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Research Article

Uterine responses to early pre-attachment embryos in the domestic dog and comparisons with other domestic animal species†

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Abstract

In the dog, there is no luteolysis in the absence of pregnancy. Thus, this species lacks any anti-luteolytic endocrine signal as found in other species that modulate uterine function during the critical period of pregnancy establishment. Nevertheless, in the dog an embryo-maternal communication must occur in order to prevent rejection of embryos. Based on this hypothesis, we performed microarray analysis of canine uterine samples collected during pre-attachment phase (days 10-12) and in corresponding non-pregnant controls, in order to elucidate the embryo attachment signal. An additional goal was to identify differences in uterine responses to pre-attachment embryos between dogs and other mammalian species exhibiting different reproductive patterns with regard to luteolysis, implantation, and preparation for placentation. Therefore, the canine microarray data were compared with gene sets from pigs, cattle, horses, and humans. We found 412 genes differentially regulated between the two experimental groups. The functional terms most strongly enriched in response to pre-attachment embryos related to extracellular matrix function and remodeling, and to immune and inflammatory responses. Several candidate genes were validated by semi-quantitative PCR. When compared with other species, best matches were found with human and equine counterparts. Especially for the pig, the majority of overlapping genes showed opposite expression patterns. Interestingly, 1926 genes did not pair with any of the other gene sets. Using a microarray approach, we report the uterine changes in the dog driven by the presence of embryos and compare these results with datasets from other mammalian species, finding common-, contrary-, and exclusively canine-regulated genes.

Summary Sentence

Pre-implantation embryos invoke functional changes in the canine uterus related to ongoing structural remodeling and immunological modulation; comparisons with different mammals reveal similarities and differences in maternal pregnancy recognition.

Key words: dog (*Canis lupus familiaris*), embryo-maternal communication, early pregnancy.

Introduction

The foundation of every successful pregnancy is the embryo-maternal cross-talk synchronizing blastocyst development and receptivity of the uterus in preparation for implantation. One of the master regulators in the underlying endocrine and molecular regulatory mechanisms is luteal progesterone (P4). It acts by inducing expression of multiple factors facilitating implantation, e.g., those involved in the secretion of endometrial histiotroph or exerting immunomodulatory effects. In most species, besides P4, estrogens are also needed for orchestrated expression of uterine growth factors, cytokines, and prostaglandins (PG) that work locally in an autocrine or paracrine manner [1]. In addition to embryotrophic effects, uterine fluids also contain signaling molecules of embryonic origin (reviewed in [2–4]). These embryo-derived factors prevent luteolysis and maintain the luteal lifespan beyond recurrent cyclic activity, which is a mechanism referred to as "maternal recognition of pregnancy" [5]. Thus, the bidirectional embryo-maternal cross-talk starts before the embryonic tissue becomes intimately attached to the uterine epithelium. Among species, this early pregnancy signal recognizing the presence of pre-attachment embryos varies, including the two best-known endocrine mechanisms comprised of estrogens produced by porcine trophoblast [6] or interferon tau (IFN tau) produced by early conceptuses in ruminants [7, 8]. Also, the duration of the pre-implantation period varies among the domestic animal species: e.g., the elongating conceptus adheres to the uterine epithelial surface and placentation starts around day 19 of gestation in ewes [9, 10], or around day 22 in cattle [11], compared with the implantation in mares that is delayed until around day 37 of gestation [12].

Interspecies differences in regulatory mechanisms involved in the onset and maintenance of pregnancy become even more apparent when the reproductive physiology of the domestic dog (*Canis lupus familiaris*) is taken into consideration. Thus, for example, the classical definition of maternal recognition of pregnancy does not apply to the dog, because in the absence of pregnancy in this species there is no luteolytic principle [13–16]. The dog is also the only domestic animal species devoid of placental steroids and, therefore, fully dependent on luteal P4 for successful pregnancy [17]. In this context, it is noteworthy that the canine uterus is physiologically exposed to relatively high amounts of circulating P4, starting with at least 5 ng/ml at the time of ovulation and reaching average levels of 30–35 ng/ml within the first 15-30 days of pregnancy [17]. Consequently, P4 is considered to be the predominant luteal steroid needed for the establishment of pregnancy in previously estrogenized dogs [17, 18]. Furthermore, in addition to the similar estradiol-17 beta (E2) profiles in pregnant and non-pregnant bitches [19, 20], and in contrast to livestock [21–23], there is no pregnancy- and/or parturition-associated increase in E2 observed in the dog [19].

It is obvious that, even in the absence of an anti-luteolytic principle, the canine embryo must be recognized, otherwise it would be rejected. Apparently, therefore, there must exist different regulatory mechanisms responsible for embryo maternal communication in the dog, or at least mechanisms that are not directed toward the suppression of luteolysis. Based on this assumption, recently, embryo-related changes in the canine uterus were described during the early embryonal free-floating phase (days 10-12 of gestation) [24]. First, clearly distinguishable morphological changes pointing toward the onset of pre-invasive uterine decidualization were, however, associated with the attachment of the embryo to the uterine surface (around day 17 of pregnancy) [25]. Thus, biochemical and functional changes appear to be detectable earlier than morphological ones [25]. The latter, i.e., biochemical responses of the pre-implantation canine uterus to the presence of embryos, are mirrored in the modulated expression of some genes related to the processes of implantation and placentation, e.g., *IGF2*, *PGR*, *ER alpha/ESR1*, and *PRLR* [24]. Among the members of the PG family system previously implicated in early canine embryo-maternal communication [26], uterine expression of *PGT*, *PTGES*, *PGFS/AKR1C3*, and their respective receptors, *FP (PTGFR)* and *PTGER2 (EP2)* was increased in the presence of free-floating embryos [24]. Following implantation, further considerable differences become obvious between the dog and other domestic animal species regarding invasive vs. non-invasive growth of the trophoblast, leading to the formation of the canine endotheliochorial placenta. This process is associated with strong morpho-functional remodeling of the uterine stromal cells, resulting in the development of species-specific decidual cells. The functional importance of these cells is underlined by the fact that they are the only cells of the canine placenta exhibiting the PR [27, 28].

Cumulatively, especially taking into account the species-specific lack of luteolysis, the decidualization and the invasive placentation type, the domestic dog appears to be an interesting model for investigating comparative aspects relating to different strategies of maternal recognition of pregnancy that have evolved in mammals. Therefore, here we used a transcriptomic approach by microarray analysis in order to obtain deeper insight into the possible underlying regulatory mechanisms involved in the cross-talk between the early pregnant canine uterus and pre-implantation embryos (days 10-12).

Materials and methods

Sample collection

For the present study, fourteen (n = 14) healthy, cross-bred bitches aged 2-8 years were used. Hormonal status was monitored by regular measurements of progesterone (P4) concentrations every 2-3 days starting with the proestrous bleeding and cytological evaluation of vaginal smears. The day of ovulation was determined by detecting circulating P4 levels of >5 ng/ml, and by vaginal cytology. Estimating the time needed for maturation of canine oocytes within the oviduct (on average 2-3 days after ovulation), bitches were mated/inseminated 2 days after ovulation (defined as day 0 of gestation). Uterine samples were collected through routine ovariohysterectomy at early pregnancy before the free-floating embryos became intimately attached to the uterine surface (pre-implantation group, days 10-12 of pregnancy). In eight (n = 8) bitches, early pregnancy was confirmed by recovering embryos by uterine flushing on days 10-12 of gestation (early pregnant group). Uterine samples from the remaining six (n = 6) dogs were used as negative controls (non-pregnant group). All experimental procedures were carried out in accordance with animal welfare legislation (permit no. 2008-25-124 from the Faculty of Veterinary Medicine, University of Ankara). Following surgery, uterine tissue samples (including all anatomical layers) were trimmed of surrounding connective tissues and immersed for 24 h at + 4°C in RNAlater (Ambion Biotechnologie GmbH, Wiesbaden, Germany), an aqueous reagent for stabilization of cellular RNA; prolonged storage was at -80°C. Subsequently, all uterine tissue samples from the two experimental groups (early-*pregnant and non-pregnant*) were used for semi-quantitative real-time PCR.

Four (n = 4) randomly chosen samples from each group were used for microarray analysis.

Microarray hybridization and data analysis

Total RNA was isolated from eight randomly chosen uterine samples (n = 4 per group, as indicated above). First, TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate RNA that was further purified using the PureLinkTM RNA Mini Kit (Cat. 12183018A) (Ambion Life Technologies, Reinach, Switzerland). Both applications were carried out according to the manufacturers' instructions. After RNA extraction, DNase treatment was performed following the supplier's protocol using RQ1 RNAse-free DNAse (Promega, Dübendorf, Switzerland). The purity and quantity of the RNA were determined with a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific AG, Reinach, Switzerland). The quality of total RNA was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) ensuring homogeneous distribution of high RIN (RNA integrity numbers) between samples. Microarray analysis was performed using a custom Agilent 8 × 60 k microarray. The design was based on the Agilent catalog array AMADID G2519F-021193 Canine Gene Expression Microarray, canine (Cfa) RefSeq transcripts from NCBI November 2012 and Ensembl 69 transcripts. In total, the chip covered 20′293 canine genes. Cy3-labeled cRNA was produced with the Low-Input Quick Amp Labeling Kit, one color (Agilent Technologies) according to the manufacturer's instructions. After hybridization and washing, the slides were measured using an Agilent DNA Microarray Scanner (G2505C; Agilent Technologies) at a resolution of 2 μm. The Feature Extraction Software 10.7.3.1 (Agilent Technologies) was used for image processing. Signals were filtered based on "well above background" flags (detection in three of four samples), and normalized with the Bioconductor package VSN [29]. Distance matrix and heatmap based on pairwise distances (Bioconductor package geneplotter) were used for quality control of the data. Analysis of significances was performed with the Bioconductor package Limma [30]. The "FDR"-method (False Discovery Rate; FDR 10%, i.e., adjusted P-value < 0.1) was applied for the correction of multiple testing. The P-value was adjusted to $P < 0.01$. Differentially expressed genes (DEG) were identified in the contrast (i.e., pairwise comparison) "pregnant samples" vs. "non-pregnant samples." Quantitatively enriched functional categories were identified using the "functional annotation clustering" tool of Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.8; http://david.abcc.ncifcrf.gov/) [31]. DAVID analysis was performed using information from the following databases: Gene Ontology (GO), biological process (BP), cellular component (CC), and molecular function (MF), single protein of protein information resource (SP_PIR), protein domains or sites (INTERPRO), and pathways extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Cytoscape analysis was prepared using the ClueGO plug-in in Cytoscape 3.0 [32] to visualize enriched functional networks. Ingenuity Pathway Analysis software (IPA, build version 366632M, content version 26127183, QIAGEN, Redwood City, USA) was further used to identify and visualize canonical pathways and main functional terms within the dataset.

Additionally, the whole microarray-derived canine gene set (microarray signals filtered on "well above background" flags, detection in at least three of four samples) was ranked according to a score based on log2 fold change and P-value ((2+log2(fold change))* -log10(P-value)) and used for gene set enrichment analysis (GSEA) with gene sets differentially expressed in other species (either up- or down-regulated). For each comparison, the gene set for dogs was compared separately with available lists of DEG in other species (cattle, pig, or horse). The following datasets were used: pregnant bovine endometrium day 15 [33], pregnant pig endometrium day 12 [34], and pregnant horse endometrium day 16 [35]. GSEA was carried out by the use of the GSEA web start tool [36]. The core-enriched genes (overlapping between canine DEGs and genes expressed in other species) were further analyzed by DAVID (6.8) to extract GO information.

Next, a multiple comparison of all datasets was performed and visualized as Venn diagrams using Venny 2.1 software [37] to identify an intersection between commonly up-regulated or down-regulated genes in the early pregnant uterus of cow, pig or horse, using the canine gene set as a background. The canine background gene set was based on the top 2000 genes with positive scores and the top 700 with negative scores of the ranked list used for GSEA analysis. To enlarge the number of overlapping genes, the dataset for the bovine species at day 15 of pregnancy was combined with that detected at day 16 of bovine pregnancy [33], and the horse gene set from day 16 of pregnancy was combined with a gene list of uterine genes detected at day 12 of equine pregnancy. The gene set of pig was not modified and was the same as for GSEA (i.e., pig endometrium day 12 [34]). Additionally, a gene list comprising a dataset generated using human endometrium during the window of implantation (WOI) [38] was included. Genes expressed in the dog and at least one another species were considered. The proportional overlap between genes expressed in dog and other species was calculated. The total numbers of genes included in this analysis were as follows: dog 2700 (2000up/700down), human (WOI) 2300 genes (982up/1318down), bovine (days 15 and 16 of pregnancy) 864 genes (586up/278down), pig (day 12 of pregnancy) 1659 genes (898up/761down), and horse (days 12 and 16 of pregnancy) 1928 genes (1013up/915down).

To identify gene clusters exclusively regulated in the dog, those canine genes that did not match in the aforementioned cumulative comparison with any of the other species (bovine, horse, pig, human) were used for DAVID analysis.

RNA isolation, reverse transcription, semi-quantitative (TaqMan) PCR and evaluation of data

Total RNA samples isolated from all uterine samples (n = 8 for early pregnant uteri and n = 6 non-pregnant controls) were used in semi-quantitative TaqMan RT-PCR in order to validate the expression of selected candidate genes detected by microarray analysis. RQ1 RNAse-free DNAse treatment (Promega) was applied following RNA isolation using TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer´s instructions. Complementary DNA (cDNA) corresponding to 200 ng RNA per sample and run was used for detection of each target gene. The reverse transcription (RT) reaction was performed with reagents from Applied Biosystems, Foster City, CA, USA. Detailed protocols were previously published [14, 39, 40]. TaqMan PCR reactions were run in duplicates with the Fast Start Universal Probe Master (ROX) (Roche Diagnostics AG, Rotkreutz, Switzerland). The negative controls consisted of autoclaved water instead of cDNA and the so-called RT-minus control [14, 40]. All analyses were performed in an automated fluorometer (ABI PRISM 7500 Sequence Detection System, Applied Biosystems). Customized canine-specific primers and 6-FAM- and TAMRA-labeled (TaqMan) probes were purchased from Microsynth, Balgach, Switzerland, and are listed in Table 1. The

Table 1. List of primers used for real-time (TaqMan) PCR.

Primer	Accession number	Primer Sequence		Product length (bp)
allograft inflammatory factor 1 (AIF1)	XM_532072.5	Forward	5′-CGA ATG CTG GAG AAA CTT GGT-3′	107
		Reverse	5′-TGA GAA AGT CAG AGT AGC TGA AGG T-3′	
		TaqMan Probe	5′-TCC CCA AGA CCC ATC TGG AGC TCA A-3′	
chemokine ligand 12 (CXCL12)	NM_001128097.1	Forward	5′-AGC CAA CGT CAA GCA TCT CA-3′	90
		Reverse	5′-CAC ACC TGT CTG CTG TTG TTC TTC-3′	
		TaqMan Probe	5′-AAC TGT GCC CTT CAG ATC GTG GCA A-3′	
chemokine ligand 16 (CXCL16)	XM_014113226.1	Forward	5′-CAG CGT CAC TGG CAG TTG TTA C-3′	105
		Reverse	5′-CGC TGA TAG ACT CGC AGA TGT T-3′	
		TaqMan Probe	5′-CCG CGG AGC TCA TGG CTC ATC-3′	
chemokine receptor 6 (CXCR6)	XM_846798.3	Forward	5′-GCT GAA GAG CCT GAC AGA TGT G-3′	91
		Reverse	5′-GCT GGC ATA GGC CCA AAA G-3′	
		TaqMan Probe	5′-CTG ATG AAC CTG CCC CTA GCT GAC CTG-3′	
chemokine receptor 7 (CXCR7)	XM_005635904.2	Forward	5′-CGG CAT GAT CGC CAA CTC-3′	94
		Reverse	5′-GAT GGC CAG GTT GAG GAT GT-3′	
		TaqMan Probe	5′-CCA AGA CCA CCG GCT ACG ACA CG-3′	
laminin alpha 2 (LAMA2)	XM_014113700.1	Forward	5′-AAA CCG GCT CAC GAT TGA G-3′	99
		Reverse	5′-AGT TGA ACG GTG GCG AAG T-3′	
		TaqMan Probe	5′-CCT GCT CTT CTA CAT GGC TCG GAT CAA-3′	
liver X receptor (LXR)	XM_014120969.1	Forward	5′-GGC CCT GCA TGC CTA CGT-3′	67
		Reverse	5′-CAT TAG CAT CCG TGG GAA CAT C-3′	
		TaqMan Probe	5′-TCC ACC ACC CCC ACG ACC GA-3′	
papilin proteoglycan-like sulfated glycoprotein (PAPLN)	XM_005623922.1	Forward	5′-GCT GAT GGG CAT CGT GTT C-3′	103
		Reverse	5′-TTC CAC GGT AGG CAC TAC ATG T-3′	
		TaqMan Probe	5′-CCA TAA CCT GCG GGC CGG AGA C-3′	
pappalysin2 (PAPPA2)	XM_537179.5	Forward	5′-ACG GGA TTG GTG CAG TGT GT-3′	90
		Reverse	5′-CCA GAG TGT CAG CAG TGA TGT TC-3′	
		TaqMan Probe	5′-ATC GTG TGT AAT TCC CCC TAG CGATCC TG-3′	
phospholipase A2 (PLA2G4A)	XM_005622454.2	Forward	5′-AGA GAA AGG GCC AGA GGA GAT T-3′	139
		Reverse	5′-GGT GAC AGG TTG TCC AGA GCT T-3′	
		TaqMan Probe	5′-CTA CAA CCC CCT TTT GCT TCT CAC ACC A-3′	
prostaglandin D2 receptor (PTGDR)	XM_848401.3	Forward	5′-CGC CTT CTG CCT GGT TTT-3′	101
		Reverse	5′-CCT CGT GCA TCA TCT GGA T-3′	
		TaqMan Probe	5′-CGC TGC CCT TCG CGG GCT-3′	
tissue inhibitor of matrix metalloproteinase-2 (TIMP2)	AF188489.1	Forward	5′ -CCT GGA CAT CGG AGG AAA GA-3′	99
		Reverse	5′ -TCC CAG GGC ACG ATG AAG T-3′	
		TaqMan Probe	5′ -CGG CAA GAT GCA CAT CAC CCT TTG T-3′	
indolamin 2,3-dioxygenase 1 (IDO1)	XM_532793.5	Forward	5′-TGA TGG CCT TAG TGG ACA CAA G-3′	116
		Reverse	5′-TCT GTG GCA AGA CCT TTC GA-3′	
		TaqMan Probe	5′-CAG CGC CTT GCA CGT CTG GC-3′	
GAPDH	AB028142	Forward:	5′-GCT GCC AAA TAT GAC GAC ATC A-3′	75
		Reverse:	5′-GTA GCC CAG GAT GCC TTT GAG-3′	
		TaqMan Probe	5′-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3′	
18S rRNA	FJ797658	Forward	5′ -GTC GCT CGC TCC TCT CCT ACT-3′	125
		Reverse	5′ -GGC TGA CCG GGT TGG TTT-3′	
		TaqMan Probe	5′ -ACA TGC CGA CGG GCG CTG AC-3′	

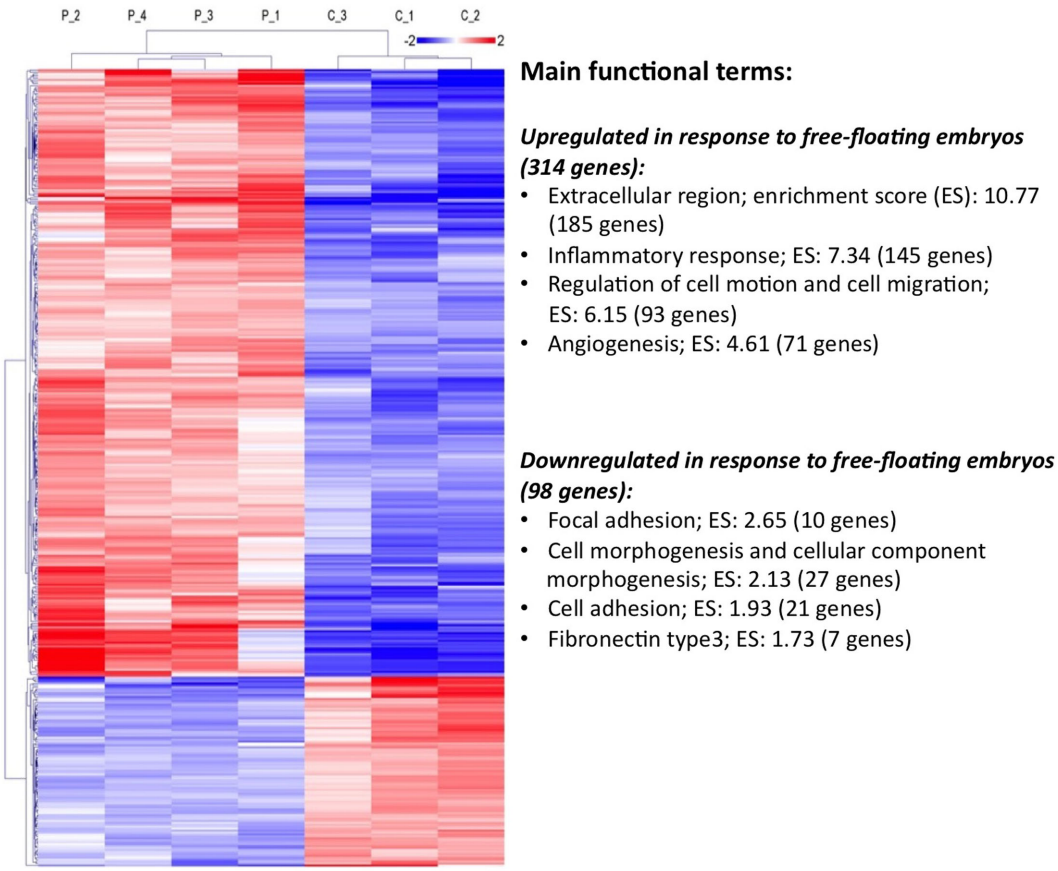


Figure 1. Heatmap showing the microarray analysis of DEG in early pregnant (P1-4) canine uterus at days 10-12, and corresponding non-pregnant controls (C1-3). In total, 412 genes (FDR: 10%) were differentially expressed between the two groups. A total of 314 genes were up-regulated and 98 down-regulated. The main functional terms determined by DAVID analysis are presented for the identification of those functional terms affected by the presence of free-floating embryos in the canine pre-implantation uterus. The full list of DEG (FDR: 10%) is provided in Supplemental File 2.

efficiency of self-designed expression assays was validated as previously described [40] ensuring approximately 100%. Three reference genes were used for normalization of data (*GAPDH*, Cyclophilin A (*PPIA*), and *18SrRNA*). The canine-specific TaqMan Gene Expression Assay for PPIA was ordered from Applied Biosystems (Prod. No. Cf03986523-gH). For relative quantification, the comparative CT method (ΔΔCT method) was applied as previously described [28, 40]. An unpaired, two-tailed Student *t*-test was performed with GraphPad 3.06 (GraphPad Software, San Diego, CA, USA). A P-value < 0.05 was considered as statistically significant.

RESULTS

Analysis of microarray data

The canine uterine response toward the presence of free-floating embryos (days 10-12 of pregnancy) was characterized using a customized Agilent microarray assay. DEG between the two experimental groups (pregnant vs. non-pregnant group) were evaluated.

The number of detected probes passing the "well above background flags" filter was 44 253. These probes were summed up on the gene annotation level, resulting in a total of 15′668 genes (provided as Supplemental File 1). These genes, ranked according to their expression level, were later used for GSEA analysis. Applying FDR of 10% (i.e., adjusted P-value < 0.1), 412 DEG between the two groups were found (Supplemental File 2). A total of 314 genes showed higher and 98 showed lower expression in the early pregnant group compared to non-pregnant uteri. The expression patterns of DEG in the two experimental groups, together with associated main functional terms, are visualized using heatmaps (Figure 1). In one of the dogs allotted to the non-pregnant group, the expression pattern of DEG exactly matched that of early pregnant dogs, suggesting that one of the samples was not properly allotted; most probably, the embryo/embryos were missed during the flushing process. This sample was excluded from further analysis. The three highest fold changes (FC) in the pairwise comparison "pregnant vs. control" were for the following genes: *IPO9/IMP9* (7.7-fold), *ITIH4/GP120/PK120* (7.6-fold), and *NOV/CCN3/IGFBP9* (7.5-fold), while the three lowest expressed genes, i.e., suppressed in early pregnant uterus, were: marker of proliferation *Ki-67 (MKI67)* (-6.4-fold), *PAPPA2* (-5.2-fold), and *DIAPH3* (-4.5-fold).

Functional annotations and networks analysis

Functional terms enriched for DEG from both experimental groups were identified by DAVID analysis. The main functional terms (Figure 1) showed over-representation of genes in the gene set as represented by the enrichment score (ES); total numbers of genes in each cluster are indicated. An increased uterine feedback in response to free-floating embryos was noted for functional networks associated with extracellular region, inflammatory response, cell motion,

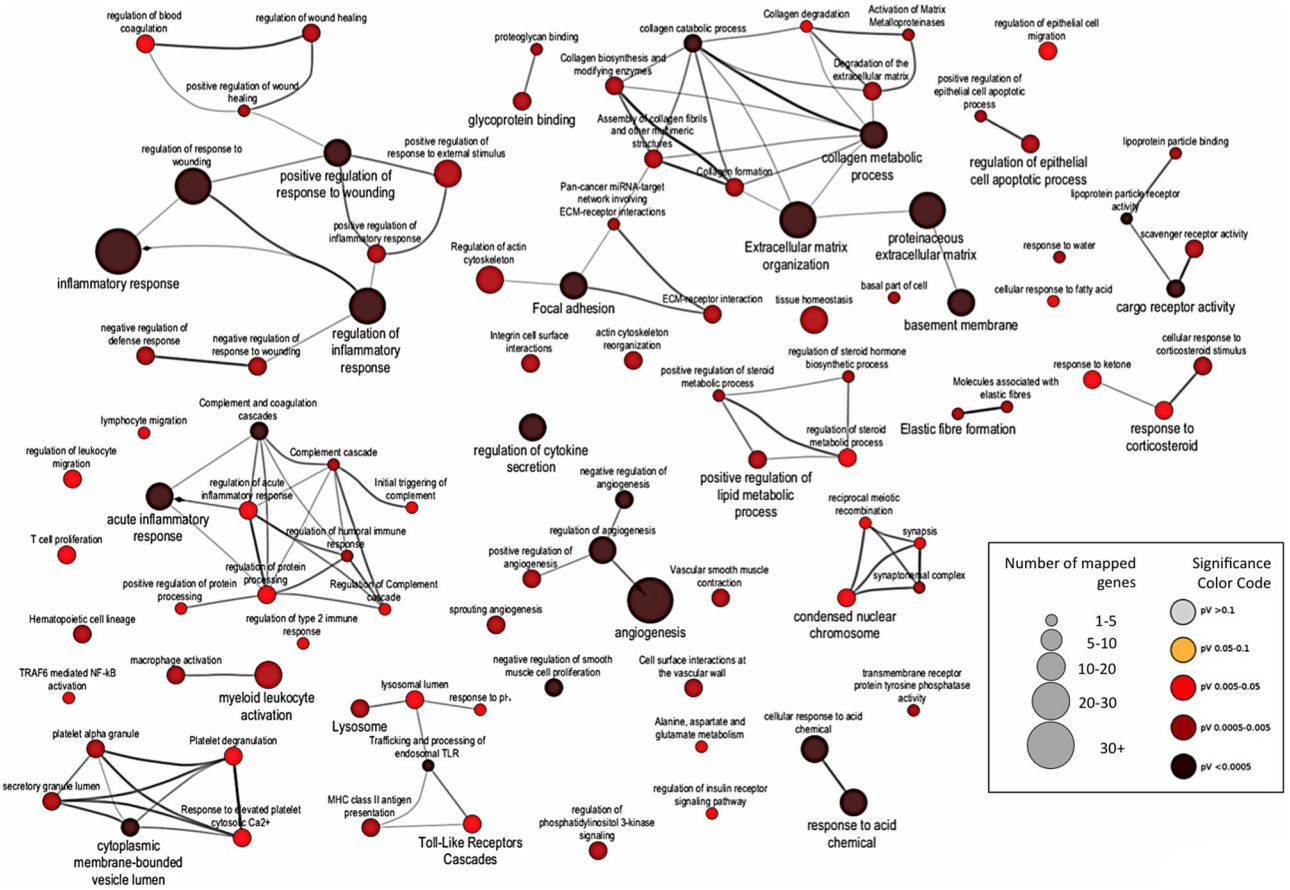


Figure 2. Cytoscape analysis of functional networks over-represented in canine uterus exposed to pre-implantation free-floating embryos. As input, DEG (FDR: 10%) were used (see Supplemental File 2). The redundant and noninformative terms were removed, and the resulting network was manually rearranged. For each network, the size of the node implies the number of genes, while the color intensity denotes the level of enrichment (see legend to illustration). Functional networks more highly represented in early pregnant canine uterus refer predominantly to ECM, immune response, and angiogenesis.

and migration and angiogenesis. The cluster "extracellular region" (ES 10.77) consisted of the following categories: glycoprotein, signal peptide, extracellular space, extracellular vesicle and secreted. Representative genes in this cluster were as follows: *ADAMTS1*, *BMP6*, *COL16A1*, *COL18A1*, *COL5A2*, *COL6A2*, *CXCL16*, *ECM1*, *IGF2*, *LAMA2*, *MMP2*, *MMP23B*, and *NOV*. The cluster "inflammatory response" (ES 7.34) comprised the following categories: defense response, response to stress, immune response, innate immune response, and positive regulation of immune system process. Representative genes were *AIF1*, *CSF1*, *CSF1R*, *ECM1*, *IDO1*, *IL15*, *IL16*, *IL1R1*, *LYZ*, *MMP2*, *NOV*, *PLA2G4A*, and *SERPINF1*. The cluster "regulation of cell motion and migration" (ES 6.15) consisted of the following categories: regulation of cellular component movement, regulation of locomotion, cell motility. Representative genes were as follows: *AIF1*, *BMP6*, *CCL15*, *CCL18*, *CSF1*, *CSF1R*, *CTSK*, *CXCL16*, *IL16*, *IL1R1*, *IL33*, and *SERPINF1*. The cluster "angiogenesis" (ES 4.61) comprised the following categories: regulation of vasculature development, regulation of angiogenesis, vasculature development, and blood vessel morphogenesis. Representative genes included *ADAMTS1*, *BMP6*, *COL18A1*, *COL5A2*, *ECM1*, *LAMA2*, *MMP2*, and *NFKB2*. The 98 down-regulated genes showed higher variability with lower ES (Figure 1). They could be connected to functional terms associated with focal adhesion, cell morphogenesis and cellular component morphogenesis, cell adhesion and fibronectin type 3. The term "focal adhesion" (ES 2.65) included categories related to regulation of actin cytoskeleton and platelet activation. Representative genes were *COL6A3*, *CRK*, *DIAPH3*, *FN1*, *ITGB1*, *MYLK*, *PARVA*, *PPP1CB*, *RRAS2*, and *TNC*. The cluster "cell morphogenesis and cellular component morphogenesis" (ES 2.13) consisted of categories: cell morphogenesis, cellular component morphogenesis, regulation of hydrolase activity, enzyme regulator activity, regulation of GTPase activity and regulation of catalytic activity. Representative genes were *ACVR1*, *CAB39*, *CDH2*, *CRK*, *FN1*, *HSPH1*, *ITGB1*, *NCAM1*, *PARVA*, and *ROBO1*. The cluster "cell adhesion" (ES 1.93) included the following categories: focal adhesion, cell-substrate adherens junction, anchoring junction, cell-cell adherens junction, actin binding, cadherin binding. Representative genes were, e.g., *ACVR1*, *CDH2*, *DIAPH3*, *FN1*, *HSPH1*, *ITGB1*, *LAYN*, *LIMS1*, *LRP8*, and *MYLK*. The cluster "fibronectin type 3" (ES 1.73) included genes such as *NCAM1*, *ROBO1*, *TNC*, *COL6A3*, *PAPPA2*, *MYLK*, and *FN1*.

For visualization of interactions between functional networks and their integration with DEG, Cytoscape analysis was performed (Figure 2). The main over-represented networks referred to extracellular matrix (ECM) (centered around matrix organization, focal adhesion, and basement membrane), immune response (centered around positive regulation of response to wounding, regulation of inflammatory response, and inflammatory response), and angiogenesis.

Using IPA on 412 DEG, the three highest over-represented canonical pathways were associated with acute phase response signaling ($P = 3.12E-07$; representative genes were: *C1R*, *HPX*, *IKBKB*, *IL33*, *IL1R1*, *NR3C1*, *C1S*, *RRAS2*, *FN1*, *NFKB2*, *C4A/C4B*, *ITIH4*, *TF*, *KLKB1*, and *SERPINF1*), liver X receptor *LXR* activation ($P = 1.44E-05$; representative genes: *ARG2*, *HPX*, *IL33*, *IL1R1*, *NR1H3*, *NFKB2*, *C4A/C4B*, *LYZ*, *ITIH4*, *TF*, and *SERPINF1*), and complement system ($P = 4.46E-05$, representative genes: *C1R*, *C1S*, *CD59*, *C4A/C4B*, *CFH*, and *ITGAM*) (Figure 3A).

The main functional terms derived from IPA analysis showed similar results, compared to DAVID analysis, with highly involved functional terms for cellular movement and immune cell trafficking (Figure 3B).

Expression of selected target genes by semi-quantitative RT-PCR

The expression of 13 selected target genes predicted to be differentially expressed was assessed by semi-quantitative (TaqMan) PCR. These were chosen related to the strongest over-represented functional networks present in the early pregnant canine uterus (i.e., ECM and immune modulation networks). Thus, increased expression of the following immunomodulatory genes was found in the early pregnant canine uterus in response to free-floating embryos: *AIF1* ($P = 0.002$), *CXCR6* ($P = 0.02$), *PTGDR* ($P = 0.001$), *IDO1* ($P = 0.02$), *CXCL16* ($P = 0.009$) and its respective receptor *CXCR7* ($P = 0.02$) (Figure 6). Pappalysin 2 *(PAPPA2)* was significantly decreased ($P = 0.04$) in the presence of free-floating embryos (Figure 6). The expression of CXCL12 and PLA2G4A, which was predicted to be elevated in early pregnancy, did not differ between the two groups in qPCR ($P > 0.05$; not shown). The validation of ECM-related genes displayed an increase for *LAMA2* ($P = 0.01$) and *TIMP2* ($P = 0.01$) (Figure 6), while *PAPLN*, which was expected to be expressed more highly did not differ significantly ($P > 0.05$) in qPCR (not shown). Following the canonical pathway analysis performed with IPA, the expression of *LXR* was evaluated and revealed an upregulation ($P = 0.002$) toward early pregnancy.

Comparison of canine differentially expressed genes (DEG) with genes expressed during early gestation in other species

Gene set enrichment analysis (GSEA)

The entire list of canine DEG derived from microarray analysis of uterine samples was compared with genes expressed in other species, i.e., bovine, pig, and horse (days 15, 12, and 16 of pregnancy, respectively) [33–35]. Therefore, genes listed in Supplemental File 1 (no FDR or *P*-value cut-off) were ranked according to a score based on log2 fold-change and *P*-value (see Methods) and compared to sets of genes either up-regulated (Figure 4A-C) or down-regulated (Figure 4D-F) in bovine, porcine, or equine uteri. For each gene set comparison, the enrichment profile, as well as the position of the matching gene on the ranked canine gene set, are indicated (Figure 4). Complete lists of core-enriched overlapping genes, identified by their official gene symbols, are provided in Supplemental File 3. To identify functional relations within the detected datasets between the dog and other species, the overlapping core enriched genes were used for DAVID analysis. The functional clusters determined by DAVID analysis are presented in Table 2. For easier interpretation of the data, information about the categories is given for each functional annotation (cluster). The over-representation of genes in the gene-set divided into functional clusters and categories, all represented by ES, including the total number of genes from the dataset is presented in Table 2. From all comparisons, only the contrast of down-regulated bovine genes at day 15 of pregnancy vs. all genes of the dog did not reveal over-represented functional categories, possibly relating to the small number of core enriched genes (n = 12 genes) (Figure 4D; Table 2). For this contrast, genes up-regulated in bovine endometrium at day 15 of pregnancy overlapped with genes that were also up-regulated in the dog (Figure 4A).

Most of the genes up-regulated in the porcine endometrium matched with canine down-regulated genes (Figure 4B; Table 2). In contrast, genes down-regulated in pig overlapped mostly with those genes up-regulated in the dog (Figure 4E; Table 2). As for the horse, whereas the up-regulated genes matched with up-regulated canine genes (Figure 4C, Table 2), down-regulated equine genes overlapped predominantly with genes up-regulated in the dog (Figure 4F; Table 2).

Venn diagrams

In addition, Venn diagrams were established to identify commonly expressed genes between early pregnant canine uterus and other species, including human during the WOI. This cumulative comparison of all datasets was performed with the background of the top 2000 positive and top 700 negative canine scores (listed in Supplemental File 4A). All datasets were analyzed for commonly (up- or down-) regulated genes (Figure 5A and B). Additionally all genes (up- and down-regulated) were cumulatively compared with each other (Figure 5C). In addition to the total number of overlapping genes, the proportional ratio of matching genes between the gene set from the different mammals (i.e., bovine, pig, horse and human) and the canine gene set is given. The full lists of overlapping genes identified in this analysis are presented in Supplemental File 5.

When all the up-regulated genes were compared with the top 2000 canine positively scoring genes (Figure 5A; Supplemental File 5), the highest proportional overlapping was found with the human and horse gene set (12.6%, 124 from a total of 982 genes, and 12.5%, 127 genes out of 1013, respectively). The cow matched with 11.6% (68 genes from a total of 586), and the up-regulated genes of pig matched with 8.9% (80 out of 898 genes) in the dog. As for the commonly down-regulated genes (Figure 5B, Supplemental File 5), the highest proportional overlapping with the top 700 down-regulated canine genes was again found for the human endometrium during the WOI. Here, 7.4% of all genes were overlapping (97 from a total of 1318 genes). The horse matched with the canine genes by 3.8% (35 out of 915), the cow overlapped with 2.9% (8 genes from a total of 278), and only 2% of genes (15 out of 716) overlapped for the pig. The combined analysis (lists of up- and down-regulated genes merged for each species and used for a cumulative comparison) showed the highest proportional overlap of identical genes with 15.7% for the porcine gene set (261 from a total of 1659 genes), 14.9% for the horse, (288 genes out of 1928), 14% for human (322 genes from a total of 2300) and 13.1% for cow (113 out of 864) (Figure 5C; Supplemental File 5). Here, the overall number of total gene hits was the highest in all three cumulative comparisons.

While 774 genes from the list of 2700 canine genes up- and down-regulated matched with genes expressed in other species in the cumulative comparison, 1926 canine genes were without a match (Supplemental File 4B). These unpaired genes specifically expressed in the dog were used for functional annotation clustering by DAVID to reveal their molecular functions. The identities of functional annotation clusters and categories, along with the respective ES and total number of genes in clusters, are presented in Table 3. The main

Figure 3. IPA of canine DEG (early pregnancy vs. non-pregnant). (A) The canonical pathways analysis with the output gene sets ranked according to log (*P*-value). The overlapping of gene sets and the trend of the status (up- or down-regulated) are indicated. (B) The main functional terms identified by IPA were determined based on the identified canonical pathways.

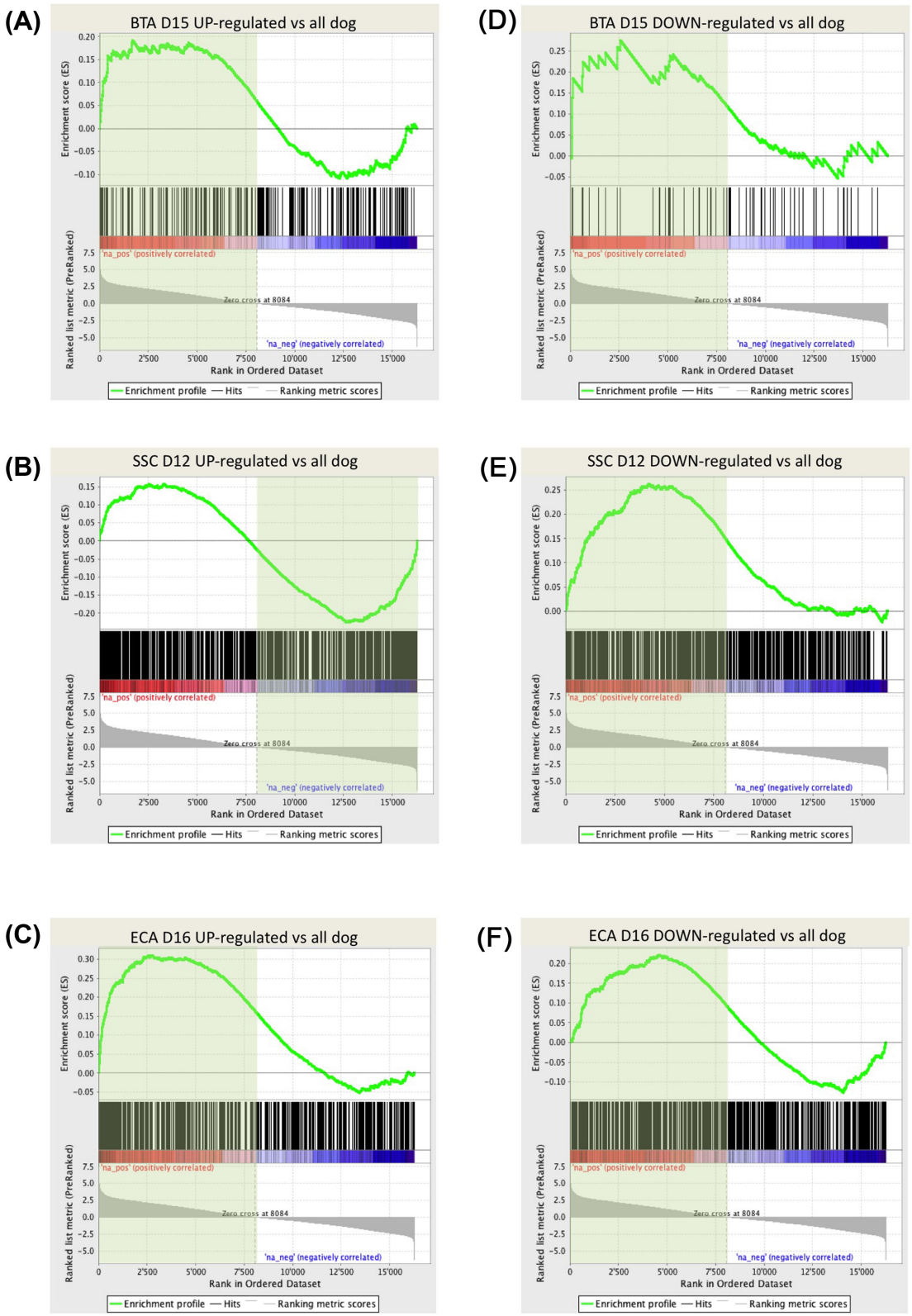
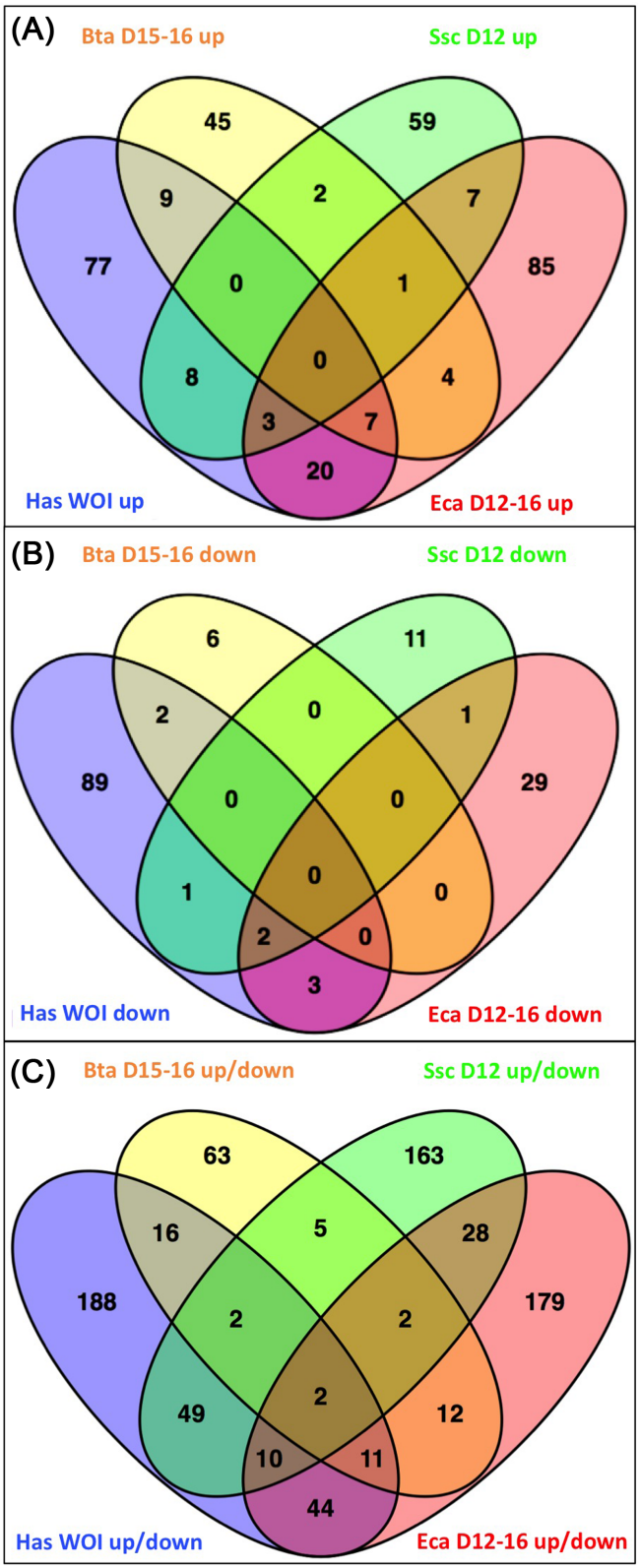


Figure 4. GSEA. The entire set of genes detected in canine uterus was ranked according to their expression levels (a score calculated from the log2 fold change and the *P*-value; see Methods). The ranked list was compared to sets of up-regulated (UP-regulated) genes (A-C) or down-regulated (DOWN-regulated) genes (D-F) from bovine (Bos taurus/BTA) uterus at day 15 of pregnancy (A and D), swine (Sus scrofa/SSC) uterus at day 12 of pregnancy (B and E) and horse (Equus caballus/ECA) uterus at day 16 of pregnancy (C and F). Enrichment scores and quantitatively strongest overlapping with the canine gene set are indicated. For details see the text. The list of overlapping genes is provided as Supplemental File 3.

Table 2. Results of functional annotation clustering analysis performed by DAVID software.

Gene set compared with canine gene set (ranked according to expression level)	Cluster	Category	Enrichment score (ES)	number of genes in the cluster
bovine upregulated (day 15 of pregnancy) 30 core-enriched genes	ion transport	carboxylic acid, organic anion transport	1.42	3
	epidermal growth factor (EGF)	EGF-like calicium binding, EGF-like domain, hydrolase	1.36	10
	extracellular matrix (ECM) signal	signal, ECM vesicle and exosome, ECM region, glycoprotein	1.32	15
	lipid transport	lipid transport, lipid localization	1.30	3
pig upregulated (day 12 of pregnancy) 245 core-enriched genes	cell cycle, division and mitosis	cell cycle process, mitotic cycle process, nuclear fusion, chromosome segregation, chromosome, regulation of mitotic cell cycle	6.27	100
	intracellular	intracellular organelle, intracellular membrane-bound organelle, cytoplasm	5.54	189
	cell selforganisation	microtubule cytoskeleton, non-membrane bound organelle, cytoskeletal part	4.24	73
pig downregulated (day 12 of pregnancy) 191 core-enriched genes	metal ion	transition metal ion homeostasis, cellular transition metal ion homeostasis, cellular iron ion homeostasis	2.04	7
	ECM	ECM-region, extracellular vesicle/exosome	1.97	70
	maturation	developmental and cell maturation	1.90	9
	mitogen-activated protein kinase kinase kinase (MAP3K)	MAP3K activity, activation of protein kinase activity	1.69	6
horse upregulated (day 16 of pregnancy) 142 core-enriched genes	ECM	ECM-region, ECM exosome, membrane-bound vesicle	6.50	95
	ECM-space	ECM-space	4.30	21
	blood vessels	blood vessel morphogenesis/development, regulation of angiogenesis, circulatory and vasculatory system development, regulation of anatomical structure morphogenesis	2.70	31
	immune system	positive regulation of immune system process, complement activation, response to external stimulus, regulation of protein activation cascade, regulation of defense response, regulation of B cell mediated immunity, regulation of humoral immune response	2.48	52
	lipoprotein	cargo receptor activity, lipoprotein particle receptor activation	2.25	4
	endocytosis	phagocytosis, endocytosis, vesicle mediated transport	2.17	26
	organ development	single-multicellular organism process, regulation of multicellular organismal process, system development, anatomical structure morphogenesis	2.01	54
horse downregulated (day 16 of pregnancy) 188 core-enriched genes	Golgi apparatus	Golgi apparatus, endomembrane system	2.31	33
	metabolic process	cellular amine metabolic process, catabolic process, small molecule catabolic process	2.09	8

Core-enriched genes derived from GSEA were used as input. For this, the entire canine gene set (no FDR, no *P*-value correction), ranked according to gene expression levels, was used as background and compared with genes differentially expressed in other species (either up- or down-regulated). The clusters of main selected over-represented functional terms are shown. The categories comprising each cluster have been additionally named. The over-representation of genes in gene sets is represented by respective ES. The number of genes in each cluster is given.



Proportional overlap [%] between genes expressed in dog and other species:
Bta = 11.6%
Ssc = 8.9%
Eca = 12.5%
Hsa = 12.6%

Proportional overlap [%] between genes expressed in dog and other species:
Bta = 2.9%
Ssc = 2.0%
Eca = 3.8%
Hsa = 7.4%

Proportional overlap [%] between genes expressed in dog and other species:
Bta = 13.1%
Ssc = 15.7%
Eca = 14.9%
Hsa = 14.0%

Figure 5. Venn diagrams showing the intersection between genes differentially expressed in the presence of pre-implantation embryos in different domestic animal species are presented using the canine gene set as reference. (A) Overlapping of the top 2000 up-regulated (2000 best positive scores) canine genes with up-regulated genes in other species; (B) comparison of the top 700 down-regulated (700 best negative scores) canine genes with genes down-regulated in other species; (C) cumulative analysis of the canine genes with genes up- and down-regulated in other species. Hsa WOI = Homo sapiens during the window of implantation (WOI), Bta = Bos taurus (days 15–16 of pregnancy), Ssc = Sus scrofa (day 12), and Eca = Equus caballus (days 12 and 16). The list of overlapping genes is provided as Supplemental File 4.

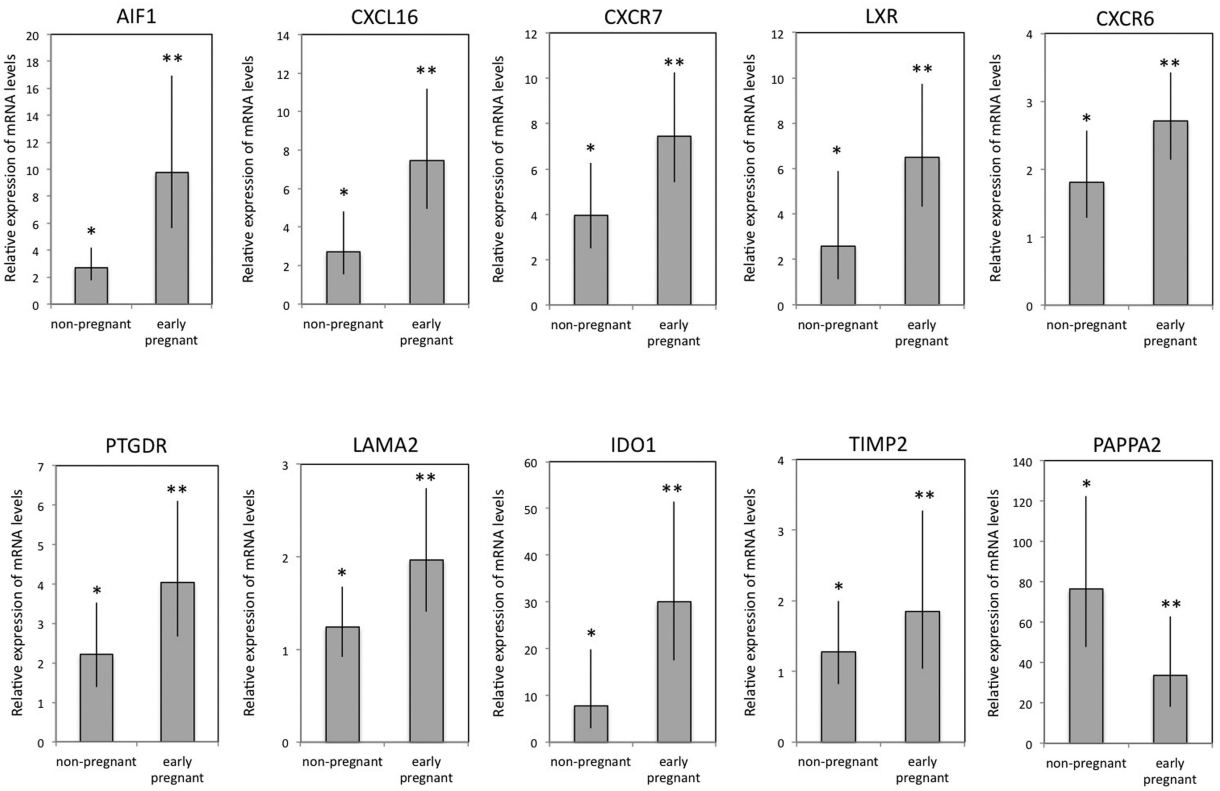


Figure 6. Expression of selected target genes as determined by real-time (TaqMan) RT-PCR. *AIF1* = allograft inflammatory factor 1, *CXCL16* = chemokine ligand 16, *CXCR7* = chemokine receptor 7, *LXR* = liver X receptor, *CXCR6* = chemokine receptor 6, *PTGDR* = prostaglandin D2 receptor, *LAMA2* = laminin alpha 2, *IDO1* = idolamin 2,3-dioxygenase 1, *TIMP2* = tissue inhibitor of matrix metalloproteinase-2, *PAPPA2* = pappalysin2. An unpaired, two-tailed Student *t*-test was applied. Bars with different asterisks differ at $P = 0.001$ (*PTGDR*), $P = 0.002$ (*AIF1, LXR*), $P = 0.009$ (*CXCL16*), $P = 0.01$ (*LAMA2, TIMP2*), $P = 0.02$ (*CXCR7, CXCR6, IDO1*), $P = 0.04$ (*PAPPA2*). Numerical data are presented as geometric means Xg ± geometric standard deviation (SD).

Table 3. DAVID analysis from 1926 genes that did not overlap in the contrast: canine genes (2000 top positive/700 top negative) vs. gene lists from other species (corresponding to Figure 5).

Comparison	Cluster	Category	Enrichment score (ES)	number of genes in the cluster
1926 genes that did not pair in the contrast 2700 canine genes (up- and downregulated) versus other species (up- and downregulated)	mitochondrion	mitochondrion, mitochondrial matrix	6.79	159
	DNA repair	DNA repair, DNA damage	2.75	55
	helicase	helicase, helicase C-terminal, ATP dependent RNA helicase activity, oligosaccharide-binding, helicase-associated domain	2.36	27
	zinc-finger	zinc, zinc-finger, metal binding	1.93	353

The clusters of main selected over-represented functional terms are shown. Additionally, functional categories comprising the main clusters are named. The over-representations of genes in the gene set are represented by the ES, the number of genes in each cluster is given.

functional clusters related to mitochondrion (ES: 6.79; 159 genes), DNA-repair (ES: 2.75; 55 genes), helicase (ES: 2.36; 27 genes) and zinc-finger (ES: 1.93; 353 genes).

Discussion

Because there is no luteolysis in the absence of pregnancy, the dog clearly lacks an endocrine signal triggering a biochemical anti-luteolytic cascade, which is present in other species to modulate uterine function during the critical early time point of pregnancy establishment. This is reflected in species-specific responses of the uterus to pre-implantation embryos. Accordingly, in our previous study we showed that in the dog, biochemical changes precede morphological modifications in the early pregnant uterus [24, 25]. The possible underlying biological mechanisms have been further explored herein using microarray analysis of uterine transcriptomes. Uteri collected between days 10-12 of pregnancy were used. It is

noteworthy that, in the dog, development of embryos and their oviductal passage are slow. It takes as long as 7-10 days for embryos to reach the tip of the uterine horns [41, 42] and are usually then at the morula or early blastocyst stages [41]. Invasion of the uterine epithelium occurs normally around days 17-18 after fertilization [43, 44]. In our study, assuming that seminal bioactive factors would have an effect on the initiation of pregnancy by modulating the uterine endocrine milieu, dogs inseminated but determined to be non-pregnant were used as controls.

Because of the prolonged oviductal transit of embryos, any disturbance in their early development leading to pregnancy failure would not originate in the uterus. This approach allowed us to determine embryo-driven effects within a similar uterine environment. The effects of seminal plasma on the canine uterus (i.e., comparing non-mated dioestric bitches with those exposed to seminal plasma) were not separately studied herein, but certainly merit attention in the future.

Microarray analysis of responses of the canine uterus to early pre-implantation embryos

The major biological processes activated by the presence of early pre-implantation embryos in the canine uterus are indicated by the main functional terms detected associated with over-represented genes. Thus, the majority of the 412 DEG was linked to ECM and cell signaling, secretory activity and matrix-cell interactions involved in cell motion and migration. The second important group was represented by inflammatory response. Higher variation and less involved genes were found among functional terms suppressed when free-floating embryos are present. These were, however, specifically linked to cell-to-cell contacts including focal and cell adhesion and fibronectins activity. These observations were confirmed by the Cytoscape analysis of functional networks and were further fulfilled by over-represented pathways detected by IPA. In particular, acute phase response signaling, the complement system and pathways associated with adhesion and diapedesis of immune cells were strongly represented.

Regarding ECM components, the initialization of structural reorganization of the uterus driven by the presence of embryos in the dog was characterized by higher representation of genes encoding for several collagen compounds (e.g., *COL16A1* or *COL18A1*). It seems also that modifications of the uterine ECM, prior to embryo attachment and trophoblast invasion, might be affected by functional changes in integrins signaling because the respective pathways were either down-regulated (e.g., *RRAS2*, *ITGB1*, *LIMS1*) or up-regulated (e.g., *ARPC1B*, *ITGAM*) in the presence of early embryos. Integrins are transmembrane receptors that interconnect cells and build links between cell and ECM proteins such as laminin or collagens [45]. Disruption of their functionality may lead to infertility [46]. A modulated, increased expression of some integrins, such as -alpha2b, -beta2 and -beta3, was previously shown in the canine early pregnant uterus [47].

Another important adhesion molecule induced by the presence of canine pre-implantation embryos was laminin alpha 2 (*LAMA2*). This glycoprotein is a constituent of the *lamina basalis* [48] and is also produced by human decidual cells [49, 50]. It could thus be seen as an early indicator of the ongoing decidualization process in the dog, even if the samples used for this study were collected before the morphological differentiation of stromal cells into decidual cells becomes visible [24, 25].

As indicated elsewhere, among the functional terms, the representation of which was suppressed by the presence of early embryos, were those related to fibronectins, in particular to FN3. Fibronectins are ECM glycoproteins interacting with membrane-bound proteins such as integrins, fibrins, or actins. Specifically, the FN3 domain is evolutionarily well conserved and can be found in a variety of extracellular proteins. Thus, NCBI's Conserved Domain Database [51] annotates the biomolecular sequence of the conserved protein domain of FN3 (cd00063) in collagens and some cell adhesion molecules like receptor signaling family leucine-rich repeat transmembrane protein (FLRT) or ITGB4. Its presence has also been confirmed in the structural protein FN1, the expression of which is modulated by pregnancy-related hormones, e.g., P4 [52]. In the rat, the early pregnancy-related uterine decrease in fibronectin has been linked to the differentiation of stromal cells during the decidualization process [53]. Accordingly, the suppression of the respective FN domain found in our study in the early pregnant canine uterus could also be seen as a further sign of the ongoing decidualization process initiated by the presence of embryos.

While the previously mentioned molecules (i.e., integrins and FN) are considered stable components of the ECM, IPA also indicated an involvement of enzymes that modulate the composition of the ECM, like inhibitors of matrix metalloproteinases (TIMPs), in the early embryo-maternal communication in the dog. Considering the high representation of the immune system in our dataset, it is worth mentioning that some of these MMPs can be stimulated by proinflammatory cytokines such as IL-1 beta, IL-6, and IL-8 [54, 55]. So far, the presence and activity of MMP2 and MMP9 have been confirmed in the early pregnant uterine compartments of the dog [56, 57].

The expression of MMPs-modulating TIMPs and their involvement in embryo-maternal communication in the dog remain to be elucidated. Here, based on the results obtained from our pathway analysis, the expression of *TIMP2* was examined. TIMP2 is among the most potent regulators of MMPs activity and was shown to be positively regulated by P4 [58, 59]. In our experiments, its uterine expression was enhanced by the presence of free-floating embryos in the uterus. Future studies should focus on the early initiation of TIMPs expression and function in the canine uterus, because these proteins could be part of a balancing system by weakening the effects of cytokine-enforced activation of MMPs. However, this hypothesis needs further verification. Interestingly, from the 98 genes down-regulated in the canine uterus in response to the presence of embryos, among the most highly suppressed genes (5.2-fold suppression) was *PAPPA2*, also referred to as pregnancy-associated plasma protein A2. PAPPA2 acts as a metalloproteinase specific for insulin-like growth factor-binding proteins, and is thereby known to modulate the bioavailability of IGFs [60]. Besides their mitogenic activity [61–63], IGFs are prominent markers of decidualization and are important regulators of embryonic development and fetal and placental growth [64, 65]. Both IGF1 and IGF2 and their main receptor IGFR1 are present in the pre-implantation canine uterus [24]. IGFs appear also to be involved in species-specific canine decidualization [39]. It thus appears plausible that by being modulated by the presence of embryos, PAPPA2 is also involved in coordinating the local availability of IGFs in the dog. However, its involvement in the decidualization process and preparation for trophoblast implantation and invasion needs to be investigated.

Cumulatively, our findings emphasize the importance of pre-attachment canine embryos in biochemical modulation of uterine ECM components in preparation for implantation and placentation in this species. It is likely that, during these processes, not only the maternal stroma cells but also changes in the composition of the

uterine ECM have an impact on canine-specific process of decidualization. This is further supported by the top score genes detected by our microarray analysis, i.e., genes responding with the highest individual fold-enrichment to the presence of free-floating embryos (*IPO9*, *ITIH4*, and *NOV*). Their functionality strongly relates to biological processes of cell differentiation, proliferation, and matrix organization as indicated by DAVID and IPA, as well as being involved in immune processes. Thus, IPO9 is a part of the nucleocytoplasmic transport system by regulating the localization and functions of substrate proteins as a generic nuclear localization sequences receptor and signal transducer [66, 67]. One of the reported signaling pathways, the Wnt/Wingless pathway, is possibly associated with decidualization [68]. By regulating the expression of some interferons, e.g., IFN epsilon [69, 70], IPO9 also exerts immunomodulatory effects. Some other importins, e.g., IPO13 and IPO5 alpha, play crucial roles in uterine cellular differentiation and proliferation [71–73]. In knockout mice lacking IPO5 alpha expression, uterine stimulation with exogenous gonadotropins results in hypertrophy, similar to the P4 receptor-deficient phenotype [71]. The interalpha trypsin inhibitors (ITIH) function as protease inhibitors and are thereby involved in maintenance of ECM [74]. More specifically, ITIH4 is an acute phase protein, and besides involvement in systemic inflammation [75], it seems to play roles during pregnancy, e.g., in pigs, where it has been implicated in the maintenance of uterine surface glycocalyx [76]. Some other functions described for ITIH4 include immunomodulatory properties due to binding and suppression of the phagocytic activity of granulocytes [77].

Finally, a member of the family of CCN genes (connective tissue growth factor, cysteine-rich protein, and nephroblastoma overexpressed), NOV plays roles in several biological processes regulating cell functions such as adhesion, migration, chemotaxis, cell proliferation, and differentiation [78, 79]. Some of its functions are mediated by direct binding and activation of integrin receptors [80, 81]. The importance of NOV during pregnancy, possibly due to its angiogenic and tissue remodeling properties, is underlined by the fact that its decreased expression has been implicated in the early onset of pre-eclampsia [82].

Apart from *PAPPA2*, the evaluation of the top down-regulated scores, i.e., the most negatively affected genes in our microarray, revealed genes linked to cellular proliferation and maintenance of cellular cytoskeleton (*Ki67/MKI67* and *DIAPH3*). Ki67 is a well-known nuclear protein frequently used as a marker of proliferation [83]. It is present during all active phases of the cell cycle except the G0-phase [84]. Interfering with its function results in inhibition of RNA synthesis [85].

Diaphanous related formin (DIAPH3) in turn has not yet been directly connected to pregnancy, but its involvement in some basic, yet pregnancy-relevant mechanisms, has been shown. Thus, it is involved in actin remodeling, erythropoiesis, vesicle trafficking, bleb formation, filopodium formation, cell invasion, and cytokinesis [86–98].

Although not targeted toward suppression of luteolysis, species-specific immunomodulation indeed seems to play a major role in the canine early embryo-maternal conversation. The involvement of acute phase factors in this cross-talk was also implied in earlier studies, in which elevated levels of some acute phase response molecules, e.g., fibrinogen and serum C-reactive protein-like CRP-LI, were found in the serum of pregnant bitches [99–101]. These were, however, associated with implantation and placentation and were observed concomitantly with increasing placental RLN levels. The breakdown and remodeling of uterine tissues during early placentation were also associated with increased expression and activation of acute phase response in other species such as rats, in which particularly the AGP was strongly increased [102]. However, similar to the situation found in dogs, since they act as mediators of inflammation, triggered, e.g., by some infections, these acute phase proteins cannot be used as highly specific markers of pregnancy.

Regarding the pre-implantation uterus, our data are in agreement with previous reports of increased availability of transcripts encoding for several cytokines possibly involved in maternal recognition of pregnancy in dogs, such as *IL2*, *IL4*, *IL10*, *IFN gamma*, *TGF beta*, and *LIF* [57]. Concomitantly, other immunomodulatory factors, such as *GM-CSF*, *IL1 beta*, *IL6*, *IL8*, and *CD4*, were present in free-floating embryos [57]. Additionally, in our recent studies, we found an increased abundance of *PTGS2* (*COX2*) and *PTGES* transcripts in free-floating-hatched blastocysts compared with unhatched ones [24]. These data also fit very well with the IPA results presented herein, pointing toward activation of the IL10-, IL4- and IL6-pathways in the early pregnant canine uterus. Some other relevant immunoreactive genes from the respective functional terms "inflammatory response" and "defense response" and "innate immune response," which were highly represented in our microarray data from the canine early pregnant uterus were *CSF1*, *CSF1R*, *AIF1*, *IDO1*, *IL15*, *IL16*, *IL1R1*, and *LYZ*.

Macrophage colony-stimulating factor (M-CSF/CSF1) is a cytokine that, through binding to its receptor CSF1R, induces differentiation, proliferation, and survival of macrophages. These cells, besides mediating immune responses, contribute to tissue remodeling during mammalian pregnancy [103, 104]. Their roles also include phagocytosis of apoptotic cells that occur during the remodeling processes and trophoblast invasion, thereby decreasing local inflammatory responses [105]. Lysozyme (LYZ) is an antibacterial enzyme catalyzing the hydrolysis of peptidoglycans. It is present in different exocrine secretions such as saliva, milk, and mucus [106]. Additionally, it is present in cytoplasmic granules of macrophages and polymorphonuclear neutrophils [107]. As part of the innate immune system, LYZ seems to provide protection against uterine infections [107]. Its uterine secretion and function is positively influenced by P4 [108].

Moreover, among the selected genes investigated in our study by semi-quantitative PCR and belonging to the immune response, we were able to confirm stimulatory effects of the presence of canine embryos on uterine expression of transcripts encoding for *IDO*, *AIF1*, *CXCL16* and its receptor *CXCR6* and *CXCR7*.

Indolamine 2,3-dioxygenase, a versatile and highly conserved protein in mammals, is expressed by a wide variety of immune cells, e.g., dendritic cells, monocytes, and macrophages [109]. Through catabolism of the essential amino acid tryptophan, it modulates the behavior of T-cells [110]. It thus has immunomodulatory and, in part, immunosuppressive functions, when the withdrawal of T-cell activity is considered [111]. An interesting aspect of IDO in both immunomodulation and immunosuppression is its central role in the activation of regulatory T cells (Treg). Namely, the IDO-derived metabolic products of tryptophan stimulate production of Treg [112]. Regarding the reproductive organs, IDO is known to be secreted by human decidual cells [113] and its immunomodulatory activity during pre-implantation and implantation stages of pregnancy have been implied, e.g., in humans [114]. Also, its involvement in regulating trophoblast invasion was suggested because inhibition of IDO function leads to embryo rejection by T lymphocytes [115]. Besides T-cells, IDO is capable of suppressing activated natural killer (NK) cells, which constitute the highest

proportion of immunomodulatory cells in human decidua during the first trimester of pregnancy [116–118]. Thus, at least in species displaying an invasive type of placentation, IDO is needed for implantation. In view of this, we felt prompted to assess its expression and, thereby, its potential involvement in early embryo-maternal communication in dogs. The quantity and types of immune cells in the canine placenta still need to be elucidated, but the elevated mRNA levels of IDO1 in the early pregnant group of dogs seem to be related to a local immunosuppression and/or modulation. The function of IDO in canine pregnancy therefore needs to be investigated further.

Similar to IDO, allograft inflammatory factor 1 (AIF1) is also an evolutionarily old and highly conserved protein in mammals. AIF1 is a cytosolic protein that plays many roles, e.g., in regulating immune responses, proliferative activities, and vasculogenesis [119–123]. It can be found in macrophages, including those residing in the uterus. [124, 125]. Here, we detected significantly elevated uterine levels of AIF1 in the early pregnant canine uterus, suggesting possible physiological functions of AIF1 during maternal responses to the presence of pre-attachment embryos in this species.

The strong, immunomodulatory uterine activity, driven by the presence of embryos, was further underlined by the increased expression of several chemokines. These are needed as regulators of maternal receptivity and embryo implantation [126, 127] and were also shown to stimulate cell proliferation and trophoblast invasion [128, 129]. CXCL16 is a cytokine belonging to the CXC chemokine family. Its receptor, CXCR6, can be found on immune cells such as T cells and natural killer cells [130]. Binding of CXCL16 to its receptor CXCR6 will lead to cell migration [130]. In the human placenta, cytotrophoblast cells coexpress CXCL16 and CXCR6, which is thought to induce their proliferation and invasion in an autocrine manner [131, 132]. Similarly, CXCR7 and its ligand CXCL12 were implicated in supporting the invasive properties of human cytotrophoblast [133]. It therefore seems plausible that this chemokine-receptor-axis is up-regulated in the pregnant canine uterus to induce proliferation, and to modulate the immune milieu in preparation for implantation and placentation.

Due to the crucial role PG plays in reproduction, including the canine reproductive system, the increased expression of PTGDR driven by the presence of embryos attracted our attention. Its ligand, PGD2, is produced in the uterus and is secreted by endometrial and myometrial compartments, including decidua, e.g., in humans [134, 135]. Acting in a receptor-dependent manner, during pregnancy PGD2 functions as a chemoattractant to recruit immune cells to the feto-maternal interface [136, 137], and inhibits antigen presentation of dendritic cells to T cells [138]. Taking this into account, and its strong expression driven by the presence of embryos, PTGDR and its ligand PGD2 appear to be attention-worthy targets for investigating the species-specific mechanisms of embryo-maternal recognition in the dog.

Interestingly, the activation of the nuclear receptor LXR pathway in early pregnant canine uterus was indicated by IPA analysis. LXR is a heterodimeric binding partner of another nuclear receptor, PPAR gamma [139], and is thus linked to metabolic pathways, such as glucose and cholesterol metabolism, insulin metabolism, and adipogenesis [140, 141]. On the other hand, PPAR gamma uses PG as endogenous ligands and can thus participate in PG-mediated biological processes, including immunomodulatory functions [142, 143]. The role of PPAR gamma has also been implied during canine pregnancy, and while it was localized solely to fetal trophoblast cells, its functions were predominantly linked to the processes of implantation, trophoblast invasion, and placentation [40].

Correlation of canine differentially expressed genes to different species

Being interested in differentiating between the common and species-specific biological pathways involved in embryo-maternal communication during establishment of pregnancy in the dog, pairwise comparisons were established with previously published datasets from other animals. The gene lists from other species were derived from the time points of pregnancy when the anti-luteolytic signals are initialized in these species. These single interspecies comparisons were performed using the whole canine gene set (both gene expression directions, up- and down-regulated), matching it with gene sets that were filtered according to their expression (either up- or down-regulated) in other species. These analyses were set up to identify if similar pathways are utilized in dogs as in other species. The lowest ES were obtained in comparison with the bovine uterus. When canine and porcine gene sets were compared, higher ES were found, however, most of the overlapping genes showed opposite patterns of expression, indicating different embryo/conceptus-mediated uterine responses prior to implantation. Thus, DAVID analysis of genes derived by GSEA related the porcine up-regulated genes to cell cycle, cell division and mitosis. This contrasted with down-regulated genes related to functional terms associated with enzyme activity, ECM, maturation, and MAP3K activity, which mapped with genes up-regulated in the dog. This reverse correlation can be plausibly explained by the species-specific estrogen-related signaling in the pig in which the embryo-controlled anti-luteolytic switch from endocrine to paracrine PGF2 alpha secretion is initiated between days 10-12 of pregnancy [6, 34, 144]. In the dog, no pregnancy-related estrogen increase occurs [19], and the estrogenic capability of canine embryos remains unknown. It is notable that, as mentioned elsewhere, in bitches P4 appears to be the only luteal steroid required for the establishment of pregnancy [145, 146]. Furthermore, the highest ES were noted when canine uterine genes were compared with genes up-regulated in the mare. Here, the commonly up-regulated genes belonged to functional terms related to secretory activity, ECM, and angiogenesis. Nevertheless, as for the pig and cow, the down-regulated equine genes matched those genes that were up-regulated in the dog with enriched functional terms relating, e.g., to Golgi apparatus and metabolic processes. This indicates that, although sharing some similar regulatory pathways regarding structural remodeling of the uterus, nevertheless, canine and equine uteri differ markedly at the level of secretory activity. Moreover, with regard to similar expression of ECM-related factors between the dog and horse, most probably, the considerably delayed implantation observed in the horse needs to be considered. In the horse, implantation does not occur until around day 37 [12]; therefore, the blastocyst is dependent on histiotrophic support.

Going one step further, in addition to the pairwise interspecies comparisons, accounting for the expected differences arising from species-specific anti-luteolytic strategies and different types of implantation and preparations for placentation, commonly regulated genes in different species were determined in multiple comparisons. Canine genes were used as references for preparation of Venn diagrams. In contrast to the single interspecies comparisons by GSEA, all gene sets (canine, bovine, porcine, equine, and human) were filtered according to their gene expression (up- or down-regulated) and compared with each other. This allowed us to identify commonly

up- or down-regulated genes. Additionally, all gene sets (regardless of gene expression direction of change) were used to identify potentially conversely regulated genes between species during the early embryo-maternal dialog. Again, a high correlation was found between genes commonly expressed (up- or down-regulated) in the canine and equine uterus.

For this comparison, the human-derived gene set was included, resulting in even higher correlation with the canine uterus. These human genes were determined during the WOI [38], which lasts for 6-10 days after ovulation and is defined as the period when the uterus is receptive to implantation of the free-floating blastocyst [147]. During this time, the estrogen- and progesterone-primed endometrium is undergoing distinct morphological and histological changes, and maternal decidual cells are formed, the latter even in the absence of an implanting blastocyst [148, 149]. Our results thus seem to be linked to the P4-mediated effects and ongoing decidualization in the dog in preparation for implantation, when morphological decidualization of stromal cells becomes obvious [25]. Preparation for the invasive type of placentation may also play an important role.

Noticeably, the more species that were overlapped, the fewer genes were found to be commonly expressed. Interestingly, however, higher numbers of genes overlapped when they were compared regardless of their expression patterns (up- or down-regulated). This implies that different strategies within similar regulatory pathways are utilized for facilitating implantation in different mammals.

In this regard, we were able to identify some genes represented in all compared species. These were *PENK* and *CYP26A1*. PENK was up-regulated in bovine, horse, and dog, but down-regulated in human and pig. Its exact involvement in pregnancy is not yet completely understood, but there are indications that PENK, as part of the opioid system, could play a role in maternal adaption to pregnancy and in supporting embryonic and fetal growth by modulating analgesia and regulating uterine motility [150, 151]. CYP26A1 is a microsomal enzyme responsible for regulating the cellular level of retinoic acid, which is involved in modulating gene expression in embryonic and adult tissues [152, 153]. In the gene sets applied here, CYP26A1 was up-regulated in human, cow, and dog, while it was down-regulated in horse and pig. It seems that functions of both genes in the uterine preparation for implantation are species-specific, and possibly also stage of pregnancy-specific, which is reflected in their divergent expression patterns among the different animals.

Interestingly, we found 1926 genes that were exclusively expressed in the canine pre-implantation uterus and, thus, did not overlap with genes expressed in other species. Most of them were specifically linked to mitochondrial functions, transcriptional activity of the genome and metabolic activities associated with DNA repair. Also, these energy- and protein-secretion-related functional terms could be related to other canine-specific up-regulated terms associated with the remodeling processes driven by the presence of embryos within the canine pre-implantation uterus.

Here, for the first time, deeper insights into species-specific pre-implantation embryo-maternal interaction in the dog are presented. Despite being devoid of a classical anti-luteolytic signal, profound biochemical changes were found, indicating the activity of biological mechanisms which are generally linked to similar processes as in other species, yet which are somehow different. These changes were predominantly linked to increased secretory activity associated with preparation for implantation, rather than to proliferative activity. Functional terms associated with ECM remodeling appeared to determine primary functions of canine embryos during the early, pre-attachment stage of pregnancy. This was followed by mobilization of the immune system. The expression and function of cytokines in the species-specific process of embryo maternal recognition in the dog, as well as the intersection between these two systems (i.e., ECM and the immune system), deserve more attention because it can reveal new mechanisms involved in the processes of decidualization and initiation of placentation in dogs. Taking into account the limited invasion of trophoblast during formation of the canine endotheliochorial placenta, such newly acquired information could extend our knowledge about the multiple pathways involved in metabolic and immunological regulation of implantation in mammals.

In summary, this study provides a basis for understanding of the uterine milieu required for proper embryo development and, thereby, for successful establishment of canine pregnancy.

Additionally, activation of some highly conserved proteins, such as IDO and AIF1, was found along with a wide range of chemokines, revealing candidate genes such as IPO, NOV, and DIAPH whose function has so far been underinvestigated. Their functions in preparation for implantation and/or decidualization should be investigated. As for the genes that are expressed jointly in different species, the function of PENK and CYP26A appears to be interesting, but both genes have so far not been recognized as candidates involved in common pathways of evolutionarily determined strategies of embryo-maternal communication.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental File 1. List of genes detectable by microarray in the uterus of early pregnant (pre-attachment) and corresponding control bitches without FDR or *P*-value correction.
Supplemental File 2. List of differentially expressed genes (DEG) between early pregnant and corresponding control bitches with 10% FDR (i.e., adjusted *P*-value < 0.1).
Supplemental File 3. List of core-enriched genes found by GSEA analysis in the pairwise contrasts between genes expressed in canine uterus vs. "other mammals" (i.e., cow, pig, or horse).
Supplemental File 4. Top 2000 genes with positive scores and top 700 with negative scores of the ranked gene list applied for GSEA, which (A) were used for Venn diagram comparisons, and (B) list of canine-specific genes without overlap with other species obtained from the Venn diagrams.
Supplemental File 5. List of genes overlapping in Venn diagrams between different species, using canine gene set as the background (2000 top positive and 700 top negative scores).
Supplemental File 6. Gene abbreviation index.

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Authors' contributions: FRG: involved in developing the concept of the study, experimental design, generating data, analysis and interpretation of data and writing of the manuscript. AG, EK: knowledge transfer, involvement in the laboratory part of the project, tissue processing. SB: knowledge transfer, analysis and critical discussion of the data. SA and ARA: involved in tissue sampling procedures, animal experiments, and knowledge transfer. AB: knowledge transfer, critical discussion of the data, editing of the manuscript.

MPK: designed and supervised the project, was involved in interpretation of the data, drafting and revising the manuscript.

Conflict of Interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. All authors read and approved the final version of the manuscript.

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