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Highlights

- . Endotheliochorial and haemochorial placentation needs adequate trophoblastic invasiveness.
- . From implantation, the trophoblast invades to extensive maternal tissue remodeling.
- . Matrix metalloproteinases (MMPs) have crucial role in carnivore and mouse trophoblastic invasiveness.

Abstract

To establish a functional placenta, its development needs adequate trophoblastic invasiveness. The intricate and complex morphological and molecular aspects regulating trophoblastic invasion during endotheliochorial placentation of domestic carnivores and their similarities and differences with the hemochorial placenta are still poorly understood. During placentation processes, from the time of implantation, trophoblast cells invade the uterine endometrium where they achieve extensive degradation and remodeling of extracellular matrix components; in this process, matrix metalloproteinases (MMPs), particularly MMP-2 and 9, have an essential role in rebuilding, cell migration, and invasiveness. This review provides an overview of comparative trophoblast invasive events and the expression and activity of MMP-2 and 9 during endotheliochorial and hemochorial placentation, emphasizing dog and mouse placental models. Understanding of trophoblastic invasiveness in two models of placentation, the intermediately invasive domestic carnivore endotheliochorial placenta, and the more highly invasive mouse hemochorial placenta, contributes to deepen knowledge of the trophoblast invasive processes and their diverse and complex human placental alterations, such as preeclampsia.

Keywords: **metalloproteinases, trophoblastic invasiveness, endotheliochorial, hemochorial**

1. Introduction

The placenta is the organ possessing the most inter-specific differences. One of the most frequently used classifications to interpret placental variability in eutherian mammals is based on the number of layers present in the placental barrier proposed by Grosser in 1909 which allows interpreting the degree of invasiveness of embryonic tissues in the uterus. Currently, following modifications generated by Wooding's studies, placentas can be classified into epitheliochorial, synepitheliochorial, endotheliochorial, and hemochorial (Wooding and Burton, 2008).

Phylogenetic studies indicate that primordial eutherian placentas had intimate maternal-fetal contact provided by trophoblast invasion. Non-invasive epitheliochorial placenta appeared on numerous occasions during the evolution of eutherians as an adaptation to facilitate prolonged pregnancies and the birth of more independent precocial offspring (Carter and Mess, 2007; Carter and Enders, 2013; Carter, 2017). In endotheliochorial and hemochorial placentation, trophoblast invades the endometrium with some differences in the invasiveness process leading to different modes of placental development and determining that the hemochorial placenta is the most invasive whereas the endotheliochorial has intermediate invasiveness (Wooding and Burton, 2008; Enders and Carter, 2012a; 2012b). Although there are differences in the degree of invasiveness of the trophoblast and transformation of the endometrium between endotheliochorial and hemochorial placentas, the need to carry out molecular studies during critical periods of pregnancy has guided the choice of suitable animal models for the study of human placental alterations, an essential point in different investigations.

Invasive trophoblast modulates maternal-fetal interaction during pregnancy by complex molecular cooperative mechanisms (Paidas et al., 2010), which leads to the uterine extracellular

matrix (ECM) remodel (Das and Basak, 2003; Lala and Nandi, 2016; Smith et al., 2016). Along with cytokines (Schäfer-Somi, 2003), growth factors (vascular endothelial growth factor (VEGF), insulin like growth factor 2 (IGF2) (Beceriklisoy et al., 2009; Ventureira et al., 2019; Hernandez et al., 2020), and galectins (Conrad et al., 2017), among other factors, the matrix metalloproteinases (MMPs) are the main regulatory molecules involved in maternal-fetal communication (Xu et al., 2000; Staun-Ram et al., 2004; Fontana et al., 2012; Li et al., 2014; Diessler et al., 2017).

Several studies have demonstrated that species with epitheliochorial placenta, such as pigs, express MMPs during the implantation period but since MMP inhibitors are expressed at high levels, these placentas have scarce invasiveness (Menino et al., 1997). In sheep, with synepitheliochorial placentation, the fall of MMP inhibitor expression is related to uterine remodeling during delivery (Vagnoni et al., 1998). These results could indicate that the increase of MMP-inhibitors and not the decrease of MMPs is involved in the loss of invasiveness in epitheliochorial and synepitheliochorial placentas.

In the invasiveness of endotheliochorial and hemochorial placentas, MMPs participate in trophoblast invasiveness and development of the feto-maternal interface. However, MMPs need to be produced in a controlled manner, since an imbalance of their expression and/or activities appears to be involved in abnormal trophoblast invasion associated with many placental alterations, such as human preeclampsia (Chen and Khalil, 2017; Cheng et al., 2019, Nikolov et al., 2021). MMP-2 and MMP-9 play a particularly key role in maternal vasculature and tissue remodeling during trophoblast invasion (Bischof and Campana, 2000). Because of their relevance in trophoblast invasiveness, we now update knowledge of MMP-2 and MMP-9 roles in endotheliochorial and hemochorial placentation, using domestic carnivores (dog and cat) and mouse as comparative models, respectively.

2. Metalloproteinases

MMPs are multigenic proteolytic zinc-dependent enzymes, secreted as zymogens with multiple roles such as tissue remodeling, protein ECM degradation**,** cell surface bioactive molecular modulation, cell-matrix, and cell-cell interactions, activation or inactivation of autocrine or paracrine signaling molecules, and cell surface receptors (Amălinei et al., 2007; Cui et al., 2017), among others.

MMPs have a common propeptide core (80 amino acids), a catalytic domain (170 amino acids), a linker peptide of variable length, and a hemopexin domain (200 amino acids) (Cui et al., 2017). Posttranslational activation of latent forms of MMP is generally processed in the extracellular compartment by proteolytic removal of the prodomain ~10-kDa propeptide (Amălinei et al., 2007; Henriet and Emonard, 2019), although MMPs are secreted as zymogens to undergo activation (Amălinei et al., 2007). MMPs are strictly controlled at transcription, secretion, and activation levels by a variety of exogenous and endogenous factors such as cytokines, growth factors, hormones, cell-matrix and cell-cell contacts, their inhibitors (tissue inhibitors of metalloproteinases (TIMP) -1 through -4), oxidative stress-related molecules**,** by nonspecific proteinase inhibitors (α1-proteinase inhibitor and α2-macroglobulin) (Amălinei et al., 2007; Beceriklisoy et al., 2007; Jacob-Ferreira and Schulz, 2013; Cui et al., 2017), phosphorylation, hypoxia-re-oxygenation, and key transcription factors (Jacob-Ferreira and Schulz, 2013). Alterations in MMP expression-activity have been involved in various placentopathies (Amălinei et al., 2007; Cui et al., 2017).

MMPs can be classified into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs (Amălinei et al., 2007; Cui et al., 2017). Different MMPs may cooperate to degrade a common protein substrate completely (Cui et al., 2017). MMPs

promote turnover of various ECM proteins including collagens (types I, II, III, IV, V, VI, VII, VIII, IX, X, and XIV), elastin, and other matrix glycoproteins and proteoglycans, such as aggrecan, entactin, fibronectin, tenascin, laminin, myelin basic protein, and vitronectin (Cui et al., 2017). Stromelysin-1 and -2 (MMP-3 and -10) can degrade other ECM protein substrates and may participate in cleaving certain proMMPs to their active form. Particularly, gelatinase A (MMP-2; 72 kDa) and gelatinase B (MMP-9; 92 kDa) are responsible for degradation of basement membrane components (collagen IV, laminin, and fibronectin) and other ECM proteins (Beceriklisoy et al., 2007; Cui et al., 2017).

During gestation, MMPs are involved in proliferation, apoptosis, migration, differentiation, ECM remodeling (Lemaître and D'Armiento, 2006; Amălinei et al., 2007; Hamutoğlu et al., 2020), survival of trophectodermal cells, and trophoblast invasion (Bischof et al., 1995; Isaka et al., 2003; Agaoglu et al., 2016). Moreover, the invasive capacity of trophoblast cells depends on MMP expression and activity (Walter and Schönkypi, 2006). In addition, MMPs participate in endothelial cell morphogenesis during placental angiogenesis-vascularization (Chandrasekar et al, 2000; Hamutoğlu, 2020) because VEGF can promote expression/activity of uteroplacental MMPs and, in turn, facilitate trophoblast invasion and vascular remodeling of the endometrium (Cui et al., 2017). Also, decidualization is partially regulated by a MMP/TIMP balance produced by trophoblastic and decidual tissues, thereby limiting excessive trophoblast invasion (Hamutoğlu et al., 2020).

Not all MMPs are equally important for trophoblast invasion during placentation. The most important gelatinases with a preponderant role in trophoblastic invasiveness and tissue remodeling of maternal tissue are MMP-2 and MMP-9 (Bischof and Campana, 2000, Staun-Ram et al. 2004). Mouse embryos without MMP-9 expression show trophoblast invasion deficiency (Plaks et al., 2013). Abundantly expressed in placental tissues, these gelatinases are capable of degrading major components of the endometrial extracellular matrix (ECM) such as collagen IV, V, VII and XI, fibronectin, laminin, elastin, proteoglycans, and entactin (Teesalu et al., 1999). Throughout

gestation, some studies showing different expression patterns of these MMPs indicate that MMP-2 is mainly involved in embryo implantation whereas MMP-9 intervenes in later trophoblast invasion (Espino et al., 2017); therefore, MMP-2 seems to be most abundant and predominates over MMP-9 in the early stages of trophoblast invasion (Xu et al. 2000; Naruse et al., 2009). However, detailed data of trophoblast MMP-2-and MMP-9 expression during invasive processes in endotheliochorial and hemochorial placenta are little reviewed.

3. Comparative trophoblast invasion and role of MMP-2 and MMP-9 during endothelial- and hemochorial placentation

First, to understand the comparative invasion processes in both endotheliochorial and hemochorial placentas, we briefly summarize the basic structure of these two placentas.

The endotheliochorial placentas of domestic carnivores develop two zones: the *zonary girdle* and the *hemophagous organs*: green-pigmented zones characteristically found on both edges or in the center of the girdle (Wooding and Burton, 2008).

In the placental girdle, the following regions are defined as: i) the chorioallantoic membrane (CAM) that forms the chorionic villi, ii) the labyrinth where the chorionic trophoblast layers develop around the maternal vessels, iii) the junctional zone, formed by the superficial glandular layer remodeled by the trophoblast cells, and iv) the maternal zone, composed of the superficial and deep glandular layers (Leiser and Koob, 1993; Miglino et al., 2006, Wooding and Burton, 2008, Aralla et al., 2013).

Quite similar, mouse hemochorial placenta consists of a trophoblastic zone composed of the junctional zone, limited by maternal tissue, where the uterine blood vessels make contact with junctional trophoblast giant cells (TGC); and by the labyrinth, a large, branched surface area of maternal-fetal exchange (Watson and Cross, 2005; Rai and Cross, 2014; Woods et al., 2018).The

other well-defined region of the hemochorial murine placenta is the maternal decidua, derived from the decidualization of uterine stromal cells, where maternal blood comes into the trophoblastic zone of the placenta through spiral arteries.

Endotheliochorial placentation has low invasiveness comparing to the hemochorial (Denker, 1993) because, although the endometrium of the labyrinth is remodeled, the maternal vessels are not fully eroded. Thus, the placental barrier in this type of placenta is constituted by vessels lined by feto-maternal endothelial cells and trophoblast layers, except in the hemophagous zone where the trophoblast contacts maternal blood. Therefore, in the endotheliochorial labyrinth, the maternal endothelium contacts chorionic trophoblasts and maternal tissue has decidual cells in cat, or decidualized stromal cells in dog. However, within endotheliochorial placenta, the canine is more invasive than the feline. The existence of more prominent and abundant decidual vimentin-positive cells in cat than in dog and because decidual cells have an anti-invasive role in the other species might partially explain the lesser invasiveness of cat placenta (Fernandez et al., 2000; Fernandez et al., 2014; Kautz et al., 2015).

In both endotheliochorial and hemochorial placentas, trophoblasts participate in the different invasiveness processes (Rossant and Cross, 2001; Carter, 2012) in which MMP-2 and MMP-9 are essential to remodel maternal tissue during gestation. A review analysis of comparative trophoblastic MMP-2 and MMP-9 expression and activity during the two placentation types is provided below.

3.1. Role of MMP-2 and MMP-9 in trophoblast invasion during endothelio- and hemochorial peri-implantation

In bitches, preimplantative blastocysts enter the uterus by 5-8 days post coito (dpc) (Graubner et al., 2017; Kowalewsk et al., 2020) (Table 1); at 10-11 dpc, the free-floating embryos

transmit signals to the uterus to reorganize the endometrial ECM composition, including the modification of MMP and TIMP expression and regulation of ECM proteins (Graubner et al., 2018). The decreased expression of several collagen compounds (Plow et al., 2000; Graubner et al., 2018) is associated with trophoblast invasion in the canine endometrium (Beceriklisoy et al., 2007]. Immediately at implantation (12-15 dpc), after blastocyst attachment, apposition, and adhesion, the first signs of trophoblast invasion are the penetration of cytotrophoblast into the uterine epithelium (Fig.1.A). During choriovitelline villi development, the allantois becomes adherent to the chorion to yield the chorioallantoic membrane (Wooding and Burton, 2008; Aralla et al., 2013; Graubner et al., 2017). At this stage, at the placental zone, the ECM of uterine epithelium and endometrium is modified and reorganized (Aralla et al., 2013; Graubner et al., 2017) (Fig.1A). In the dog, expression and activity of MMP-2 and MMP-9 have been confirmed in the uterine compartments during implantation (Table 2) (Beceriklisoy et al., 2007; Graubner et al., 2018). However, the expression of TIMP-2, the stronger regulator of MMP activity enhanced by the presence of embryos in the uterus (Graubner et al., 2017), could explain the minor invasiveness at the implantation of endotheliochorial placentas compared to hemochorial placentas. Unlike the canine placenta, MMP-2 and MMP-9 protein expression and activity in cat invasive endotheliochorial placenta seem to be less known (Table 2) (Walter and Schönkypi, 2006; Agaoglu et al., 2016).

During hemochorial mouse implantation (5-5.5 dpc), embryo-maternal interaction begins with blastocyst attachment, adhesion to the apical uterine epithelial surface, and endometrial penetration (Aplin and Kimber, 2004) (Table 1). Mural trophectodermal cells of the blastocyst antimesometrially invade the uterine basement membrane and underlying stroma (Rossant and Cross, 2001; Hu and Cross, 2010; Woods et al., 2018) while becoming polyploid to form the primary trophoblast giant cells (TGCs). These invasive cells extensively lead ECM degradation and

remodeling when attaching to uterine ECM proteins (fibronectin, laminin, vitronectin, and collagen) (Chen et al., 2007). Trophoblast secretion of proteinases, such as urokinase-type plasminogen activator (uPA), stromelysin, and MMPs, suggests that the high migration and invasiveness of these cells are linked to the expression of these proteolytic factors (Librach et al., 1991).

Trophoblast invasion inhibited by administration of MMP inhibitors leads to retardation of decidua remodeling (Estella et al., 2012), indicating that successful implantation is closely associated with degradation of the uterine ECM by embryonic MMP-2 and MMP-9 expression (Bany et al., 2000; Bai et al., 2005). Recent data demonstrated that the use of MMP-9 neutralizing antibodies during blastocyst culture inhibits trophoblast invasiveness (Zhang et al., 2020). In mice, around 5.5 dpc, MMP-9 protein expression was detected in primary TGCs simultaneously with the increase of their differentiation and invasiveness (Alexander et al., 1996; Bany et al., 2000). Mainly during implantation *in vitro*, a weak signal of MMP-2 mRNA was detected in mouse blastocysts undergoing outgrowth at 72 h; expression much lower than that of MMP-9 (Chen et al., 2007) (Table 2). Moreover, very strong MMP-9 activity was found in blastocyst outgrowth (24-72 h) in zymography, with no detection of MMP-2 activity (Alexander et al., 1996; Chen et al., 2007). Consistently, treatment of mouse blastocyst outgrowths with MMP-9 antisense oligonucleotides results in a reduction of ECM degradation (Whiteside et al., 2001). However, a broad-spectrum of TIMPs does not inhibit implantation in rats. Likewise, implantation still occurs in MMP-2 and MMP-9-deficient mice (Bany et al., 2000), suggesting that other proteinases must be sufficient to compensate for the deficiency of these MMPs.

3.2. Early endothelial- and hemochorial placentation and participation of MMP-2 and MMP-9 in trophoblast invasion

In the first stage of the early endotheliochorial placenta, around 18 dpc, the labyrinth begins to form with incipient chorionic villi development. The cytotrophoblast cells contact the epithelium of glands to begin the remodel (Fig.1.A, Table 1). Around 22–25 dpc, high columnar trophectoderm lines the edge of the placenta where it degrades the endothelial vessels to form the marginal hematoma, a zone of active phagocytosis and digestion of erythrocytes to deliver iron to the embryo (Miglino et al., 2006). Comparatively, in the first stage of mouse placenta (6.5-7.5 dpc) (Fig.1B), primary TGCs invade the maternal blood sinusoidal capillaries around the already established yolk sac (Malassine et al., 2003). At this time, mesometrial polar trophectodermal cells proliferate to form the extraembryonic ectoderm, from which the ectoplacental cone (EPC) and the chorionic ectoderm arise (Table 1). At 8-8.5 dpc, the chorion and the EPC roof adhere, after which the growing allantois fuses with the chorion for final chorioallantois attachment (Croy et al., 2014; Woods et al., 2018). Meanwhile, trophoblasts at the margin of the EPC differentiate into secondary TGCs, becoming highly invasive to remodel the mesometrial uterine luminal epithelium and the surrounding maternal stroma (Malassine et al., 2003; Croy et al., 2014; Rai and Cross, 2014; Woods et al., 2018) (Fig.1.B).

In the second early stage of endotheliochorial carnivore placentation (Fig 1.C), the differentiated syncytiotrophoblast from cytotrophoblast invades the maternal endometrium and the glandular epithelium to establish the junctional zone (Wooding and Burton, 2008; Aralla et al., 2013) Here, a significant part of the superficial glandular connective tissue is degraded; the trophoblast establishes around the maternal capillaries with only some stromal decidualized cells in dog and decidual cells in cat (Table 1).

Similarly, by the early stage of mouse placenta, around 9-10 dpc, the chorioallantoic fusion determines the labyrinth's development (Table 1, Fig.1.D) (Hu and Cross, 2010; Croy et al, 2014; Rai and Cross, 2014; Woods et al., 2018). Immediately, with the labyrinthine branching morphogenesis,

the chorionic trophoblasts differentiate into sinusoidal TGCs (S-TGC), and by fusion, into the syncytiotrophoblast. The S-TGCs, two layers of syncytiotrophoblast, the fetal endothelium, and the basement membrane form the labyrinthine interhemal membrane. Between the labyrinth and the decidua, the other fetal layer of the junctional zone (JZ) is constituted by spongiotrophoblasts (SpT) (non-syncytial cells), the spiral artery-associated TGCs (SpA-TGCs), some early differentiated glycogen cells and the parietal TGCs (P-TGC) (Rossant and Cross, 2001; Malassine et al., 2003; Hu and Cross, 2010; Croy et al., 2014; Rai and Cross, 2014; Woods et al., 2018). In terms of invasion in the mouse, the trophoblasts have different invasive functions. Strictly, P-TGCs of the junctional-decidual interface remodel maternal tissue by interstitial invasion (Knöfler et al., 2001; Rai and Cross, 2014) (Fig.1.D). From 10.5 dpc, also, the junctional SpA-TGCs migrate around and into the partially remodeled decidual spiral arteries that reach JZ, and replace the vascular endothelium (Rai and Cross, 2014) (Fig.1.D). Also, in the fetal face, the displacement of endothelial cells from the maternal arterial blood vessels by TGCs associated with maternal canal (C-TGC) (Malassine et al., 2003; Croy et al., 2014; Rai and Cross, 2014) can be considered an event of trophoblast invasion. Therefore, invasive TGCs erode maternal vessels, determining direct contact between trophoblasts and maternal blood. However, although early mouse invasion is comparable to human EVT invasion about two weeks after fertilization, the EVTs reach the uterine glands in humans (Fitzgerald et al., 2010) an event more like the ST-invasion of the uterine glandular layer of early EC-placentation.

In endotheliochorial placentas, structural remodeling processes and trophoblast invasion are associated with decreased collagens 1 and 3 in the utero-placental stroma (Graubner et al., 2018), indicating the role of MMP-2 and MMP-9 in invasiveness during early placentation. However, the specific roles of MMP-2 and MMP-9 in trophoblast invasion capacity remain little known. Some authors only reported high activity of the active form of MMP-2 and moderate MMP-9 activity in the endometrium of early canine placenta (Beceriklisoy et al., 2007), and consequently

the expression and activity of these proteases in trophoblastic cells was not completely described. Recently, we reported strong MMP-2 immunoexpression, cytotrophoblast, and syncytiotrophoblast cells of canine placental labyrinth (Table 3), whereas this expression was found moderately in fetal endothelium and weakly in the mesenchyme, indicating the major role of MMP-2 in trophoblastic cell remodeling functions in the labyrinth. Conversely, MMP-9 was weakly expressed in cyto- and syncytiotrophoblast but strongly in fetal mesenchyme (Diessler et al., 2017). In addition, the expression of trophoblastic MMP-2 is linked to processes of invasion of maternal endometrium to direct fetal nutrition. That the marginal hematoma has a very high activity of the active MMP-2 (Diessler et al., 2017) suggests that this protease has the main role in the trophoblastic invasion of maternal tissues and vascular remodeling (Diessler et al., 2017). These results indicate that MMP-2 is more important than MMP-9 for trophoblastic invasiveness during early canine placentation.

In early feline placenta, MMP-2 expression was negative in cytotrophoblast and syncytiotrophoblast (Walter and Schönkypi, 2006), while no data on localization or expression of MMP-9 in these cells were reported (Table 3). Moreover, only MMP-2 is moderately expressed in allantois and decidual cells, is weak in labyrinthine maternal stroma and endothelium, and very low in the epithelium of superficial glands (Walter and Schönkypi, 2006). Although this protease was highly expressed in the non-implantative endometrium (Walter and Schönkypi, 2006; Agaoglu et al., 2016), in this maternal tissue only predominantly inactive forms of MMP-2 and MMP-9 were demonstrated by zymography in cat placenta (Walter and Schönkypi, 2006). Therefore, to date, little information about the expression and activity of these two metalloproteinases in early feline placenta is available.

In the endotheliochorial placenta, higher trophoblastic expression of MMP-2 and MMP-9 and their activity in maternal tissues of canine than in feline placenta might indicate greater

invasiveness in the former; this difference could be related to the occurrence of the disease known as subinvolution of the placental sites in bitches but not in queens (Fernandez et al., 1998).

In early mouse placenta (6.5-7 dpc), only primary TGCs are positive for mRNA and protein MMP-9 expression (Alexander et al., 1996) (Table 3). By 8 dpc, secondary TGCs become very invasive into the decidua while expressing both MMP-2 and MMP-9 (Bany et al., 2000; Bai et al., 2005) (Table 3). These proteases continuously increase to high levels at day 8.5 (Alexander et al., 1996), simultaneously with the declination of TIMP-3 mRNA expression, which in turns produces uterine apoptosis and remodeling of the underlying basement membrane (Alexander et al., 1996). Therefore, during this stage, a strong invasion of decidua by primary and secondary TGC takes place through MMP-2 and MMP-9 secretion, by which MMPs are considered markers of differentiation and invasiveness of ectoplacental TGCs (Hamutoğlu et al., 2020).

Crosstalk between MMP-2 and -9 and VEGF in junctional and labyrinthine cells around 10 dpc is critical for adequate labyrinthine vascularization at mid-gestation in mice (Staun-Ram et al., 2004; Gualdoni et al., 2021). Trophoblastic cells produce MMPs as part of a complex angiogenic pathway related to the VEGF system of feto-maternal interface and uteroplacental ECM remodeling and apoptosis (Heo et al., 2010). In this regard, we recently evaluated decidual and trophoblast expression of VEGF and MMP-2 and MMP-9 during early placentation in the organogenesis of a mouse model for abnormal angiogenesis (Ventureira et al., 2019, Gualdoni et al., 2021), suggesting the importance of an adequate balance of MMP-VEGF for feto-maternal angiogenesis-vascularization, and trophoblastic invasiveness at mid-gestation. Regarding the expression and activity of MMP-2 and MMP-9 in mouse implantation sites on day 10 of gestation, MMP-2 expression was shown in P-TGCs as a decrease from high to very low levels at 9.5 to 10 dpc, although MMP-9 expression remains relatively high in both these periods (Fontana et al., 2012; Gualdoni et al., 2021) (Table 3). However, other authors demonstrated in mice the up-regulation of

MMP-3, -9, and -14 in trophoblasts that undergo differentiation to the invasive P-TGC type (Rai and Cross, 2014). Specifically, around mouse 10 dpc, although no infiltration of trophoblasts into deep decidua has taken place, the strong MMP-9 immunoexpression in P-TGC suggests the relationship between the degree of differentiation and potential capacity of invasion into decidua (Gualdoni et al., 2021). Moreover, MMP-9 ARNm was found in TGC at feto-maternal interface (Teesalu et al., 1999), confirming that this protease may be an important player in early P-TGC-invasion at mouse mid-gestation. On the other hand, high MMP-9 expression in spongiotrophoblasts compared to weak expression of MMP-2 in junctional trophoblasts (table 3), indicates that this protease has a major functional role in VEGF-dependent maternal vascular remodeling in the junctional zone of murine early placenta (Solberg et al., 2003; Gualdoni et al., 2021).

3.3. Trophoblastic MMP-2 and MMP-9 and invasion in mid- and late mature endotheliochorial and hemochorial placenta.

By mid-gestation of endotheliochorial placenta (30-45 dpc), the expanded labyrinth consists of mesenchyme containing fetal capillaries, a single layer of cuboidal cytotrophoblast cells surrounding the mesenchymal axis, and outer invasive syncytiotrophoblast cells arranged around maternal capillaries and in contact with the maternal epithelium and endometrium of glands (Fig. 2.A). In a parallel comparison, in mature mouse placenta (Hu and Cross, 2010; Furukawa et al., 2014; Rai and Cross, 2014; Woods et al., 2018) (Fig.2.B), once established around 11.5 dpc (Table 1), various subtypes of trophoblast cells migrate and invade far away from JZ to decidual vascular region to locate in different positions in relation to the maternal vascular space (Simmons et al., 2007). The trophoblastic group of glycogenic cells (GCs) increases in number 250-fold from day 10 to 12.5 dpc, becoming highly invasive (Cross et al., 2002; Hu and Cross, 2010; Rai and Cross, 2014) and offering an energy supply that provides additional nutrition to the placenta and/or fetus (Rai

and Cross, 2014; Woods et al., 2018). For this reason, these cells, earlier located at junctional maternal blood canals and sinuses, after day 12.5 pc, leave the confines of JZ to invade the decidua (Croy et al., 2014) (Fig.2.B) and progressively form multiple GC islands, afterward decreasing between E16.5 and E18.5 (Malassine et al., 2003; Croy et al., 2014; Woods et al., 2018).

Regarding MMPs and invasiveness, in dog mid-mature placenta, moderate labeling of MMP-2 is found in cytotrophoblasts and syncytiotrophoblasts whereas MMP-9 is expressed only weakly in syncytiotrophoblast (Table 4). However, moderate to low expression of MMP-2 is detected in fetal and maternal endothelium and the stroma of superficial glands, while MMP-9 is expressed mainly in tissues of the superficial glandular layer (Beceriklisoy et al., 2007). However, both MMP activities were reported as being high in the whole placental tissues (Beceriklisoy et al., 2007). In mature feline placenta, no data is available yet on the expression of MMP-2 and MMP-9, zymograms revealing only latent MMP-2 and MMP-9 in feline placenta (Walter and Schönkypi, 2006). In 45 dpc cat placenta, only moderate MMP-13 expression is detected in syncytiotrophoblast and maternal endothelium (Walter and Schönkypi, 2006).

In canine pre-term placenta (Table 1), when the labyrinth is fully developed, the superficial glandular area is diminished, and deep uterine glands increase in size, reaching maximum expansion (Wooding and Burton, 2008; Aralla et al., 2013), trophoblasts expressing more MMP-9 mRNA than the weak expression of MMP-2 (Fellows et al., 2012) (Table 4). This is consistent with the human trophoblast, where MMP-9 predominates in late gestation, having a role in ECM degradation for successful detachment of placental membranes, whereas MMP-2 is expressed predominantly in early gestation (Shimonovitz et al., 1994; Ioannidis et al., 2010; Xu et al., 2002). Further studies on the localization, expression, and activation of MMP-2 and MMP-9 in late canine and feline placenta could clarify the role of these proteases at term.

In mature mouse placenta, from 10.5 dpc, decidual areas enriched with ECM components undergo intense tissue remodeling by invasive trophoblasts, probably by the action of higher moderate MMP-9 expression/activity of P-TGCs rather than the weak expression of MMP-2 from the same cells (Teesalu et al., 1999) (Table 4).

To date, MMP-2 and MMP-9 expression in late mouse placenta remains unclear (Table 4), and only membrane-type matrix metalloproteinase (MT-MMP) expression was sparsely reported. These proteases are essential for pericellular matrix remodeling in late gestation (Szabova, et al., 2010). The labyrinth displays strong overlapping expression of MT1-MMP and MT2-MMP, both critical for syncytiotrophoblast formation and placental vasculogenesis, although the knocking-down of MT-MMP activity after labyrinthine formation is compatible with development to term (Szabova, et al, 2010).

Overall, little is still known about trophoblast expression and MMP-2 and -9 activity in mature and late mouse placenta. Further studies are needed to clarify MMP roles in trophoblast invasion from mid-gestation to term in mouse placenta.

4. Comparative dynamics of trophoblastic MMP-2 and MMP-9 expression during endotheliochorial and hemochorial placentation

Regarding dynamic trophoblastic expression of MMP-2, the canine cytotrophoblast has the highest MMP-2 expression both at implantation and in the first stage of the early placenta, its expression declining to low levels toward the end of gestation (Fig.3.A). Similarly, canine MMP-2 expression of syncytiotrophoblast is increased around the beginning of the early stage of placenta, falling to moderate levels in the mature placental stage (Fig.3.A). Considering these data, it seems that MMP-2 expressed by cytotrophoblast directs the initial events of canine placental invasiveness whereas the expression of this protease by ST is involved in pregnancy termination processes.

However, in mouse placenta, MMP-2 expression by primary TGCs during implantation is lower than in the same canine stage, although its expression increases during early placenta, reaching values similar to canine MMP-2 expression of syncytiotrophoblast in the second stage of early placenta. In parallel, MMP-2 expression of secondary-TGCs, low in the very early mouse placenta, also reaches its maximum in the second stage of early placenta, then falls in the mature placenta. Comparatively as a whole, and considering the data overlap, MMP-2 expression is maximum and has similar maximum levels in mouse primary TGCs and P-TGC and in canine syncytiotrophoblast in the first and second stages of the early placenta (Fig.3.A). Probably, during the first early stages of gestation, these differences in MMP-2 expression are related to the degree of invasion of cytotrophoblasts into maternal stroma and epithelium of glands, required to remodel ECM components of maternal tissue of EC-placenta, on which very early fetal nutrition depends. In the second phase of the early mouse placenta, invasion of P-TGC into decidual matrix and intense erosion of decidual microvasculature around the conceptus seems to need a high expression of MMP-2, probably more than of syncytiotrophoblast-MMP-2 expression of canine placenta, to sustain incipient embryo organogenesis and development of the labyrinth (Fig.3.A). Therefore, we suggest that MMP-2 of invasive canine cytotrophoblasts and mouse TGCs has an important role in the invasion and remodeling of maternal tissues during the first stages of early placentation. However, more studies are needed to elucidate the participation of MMP-2 at advanced stages of these two placentas.

In contrast to MMP-2 expression, cytotrophoblast MMP-9 expression is low at implantation, decreasing further at the early placentation stage (Fig.3.B). MMP-9 from endotheliochorial syncytiotrophoblast is very low in the early and mature placenta, remaining constant through these stages in canine. However, in mouse, moderate to high levels of MMP-9 expression were reported in primary TGCs at implantation and early placenta, although its expression is not detected or not reported in mature and late placenta (Fig.3.B). However, the gradual increment of MMP-9 of P-

TGCs from the mouse early placental stage up to its maximum in the second phase of the early placenta, then continuing with moderately high levels until term gestation (Fig.3.B), could indicate a greater role of this mouse MMP in several invasive events throughout mouse placentation, such as those depending on C-TGC and SpA-TGCs, compared to the role of trophoblastic MMP-9 expression of endotheliochorial placentation. Therefore, the relatively lower expression of MMP-9 in endotheliochorial than in hemochorial placentation throughout pregnancy perhaps makes the hemochorial more invasive than the endotheliochorial placenta. However, to date, we have very little information about MMP-9 expression in advanced mature endotheliochorial and hemochorial placentas.

In synthesis, data comparing the first stage of endotheliochorial and hemochorial gestation reveal that MMP-2 is more important than the MMP-9 expression in the endotheliochorial placenta, whereas MMP-2 and MMP-9 may have similar expressions and roles during early trophoblastic invasiveness in mouse hemochorial placentas. However, similar to what occurs in humans, only MMP-9 mRNA is strongly expressed in canine placenta at term. Additional studies are needed to fully clarify the participation of these MMPs in trophoblast invasion in mature and late endotheliochorial and hemochorial placentas.

5. Discussion and concluding remarks

This review aims to highlight differences in MMP-2 and MMP-9 expression and potential activity in invasiveness processes of endoteliochorial and hemochorial placentas, using canine and mouse models, and to stress the potential implications of these studies for research of human placental complications.

The MMP/TIMP system is a very important regulator of placenta invasiveness. MMPs are present in all eutherian mammal studies during the stages of placentation (Menino et al, 1997).

Specifically, expression and activity of MMP-2 and MMP-9 is evident in endotheliochorial and hemochorial placenta during gestation, the temporal differences in expression and activity of these enzymes in these types of placentas appearing to be related to some characteristics of the placentation process; for example, the early higher expression of MMP-2 in canine placenta compared to MMP-2 of mouse placenta is related to glandular resorption produced during this period in the former placenta. In the last stages of mature placenta, decreased expression and activity of MMP-2 and MMP-9 correlates with a lower rate of invasiveness, this being indicative of placental maturation. In this regard, although the role of MMP-2 and MMP-9 in later gestation has not been fully determined, MMP-9 could have more significant participation in the separation of the placenta during delivery. New studies are needed to better understand the role of these MMPs during the last phases of endotheliochorial and hemochorial mature placenta, including their relevance forpostpartum pathophysiology.

The great variability of the placentas of different mammals makes it impossible for us to use any single species as a model to study this organ in humans. The small size and extremely altricial pups are negative aspects of using the mouse as a model. For this reason, it is important to search for models with larger body size that deliver more developed offspring (Carter, 2020). Although both hemochorial and endotheliochorial trophoblast invasion induces transformation of the endometrium, the placenta of *Canis familiaris* has a higher degree of invasiveness than other endotheliochorial placenta, and therefore has been proposed as a model to study some aspects of human pre-eclampsia (Kutzler et al., 2012). On the other hand, subinvolution of placentation sites is a disorder that produces late postpartum uterine bleeding, among other obstetric complications, during the puerperium of women (Weydert and Benda 2006). In this entity, the trophoblast is found in the postpartum uterus as a result of a situation opposite to that of preeclampsia, which is characterized by greater invasiveness of the trophoblast in the uterus (Ramkumar and Karshining

2021). Subinvolution is not described in rodents except in the case of a capybara in captivity (Juan-Sallés et al., 2005), nor in any other mammal except the bitch, in which it generates puerperal disorders and whose pathogenesis also includes the persistence of trophoblastic cells in the postpartum uterus (Fernández et al., 1998). Therefore, the female dog is the only model available to study this human postpartum disorder. Interestingly, no studies in dogs or women have been reported on MMP expression or activity in the subinvolution of placentation sites. We consider that the study of these enzymes in the disease in bitches may be important to increase its understanding in women since MMP-9 is essential for separation of the placenta from the uterus during human delivery (Demir-Weusten et al., 2007; Sundrani et al., 2017) and the mRNA of this metalloprotease is increased in the placenta at canine term (Fellows et al., 2012). Therefore, *Canis familiaris* could provide a model to study some aspects of invasiveness of early and term placenta with results that could potentially be extrapolated to human placentas.

However, since the first trimester of feto-placental pathologies in humans has been extraordinarily difficult to study because of logistical and ethical challenges associated with their examination, animal models with human placental homologies in the first trimester are critically important for longitudinal investigation of early events of healthy pregnancy outcomes. Despite some differences in pregnancy physiology, rodents have been widely used for studying human pregnancy disorders, such as preeclampsia (Sones and Davisson, 2016). In this regard, a timeline of mouse gestation and analogous trimester demarcations in human pregnancy is given, showing that mice have a relatively short gestation period (20 days) compared to 9 months for humans. Importantly, 1st-trimester early pregnancy milestones leading to placenta formation make up half of the gestation time in mice (Sones and Davisson, 2016). A thorough understanding of the significance of each day of mouse gestation leads to crucial translation of findings to the human gestational disease process. On the other hand, the applicability and use of murine KO models also

aim to study participation of MMPs in gestation outcomes. Therefore, single gene mutations in mice that develop specific pregnancy-associated pathologies, such as the matrix metalloproteinase 9 (MMP9)-null mouse, have been shown to phenocopy features of preeclampsia and intrauterine growth restriction (IUGR) (Dubois et al., 2000; Plaks et al., 2013). Homozygous matings between MMP9-null mice resulted in pregnancies with resorptions and poorly developed feto-placental units at midgestation which was attributed to impaired differentiation-invasion of trophoblast cells (Plaks et al., 2013). Therefore, MMP-9 production by trophoblast giant cells is needed for proper trophoblast invasion into the decidua; this MMP imbalance also leads to inadequate remodeling of the decidua and morphologically abnormal maternal-embryonic connections early at 7.5 dpc (Plaks et al., 2013). These results underscore the importance of shifting the focus of human gestational disease research toward the origins of abnormal placental processes in maternal complications.

Studies on MMP-2 and -9 expression in animal models should be continued, highlighting the importance of analyzing changes in MMP-9 in the subinvolution of human and canine placentation sites and the role of expression/activity alterations of these proteases in mouse hemochorial placentation as a potential model to elucidate the etiologies of human placental alterations and gestational complications during pregnancy and puerperium.

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Conflict of interest

The authors have no conflict of interest or competing interests. All co-authors have read, approved, and concur with the submitted manuscript. No conflict of interest that could be perceived as prejudicing the impartiality of the research reported. There is no potential conflict of interest with any financial aid.

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Legends of figures

Figure 1. Endotheliochorial and hemochorial (mouse) early placenta

Representative figures of the first and second stages of early endotheliochorial (A, C) and hemochorial mouse (B, D) placenta. CT: cytotrophoblast cells; s-TGCs: secondary giant trophoblast cells; e-Tb: ectoplacental proliferative trophoblast cells; p-TGCs: primary giant trophoblast cells; ST: syncytiotrophoblast cells; P-TGCs: parietal trophoblast giant cells, at the decidual interface; SpA-TGCs: spiral artery trophoblast giant cells; SpT: spongiotrophoblast cells; GCs: glycogen cells, in their early differentiation stage; lab-ST: labyrinthine syncytiotrophoblast cells in their early differentiation stage; cTB: labyrinthine chorionic trophoblast cells; SpA: decidual spiral arteries, showing the endothelial cells and partially remodeled smooth muscle. YS: yolk sac. ml: maternal blood lacunae. JZ: junctional zone. Lab: labyrinth. ec: maternal endothelial cells. sm: smooth muscle of spiral arteries. Inserts represent a transverse section of placenta showing the position of the embryo and its extraembryonic membranes. Arrow in the insert of D indicates the orientation of mesometrial decidua in the mouse implantation site.

Figure 2. Mature definitive endotheliochorial and hemochorial placenta

Representative figures of endotheliochorial (A) and hemochorial (mouse) (B) mature placenta. CT: cytotrophoblast cells; ST: syncytiotrophoblast cells; gl-ep: remodeled glandular epithelium; P-TGCs: parietal trophoblast giant cells at the decidual interface; GCs: glycogen cells; SpT: spongiotrophoblast cells; SpA-TGCs: spiral artery trophoblast giant cells; ST; syncytiotrophoblast cells; 9: S-TGCs: sinusoidal trophoblast giant cells; 10: lab-TB: labyrinthine trophoblast cells, including S-TGCs and ST; C-TGCs: canal-trophoblast giant cells; SpA: decidual spiral arteries showing remodeled endothelium and replaced by trophoblast cells; fe: fetal endothelium;

ml: maternal lacunae. JZ: junctional zone. Lab: labyrinth. Inserts represent a transverse section of placenta showing the position of the embryo and its extraembryonic membranes.

Figure 3. Comparative dynamics of MMP-2 and MMP-9 expression in invasive trophoblastic cells from endotheliochorial and hemochorial (mouse) placenta

Graphics show the dynamic changes of trophoblastic MMP-2 (A) and MMP-9 (B) expression levels in major placental stages during endotheliochorial and hemochorial (mouse) gestation. CT: cytotrophoblast cells; ST: syncytiotrophoblast cells; Sec- or P-TGCs: secondary or parietal trophoblast giant cells.

Figure 1

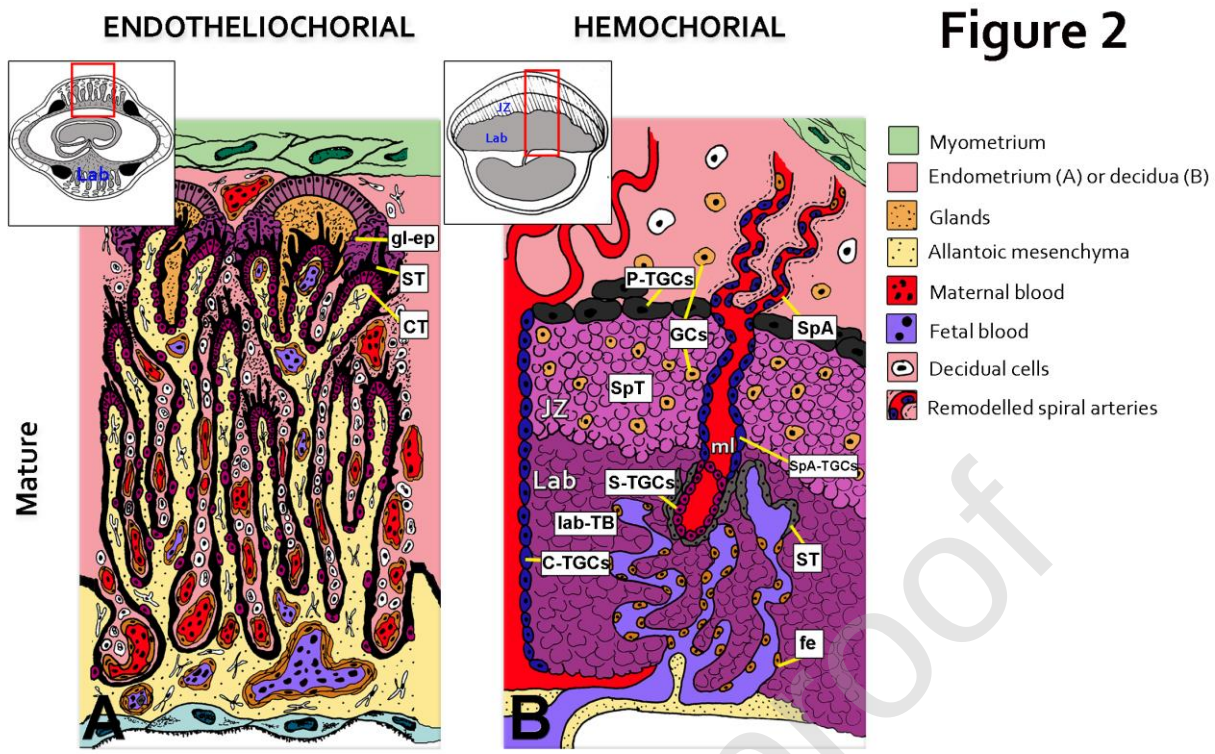

Figure 2

Figure 3

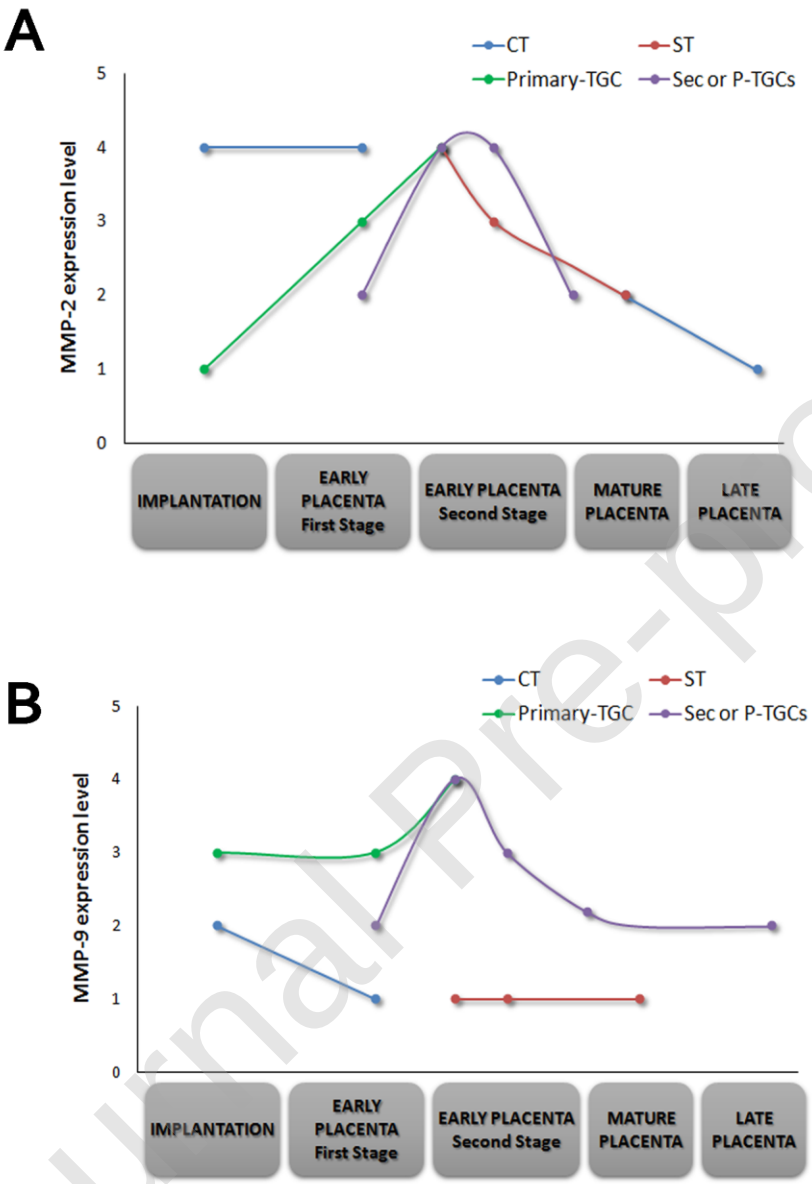

Table 1: Summary of endotheliochorial (canine) and haemochorial (mouse) placental developmental events and trophoblast invasiveness

Gestational stage	Endotheliochorial (canine) gestation			Haemochorial (mouse) gestation		
	Days p.c	Gestational process	Trophoblast invasive process	Days p.c	Gestational process	Trophoblast invasive process
Preimplantation	**1-11 dpc**	Pre-implantation		**1-4.5 dpc**	. Pre-implantation	
Implantation	**12-15 dpc**	. Superficial; central implantation . Apposition, TB-uterine contact . TB penetration to maternal glandular layer . Yolk sac, amnios and allantoic development . Chorioallantoic membrane formation	. First invasion of TB to epithelium and endometrium of glandular layer	**5-6 dpc**	Implantation by displacement . Attachment-penetration of blastocyst into endometrium	**Uterine TB invasion and remodeling:** Apoptosis of uterine epithelium Rupture of epithelial basement membrane Modification of uterine stroma (ECM)
Early placenta	**18-30 dpc**	**Labyrinth**		**6.5-8 dpc**	. Chorion development (from EXE and mesEXE)	**Primary TGCs:**
		. Early chorionic villi development (Primary villi) . Initial differentiation of ST from CT . Chorionic villi growth and expansion	CT and ST: remodeling of connective maternal tissue CT and ST-remodeling of maternal smooth muscle of maternal arteries		. EPC development . Differentiation of secondary TGC in EPC . Allantoic bud formation . Occlusion of the EPC . EPC: expansion and patterning of progenitors of TB of JZ . Attachment of allantois with chorion	invasion of maternal stroma and capillaries **Secondary TGC:** Mesometrial invasion of uterine luminal epithelium Mesometrial endometrial remodeling
		Junctional zone: . Increased trophoblast expansión . Glandular epithelial and stromal remodeling	ST invasion into glandular layer Intense invasion of ST into endometrial glandular cavity High ST-epithelial, endometrial stromal and vascular remodeling	**8.5-9 dpc**	.Establishment of chorioallantoic placenta	
				9.5-11.5 dpc	**. Labyrinth:** **.Development of** branching morphogenesis, . TB differentiation (ST, S-TGC)	
					. Junctional zone .Differentiation of P-TGC, SpA-TGC, SpT, GCs, C-TGCs . Canals are evident in center and basis of placenta	. Interstitial invasion of P-TGC to decidual stroma . Endo/perivascular invasion of SpA-TGC and C-TGCs
Mature placenta	**30-45 dpc**	**Labyrinth:** Maximum development of the placental labyrinth Increase of fetal-maternal vascularization	Continue remodeling of lamellar maternal connective tissue	**11.5-13.5 dpc**	**Labyrinth:** .Expansion and growth in size and complexity . Complete interhemal membrane development	. P-TGC at interface with decidua . ST layers, S-TGCs, C-TGCs
		Junctional zone Remodeled glandular endometrium and epithelium	Continue ST invasion		**Junctional zone:** . SpT projections within the Lab (E13.5) . Continue GC differentiation and expansion	. GCs invasion into decidua, around SpA . Endo/perivascular continue invasion of SpA-TGC
Late placenta	**45-60 dpc**	**Placental fully developed labyrinth**		**14-18 dpc**		

Summary of comparative events of canine endotheliochorial and mouse hemochorial placenta and their trophoblast invasive processes during gestational stages. Days pc: days post-coitus. EPC: ectoplacental cone; TB: trophoblast; CT: cytotrophoblast; ST: syncytiotrophoblast; TGC: trophoblast giant cell; P-TGC: parietal-TGC; C-TGC: canal trophoblast giant cells; SpT: spongiotrophoblast; SpA-TGC: spiral artery trophoblast giant cell; S-TGC: sinusoidal trophoblast giant cells; GCs: glycogenic cells; SpA: spiral artery; EXE: extraembryonic ectoderm; mesEXE: extraembryonic mesoderm; YS: yolk sac; CAM: chorioallantoic membrane; ECM: extracellular matrix; Lab: labyrinth; JZ: junctional zone.

Table 2. **Expression and activity of MMP-2 and MMP-9 during endotheliochorial and hemochorial (mouse) implantation**

	MMP-2			MMP-9		
	Canine	**Feline**	**Mouse**	**Canine**	**Feline**	**Mouse**
Gene expression	ND	+	+/-	ND	ND	ND
Protein expression	+++	ND	+/-	+	ND	++
MMP activity	+++	ND	ND	+	ND	++

Summary of gene (mRNA) and protein expression and activity of active form (zymography) of MMP-2 and MMP-9, in whole canine and feline placental tissues during implantation. Relative levels are represented as follows: −: negative (without MMP expression/activity or non-detected), +/−: very weakly positive reaction, +: low-moderate reaction; ++: moderate reaction, +++: strong reaction. ND: not reported data.

Table 3. MMP-2 and MMP-9 expression in subtypes of trophoblastic cells from early endotheliochorial and hemochorial placentas

MMP-2				MMP-9			
Canine	**Feline**	**Mouse**		**Canine**	**Feline**	**Mouse**	
CT (+++) ST(+++)	CT (-) ST (-)	6.5 dpc	ND	CT (+/-) ST (+/-)	ND	6.5 dpc	Pr-TGCs (++)
		7.5 dpc	Pr-TGCs (-)? Sc-TGC (-)?			7.5 dpc	Pr-TGCs (++) Sc-TGC (+/-)
		8 dpc	Pr-TGCs (++) Sc-TGC (+)			8 dpc	Pr-TGCs (++) Sc-TGC (+)
		8.5 dpc	Pr-TGCs (+++) P-TGC (+++)			8.5 dpc	Pr-TGCs (+++) P-TGC (+++)
		9.5 dpc	P-TGCs (+++)			9.5 dpc	P-TGCs (+++)
		10 dpc	P-TGC (+/-) SpT (+/-) cT (-)			10 dpc	P-TGC (++) SpT (++) cT (+/-)
		10.5 dpc	P-TGC (+/-)			10.5 dpc	P-TGC (+)

Summary of comparative expression (immunohistochemistry) of matrix metalloproteinases (MMPs) in subtypes of trophoblastic cells of canine, feline and mouse placenta during early gestation. Relative intensity of MMP expression is represented as follows: −: negative (without MMP reaction or non-detected), +/−: very weakly positive reactivity, +: low-moderate reactivity; ++: moderate reactivity, +++: strong reactivity. ?: indicates not confirmed reported data. ND: not reported data. dpc: day post-coitus. CT: cytotrophoblast; ST: syncytiotrophoblast, TGC: trophoblast giant cell, Pr-TGCs: primary TGCs; Sc-TGC: secondary TGC; P-TGC: parietal-TGC; cT: chorionic trophoblastic cells; SpT: spongiotrophoblastic cells.

Table 4. MMP-2 and MMP-9 expression in subtypes of trophoblastic cells from mid-mature endotheliochorial and hemochorial placentas

	MMP-2				MMP-9			
	Canine	**Feline**	**Mouse**		**Canine**	**Feline**	**Mouse**	
Mid-mature placenta	CT (+) ST(+)	ND	10.5-11 dpc 11.5-13 dpc	P-TGC (+/-) ND	ST (+/-)	ND	10.5-11 dpc 11.5-13 dpc	P-TGC (+) ND
Late placenta	(+/-)	ND	16.5 dpc 18.5 dpc	P-TGC (-)? P-TGC (-)?	(+)	ND	16.5 dpc 18.5 dpc	P-TGC (+) ? P-TGC (+) ?

Summary of comparative expression (immunohistochemistry) of matrix metalloproteinases (MMPs) in subtypes of trophoblastic cells of canine, feline, and mouse placenta during mid-gestation. Relative intensity of MMP expression is represented as follows: −: negative (without MMP reaction or non-detected), +/−: weakly positive reactivity, +: moderate reactivity, ++: strong reactivity. ?: indicates not confirmed reported data. ND: not reported data. dpc: day post-coitus. CT: cytotrophoblast; ST: syncytiotrophoblast, TGC: trophoblast giant cell; P-TGC: parietal-TGC.