0.82	-3.21	0.01	-1.72	-5.57	<0.001	Transgressive lower
Psg25	7	-0.76	-3.45	<0.001	-1.44	-5.54	<0.001	Transgressive lower
Cts3	13	-0.76	-3.01	0.01	-1.55	-5.94	<0.001	Transgressive lower
Prl2c3	13	-0.75	-4.64	<0.001	-1.36	-3.58	<0.001	Transgressive lower
Psg28	7	-0.73	-3.26	0.01	-0.62	-2.38	0.04	Transgressive lower
Prl4a1	13	-0.72	-2.80	0.02	-1.46	-5.20	<0.001	Transgressive lower
Prl7a2	13	-0.71	-2.91	0.01	-1.69	-6.09	<0.001	Transgressive lower
Prl2b1	13	-0.64	-3.64	<0.001	-1.26	-7.05	<0.001	Transgressive lower
Prl2a1	13	-0.59	-3.45	<0.001	-1.41	-5.30	<0.001	Transgressive lower
Cts7	13	-3.68	-13.40	<0.001	3.34	7.92	<0.001	Intermediate
Prl3d1	13	-3.39	-21.70	<0.001	2.41	5.89	<0.001	Intermediate
Ceacam5	7	-1.47	-8.01	<0.001	2.25	10.25	<0.001	Intermediate
Ctsr	13	-0.68	-6.29	<0.001	0.83	6.43	<0.001	Intermediate
Psg20	7	1.43	6.58	<0.001	-2.13	-7.59	<0.001	Intermediate
Psg22	7	2.26	11.63	<0.001	-2.17	-9.72	<0.001	Intermediate
Ceacam3	7	2.73	11.03	<0.001	-1.67	-6.30	<0.001	Intermediate
Cts6	13	-0.97	-7.65	<0.001	-0.12	-0.97	0.46	*Spret-like* expression
Prl2c1	13	-1.49	-7.78	<0.001	0.43	1.39	0.26	*Spret-like* expression
Psg26	7	-0.92	-3.80	<0.001	0.46	1.97	0.10	*Spret-like* expression
Psg27	7	-0.90	-4.08	<0.001	-0.36	-1.50	0.22	*Spret-like* expression
Prl7c1	13	-0.84	-2.54	0.03	0.05	0.11	0.95	*Spret-like* expression
Psg19	7	-0.79	-3.39	<0.001	-0.08	-0.34	0.82	*Spret-like* expression
Prl3d2	13	-0.74	-2.54	0.03	-0.48	-1.11	0.39	*Spret-like* expression
Ceacam11	7	-0.73	-3.91	<0.001	-0.43	-1.88	0.12	*Spret-like* expression
Psg29	7	-0.67	-2.85	0.02	-0.30	-0.96	0.46	*Spret-like* expression
Ceacam15	7	1.24	4.17	<0.001	-0.50	-1.79	0.14	*Spret-like* expression
Prl3b1	13	-1.10	-10.71	<0.001	0.41	3.05	0.01	*Spret-like* expression
Tpbpa	13	-0.58	-3.64	<0.001	-0.49	-2.38	0.04	*Spret-like* expression

Chr.=Chromosome, DE=differential expression, LFC=log2 Fold Change of expression, Padj=adjsted p-value according to Benjamini-Hochberg method, Stat=Wald test (DESeq2).

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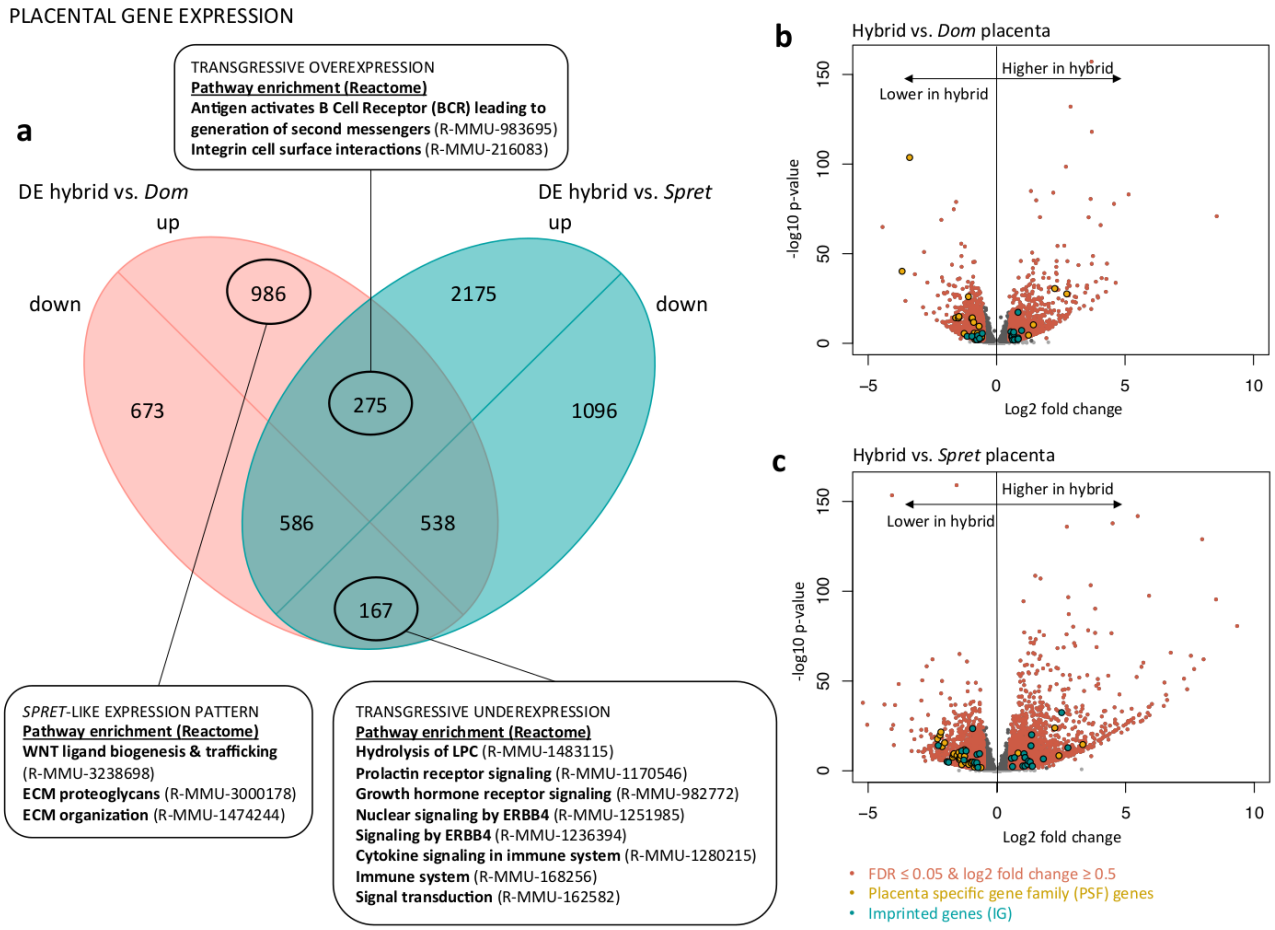

Figure 2. Placental gene expression. Summary of results of differential gene expression (DE) analysis between *Mus m. domesticus* (*Dom*), *Mus spretus* (*Spret*) and hybrid placentas. (A) Venn diagram indicating the overlap of differentially expressed genes between the comparisons hybrid vs. *Dom* and hybrid vs. *Spret*; up = genes expressed higher in hybrids compared to parental species, down = genes expressed lower in hybrids compared to parental species. Genes expressed higher or lower compared to both parental species (transgressive expression) and genes with *Spret*-like expression in the hybrid are marked in the diagram. Results of pathway overrepresentation (Reactome, version 58,Mi et al. 2017) are provided in connected text boxes. Volcano plot of DE analysis results of (B) hybrid vs. *Dom* and (C) hybrid vs. *Spret* placentas. Significantly differentially expressed genes with FDR ≤ 0.05 and log2 fold change ≥ 0.5 are depicted in red. Imprinted genes are indicated in blue and placenta-specific gene family genes in yellow.

217
218

Table 2. Differential expression of imprinted genes in hybrid placenta compared to both parental species

	IC/Chr.	Hybrid vs. *Dom* placenta			Hybrid vs. *Spret* placenta			Hybrid vs. both parental species	expressed allele
		LFC	Stat	padj	LFC	Stat	padj		
Phlda2	dist7-IC2	0.97	5.39	<0.001	1.09	5.44	<0.001	Transgressive higher	Maternal
Klf14	prox6	0.67	5.01	<0.001	1.06	6.29	<0.001	Transgressive higher	Maternal
Tnfrsf23	dist7-IC2	0.59	3.57	<0.001	1.97	9.02	<0.001	Transgressive higher	Maternal
Tspan32	dist7-IC2	-0.66	-3.11	0.01	2.01	6.43	<0.001	Intermediate	Maternal
Th	dist7	0.69	2.48	0.04	-2.28	-7.76	<0.001	Intermediate	Maternal
Ascl2	dist7-IC2	-0.96	-3.85	<0.001	-1.28	-4.90	<0.001	Transgressive lower	Maternal
Sfmbt2	2	-0.56	-4.69	<0.001	-0.67	-6.30	<0.001	Transgressive lower	Paternal
Magel2	cent7	0.61	3.81	<0.001	0.48	2.25	0.06	*Spret-like* expression	Paternal
Dcn	10	0.63	2.45	0.04	-0.06	-0.16	0.92	*Spret-like* expression	Maternal
Nap1l5	prox6	0.82	2.63	0.03	0.31	0.91	0.49	*Spret-like* expression	Paternal
Grb10	prox11	0.83	8.65	<0.001	-0.06	-0.47	0.74	*Spret-like* expression	Maternal
Nnat	dist2	0.84	3.29	<0.001	-0.07	-0.27	0.86	*Spret-like* expression	Paternal
Igf2	dist7-IC1	0.84	2.89	0.01	0.02	0.08	0.96	*Spret-like* expression	Paternal

IC=imprinting cluster (https://www.mousebook.org (03.22.18)), Chr.=Chromosome, DE=differential expression, LFC=log2 Fold Change of expression, Padj=adjsted p-value according to Benjamini-Hochberg method, Stat=Wald test (DESeq2), expressed allele according to https://www.mousebook.org (03.22.18).

219 **Overlap of placental DE genes with genes involved in preeclampsia**

220 Among the misexpressed genes in the hybrid placenta we noticed several that are also
221 misexpressed in the human pregnancy pathology preeclampsia. Although mice do not develop
222 preeclampsia, preeclampsia-like phenotypes can occur and several rodent models have been
223 developed to study key symptoms such as hypertension, proteinura, and altered inflammatory
224 response (Podjarny et al. 2004; Dokras et al. 2006). To test if DE genes in the hybrid overlap
225 with genes related to preeclampsia we extracted genes associated with preeclampsia from the
226 database for preeclampsia (http://ptbdb.cs.brown.edu/dbpec/ (Uzun et al. 2016)) and obtained the
227 mouse orthologs for these from biomart (R-package biomaRt (Durinck et al. 2005)). Of the 490
228 mouse orthologs we obtained, 26 were transgressively misexpressed in the hybrid placenta.
229 Preeclampsia related genes were significantly overrepresented among transgressively
230 misexpressed genes (Fisher's exact test: p=0.003, odds ratio=1.91). An altered inflammatory
231 response at the fetal-maternal interface is involved in preeclampsia in humans (Harmon et al.
232 2016); in the hybrid placenta, both up- and down-regulated transgressively expressed genes were
233 enriched for immune-related and cytokine signaling pathways (Supplemental Dataset S1).

234

235 **Differential expression in the MPoA**

236 To explore maternal gene expression in response to placental genotype we compared late
237 gestation MPoA of *Dom* females during *Dom* pregnancies (MPoA-*dom*), *Dom* females during
238 hybrid pregnancies (MPoA-*hy*) and *Spret* females during *Spret* pregnancies (MPoA-*spret*).
239 Neural and placental tissues were collected from the same females. Gene expression in MPoA-*hy*
240 vs. MPoA-*dom* is expected to be highly similar, with any DE attributable to carrying a hybrid
241 litter, while the other comparisons should result in a large number of DE genes attributable to

242 interspecific differences. For the intraspecific comparison we report significantly DE genes with
243 log2 fold change (LFC) ≥ 0.2, since differences are expected to be subtle. For all other
244 comparisons we report significantly DE genes with LFC ≥ 0.5.

245 Four genes were DE between MPoA-*hy* and MPoA-*dom*: Cathepsin-R (*Ctsr*) was
246 expressed higher in MPoA-*hy* compared to MPoA-*dom*, and Dopamine receptor 3 (*Drd3*),
247 Calneuron 1 (*Caln1*) and Formin 1 (*Fmn1*) were expressed lower (up: 1/18,779; down: 3/18,779)
248 (Fig. 3, Table 3*,* Supplemental Fig. S4 and Supplemental Dataset S2). In MPoA-*hy* compared to
249 MPoA-*spret* 10.61% of all tested genes were expressed higher and 10.32% lower (up:
250 1,608/15,154; down: 1,565/15,154) (Fig. 3*,* Supplemental Fig. S5, Supplemental Dataset S2). In
251 the interspecific comparison of conspecific pregnancies, 11.32% of all tested gene were
252 expressed higher and 10.76% lower in MPoA-*dom* compared to MPoA-*spret* (up: 1,757/15,515;
253 down: 1,670/15,515) (Fig. 3*,* Supplemental Fig. S6, Supplemental Dataset S2).

254 Hybrid placentas express both *Spret* and *Dom* alleles and 1,659 genes had *Spret*-like
255 expression. Therefore, the MPoA in *Dom* females carrying hybrid litters might exhibit
256 expression patterns more similar to *Spret* female MPoA for some maternal-fetal communication
257 genes. We extracted a list of 959 genes that were DE in MPoA-*dom* vs. MPoA-*spret* but not in
258 MPoA-*hy* vs. MPoA-*spret.* This list includes *Drd3,* which was expressed lower in MPoA-*hy*
259 compared to MPoA-*dom* (Fig. 3*,* Supplemental Dataset S2). Thus, *Drd3* could be defined as
260 having *Spret*-like expression in MPoA-*hy. Fmn1* and *Ctsr* were expressed lower and higher,
261 respectively, in MPoA-*hy* compared to both MPoA-*dom* and MPoA-*spret*, and were not DE
262 between *Dom* and *Spret* MPoA during regular pregnancies (Fig. 3*,* Supplemental Dataset S2).
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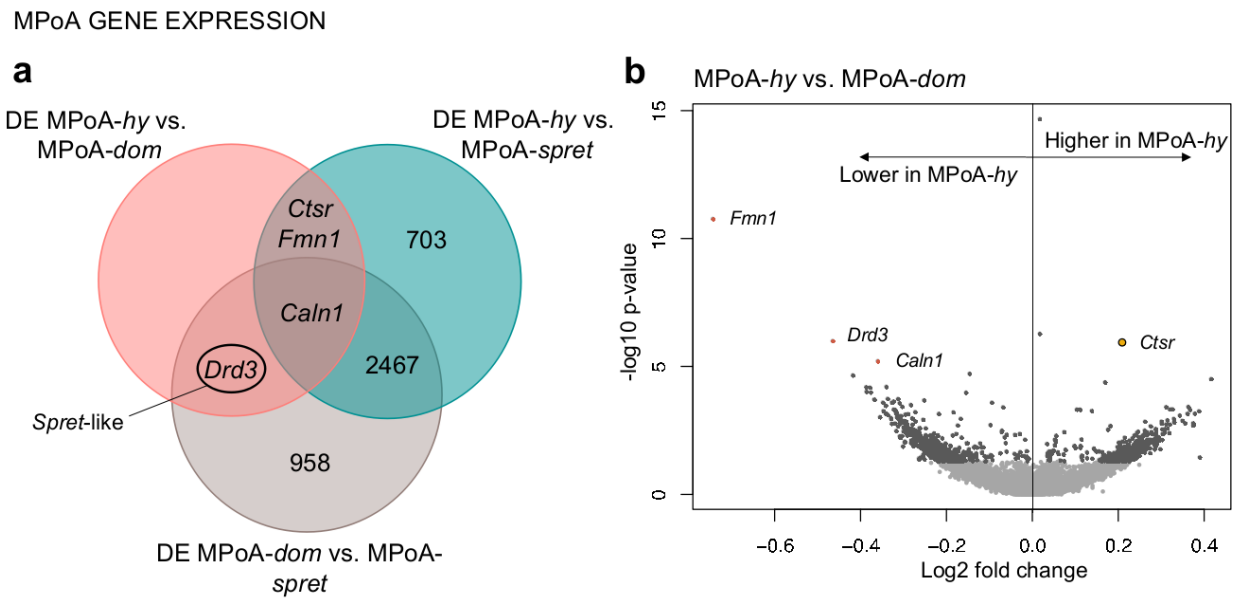


Figure 3. Maternal medial preoptic area (MPoA) gene expression. Summary of results of differential gene expression (DE) analysis between *Mus m. domesticus* MPoA during normal pregnancy (MPoA-*dom*), *Mus m. domesticus* MPoA during hybrid pregnancy (MPoA-*hy*) and *Mus spretus* MPoA during normal pregnacy (MPoA-*spret*). (A) Venn diagram indicating the overlap of differentially expressed genes between the comparisons MPoA-*hy* vs. MPoA-*dom*, MPoA-*hy* vs. MPoA-*spret* and MPoA-*dom* vs. MPoA-*spret*. Genes with *Spret*-like expression in MPoA-*hy* are marked in the diagram. (B) Volcano plot of DE analysis results of MPoA-*hy* vs. MPoA-*dom*. Significantly differentially expressed genes with FDR ≤ 0.05 and log2 fold change ≥ 0.2 are depicted in red, placenta-specific gene family genes in yellow.

Table 3. Differential expression in MpoA-*hy* compared to MPoA-*dom*.

	Chr.	LFC	Stat	padj
Fmn1	2	-0.74	-6.73	<0.001
Drd3	16	-0.47	-4.89	<0.001
Caln1	5	-0.36	-4.52	0.02
Ctsr	13	0.21	4.86	<0.001

Chr.=Chromosome, LFC=log2 Fold Change of expression, Padj=adjsted p-value according to Benjamini-Hochberg method, Stat=Wald test (DESeq2).

Co-expression between the placenta and MPoA

The MPoA is thought to be an important target of placenta-secreted molecules and we found the placenta-specific gene, *Ctsr*, to be expressed in the maternal MPoA during hybrid pregnancies. Therefore, co-expression between placenta and MPoA is of particular interest. We determined

279 the level of placenta-MPoA co-expression for the three pregnancy types (hybrid, *Dom*, *Spret*),
280 and assessed differences between them.

281 10,839 genes were co-expressed between placenta and MPoA in all 3 comparisons. 172
282 genes were only co-expressed in *Dom* and 494 only in *Spret* pregnancies (Fig. 4*,* Supplemental
283 Dataset S3). 176 genes were uniquely co-expressed in hybrid pregnancies. This gene set is of
284 specific interest since these genes are indicators of MPoA response to abnormal placental
285 expression. Uniquely co-expressed genes in hybrid pregnancies included 45 genes that were DE
286 between hybrid and *Dom* placentas and 16 that were DE compared to both parental species'
287 placentas. *Ctsr*, which was significantly higher expressed in MPoA-*hy* compared to MPoA-*dom*,
288 was uniquely co-expressed in hybrid pregnancies (Fig. 4*,* Supplemental Dataset S3).

289 228 genes were co-expressed in *Spret* and hybrid pregnancies but not in *Dom*
290 pregnancies. Thus, these genes exhibit *Spret*-like co-expression in the MPoA of *Dom* females
291 carrying a hybrid litter. There was pathway overrepresentation overlap between this gene set and
292 genes with transgressive misexpression in hybrid placenta (Fig. 2*A*), including prolactin and
293 growth hormone receptor signaling, and ERBB signaling (Fig. 4*,* Supplemental Dataset S3).
294 Moreover, PSFs were significantly overrepresented among these co-expressed genes (Fisher's
295 exact test: $p<0.001$, odds ratio=12.57). Although expression levels were far lower in the MPoA
296 (range=10-589, mean=100 normalized counts, LFC to placenta: range=-9.54 to -14.13, mean=-
297 11.12) than in the placenta (range=20-1,127,603, mean=97,988 normalized counts), these results
298 are striking. To explore this relationship further, we tested for correlated expression of PSF genes
299 between placenta and MPoA. We found very strong, positive correlations for hybrid
300 (R^2adj=0.95, $p<0.001$) and *Spret* (R^2adj=0.9, $p<0.001$) and a significant but, surprisingly, weaker
301 positive correlation for *Dom* (R^2adj=0.38, $p=0.01$) (Fig. 4*B*, Table 3). Additionally, we found

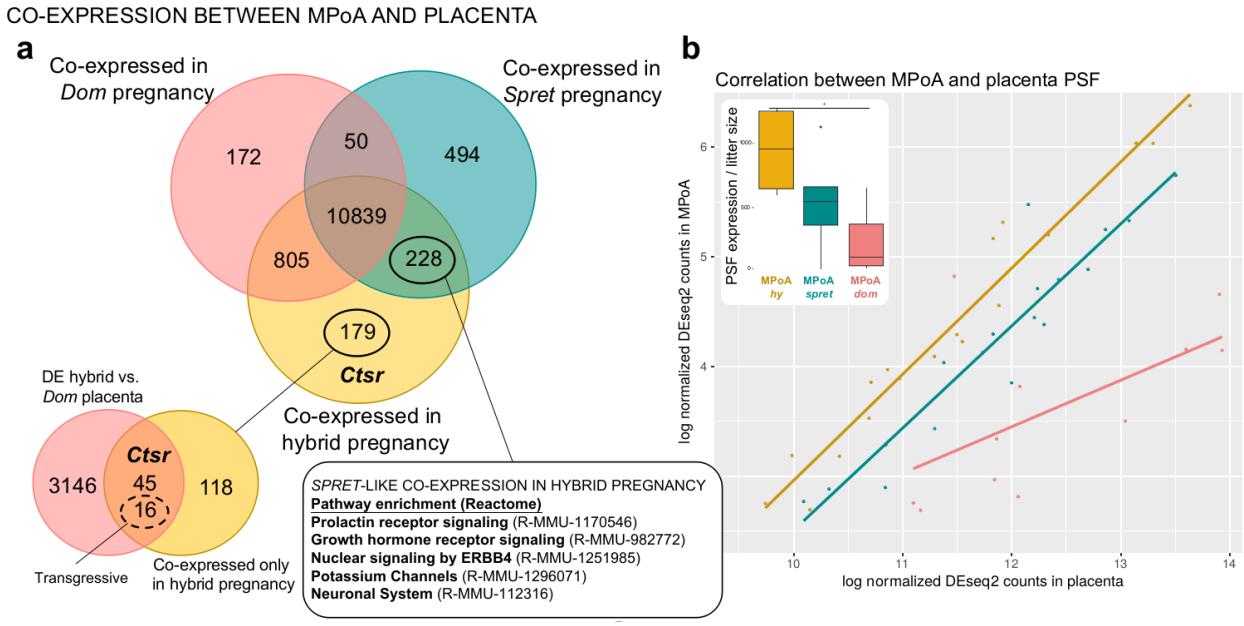
302 that total PSF expression (sum of all PSF read counts divided by litter size) is significantly

303 higher in MPoA-*hy* relative to MPoA-*dom* but is statistically indistinguishable fromMPoA-*spret*

304 (One-way ANOVA: $F_{2,12}$=5.44, p=0.02, Tukey HSD: MPoA-*hy* vs. *MPoA-dom*: p=0.016,

305 MPoA-*hy* vs. MPoA-*spret*: p=0.18, MPoA-*dom* vs. MPoA-*spret*: p=0.36) (Fig. 4*B*).

306



307

308 **Figure 4**. Co-expression between maternal medial preoptic area (MPoA) and placenta during late gestation.
309 Summary of results of co-expression analysis for *Mus m. domesticus* during normal pregnancy (*Dom*-pregnancy),
310 *Mus m. domesticus* during hybrid pregnancy (hybrid pregnancy) and *Mus spretus* during normal pregnancy (*Spret*
311 pregnancy). (A) Venn diagram indicating the overlap of co-expressed genes between the three pregnancy types.
312 Genes that are only co-expressed in hybrid pregnancy are marked in the diagram. A secondary Venn diagram for
313 this gene set shows its overlap with differentially expressed genes in hybrid vs. *Dom* placentas. Transgressively
314 expressed genes contained in this overlap are marked. Genes that are co-expressed in *Spret* and hybrid pregnancies
315 but not in *Dom* pregnancies (*Spret*-like co-expression) are marked in the primary Venn diagram. Results of pathway
316 overrepresentation (Reactome, version 58, Mi et al. 2017) for this gene set are provided in the connected text box.
317 (B) Scatterplot showing the correlation between placenta specific gene family (PSF) gene expression in placenta and
318 MPoA for the three pregnancy types. Red = *Dom* pregnancy (R^2_{adj}=0.38, p=0.01), Blue = *Spret* pregnancy
319 (R^2_{adj}=0.9, p<0.001), Yellow = *hybrid* pregnancy (R^2_{adj}=0.95, p<0.001). Inset boxplot shows total PSF gene
320 expression in MPoA (sum of normalized PSF counts/litter size), asterix indicates significant difference between
321 MPoA-*hy* and MPoA-*dom*.

322

323 **Pairwise evolutionary rates of selected PSF genes**

324 PSF genes were previously shown to exhibit accelerated evolutionary rates, potentially driven by

325 maternal-fetal conflict (Chuong et al. 2010). We selected the top 10 co-expressed PSF genes with

326 the highest expression in MPoA and extracted pairwise evolutionary rates (dN/dS) from Biomart
327 (R-package biomaRt, (Durinck et al. 2005)) to test for evidence of positive selection. We also
328 included the PSF gene *Ctsr*, which was significantly differentially expressed between MPoA-*hy*
329 and MPoA-*dom*.

330 dN/dS, the per site ratio of nucleotide substitutions that change amino acid identity to
331 those that do not, is an indicator of selective pressure, with dN/dS = 1 indicating neutral
332 evolution, dN/dS < 1 purifying selection, and dN/dS > 1 diversifying positive selection
333 (Goldman and Yang 1994). Of the 11 genes, three (*Prl8a6*, *Tpbpb* and *Ctsr*) had pairwise dN/dS
334 >1 between *Dom* and *Spret* (Fig. 5*,* Supplemental Table S1)*.* To infer which lineage experienced
335 selection, we analyzed these three genes using the application CodeML (implemented in PAML
336 4; (Yang and Rannala 1997; Yang 2007)), and including sequences from additional *Mus*
337 subspecies and species (*Mus m. musculus* (*Musc*), *Mus m. castaneus* (*Cast*), *Mus caroli* (*Car*)
338 and *Mus pahari* (*Pah*)). CodeML fits alternative models to the data; the best-fit model is chosen
339 based on likelihood ratio tests (LTRs). The two main models tested were M0, one evolutionary
340 rate for the whole tree, and MC, selected branches (foreground) evolve at a different rate than the
341 rest of the tree (background).

342 For all three genes, we found evidence for positive selection on *Dom* (*Prl8a6*) or *Spret*
343 branches (*Ctsr*), or both (*Tpbpb*) (Fig. 5*,* Supplemental Table S1). Specifically, there was
344 evidence for positive selection on *Prl8a6* in the *Mus musculus* subspecies clade ($LRT_{(M0-}$
345 $_{MC)}$=7.50, p=0.01, foreground $dN/dS_{(MC)}$=3.78, background $dN/dS_{(MC)}$=0.25). Evolutionary rates
346 for *Ctsr* were elevated on branches leading to *Pah, Spret* and *Musc* relative to the rest of the tree
347 ($LRT_{(M0-MC)}$=6.93, p=0.01, foreground $dN/dS_{(MC)}$>10, background $dN/dS_{(MC)}$=0.26). Results for

348 *Tpbpb* suggest high evolutionary rates across the whole tree ($LRT_{(M0-MC)}$=3.56, p=0.1,

349 $dN/dS_{(M0)}$=1.43) (Fig. 5, Supplemental Table S1).

350

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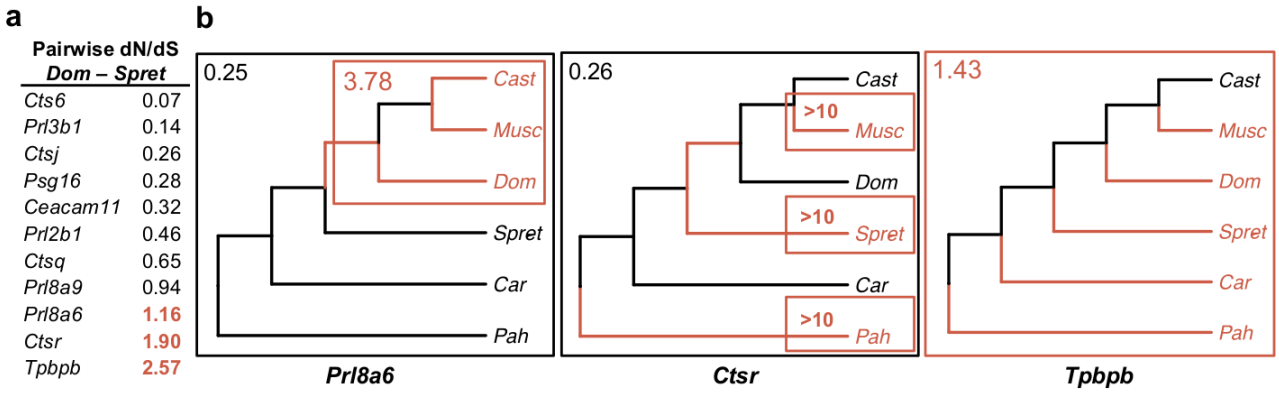

352

353 **Figure 5**. Evolutionary rates of placenta-specific gene family (PSF) genes expressed in the maternal medial preoptic
354 area (MPoA). (A) Pairwise evolutionary rate between *Mus m. domesticus (Dom)* and *Mus spretus (Spret)* for *Ctsr*
355 and the 10 co-expressed PSF genes with the highest expression in MPoA. Evolutionary rate (dN/dS) > 1 is marked
356 in red throughout the figure and is indicative of positive selection. (B) PAML4 CodeML analysis results for the
357 three genes with pairwise dN/dS>1. Species are *Mus m. castaneus (Cast), Mus m. musculus (Musc), Mus m.*
358 *domesticus (Dom), Mus spretus (Spret), Mus caroli (Car), Mus pahari (Pah).* dN/dS values are indicated for groups
359 of branches depending on which CodeML model provided the best fit for the data (M0: one evolutionary rate for the
360 whole tree, MC: selected branches evolve at a different rate than the rest of the tree). dN/dS values depicted in black
361 for *Prl8a6* and *Ctsr* indicate background evolutionary rates.

362

363

364 **Discussion**

365 Molecular communication between the placenta and the maternal brain is crucial for the

366 expression of maternal behavior in rodents (Bridges et al. 1996; Larsen and Grattan 2012; Creeth

367 et al. 2018). In humans, altered regulation of placental imprinted genes is associated with

368 prenatal depression (Janssen et al. 2016b), a predictor of lower growth rate and higher disease

369 risk in infants (Rahman et al. 2004). In this study, we used a hybrid mouse model to characterize

370 the extent to which placental disruption influences gene expression in the maternal brain. Several

371 maternally expressed imprinted genes were transgressively misexpressed in the hybrid placenta.

372 In *Mus m. domesticus* females carrying hybrid litters we found altered placenta-specific gene
373 family expression in the placenta, and in the maternal MPoA. Surprisingly, the expression of
374 these genes was highly correlated between the two tissues, and was *Mus spretus*-like in the
375 MPoA. This suggests that paternally inherited alleles in the placenta exert substantial influence
376 on expression in the maternal brain. Collectively, our results reveal reciprocal effects of mothers
377 on offspring and offspring on mothers, mediated in both cases by the placenta. We discuss these
378 findings in light of maternal-fetal coevolution and parental conflict, and identify potential
379 implications for placental pathologies.
380

381 **Maternal effects on placental expression**

382 Global patterns of expression in the placenta were strongly associated with maternal genotype. In
383 hybrids, half as many genes were DE relative to normal *Dom* placentas as opposed to *Spret*
384 placentas. Notably, the >1,600 genes with *Dom*-like expression in hybrid placenta were highly
385 enriched for terms associated with immunity and regulation of blood flow, both of which are
386 essential to placental mediation between mother and embryo (Cross et al. 1994). Because
387 maternal vasculature is incorporated into the placenta, whole placenta transcriptomes necessarily
388 include some transcripts of maternal origin and detection of *Tnfsrs23* transcripts in all samples
389 indicates that some cells from the maternal decidua remained following dissection. However,
390 maternal blood flow within the placenta is under the direct control of placental cell lineages;
391 trophoblast giant cells invade and replace maternal vascular endothelium, limiting maternally-
392 derived tissue to blood (Rai and Cross 2014). Likewise, *Tnfsrs23*-expressing cells are adjacent to
393 fetal trophoblast giant cells at the junction between fetal and maternal tissues (Clark et al. 2002).
394 Therefore, while contamination from maternal transcripts may contribute to *Dom*-like expression

395 in hybrid placenta, it is unlikely to bias the expression of such a large number of genes. The
396 regulatory effects of maternal hormones, and of maternally inherited genes in the placenta, are
397 non-mutually exclusive alternative explanations. For example, because paternal X chromosome
398 inactivation is maintained in mouse placenta (Tagaki and Sasaki 1975), maternally inherited X-
399 linked genes are strong candidates for modulating autosomal expression in both sexes. While
400 disentangling maternal effects (*sensu* Wolf and Wade 2009) from the effects of maternally
401 inherited genes is a challenge for future studies, we note that the match between maternal
402 genotype and placental expression of genes that modulate maternal immune tolerance and
403 angiogenesis is consistent with the expectation of molecular coadaptation between mother and
404 offspring (Wolf and Brodie 1998, Keverne and Curley 2008), and the well-established effect of
405 maternal environment on placental function (Cottrell and Seckl 2009; Monk et al. 2012).
406

407 **Altered PSF and IG expression in the placenta**

408 Maternal adaptation to pregnancy relies to a great extent on placental signaling. Thus, altered
409 expression of genes encoding or influencing placental signaling molecules can ultimately affect
410 maternal physiological and behavioral response to pregnancy (Bridges et al. 1996; Creeth et al.
411 2018). Misexpression in the hybrid placenta was substantial. However, the most striking pattern
412 we found was the reduced expression of a large number of PSFs. In mice, most of these genes
413 are expressed from the placental endocrine compartment and many are found in maternal plasma
414 during pregnancy (Rawn and Cross 2008). In this hybrid mouse model, the endocrine
415 compartment is markedly reduced when the mother is *Dom* (Zechner et al. 1996). Thus, reduced
416 abundance of PSF producing cell types likely contributes to overall reduction in PSF expression.

417 IGs are thought to modulate PSF expression, primarily through effects on placental
418 endocrine cell abundance, with maternally expressed genes (MEGs) repressing and paternally
419 expressed genes (PEGs) promoting cell proliferation (John 2013, 2017). Two such MEGs,
420 *Phlda2* and *Ascl2*, were transgressively misexpressed in hybrid placentas. Altered expression of
421 either of these genes in lab mouse models results in an undersized endocrine compartment,
422 altered glycogen energy stores and reduced PSF gene expression (Tunster et al. 2010, 2016a,b).
423 Indeed, *Phlda2* and *Ascl2* seem to be critical co-regulators of placental endocrine compartment
424 development (John 2017). Two other MEGs, *Dcn* and *Grb10*, were overexpressed when
425 compared to *Dom* placentas. While neither is specifically implicated in placental endocrine
426 function, both are key modulators of placental growth, and *Dcn* overexpression represses cellular
427 proliferation (Yamaguchi and Ruoslahti 1988; Kresse and Schönherr 2001; Garfield et al. 2011).
428 Collectively, our results are consistent with the proposed role of IGs in placental signaling (Haig
429 1996; Tunster et al. 2013; John 2013,2017), and identify MEG misexpression as a candidate
430 mechanism for the undersized endocrine compartment and consequent global reduction in PSF
431 expression in hybrid placenta.
432

433 **The effects of hybrid placental dysfunction on the maternal brain**

434 Altered signaling in hybrid placentas has the potential to affect the maternal brain. We found
435 subtle but significant differences in the expression of four genes in the MPoA of *Dom* females
436 exposed to hybrid relative to conspecific placentas. Both *Fmn1* (Formin1) and *Caln1*
437 (Calneuron1) were underexpressed. In the brain, *Fmn1* is involved in the formation of adherens
438 junctions and in linear actin cable polymerization (Kobielak et al. 2004). The formation of
439 adherens junctions is important in the maintenance of the blood brain barrier (BBB), a highly

440 specialized structure that regulates the influx of molecules into the brain (Stamatovic et al. 2008).
441 During pregnancy, the permeability of the BBB is increased by placenta-derived factors to which
442 the maternal brain must respond in order to maintain this barrier (Cipolla 2007; Schreurs et al.
443 2012). Reduced expression of *Fmn1* therefore suggests alterations in BBB adaptation during
444 hybrid pregnancies. *Caln1* encodes a neuron-specific protein with sequence similarities to
445 calcium-binding calmodulins. While the function of *Caln1* is uncharacterized, homology to
446 calmodulin suggests a role in neuronal calcium signaling (Wu et al. 2001). Decreased expression
447 of a calcium-binding protein could indicate alterations in neuronal activity in the MPoA exposed
448 to hybrid placentas.

449 *Drd3* (Dopamine receptor D3) was also underexpressed compared to *Dom* mothers, but
450 not to *Spret* mothers, in the hybrid pregnancy MPoA. DRD3, a D2-like receptor with a generally
451 inhibitory role, is implicated in treatment-resistant major depression (Lattanzi et al. 2002) and
452 *Drd3* knock-out mice exhibit a suite of anxiety- and depressive-like behaviors with similar but
453 milder effects in heterozygous knock-outs (Accili et al. 1996; Moraga-Amaro et al. 2014). Given
454 that the action of dopamine in the MPoA is critical for the expression of maternal behavior in
455 rats (Numan and Stolzenberg 2009), and hypothalamic dopamine is altered in a mouse model for
456 post-partum depression (Avraham et al. 2017), reduced *Drd3* expression in the MPoA might
457 cause deficits in maternal behavior. However, *M. spretus* mothers are more responsive to pups
458 than *M. m. domesticus* mothers (Cassaing et al. 2010) and *Drd3* expression in the hybrid
459 pregnancy was statistically indistinguishable from the normal *Spret* pregnancy. How, and to what
460 extent placental expression of paternally inherited alleles promotes maternal behaviors is an
461 intriguing question for in-depth studies.

462 *Ctsr* (Cathepsin R), a placenta-specific cathepsin, was the only gene that was
463 overexpressed in the MPoA exposed to hybrid placentas. The difference in expression, although
464 significant, was so small that the biological relevance is questionable. However, unlike other PSF
465 genes, expression of *Ctsr* in the maternal brain was unique to the hybrid pregnancy.
466 Interestingly, loss of the IG *Peg3* leads to de-repression of several PSF members, including *Ctsr*,
467 in the fetal and adult brain (Kim et al. 2013). While *Peg3* was not misexpressed in the hybrid
468 placenta, the transgressively overexpressed MEG, *Phlda2*, was recently shown to perturb
469 maternal behavior and neural gene expression when its dosage was altered in mouse placenta
470 (Creeth et al. 2018). Specifically, overexpression of placental *Phlda2* reduced postpartum
471 nurturing behavior, while underexpression increased maternal behavior (Creeth et al. 2018).
472 Since *Phlda2* and *Ascl2* jointly regulate development of the endocrine compartment (John 2017),
473 it is likely that transgressive misexpression of both genes in hybrid placenta impacts the maternal
474 brain via effects on placental hormone expression.
475
476 **Paternal effects on the maternal brain**
477 The hybrid placenta expresses both maternally derived (*Dom*) and paternally derived (*Spret*)
478 alleles. Thus, females pregnant with hybrids are exposed to gene products from a foreign
479 paternal genome. In *Dom* females exposed to hybrid placentas we found a substantial subset of
480 genes, including PSF genes and *Drd3*, with expression patterns that differed from *Dom* mothers
481 with conspecific litters, but closely matched those of *Spret* mothers.
482 A surprisingly large number of genes were co-expressed between placenta and MPoA in
483 hybrid and *Spret* pregnancies but not in *Dom* pregnancies. In particular, placental and MPoA
484 PSF gene expression was highly correlated in hybrid pregnancies and in *Spret* pregnancies, while

485 *Dom* pregnancies showed a weaker correlation. Likewise, total MPoA PSF gene expression was
486 *Spret*-like in hybrid pregnancies. These results suggest that PSF expression levels in the maternal
487 MPoA are driven by placental expression levels of the same genes, and that PSF, and potentially
488 *Drd3*, expression in the MPoA is influenced by paternally inherited alleles in the placenta. Thus,
489 natural differences between the parental species used in this study reveal an unanticipated effect
490 of the paternal genome on the maternal brain. This hypothesis is illustrated in Fig. 6.

491 A reciprocal shift towards *Dom*-like PSF expression in the brains of *Spret* females
492 carrying litters sired by *Dom* males would provide strong support for this hypothesis.
493 Unfortunately, generating hybrid pregnancies in this direction of the cross is notoriously difficult
494 (Zechner et al. 1996) and our attempts failed (see Methods). However, the generality of
495 paternally-mediated placental effects on mothers could be tested in other species crosses
496 characterized by parent-of-origin-dependent placental abnormalities (e.g. *Phodopus* hamsters
497 (Brekke and Good 2014) and *Peromyscus* mice (Vrana et al. 1998)).

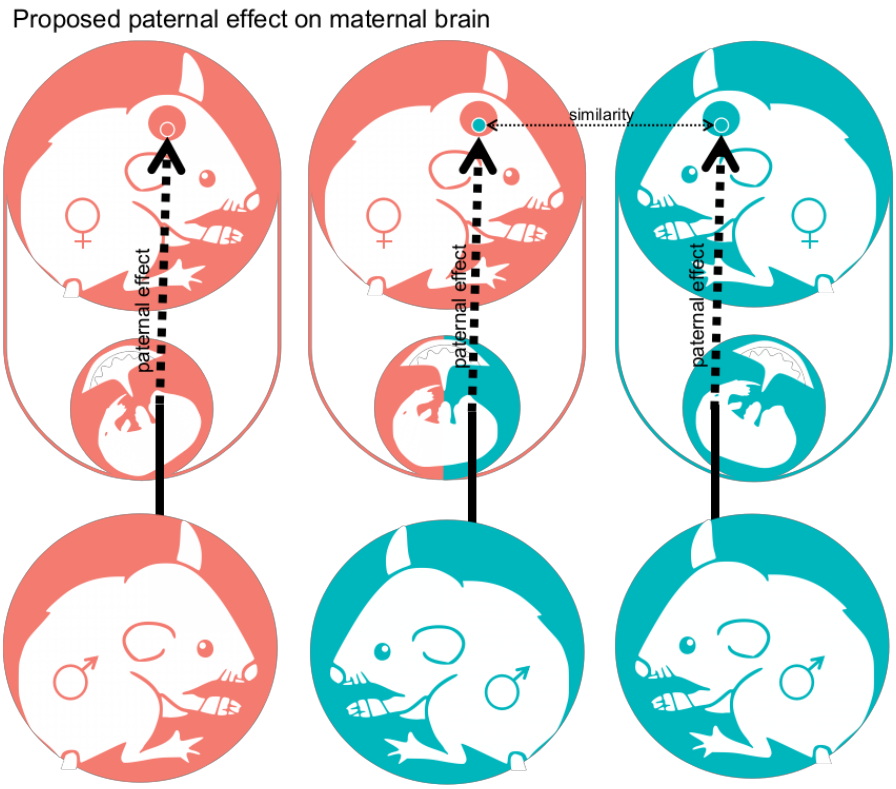

Figure 6. Proposed paternal effect on maternal brain. Schematic visualization of the three different crosses and the potential indirect effect of the paternally inherited allele through the placenta on the maternal brain. *Mus m. domesticus* is depicted in red and *Mus spretus* in blue. Hybrid tissue is indicated by a combination of red and blue.

A signature of conflict in PSF evolution and expression in the maternal brain

Pregnancy requires substantial investment from the mother, which is offset by costs to her capacity to invest in future offspring (Trivers 1972). However, when offspring are sired by multiple males, selection favors fathers who extract maximal maternal resources for their own offspring (Trivers 1972). Haig and colleagues proposed that these asymmetries in the reproductive interests of males and females, and the coefficients of relatedness between mothers and offspring (always 0.5) vs. fathers and offspring (0.5 or 0), should promote parental antagonism, played out at the molecular level between maternally and paternally expressed IGs in the placenta (Moore and Haig 1991; Haig and Graham 1991; Haig 2000). Because placental endocrine signals promote maternal investment in current offspring, placental hormones are also

513 proposed players in both parental and mother-offspring conflicts (Trivers 1985; Haig 1996).
514 Consistent with a history of antagonistic coevolution, PSFs in general are the fastest evolving
515 genes in the rodent placenta (Chuong et al. 2010). We report a similar signature of selection on
516 three PSF genes that are co-expressed in the hybrid placenta and the maternal MPoA.
517 Trivers (1985) described placental hormones as the molecular equivalent of begging
518 calls. Here we show for the first time that the expression of Prls and other PSFs is highly
519 correlated between placenta and maternal brain. While the function of PSFs in the brain is
520 undefined, placental genotype-dependent differences between *Dom* females in the strength of the
521 correlation and the number of co-expressed genes indicate that the relationship is driven by the
522 placenta not the mother. Moreover, the *Spret*-like co-expression patterns of PSF genes in
523 mothers of litters sired by *Spret* males implicate the paternally inherited genome as the driver of
524 these placental begging calls, which are echoed in the maternal brain. Given that these patterns
525 of expression are consistent with parental conflict, it is noteworthy that the opportunity for sperm
526 competition (inferred from testis-body mass ratios) is higher in *M. spretus* than in *M. m.*
527 *domesticus* (Gomez Montoto et al. 2011).
528
529 **Preeclampsia related gene expression in hybrid pregnancies**
530 Preeclampsia is a serious pregnancy complication and the lead cause of maternal and fetal
531 morbidity and mortality (Burton and Jauniaux 2004; Redman and Sargent 2009). We found
532 significant overlap between transgressively misexpressed genes in hybrid placentas and
533 preeclampsia related genes and pathways. Haig (1993) interpreted preeclampsia as a
534 consequence of conflicts between maternal and paternal genomes, played out in the placenta and
535 potentially involving IGs. While the genetic basis of preeclampsia is complex (Uzun et al. 2016),

536 involvement of IGs is supported in both humans and mouse models. For example, human
537 chromosome 10 regions containing imprinting clusters are associated with preeclampsia
538 (Oudejans et al. 2004), and loss of the MEG *Cdkn1c* causes preeclampsia-like symptoms in mice
539 (Kanayama et al. 2002). Interestingly, *Cdkn1c* is in the same imprinting cluster as *Phlda2* and
540 *Ascl2* (dist7, IC2), both of which were misexpressed in hybrid placentas. While *Cdkn1c* was not
541 differentially expressed in the hybrid placenta, it is possible that misexpression in this imprinting
542 cluster is a general contributor to preeclampsia-like placental phenotypes. Because preeclampsia
543 can significantly alter permeability of the BBB (Cipolla 2007), it is also notable that *Fmn1*, a
544 gene implicated in BBB maintenance, was underexpressed in brains of mothers exposed to
545 hybrid placentas.
546

547 **Conclusions**

548 Evolutionary theoreticians have modeled mammalian pregnancy as both intimate cooperation
549 and antagonistic struggle between two genetically distinct organisms (Trivers 1974; Haig 1993;
550 Wolf and Hager 2006). Whether driven by conflict or coadaptation, it is clear that the placenta is
551 the mediator of these complex interactions between mother and offspring. Here we concentrated
552 on placental effects on the maternal brain during the final stages of pregnancy, when it is
553 believed to be a critical source of signal molecules that prime female physiology and behavior
554 for motherhood (Larsen and Grattan 2012; Bridges 2015). We found both hybrid placental
555 misexpression with the potential to disrupt maternal-fetal communication, and altered expression
556 in the brains of mothers exposed to hybrid placentas. Expression in the hybrid placenta seems to
557 be dominated by the maternally derived genome and/or driven by maternal effects. Maternal-
558 placental communication genes co-expressed in maternal brain and placenta show elevated

559 evolutionary rates, consistent with antagonistic coevolutionary processes. The expression of a
560 proportion of transcripts of these genes from a foreign paternal genome in the placenta has the
561 potential to affect the maternal brain and alter maternal behavior. In addition to the effects of
562 placental disruption on the maternal brain, natural differences between the parental species in
563 this hybrid system suggest a previously undescribed influence of the paternal placental genome
564 on the maternal brain. These paternal effects on the maternal brain could play a major role in the
565 expression of maternal behavior and the quality of maternal care, and open novel avenues of
566 research in both evolutionary and biomedical fields.
567

568 **Methods**

569 **Animals and tissue collection**

570 Mice used in this study were maintained on a 12:12 light:dark cycle with lights on at 9:00 AM,
571 and were provided with 5001 Rodent Diet (LabDiet, Brentwood, MO, U.S.A.) and water ad lib.
572 All animal procedures were approved by the Oklahoma State University IACUC under protocol
573 #141-AS. *Mus m. domesticus* (*Dom*) was represented by the wild-derived inbred strain WSB/EiJ
574 (Jackson Laboratory) and *Mus spretus* (*Spret*) was represented by the wild-derived inbred strain
575 SFM/Pas (Montpellier Wild Mice Genetic Repository). We conducted three crosses (female
576 shown first): *Dom* X *Dom* (n=5), *Dom* X *Spret* (n=5), *Spret* X *Spret* (n=5). The reciprocal cross
577 (*Spret* X *Dom*) was attempted 25 times but was never successful. Prior to pairing, females were
578 placed in a cage with soiled conspecific male bedding for ~48 hrs to induce receptivity to mating
579 (Whitten 1956). Mice were paired between 5:00 and 6:00 PM, left undisturbed for two nights,
580 and split on the morning of the second day. The second night was counted as embryonic day 0
581 (e0). Females were weighed after two weeks to confirm pregnancy but were otherwise left

582 undisturbed. Pregnant females (n=5/type of pregnancy) were euthanized by cervical dislocation
583 between 10:00 and 11:00 AM on embryonic day 17-18 (e17.5) and the maternal brain was
584 extracted. Embryos were separated from placentas and placentas were weighed, and the
585 maternally-derived decidual layer was removed as previously described (Qu et al. 2014) and
586 discarded. Only fetally-derived placental tissue was used in this study. All tissues were
587 transferred immediately to RNAlater (Thermo Fisher, USA), kept at 4°C overnight to allow
588 RNAlater perfusion, and stored at -20°C until microdissection and RNA extraction.
589

590 **Brain microdissection and RNA extraction**

591 The maternal MPoA was localized using the Mouse Brain atlas (Figs. 26-33, (Paxinos and
592 Franklin 2013)), and microdissected by sectioning the RNAlater perfused brain at 100µm on a
593 Leica CM 1950 cryostat, followed by dissection under a dissecting microscope in chilled PBS
594 droplets for improved visibility of brain microstructure. DNA was extracted from embryonic
595 tissue using the DNeasy Blood & Tissue Kit (Qiagen, USA) followed by PCR for the Y-linked
596 gene, *Zfy1*, to determine sex. Placentas from one male and one female per litter were used for
597 RNA extraction (n=5 males/cross, n=4 females/hybrid cross, n=5 females/conspecific cross).
598 RNA was extracted from all tissues immediately after microdissection using the RNeasy Plus
599 Universal Mini Kit (Qiagen) for MPoA, and the AllPrep RNA/DNA Mini Kit (Qiagen) for
600 placenta. RNA was stored at -80°C until sequencing.
601

602 **RNAseq pipeline**

603 <u>Sequencing (RNAseq):</u> RNA integrity (RIN) was determined by the sequencing facility
604 (Novogene, Sacramento, CA) using the RNA Nano 6000 Assay Kit with the Agilent Bioanalyzer

605 2100 system (Agilent Technologies, Santa Clara, CA, USA). RIN values ranged from 8.2-10 for
606 all samples. Library preparation was performed by the sequencing facility, using the NEB Next
607 Ultra RNA library prep kit for Illumina. RNAseq was performed on the Illumina HiSeq 4000
608 platform, producing >30 million, 150bp paired-end reads per sample.
609 Mapping: QC of raw sequencing reads and trimming were performed in Trim Galore! 0.4.5
610 (Brabraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore),
611 using a phred score cutoff of 30 and minimum sequence length of 100 after trimming. In order to
612 map hybrid placenta reads we generated a pseudo-hybrid genome using the genome preparation
613 tool of the program SNPsplit (Brabraham Bioinformatics, (Krueger and Andrews 2016)). Briefly,
614 SNPs from both *Dom* (WSB/EiJ) and *Spret* (SPRET/EiJ) relative to the mouse genome
615 (GRCm38.89) available from the Ensembl FTP server (ftp://ftp.ensembl.org) were introduced
616 into the mouse genome (GRCm38.89). SNPs between *Dom* and *Spret* were then N-masked to
617 allow mapping of both *Dom*- and *Spret*-derived reads. To improve comparability, all placenta
618 samples (*Dom*, *Spret* and hybrid) were mapped to the pseudo-hybrid genome. MPoA samples
619 were mapped to their corresponding genomes (WSB/EiJ_v1 for *Dom* MPoA, SPRET/EiJ_v1 for
620 *Spret* MPoA, (Keane et al. 2011)). Mapping was done using HISAT2 2.1 (Kim et al. 2015). After
621 mapping we filtered the resulting alignment files using SAMtools 0.1.19 (Li et al. 2009),
622 retaining only high quality (HISAT2 MAPQ score 40), uniquely mapped, paired reads for
623 analysis.
624 Post-processing of alignments: Before filtering, average alignment rate for MPoA samples was
625 87% for *Spret* MPoA samples and 88% for *Dom* MPoA samples. For placenta samples the
626 alignment rate was slightly lower for *Spret* samples (88%) compared to *Dom* (91%) and *hybrid*
627 (91%). We therefore randomly downsampled all alignment files to ~40 million reads using

628 SAMtools 0.1.19 (Li et al. 2009) to account for a possible mapping bias and to improve
629 comparability.
630 Allele-splitting of hybrid alignments: To confirm ~equal expression from *Dom* and *Spret* alleles
631 in the hybrid placenta we split the hybrid alignment files, separating reads originating from the
632 *Spret* and *Dom* alleles. Splitting was performed using the program SNPsplit (Brabraham
633 Bioinformatics, (Krueger and Andrews 2016)). The pseudo-hybrid genome was produced by
634 introducing SNPs between both Spret and mouse and Dom and mouse into the mouse genome
635 and then N-masking those SNPs that are present between Dom and Spret. Mapped reads can
636 therefore be assigned to the *Dom* or *Spret* allele based on the list of SNPs between *Dom* and
637 *Spret* and overlap with N-masked positions in the mouse genome. Using this procedure, SNPsplit
638 produced separate alignment files for each allele containing clearly assignable reads. These were
639 then quantified and used to produce a diagnostic boxplot, demonstrating ~equal expression of
640 both alleles in the hybrid placenta (Supplemental Fig. S8).
641 Quantification: Transcript quantification and annotation was done using StringTie 1.3.3 (Pertea
642 et al. 2015). Gene annotation information was retrieved from the Ensembl FTP server
643 (ftp://ftp.ensembl.org) for *Spret* (SPRET/EiJ_v1.86) and *Dom* (WSB/EiJ_v1.86). Mouse genome
644 annotation was used for samples mapped to the pseudo-hybrid genome (GRCm38.89). We used
645 the python script (preDE.py) included in the StringTie package to prepare gene-level count
646 matrices for analysis of differential gene expression.
647 Differential expression (DE) analysis: Differential expression was tested with DESeq2 1.16.1
648 (Love et al. 2014). Pseudogenes were removed from the count matrices based on "biotype"
649 annotation information extracted from Biomart (R-package biomaRt, (Durinck et al. 2005)). Low
650 counts were removed by the independent filtering process implemented in DESeq2 (Bourgon et

651 al. 2010). The adjusted p-value (Benjamini-Hochberg method) cutoff for DE was set at 0.05. Due
652 to variation in litter size, especially in females carrying hybrid litters (range=3-6), and its
653 potential effect of on MPoA expression, we corrected for litter size in all MPoA sample
654 comparisons. To analyze DE between *Spret* and *Dom* MPoA, which were mapped to their
655 respective genomes, we extracted homologous gene names from the mouse genome database
656 using Ensembl Biomart (R-package biomaRt, (Durinck et al. 2005)) and merged the dataset
657 based on the genes that had a clear mouse homolog in both. Normalized read count tables
658 produced by DESeq2 were used in subsequent co-expression analyses.
659 Co-expression: To determine co-expression between placenta and MPoA we set a cutoff of 10
660 normalized counts for at least 4 out of 5 observations each tissue type (MPoA, male placenta and
661 female placenta). Based on this cutoff we report genes expressed in both placenta and MPoA for
662 each type of pregnancy. We then determined differences in co-expression between the three
663 pregnancy types.
664 Gene ontology (GO) term and pathway overrepresentation analysis: We performed GO term and
665 pathway overrepresentation analyses on relevant lists of genes from DE and co-expression
666 analyses using the PANTHER gene list analysis tool with Fisher's exact test and FDR correction
667 (Mi et al. 2017). We tested for overrepresentation based on the GO annotation database
668 (Biological Processes) (released 07-Jan-2017, (Ashburner et al. 2000; The Gene Ontology
669 Consortium 2017)) and the Reactome pathway database (version 58 (Fabregat et al. 2017)).
670

671 **Evolutionary rates for selected genes**

672 We extracted the pairwise evolutionary rate (dN/dS = nonsynonymous to synonymous
673 substitution rate ratio) between *Dom* and *Spret* from Biomart (R-package biomaRt, (Durinck et

674 al. 2005)). dN/dS is an index of selective pressure on coding sequence, with dN/dS = 1 indicating
675 neutral evolution, dN/dS < 1 purifying selection, and dN/dS > 1 diversifying positive selection
676 (Goldman and Yang 1994). Further analysis of genes with dN/dS > 1 (*Prl8a6*, *Ctsr*, *Tpbpb*) was
677 performed with CodeML implemented in PAML 4.8 (Yang and Rannala 1997; Yang 2007),
678 including sequences from related *Mus* subspecies and species (*Mus m. musculus (Musc)*, *Mus m.*
679 *castaneus (Cast)*, *Mus caroli (Car)* and *Mus pahari (Pah)*). Coding sequences for all three genes
680 for *Musc*, *Cast* and *Car* were available from Ensembl. For *Pah*, coding sequences for *Prl8a6* and
681 *Ctsr* were downloaded from NCBI Genbank. For *Tpbpb*, we ran blastn on NCBI with the *Dom*
682 *Tpbpb* coding sequence against the nr/nt database and found two matches for *Pah*, of which one
683 showed higher similarity to *Dom Tpbpa* and the other to *Dom Tpbpb*. The latter was included in
684 the CodeML analysis (Supplemental Table S5). CodeML calculates evolutionary rates by
685 applying different models to an alignment and a phylogeny. To prepare the alignments,
686 sequences were visualized with Geneious 9.1.8 (Biomatters, http://www.geneious.com/) and
687 trimmed to coding sequence. Translation alignments were performed using the MUSCLE
688 alignment algorithm, implemented in Geneious. The phylogeny was built based on recent
689 phylogenomic analyses of house mice and related species (White et al. 2009; Sarver et al. 2017).
690 For the CodeML codon frequency setting we used the setting with the best fit for each analysis
691 according to the preliminary likelihood ratio analysis.

692 To calculate individual evolutionary rates for each branch in the tree we used CodeML's
693 "free-ratio" model. This served as an initial indication as to which branches might show higher
694 evolutionary rates. After this, two models were computed: Model M0 "one ratio" in which all
695 branches were constrained to evolve at the same rate and MC "two-ratio" in which selected
696 branches are allowed to evolve at a different rate than the rest of the tree. Branches with

697 potentially higher evolutionary rate based on the "free-ratio" model result were marked as
698 foreground branches and were allowed to evolve differently from the background. To test if MC
699 provides a better fit for the data than M0 we performed Likelihood Ratio Tests. When MC
700 provided the better fit, and dN/dS calculated for the foreground branches was > 1 and dN/dS
701 calculated for the background branches was < 1, we inferred positive selection on the foreground
702 branches. When M0 provided the better fit and dN/dS for the whole tree was > 1, we inferred
703 positive selection for the whole tree (Yang 1998).
704

705 **Data Access**

706 RNA-seq data from this study have been submitted to the NCBI Gene Expression Omnibus
707 (GEO; https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE126469.
708

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