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Endometrial recognition of pregnancy occurs in the grey short-tailed opossum (*Monodelphis domestica*)

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In human pregnancy, recognition of an embryo within the uterus is essential to support the fetus through gestation. In most marsupials, such as the opossums, pregnancy is shorter than the oestrous cycle and the steroid hormone profile during pregnancy and oestrous cycle are indistinguishable. For these reasons, it was assumed that recognition of pregnancy, as a trait, evolved in the eutherian (placental) stem lineage and independently in wallabies and kangaroos. To investigate whether uterine recognition of pregnancy occurs in species with pregnancy shorter than the oestrous cycle, we examined reproduction in the short-tailed opossum (*Monodelphis domestica*), a marsupial with a plesiomorphic mode of pregnancy. We examined the morphological and gene expression changes in the uterus of females in the non-pregnant oestrous cycle and compared these to pregnancy. We found that the presence of an embryo did not alter some aspects of uterine development but increased glandular activity. Transcriptionally, we saw big differences between the uterus of pregnant and cycling animals. These differences included an upregulation of genes involved in transport, inflammation and metabolic-activity in response to the presence of a fetus. Furthermore, transcriptional differences reflected protein level differences in transporter abundance. Our results suggest that while the uterus exhibits programmed changes after ovulation, its transcriptional landscape during pregnancy responds to the presence of a fetus and upregulates genes that may be essential for fetal support. These results are consistent with endometrial recognition of pregnancy occurring in the opossum. While the effects on maternal physiology appear to differ, recognition of pregnancy has now been observed in eutherian mammals, as well as, Australian and American marsupials.

1. Introduction

In humans, the establishment of pregnancy after fertilization requires a signal from the fetus to the mother (human chorionic gonadotropin) to prevent the degeneration of the *corpus luteum* (CL), the principal source of progesterone during early pregnancy. If no conceptus is present or if it is unable to elicit the pregnancy recognition cascade, the CL degenerates and the endometrium undergoes menstruation or early pregnancy loss, respectively [1,2]. This prolonging of the availability of progesterone (by prevention of CL regression and/or shift in the source of progesterone) for the duration of pregnancy has been called 'maternal recognition of pregnancy', and is necessary for pregnancy in many eutherian mammals (e.g. cattle, pigs and horses), although the mechanisms of

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fetal signalling to mother are diverse [3–5]. Some eutherian mammals, however, do not require a fetal signal for the survival of the CL. For instance, in the mouse copulation alone, without fertilization, is sufficient to extend the lifespan of the CL, leading to a situation called 'pseudopregnancy' [6]. The impact of the fetus on the initial maternal physiology of pregnancy is then limited to causing the decidualization and remodelling of the endometrium [7]. Hence, even in eutherians in which recognition of pregnancy is well established, the maternal responses to fetal signals vary, ranging from trophoblast–endometrial interaction only to more systemic effects including maintenance of the CL.

In most marsupials, with the exception of macropodids (kangaroos and wallabies), gestation is shorter than the sterile sexual cycle, therefore, the CL does not need to be maintained beyond its lifetime in a sterile sexual cycle. Neither does the conceptus depend on the CL after the first quarter of gestation [8,9]. Although the existence of fetal-maternal signalling has been hypothesized for the dasyurid *Smithopsis macroura* [10], previous studies have failed to find strong evidence of any maternal recognition of pregnancy in non-macropod marsupials [9]. Several aspects of maternal physiology during pregnancy do not require the recognition of pregnancy in non-macropodid marsupials, including the production of progesterone from the CL and the growth of the uterus [11–13]. However, endometrial inflammation during pregnancy in *Monodelphis domestica* suggests that there may be aspects of uterine physiology that result from specific maternal–fetal interactions [14], hinting at the potential for some kind of local uterine recognition of pregnancy.

Here, we investigate whether the presence of the fetus affects the morphology and gene expression of the endometrium of the grey short-tailed opossum, *M. domestica*. The opossum is an important model for understanding the evolution of mammalian pregnancy because it shares important reproductive traits with monotreme and eutherian mammals, and its mode of pregnancy is probably plesiomorphic to therian mammals (see [12,15,16]). Opossum pregnancy, like that of most other marsupials, is short, lasting only 14.5 days. Furthermore, for most of gestation the conceptus is covered by a shell-coat which prevents physical contact between maternal and fetal tissues. On day 12.5, this shell coat is lost allowing the formation of a placenta. This period of placentation involves the production of a uterine inflammatory reaction, which is followed by birth just 2 days later. We examined the uterine changes during the oestrous cycle and compared them with the previously published changes during pregnancy [14]. If the mother does not identify the presence of a fetus *in utero*, then the endometrial changes during the oestrous cycle should be the same as those during pregnancy. Our transcriptomic, histological and immunohistochemical comparisons indicate otherwise, providing evidence for endometrial recognition of pregnancy in *M. domestica*. These results suggest that *endometrial* recognition of pregnancy may be widespread in therian mammals, as it is found, not only in eutherian mammals and macropodid marsupials that have a *physiological* recognition of pregnancy but also in opossum, a marsupial with a plesiomorphic mode of pregnancy.

2. Methods

(a) Animal husbandry

All animal procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee of Yale University (protocol no. 15-11313). Opossum uterine tissue was collected from a *M. domestica* colony housed at Yale University. To collect pregnant tissues, we housed males and females separately and then put them together following a slow introduction process as used in Kin *et al*. [17]. Paired opossums were filmed and pregnancy was timed from the observed time of copulation. We developed a strategy to collect timed oestrous cycle animals for comparison with pregnancy (see the electronic supplementary material). In short, females were exposed to male scents and then we examined urogenital epithelial cells from cloacal swabs to time the oestrous cycle, which has been well characterized by Fadem & Rayve [18]. We examined females on 6, 11 and 13 days post-oestrus (dpe) which correspond to 6.5, 11.5 and 13.5 days post-copulation (dpc) in pregnancy. At the time of dissection, the uterus of oestrus cycle animals was observed to be enlarged, and the ovary was inspected for the presence of *corpora lutea*.

(b) RNA sequencing and analysis

For RNA sequencing analysis, we examined uterine tissue from females on day 6 dpe ($n = 2$) and 13 dpe ($n = 3$), which is equivalent in timing to days 6.5 and 13.5 dpc in a normal pregnancy. RNA extraction, quality control, sequencing library preparation and sequencing were performed using the same methods as Griffith *et al*. [14]. A mean of 2.9×10^7 reads were sequenced per sample.

We quantified gene expression by aligning raw sequencing reads to the *M. domestica* genome (release 79) with TOPHAT2 [19], then gene counts were calculated using HTSEQ [20]. A mean mapping rate of 75.6% was achieved. We performed hierarchical clustering and principal component analysis (PCA) in the R *stats* package [21] to confirm that treatment groups broadly had distinct gene expression profiles. Hierarchical clustering and PCA were performed on the square root transcripts per million (TPM) for each gene. We weighted each gene equally by subtracting the mean expression for that gene across samples from the expression of that gene in each sample. We compared differential gene expression between non-pregnant and 13 dpe females, and 13 dpe with late gestation (LG) using DESEQ2 [22]. Differential expression was called with a false discovery rate of 0.05. Raw RNA sequencing reads were uploaded to the Sequence Read Archive (PRJNA543903).

To explore how the presence of a fetus may be facilitating endometrial recognition of pregnancy, we compared known receptor–ligand relationships independent of whether they are known to function in pregnancy [23] (figure 5). Specifically, we compared the ligands and receptors expressed in our uterine samples with previously published gene expression profiles from the opossum trophoblast [24].

(c) Histology and immunostaining

We embedded paraformaldehyde fixed uterine tissue by first dehydrating tissue through a graded ethanol series, clearing in toluene, and then embedding in paraffin. Haematoxylin and eosin staining, and immunohistochemistry was performed with a standard protocol outlined in Kin *et al*. [17]. We characterized changes in basic cell morphology through the oestrous cycle by examining days 6, 11 and 13 dpe and equivalent stages of pregnancy ($n = 3$). We localized the expression of liver fatty acid binding protein (FABP1) and aquaporin 8 (AQP8) to the uterus of LG and the oestrous cycle equivalent of late gestation (ELG) animals ($n = 3$). For FABP1, we used a mouse monoclonal antibody raised against amino acids 7–126 of the human FABP1 peptide (1 : 50 dilution, sc-374537, Santa Cruz Biotechnology Inc.). For AQP8, we used a mouse monoclonal antibody raised against the recombinant AQP8 of human origin (1 : 50 dilution, sc-81870, Santa Cruz Biotechnology Inc.).

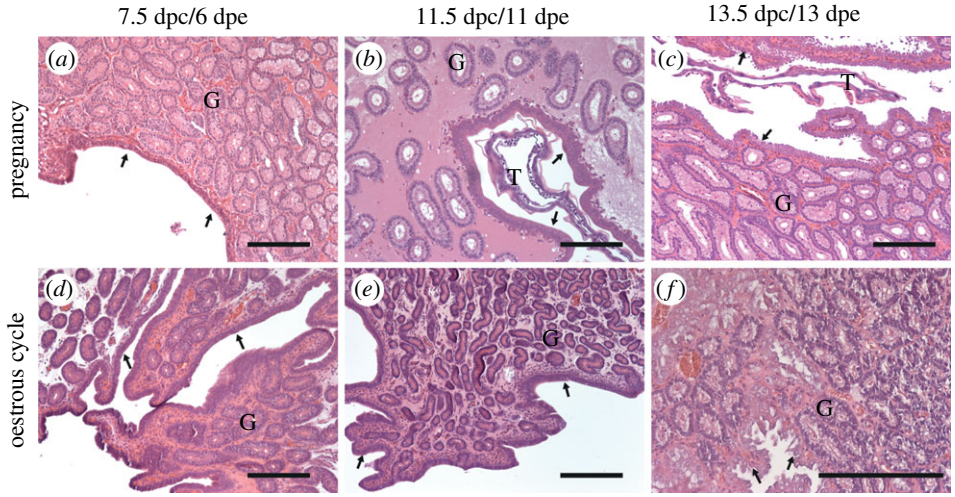

Figure 1. Histological comparison of the uterus through pregnancy and at consistent stages of the oestrous cycle. Staging is listed as days post-copulation (dpc) in the case of pregnant animals and days post-oestrus (dpe). G, uterine glands; T, trophoblast; arrows, luminal epithelial cells.

3. Results

(a) Histological comparison between pregnancy and non-pregnant cycle in the opossum

Changes in the endometrial tissue during pregnancy of *M. domestica* have been described before [14,17,25–27]; also see figure 1*a–c*). In pregnancy, to a surprising degree, the cellular changes to the endometrium largely track the changes that happen at equivalent time points in the oestrous cycle (figure 1). These changes include an expansion of the amount of glandular tissue, which appears to replace or dilute the endometrial stromal cell population. This process even includes the formation of endometrial folds, that are folds of the luminal epithelium folded onto itself, with very little stroma or uterine glands in between (electronic supplementary material, figure S1A,D). This is notable in the oestrous cycle, because this tissue configuration has been interpreted as being induced by the fetus [25]. By the last day of pregnancy and at the corresponding day of the non-pregnant cycle, the luminal epithelial cells have changed and appear to be shedding vesicles into the uterine lumen reminiscent of apocrine secretion.

One important difference between the late-pregnant uterus (after maternal–fetal attachment) and the equivalent time in the oestrous cycle is that the lumen of glandular tissue during pregnancy (figure 1*b*; electronic supplementary material, figure S1B) appears to be more open than in cycling females, hinting at greater glandular activity (figure 1*c*; electronic supplementary material, figure S1E). Furthermore, in the oestrous cycle, the glandular epithelial cells at 13 dpe do not look actively secretory, as their apical cytoplasm is reduced to the point where the cells are barely larger than the nucleus (figure 1*f*; electronic supplementary material, figure S1E). In addition, in pregnancy, the sub-epithelial stroma is replaced by a sub-epithelial capillary network, while in the non-pregnant cycle, the sub-epithelial layer of endometrial stromal cells remains compact (electronic supplementary material, figure S1C,F) suggesting an angiogenic effect of the presence of the fetus.

(b) Transcriptomic comparison of the uterus in pregnancy and oestrous

The gene expression profiles of the uterus in *M. domestica* change substantially throughout pregnancy, with thousands of differentially expressed genes between non-pregnant and mid-pregnancy, as well as thousands of differences between mid-pregnancy and late pregnancy [14,28]. PCA shows that most samples can be discretely separated by their reproductive state (figure 2*a*). Notably, oestrous cycle samples that correspond to mid-gestation cannot be separated from non-reproductive samples (NR) and do not cluster with mid-gestation samples. This suggests that even prior to the formation of a placenta, the *in utero* presence of an embryo impacts uterine gene expression. Principal component (PC) 1 (explaining 46% of the variance) separates late pregnant (13.5 dpc) and the ELG samples from other uterine tissues, showing that, in the sterile cycle, towards the time when pregnancy would normally end there is a component of uterine gene expression that is programmed, i.e. it is induced after initiation of oestrus regardless of the presence of embryos. Despite this, late pregnant and oestrous cycle samples are separated from each other along PC2 (explaining 25% of the variance), which suggests gene expression differences caused by the presence of the fetus.

When we cluster genes by their expression levels across samples, we see a remarkable similarity of gene expression between NR and oestrus samples that correspond to mid-gestation (EMG), gene clusters A and B, as already indicated in the PCA projection (figure 2*a*). In addition, gene cluster C is upregulated in EMG which is not shared with the non-reproductive stage, but shared with ELG. Furthermore, gene cluster D seems to be specifically upregulated in ELG but not shared with LG. Gene cluster F is shared between LG and its corresponding oestrus sage (ELG), and gene cluster G is unique to LG. Finally, gene cluster E is unique to mid-gestation, not shared with the corresponding oestrus stage. Overall the pattern of gene expression is a mosaic of shared gene expression among corresponding late stages (cluster F) and unique to gestational and oestrus stages (clusters E and G). More surprising is the existence of gene clusters that are unique to oestrus stages such as clusters D and C, where the latter is shared between mid- and late oestrus stages. This pattern suggests oestrus-specific gene expression dynamics that is not shared with non-reproductive stages and suppressed during pregnancy.

Between each pairwise group of samples, thousands of genes are differentially expressed (figure 2*c*), with more

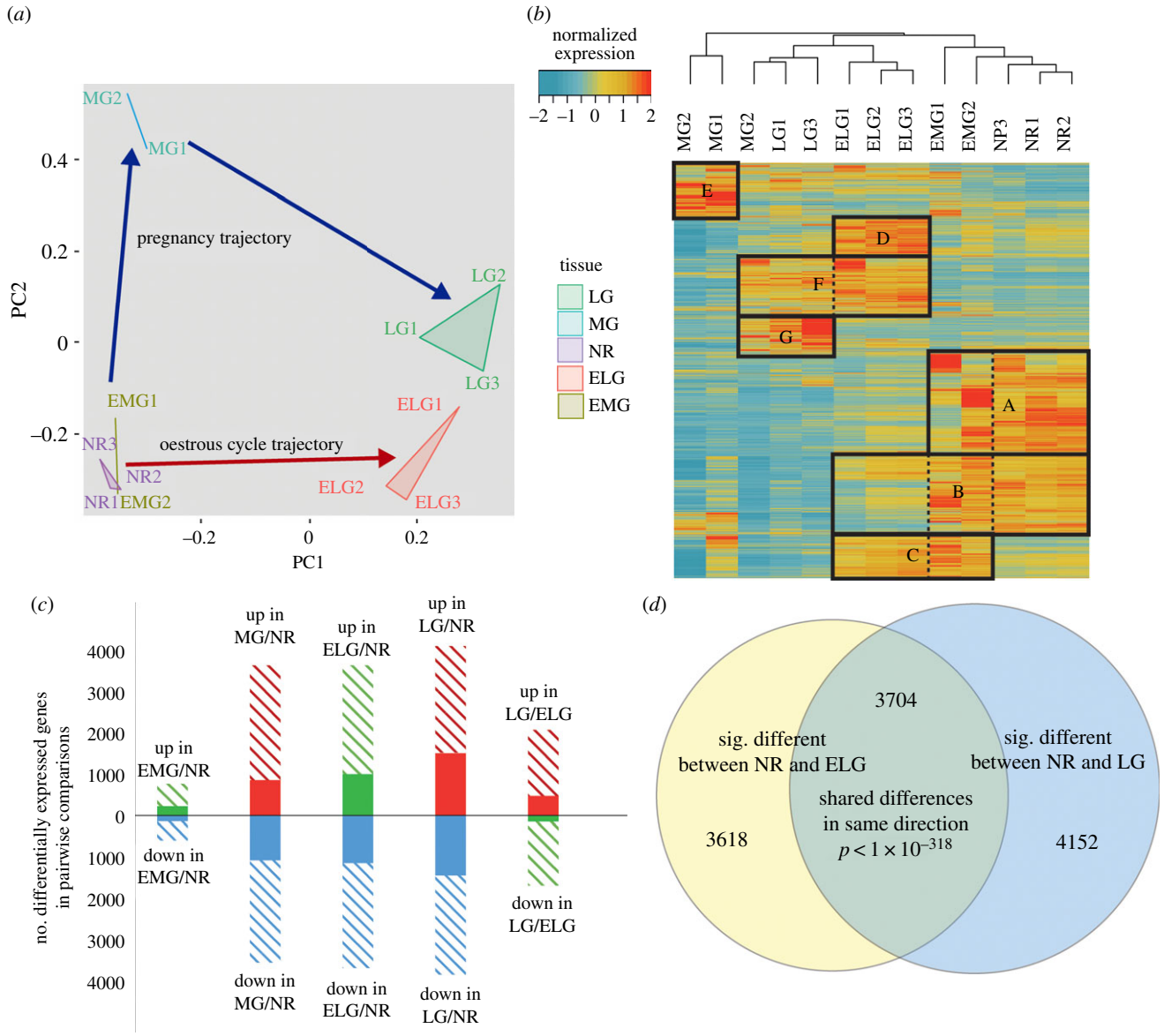

Figure 2. Transcriptomic comparison of the uterus from non-reproductive (NR), mid-gestation (MG) and late, i.e. 13.5 dpc, gestation (LG) females, as well as oestrous cycle females that are 7 dpe and 13 dpe, these correspond to mid-gestation (EMG) and late gestation (ELG) in a pregnant cycle respectively. (*a*) Principal component analysis (PCA) of uterine transcriptomes in various stages of reproduction. While PC1 separates late pregnant and the equivalent stage of the oestrous cycle samples from the others, PC2 does not separate any of the non-pregnant samples from each other, but separates both mid- and late-gestation samples from each other and the non-pregnant samples. (*b*) Heatmap of Z transformed square root TPM expression data. Boxes highlight clusters of genes that are either more highly expressed in non-pregnant samples compared to pregnant samples, late pregnant and oestrous cycle samples compared to other samples, or are uniquely upregulated in either late pregnancy or the equivalent stage of the oestrous cycle. (*c*) Number of differentially expressed genes between pairwise comparisons of NR, MG, EMG, LG and ELG. Striped bars represent the total number of significantly differentially expressed genes, while the solid bars represent significantly differentially expressed genes with a fourfold difference in expression. (*d*) There is a highly significant overlap in the genes differentially expressed between LG and NR with ELG and NR (hypergeometric test, $p < 1 \times 10^{-318}$). (Online version in colour.)

differences between the non-reproductive tissue compared to late oestrous cycling samples (ELG), than between the late pregnant (LG) and late oestrous cycle (ELG) samples. Of the genes differentially expressed between non-reproductive and late oestrous cycle (ELG) tissue, about half are differentially expressed in the same direction between LG and non-reproductive uterus (figure 2*d*), this overlap is much higher than would be expected by chance (hypergeometric test, $p < 1 \times 10^{-318}$).

To understand how the uterus prepares for pregnancy even without fetal inputs, we can look at differentially expressed genes between oestrous cycling samples (at the equivalent stage to LG) and NR. When we look at the genes which are upregulated in the oestrous cycle compared to NR, we see two major clusters of over-represented gene ontology terms, those related to 'regulation of hormone levels' and 'response to lipid' (figure 3*a*). This may be a hallmark of the impacts of progesterone on uterine development. Progesterone production is much higher in the oestrous cycle than in non-pregnant animals but would require manipulative experiments to confirm [11,29]. Unlike in pregnancy, in the oestrous cycle, there is little evidence of inflammation. We do see the upregulation of the interleukin 6 receptor IL6R, the tumour necrosis factor receptors TNFRSF9, TNFRSF11B and prostaglandin E2 receptor PTGER4 relative to the non-reproductive uterus, but we do not find significant expression of the ligands of these receptors in the oestrous cycle while these ligands are progressively

Figure 3. Gene ontology analysis of differentially expressed genes. (*a*) Significantly over-represented gene ontology terms from the list of significantly upregulated genes in the late-oestrous cycle (ELG) compared to the non-reproductive uterus. (*b*) Significantly over-represented gene ontology terms from the list of significantly upregulated genes in LG uterus compared to the ELG. (Online version in colour.)

expressed in LG [14,30]. These results suggest that in the oestrous cycle the uterus is primed to respond to inflammatory signals even in the absence of an embryo and inflammatory mediators.

To determine what genes are influenced by the presence of the fetus, we compared gene expression between LG and ELG. In LG, we see a significant upregulation of genes involved in nutrient transport, nutrient metabolism and acute inflammation compared to ELG (figure 3*b*). The finding of genes involved in nutrient transport is consistent with pregnancy-specific gene expression being associated with increased potential for uterine nutrient transport to the fetus and also with our finding of a stronger secretory morphology of the uterine gland epithelium (figure 1). To identify if this differential gene expression resulted in different localization of nutrient transport molecules, we used immunohistochemistry to localize two proteins associated with nutrient transport to the pregnant uterus (figure 4). Liver fatty FABP1 is involved in fatty acid uptake, transport and metabolism [31]. In our transcriptome data, there are no transcripts of this gene in any sample other than LG (where it is expressed at approximately 12 TPM. Immunohistochemistry shows that liver fatty acid binding protein localizes to the glandular epithelial cells in late pregnancy but not in the oestrous cycle (figure 4*a*,*b*), which is consistent with glandular tissue being involved in lipid and fatty acid transport in pregnancy. This result is also consistent with the histological observation above, which suggests that uterine glands are more active during pregnancy than in the oestrous cycle.

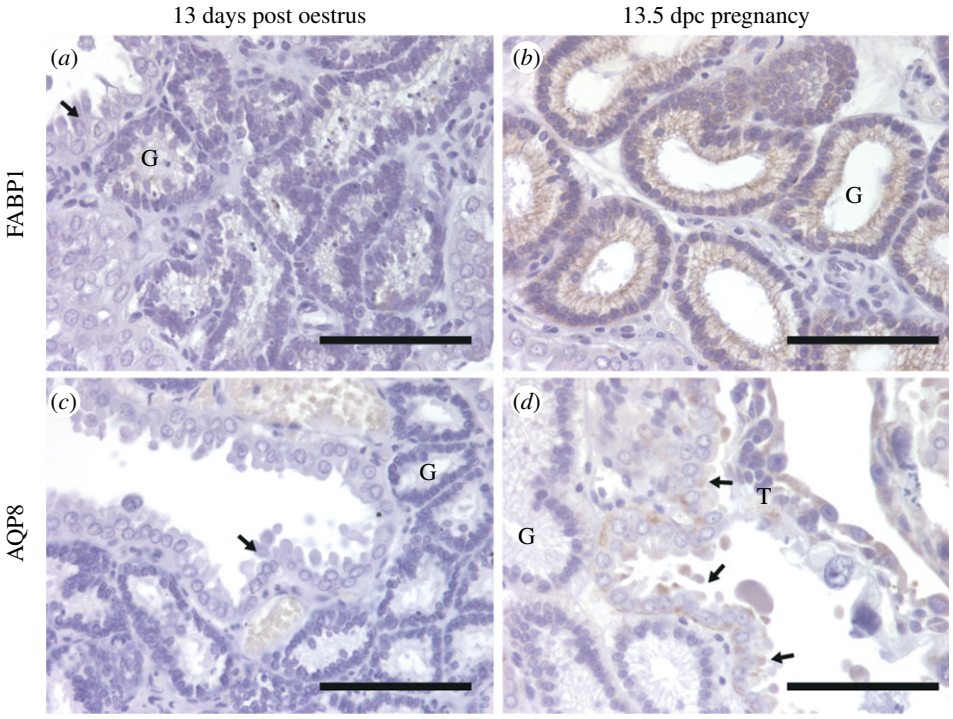

Figure 4. Expression of nutrient transport molecules liver fatty acid binding protein (FABP1) (*a*,*b*) and aquaporin 8 (AQP8) (*c*,*d*), in oestrous cycle (13 dpe) (*a*,*c*) and late pregnancy (*b*,*d*). FABP1 can be localized to the cytoplasm of the glandular epithelium as seen by brown 3,3′-diaminobenzidine (DAB) staining, in pregnancy and not at the equivalent stage of the oestrous cycle. Similarly, AQP8 can be localized to the luminal epithelium of late-pregnant opossum, as seen by brown DAB staining, but is not found in the uterus during the oestrous cycle. These results are consistent with the uterus having a greater potential for nutrient transport when an embryo is present from both the glandular tissue and across luminal epithelial cells. This suggests that the transcriptional changes that occur in response to the presence of an embryo *in utero* may be associated with the processes of pregnancy such as nutrient transport. G, uterine glands; T, trophoblast; arrows, luminal epithelial cells.

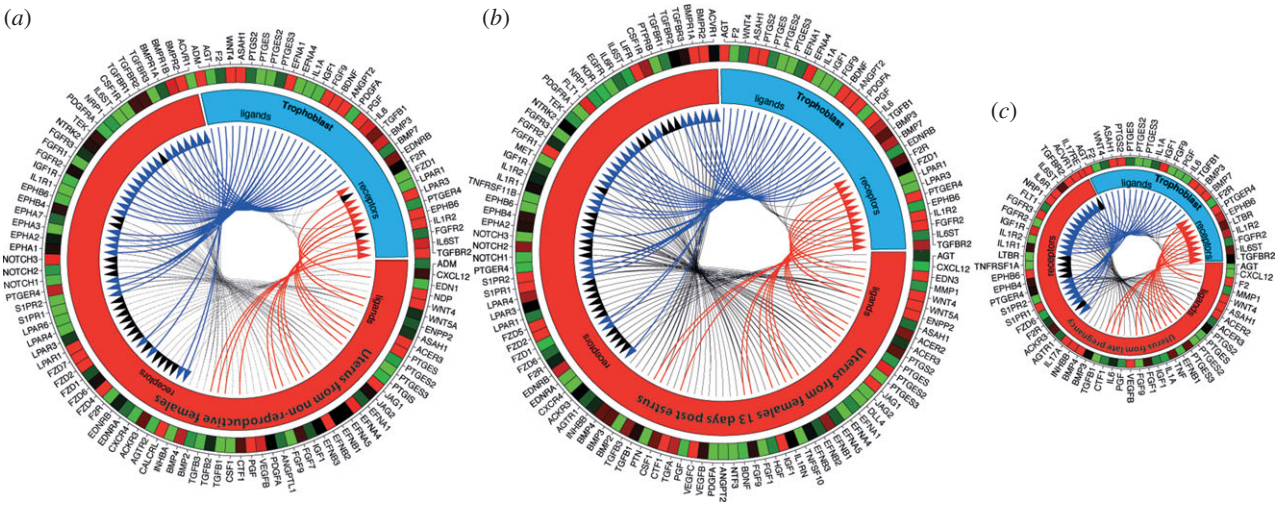

Figure 5. Signalling potential between the trophoblast (data collected from [24]) and the uterus from non-reproductive (*a*), late oestrous cycle (*b*) and LG (*c*) opossums, as measured by the expression of genes responsible for ligand and receptor synthesis. These network maps, include ligand and receptor genes with more than 10 TPM reads. Only ligands and receptors with active connections are included in the plot. Ligand–receptor relationships were taken from Pavlicev *et al*. [23].

AQP8 facilitates the transport of water along an osmotic gradient. In our transcriptome data, *AQP8* is expressed at approximately 6 TPM in LG, with no transcripts in all other samples (except for a single mapped read in each non-reproductive sample). AQP8 localizes to the luminal epithelial cells of the uterus in late pregnancy but not in the equivalent stage of the oestrous cycle (figure 4*c*,*d*). Interestingly, AQP8 is also upregulated in the endometrium during early placenta formation in the horse, and is not produced in the equivalent stage of cycling females [32], suggesting that it is influenced by the presence of an embryo in horse pregnancy as well. While the TPM values for both FABP1 and AQP8 appear low, immunostaining shows that this gene expression is probably coming from a very small subset of the uterine cells; therefore, the expression levels within these cells are likely to be much higher than observed from the whole tissue transcriptomes.

If we look at genes that are downregulated in the oestrous cycle compared to non-reproductive tissue, and genes that are downregulated in pregnancy compared to the oestrous cycle there is an over-representation of genes involved in

normal cellular processes, including transcription, DNA regulation and metabolic processes (electronic supplementary material, figure S2). We suspect this might be owing to an overall increase in transcription of genes for those particular reproductive states, resulting in a decrease in the fraction of transcripts from genes expressed at stable levels between comparisons (housekeeping genes).

(c) Maternal–fetal interaction and the origin of endometrial recognition of pregnancy

The oestrous cycle uterus, relative to other stages of reproduction, expresses the highest number of receptors for ligands that are produced by the trophoblast, suggesting that it has the highest potential to receive fetal signals. The LG uterus has substantially fewer corresponding ligand–receptor pairs expressed for maternal–fetal signalling (only 21 potential trophoblast to uterus signalling connections as opposed to 33 in non-reproductive, and 39 in the oestrous cycle 13 dpe). The decrease in the potential for maternal–fetal interactions during pregnancy suggests the existence of negative feedback from these signalling networks, which may stabilize the pregnancy-specific state of gene regulation. A particularly interesting set of receptors that are down-regulated in the LG uterus compared to other tissues are BMPR1A, BMPR1B and BMPR2. These proteins form dimers with each other, and are expressed by the endometrium of cattle at the time of maternal recognition of pregnancy [33]. Furthermore, BMP2 signalling is essential for implantation and decidualization in the mouse endometrium, while it is not necessary for attachment [34]. Note that decidualization is a process that does not seem to exist in opossums [17,35].

4. Discussion

(a) Pregnancy involves programmed changes to the uterus in the opossum

We found evidence that several uterine changes that occur during pregnancy are programmed responses that follow ovulation rather than being induced by the conceptus. These changes include the proliferation of uterine glands and the transformation of the uterine luminal epithelium including the formation of endometrial folds, which is consistent with the changes observed by electron microscopy [26,36]. These morphological changes are accompanied by a suite of changes to uterine gene expression, including the upregulation of genes involved in nutrient metabolism, nutrient transport and gene regulation (figure 3*a*). The two most significantly over-represented gene ontology terms for genes upregulated in the oestrous cycle compared to the non-cycling uterus are 'response to lipid' and 'regulation of hormone levels'. This suggests that lipid hormones (such as steroid hormones and prostaglandins) may play a part in the maternally driven aspects of the uterine cycle. Both progesterone and oestrogen have been shown to induce pregnancy-like changes to the endometrium of another marsupial, the fat-tailed dunnart [37]. Progesterone levels in *Monodelphis* peak at day 8 of pregnancy and then slowly reduce until the time of parturition [29]. This pattern is also present in the oestrous cycle, and is thus not owing to the presence of a fetus, but correlates with CL size [29]. As the levels of progesterone drop, the ratio of oestrogen to progesterone increases, peaking at the time of parturition [29].

(b) Significance of endometrial recognition of pregnancy in the opossum

Previously, recognition of pregnancy was recognized in two clades of therian mammals: the eutherian (so-called placental) mammals and the macropodid marsupials [38–40]). Macropods are nested within the Austro-marsupial clade [41]. For this reason, recognition of pregnancy in macropods was thought to be independently derived from that seen in eutherian mammals [15]. Our data demonstrate, at a minimum, that there are morphological, transcriptional and protein level responses in the endometrium that are consistent with an endometrial recognition of pregnancy in the opossum.

By having a mechanism for endometrial recognition of pregnancy in *Monodelphis* it is possible for the uterus to behave differently in pregnancy compared to a sterile oestrous cycle. In mammals that have uterine provisioning of nutrients, this means that uterine nutrient secretion can be supplied only when embryos are present, avoiding waste. The fact that we see differences in transporter expression between pregnancy and the oestrous cycle is particularly important because the ability to regulate maternal nutrient provisioning is probably one of the major advantages for having a mechanism for recognizing pregnancy. The mechanisms of nutrient allocation to offspring are major drivers of organismal fitness and are prone to selection through parent-offspring conflict, so even small effects of placentotrophy on maternal reproductive output and offspring survival are probably subject to strong natural selection [42,43]. We expect that constraining placentotrophy to only pregnant cycles, may be a significant driver for the evolution of recognizing pregnancy.

As has been proposed in wallabies the exact mechanism for maternal recognition of pregnancy may include hormonal secretions by the embryo or its membranes, immunological interaction of the mother and fetus, or the physical interaction of a fetus with the endometrial tissue [38]. Our transcriptome data suggest that in the opossum there is significant potential for maternal–fetal signalling (figure 5), which is not in its own right surprising, as the extra-embryonic membranes of amniotes ancestrally have significant endocrine activity [44–47], along with the uterus of even oviparous amniotes expressing a range of receptors and signals [48]. The apposition of maternal and fetal tissues is thus likely to be a primer for the initiation of maternal recognition of pregnancy [49]. However, the physical cues that could facilitate maternal recognition of pregnancy are probably important too.

(c) Specificity of embryonic impact for inducing uterine reaction

Our results show that the presence of a fetus *in utero*, elicits transcriptional and cellular responses in the endometrium. What is not clear is how this response is achieved. One possible explanation is that this is the result of mechanical stimulation of the endometrium by the presence of a conceptus. In mice, the insertion of plastic beads into the uterus can induce similar, but not identical, endometrial changes to those that occur in pregnancy [50]. A difficulty in performing a similar experiment in the opossum, is that the surgical

manipulation required for implanting a bead in the uterus, is likely to naturally lead to inflammation, which may mimic the inflammation observed during placentation. However, even if the endometrium is not able to differentiate between a developing embryo and an unnatural condition such as the presence of an inert plastic bead, it does not follow that the endometrial reaction is not an evolutionarily implemented strategy for recognizing pregnancy, if the impact is unlikely to occur in any natural circumstances other than pregnancy. As long as a cue is sufficiently specific to pregnancy, such as uterine stretch may be, it need not itself be complex to be coopted into inducing a maternal response.

(d) Inflammation as an early mechanism for the recognition of pregnancy

One of the major differences observed in the endometrium between pregnancy and the oestrous cycle, is inflammatory signalling. Inflammatory signalling within the opossum uterus only occurs in pregnancy, when both maternal and fetal tissues directly interact (figure 3). As inflammatory signalling can have wide transcriptional impacts, we predict that this inflammation may be a key mechanism by which the uterus recognizes the presence of a developing embryo if it occurs coincidental with elevated progesterone levels, the latter being an indication that ovulation happened.

Inflammation is a convenient mechanism for an early form of recognizing pregnancy, because (i) it is probably a direct consequence of exposure of the uterine epithelium to the yolk-sac membrane following breakdown of the eggshell barrier [14], (ii) even in the absence of genetic re-wiring it is likely to result in endometrial changes that are advantageous to the developing fetus, such as angiogenesis, vascular leakage and oedema [16,51,52], which may enhance the supply and transport of respiratory gasses and nutrients to the fetus, and (iii) inflammatory signalling may have further supported the parturition process which occurs shortly after the recognition of pregnancy in the opossum and probably the common ancestor of today's therian mammals [30]. In a normal physiological setting, inflammatory signalling has thus widespread consequences for the tissue in which it occurs, including oedema, angiogenesis, and it induces changes to gene regulation through the activation of transcription regulators including NF-κB and STAT3 [53].

Based on this hypothesis, we predict that the suppression of inflammation, particularly the suppression of cytokine signalling and the NF-κB pathway will result in a failure to recognize pregnancy, a reduced rate of maternal nutrient provisioning, and pregnancy failure in marsupials.

5. Conclusion

Our results show both, parallels between uterine changes in the oestrous cycle and during pregnancy as well as pregnancy-specific changes in the opossum. The latter affect the physiological state of the endometrial glands and epithelium as well as the expression of genes related to nutrient transfer and inflammation. While there is little if any change in the systemic steroid hormone levels of the mother during pregnancy compared to the oestrous cycle, these findings indicate a direct impact of the presence of embryos on the uterus, representing a case of 'endometrial recognition of pregnancy'. Establishing the route of endometrial recognition of pregnancy in marsupials is an essential next step in this research programme and could provide important insights into the origins of the signalling dynamics essential for normal pregnancy.

Ethics. All animal procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee of Yale University (protocol no. 15-11313).

Data accessibility. Raw RNA sequencing are available on the Sequence Read Archive (PRJNA543903). Available at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA543903

Authors' contributions. O.W.G., S.P. and G.P.W. designed research; O.W.G., S.P., R.C. and J.M. performed research; O.W.G., A.R.C. and M.P. analysed data; and O.W.G. and G.P.W. wrote the paper.

Competing interests. We declare we have no competing interests.

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