REPRODUCTION
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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. *PGES* (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.

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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*. 2006*a*, 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,

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2009, 2013). 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), gonadotropic 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*. 2011*a*), 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 diestrual 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 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*. 2006*a*, 2011*a*). 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′-GTT GTG GCG 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*. 2006*a*, 2011*a*). 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 system (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, 2011*b*). 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 *C*T method (ΔΔ*C*T method) as described previously (Kowalewski *et al*. 2010, 2011*b*) 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. $DF^{\pm 1}$). The statistical software program

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

Primer	Accession numbers	Primer sequence	Product lenght (bp)
GAPDH	AB028142	Forward: 5′-GCT GCCAAATAT GACGACATC A-3′ Reverse: 5′-GTA GCC CAG GAT GCC TTT GAG-3′ TaqMan probe: 5′-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3′	75
18SrRNA	FJ797658	Forward: 5′-GTC GCT CGC TCC TCT CCT ACT-3′ Reverse: 5′-GGC TGA CCG GGT TGG TTT-3′ TaqMan probe: 5′-ACA TGC CGA CGG GCG CTG AC-3′	125
IGF1R	XM545828	Forward: 5′-GGA CGT TGA GCC TGG CAT T-3′ Reverse: 5′-CAC TCT TAG CCC CAC GGA TGT-3′ TaqMan probe: 5′-AGC CCT GGA CGC AGT ATG CGG-3′	119
PRLR	HQ267784	Forward: 5′-GGA TCT TTG TGG CCG TTC TTT-3′ Reverse: 5′-AAG GAT GCA GGT CAC CAT GCT AT-3′ TaqMan probe: 5′-ATT ATG GTC GTA GCA GTG GCT TTG AAA GGC-3′	92
PRL	NM_00108275	Forward: 5′-CAA GCC CAA CAG ATC CAC CAT-3′ Reverse: 5′-ATC CCC CGC ACT TCT GTG A-3′ TaqMan probe: 5′-CTG AGG GTG CTG CGC TCC TGG-3′	104
PGR	NM_001003074	Forward: 5′-CGA GTC ATT ACC TCA GAA GAT TTG TTT-3′ Reverse: 5′-CTT CCA TTG CCC TTT TAA AGA AGA-3′ TaqMan probe: 5′-AAG CAT CAG GCT GTC ATT ATG GTG TCC TAA CTT-3′	113
ESR1	XM533454	Forward: 5′-CCC ATG GAG GAG ACA AAC CA-3′ Reverse: 5′-CCC TGC CTC CTC GGT GAT ATA-3′ TaqMan probe: 5′-CAC GGG CCC AAC TTC ATC ACA TTC C-3′	93
ESR2	XM861041	Forward: 5′-CCC AGC ACG CCC TTC A-3′ Reverse: 5′-AAT CAT ATG CAC GAG TTC CTT GTC-3′ TaqMan probe: 5′-CCT CCA TGA TGA TGT CCC TGA CC-3′	78
PTGS2	HQ110882	Forward: 5′-GGA GCA TAA CAG AGT GTG TGA TGT G-3′ Reverse: 5′-AAG TAT TAG CCT GCT CGT CTG GAA T-3′ TaqMan probe: 5′-CGC TCA TCA TCC CAT TCT GGG TGC-3′	87
AKR1C3	NM_001012344	Forward: 5′-AGG GCT TGC CAA GTC TAT TGG-3′ Reverse: 5′-GCC TTG GCT TGC TCA GGA T-3′ TaqMan probe: 5′-TCC AAC TTT AAC CGC AGG CAG CTG G-3′	74
PTGES	NM_001122854	Forward: 5′-GTC CTG GCG CTG GTG AGT-3′ Reverse: 5′-ATG ACA GCC ACC ACG TAC ATC T-3′ TaqMan probe: 5′-TCC CAG CCT TCC TGC TCT GCA GC-3′	89
PTGFR (FP)	NM_001048097	Forward: 5′-ACC AGT CGA ACA TCC TTT GCA-3′ Reverse: 5′-GGC CAT CAC ACT GCC TAG AAA-3′ TaqMan probe: 5′-CAT GGT GTT CTC CGG TCT GTG CCC-3′	86
PTGER2	AF075602	Forward: 5′-CAC CCT GCT GCT GCT TCT C-3′ Reverse: 5′-CGG TGC ATG CGG ATG AG-3′ TaqMan probe: 5′-TGC TCG CCT GCA ACT TTC AGC GTC-3′	78
PTGER4	NM_001003054	Forward: 5′-AAA TCA GCA AAA ACC CAG ACT TG-3′ Reverse: 5′-GCA CGG TCT TCC GCA GAA-3′ TaqMan probe: 5′-ATCCGA ATT GCT GCT GTG AAC CCT ATC C-3′	96
SLCO2A1	NM_001011558	Forward: 5′-TGC AGC ACT AGG AAT GCT GTT C-3′ Reverse: 5′-GGG CGC AGA GAA TCA TGG A-3′ TaqMan probe: 5′-TCT GCA AAC CAT TCC CCG CGT G-3′	116
HPGD	NM_001284477	Forward: 5′-GGC AGC GAA TCT CAT GAA CAG-3′ Reverse: 5′-TCT TCT TTC TCA ATG GAT TCA AGGA-3′ TaqMan probe: 5′-TGA ATG CCA TTT GCC CAG GCT TTG-3′	93
OTR	NM_001198659	Forward: 5′-GGA TCA CGC TCT CCG TCT ACA-3′ Reverse: 5′-CGT CTT GAG TCG CAG GTT CTG-3′ TaqMan probe: 5′-CCT GCT ACG GCC TCA TCA GCT TCA A-3′	98
LHR	XM538486	Forward: 5′-TCA TCA TTT GTG CTT GCT ACA TTA AA-3′ Reverse: 5′-CGC CAT TTT CTT AGC AAT CTT TG-3′ TaqMan probe: 5′-TGC AGT TCA AAA TCC AGA GCT GAT GGC-3′	98

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*. 2006*b*, 2010, Gram *et al*. 2013*a*). 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.

Name/ antigen	Clone	Company	Immunogen	Concentration	Species/type
IGFR1	bs-0227R	Bioss, Inc., Woburn, MA, USA	Ab against human IGFR1	1:800	Rabbit polyclonal
IGF2	IGF2 (H103) Sc-5622	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	Ab against human IGF2	1:200	Rabbit polyclonal
PGR	Clone 10A9	Immunotech, Hamburg, Germany	Ab against human C-terminal PGR	1:100	Mouse monoclonal
ESR1	NCL-ER-6F11	Novo Castra, Newcastle, UK	Ab against human ESR1	1:10	Mouse monoclonal
ESR2	MCA 1974	Serotec, Puchleim, Germany	Ab against human ESR2	1:20	Mouse monoclonal
PRLR	Catalog no. AF 1167	R&D Systems Europe Ltd. Abingdon, UK	NSO-derived rhProlactin R extracellular domain	1:50	Goat polyclonal
PTGS2	Clone 33	BD Pharmingen, Heidelberg, Germany	Anti-rat COX2 IgG	1:100	Mouse monoclonal
PGT	(G-17) Sc-103085	Santa Cruz Biotechnology	IgG against human PGT	1:100	Goat polyclonal
AKR1C3	Custom made canine-specific antibody Gram *et al.* (2013*a*)	Eurogentec, Seraing, Belgium	IgG against canine-specific peptide sequence DTLFATHPDYPFNDED, C-terminal amino acids 309–324	1:750	Guinea pig polyclonal
PTGES	Custom made canine-specific antibody	Eurogentec	IgG against canine-specific peptide sequence RSDQDVDRCLRAHRND, C-terminal amino acids 61–76	1:300	Guinea pig polyclonal
HPGD	Custom made canine-specific antibody Gram *et al.* (2013*a*,*b*)	Eurogentec	IgG against canine-specific peptide sequence HFQDYETTPFHAKTQ, C-terminal amino acids 252–266	1:750	Guinea pig polyclonal
PTGER2	Catalog no. 101770	Cayman Chemicals, Ann Arbor, MI, USA	IgG against human PTGER2, C-terminal amino acids 335–358	1:200	Rabbit polyclonal
PTGER4	Catalog no. 101775	Cayman Chemicals, MI, USA	IgG against human PTGER4, C-terminal amino acids 459–488	1:100	Rabbit polyclonal
Isotype control	IgG	Vector Laboratories, Inc., Burlingame, CA, USA	–	Same protein concentration as primary antibody	Mouse
Isotype control	IgG	Vector Laboratories, Inc.	–	Same protein concentration as primary antibody	Rabbit
Isotype control	IgG	Vector Laboratories, Inc.	–	Same protein concentration as primary antibody	Goat
Isotype control	IgG	Vector Laboratories Inc.	–	Same protein concentration as primary antibody	Guinea pig

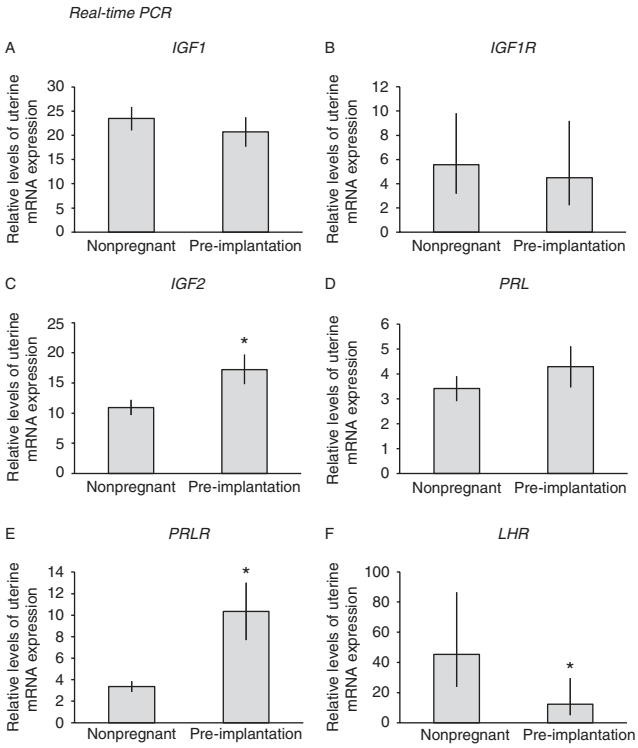

Figure 1 Expression of *IGF1*, *IGF2*, *IGF1R*, *PRL* and its receptor (*PRLR*), and *LHR* as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its nonpregnant counterparts. Numerical data are presented either as the mean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

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.* 2006*b*, Gram *et al.* 2013*a*), 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 CGC 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′-GTG CTG CTT TGC TGC TTA C-3′, reverse: 5′-GGG TAT CTG GGG 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.

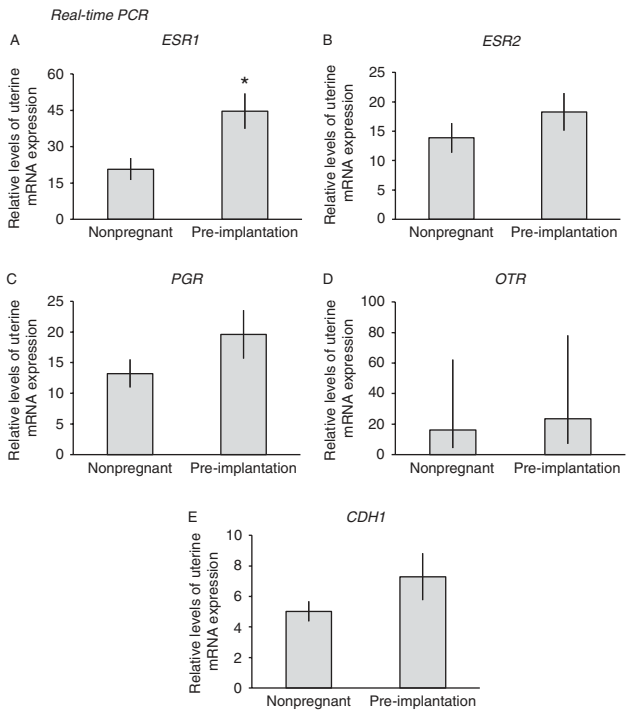

Figure 2 Expression of *ESR1*, *ESR2*, *PGR*, *OXTR*, and *CDH1* as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its nonpregnant counterparts. Numerical data are presented either as the mean ± S.D. (A, B, C, and E), or as geometric means with deviation factor (Xg. DF$^{\pm}$1) (D). Bar with (*) in (A) differs at: $P=0.03$.

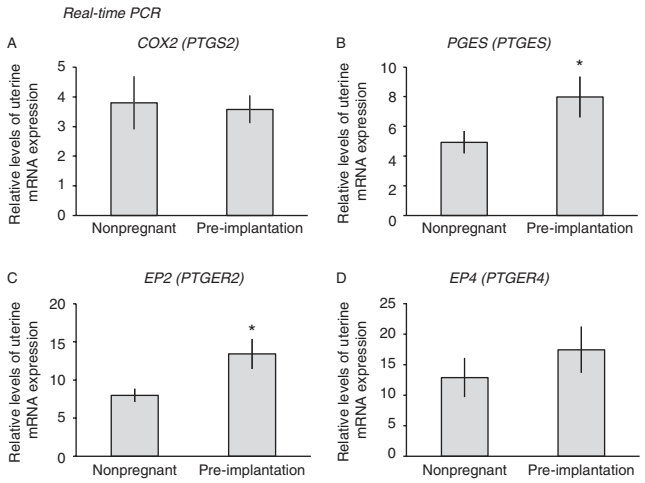

Figure 3 Expression of prostaglandin-endoperoxide synthase 2 (PTGS2, COX2), prostaglandin E2 (PGE2)-synthase (*PTGES*) and of PGE2 receptors, *PTGER2* and *PTGER4* as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its nonpregnant counterparts. Numerical data are presented as the mean±S.D. Bars with (*) differ at: (B) $P=0.04$, (C) $P=0.02$.

Among the steroid hormone receptors, only the expression of estrogen receptor α (*ESR1*) was significantly ($P=0.03$) elevated in the early-pregnant uterus (Fig. 2), while expression of the *PGR* and of *ESR2* did not differ between the two groups ($P=0.27$ and $P=0.23$ respectively). Similarly, expression of the *OTR*, which varied widely among individuals in both groups, was unaffected ($P=0.5$) by the reproductive status of the animals. The expression of *CDH1* did not differ ($P=0.25$).

Concerning the expression of the major members of the PG system, no statistically significant differences were observed for the expression of mRNA encoding for the prostaglandin-endoperoxide synthase 2 (*PTGS2*, formerly known as *COX2*) ($P=0.8$), the PGE2 receptor designated as *PGER4* ($P=0.21$), and 15-hydroxy prostaglandin dehydrogenase (*HPGD*) ($P=0.29$) (Figs 3A, D, and 4D). In contrast, a significant upregulation was noted for mRNA expression of PGE2-synthase *PTGES* ($P=0.04$) and *AKR1B3* ($P=0.007$), as well as of their respective receptors, *PTGER2* ($P=0.02$) and *PTGFR* ($P=0.02$) and of the PG-transporter (*PGT*) ($P=0.02$), in the pre-implantation uterus compared with nonpregnant uterus (Figs 3B, C, and 4A, B, C).

Localization 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, *IGF1* 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 IGF1R protein expression followed the *IGF1* 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 uteri 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 for the other nuclear receptors (Fig. 7E and F).

Whereas endometrium stained negatively for COX2, myometrial signals were strong (Fig. 8A), but no or only

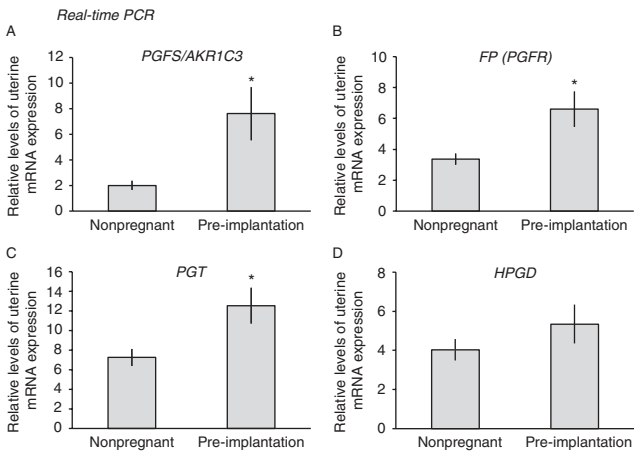

Figure 4 Expression of (A) prostaglandin F2α (PGF2α)-synthase (*AKR1C3*), (B) PGF2α-receptor (*FP, PTGFR*), (C) prostaglandin transporter (*PGT*) and of (D) 15-prostaglandin dehydrogenase (*HPGD*) as determined by Real Time (TaqMan) PCR in uterine samples from early-pregnant uterus (pre-implantation; embryo-induced effects), and its nonpregnant counterparts. Numerical data are presented as the mean±S.D. Bars with (*) differ at (A) $P=0.007$, (B) $P=0.02$, (C) $P=0.02$.

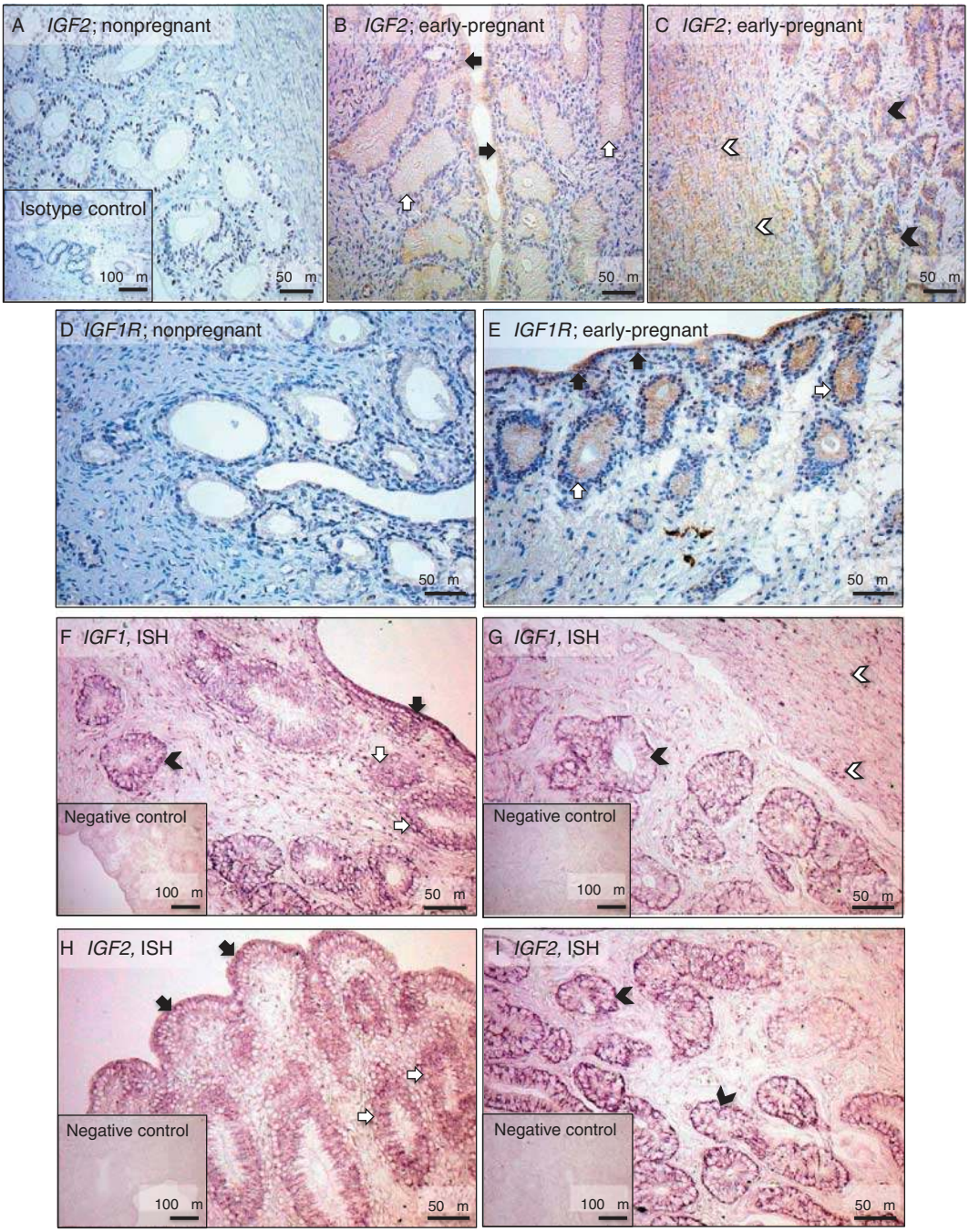

Figure 5 IHC localization of *IGF2* (A, B, and C) and *IGF1R* (D and E), in early-pregnant (pre-implantation) canine uterus and corresponding nonpregnant uterus. The localization of *IGF1* (F and G) and *IGF2* (H and I) mRNA expression is presented by *in situ* hybridization (ISH). Solid arrows, superficial (luminal) uterine epithelium; open arrows, superficial uterine glands; solid arrowheads, deep uterine glands; open arrowheads, myometrium. The inset to (A) shows a representative IgG isotype control for anti-rabbit immune serum.

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 myometrium, 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

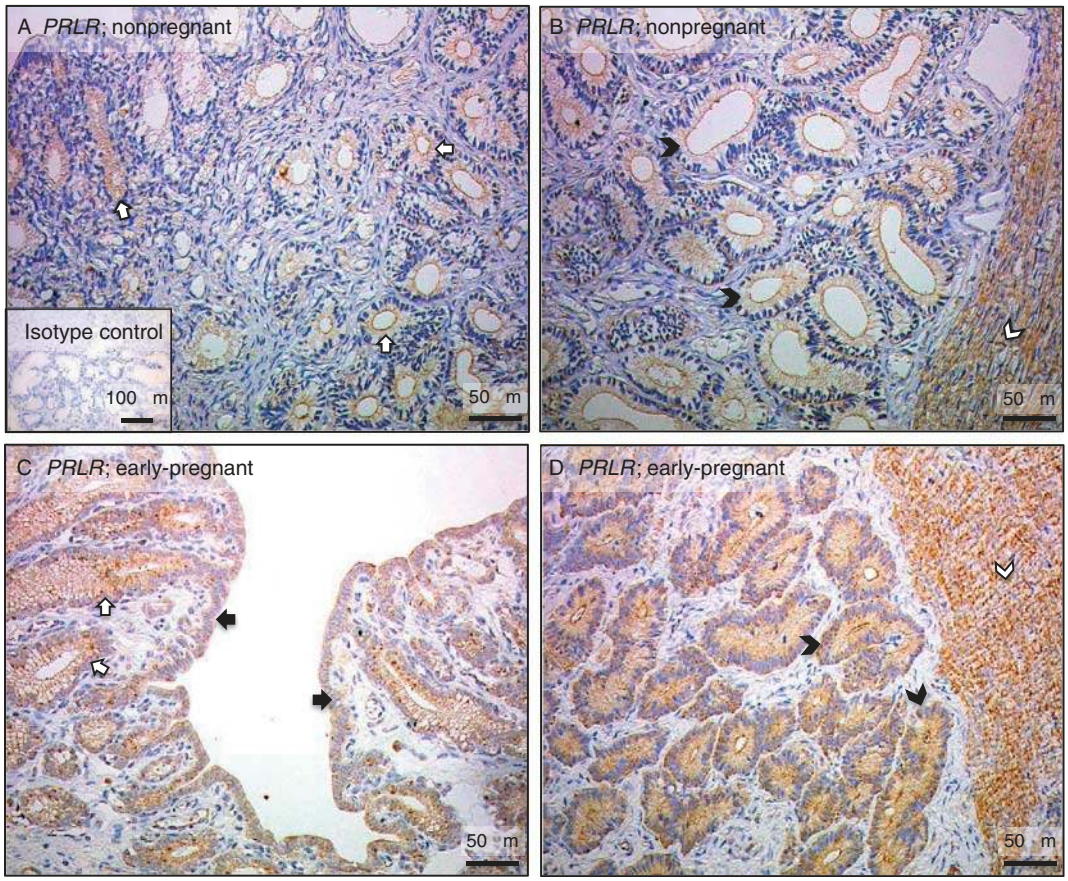

Figure 6 Immunohistochemical localization of *PRLR* in early-pregnant (pre-implantation) canine uterus (C and D) and corresponding nonpregnant uterus (A and B). Solid arrows, superficial (luminal) uterine epithelium; open arrows, superficial uterine glands; solid arrowheads, deep uterine glands; open arrowheads, myometrium. The inset to (A) shows a representative IgG isotype control for anti-goat immune serum.

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

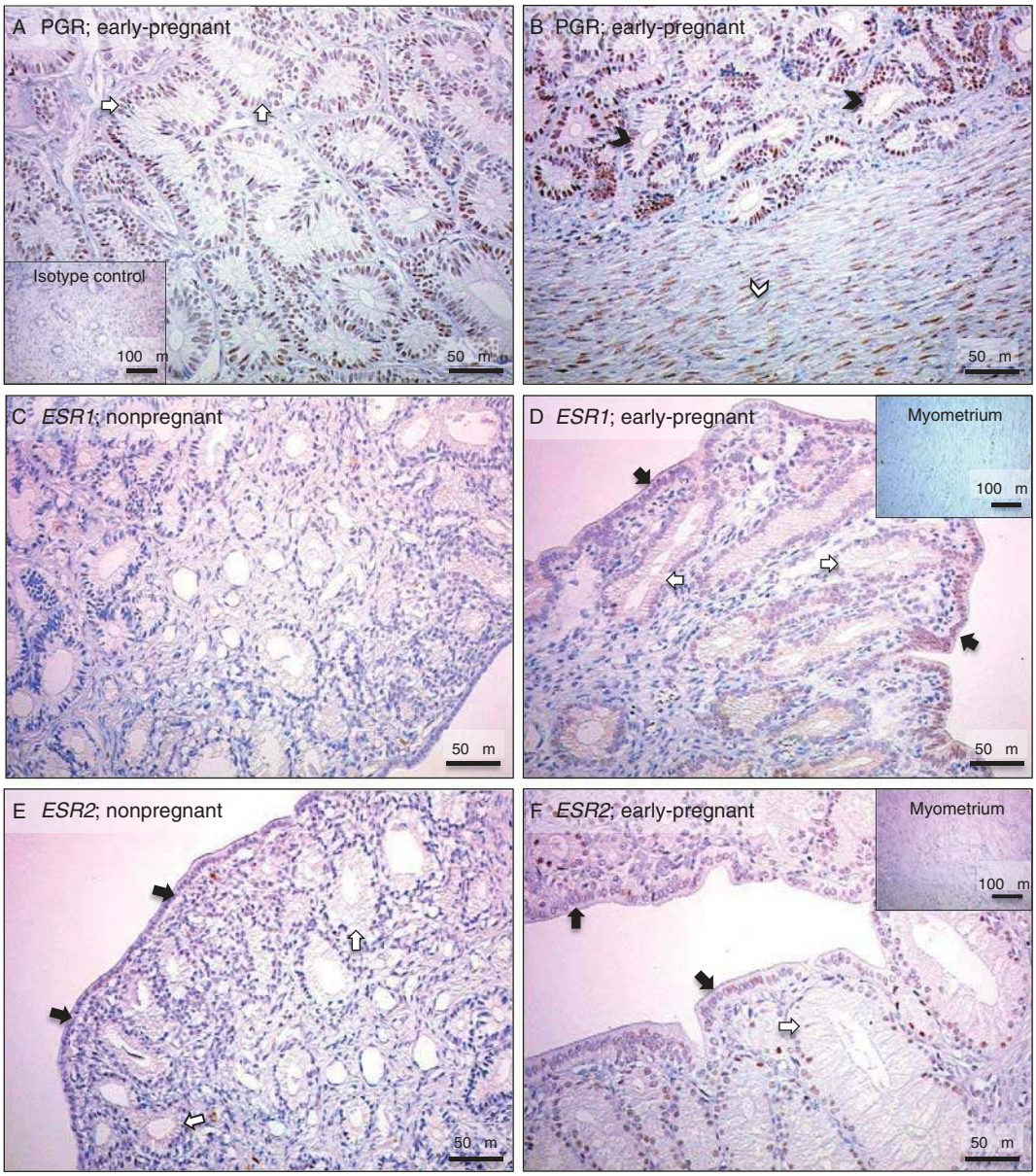

Figure 7 Immunohistochemical localization of PGR (A and B), *ESR1* (C and D) and *ESR2* (E and F) in early-pregnant (pre-implantation) canine uterus and corresponding nonpregnant uterus. Solid arrows, superficial (luminal) uterine epithelium; open arrows, superficial uterine glands; solid arrowheads, 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.

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

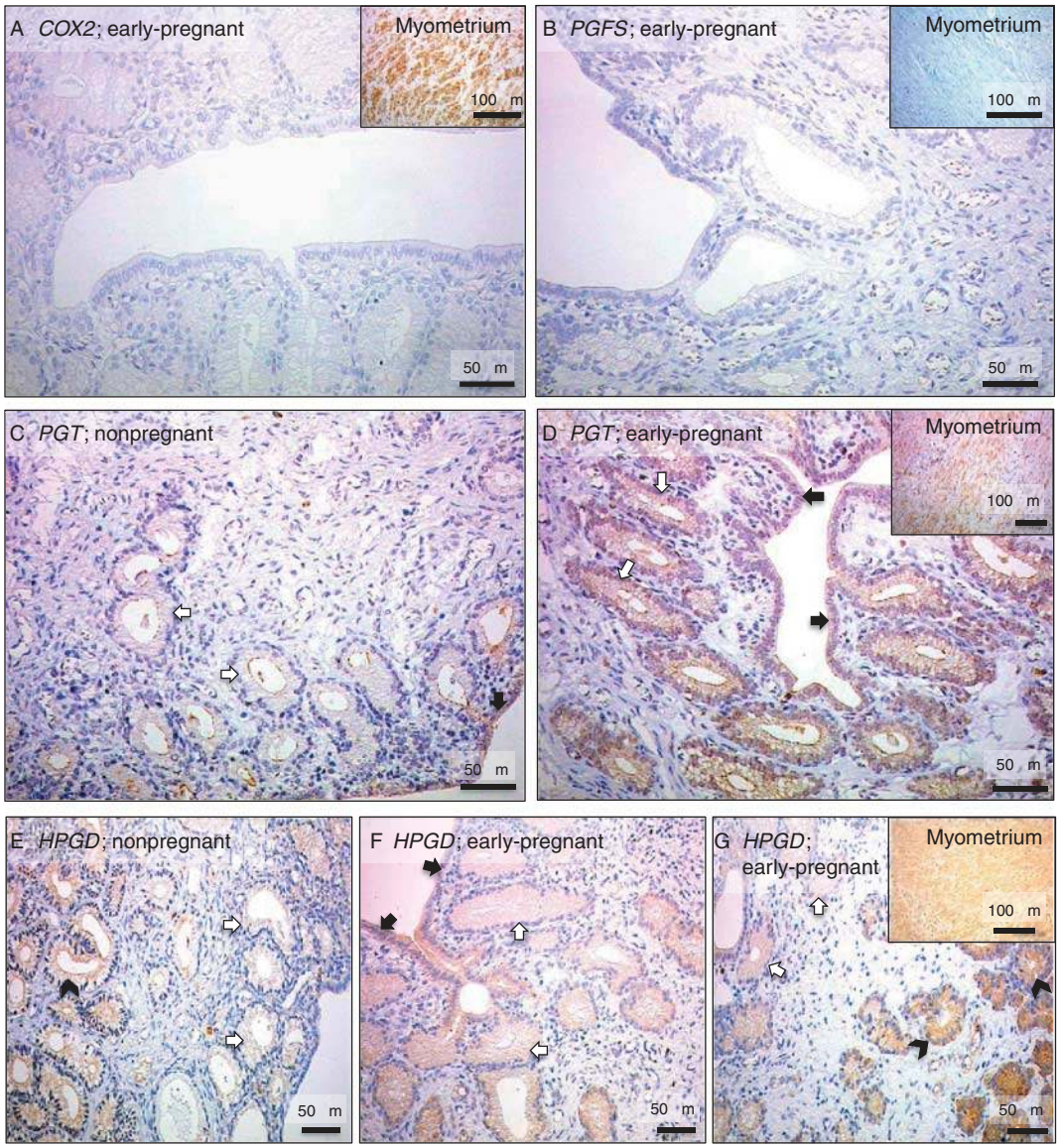

Figure 8 Immunohistochemical localization of *COX2*, (*PTGS2*) (A), *PGFS*/*AKR1C3* (B) in early-pregnant (pre-implantation) canine and of *PGT* (C and D) and *HPGD* (E, F, and G) in early pre-implantation uterus and corresponding nonpregnant uterus. Solid arrows, superficial (luminal) uterine epithelium; open arrows, superficial uterine glands; solid arrowheads, deep uterine glands. Insets in (A, B, D and G) show the myometrial expression of *COX2*, *AKR1C3*, *SLCO2A1*, and *HPGD* respectively.

pre-implantation. This finding agrees with our previous report about the uterine and placental expression of *PRLR* (Kowalewski *et al*. 2011*a*), 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*. 2011*a*). 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 (Gridelet *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

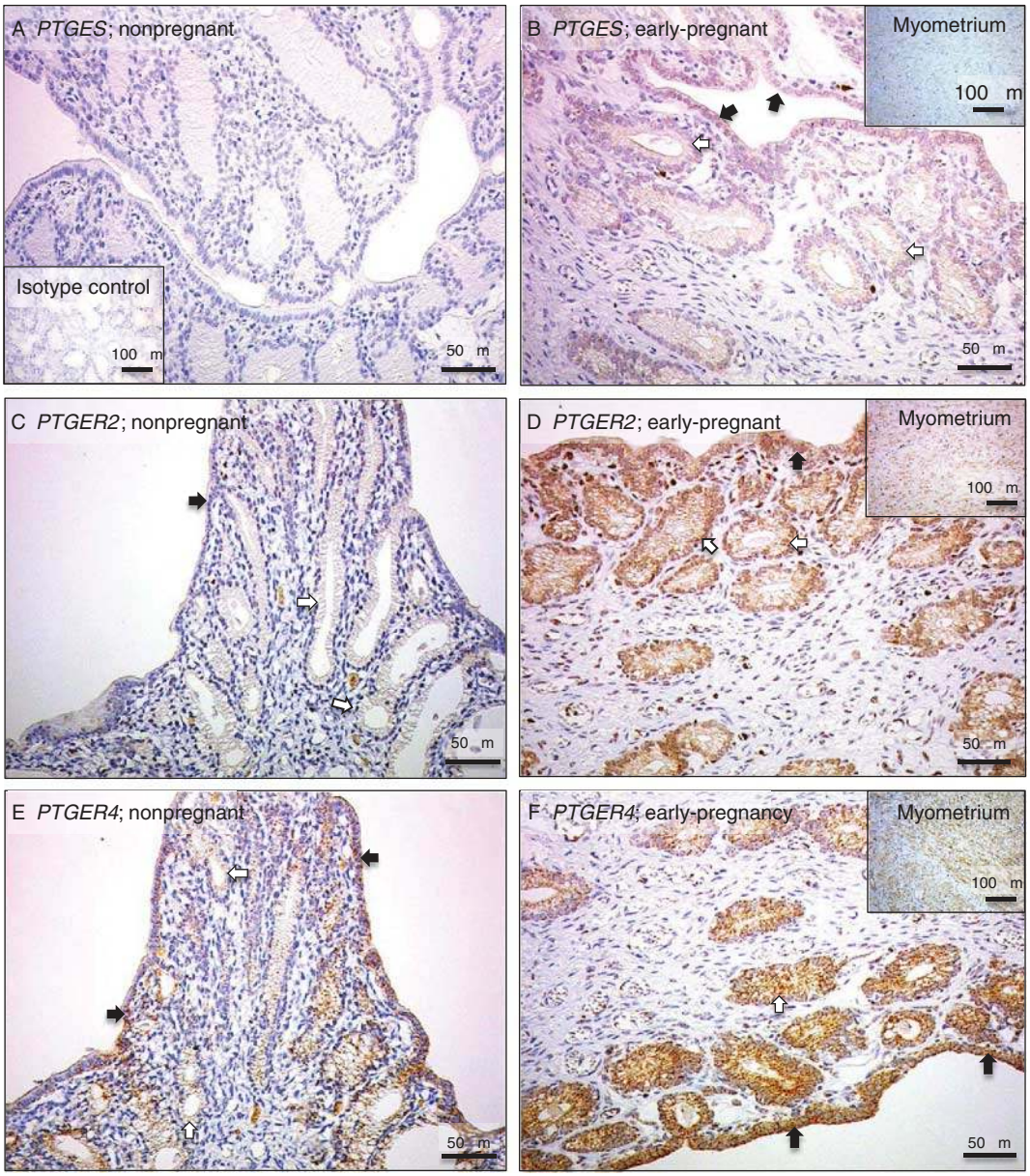

Figure 9 Immunohistochemical localization of prostaglandin E2-synthase (PGE2-synthase, PTGES) (A and B), PGE2-receptors *PTGER2* and *PTGER4* (C and D) and (E and F), respectively, in the early-pregnant (pre-implantation) canine uterus and corresponding nonpregnant uterus. Solid arrows, superficial (luminal) 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.

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.* 2013*b*). 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.* 2013*a*). As the expression of the

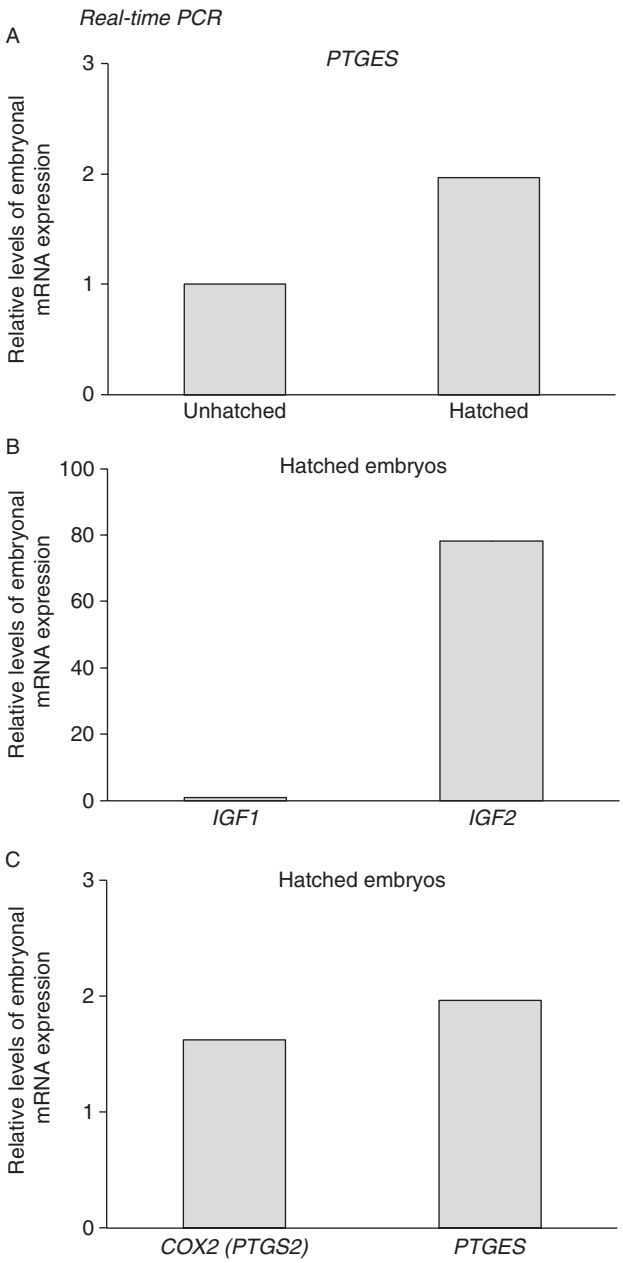

Figure 10 Embryonal expression of prostaglandin E2-synthase (*PTGES*), cyclooxygenase 2 (*PTGS2*), insulin-like growth factor 1 (*IGF1*) and *IGF2* as determined by Real Time (TaqMan) PCR.

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 the 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*. 2013*a*). 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 PGE2 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 preimplantation 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

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.

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