Expression of genes involved in the embryo–maternal interaction in the early-pregnant canine uterus

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Abstract

Although there is no acute luteolytic mechanism in the absence of pregnancy in the bitch, a precise and well-timed embryo–maternal interaction seems to be required for the initiation and maintenance of gestation. As only limited information is available about these processes in dogs, in this study, the uterine expression of possible decidualization markers was investigated during the pre-implantation stage (days 10–12) of pregnancy and in the corresponding nonpregnant controls. In addition, the expression of selected genes associated with blastocyst development and/or implantation was investigated in embryos flushed from the uteri of bitches used for this study (unhatched and hatched blastocysts). There was an upregulated expression of prolactin receptor (PRLR) and IGF2 observed pre-implantation. The expression of PRL and of IGF1 was unaffected, and neither was the expression of progesterone- or estrogen receptor β (ESR2). In contrast, (ESR1) levels were elevated during early pregnancy. Prostaglandin (PG)-system revealed upregulated expression of PGE2-synthase and its receptors, PTGER2 and PTGER4, and of the PG-transporter. Elevated levels of AKR1C3 mRNA, but not the protein itself, were noted. Expression of prostaglandin-endoperoxide synthase 2 (PTGS2) remained unaffected. Most of the transcripts were predominantly localized to the uterine epithelial cells, myometrium and, to a lesser extent, to the uterine stroma. PGE5 (PTGES) mRNA was abundantly expressed in both groups of embryos and appeared higher in the hatched ones. The expression level of IGF2 mRNA appeared higher than that of IGF1 mRNA in hatched embryos. In unhatched embryos IGF1, IGF2, and PTGS2 mRNA levels were below the detection limit.

Reproduction (2014) 147 703–717

Introduction

Establishment and maintenance of pregnancy require synthesis and well-orchestrated secretion of a plethora of regulatory factors that establish the uterine milieu needed for embryo implantation and development. The vast majority of these factors remain under the control of progesterone, which is an essential pleiotropic regulator of uterine function.

Because there is no placental steroidogenic activity in the dog, the provision of circulating progesterone depends on corpora lutea (CL) as the major source of this hormone throughout gestation (Concannon et al. 1989). In livestock, e.g., cattle, pigs, and horses, it is well established that uterine prostaglandin F2α (PGF2α) is luteolytic and is responsible for terminating the luteal phase of the estrous cycle in nonpregnant females; therefore, there is no pseudopregnancy in these animals. In contrast to livestock, at least in nonpregnant bitches, there is no uterine luteolysin that could be required for normal ovarian cyclicity, because normal ovarian function is observed following hysterectomy (Olson et al. 1984, Hoffmann et al. 1992). Furthermore, a luteolytic role of intraluteally produced prostaglandins (PGs) can be ruled out (Kowalewski et al. 2006a, 2009). Thus, the absence of an acute luteolytic mechanism in the nonpregnant bitch (Concannon et al. 1989, Hoffmann et al. 1992) results in a physiological pseudopregnancy and a luteal life span similar to, or even longer than, that observed in pregnant bitches. In contrast, in pregnant bitches, the steep prepartum progesterone decline is associated with strongly increased PGF2α concentrations in the maternal circulation (Nohr et al. 1993), implying its role during prepartum luteolysis and/or parturition.

Prostaglandin E2 (PGE2) is one of the important luteotropic factors in the dog (Kowalewski et al. 2008, 2009a, 2009b).
As recently shown, PGE2 is capable of activating progesterone synthesis in canine luteal cells isolated from early developing CL acting at the level of STAR protein expression and function (Kowalewski et al. 2013). Although both luteinizing hormone (LH) and prolactin (PRL) are luteotropic factors, with PRL being the predominant one (Concannon 1980, Okkens et al. 1990, Onclin et al. 1993, 2000), gonadotrophic support does not seem to be required for luteal maintenance during the early-CL phase (Okkens et al. 1986).

Consequently, the canine CL seems to possess an inherent life span, resulting in a similar progesterone secretion pattern in pregnant and nonpregnant animals that is mirrored in circulating progesterone levels that do not differ significantly until shortly before parturition; at that time, when a dramatic prepartum progesterone decline is observed, signaling the onset of parturition (Concannon et al. 1989). This hormone profile precludes progesterone as a usable marker for detection of pregnancy in the bitch. Moreover, no pregnancy-associated increase in estrogens is observed in the dog (Hoffmann et al. 1994).

Even though knowledge concerning the endocrine control of the canine reproductive cycle has greatly improved, there is still a lack of information concerning the progesterone-dependent establishment of the intimate, initial embryo–maternal contact and the role of the early canine embryo during this process. In particular, knowledge about endocrine mechanisms regulating the uterine microenvironment before implantation is still limited for the dog. This aspect is important for the entire early gestational period, up until days 17–18 after mating, at which time implantation takes place in dogs, immediately followed by the start of placenta formation (Amoroso 1952).

Before that, the progesterone-dependent decidualization process starts, which is characterized by a very strong, species-specific remodeling of the uterine tissues, especially at the implantation sites. As a result of this change, maternal stroma-derived, so-called decidual cells, are the only cells of the canine placenta expressing the progesterone receptor (PGR; Vermeirsch et al. 2000, Kowalewski et al. 2010). Interfering with PGR function, e.g., by application of an anti-gestagen, will unequivocally lead to preterm parturition/abortion (Baan et al. 2008, Kowalewski et al. 2010).

In some earlier studies aimed at detecting factors possibly contributing to embryo implantation in the dog, no differences were found in the expression of heat-shock proteins and acute phase proteins between the uteri of early-pregnant and nonpregnant dogs (Evans & Anderton 1992, Buhi et al. 1993, Concannon et al. 1996). Recently, CD8, IL4 and IFNγ mRNA were found as being abundantly expressed in the early-pregnant uterus, while the expression of CD4, TNF and IL6 mRNA seemed to be targeted to the nonpregnant uterus (Schafer-Somi et al. 2008, Beceriklisoy et al. 2009). In contrast to insulin-like growth factor 1 (IGF1), the expression of IGF2 mRNA was found both during early pregnancy and in the nonpregnant uterus (Schafer-Somi et al. 2008). Even though these data, which are mostly based on qualitative transcriptional analysis studies, still need further confirmation, they indicate the differential regulation of the uterine function in the pregnant vs nonpregnant dogs and suggest a possible role of the pre-implantation embryo in this process.

Together with IGF1 and 2, the increased endometrial expression of PRL belongs to the so-called markers of decidualization (Irwin et al. 1994, Ramathal et al. 2010). Recently (Kowalewski et al. 2011a), we have speculated that PRL acting through endo- and/or paracrine mechanisms might be involved in endometrial glandular secretory function in the dog. Furthermore, Bukowska et al. (2011) reported an increased expression of ITGA2B, ITGB2, and ITGB3 and of VEGF -165, -182, and -188 in the uterus of early-pregnant bitches.

Nevertheless, the factors and endocrine pathways regulating the functions of the pregnant uterus during the onset of canine pregnancy require further elucidation. Improving our knowledge about the establishment and composition of the proper uterine pre-implantation milieu could improve understanding of the etiopathogenesis of some frequently occurring diestral disorders of the uterus, such as endometrial hyperplasia complex. This disorder seems to originate from a dysregulated endocrinological response of the uterus to hormonal stimulation during the luteal phase of the estrous cycle and is considered by many authors as an initial phase in the development of pyometra. Furthermore, the poor outcome of IVF procedures in canids may also be related to an inappropriate environment for oocyte maturation, lacking growth factors required for the acquisition of full embryo developmental competence (Luvoni et al. 2006).

Consequently, the expression and cellular localization of several genes that are possibly differentially regulated during canine early pregnancy, including the so-called decidualization markers, were investigated during the pre-implantation stage of pregnancy and in corresponding uterine tissues from nonpregnant dogs. During this early stage of pregnancy, the survival and development of free-floating embryos are dependent on the intrauterine environment. The expression of CDH1, a cell adhesion protein, whose decreased expression is frequently associated with increased migratory activity of different cell types, was also evaluated. In addition, while limited by availability of the experimental material, the expression of selected genes was investigated when possible in embryos flushed from the early-pregnant uteri of bitches used for this study.
Materials and methods

Tissue collections

Uterine tissues from eight (n=8) early-pregnant (pre-implantation group, days 10–12 of pregnancy), crossbreed, healthy bitches were used for this study. The day of mating (day 0) was 2–3 days after ovulation, which was determined by vaginal cytology and by progesterone measurements (>5 ng/ml in peripheral blood). The pre-implantation stage of pregnancy was confirmed by flushing embryos from uteri. Dogs determined as nonpregnant in the uterine flushings served as negative controls (n=6). Uterine samples were collected via ovariohysterectomy. All experimental procedures were carried out in accordance with animal welfare legislation.

For the isolation of RNA, immediately after surgery, uterine tissues (including all anatomical layers) were trimmed of surrounding connective tissues and shock-frozen in liquid nitrogen; longer storage was at −80 °C.

For immunohistochemistry (IHC) and in situ hybridization (ISH), after surgery tissue samples were fixed for 24 h at +4 °C in 10% neutral phosphate buffered formalin. Afterwards, they were washed daily with PBS for one week, subsequently dehydrated in a graded ethanol series, and subsequently dehydrated in a graded ethanol series, and embedded in paraffin-equivalent HistoComp (Vogel, Giessen, Germany).

In addition, embryos sampled from five uteri (n=19) were available for this study. After careful evaluation under a stereomicroscope, embryos were classified into two groups: hatched blastocysts (n=12, 63%) and unhatched blastocysts (n=7, 37%), and immediately frozen and stored at −80 °C.

RNA isolation and reverse transcription (RT)

TRIZOL reagent (Invitrogen) was used following the manufacturer’s protocol in order to isolate total RNA from all samples investigated. The RNA content was measured with a NanoDrop 2000 u.v.–Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

For further purification of the RNA content, DNase treatment with RQ1 RNase-free DNase (Promega) was carried out following the manufacturer’s instructions. For each sample, 100–200 ng DNase-treated total RNA were used in the RT, and cDNA was synthesized using RT reagents purchased from Applied Biosystems, with random hexamers used as primers according to our previously published protocol (Kowalewski et al. 2006a, 2011a). All reactions were carried out in an Eppendorf Mastercycler (Vaudaux-Eppendorf AG, Basel, CH, Switzerland). The following RT conditions were applied: 8 min at 21 °C, then 15 min at 42 °C, after which the reaction was stopped by incubation for 5 min at 99 °C.

Homology cloning of canine-specific IGF1R

The canine-specific IGF1R cDNA had not been characterized before this study. Thus, to provide required data on the mRNA level, molecular cloning and sequencing were carried out. Using an online available predicted sequence, canine-specific IGF1R primers were designed and ordered from Microsynth AG (Balgach, CH, Switzerland): forward 5′-CTC GAC AAC CAG AAC TTG C-3′ and reverse 5′-GTG GTG CCC GTA AAG GTA AC-3′.

The GeneAmp Gold RNA PCR Kit from Applied Biosystems was used in a hot-start PCR according to our previously described protocol (Kowalewski et al. 2006a, 2011a). The annealing temperature was 58 °C. Total RNA obtained from at least three uterine samples was used, and PCR fragments comprising 717 bp of partial canine IGF1R were successfully amplified. The following negative controls were run for each experiment: autoclaved water used instead of cDNA (no template control) and the so-called RT-minus control, i.e., samples in which no RT reaction was carried out. The PCR products were separated on a 2% ethidium bromide-stained agarose gel extracted using a Qiaex II gel extraction kit (Qiagen GmbH), subcloned into pGEM-T vector (Promega), and transformed and amplified in XL1 Blue competent cells (Stratagene, La Jolla, CA, USA). After being purified with Pure Yield Plasmid MidiPrep System (Promega), bacterial plasmids were sequenced on both strands with T7 and Sp6 primers (Microsynth). Finally, the cloned sequence was submitted to GenBank with the following accession number: KF793925.

Real-time (TaqMan) PCR and data evaluation

Real-time (TaqMan) PCR analysis was carried out in an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems), in accordance with the manufacturer’s instructions and following our previously described protocol (Kowalewski et al. 2010, 2011b). The cDNA synthesis and negative controls were as described earlier for qualitative PCR. Fast Start Universal Probe Master (ROX) (Roche Diagnostics AG) was used. The semi-quantitation of target gene expression was performed using three independent endogenous reference genes (GAPDH, 18SrRNA, and cyclophilin A) in the comparative CT method (ΔΔCT method) as described previously (Kowalewski et al. 2010, 2011b) and according to the ABI 7500 Fast Real-Time PCR System manufacturer’s protocol. The efficiencies of the PCR assays were established by the CT slope method assuring ~100% reaction efficiency. Selected PCR products were sent for sequencing (Microsynth). Primers and 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethylrhodamine (TAMRA)-labeled TaqMan probes, provided by Microsynth, are listed in Table 1. The following canine-specific TaqMan Gene Expression Assays are commercially available and purchased from Applied Biosystems: cyclophilin A (Prod. No. Cf03986523_gH), CDH1 (Prod. No. Cf02624268_m1), IGF1 (Prod. No. Cf02627846_m1), and IGF2 (Prod. No. Cf02647136_m1).

An unpaired, two-tailed Student’s t-test was performed to compare the levels of target genes in uterine samples from early-pregnant and nonpregnant control dogs. Numerical data are presented as the mean ± s.d. Due to the uneven distribution of the RT-PCR data obtained for the expression of LHR, IGF1R and OTR, results are presented as geometric means with deviation factor (Xg. DF±).

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Table 1 List of primers and TaqMan probes used for the semi-quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession numbers</th>
<th>Primer sequence</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AB028142</td>
<td>Forward: 5'-GCT GCCAAATAT GACGACATC A-3'</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-GTA GCC CAG GAT TTT GCC GGG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TaqMan probe: 5'-TCC TCT GTA TCG GCT TTT CTT A-3'</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>FJ97658</td>
<td>Forward: 5'-GTC GCT CCG TCC TCT TCT ACT-3'</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-GGC TGA CCC GGT TGG TTT-3'</td>
<td></td>
</tr>
<tr>
<td>IGF1R</td>
<td>XM545828</td>
<td>Forward: 5'-ACA TGC CGA CCG GGC CGC TAC A-3'</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-CAC TCT CAC GAC CAA TGT TGT-3'</td>
<td></td>
</tr>
<tr>
<td>PRL</td>
<td>HQ267784</td>
<td>Forward: 5'-AGC CCT GGA CCG AGT CGG GCG-3'</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-AGA GTG CAT GCC TAC ACG TAT-3'</td>
<td></td>
</tr>
<tr>
<td>PGR</td>
<td>NM_001003074</td>
<td>Forward: 5'-AGG ATC ACC TCA GAA GAT TTG TGG-3'</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-ATT CAT GAG CTG GTC CAT TTC C-3'</td>
<td></td>
</tr>
<tr>
<td>ESR1</td>
<td>XM533454</td>
<td>Forward: 5'-AGG GCT GCC GGC ACC ACC AAC CA-3'</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-CCC TGC CTC GCG GGT GAT ATA-3'</td>
<td></td>
</tr>
<tr>
<td>ESR2</td>
<td>XM861041</td>
<td>Forward: 5'-AAC AGC CCC AAT TTC ATC ACA TAC C-3'</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-ATG CAT ATG CAT GAG TGC TTC CTT-3'</td>
<td></td>
</tr>
<tr>
<td>PTGER2</td>
<td>AF075602</td>
<td>Forward: 5'-ACT CCA TGA TGA TGA CCC TGA CC-3'</td>
<td>87</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>NM_001012344</td>
<td>Forward: 5'-AGG GCT TGC CAA GAT TGC-3'</td>
<td>74</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: 5'-GCC TGC GCT GCC TCA GGA-3'</td>
<td></td>
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<tr>
<td>PTGES</td>
<td>NM_001122854</td>
<td>Forward: 5'-AGG GCT GCC GGC TCG GCC AGT GAG-3'</td>
<td>89</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: 5'-ATG ACA GGC ACC ACC ACG GAG TAC TAC-3'</td>
<td></td>
</tr>
<tr>
<td>PTGER4</td>
<td>NM_001003054</td>
<td>Forward: 5'-ATC TCC CTC CCA ACC GCA TGC C-3'</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-GGA CGG TCC TCC CCA CCG GAC-3'</td>
<td></td>
</tr>
<tr>
<td>SLCO2A1</td>
<td>NM_001014967</td>
<td>Forward: 5'-TCC CTG CGA GGT GCA ACC AGT CAG-3'</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-AGT CCA TGC TGC TCC TCA AGT TGC ACT-3'</td>
<td></td>
</tr>
<tr>
<td>HPGD</td>
<td>NM_001284477</td>
<td>Forward: 5'-GGC GAG TGA TGG GCG GGA TCA TGA-3'</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-TTG TCT TCC ATC GAG TGA AGC AAT-3'</td>
<td></td>
</tr>
<tr>
<td>OTR</td>
<td>NM_001198659</td>
<td>Forward: 5'-TGC TGC GAG TGC AGT CGG GCG-3'</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-CTG CGT GAG TGC TCC CAC CCG CAC-3'</td>
<td></td>
</tr>
<tr>
<td>LHR</td>
<td>XM538486</td>
<td>Forward: 5'-ATG AGC AGA ATG CAG TGC TCA TCA-3'</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-AGG CCA TAT TCA TAT TAT GAG TCA-3'</td>
<td></td>
</tr>
</tbody>
</table>

GraphPad 3.06 (GraphPad Software, San Diego, CA, USA) was used. P < 0.05 was considered statistically significant.

**Immunohistochemistry (IHC)**

Formalin-fixed, paraffin-embedded uterine cross-sections (2–3 μm thick) from early-pregnant and nonpregnant bitches, mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany), were subjected to the standard immunoperoxidase detection method following our previously described protocol (Kowalewski et al. 2006b, 2010, Gram et al. 2013a). The list of primary antibodies and of the respective IgG irrelevant antibodies (negative/isotype controls) is presented in Table 2. Following biotinylated secondary antibodies were used (all at 1:100 dilution): horse anti mouse IgG BA-2000, goat anti guinea pig IgG BA-7000, goat anti rabbit IgG BA-1000 and horse anti goat IgG BA-9500, all from Vector Laboratories Inc. (Burlingame, CA, USA). Additionally, slides omitting the
Table 2 List of primary antibodies and isotype controls used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Name/antigen</th>
<th>Clone</th>
<th>Company</th>
<th>Immunogen</th>
<th>Concentration</th>
<th>Species/type</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFR1</td>
<td>bs-0227R</td>
<td>Bioss, Inc., Woburn, MA, USA</td>
<td>Ab against human IGFR1</td>
<td>1:800</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>IGFR2</td>
<td>IGF2 (H103) Sc-5622</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA</td>
<td>Ab against human IGF2</td>
<td>1:200</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>PGR</td>
<td>Clone 10A9</td>
<td>Immunotech, Hamburg, Germany</td>
<td>Ab against human C-terminal PGR</td>
<td>1:100</td>
<td>Mouse monoclonal</td>
</tr>
<tr>
<td>ESR1</td>
<td>NCL-ER-6F11</td>
<td>Novo Castra, Newcastle, UK</td>
<td>Ab against human ESR1</td>
<td>1:10</td>
<td>Mouse monoclonal</td>
</tr>
<tr>
<td>ESR2</td>
<td>MCA 1974</td>
<td>Serotec, Puchheim, Germany</td>
<td>Ab against human ESR2</td>
<td>1:20</td>
<td>Mouse monoclonal</td>
</tr>
<tr>
<td>PRLR</td>
<td>Catalog no. AF 1167</td>
<td>R&amp;D Systems Europe Ltd., Abingdon, UK</td>
<td>NSO-derived rhProlactin R extracellular domain</td>
<td>1:50</td>
<td>Goat polyclonal</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Clone 33</td>
<td>BD Pharmingen, Heidelberg, Germany</td>
<td>Anti-rat COX2 IgG</td>
<td>1:100</td>
<td>Mouse monoclonal</td>
</tr>
<tr>
<td>PGT</td>
<td>(G-17) Sc-103085</td>
<td>Santa Cruz Biotechnology</td>
<td>IgG against human PGT</td>
<td>1:100</td>
<td>Goat polyclonal</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>Custom made canine-specific antibody Gram et al. (2013a)</td>
<td>Eurogentec, Seraing, Belgium</td>
<td>IgG against canine-specific peptide sequence DTLFATHPDYPFNDDED, C-terminal amino acids 309–324</td>
<td>1:750</td>
<td>Guinea pig polyclonal</td>
</tr>
<tr>
<td>PTGES</td>
<td>Custom made canine-specific antibody</td>
<td>Eurogentec</td>
<td>IgG against canine-specific peptide sequence RSDQDVDRCLRRAHRND, C-terminal amino acids 61–76</td>
<td>1:300</td>
<td>Guinea pig polyclonal</td>
</tr>
<tr>
<td>HPGD</td>
<td>Custom made canine-specific antibody Gram et al. (2013a,b)</td>
<td>Eurogentec</td>
<td>IgG against canine-specific peptide sequence HFQDYETTPFHAKTQ, C-terminal amino acids 252–266</td>
<td>1:750</td>
<td>Guinea pig polyclonal</td>
</tr>
<tr>
<td>PTGER2</td>
<td>Catalog no. 101770</td>
<td>Cayman Chemicals, Ann Arbor, MI, USA</td>
<td>IgG against human PTGER2, C-terminal amino acids 335–358</td>
<td>1:200</td>
<td>Rabbit polyclonal</td>
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<tr>
<td>PTGER4</td>
<td>Catalog no. 101775</td>
<td>Cayman Chemicals, MI, USA</td>
<td>IgG against human PTGER4, C-terminal amino acids 459–488</td>
<td>1:100</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>Isotype control IgG</td>
<td>Vector Laboratories, Inc., Burlingame, CA, USA</td>
<td>Same protein concentration as primary antibody</td>
<td>Mouse</td>
<td></td>
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<tr>
<td>Isotype control IgG</td>
<td>Vector Laboratories, Inc.</td>
<td>Same protein concentration as primary antibody</td>
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<td>Isotype control IgG</td>
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<td>Isotype control IgG</td>
<td>Vector Laboratories Inc.</td>
<td>Same protein concentration as primary antibody</td>
<td>Guinea pig</td>
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</table>
primary antibodies served as negative controls. The nonspecific binding sites were blocked with either 10% horse serum or 10% normal goat serum, depending on the secondary antibody used in experiments. Peroxidase activity was detected using Liquid DAB+ substrate kit (Dako Schweiz AG, Baar, CH, Switzerland). The sections were counterstained with hematoxylin and embedded in Histokit (Assistant, Osterode, Germany).

In situ hybridization (ISH)

According to the protocol described previously (Kowalewski et al. 2006b, Gram et al. 2013a), nonradioactive ISH on paraffin-embedded sections was carried out in order to investigate the uterine cellular localization of IGF1 and IGF2 at the mRNA level.

PCR products generated with the following primers were used for subsequent synthesis of the digoxigenin (DIG)-labeled cRNA probes: IGF1 forward: 5'-GGT GGA GCC TCT TCA GTT C-3', reverse: 5'-TCC TGC ACT CCC TCT ACT TG-3' (product length 268 bp, annealing temperature 60°C) and IGF2 forward: 5'-GTT CTG TCT TGC TGC TTA C-3', reverse: 5'-GGT TAT CGT CGG AAG TTG TC-3' (product length 251 bp, annealing temperature 60°C). The DIG-labeled cRNA was detected using alkaline phosphatase-conjugated sheep anti-DIG Fab Fragments (Roche Diagnostics) at 1:5000 dilution in 1% ovine serum, according to the manufacturer's instructions. Signals were detected with the substrate 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (NBT/BCIP; Roche Diagnostics).

Results

Temporal expression of selected genes in canine uterine tissues during the pre-implantation stage of pregnancy

Expression of several genes, as listed in Table 1, was investigated at both the mRNA and protein levels in the uteri of early-pregnant animals (pre-implantation) and compared with their expression in the corresponding tissues from nonpregnant dogs (nonpregnant controls).

Expression of all the selected genes was detectable in tissue samples obtained from early-pregnant and nonpregnant dogs. A significantly higher expression of IGF2 and PRLR mRNA was observed in the early-pregnant uterus (P = 0.04 and P = 0.02 respectively) compared with the controls (Fig. 1). The opposite effect was observed for the uterine expression of LHR, which was significantly downregulated during early pregnancy (P = 0.01) (Fig. 1).

However, the expression of IGF1, IGF1R and PRL mRNA remained unaffected (P = 0.5, P = 0.47 and P = 0.42 respectively) by uterine exposure to embryos (Fig. 1). In addition, PRL mRNA was generally expressed at a very low level, and was frequently below the detection limit in both groups of animals.

Figure 1 Expression of IGF1, IGF2, IGF1R, PRL and its receptor (PRLR), and LHR as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its nonpregnant counterparts. Numerical data are presented either as the mean±s.d. (A, C, D, and E), or as geometric means with deviation factor (Xg, DF±1) (B and F). Bars with (*) differ at (C) P = 0.04, (E) P = 0.02, (F) P = 0.01.

Figure 2 Expression of ESR1, ESR2, PGR, OXTR, and CDH1 as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its nonpregnant counterparts. Numerical data are presented either as the mean±s.d. (A, B, C, and E), or as geometric means with deviation factor (Xg, DF±1) (D). Bar with (*) in (A) differs at: P = 0.03.
In contrast, a significant upregulation was noted for mRNA expression of PGE2-synthase PTGES in the endometrial stroma (Fig. 5B and C). No, or only very weak IHC signals were observed for the expression of IGF2 protein to the surface epithelial cells of the superficial and deep endometrial glands; clearly detectable signals were also localized in the myometrium. Weaker signals were observed in the endometrial stroma (Fig. 5B and C). No, or only very weak IHC signals were observed for IGFR1 protein expression to the surface epithelial cells of the superficial and deep endometrial glands.

Localisation of gene expression

In the pre-implantation uterus, IHC clearly localized IGF2 protein expression to the surface epithelial cells and to the epithelial cells of the superficial and deep uterine glands; clearly detectable signals were also localized in the myometrium. Weaker signals were observed in the endometrial stroma (Fig. 5B and C). No, or only very weak IHC signals were observed for IGF2 expression in the uteri of nonpregnant dogs (Fig. 5A). A localization pattern similar to this but in both early-pregnant and nonpregnant uteri was observed at the mRNA level by using ISH (Fig. 5H and I). As also determined by ISH, IGFI expression was co-localized with uterine IGF2 expression (Fig. 5F and G). There was no anti-IGF1 canine-specific antibody available for the IHC studies. The IGFR1 protein expression followed the IGFI and IGF2 distribution pattern with stronger signals observed in the early-pregnant uterus (Fig. 5D and E).

While weaker endometrial signals were observed for PRLR protein expression in the nonpregnant animals (Fig. 6A and B), stronger signals were detected in the surface and glandular endometrial epithelial cells of the early-pregnant uterus (Fig. 6C and D). Clear myometrial staining was noted in both groups of animals (Fig. 6B and D).

Expression of PGR was detectable in the uteri of both early-pregnant and control animals but did not vary widely between the two groups. The IHC signals were localized to the nuclei of both superficial and glandular epithelial cells, as well as the smooth muscle cells of the myometrium and to a lesser extent in the endometrial stroma (Fig. 7A and B).

The ESR1 protein was co-localized with PGR; however, signals were distinctly stronger in the uterus after exposure to embryos than in the nonpregnant controls (Fig. 7C and D). Much weaker staining was observed for uterine ESR2 expression which did not differ between the two groups and showed a similar localization pattern as the other nuclear receptors (Fig. 7E and F).

Whereas endometrium stained negatively for COX2, myometrial signals were strong (Fig. 8A), but no or only...
very weak uterine signals were observed for AKR1C3 protein, in both groups of animals (Fig. 8B).

The expression of SLCO2A1, PTGES, PTGER2, and PTGER4 revealed a similar protein distribution pattern as that of PRLR, showing their co-localization and higher abundance in endometrial epithelial cells, the myo-
meterium, and the stromal cells in the pre-implantation uterus (Figs 8C, D, and 9A, B, C, D, E, F). For all these factors, staining in the endometrial stroma was weaker than in the epithelial compartments. High variability for the HPGD IHC signals was observed between individual animals in both groups. They tended, however, to be stronger in the early-pregnant uteri and revealed a similar localization pattern, but with
distinctly stronger staining in the deep uterine glands (Fig. 8E, F, and G).

Expression of selected genes in early, free-floating canine embryos before implantation

Due to the limited availability of the embryo material, investigations were restricted for detecting the expression of IGF1, IGF2, PTGS2, and PTGES in the two groups of embryos (unhatched and hatched blastocysts) collected from five early-pregnant bitches. Embryos were pooled in order to reach the required limits of detection. Consequently, no statistical analysis of gene expression was possible.

Whereas the expression of IGF1, IGF2, and PTGS2 mRNA was below the detection limit in the unhatched embryos, the PTGES mRNA was abundantly expressed and detectable in both unhatched and hatched blastocysts, with apparently higher expression level in the latter ones (Fig. 10A). The expression of IGF2 mRNA seemed to be higher than that of IGF1 mRNA in the group of hatched embryos (Fig. 10B), while PTGS2 and PTGES showed similar transcript abundance (Fig. 10C).

Discussion

The uterine response to early embryo exposure was investigated during the pre-implantation stage of canine pregnancy by measuring the expression of several target genes. Our investigations were based on the assumption that, in view of the lack of an anti-luteolytic signal in the dog resulting in similar hormonal status in early-pregnant and nonpregnant dioestric bitches, some local effects would be exerted by the early pre-implantation embryos that modify the uterine milieu, serving as a prerequisite for a successful implantation and ensuring embryo survival before attachment. The effects of seminal plasma-derived bioactive factors in the modulation of the uterine endocrine milieu, and possibly having an impact on the initiation of pregnancy in dogs, were not separately investigated in this study, but would certainly merit future investigations.

Among the genes investigated, IGF1, IGF2, and PRL count as the most prominent and well-characterized markers of the decidualization process (Irwin et al. 1994, Ramathal et al. 2010). By interacting mainly with the IGF1R (Wang & Chard 1999), both IGF1 and IGF2 are mitogenic factors whose uterine expression is regulated...
by steroidogenic hormones (De Cock et al. 2002, Bhatti et al. 2007, Dantzer & Swanson 2012). They possess differentiation properties capable of influencing embryonic development, as shown, e.g., in humans and ruminants (Wathes et al. 1998, Irwin et al. 1999, Kim et al. 2008). In addition, in human decidua, for example, IGF1 regulates PRL and arachidonic acid secretion (Handwerger et al. 1991), the latter serving as a common precursor for PG synthesis.

As for the early-pregnant canine uterus investigated in this study, only the expression of IGF2 was significantly upregulated. This was concomitant with the greater abundance of IGF2 transcripts in the hatched embryos collected at days 10–12 of canine pregnancy, suggesting the predominant role of IGF2 compared with IGF1, during this very early stage of pregnancy in the dog. In contrast to the unaffected IGF1R-mRNA expression levels, which varied widely among individuals, expression of the respective protein was clearly detectable and seemed to be more strongly expressed during early pregnancy, implying the involvement of posttranscriptional regulatory mechanisms in its expression.

The uterine expression of PRLR, but not of PRL, was strongly upregulated after embryo exposure.

Figure 7 Immunohistochemical localization of PGR (A and B), ESR1 (C and D) and ESR2 (E and F) in early-pregnant (pre-implantation) canine uterus and corresponding nonpregnant uterus. Solid arrows, superficial (luminal) uterine epithelium; open arrows, superficial uterine glands; solid arrowheads, deep uterine glands, open arrowhead in (B) indicates myometrium. Insets to (D and F) show the myometrial expression of ESR1 and ESR2, respectively. The inset to (A) shows a representative IgG isotype control for anti-mouse immune serum.
pre-implantation. This finding agrees with our previous report about the uterine and placental expression of PRLR (Kowalewski et al. 2011), implying the possible role of PRLR-mediated effects in endometrial glandular secretory activity during the production of uterine milk (histiotrophe), a mechanism that was also suggested for humans (Jabbour et al. 1998). Our previous observation that interfering with PGR function by applying an anti-gestagen results in a decreased utero/placental PRLR expression suggests that this involves progesterone-mediated effects (Kowalewski et al. 2011a). In this study, although uterine PRL expression was relatively low, and frequently even below the detection limit, possible paracrine effects of locally produced PRL cannot be ruled out. Its contribution to circulating PRL levels does not seem, however, very likely. In contrast to PRLR expression, the expression of LHR was down-regulated in the early-pregnant canine uterus. Recently, the role of LHR was suggested as a possible important factor contributing to the implantation process in mice (Grîdelet et al. 2013). While any final conclusion concerning LHR function during the onset of canine pregnancy would be premature, we believe that this warrants further investigations.

Besides acting as one of the most potent uterotonic hormones, oxytocin also regulates secretion of other...
hormones, e.g., PGs (Meier et al. 1995, Fuchs et al. 1999). This prompted us to investigate the expression of the OTR in the early-pregnant uterus and its corresponding nonpregnant counterpart. Most recently we have localized OTR to the uterine surface epithelium of the pre-implantation uterus, specifically in the superficial and deep glands and the vascular endothelial and stromal cells (Gram et al. 2013b). In this study, however, OTR expression varied widely among individuals and, consequently, did not differ significantly between the two groups, thereby not allowing any further conclusions to be drawn regarding its potential secretory or constrictory activity during the onset of canine pregnancy. Such activity could relate, e.g., to mechanisms involved in the distribution and positioning of free-floating embryos before attachment, or to the role of oxytocin as a mediator of local PG effects.

The expression of PTGS2, the rate-limiting factor in the provision of PGs, and of AKR1C3 protein, was low in the early dioestric uterus and remained unaffected by the presence of embryos. The AKR1C3 is the only canine-specific isoform of PGFS known to date and is responsible for the direct conversion of PGH2 to PGF2α (Gram et al. 2013a). As the expression of the

Figure 9 Immunohistochemical localization of prostaglandin E2-synthase (PGE2-synthase, PTGES) (A and B), PGE2-receptors PTGER2 and PTGER4 (C and D) and (E and F), respectively, in the early-pregnant (pre-implantation) canine uterus and corresponding nonpregnant uterus. Solid arrows, superficial (luminal) uterine epithelium; open arrows, uterine glands. Insets to (B, D, and F) show the myometrial expression of PTGES, PTGER2 and PTGER4, respectively. The inset to (A) shows a representative IgG isotype control for anti-guinea pig immune serum.
respective mRNA was significantly increased in early pregnancy, some local effects and possible involvement of posttranscriptional regulatory mechanisms cannot be excluded, especially in view of the concomitantly increased expression of PGF2α-receptor (PTGFR).

In contrast, the expression of PTGES was significantly affected both at the mRNA and protein levels in response to early embryo exposure, which together with the upregulated expression of SLCO2A1 further implies local effects of PGs. This conclusion also agrees with our previous report suggesting a role of PGs in canine decidualization, placentation and, later on, in trophoblast invasion (Kowalewski et al. 2010, Gram et al. 2013a). The low levels of PTGS2 expression, together with clearly detectable uterine HPGD expression (the enzyme responsible for conversion of PGE2 and PGF2α to their inactive metabolites) in pregnant and nonpregnant uteri, could additionally coordinate and restrict the effects of PGs as local regulatory factors.

By acting through its two G-protein-coupled receptors designated as PTGER2 and PTGER4, PGE2 exerts its roles mostly through the cAMP/PKA signaling pathway (Christenson et al. 1994, Boiti et al. 2001, Harris et al. 2001, Arosh et al. 2004). Also, progesterone-dependent decidualization is cAMP-mediated and this process is accelerated by PGF2 in human endometrial stromal cells (Brar et al. 1997). Similar effects of PGE2 and its potential to stimulate the decidual cell reaction were observed in rats (Kennedy & Doktorcik 1988). This could also be true for the canine species, as indicated in the present study by the increased uterine synthesis of PTGES, PTGER2, and also PTGER4 protein, concomitant with the higher PTGES expression in the hatched embryos, possibly actively contributing to the decidualization process.

It is noteworthy that in the uterine samples investigated in this study, derived from both early-pregnant and nonpregnant bitches, stronger IHC signals were localized in the endometrial epithelial compartments. On the other hand, weaker signals were observed in the uterine stromal cells that undergo a strong, species-specific decidualization process later on in canine gestation. This, together with the unaffected IGF1 and strongly varying IGF1R mRNA expression in the pre-implantation uterus, seems to be an indicator of the early stage of uterine differentiation at the beginning of pregnancy (gestational days 10–12) observed in our study. At this time, the uterine morphology is characterized by obvious embryo-induced functional changes reflected in the modified uterine milieu observed here, but does not yet exhibit the very strong structural changes related to the intense remodeling of uterine tissues that occur later in gestation, especially during decidua formation at the implantation sites. This is also indicated by the unaffected expression of CDH1.

Taken together with some earlier studies, our investigations describe the expression of genes that are differentially regulated in response to the presence of free-floating embryos in the uterine lumen of early-pregnant dogs and provide a basis for better understanding of the uterine milieu required for proper embryo development and, thereby, for successful establishment of canine pregnancy. Elucidating possible functional interactions between these factors, e.g., their role in uterine growth and secretory activity, could be helpful in understanding some pathological conditions connected with dysregulated endocrinological responses of the canine uterus.

Figure 10 Embryonal expression of prostaglandin E2-synthase (PTGES), cyclooxygenase 2 (PTGS2), insulin-like growth factor 1 (IGF1) and IGF2 as determined by Real Time (TaqMan) PCR.
uterus, which are frequently caused by impaired cross-talk between growth factors and hormones.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The research work was supported by The Swiss National Science Foundation (SNSF) grant number 31003A_140947 to MPK.

Acknowledgements

The authors are grateful to Dr Barry Bavister for careful editing of the manuscript. The technical expertise and contributions of Elisabeth Högger and Stefanie Ihle are gratefully appreciated.

References

Irwin JF, de las Fuentes L & Giudice LC 2001a Effect of interleukin-1α on the decidual cell reaction in the rat. Reproductive Toxicology 15 207–219. (doi:10.1016/S0890-6238(00)00088-3)


Received 6 December 2013
First decision 8 January 2014
Revised manuscript received 17 January 2014
Accepted 30 January 2014