Embryo implantation evolved from an ancestral inflammatory attachment reaction

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Edited by Hopi E. Hoekstra, Harvard University, Cambridge, MA, and approved July 5, 2017 (received for review January 24, 2017)

The molecular changes that support implantation in eutherian mammals are necessary to establish pregnancy. In marsupials, pregnancy is relatively short, and although a placenta does form, it is present for only a few days before parturition. However, morphological changes in the uterus of marsupials at term mimic those that occur during implantation in humans and mice. We investigated the molecular similarity between term pregnancy in the marsupials and implantation in eutherian mammals using the gray short-tailed opossum (*Monodelphis domestica*) as a model. Transcriptomic analysis shows that term pregnancy in the opossum is characterized by an inflammatory response consistent with implantation in humans and mice. This immune response is temporally correlated with the loss of the eggshell, and we used immunohistochemistry to report that this reaction occurs at the materno–fetal interface. We demonstrate that key markers of implantation, including Heparin binding EGF-like growth factor and Mucin 1, exhibit expression and localization profiles consistent with the pattern observed during implantation in eutherian mammals. Finally, we show that there are transcriptome-wide similarities between the opossum attachment reaction and implantation in rabbits and humans. Our data suggest that the implantation reaction that occurs in eutherians is derived from an attachment reaction in the ancestral therian mammal which, in the opossum, leads directly to parturition. Finally, we argue that the ability to shift from an inflammatory attachment reaction to a noninflammatory period of pregnancy was a key innovation in eutherian mammals that allowed an extended period of intimate placentation.

placenta | marsupial | inflammation | pregnancy | evolution

In eutherian (so-called "placental") mammals, pregnancy begins when the blastocyst attaches to the uterine wall, followed by the establishment of a stable fetal–maternal interface. Implantation involves the apposition, attachment, and, in many species, the invasion of the blastocyst into the uterus (1). In particular, structural and molecular changes occur in the luminal epithelia that allow the embryo to attach and invade the uterus (2). Epithelial changes are followed by remodeling of the endometrial stroma, generally known as "decidualization," i.e., the transformation of endometrial stromal fibroblasts into decidual stromal cells, as well as modifications of the endometrial vascular bed (3). When these changes do not occur or occur incompletely, blastocysts fail to implant, resulting in early pregnancy failure (4, 5). In humans, 75% of unsuccessful pregnancies are the result of failures of implantation, and implantation failure is the limiting factor for in vitro fertilization treatment. Furthermore, decades of research have failed to produce clinically effective treatments that increase uterine receptivity to implantation (6–8). Given the magnitude of this problem, extensive efforts have been made to characterize the endometrium for signatures of receptivity (9, 10).

Implantation in humans and rodents paradoxically relies on a proinflammatory mechanism. During the "implantation window," the uterus is primed to produce several proinflammatory signals, such as prostaglandin E_2 (PGE_2), and a range of proinflammatory cytokines, including TNF and IL6 (11–13). This inflammatory reaction is essential for successful implantation, and in humans, the consumption of antiinflammatory drugs during the implantation window is associated with an increased rate of miscarriage (14). Following implantation, the endometrium switches to an antiinflammatory state, which is necessary to prevent rejection of the fetus, because the fetus contains paternal genetic material and perturbs the tissue integrity of the endometrium (15). Given that an antiinflammatory state is essential for the maintenance of pregnancy, the evolutionary origins of implantation as a proinflammatory process is paradoxical and requires explanation. Here we present evidence that an inflammatory reaction occurs during the short-lived attachment of the opossum placenta, but no switch to an antiinflammatory regime is evident, a fundamental difference from what occurs in eutherians.

Opossum as a Model for Understanding Early Therian Mammal Reproductive Biology

The first mammals were egg-laying (oviparous) and therefore did not undergo any form of implantation (Fig. 1). This inference is supported by the facts that most reptiles (the sister taxon of mammals) are egg-laying and that the most basally branching lineage of mammals, i.e., extant monotremes, are also egg-laying.

Significance

Our data suggest that implantation in eutherians is derived from an ancestral inflammatory reaction to embryo attachment in the therian ancestor. These results explain the paradoxical role of inflammation at the beginning and the end of pregnancy in humans: Inflammation is necessary for implantation and parturition, but for most of pregnancy, inflammation threatens the continuation of pregnancy. We argue that the role of inflammation during implantation is an ancestral response to the embryo as a foreign body. By changing the way investigators think about implantation, we expect this research to contribute to new ways to study and treat implantation disorders, the most vulnerable step of assisted reproductive technology, in women.

Author contributions: O.W.G. and G.P.W. designed research; O.W.G., S.P., and J.M. performed research; O.W.G. and A.R.C. analyzed data; and O.W.G., R.R., and G.P.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1701129114/-/DCSupplemental.

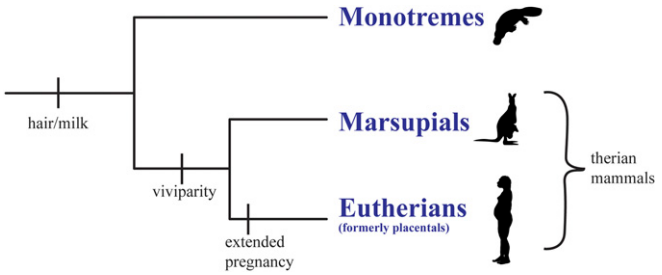

Fig. 1. Relationship between major groups of mammals. Oviparity is still observed in the extant monotremes. Viviparity evolved in the stem therians and is present in almost all mammals today; however, marsupial pregnancy is short, and an extended pregnancy is observed only in eutherian mammals.

Monotremes have a short, 15- to 21-d gestation following ovulation and a short, ~10-d period of development in the egg after laying. Monotreme hatchlings are highly altricial (16, 17). Two major viviparous mammalian lineages exist today: eutherians and marsupials. Thus, viviparity likely evolved before the most recent common ancestor of marsupials and eutherians within the therian stem lineage 180–150 million years ago. Although viviparity has evolved many times in the animal kingdom, it appears to have occurred only once in mammals (18, 19). In eutherians, following fertilization, blastocysts attach to the uterine wall, and in most species, including humans, this attachment is followed by the formation of an invasive placenta. This invasive mode of development, although not universal in eutherians, is nevertheless their ancestral mode of development (20–22). Embryonic development occurs during an extended period of gestation following attachment.

Marsupials, although viviparous, have retained many features of monotreme reproductive biology. Like monotremes, marsupial young are highly altricial. Further, they have a shelled egg, which is breached only shortly before birth (23). However, once the eggshell is breached, the choriovitelline membrane of the embryo attaches to the uterine endometrium, forming a short-lived placenta. As in eutherians, placenta formation involves remodeling of the uterus, maternal–fetal interaction, and attachment (24–27). Because marsupials share features with eutherian and monotreme reproduction, marsupials are likely close to the style of reproduction that existed in the first live-bearing mammals. Opossums, in particular, are the best model for the configuration from which the eutherian mode of pregnancy evolved, because they retain ancestral features of marsupial pregnancy and, likely, therian pregnancy (25). For these reasons, the opossum has been used to understand the evolutionary origins of pregnancy in live-bearing mammals (28, 29).

Although marsupial pregnancy is short, important structural changes to the uterus take place during the formation of the placenta that are similar to those that occur at implantation in eutherian mammals. To understand the evolutionary origins of implantation, we examined the molecular changes coincident with the attachment reaction in the gray short-tailed opossum (*Monodelphis domestica*). We tested the hypothesis that the opossum attachment reaction is homologous to the inflammatory phase of eutherian implantation but lacks the switch to an antiinflammatory phenotype characteristic of human and rodent pregnancy by examining gene-expression changes in response to the attachment reaction in *M. domestica* and found that the production of inflammatory mediators is associated with attachment. We then investigated the expression of key implantation markers, Heparin-binding EGF-like growth factor (HBEGF) and Mucin 1 (MUC1), and found that their expression and localization correlate with attachment of the embryo. These molecular changes are followed immediately by parturition, showing that the opossum lacks the antiinflammatory phase known from eutherian pregnancy.

Results

Histological Description of Placental Contact in *Monodelphis*. *Monodelphis* gestation lasts for 14.5 d post copulation on average. Throughout most of gestation, the embryonic capsule is small, 500–1,000 μm across, and is covered by a shell coat (Fig. 2*A*). By 11.5 d post copulation, the embryonic membranes have grown extensively, and there is a high degree of folding and substantial interdigitation between the folds of the trophoblast and endometrium, but the shell coat is still intact, and no direct physical contact between maternal and embryonic tissue occurs (Fig. 2*B*). Up to this gestational stage, the luminal epithelium remains pseudostratified and columnar, with dense nuclei and a smooth, partially ciliated surface. The subepithelial layer of endometrial stromal cells, typical for nonpregnant opossum epithelium (30), disappears and is replaced by an oedematic extracellular matrix and a net of subepithelial capillaries. On day 12.5 post copulation this shell coat is breached (Fig. 2*C*). The luminal epithelium becomes less pseudostratified, and the nuclei align at one level, suggesting an expansion of the epithelial surface area. On day 13.5 post copulation (the last day of pregnancy), luminal cells become lower, the nuclei become lighter, and thus the luminal epithelial cells show signs of strong metabolic activation. At the luminal surface, large vesicles (5–15 μm across) actively bud from the apical surface of epithelial cells. This apocrine secretion is associated with an apparently less intimate contact between fetal and maternal surfaces and likely hinders strong attachment by the fetus. At this stage the luminal epithelial surface seems to enlarge even further so that, in some places, the luminal epithelium folds back onto itself, forming epithelial septa. Direct physical contact between the fetal membrane and the endometrium thus only starts to develop on the 12th day of a 14-d gestation. On day 14, parturition occurs.

We used these histological findings as a foundation for investigating gene-expression changes associated with these alterations in the fetal–maternal relationship, the presence of a conceptus confined in the shell coat, and the eventual (although brief) direct contact between the chorion and the luminal epithelium. We first pursued this objective with whole-uterus transcriptome analysis.

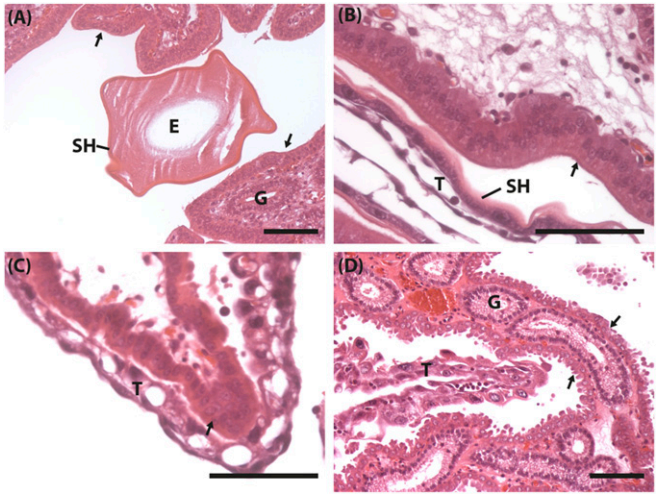

Fig. 2. Histological examination of the fetal–maternal interface in the opossum through the second half of gestation. H&E-stained uterine sections from females 7 (*A*), 11.5 (*B*), 12.5 (*C*), and 13.5 (*D*) d post copulation. Parturition occurs 14.5 d post copulation. Before day 12.5 there is an intact shell coat (SH) that separates the embryonic trophoblast (T) from the uterine luminal epithelial cells (arrows). On day 13.5 of gestation large vesicles actively bud from the apical surface of the uterine lumen. (Scale bars: 100 μm.) Arrows point to uterine luminal epithelial cells. E, developing egg/embryo; G, uterine glandular tissue.

Gene-Expression Changes Associated with the Attachment Reaction. We examined uterine gene expression by sequencing the transcriptomes of uteri of nonpregnant, preattachment (7 d post copulation), and postattachment (13.5 d post copulation) females. Hierarchical clustering and principal component analysis of all mapped genes (n = 23,899 genes) suggest there are gene-expression changes in the uterus of *M. domestica* throughout the reproductive cycle (Fig. 3). Principal component analysis (Fig. 3*B*) separates the three groups of uterine samples. The first principal component separates postattachment samples from other uterine samples and explains 51% of the variance (Fig. 3*B*).

Gene-expression changes throughout the reproductive cycle clearly show that there are clusters of genes that are uniquely up-regulated in mid or late pregnancy as well as a large number of genes that are up-regulated in both stages of pregnancy (Fig. 3*C*). Differential gene-expression analysis using DESeq2 identified massive gene-expression changes throughout pregnancy (Fig. 3*C*). The full output of the differential expression analysis is shown in Dataset S1, Tables S1 and S2. Dataset S1, Tables S3–S6 demonstrate the full output of gene ontology (GO) analysis of differentially expressed genes.

There is greater expression of genes involved in nutrient metabolism and transport in pregnant samples than in nonpregnant samples (Fig. 4). In the postattachment uterus compared with preattachment uterus, there is strong up-regulation of genes involved in inflammation and wound healing, including an overrepresentation of genes involved in inflammatory response (GO:0006954, 2.43-fold, $q = 9.72 \times 10^{-8}$), immune response (GO: 0006955, 1.77-fold, $q = 5.8 \times 10^{-5}$), and cytokine secretion (GO:0050663, 3.86-fold, $q = 6.75 \times 10^{-3}$). Inflammatory markers include *PTGS2*, also known as "cyclooxygenase-2" (COX2), the key enzyme of prostaglandin synthesis, several proinflammatory cytokines (*TNF*, *IL6*, *IL1A*, *IL19*, and *IL17A*), and the antiinflammatory cytokine *IL10*.

Despite the increase in inflammatory markers, differential gene-expression analysis does not identify a significant increase in known leukocyte marker genes. Protein tyrosine phosphatase receptor type C (the pan-leukocyte marker *CD45*) has lower expression at midgestation than in nonpregnant or late-gestation females, suggesting that the leukocyte density changes throughout pregnancy. There is down-regulation of the B-cell marker *MS4A1*, low but stable expression of the T-cell markers *CD3D*, *CD3E*, *CD3G*, *CD4*, and *CD8B*, significant decrease of the natural killer cell marker *NCAM1*, and stable levels of the macrophage markers *CD68*, *IL3RA1*, and *IL4R*. However, there is a significant 40% increase in the level of *CD14* during pregnancy; *CD14* is primarily produced by macrophages, so this increase suggests there could be some additional macrophage activity. CD14 and its interaction partner lipopolysaccharide-binding

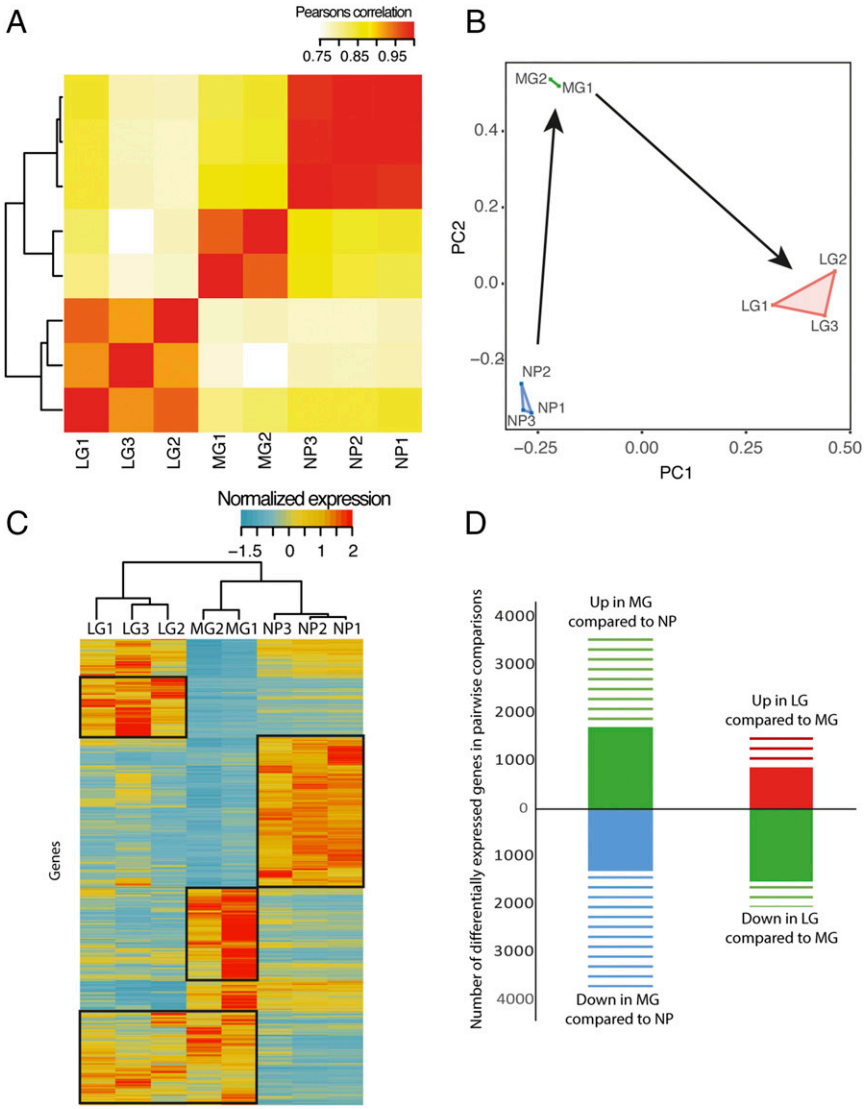

Fig. 3. Correlations of transcriptome samples. (*A*) A heatmap showing the correlations between each pair of transcriptome samples. (*B*) Principal component analysis of uterine gene expression between samples at different stages of the reproductive cycle. LG, late gestation (postattachment, day 13.5 of pregnancy); MG, midgestation (preattachment, day 7 of pregnancy); NP, nonpregnant tissue. (*C*) Heatmap of clustering of differentially expressed genes in each uterine tissue sample. Gene-expression values are normalized by using a z-score transformation on TPM. (*D*) Number of differentially expressed genes in pairwise comparisons between each reproductive stage.

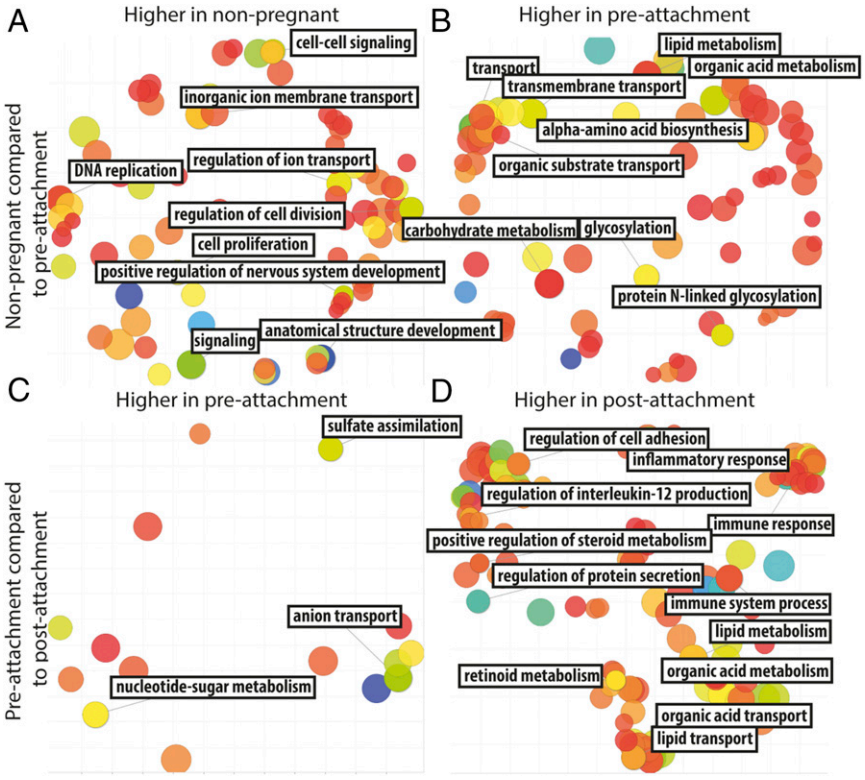

Fig. 4. Semantic clustering of significantly over-represented GO terms in differentially expressed genes in comparisons between nonpregnant (*A*) and midgestation (*B*) tissue and between preattachment (*C*) and postattachment (*D*) tissue. GO analysis was performed by GOrilla (77), and semantic clustering was achieved by ReviGO (78). In each gene set there is significant enrichment of GO terms.

protein (LBP) have also been detected in opossum endometrial stromal cells (31). Further, there is a qualitative increase in neutrophil-specific elastase, *ELANE*, with few mapped transcripts in nonpregnant and midgestation samples but expression of this gene in all late-gestation samples, with a mean level of 1.1 transcripts per million (TPM), suggestive of neutrophil infiltration of the uterus during the attachment reaction. GO terms associated with inflammation or the immune system are found only in the list of genes up-regulated at late gestation.

GO analysis of genes with increased expression in late gestation also identified an overrepresentation of genes involved in the response to hormone (GO: 0009725, 1.86-fold, $q = 2.53 \times 10^{-5}$); genes present include the oxytocin receptor *OXTR*, angiogenic factors such as *VEGFA*, and several genes involved in retinol metabolism, including *RDH12*, *RDH8*, *RBP4*, and *RPE65*. Although RNA levels for genes involved in prostaglandin metabolism and synthesis, including *PTGS2* and Prostaglandin E_2 synthase (*PTGES*) increase, the levels of RNA encoding the estrogen receptor *ESR1* and progesterone receptor *PGR* decrease through pregnancy.

Implantation Marker Expression Is Induced Following Uterine–Trophoblast Contact. To test the hypothesis that the expression of inflammatory signals is homologous to implantation-related inflammation in eutherians, we examined whether the expression of eutherian implantation markers is correlated with the loss of the shell-coat barrier and thus with the initiation of direct fetal–maternal contact. Specifically, we examined the expression of these implantation markers at a finer scale using qPCR (Fig. 5). As can be seen, the up-regulation of several inflammatory markers (*IL6*, *TNF*, and *PTGS2*) does not occur until after attachment, i.e., after 11.5 d post copulation (Fig. 5 *A–C*). In addition, MUC1, HBEGF, and PTGES show moderate increases in expression following the breach of the shell coat and a consistent pattern of increasing expression through successive days of pregnancy. Given the temporal correlation between the expression of these genes and the attachment reaction, we predict that these genes will be localized to the tissues of the attachment reaction.

Implantation Markers Are Localized to the Maternal–Fetal Interface. To investigate the hypothesis that the opossum attachment reaction is homologous with the eutherian implantation process, we immunolocalized several known markers of implantation that share expression profiles in the transcriptome data with uterine tissue at key stages of gestation (Fig. 6). Although there were many genes that we would have wished to investigate, many interesting molecules could not be studied because of limitations of available antibodies that would be cross-reactive with *Monodelphis*. Validation of the antibodies we used is provided in the *SI Appendix*.

Each of the examined proteins showed expression levels that were broadly consistent with the expression levels observed in transcriptomics (Figs. 5 and 6). PTGS2 is the rate-limiting enzyme in the prostaglandin synthesis pathway, and strong cytoplasmic staining was seen in uterine luminal epithelial cells in the post-attachment phase of pregnancy (Fig. 6*C*). This finding is consistent with the uterine tissue expressing inflammatory markers as a component of the attachment reaction and is consistent with PTGS2 expression in eutherian implantation (32).

HBEGF facilitates the interaction between the trophoblast and endometrium in humans and rodents and other eutherians (33). HBEGF reactivity was strongly localized to the cytoplasm of epithelial cells in the postattachment uterus (Fig. 6*F*). We also saw HBEGF nuclear staining of some glandular epithelial cells throughout the reproductive cycle; however, staining did not appear in all nuclei (Fig. 6 *D–F*).

Mucins are glycoproteins that line organ surfaces exposed to the external environment, including lung, gut, and eyes. MUC1 inhibits cell–cell interactions in vitro and can act as a physical barrier to implantation (34). MUC1 is degraded as a barrier to implantation by the trophoblast in rabbits (35), is down-regulated by the blastocyst in vitro in humans (36), and is secondarily down-regulated at implantation in mice by steroid hormones (37). In humans, MUC1 acts as a scaffold and ligand for selectins present on the blastocyst, facilitating attachment (38, 39). MUC1 reactivity was consistently found on the apical surface of luminal epithelial cells in the opossum (Fig. 6 *G–I*) and was strongest in nonpregnant and postattachment females. In postattachment females, MUC1

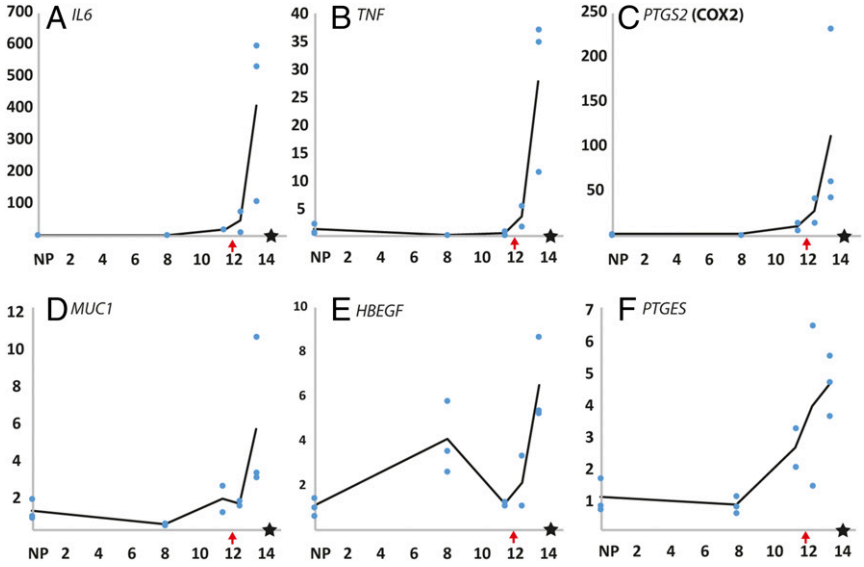

Fig. 5. Expression of key markers (as labeled in *A*–*F*) of implantation in *M. domestica* through key days of pregnancy, as measured by real-time qPCR. The time of shell-coat breach is marked by a red arrow, and parturition is marked by a star. Expression levels are reported relative to expression in nonpregnant uterine tissue and to the internal reference gene *TBP*. The change in mean value is indicated by a black line.

surrounds budding vesicles as they separate from the luminal epithelia. In humans, MUC1 is secreted by uterine luminal epithelial cells, and maximal expression is observed during the implantation window of the estrous cycle (40). The presence of MUC1 does not appear to be weakened by the trophoblast, because it is expressed in regions that both are and are not in contact with embryonic trophoblast. The persistence of MUC1 in postattachment luminal epithelium may act to decrease the ability of the trophoblast to damage and invade the endometrium.

In addition to uterine markers, we localized two molecules to the fetal side of the placenta. Serine protease 8 is present in trophoblast cells of the placenta 14.5 d post copulation (*SI Appendix*, Fig. S1). The expression of PGE_2 synthase (PGES) has been found in the late-gestation uterine RNA, but the protein localization is limited to the trophoblast (Fig. 6*L*), suggesting that the RNA in the late-gestation samples is, in part, the result of contamination with trophoblast cells. Given the potential for trophoblast contamination of uterine tissue, future work should aim to identify the cell types responsible for inflammatory gene expression using either laser-captured microdissection and RNA-sequencing (RNA-seq) or in situ hybridization on uterine sections. It is interesting that the two key steps in PGE_2 synthesis are distributed in different tissues: PTGS2 in the uterine luminal epithelium and PTGES in the trophoblast, suggesting that the production of PGE_2 can signal the presence of a fetus.

Transcriptome-Wide Similarities Between the Opossum Attachment Reaction and Eutherian Implantation. To identify whether the similarities between implantation in eutherian mammals and term pregnancy in the opossum were limited to a small number of implantation biomarkers or whether there is evidence for global transcriptome overlaps, we performed transcriptome-wide comparisons with a number of existing eutherian implantation transcriptome datasets. In eutherians, various experimental designs have been used to identify implantation-specific gene-expression changes. In rodents and rabbits, experimental designs typically compare gene expression in implantation sites and interimplantation sites within the uterus.

There is a highly significant overlap in the genes expressed in the implantation sites of rabbits (41) and in the genes up-regulated during the opossum attachment reaction ($P = 3.7 \times 10^{-20}$, χ^2 test) (Fig. 7*D*). The detected overlap is substantial and includes 83 genes that are up-regulated in both the rabbit implantation sites and the postattachment opossum uterus. This overlap set is highly enriched in genes related to hormone response (GO:0009725, 6.97-fold, $q = 2.42 \times 10^{-7}$), cellular secretion (GO:0032940, 5.19-fold, $q = 2.5 \times 10^{-7}$), and inflammatory response (GO:0006954, 4.98-fold, $q = 8.74 \times 10^{-3}$). We also find that genes involved in embryo implantation are enriched (GO:0007566, 15.2-fold, $q = 6.2 \times 10^{-2}$). These genes include the implantation biomarkers osteopontin (*SPP1*), stanniocalcin 1 (*STC1*), and fibulin 1 (*FBLN1*). In contrast, studies that used a similar design in mice (42, 43) showed a degree of overlap that is not statistically significant (*SI Appendix*, Fig. S2 *A* and *B*).

In humans, studies have conducted uterine tissue biopsies either within or outside the window of implantation. There are substantial discrepancies among the many studies that have been conducted (44). These discrepancies might reflect real biological variation, heterogeneity within the uterus, and/or biopsy technique. Furthermore, the human transcriptome data were generated using microarrays that limit the ability to detect gene-expression changes, so the number of identified implantation genes is lower than in RNA-seq experiments. Despite these differences, there is a significant overlap between the genes up-regulated during the attachment reaction in the opossum and the genes up-regulated in the human implantation window as identified by Riesewijk et al. (45) ($P = 1.5 \times 10^{-6}$, χ^2 test) (Fig. 7*E*), Mirkin et al. (46) ($P = 3.2 \times 10^{-3}$, χ^2 test) (Fig. 7*F*), and Kao et al. (47) ($P = 9.3 \times 10^{-4}$, χ^2 test) (Fig. 7*G*). Specifically, we see an overlap in the key implantation biomarker SPP1, which is the only consistent implantation window biomarker identified from all human microarray experiments (44). Because the number of identified implantation window genes in the human studies is low, there is limited power to perform GO analysis on each of the studies. However, when we pooled the genes from all the human studies, we found the overrepresentation of some interesting GO terms, namely, response to external stimulus (GO:0009605, 4.10-fold, $q = 2.34 \times 10^{-2}$) and regulation of apoptotic processes (GO:0042981, 3.70-fold, $q = 1.25 \times 10^{-2}$). Several of these overlapping genes, including *G0S2* (48) and *SGK1* (49), have been implicated in diseases associated with implantation pathologies.

Together, these global comparisons between implantation and the opossum attachment reaction suggest that there is a transcriptome-level similarity between the opossum attachment reaction and gene expression during rabbit implantation as well as in the human window of implantation, supporting our suggestion that they are

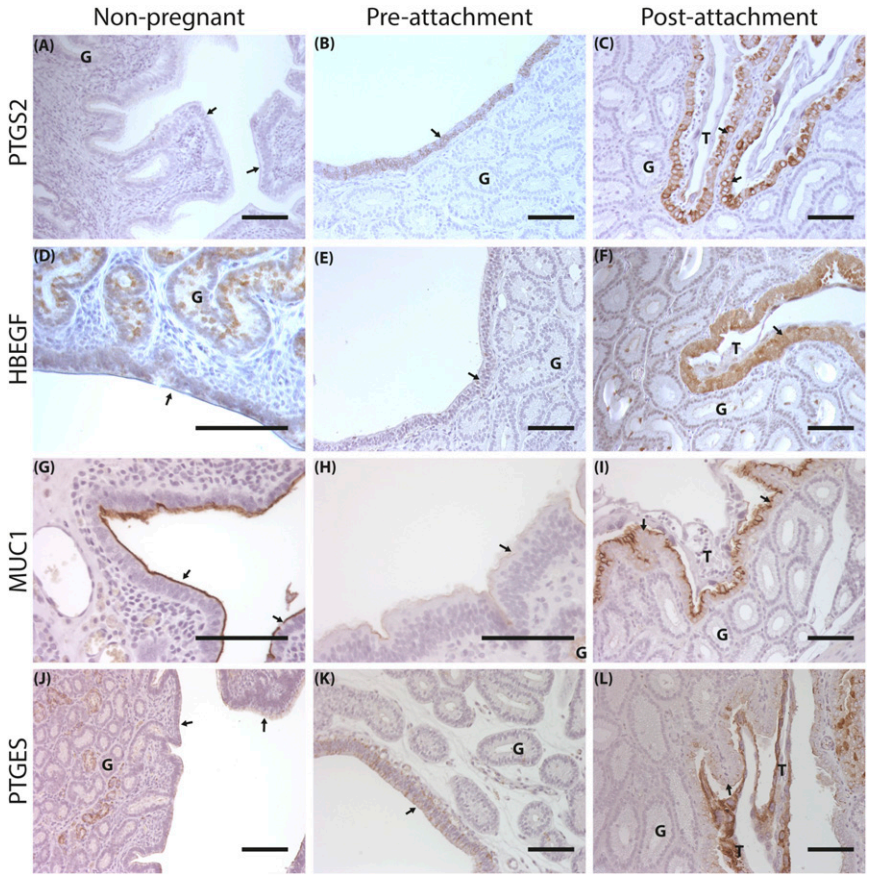

Fig. 6. Immunolocalization of key implantation markers to the uterine tissue of *M. domestica*. Images present the localization of PTGS2, also known as COX2 (*A*–*C*), HBEGF (*D*–*F*), MUC1 (*G*–*I*), and PTGES (*J*–*L*). Uterine tissue was collected at different stages of the reproductive cycle from nonpregnant (*A*, *D*, *G*, *J*), preattachment (*B*, *E*, *H*, *K*), and postattachment (*C*, *F*, *I*, *L*) females. Localization was visualized with 3,3′-diaminobenzidine-tetrahydrochloride (DAB), which appears as a brown precipitate in sections. (Scale bars: 100 μm.) Arrowheads point to uterine luminal epithelial cells. G, uterine glandular tissue; T, trophoblast tissue.

homologous. The lack of a statistically significant overlap with gene expression in mouse studies is interesting, because specific genes with an experimentally verified role in implantation (HBEGF, COX2, and others) are seen in both mouse implantation and opossum attachment reaction. These admittedly limited comparisons may suggest that implantation in the mouse is more highly derived from the ancestral attachment reaction than is implantation in rabbits and humans.

Discussion

The Attachment Reaction in *Monodelphis* Has Molecular Signatures Consistent with Human and Rodent Implantation. In humans, implantation follows a series of structural and molecular changes to the endometrial lining; in particular, these changes involve the production of proteins to regulate maternal–embryo interactions (Fig. 7*A*). The changes to the luminal epithelial cells and the density of cell-adhesion molecules in the opossum attachment reaction are similar to the implantation reaction in human and rodent pregnancy (50, 51). Several of the changes that support implantation in eutherians also occur at term in *M. domestica* (Fig. 7*B*). The most striking similarity is that, despite there being normal physiological processes and despite the absence of an infection or pathogens, both implantation in eutherians and parturition in *M. domestica* involve a conserved set of inflammation markers and immune signaling pathways (Fig. 7*A*). The expression of key implantation markers and consistent patterns of inflammation suggest that the attachment reaction in *M. domestica* is homologous with the molecular processes of implantation in human and rodent pregnancies and also support the hypothesis that viviparity evolved before the most recent common ancestor of eutherians and marsupials. Homology implies that the human implantation process evolved through the modification of an ancestral attachment reaction similar to that documented here in the opossum. This view is consistent with the interpretation of human implantation as a modified inflammatory reaction (11).

In the opossum, HBEGF localization patterns are consistent with this molecule being a key component of the attachment reaction. HBEGF is most abundant in luminal epithelial cells during implantation in rodent and human pregnancy (33, 52). An interesting difference is that in the mouse HBEGF is also expressed in the trophoblast; we did not find any staining in the opossum trophoblast. Although the functional significance of HBEGF in *Monodelphis* pregnancy cannot be inferred from our data, it is likely to be important for maternal–embryo communication following placenta formation. Although HBEGF localization correlates with uterine gene expression levels in nonpregnant and postattachment *Monodelphis* (Fig. 6 *D* and *F*), on day 8 of pregnancy there is an increase in *HBEGF* mRNA expression that does not appear to result in the presence of HBEGF protein in tissues (Fig. 5*E*). We suspect that this gene exhibits posttranscriptional regulation in the preattachment uterus. HBEGF exists in a membrane-bound form (which facilitates interactions with adjacent cells) and as a secreted molecule (which signals to nearby cells, such as stromal fibroblasts) (53). Localization of HBEGF to the cytoplasm of cells suggests that it exists in a secretable form and may have a signaling function in the attachment reaction. In mice, uterine production of HBEGF is dependent on hormonal signals from the blastocyst and does not occur during pseudopregnancy (33, 54). Blastocyst-sized beads laced with HBEGF were sufficient to evoke implantation-like responses in the mouse

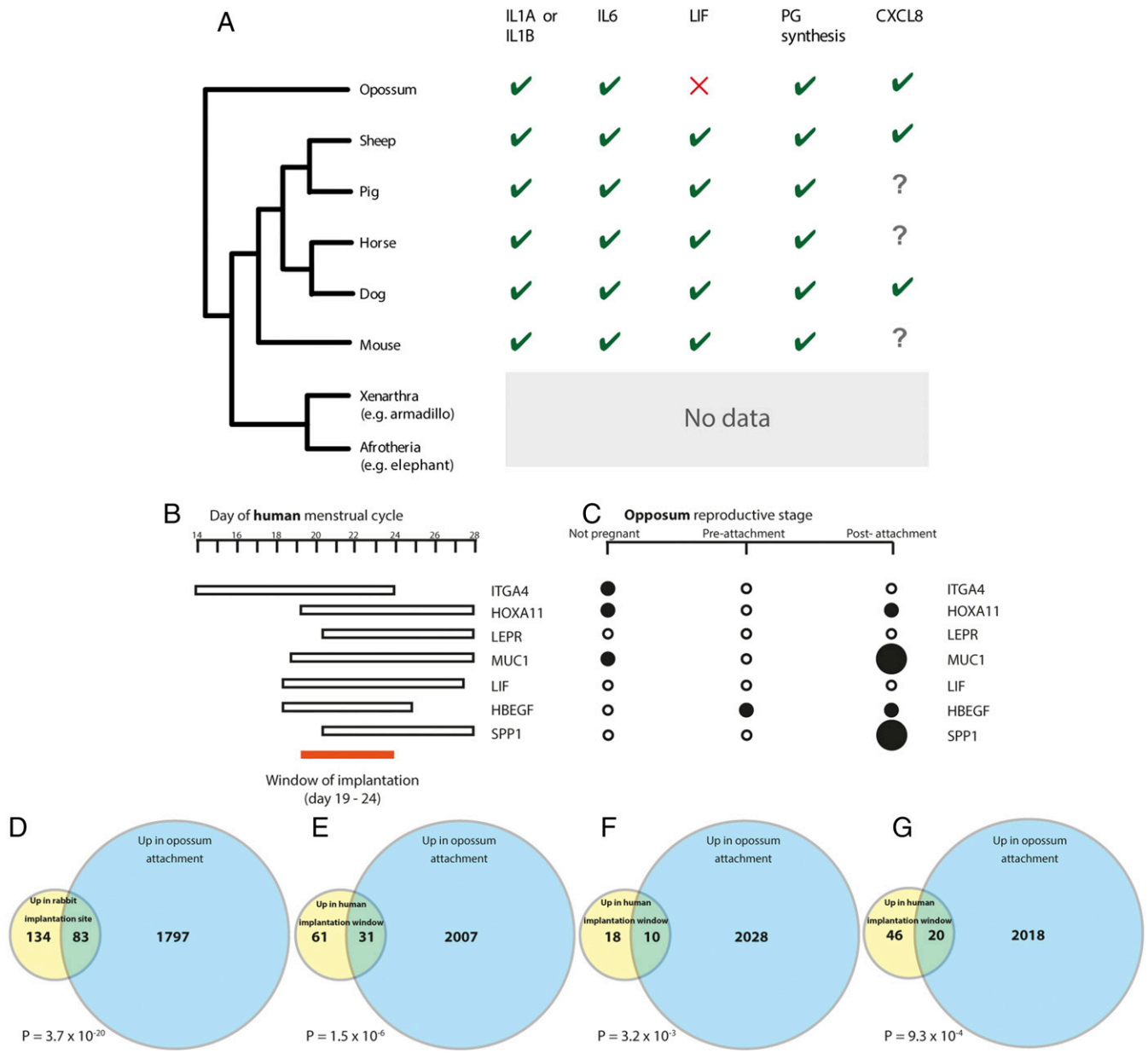

Fig. 7. Transcriptomic comparisons between the opossum attachment reaction and eutherian implantation. (*A*) Comparison between known inflammatory molecules produced during implantation in several eutherian lineages and their production during attachment in the opossum. PG, prostaglandin. References: sheep (79–81), pig (79, 82–86), horse (87–89), dog (90, 91), mouse (92–94). (*B* and *C*) The timing of implantation marker expression relative to the menstrual cycle in humans (*B*) and in opossum in days following copulation (*C*). (*B*) The periods of expression of key markers of implantation during the menstrual cycle are indicated by the horizontal bars (5). The window for successful implantation (orange bar) occurs when there is coexpression of each protein. (*C*) The expression of key implantation markers in different stages of the reproductive cycle in *M. domestica*. No expression (<3 TPM) is indicated by empty circles; solid circles indicate that expression was observed (>3 TPM); large circles indicate that expression was very high (>30 TPM). (*D*–*G*) Statistical tests of overlap between transcriptome-wide changes in gene expression during implantation in eutherians and during the opossum attachment reaction. (*D*) Overlap between genes enriched in the rabbit implantation site compared with the interimplantation site (41) and opossum attachment. (*E*–*G*) Overlap of genes more highly expressed in the human endometrium during the implantation window (45–47) and during opossum attachment. *P* values were calculated using the χ^2 test.

endometrium, including the production of PTGS2 and the decidualization of endometrial stromal cells in vivo (55, 56). Ectopic HBEGF is also sufficient to induce the expression of HBEGF in the endometrium, suggesting that it may positively regulate itself.

Implantation in eutherian mammals is an inflammatory process (11). During normal pregnancy, this inflammation is initiated by the trophoblast and involves the recruitment of natural killer cells to the site of implantation as well as the production of a range of proinflammatory cytokines including IL6 and TNF (57). Localized injury and endometrial biopsies before in vitro fertilization treatment result in greater implantation rates; these increased rates are suspected to be caused by an induction of the proinflammatory molecules necessary for implantation (58, 59). Term pregnancy in *M. domestica* is also inflammatory, with many of the same cytokines (including IL6, TNF, and IL19) being produced in the uterus. PGE_2 is a lipid that promotes inflammation locally and generally in vertebrates. PTGS2 is the rate-limiting enzyme in PGE_2 synthesis, and it is expressed only during inflammation in normal tissues. We localized the expression of PTGS2 to opossum luminal

epithelial cells at the placental interface, indicating that this interface is inducing a proinflammatory state (Fig. 6*I*). In mice, localized repression of PTGS2 results in multiple forms of reproductive failure, including implantation and decidualization failure of the uterus (60).

PTGES is another enzyme crucial for the production of PGE_2. Unlike PTGS2, PTGES is localized to trophoblast tissue (Fig. 6*L*). The expression of PTGS2 in the uterus and PTGES in the trophoblast suggests that the induction of placental inflammation is likely a signal indicating the presence of a fetus, because both the activation of the uterus providing PTGS2 and the trophoblast providing PTGES seem to be necessary to produce PGE_2. We would expect only inflammatory PGE_2 signaling when the primed uterus expressing PTGS2 is adhered to the trophoblast, which expresses PTGES, the final enzyme responsible for PGE_2 synthesis.

Endometrial inflammation is likely induced by a combination of two processes: tissue damage caused by the trophoblast, which may induce inflammation, and a concerted effort (by the mother and embryo), which may evolve to induce inflammation as a mechanism to induce parturition. In humans and mice, the trophoblast produces a range of serine proteases responsible for up-regulating inflammatory molecules such as PTGS2 (61). Similarly, the opossum trophoblast produces serine protease 8 (*SI Appendix*, Fig. S1) and cathepsins (62) associated with the degradation of the shell membrane. Serine protease 8 also is among the most induced proteases in the mouse and the human blastocyst (61). Following the breakdown of the shell, these proteases may damage the uterine tissue, inducing inflammation.

The immune consequences of pregnancy appear to be milder in viviparous reptiles, in which some specific interleukin genes appear to be down-regulated and no whole-scale up-regulation of inflammatory markers has been observed (19, 63, 64). This difference could be caused, in part, by the absence of an invasive trophoblast or other properties of the chorionic tissue unique to mammals and not associated with the evolution of viviparity (65, 66). The absence of a strong inflammatory and immune response in viviparous reptiles with a placenta suggests that derived properties of the therian trophoblast may induce the inflammatory response observed in *M. domestica*. In particular, the production of components of the PGE_2 synthesis pathway in maternal and embryonic tissue (Fig. 6) suggests that the inflammatory response seen at term is the result of a cooperative process between mother and embryo to induce parturition.

Evolution and Function of the Attachment Reaction During the Evolution of Therian Pregnancy. The reason for the marsupial reproductive mode—short gestation with very short placental attachment and extended postpartum development and growth—is a question fundamental to understanding marsupial evolution. The trophoblast of *M. domestica* invades between maternal uterine epithelial cells but does not breach the basal membrane (67). This tissue damage is likely to induce inflammation in maternal tissues, thus raising the question: "Why have trophoblast cells evolved to penetrate between uterine epithelial cells in marsupials at all?" Unlike eutherians, marsupials gain scant resources from the mother before birth, so invasion as a means to increase embryonic control over placental transport seems unlikely.

Alternatively, quasi-invasion may have evolved specifically to induce an inflammatory process to bring about parturition. Inflammation is an important component of the parturition process in eutherian mammals, and infection can cause preterm labor (68, 69). In wallabies, inflammatory prostaglandins are sufficient to induce parturition behavior, and blocking prostaglandin activity late in gestation results in the extension of pregnancy (70, 71). Our data suggest that in the opossum prostaglandin E_2 synthesis via the production of PTGS2 and PTGES is a consequence of the attachment reaction, and these processes may be linked to the induction of parturition.

Given the inflammatory nature of the attachment reaction in marsupials and eutherians alike, and presumably in the therian ancestor, the extension of pregnancy in eutherians must require a mechanism to suppress the immune system to prevent maternal rejection of the baby. Pregnancy in humans and mice is sustained by an extended antiinflammatory period between implantation and parturition (11), suggesting that the switch to an anti-inflammatory state following implantation was an innovation of fundamental importance to the extension of pregnancy in eutherians (Fig. 8). This hypothesis can be tested by identifying whether the more basal eutherian clades Afrotheria and Xenarthra also have mechanisms to control the inflammatory processes induced during implantation (72, 73).

Given that eutherian parturition, like implantation, is an inflammatory process (11, 68), and the attachment reaction in the opossum is inflammatory, an interesting hypothesis is that both implantation and parturition are proinflammatory reactions because they are both derived forms of the therian attachment reaction (Fig. 8). Therefore, only the noninflammatory phase of pregnancy is unique to eutherian mammals. Alternatively, the inflammation occurring at parturition in eutherians might be an independently derived strategy unique to this group (Fig. 8). To disentangle these hypotheses, future research should test whether (*i*) inflammation facilitates parturition in the opossum, (*ii*) the inflammation during eutherian parturition, like implantation, shows an overlap at the transcriptome level with the marsupial

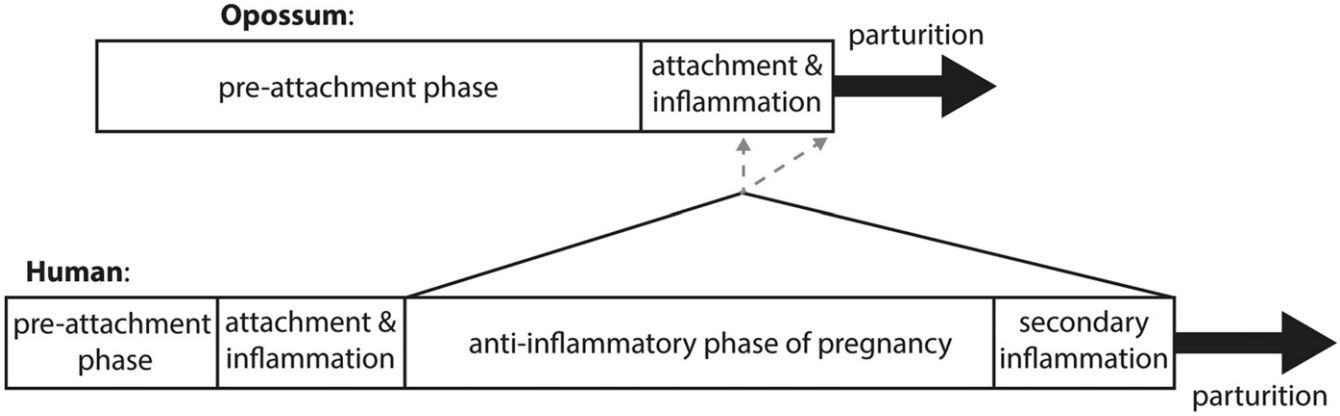

Fig. 8. Comparison between the key stages of pregnancy in the opossum and human. The eutherian reproductive condition has evolved by inserting an anti-inflammatory period of gestation between the inflammatory attachment reaction and the inflammatory parturition reaction. Dashed arrows represent the two hypotheses that could explain how secondary inflammation arose in eutherians: 1) eutherian pregnancy can be understood either through the insertion of an antiinflammatory phase of pregnancy into what was one inflammatory process in the ancestral therian; or 2) by the addition of both an antiinflammatory phase and an independently derived secondary inflammation at parturition.

attachment reaction, and (*iii*) the type of inflammation at implantation and parturition have conserved elements.

A caveat of our study is that it assumes that the reproductive mode observed in *Monodelphis* is representative of the stem therian mammal. Although it can be argued that the broad reproductive pattern of the opossum likely reflects the ancestral therian mammal, it is not possible to test this hypothesis using ancestral state reconstructions because of the topology of the mammalian phylogeny, i.e., there is only one viviparous mammalian clade outside the eutherians. Furthermore, fossils that could identify whether the marsupial or eutherian reproductive strategy more closely reflects that of the ancestral therian have not been found. Therefore, an alternative explanation of our findings can be proposed if we assume that eutherians more closely represent the ancestral live-bearing mammal's reproductive strategy. Under this model, the gene-expression changes observed in the uterus at term in the opossum are homologous to that of eutherian implantation because marsupial pregnancy can be viewed as an early termination of pregnancy at implantation. Although we do not think this scenario is likely, it is important to state it as an alternative interpretation.

Conclusions

Our data support the hypothesis that the molecular processes that occur at the maternal–fetal interface during embryo attachment in the opossum are homologous to those that occur at implantation in human and rodent pregnancies. This hypothesis suggests that the inflammatory nature of implantation in eutherians is an evolutionary heritage from the ancestral condition for live-bearing mammals. We propose that the inflammatory processes induced by the attachment reaction are likely to be a barrier to the extension of pregnancy in marsupials, possibly explaining the limited diversity in gestation length and stage of development at birth in this group. We further suggest that a modification of these inflammatory processes was a key innovation in the evolution of eutherian reproduction and is worthy of further investigation.

Methods

Animal Husbandry and Tissue Collection. All animal procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee of Yale University. Opossum uterine tissue was collected from a *M. domestica* colony housed at Yale University. Tissue from specific stages of the reproductive cycle were collected by following a standard breeding protocol outlined in Kin et al. (30). Once collected, tissue was stored for RNA analysis, histology, and Western blot analysis.

RNA-Seq and Analysis. For RNA-seq analysis we examined uterine tissue from nonpregnant females ($n = 3$) and from females at the preattachment (7 d post copulation, $n = 3$) and postattachment (13.5–4 d post copulation, $n = 3$) stage. Illumina sequencing libraries were generated by Yale Centre for Genome Analysis, using strand-specific, Poly-A–selected, in-house library preparation methods. Sequencing of libraries was performed on the Illumina HiSeq 2500 System.

Gene expression was quantified by aligning raw sequencing reads to the *M. domestica* genome (release 79) with Tophat2 (74), and gene counts were calculated with HTSeq (75). We performed hierarchical clustering and principal components analysis in the R *stats* package to confirm that treatment groups broadly had distinct gene-expression profiles. We compared differential gene expression between nonpregnant and preattachment females as well as between preattachment and postattachment females using DESeq2 (76).

GO analysis was performed in GOrilla (77); the list of all genes in *M. domestica* was used as a background for analyses. GO analysis was visualized with ReviGO (78).

qPCR. To test the association between the expression of key genes and the attachment reaction, we performed real-time qPCR for IL6, TNF, PTGS2, MUC1, HBEGF, and PTGES over a finer time scale than was used in transcriptome analysis. RNA was extracted and reverse transcribed; then qPCR reactions were performed for each sample and gene. The expression of each gene was normalized using the internal reference gene TBP.

Immunostaining. We localized the expression of PTGS2, HBEGF, MUC1, and PTGES using immunohistochemistry. Fixed tissues were embedded in paraffin, sectioned, deparaffinized, cleared, and then incubated with a primary antibody specific to the protein of interest. Sections then were washed and stained with a secondary antibody, and staining was visualized with 3,3′-diaminobenzidine-tetrahydrochloride. The specificity of each primary antibody was confirmed with Western blot analysis.

ACKNOWLEDGMENTS. This research was funded by a Gaylord Donnelley Postdoctoral Environmental Fellowship (to O.W.G.) and John Templeton Foundation Grant 54860 (to G.P.W.), and, in part, by the Perinatology Research Branch, Division of Obstetrics and Maternal-Fetal Medicine, Division of Intramural Research, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, US Department of Health and Human Services (NICHD/NIH/DHHS); and, in part, with Federal funds from NICHD/NIH/DHHS under Contract HHSN275201300006C.

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