

Gene Expressions in the Persistent Corpus Luteum of Postpartum Dairy Cows: Distinct Profiles from the Corpora Lutea of the Estrous Cycle and Pregnancy

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Abstract. Persistence of the corpus luteum (CL) in cattle usually occurs during the puerperium and is associated with interference of prostaglandin (PG) F_{2α} release from the uterus. The objective of the present study was to determine for the first time the gene expressions in the persistent CL compared with the CL of pregnancy and cyclic CL. Three types of CL biopsy samples were collected from 32 lactating Holstein cows: (1) CL persisting for 29 to 33 days after the first ovulation postpartum (persistent CL, n=9), (2) CL between days 29 and 33 of early pregnancy (CL of pregnancy, n=8) and (3) CL between days 10 and 13 of the estrous cycle (cyclic CL, n=27). mRNA expression of 2',5'-oligoadenylate synthetase-1 was upregulated only in the CL of pregnancy, confirming exposure to interferon-τ (IFNT) produced by trophoblasts in pregnant cows. mRNA expressions of immune tolerance-related factors (PGES and forkhead/winged helix transcription factor 3) were upregulated in the CL of pregnancy but not in the persistent CL, suggesting that IFNT controls upregulation of these genes. mRNA expression relating to some of the major systems such as lymphangiogenesis, inflammation and apoptosis were similarly upregulated in the persistent CL and the CL of pregnancy but not in the cyclic CL. The results suggest that the persistent CL may survive for a long period without changes in local immune tolerance but develops several major systems required for CL maintenance similar to the CL of pregnancy.

Key words: Dairy cow, Early pregnancy, Persistent corpus luteum, mRNA expression

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In mammals, the corpus luteum (CL) develops rapidly after ovulation and plays a crucial role in survival of the embryo and maintenance of pregnancy by producing progesterone (P4). Luteinization of steroidogenic cells and angiogenesis are essential processes during CL development and maturation [1]. On the other hand, prostaglandin (PG) F_{2α} released by the uterine endometrium induces regression of the CL if pregnancy is not established. Formation, maintenance and regression of the CL are regulatory mechanisms controlled by various luteotropic and luteolytic factors that are mediated by the cyclic expression of key genes during the lifespan of the CL [2].

Luteolysis in cattle is generated by the pulsatile release of PGF_{2α} by the endometrium between days 16 and 19 of the estrous cycle [3]. The persistence of the CL in cattle usually occurs postpartum in association with uterine disorders, such as fetal mummification, endometritis or pyometra [4]. In addition, mastitis [5] and negative energy balance [6] can cause a higher incidence of a prolonged luteal phase. It is generally thought that interference with the production or

release of PGF_{2α} causes persistent CL [7]. Indeed, the lifespan of the CL can be prolonged for at least 145 days by immunization against PGF_{2α}, thereby maintaining plasma P4 concentrations above 4 ng/ml [8]. Although a prolonged luteal phase due to CL persistence is one of the major inhibitors of reproductive performance because it prevents normal estrous cycling [4, 9], there have been no previous reports that have investigated the characteristics of persistent CL.

Interferon-τ (IFNT) is recognized as a pregnancy recognition signal that is secreted by embryonic trophoblast cells [10]. IFNT downregulates the expression of uterine receptors for estrogen and oxytocin, thereby inhibiting the oxytocin-induced pulsatile release of PGF_{2α}, which, in turn, prevents luteolysis [11]. IFNT induces the expression of IFN response genes such as 2',5'-oligoadenylate synthetase 1 (OAS-1) and IFN-stimulated gene 15 (ISG-15) [12]. Oliveira *et al.* have demonstrated that IFNT is released into the uterine vein, which induces OAS-1 in extrauterine tissues (such as the CL) during the time of maternal recognition of pregnancy (MRP) [13]. Additionally, we recently reported that lymphangiogenesis occurred in the bovine CL during MRP by upregulating mRNA expression of the lymphangiogenic factor VEGF receptor 3 (VEGF-R3) [14]. That study revealed that IFNT induced lymphangiogenesis *in vitro*, suggesting that IFNT possibly regulates the occurrence of new lymphangiogenesis in the bovine CL during MRP, thereby contribut-

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ing to the transition into a CL of pregnancy. The physiological significance of the lymphatic system in the CL for the establishment and maintenance of pregnancy in cows remains to be determined. Theoretically, it could be assumed that a lack of exposure to IFNT is the main difference between the persistent CL and CL of pregnancy, although both have a longer lifespan than that of the cyclic CL.

We hypothesized that the regulatory mechanism of the persistent CL is similar to that of the cyclic mature CL but different from that of the CL of pregnancy and that exposure to IFNT contributes to the differences between the persistent CL and CL of pregnancy. Therefore, the present study investigated the expressions of genes related to the regulation of CL functions in the persistent CL compared with the CL of pregnancy and cyclic CL.

Materials and Methods

Animals

Twenty-six lactating Holstein Friesian cows (age 4.0 ± 1.7 , body weight 638 ± 72 kg; mean \pm SD) were used. The animals belonged to the Farm for Education and Research in Ruthe, University of Veterinary Medicine Hannover, Germany, and the herd average milk production was $10,082 \pm 1054$ kg/year (mean \pm SD). The present study was approved in accordance with the German legislation on animal welfare (Lower Saxony Federal State Office for Consumer Protection and Food Safety, file reference number 33.9-42502-04-09/1782).

Study design

The resumption of ovarian cyclicity was monitored 3 times per week (Monday, Wednesday, Friday) starting on day 12 postpartum by transrectal ultrasonography (HS-101V, Honda Electronics, Tokyo, Japan) using a 7.5 MHz linear transducer. After the first ovulation, the presence of the CL was determined 3 times per week starting on day 14 (day 1 = ovulation). In animals that did not show any symptoms of CL regression by day 29 after ovulation, the plasma concentration of P4 was determined. The CL was considered to be persistent when P4 concentrations were higher than 1 ng/ml, after which CL biopsy was performed between days 29 and 33 ($n=9$). After biopsy, regression of the persistent CL was induced using 0.5 mg of a $\text{PGF}_{2\alpha}$ analogue (2.0 ml cloprostenol; EstrumateTM; Intervet, Unterschleißheim, Germany). In cyclic cows, ultrasonographic examination and blood sampling were performed, and CL biopsy samples were collected between days 10 and 13 of estrous (2nd cycle at day 45 to 65 postpartum, $n=17$; 4th cycle at day 90 to 118 postpartum, $n=10$).

Artificial inseminations (AI) were performed after the 4th cycle, and pregnancy diagnosis was carried out using ultrasonography between days 29 and 33 post insemination. When the cow was diagnosed as pregnant with the proof of an embryonic heartbeat, CL biopsy samples were collected ($n=8$). Pregnancy diagnosis was performed again between days 37 and 43. The time schedule for collection of the CL biopsies is shown in Fig. 1.

Progesterone determination

Blood samples were collected from the jugular vein into serum tubes (Serum tube, Sarstedt, Nümbrecht, Germany). The tubes were immediately placed on ice, and plasma was separated by

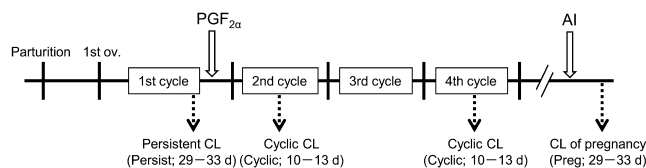


Fig. 1. The time schedule of CL biopsy collection. If the CL did not regress by day 29 after the first postpartum ovulation and the plasma P4 concentration was >1 ng/ml, the CL was considered to be persistent ($n=9$), and CL biopsy was performed between days 29 and 33. CL regression was then induced using 0.5 mg $\text{PGF}_{2\alpha}$. In the 2nd and 4th cycle, CL biopsy samples were collected from normal cyclic cows ($n=27$) between days 10 and 13 of estrous. Artificial inseminations were performed after the 4th cycle, and pregnancy diagnosis was performed using ultrasonography between days 29 and 33 post insemination. When a cow was diagnosed as pregnant, a CL biopsy sample was collected ($n=8$).

centrifugation ($3000\times g$, 15 min) within 90 min and frozen at -20 C until P4 levels were determined. Plasma P4 concentrations were determined by radioimmunoassay (Progesterone Coat-a-Count, TKPG1, Siemens Medical Diagnostics, Berkeley, CA, USA). The interassay coefficient of variation was 4.0%.

Corpus luteum biopsy

Prior to biopsy, the cows received epidural analgesia (4.0 ml 2% procaine hydrochloride; ProcaselTM; Selectavet, Weyarn-Holzolling, Germany) to eliminate any pain or rectal contractions. A sample of the CL was collected using an RNase-free (RNase-ExitusPlusTM; AppliChem, Darmstadt, Germany) semi-automatic high-speed biopsy needle (TEMNO EvolutionTM; Fa. Walter, Baruth/Mark, Germany). A portable ultrasound device (LOGIQ Book XP; General Electric Medical Systems, Solingen, Germany) equipped with a 7.5 MHz convex transducer was used to guide the needle. The biopsy needle and the ultrasound transducer were guided transvaginally using a bearing system (type Hannover), while the CL was placed in front of the vaginal fornix using transrectal manual assistance. Tissue samples were immediately placed in a sterile DNase- and RNase-free cryotube (Fa. Brand, Wertheim, Germany), frozen in liquid nitrogen and stored at -80 C until mRNA expression analysis. This method allowed for repeated biopsy sampling from a single CL without impairing its subsequent function, as described previously [15].

Extraction of RNA

Total RNA was extracted from the CL tissue following the protocol of Chomczynski and Sacchi using TRIzol reagent [16]. The yield of extracted total luteal RNA for each sample was determined by ultraviolet (UV) spectroscopy (optical density, 260). The RNA concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at 260 and 280 nm absorbances. The extracted total RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at -80 C until it was used for cDNA production.

Production of cDNA

DNase treatment was carried out using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA). Two microliters of the tissue sample was incubated for 30 min at 37 C with 1 μ l RQ1 RNase-Free

DNase 10× Reaction Buffer and 2 µl of 1 µg/µl RNase-Free DNase, respectively. Then 1 µl RQ1 DNase Stop solution (20 mM EDTA) was added, and the mixture was incubated for 10 min at 65 C to terminate the reaction. First-strand cDNA synthesis was conducted according to the commercial protocol described in the SuperScript™ II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The first cocktail was prepared using 2 µl total RNA extracted from the tissue sample, 1.5 µl of 50 ng/µl random primer (Invitrogen), 1.5 µl of 10 mM PCR Nucleotide Mix (dNTP; Roche Diagnostics, Indianapolis, IN, USA) and 12 µl H₂O, yielding a final volume of 18 µl per tube. This cocktail was then incubated at 65 C for 5 min in a thermal cycler (Bio-Rad, Munich, Germany). The samples were kept on ice while the second cocktail, 3 µl of 0.1 M DTT (Invitrogen), 1.5 µl of 40 units/µl RNasin™ Ribonuclease Inhibitor (Promega, Madison, WI, USA) and 6 µl of 5× First-Strand Buffer (Invitrogen), was added to each tube. The samples were incubated for 2 min at 42 C, and 0.2 µl of 200 units/µl SuperScript™ II Reverse Transcriptase was added to each tube. The thermal cycler was programmed at 25 C for 10 min, 42 C for 50 min and then 70 C for 15 min. The synthesized cDNA was stored at –30 C.

Real-time polymerase chain reaction (real-time PCR)

The following factors were analyzed in the present study: OAS-1, steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), cyclooxygenase-2 (COX-2), PGE synthase (PGES), PGF synthase (PGFS), PGI synthase (PGIS), PG dehydrogenase (PGDH), VEGF-A, VEGF-C, VEGF-R3, endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), tumor necrosis factor α (TNFα), caspase-3, transforming growth factor β (TGFβ), forkhead/winged helix transcription factor (Foxp3), β-actin and GAPDH. Quantifications of mRNA expression were performed using synthesized cDNA via real-time PCR with a LightCycler (Roche Diagnostics, Mannheim, Germany) using either a QuantiTect™ SYBR Green PCR kit (QIAGEN GmbH, Hilden, Germany), LightCycler™ FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) or KAPA SYBR™ FAST qPCR kit (NIPPON Genetics, Tokyo, Japan). The primers were designed using Primer3 based on bovine sequences, and the primers used for real-time PCR are listed in Table 1. The amplification program consisted of 15 min activation at 95 C, followed by 40 cycles of PCR steps (15 sec denaturation at 94 C, 30 sec annealing at 54–58 C and 20 sec extension at 72 C). The values of mRNA expression were assayed by normalization to β-actin and GAPDH, which were used as internal controls. The Delta–Delta Comparative Threshold method [17] was used to quantify the fold change between the samples.

Statistical analysis

All data are presented as means ± SEM. Statistical analysis was performed using Stat View 5.0 (SAS Institute, Cary, North Carolina, USA). One-way ANOVA followed by Fisher's multiple comparison test was performed, and all analyses were considered to be statistically significant at P<0.05.

Results

OAS-1

mRNA expression of OAS-1 in the CL tissues is shown in Fig. 2A. The mRNA expression of OAS-1 was significantly higher in the CL of pregnancy than in the cyclic and persistent CLs (P<0.05). This result confirms that the CL of pregnancy at Day 30 of early pregnancy is exposed to IFNT.

Steroidogenesis-related factors

mRNA expressions of StAR, P450scc and 3β-HSD in the CL tissues are shown in Fig. 2B–D. StAR, P450scc and 3β-HSD are known to regulate the synthesis of P4 as it is converted from cholesterol [18]. Although the mRNA expression of 3β-HSD was higher in the persistent CL and CL of pregnancy than in the cyclic CL (P<0.05), there were no significant differences in the StAR and P450scc mRNA expressions among the groups. The plasma concentration of P4 did not differ among the cyclic CL, persistent CL and CL of pregnancy (4.8 ± 1.2 ng/ml, mean ± SD).

Prostaglandin-related factors

mRNA expressions of COX-2, PGES, PGFS, PGIS and PGDH in the CL tissues are shown in Fig. 3. To produce various forms of PGs, such as PGE₂, PGF_{2α} and PGI₂, COX-2 converts arachidonic acid into PGH₂. The downstream enzymes PGES, PGFS and PGIS then catalyze the conversion of PGH₂ into PGE₂, PGF_{2α} and PGI₂, respectively. Catabolism of PGs is governed by PGDH [19]. In the present study, there were no differences in COX-2 and PGDH mRNA expressions among the groups. The mRNA expression of PGES was significantly higher in the CL of pregnancy than in the cyclic and persistent CLs (P<0.05), whereas the mRNA expressions of PGFS and PGIS were significantly higher in both the persistent CL and CL of pregnancy compared with the cyclic CL (P<0.05).

Angiogenesis and lymphangiogenesis-related factors

mRNA expressions of VEGF-A, VEGF-C and VEGF-R3 in the CL tissues are shown in Fig. 4. VEGF-A is known as the major factor that regulates angiogenesis, a potent mitogen for endothelial cells [20] and a stimulator of vascular permeability [21]. VEGF-C acts as the ligand of VEGFR-3 [22], and VEGFR-3 activation leads to proliferation and migration of lymphatic endothelial cells, thereby stimulating lymphangiogenesis [23]. The mRNA expressions of VEGF-A and VEGF-C did not differ significantly among the groups, whereas VEGF-R3 mRNA expression was higher in both the persistent CL and CL of pregnancy (P<0.05).

Inflammation and apoptosis-related factors

mRNA expressions of eNOS, iNOS, TNFα and caspase-3 in the CL tissues are shown in Fig. 5. Nitric oxide (NO) is an important signaling molecule that plays various physiological roles in the reproductive system [24]. NO is produced by 3 isoforms of synthases: neuronal NOS, iNOS and eNOS [25]. The presence of iNOS and eNOS has been reported in the bovine CL [26]. The mRNA expressions of eNOS and iNOS were significantly higher in the persistent CL and CL of pregnancy compared with the cyclic CL (P<0.05).

TNFα is a pleiotropic cytokine whose cellular effects depend on

Table 1. Primers used in real-time PCR

Gene		Sequence of nucleotide (5'-3') *	Accession No.	Product size (bp)
<i>OAS-1</i>	F	TAGGCCTGGAACATCAGGTC	NM001040606	253
	R	TTTGGTCTGGCTGGATTACC		
<i>StAR</i>	F	GTGGATTTTGCCAATCACCT	MN174189	203
	R	TTATTGAAAACGTGCCACCA		
<i>P450_{scc}</i>	F	CTGCAAATGGTCCCCTTCT	K02130	208
	R	CACCTGGTTGGGTCAAACCT		
<i>3β-HSD</i>	F	TCCACACCAGCACCATAGAA	X17614	178
	R	AAGGTGCCACCATTTTTCAG		
<i>COX-2</i>	F	TCCTGAAACCCACTCCCAACA	AF031698	241
	R	TGGGCAGTCATCAGGCACAG		
<i>PGES</i>	F	AGGACGCTCAGAGACATGGA	NM174443	142
	R	TTCGGTCCGAGGAAAAGAGTA		
<i>PGFS</i>	F	GATCAAAGCGATTGCAGACA	S54973	113
	R	CAATGCGTTCAGGTGTCCT		
<i>PGIS</i>	F	AGGATGAAGGAGAAGCATGG	MN174444	226
	R	TGTGGAGGAGAGTCGGTTTC		
<i>PGDH</i>	F	GAACCTACTGGGCTTGGAT	NM001034419.1	323
	R	CGAGCGTGTGAATCCAATA		
<i>VEGF-A</i>	F	CCCAGATGAGATTGAGTTCATTTT	M32976	245
	R	AGCAAGGCCACAGGGATT		
<i>VEGF-C</i>	F	CTCAAGGGCCCCAAACCAGT	NM174488	71
	R	CATCCAGCTTAGACATGCATCG		
<i>VEGF-R3</i>	F	TGAGGATAAAGGCAGCATGGA	AF030379	66
	R	CCCAGAAAAAGACAGCGATGA		
<i>eNOS</i>	F	GGAAATCGGGGTCTGGAGT	M89952	219
	R	TTGGCGAGCTGAAAGCTGTG		
<i>iNOS</i>	F	TCATCTTCGCCACCAAGCAG	NM001076799	167
	R	CAGTGATGGCCGACCTGATG		
<i>TNFα</i>	F	TCTACCAGGGAGGAGTCTTCCA	AF348421	221
	R	GTCCGGCAGGTTGATCTCA		
<i>caspase-3</i>	F	AAGCCATGGTGAAGAAGGAA	NM001077840.1	182
	R	CCTCAGCACCCTGTCTGTC		
<i>TGFβ</i>	F	CTGCTGAGGCTCAAGTAAAAGTG	NM001166068.1	90
	R	CAGCCGGTTGCTGAGGTAG		
<i>Foxp3</i>	F	CACAACCTGAGCCTGCACAA	NM001045933.1	233
	R	TCTTGCGGAACTCAAACCTCATC		
<i>β-actin</i>	F	CCAAGGCCAACCGTGAGAAAAAT	K00622	256
	R	CCACATTCCGTGAGGATCTTCA		
<i>GAPDH</i>	F	CTCTCAAGGGCATTCTAGGC	NM001034034	160
	R	TGACAAAAGTGGTCGTTGAGG		

* F, forward; R, reverse.

cell type, concentration and receptor type [27]. Caspase-3 is a pivotal mediator of apoptosis during regression of the CL [28]. In the bovine CL, TNF α induces apoptosis via caspase-3 activation in luteal cells and luteal endothelial cells [29]. Although TNF α mRNA expression was higher in the persistent CL and CL of pregnancy than in the cyclic CL ($P < 0.05$), there were no significant differences among the groups in regard to mRNA expression of caspase-3.

Immune tolerance-related factors

mRNA expressions of TGF β and Foxp3 in the CL tissues are shown in Fig. 6. TGF β is a multifunctional cytokine regulating T-cell

growth and development, and it induces CD4⁺ T cells to become CD4⁺ CD25⁺ regulatory T cells (Tregs) [30]. Foxp3 is specifically expressed in Tregs, and retroviral gene transfer of Foxp3 converts naive T cells to Tregs [31]. Tregs are essential for immune tolerance, modulation of inflammatory responses [32] and protection of the fetus from rejection by the maternal immune system during pregnancy [33]. TGF β mRNA expression was higher in the persistent CL and CL of pregnancy compared with the cyclic CL ($P < 0.05$). The mRNA expression of Foxp3 was significantly higher in the CL of pregnancy than in the cyclic and persistent CLs ($P < 0.05$).

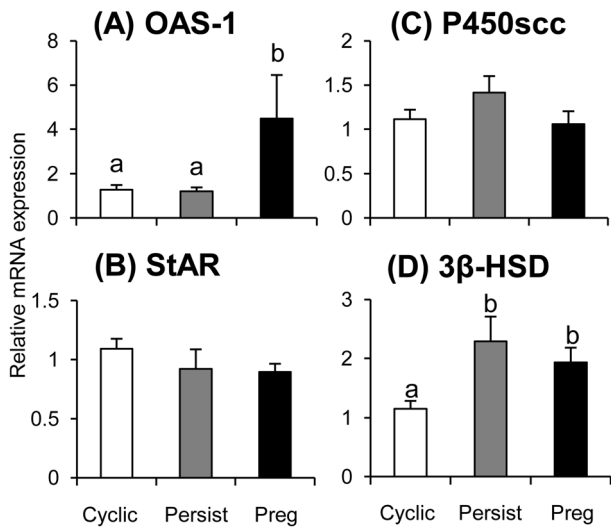


Fig. 2. The relative mRNA expression of OAS-1 (A) and steroidogenesis-related factors, StAR (B), P450scc (C), and 3β-HSD (D), in the cyclic CL (n=27), persistent CL (n=9), and CL of pregnancy (n=8). In each figure, the white bar indicates cyclic CL, gray bar indicates persistent CL, and black bar indicates CL of pregnancy. All values are shown as means ± SEM. Values with different letters (a, b) are different between groups (P<0.05).

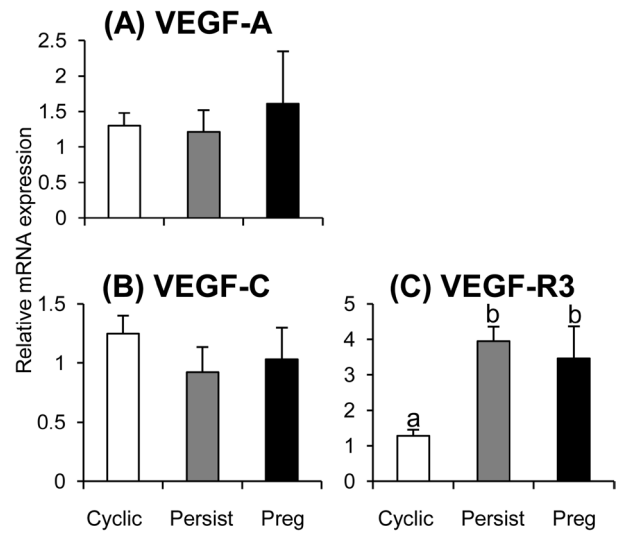


Fig. 4. The relative mRNA expression of an angiogenesis-related factor, VEGF-A (A), and lymphangiogenesis-related factors, VEGF-C (B) and VEGF-R3 (C) in the cyclic CL (n=27), persistent CL (n=9), and CL of pregnancy (n=8). In each figure, the white bar indicates cyclic CL, gray bar indicates persistent CL, and black bar indicates CL of pregnancy. All values are shown as means ± SEM. Values with different letters (a, b) are different between groups (P<0.05).

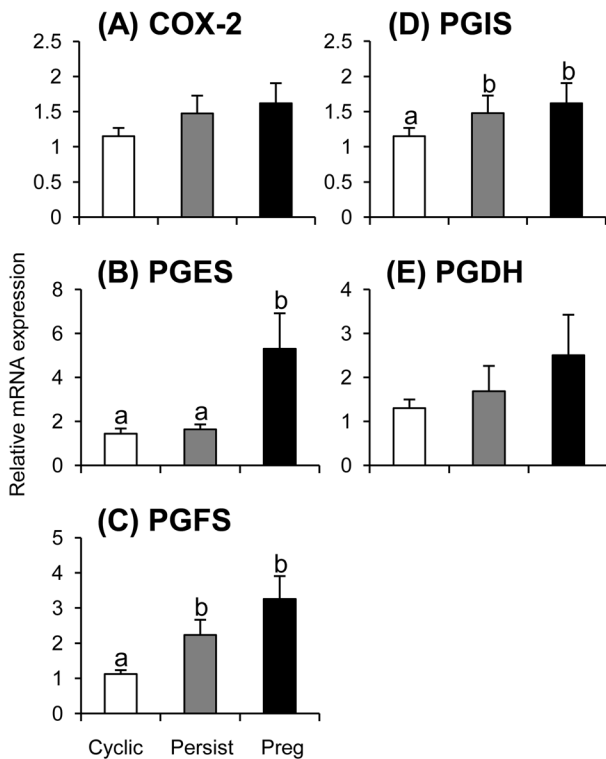


Fig. 3. The relative mRNA expression of prostaglandin-related factors, COX-2 (A), PGES (B), PGFS (C), PGIS (D) and PGDH (E), in the cyclic CL (n=27), persistent CL (n=9), and CL of pregnancy (n=8). In each figure, the white bar indicates cyclic CL, gray bar indicates persistent CL, and black bar indicates CL of pregnancy. All values are shown as means ± SEM. Values with different letters (a, b) are different between groups (P<0.05).

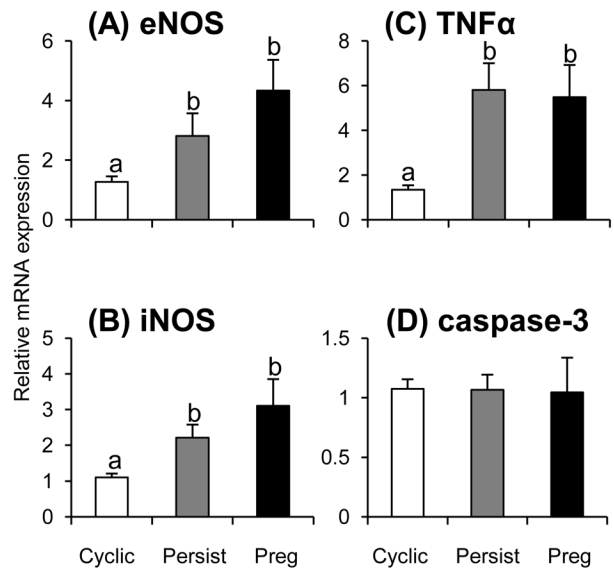


Fig. 5. The relative mRNA expression of inflammation-related factors, eNOS (A) and iNOS (B), and apoptosis-related factors, TNFα (C) and caspase-3 (D) in the cyclic CL (n=27), persistent CL (n=9), and CL of pregnancy (n=8). In each figure, the white bar indicates cyclic CL, gray bar indicates persistent CL, and black bar indicates CL of pregnancy. All values are shown as means ± SEM. Values with different letters (a, b) are different between groups (P<0.05).

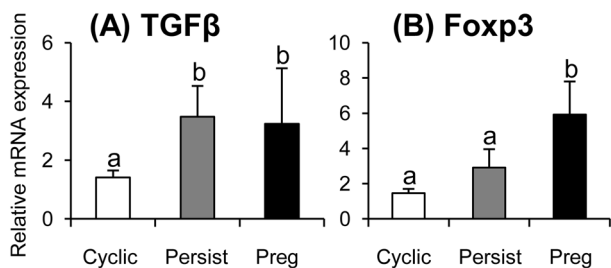


Fig. 6. The relative mRNA expression of immune tolerance-related factors, TGF- β (A) and Foxp3 (B) in the cyclic CL (n=27), persistent CL (n=9), and CL of pregnancy (n=8). In each figure, the white bar indicates cyclic CL, gray bar indicates persistent CL, and black bar indicates CL of pregnancy. All values are shown as means \pm SEM. Values with different letters (a, b) are different between groups ($P < 0.05$).

Discussion

The present study gives the first information on mRNA expressions for regulating the function of the persistent CL in cows. The expressions of immune tolerance-related factors, which might play a crucial role in the establishment and maintenance of pregnancy, were upregulated in the CL of pregnancy but not in the persistent CL, suggesting that exposure to IFNT is responsible for this difference. In contrast to our hypothesis, results indicate that some of the major systems such as lymphangiogenesis-, inflammation- and apoptosis-related factors found in the persistent CL are similar to those in the CL of pregnancy but different from those in the cyclic CL.

The present study revealed the critical difference between the persistent CL and CL of pregnancy; the mRNA expressions of OAS-1, PGES and Foxp3 were upregulated in the CL of pregnancy but not in the persistent CL. During pregnancy, there are some dynamic changes in maternal immune functions that prevent immunological destruction of the conceptus. Among the immunological changes, circulating Tregs, defined as CD4⁺ CD25⁺ FOXP3⁺ cells, increase to downregulate T lymphocyte function [34, 35]. Foxp3 is specifically expressed in Tregs and is a critical regulator of their development and function [36]. Several *in vitro* studies have reported an immunosuppressive role of PGE₂, which is known to induce Foxp3 gene expression and modulate Treg function in human lymphocytes [37]. Importantly, IFNT activates PGE₂ biosynthesis in the CL at the time of recognition and establishment of pregnancy in cattle [38]. The findings in the present study suggest that immune tolerance by Treg upregulation may occur only in the CL of pregnancy but not in the persistent CL. In turn, the persistent CL might survive for a long period without changes in the tolerance of the local immune system. In fact, Oliveira *et al.* [35] reported that Treg concentrations were elevated in the peripheral blood lymphocytes of pregnant cows between days 33 and 34 of gestation, the same period of pregnancy examined in the present study. TGF β is a multifunctional cytokine and regulates T-cell growth and development; TGF β converts naive T cells to Tregs with upregulation of IL-2, while TGF β and inflammatory cytokine IL-6 induce differentiation of Th 17 to stimulate inflammation [39]. In the present study, the mRNA expression of TGF β was higher in both

the persistent CL and CL of pregnancy, although it is possible that TGF β has different effects on the 2 types of CL.

Although the persistent CL as well as CL of pregnancy expressed higher levels of 3 β -HSD mRNA than did the cyclic CL, the mRNA expressions of StAR and P450scc did not differ among the groups. The StAR protein acts as the rate-limiting enzyme in luteal P4 production [40], which may be associated with the observation that the plasma P4 concentration did not differ among the persistent, cyclic and pregnant groups of cows in this study. Crowe *et al.* [8] induced the formation of persistent CLs in heifers by actively immunizing them against PGF_{2 α} ; the lifespan of the CL was prolonged at least 145 days, with plasma P4 concentrations remaining elevated above 4 ng/ml, which is comparable to that in the luteal phase of cyclic cows [41]. The data indicate that CL function is able to persist for a significant amount of time with the interference of PGF_{2 α} . We have previously shown that luteal mRNA expressions of angiogenic factors such as VEGF₁₂₀ and VEGF₁₆₄, fragments of VEGF-A, do not differ between the late luteal phase and early pregnancy, while histological examination showed that angiogenesis does not occur in the CL during early pregnancy [42]. This finding is in agreement with the present study, where VEGF-A did not differ among the persistent CL, cyclic CL and CL of pregnancy, indicating that angiogenesis does not occur in the persistent CL or CL of pregnancy. In the present study, the persistent CL, cyclic CL and CL of pregnancy all showed the same level of mRNA expression of caspase-3, suggesting that there were no differences in the occurrence of apoptosis in the luteal tissues of the 3 groups. Collectively, the present findings indicate that the function and structure of the persistent CL are similar to those of the CL of pregnancy and cyclic CL in terms of P4 synthesis, angiogenesis, and apoptosis.

In addition to the uterus, the bovine CL itself expresses all of the components necessary for complete PG biosynthesis, transport and signaling, which helps determine its life span and function [43]. PGs are key mediators of embryo implantation during early pregnancy. PGE₂ has been proposed to have multiple roles as a temporary luteotropic or luteoprotective signal [38, 44]. The biosynthetic and signaling pathways of PGE₂ and PGF_{2 α} are selectively activated at the time of pregnancy recognition; thus, the balance between PGE₂ and PGF_{2 α} production plays a critical role in CL function. It has been shown that IFNT upregulates PGES expression but not PGFS expression in the CL, leading to an increase in the PGES to PGFS ratio [38]. In the present study, the mRNA expression of PGFS was higher both in the persistent CL and CL of pregnancy, whereas PGES expression was upregulated only in the CL of pregnancy, i.e., the PGES to PGFS ratio was increased in the CL of pregnancy but not in the persistent CL. The findings suggest that the characteristics of the PG system in the persistent CL are distinct from those of both the cyclic CL and CL of pregnancy. Moreover, PGIS mRNA expression was higher in the persistent CL and CL of pregnancy compared with the cyclic CL. It is well accepted that PGI₂ stimulates P4 production [45] and regulates vascular tone [46]; however, the role of PGI₂ (PGIS) in the persistent CL and CL of pregnancy needs to be further determined.

VEGF-C stimulates lymphangiogenesis via the receptor VEGFR3 [22, 23]. In the present study, mRNA expression of VEGF-R3 was remarkably high in both the persistent CL and CL of pregnancy,

suggesting that the system of lymphangiogenesis is activated in the persistent CL as well. Interestingly, it has been demonstrated that inflammatory cytokines including TNF α [47, 48], NO *via* iNOS [49, 50] and eNOS [25] induce VEGF-C expression and may promote lymphangiogenesis in tumors. The mRNA expressions of TNF α , iNOS and eNOS were higher in the persistent CL and CL of pregnancy compared with the cyclic CL in this study, potentially contributing to the activation of lymphangiogenesis. Our recent study [14] has shown that IFNT directly stimulates proliferation of lymphatic endothelial cells and increases VEGF-C mRNA expression, suggesting that IFNT is involved in lymphangiogenesis. However, the low OAS-1 mRNA expression in the persistent CL indicates a lack of exposure to IFNT; thus, lymphangiogenesis might be simply required for the long survival of the CL.

mRNA expressions for PGFS and PGIS, as well as factors relating to lymphangiogenesis and inflammation, were upregulated both in the persistent CL and CL of pregnancy compared with the cyclic CL. The longer survival of the persistent CL and CL of pregnancy (29 to 33 days) might require upregulation of several systems regulating their structure and function. The development of immune tolerance seems to be particularly crucial for the transition into a CL of pregnancy but is perhaps not necessary for the CL survival.

In conclusion, the results demonstrate that the persistent CL has similar characteristics to the CL of pregnancy but not the cyclic CL with regards to gene expression in several major systems that regulate CL function. The critical difference between the persistent CL and CL of pregnancy was the immune tolerance system that was upregulated only in the CL of pregnancy, which may be due to a lack of exposure to IFNT in the persistent CL.

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