Differential Spatiotemporal Patterns of Galectin Expression are a Hallmark of Endotheliochorial Placentation

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Problem
Galectins influence the progress of pregnancy by regulating key processes associated with embryo-maternal cross talk, including angiogenesis and placentation. Galectin family members exert multiple roles in the context of hemochorial and epitheliochorial placentation; however, the galectin prolife in endotheliochorial placenta remains to be investigated.

Method of study
Here, we used immunohistochemistry to analyze galectin (gal)-1, gal-3 and gal-9 expression during early and late endotheliochorial placentation in two different species (dogs and cats).

Results
We found that during early feline gestation, all three galectin members were more strongly expressed on trophoblast and maternal vessels compared to the decidua. This was accompanied by an overall decrease of gal-1, gal-3 and gal-9 expressions in late feline gestation. In canine early pregnancy, we observed that gal-1 and gal-9 were expressed strongly in cytotrophoblast (CTB) cells compared to gal-3, and no galectin expression was observed in syncytiotrophoblast (STB) cells. Progression of canine gestation was accompanied by increased gal-1 and gal-3 expressions on STB cells, whereas gal-9 expression remained similar in CTB and STB.

Conclusion
These data suggest that both the maternal and fetal compartments are characterized by a spatiotemporal regulation of galectin expression during endotheliochorial placentation. This strongly suggests the involvement of the galectin family in important developmental processes during gestation including immunemodulation, trophoblast invasion and angiogenesis. A conserved functional role for galectins during mammalian placental development emerges from these studies.

Introduction

In eutherian mammals, the placenta is a transitory organ that invades the maternal uterus to provide nutrition and gas exchange for the developing fetus. As a consequence of this process, tolerance mechanisms must be employed at the maternal–fetal interface to protect the developing semiallogenic fetus

from recognition by the maternal immune system.[1] According to the extent of trophoblast invasion into the uterus, placentation can be classified as hemochorial, endotheliochorial or epitheliochorial. Humans and mice exhibit hemochorial placentation; the most invasive placental type in which trophoblast cells penetrate the maternal blood vessels and come into direct contact with maternal blood.[2] In contrast, epitheliochorial placentation, characteristic of large farm animals such as cows, pig and sheep, is non-invasive, and trophoblast cells simply attach to the uterine epithelium.[3] The third type of placentation, endotheliochorial, is found in dogs and cats; here trophoblast cells invade the uterus until they border the maternal blood vessels.[4–6] Regardless of the type of placentation, this organ ensures normal growth and development of the embryo and fetus while supporting pregnancy-related changes in maternal physiological systems.

Galectins are a family of highly conserved proteins that mediate numerous biological processes by cross-linking glycans on cell membranes.[7] All members of this family are composed of a distinct carbohydrate recognition domain (CRD) with conserved consensus regions and beta-galactoside specific lectin activity. While some galectins contain one CRD and are biologically active as either homodimers (e.g., galectin-1) or oligomers that aggregate through their non-lectin domain (galectin-3); other galectins contain two CRDs connected by a short linker peptide (e.g., galectin-9).[8] Several members of this family play an active role in pregnancy maintenance by regulating trophoblast cell growth and differentiation, angiogenesis and inflammation throughout gestation.[9–14] Within this family, galectin-1 (gal-1), gal-3 and gal-9 are of particular interest as they are highly expressed at the maternal–fetal interface.[15] Their expression is spatiotemporally regulated during pregnancy, and the biological function of a particular galectin may vary according to the site of expression and availability of particular ligands.[8] For instance, gal-1 appears to play specific roles during implantation, decidualization, vascularization and later on, during placentation.[14,16–18] During early gestational stages, gal-1 exhibits proangiogenic functions by promoting decidual vascular expansion through vascular endothelial growth factor (VEGF) receptor 2 signaling.[12] Putative roles played by gal-1 during hemochorial placentation include organization of the extracellular matrix, regulation of trophoblast differentiation and cell motility[17,19,20] and modulation of class Ib human leukocyte antigen (HLA) expression;[11] the later representing a potent immunosuppressive mechanism triggered by trophoblast cells.[21–23] In addition, gal-1 is also strongly expressed in immune cells such as decidual natural killer (NK) cells[24] and T regulatory cells (Tregs)[25] and we have demonstrated that this lectin plays a critical role in immune tolerance by inducing tolerogenic dendritic cells and the subsequent expansion of IL-10 producing regulatory T cells.[9] During epitheliochorial placentation gal-1 is present in decidual stromal cells and early gestational trophoblast giant cells.[26]

Regarding the function of gal-3 and gal-9 during pregnancy, specific roles for these proteins remain to be elucidated. Human reports demonstrate that gal-3 expression at the fetal–maternal interface partially overlaps that of gal-1.[17,27] In mice, uterine gal-3 expression is selectively upregulated within the decidua during early stages of pregnancy, whereas at later stages it is predominantly expressed in the placenta.[28] Furthermore, decreased implantation rates were observed upon tissue-specific gal-3 knockdown in the murine uterus,[29] suggesting a role for this lectin during the embryo-maternal cross talk driving implantation. Little information is available on epitheliochorial placentation where gal-3 is confined to uterine epithelial cells.[26] As for gal-9, this lectin is substantially upregulated in human decidua compared to proliferative endometrium.[30] We recently showed the expression of at least six gal-9 isoforms in decidual tissue, and that the *Lgals9* D5 variant can selectively suppress IFN-γ production by uterine NK cells.[13] Finally, expression of gal-9 has also been reported in the epitheliochorial placenta, though at lower levels than in the decidual compartment.[26]

Although the role that galectins play in placentation is presently a topic of much interest, the information concentrates mainly on the hemochorial placental type. Information on the involvement of galectins in endotheliochorial and epitheliochorial placental types is minimal, and limited mainly to farm relevant animals such as cows,[26] sheep[31,32] and goats.[33] To our knowledge there are presently no studies investigating galectin expression in endotheliochorial placental mammals such as dogs and cats. As a first step in illuminating this subject, the objective of this research was to examine gal-1, gal-3 and gal-9 expression patterns at the feto-maternal interface in cats and dogs, and to identify changes in expression between early and late gestation. This new knowledge will provide a strong foundation for galectin research and pregnancy in veterinary relevant species.

Materials and methods

Tissue Collection

The cat (*Felis catus*) and dog (*Canis lupus familiaris*) specimens for this study were obtained with owners' approval, during hysterectomy or Cesarean section at the University of La Plata. All procedures were approved by the Universidad Nacional de La Plata ethical committee. Gestational ages were estimated by embryo or fetal length. Average gestation length for dogs and cats is 63–65 days. Accordingly, samples were collected at estimated period of gestation: early (<30 days) or late (>45 days). For each estimated period of gestation, more than one visible embryonic vesicle was used in the study: early (n = 7 dogs and 4 cats), late (n = 5 dogs and 4 cats). We collected samples of different fetal sacs from each mother. The samples included central areas of placental girdle and excluded marginal hematoma. Maternal and embryo or fetal placental structures were collected and fixed in buffered formalin for 24 h following our standard protocol.[12]

Immunohistochemistry

After deparaffinization and rehydration, serial dog and cat paraffin-embedded tissue sections (4 μm) were washed in TBS, followed by blocking of endogenous peroxidase (PO) through incubation with 3% H_2O_2 in methanol for 30 min at room temperature. After incubation with 2% normal serum for 20 min, primary antibodies rabbit against gal-1 (1:500, sc-28248), gal-3 (1:200, sc-20157) and goat against gal-9 (1:100, sc-19292, all purchased from Santa Cruz Biotechnology) were incubated overnight at 4°C. The slides were then washed and incubated for 1 hr at room temperature with goat anti-rabbit PO-conjugated secondary Ab (1:200, cat. #111-035-003, ImmunoResearch) for gal-1 and gal-3 determination or donkey anti-goat PO-conjugated secondary Ab (1:200, cat. #705-035-147 Jackson ImmunoResearch) for gal-9. The signal was detected using a liquid DAB + Substrate Chromogen System (cat. #K3467, DAKO) at room temperature. After washing, nuclei were counterstained with 0.1% Mayer's hematoxylin followed by a standard dehydration procedure and mounting in Vitro-Clud medium (R. Langenbrinck, Germany). Negative controls were run in parallel for each galectin primary antibody. Briefly, we pre-absorbed the rabbit anti-gal-1, gal-3 or gal-9 primary antibody with the respective blocking peptide (overnight at 4°C), and then proceeded with the above described protocol. Negative controls showed no specific immuno-reactivity. For overview, image tissue sections were stained with hematoxylin and eosin (H&E).

Analysis

Analysis of galectin-stained slides was performed using a Keyence BZ9000 microscope and by three independent observers. Images were taken at different magnifications for each staining. In both species, galectin expression of labyrinthine structures was described, taking into account: maternal vessels (mv), syncytiotrophoblast (STB) and cytotrophoblast (CTB), fetal mesenchyma (fm) and fetal vessels (fv). Galectin expression in the decidual cells of the cat placenta was also assessed. Each observer quantified staining of the respective galectins in the mentioned placental structures with the following score: strong (+++), moderate (++), weak (+) staining and negative (−). Furthermore, positive cells were quantified by counting within the different areas in each tissue sample including mv, STB, CTB, fm, fv and decidua (Dec). For each sample, at least four different regions (i.e., fields) were recorded. Percentage of positive cells was expressed as a mean count for each of the analyzed group.

Results

Differential Expression of gal-1, gal-3 and gal-9 Between Early and Late Healthy Feline Gestation

Early feline gestation samples showed expression of gal-1, gal-3 and gal-9 within the trophoblast cytoplasm, maternal vessels and decidual cytoplasm (Fig. 1a–d; Table I, Figure S1a). In all galectin stainings, the cyto- and syncytiotrophoblasts and maternal vessels stained more strongly than decidual tissue (Fig. 1b–d). Interestingly, comparing between the different galectin stainings, gal-3 and gal-9 were more strongly expressed than gal-1 in early gestation. The fetal mesenchyma only expressed low levels of gal-1 (Fig. 1b). The fetal vessels were negative for all three galectins tested. Also noteworthy, strong cytoplasmic expression of gal-9 was observed in approximately 25% of decidual stromal cells (Figs 1d and S1a). No nuclear staining was evident in any of the cell types observed.

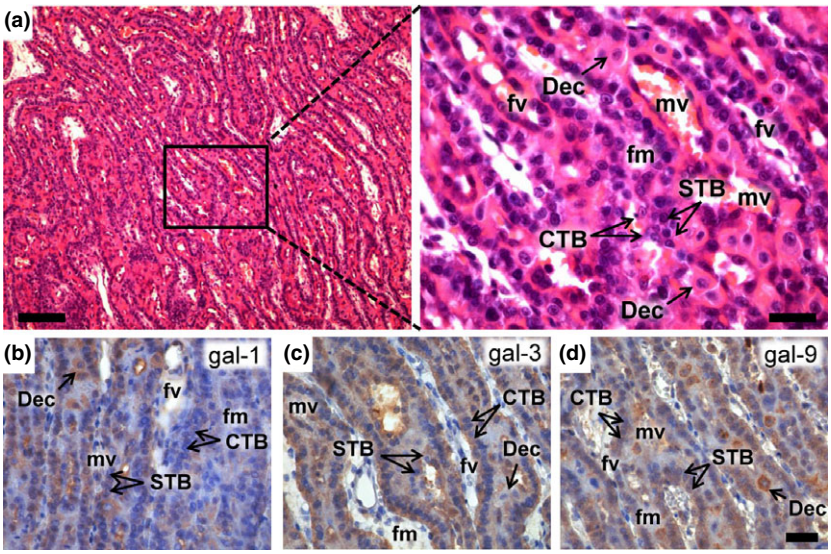

Fig. 1 Galectins expression during early feline gestation. (a) Left panel: Representative overview image of hematoxylin and eosin (H&E) staining in the labyrinth of endotheliochorial early (<30 days) feline placenta. Scale bar = 100 μm; magnification 100×. Right panel: Scale bar = 25 μm; magnification 400×. (b–d) Representative photomicrographs of gal-1 (b), gal-3 (c) and gal-9 (d) staining at the maternal–fetal interface of early (<30 days) healthy feline gestations. Bar represents 25 μm; magnification 400×. Dec, decidua; mv, maternal vessel; CTB, cytotrophoblast; STB, syncytiotrophoblast; fv, fetal vessels; fm, fetal mesenchyme.

Table I Quantification of Galectin Distribution in Early and Late Feline Placenta Samples

Placental structure	Gestation	gal-1	gal-3	gal-9
Maternal vessels	Early	++	++	++(+)
	Late	++	+++	+(+)
Syncytiotrophoblast	Early	++	++	++
	Late	++	+++	++
Cytotrophoblast	Early	+	++	+++
	Late	++	+++	++
Fetal mesenchyma	Early	–	–	–
	Late	+	+	++
Fetal vessels	Early	+	–	–
	Late	–	–	–
Decidua	Early	+	+	++(+)
	Late	++	+	++(+)

Quantification of staining: strong +++, moderate ++, weak +, negative –. Brackets are indicative of heterogeneity of labeling intensities [i.e., ++(+) weak to moderate staining].

Analysis of mature feline placenta revealed that gal-1 was moderately expressed at similar levels in maternal vessels, the trophoblast and the cytoplasm of decidual cells (Fig. 2b; Table I, Figure S1b). Interestingly, the nuclei of decidual cells were also stained. However, gal-1 expression was almost absent from the fetal vessels during late gestation, while the fetal mesenchyma started to express gal-1 at low levels (Fig. 2b). Gal-3 expression in mature placenta was also increased overall when compared with early pregnancy samples; however, the staining pattern remained the same with the trophoblast cell cytoplasm and maternal blood vessels staining more strongly than decidual cell cytoplasm. The fetal mesenchyma was weakly stained for gal-3 (Figs 2c and S1b). As for gal-9, a differential pattern of expression was observed between early and late stages of feline gestations. Maternal vessel and trophoblast expression of gal-9 decreased in the mature placenta but decidual cells maintained strong expression. Additionally, the decidual cells presented a nuclear staining. The syncytio- and cytotrophoblasts displayed a vesicular rather than uniform staining in the cytoplasm. The fetal mesenchyma exhibited moderate gal-9 expression in all areas of the mature placenta (Figs 2d and S1b), whereas the fetal vessels were negative for all galectins during late gestation.

Spatiotemporal Expression of gal-1, gal-3 and gal-9 During Normal Canine Gestation

Staining of early canine gestation placental samples revealed that the CTB expresses all three galectins: gal-1 and gal-9 were strongly stained while gal-3 was only weakly expressed (Fig. 3a–d, Table II, Figure S1c). No galectin expression was observed in the STB in early gestation. Maternal blood vessels also expressed gal-3 and gal-9 and no staining of fetal vessels was observed (Fig. 3c,d). When analyzing late canine gestation samples, gal-1 expression was evident in maternal vessels and surrounding STB cells (Fig. 4a–d, Table II, Figure S1d). This lectin was also expressed in CTB, albeit more weakly than in

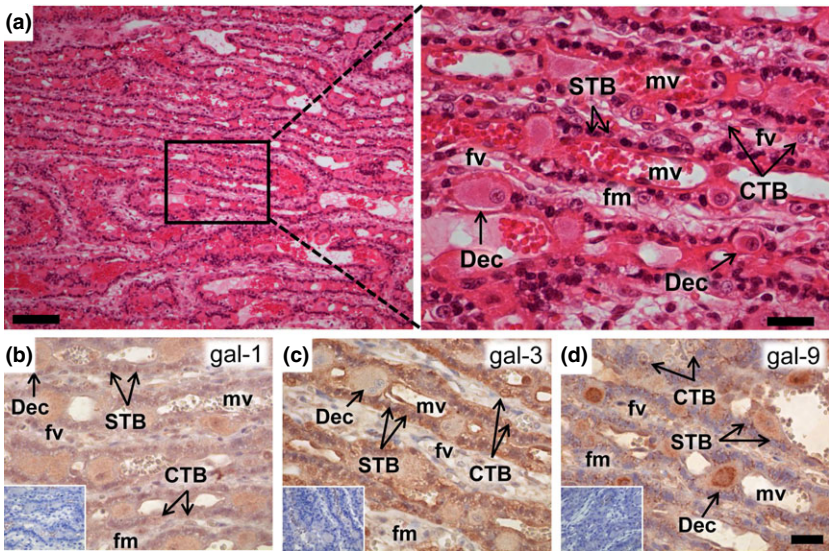

Fig. 2 Galectins expression pattern in late feline endotheliochorial placentation. (a) Left panel: Representative overview image of hematoxylin & eosin (H&E) staining in the labyrinth of endotheliochorial late (>35 days) feline placenta. Scale bar = 100 μm; magnification 100×. Right panel: Scale bar = 25 μm; magnification 400×. (b–d) Representative examples of gal-1 (b), gal-3 (c) and gal-9 (d) staining at the maternal–fetal interface of late (>45 days) healthy feline gestations. Insert on the bottom left of each panel show negative controls for gal-1 (b), gal-3 (c) and gal-9 (d) staining. Bar represents 25 μm; magnification 400×. Dec, decidua; mv, maternal vessel; CTB, cytotrophoblast; STB, syncytiotrophoblast; fv, fetal vessels; fm, fetal mesenchyme.

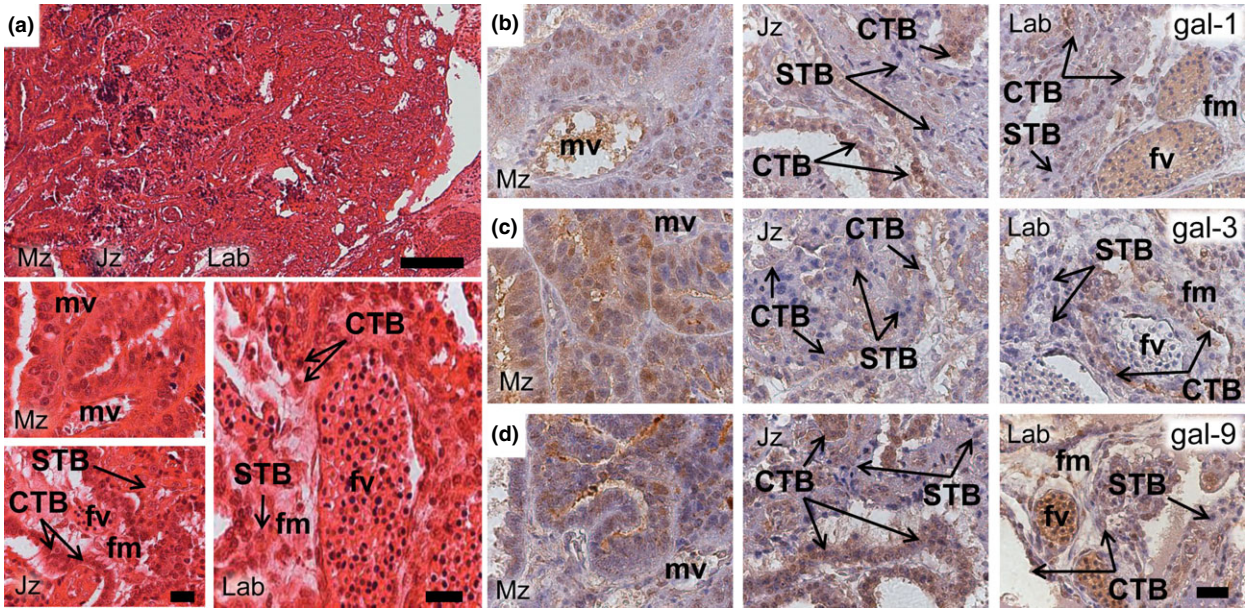

Fig. 3 Characterization of galectin expression patterns during early canine endotheliochorial placentation. (a) Top panel: Representative H&E staining shows the maternal zone (Mz), Junctional zone (Jz) and labyrinth (Lab) of the endotheliochorial early canine placenta (<30 days). Scale bar = 250 μm; magnification 50×. Bottom panels: Details of the maternal zone (Mz), Junctional zone (Jz) and labyrinth (Lab) of the endotheliochorial canine placenta during early are shown. Bars represent 25 μm; magnification 400×. (b–d) Representative examples of gal-1 (b), gal-3 (c) and gal-9 (d) staining showing the maternal zone (left), junctional zone (middle) and labyrinth (right) panels of the maternal–fetal interface during early stage (<30 days) healthy canine gestation. Scale bar represent 25 μm; magnification 400×. CTB, cytotrophoblast; STB, syncytiotrophoblast; fv, fetal vessels; fm, fetal mesenchyme; mv, maternal vessel.

early gestation. Regarding gal-3 expression in late canine gestation, we found immunoreactivity only in STB cytoplasm, whereas no CTB staining was observed. In addition, maternal vessels and fetal mesenchyma also weakly expressed gal-3 (Fig. 4c). Finally, gal-9 expression was detected in maternal vessels and all trophoblast cell populations, with most strong labeling in the CTB and STB cytoplasm. However, the CTB gal-9 expression was heterogeneous (Fig. 4d).

Table II Quantification of Immunohistochemical Detection of Galectin Expression in Early and Late Canine Gestation

Placental structure	Gestation	gal-1	gal-3	gal-9
Maternal vessels	Early	–	++	++
	Late	++	+	+
Syncytiotrophoblast	Early	–	–	–
	Late	++	++	+++
Cytotrophoblast	Early	+++	+	+++
	Late	+	–	++(+)
Fetal mesenchyma	Early	–	–	–
	Late	–	–	–
Fetal vessels	Early	–	–	–
	Late	–	+	–

Quantification of staining: strong +++, moderate ++, weak +, negative –. Brackets are indicative of heterogeneity of labeling intensities [i.e., ++(+) weak to moderate staining].

Discussion

Studies in reproductive tissues from different species have provided important insights into the role played by galectins in pregnancy. As a delicate interplay between maternal and fetal galectin expression is critical for healthy gestation, identification of galectin profiles in placental types with different invasive capacities can help to understand the roles of these important molecules. In this study, we provide the first evidence that endotheliochorial placentas (both canine and feline) have a temporal and cell specific expression of three galectin members from early to late gestation.

Galectins display a unique combination of biological functions, with immunemodulation at the feto-maternal interface being one of the best-described mechanisms of this lectin family.[34] Here, we show that gal-1, gal-3 and gal-9 are expressed during early gestation in endotheliochorial placentation, implying that these galectins could also contribute to the protection of canine and feline embryos from attack by the maternal immune system. In this context, canine embryos do not express major histocompatibility complex (MHC) I and II but express FasL, preventing their recognition as foreign antigens and eliminating Fas-bearing cytotoxic maternal T cells, respectively.[35] Interestingly, Fas/Apo-1 has been identified as a target for gal-1 recognition in Jurkat cells, sensitizing them to caspase-8 mediated apoptosis.[36] Thus, gal-1 expression in early gestation endotheliochorial placentas could constitute a complementary mechanism contributing to feto-maternal tolerance, which is consistent with its described role facilitating the apoptosis of activated maternal T cells and inducing Treg cells in hemochorial placentation.[9,10] Similarly to gal-1, gal-9 also functions as an anti-inflammatory signal that influences T-cell survival by promoting Treg and suppressing Th17 cell activity,[37] and therefore, its expression during early endotheliochorial placentation could be involved in promoting feto-maternal tolerance. Additionally, our study showed that gal-3 expression was abundant during early endotheliochorial gestation, whereas its expression decreased at later stages. This distribution could imply a role of this lectin in the embryo-maternal cross talk driving implantation, as a similar pattern of gal-3 expression has been found in hemochorial placentation during early gestation.[18,27,28]

Besides their roles in immunemodulation, galectins also modulate angiogenic responses *in vitro* and *in vivo* by influencing endothelial cell activation, proliferation, migration and tube formation.[38] During early endotheliochorial placentation, localization of gal-1, gal-3 and gal-9 to the endothelia of maternal blood vessels within the labyrinth could represent a marker of endothelial cell activation. This is in agreement with studies that show increased galectin expression in the maternal associated vasculature during hemochorial placentation[39] and tumorigenesis.[40–42] As VEGF signaling is increased during the early stages of endotheliochorial placentation,[35] it is tempting to speculate that the expression of these three galectins in the maternal vessel endothelia could contribute to the angiogenesis process. Supporting this notion, gal-1 interacts with neuropilin to promote signaling via the VEGF receptor 2,[43] enhances H-Ras signaling in endothelial cells[44] and is associated with fetal growth delay in *Lgals-1* deficient mice due to hampered placental vascularization.[12]

Similarly to gal-1, gal-3 also exhibits pro-angiogenic functions. Gal-3 binding to αvβ3 integrin modulates VEGF and basic fibroblast growth factor (bFGF)-mediated angiogenesis,[45] and can also activate VEGFR2 by regulating receptor internalization.[46] Notably, studies profiling the expression of the VEGF system in canine and mink endotheliochorial placentas have demonstrated that the VEGFR2 is the predominant receptor expressed on microvascular endothelial cells during early pregnancy.[47,48] This expression largely correlates with the pattern described for several matrix metalloproteases (MMP) in canine and feline pregnancies,[49,50] particularly with MMP-2 and MMP-9. MMP-2 and MMP-9 target

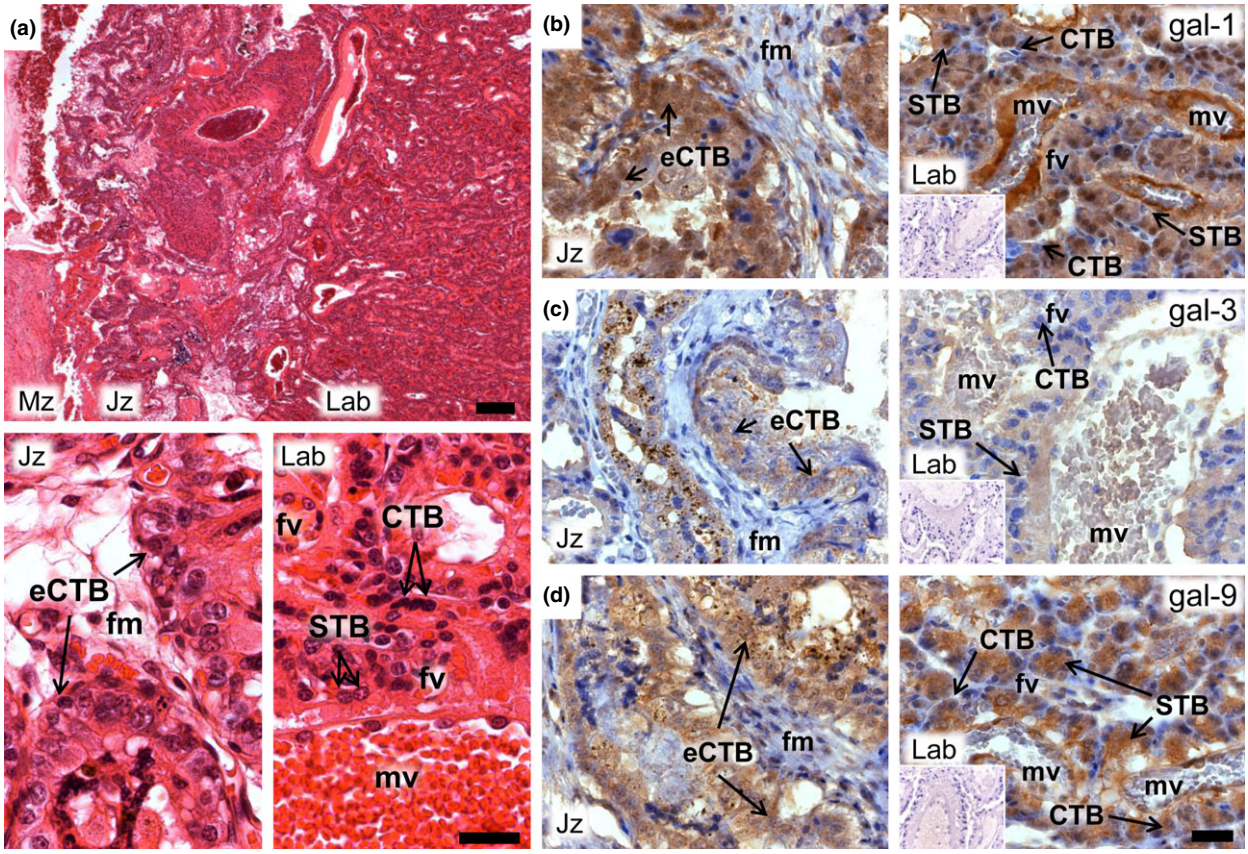

Fig. 4 Distribution of galectins at the maternal–fetal interface during late (>35 days) normal canine pregnancies. Top panel: Representative H&E staining shows the maternal zone (Mz), Junctional zone (Jz) and labyrinth (Lab) of the endotheliochorial late canine placenta during late (>45 days). Scale bar = 250 μm; magnification 25×. Bottom panels: Details of the Junctional zone (Jz) and labyrinth (Lab) of the endotheliochorial canine placenta during late are shown. Bars represent 25 μm; magnification 400×. (b–d) Representative images of gal-1 (b), gal-3 (c) and gal-9 (d) immunohistochemical staining in the junctional zone (left) and labyrinth (right) of the endotheliochorial canine placenta are shown. Inserts show negative controls. CTB, cytotrophoblast; STB, syncytiotrophoblast; eCTB, extra villous CTB; fv, fetal vessels; fm, fetal mesenchyme; mv, maternal vessel.

the non-galectin domain of gal-3, enhancing the chemotactic properties of this lectin toward endothelial cells and promoting migration and onset of angiogenesis.[51] While pro-angiogenic functions of gal-9 are still elusive, Heusschen et al.[42] demonstrated that the endogenous Lgals9Δ5 is able to influence human endothelial cell proliferation *in vitro,* suggesting that this splice variant of gal-9 can modulate angiogenesis *in vivo*. Together, these findings suggest that galectin expression on maternal blood vessel endothelia in the labyrinth is likely associated with angiogenesis in the canine and feline placenta. These results encourage further studies on galectin interaction with the VEGF system during early endotheliochorial placentation.

Within endotheliochorial placentation, comparison of galectin expression from early to late gestation in feline and canine placentas revealed some striking differences. In the feline placenta, gal-1, gal-3 and gal-9 were expressed in both CTB and STB, whereas at later stages these galectins were more weakly expressed in the trophoblast. This suggests that progression of endotheliochorial placentation leads to a decrease in galectin expression. Contrasting this, analysis of canine early gestation trophoblast cells revealed that only the CTB expressed gal-1, gal-3 and gal-9. In late canine gestation, galectin expression decreased in the CTB and appeared in the STB, suggesting a gain of galectin expression in canines as CTB differentiates into STB. These results appear to contradict findings in human hemochorial placentation, where gal-1 expression is lost during the differentiation of CTB to STB.[11] These contradictory results may be due to the different degree of

trophoblast invasiveness in hemochorial and endotheliochorial placentation and may also account for the finding that decidual galectin expression (-1, -3 and 9) appears to be lower in canine and feline specimens than in species with deeper invasion such as mice and humans.

The spatiotemporal expression of gal-1, gal-3 and gal-9 in the endotheliochorial placenta provides clues into the roles of these molecules in pregnancy. Research into species with alternative placental strategies can help to unravel the fine details of galectin function, specifically with regard to immune tolerance and angiogenesis at the maternal–fetal interface.

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Conflict of interest

The authors declare that no conflict of interest exists.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Quantification of the galectins immunohistochemical detection during endotheliochorial placentation.