This research investigated the development of a novel canine model to study preeclampsia. Normal canine placental development has morphologic and histologic similarities to the shallow trophoblast invasion occurring with preeclampsia in humans, which makes the dog a particularly good choice for modeling this disease and will be an improvement on existing animal models. Preeclampsia is a pregnancy-specific syndrome, occurring in mild (late onset) and severe (early onset) forms. Severe preeclampsia is a major cause of maternal, fetal, and neonatal morbidity and mortality worldwide. It affects 0.35-1.40% of human pregnancies. Despite intense investigation, the cause (and therefore the prevention or treatment) of shallow trophoblast invasion in preeclampsia remains largely unknown. In a normal human pregnancy, trophoblasts invade the endometrium and myometrium as well as the maternal blood vessels (hemochorial placentation). In preeclampsia, trophoblast invasion is shallow and vascular transformation incomplete. In contrast to the normal human placenta, trophoblasts within the canine placenta only invade to the level of the endothelial cells within the maternal blood vessels (endotheliochorial). In this way, normal canine placental development is similar to preeclampsia. The hypothesis of this research was that isolated canine trophoblasts will express similar proteins as human preeclamptic trophoblasts. The objectives of the research were to (1) isolate canine trophoblasts from fresh and cryogenically frozen placenta and (2) perform
immunocytochemistry and immunohistochemistry on canine trophoblasts for proteins expressed in human preeclamptic trophoblasts. Cellular morphology was similar to that reported for trophoblasts. More than 97% of the cells cultured expressed cytokeratin-7. Although both matrix metalloproteinases (MMPs) were immunolocalized to the cytoplasm, MMP2 was found in large, coalescing granules, whereas MMP9 was more diffusely expressed throughout the cell. More cultured canine trophoblasts expressed MMP9 (54.7±3.4%) compared to MMP2 (40.3±1.8%) (p=0.02). Cryopreserving placental tissue prior to primary cell culture had no effect on cell proliferation (p=0.37). Relaxin, vascular endothelial growth factor, and tissue inhibitor of metalloproteinase 2 were positively expressed in primary canine trophoblasts. Immunohistochemical results revealed CK-7, MMP9, TIMP2 and relaxin was expressed in trophoblasts along the villous margin with MMP9, TIMP2 and relaxin extending towards the basement membrane. S100A4 was minimally expressed in the basement membrane. MMP2 was strongly expressed within the basement membrane. CK-7, MMP2, MMP9 & TIMP2 were all immunolocalized to the same cells in canine placental sections as previously described in human preeclamptic placental sections. These results have demonstrated the cellular similarities in protein expression between normal canine and human preeclamptic trophoblasts thereby confirming this model is suitable for further studies.
Cellular Characteristics of Canine Trophoblasts

by
Laura Sahlfeld

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

__________________________________________________________________
Laura Sahlfeld, Author
I still remember the first time I met Dr. Michelle Kutzler. She was working at Oregon State University College of Veterinary Medicine and gave a seminar one night on breeding older mares. Of course I had to talk horse talk with her afterwards but I never knew that two years later I would be sitting in her office and realizing I was going to be her graduate student. I was fortunate to have a driven graduate advisor. She knew the goals I had set for myself and assisted me in achieving them. We have spent many long hours together working on research and I appreciate her knowledge and guidance.

My mother has always supported me and without her support, I would not be where I am today. Thank you for sharing your thoughts and concerns I had about what I was doing with my life. You were my greatest support system.

My father has personally witnessed how one can spend many hours in a laboratory to get the tiniest result. He never objected to spending his time with me in the laboratory. He understood that my research was important to me. Dad, I hope you never have to witness me dissecting a placenta again.

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CONTRIBUTION OF AUTHORS

Dr. Timothy Hazzard assisted in the collection of placentas and edited the manuscript.
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CHAPTER I

INTRODUCTION

1.1 PREECLAMPSIA

1.1.1 Summary

There are two categories of preeclampsia: severe (early onset) and mild (late onset), with the latter condition unrelated to placental disease (Huppertz, 2008). For the purposes of this review, only severe (early onset) preeclampsia will be discussed. Severe preeclampsia is a major cause of maternal, fetal, and neonatal morbidity and mortality worldwide (Norwitz et al., 1999; Haddad and Sibai, 2005; Huppertz, 2008; Orendi et al., 2011). It affects 0.35-1.40% of human pregnancies (Norwitz et al., 1999; Haddad and Sibai, 2005; Huppertz, 2008; Orendi et al., 2011). Risk factors for developing both forms of preeclampsia include prior history of preeclampsia, diabetes mellitus, chronic hypertension, and primaparity. The only treatment for severe preeclampsia requires immediate delivery.

1.1.2 Historical Perspective

Preeclampsia has been a recognized disorder since the time of the ancient Greeks (Chesley, 1984). In 1596, Gabelchoverus described preeclampsia as “epilepsy of the pregnant uterus”, which was also later described by Chaussier in 1824 (Chesley, 1984). Between 1837 and 1867, 27 to 37% of maternal mortality was attributed to preeclampsia (Chesley, 1984). In 1843 and 1844, proteinuria (by Lever) and high blood pressure (by Vinay) were identified as symptoms of preeclampsia, which was later confirmed by Vaquez and Nobécourt in 1897 (Chesley, 1984).
Treatment for preeclampsia was often extreme. Protein was postulated to cause preeclampsia so low protein diets were prescribed to pregnant women (Chesley, 1984). Others believed a large uterus was to blame for compressing the kidneys resulting in proteinuria so preeclamptic women were treated by lying belly down on a mattress with a hole cut out (Chesley, 1984). For centuries, phlebotomy and purgation were the main treatments of preeclampsia (Chesley, 1984). Bovine parturient paresis was believed to be similar to preeclampsia so obstetricians applied techniques used by veterinarians (e.g. injecting potassium chloride into the breasts of preeclamptic women) (Chesley, 1984). Further treatment lead to bilateral mastectomies but results were unsuccessful (Chesley, 1984). Starting in 1906, magnesium sulfate became crucial in the management of preeclampsia because it was thought that magnesium sulfate would have the same effect of controlling “uterine” convulsions in preeclampsia as it did with tetanus (Chesley, 1984). Historical treatments for preeclampsia seem outlandish but even today obstetricians try new drugs that in time may also seem just as bizarre.

1.1.3 Etiology

With the exception of nulliparity, there is no known prevention of preeclampsia (Norwitz et al., 1999). In 1694, Mauriceau found that women pregnant with their first child were more likely to develop preeclampsia (Chesley, 1984). In addition, in 1775, Hamilton found that plural (e.g. twin) pregnancies greatly increased the risk of developing preeclampsia (Chesley, 1984). Many authors agree that diabetes also increases the incidence of preeclampsia (Chesley, 1984). Socioeconomic statuses, illegitimate pregnancy, rural versus urban living, race, fetal malformation, hair color and weather have all been speculated as risk factors. It has also been speculated that a recessive gene could predispose to the development of preeclampsia (Chesley, 1984).
1.1.4 Pathophysiology

The pathophysiology of severe preeclampsia is not well understood but its development occurs early in pregnancy (Norwitz et al., 2002). In normal pregnancy, trophoblasts invade the decidua and myometrium and invoke physiological changes in the walls of the spiral arteries (Brosens et al., 1972; De Wolf et al., 1980; Pijnenborg et al., 1980). In preeclampsia, trophoblasts invade shallowly (Brosens et al., 1972; Gerretsen et al., 1981, Sheppard and Bonnar, 1981, Pijnenborg et al., 1991). Meekins and colleagues (1994) found that trophoblasts invaded 100% of decidual and 76% of myometrial spiral arteries in normal pregnancies while only 44% of decidual and 18% of myometrial spiral arteries were invaded in preeclampsia. In fact, some decidual spiral arteries from women with preeclampsia resembled those of non-pregnant women (Khong et al., 1986). However, the extent of trophoblast invasion in preeclampsia is variable (Pijnenborg et al., 1991; Meekins et al., 1994). As pregnancy progresses, there is an increase in demand for oxygen and nutrients from the fetoplacental unit (Norwitz et al., 2002). Due to shallow trophoblast invasion in preeclampsia, spiral arteries are not able to accommodate the needed increase in blood flow, which results in the clinical syndrome of preeclampsia (Norwitz et al., 2002).

1.1.5 Clinical Syndrome

There are two clinical syndromes of preeclampsia: severe (early onset) and mild (late onset), with the latter condition unrelated to placental disease (Huppertz, 2008). For the purposes of this review, only severe (early onset) preeclampsia will be discussed. Severe preeclampsia occurs before 34 weeks gestation (Huppertz, 2008). Severe preeclampsia comprises about 5 to 20% of all preeclamptic cases and affects 0.35-1.4% of all human pregnancies (Huppertz, 2008). Some features of severe preeclampsia include fetal growth restriction and changes in blood flow within the spiral, uterine, and umbilical arteries (Huppertz, 2008). According to the American
College of Obstetricians and Gynecologists (ACOG), the clinical diagnosis of preeclampsia is based upon two symptoms in previously normal women: hypertension (sustained resting blood pressure ≥140/90 mm Hg) and proteinuria (≥0.3 g/24 hours or ≥2+ on a clean-catch urinalysis in the absence of urinary tract infection) (ACOG, 2002). In addition to hypertension and proteinuria, severe preeclampsia is characterized by one or more of the following: cerebral/visual disturbances, thrombocytopenia, impaired liver function, epigastric/right upper-quadrant pain, oliguria <500 mL/24 hours, pulmonary edema/cyanosis, fetal growth restriction and HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome (ACOG, 2002). A complication of high blood pressure resulting from severe preeclampsia is cortical blindness, which is characterized by headaches and seizures (Norwitz et al., 2002). About 15-20% of deaths from preeclampsia are from cerebrovascular accidents (Norwitz et al., 2002). One of the most severe consequences of severe preeclampsia with HELLP syndrome is liver rupture, which results in greater than 30% maternal death rate (Smith et al., 1991). Preeclampsia cases complicated by HELLP syndrome also have significantly increased fetal death rates (Norwitz et al., 2002).

1.2 MODELS OF PREECLAMPSIA

1.2.1 Summary

Preeclampsia involves how trophoblasts interact with and change the maternal endometrium (Pennington et al., 2012). Although many aspects of preeclampsia can be studied in vitro, in vivo models are necessary to understand this complex disorder (Pennington et al., 2012). The ideal animal model would be one that mimics the cause of preeclampsia (shallow trophoblast invasion) as well as displays the symptoms (proteinuria, hypertension) (McCarthy et al., 2011). Animal models that only mimic the symptoms of preeclampsia will not be discussed in this review.
1.2.2 Animal Models of Preeclampsia

The differences in structure and form of placentation between animals makes finding an ideal in vivo model particularly difficult (Pennington et al., 2012). Human trophoblasts are normally highly invasive and penetrate the endometrium (interstitial) where they invade the lumen of the spiral arteries (endovascular) and then continue to invade into the myometrium (James et al., 2012). Non-human primates have the most closely related type of placental development as humans but the cost, availability and ethical consideration of using these species do not make them an ideal model choice (Pennington et al., 2012). The most commonly used animal models are rats and mice (Pennington et al., 2012). Both of these rodents have a hemochorial-type placenta that has interstitial and endovascular trophoblast invasion but does not continue into the myometrium (Pennington et al., 2012). There are rodent models that display symptoms of preeclampsia (like the BPH/5 mice, an inbred mouse strain with mildly elevated blood pressure, or CBA/J x DBA/2 mice, a model of recurrent miscarriage and immunologically-mediated preeclampsia) but neither of these models develop placental lesions (Davisson et al., 2002; Ahmed et al., 2010). The administration of doxycycline to transgenic Angiotensin II–dependent preeclamptic rats during early pregnancy mimics actions of matrix metalloproteinase inhibitors by decreasing trophoblast invasion and spiral artery remodeling and resulting in mild intrauterine growth retardation (Verlohren et al., 2010). The overexpression of anti-angiogenic factors has been explored in rodent models (Pennington et al., 2012). However, this type of model is only useful for understanding the pathophysiology and treatment of the symptoms of preeclampsia and not the underlying cause (Pennington et al., 2012).

1.2.3 In Vitro Models of Preeclampsia

In vitro models allow the study of cellular interactions related to trophoblast proliferation, migration and invasion (Pennington et al., 2012). These include primary
trophoblast cell culture, immortalized trophoblast cells in culture, and cultured placental explants (Pennington et al., 2012). The options are to either use primary cells that have been isolated from fresh tissue or an established cell line (Whitley, 2006). Primary cultures are derived from tissue and cultured as either an explant or single cell suspension following dissociation by enzyme digestion (Sigma-Aldrich, n.d.). Primary cultures usually retain many of the characteristics of the cells in vivo (Sigma-Aldrich, n.d.). Primary cultures have not been passaged. If they are passaged, they become a cell line and are no longer described as “primary” (Sigma-Aldrich, n.d.). Primary cells do not proliferate indefinitely but instead are “mortal” (Kuilman et al., 2010). Their proliferative capacity displays three phases (Hayflick and Moorhead, 1961). In phase one, before the first passage, there is a period of little proliferation, during which the culture is established (Hayflick and Moorhead, 1961). In phase two, there is rapid cell proliferation, which is followed by phase three where proliferation gradually grinds to a halt (Hayflick and Moorhead, 1961).

Cell lines may be obtained from out-growths or from manipulated primary cultures (Whitley, 2006). A disadvantage of an established cell line is that the methods used to transfect/immortalize could alter the regulation of cell division hence affecting differentiated functions and gene expression (Whitley, 2006). There are more than 21 immortalized cell lines derived from human trophoblasts (Whitley, 2006; Orendi et al., 2011). Some early trophoblast lines derived from choriocarcinomas include BeWo, JEG and JAR (Whitley, 2006). However, cell lines like the HTR-8/SVneo and SGHPL, derived from first trimester trophoblasts and transfected with the SV40 virus, are not useful as surrogates in studying trophoblasts since some have dedifferentiated into a fibroblast phenotype as determined by vimentin expression (Orendi et al., 2011). On a related topic, hypoxia enhances invasion of HTR-8/SVneo cells (Graham et al., 1998), which has been confirmed in pregnant hypoxic rats that had increased trophoblast invasion (Rosario et al., 2008). However, it has been shown that hypoxic conditions inhibit primary first trimester placental cells invasion through Matrigel (Hunkapiller and Fisher, 2008).
Cultured placental explants have been used to study the materno-fetal interface (Miller et al., 2005). The use of placental explants allows for examination of placental tissue function and direct assessment of the effects of novel therapeutic modalities (Miller et al., 2005). Placental explants, delivered at term, can provide information about how the placenta behaves in late gestation (Miller et al., 2005). Cultured term placental explants from women affected by preeclampsia demonstrate the long-term consequence of this disease on tissue turnover, secretory function and nutrient function (Miller et al., 2005).

1.2.4 Conclusion

All the in vitro models discussed provide insight into factors that contribute to preeclampsia. In vivo models and in vitro models complement each other but no existing model replicates all aspects (cause and clinical signs) of preeclampsia (Pennington et al., 2012). Pennington and colleagues (2012) emphasized the consensus of researchers that “the field remains in desperate need of bold investigators, innovative modeling approaches and new insights into pathophysiology”.

1.3 TROPHOBLAST CELL CULTURE

Trophoblast isolation for primary cell culture is based upon classical protocols including enzymatic digestion and purification steps (Orendi et al., 2011). In the mid-1980s, a standard trypsinization protocol was developed (Kliman et al., 1986). Trypsinization removes trophoblasts from placental villous tissue (Hunkapiller and Fisher, 2008). Huppertz and colleagues (1999) demonstrated that trypsinization of placental tissue results in the isolation of grouped mononuclear cytotrophoblasts. After this discovery, researchers developed methods to separate grouped cytotrophoblasts (Guilbert et al., 2002; Tannetta et al., 2008). In 1986, Kliman and
associates developed a Percoll gradient procedure that yielded 80% purity of
trophoblasts (Kliman et al., 1986). A Percoll gradient separates cells according to
their size (density) (Hunkapiller and Fisher, 2008). Magnetic beads are commonly
used to purify trophoblast isolates (Loke et al., 1989; Hunkapiller and Fisher, 2008;
Stenqvist et al., 2008; Douglas and King, 1989). Magnetic beads can be used for
positive and negative cell isolation (Life Technologies, 2012). The positive method
involves covering the beads with an antibody that binds the target cells then uses a
magnet to attract the bead/cell combination and the supernatant is discarded (Life
Technologies, 2012). With the negative method, the beads attract the unwanted cell
population while the supernatant retains the target cells (Life Technologies, 2012).
Monoclonal anti-CD45 is a common leukocyte antibody used to coat beads to attract
contaminating leukocytes in human trophoblast isolates (Hunkapiller and Fisher,
2008; Stenqvist et al., 2008).

Fluorescence activated cell sorting (FACS) separates groups of cells into sub-
populations based upon fluorescent labeling (Abcam, 2012). The antibody binds a
protein that is expressed in the cells (Biology at Davidson, 2001). Cells are stained
using fluorophore-conjugated antibodies and are separated based on their fluorophore
(Abcam, 2012). Each individual cell enters a single droplet which becomes
electronically charged based upon the fluorescence of the cell from a laser that excites
the dye and emits a color that is detected by a light detector (Biology at Davidson,
2001, Abcam, 2012). Deflection plates attract or repel the cells into collection tubes
(Abcam, 2012). Fluorescence-activated cell sorting requires special equipment and
the cell yields are very low (Whitley, 2006).

1.3.1 Selected Markers for Human Trophoblasts

Understanding trophoblast physiology is key to developing a specific treatment
or prevention of preeclampsia. Cytokeratins are intermediate filament proteins. There
are more than nineteen cytokeratins, which differ in their cellular protein expression
Cytokeratin-7 (CK-7) is only expressed in epithelial cells (not mesenchymal cells) and its placental expression is limited to trophoblasts (Blaschitz et al., 2000).

GB25 is a monoclonal antibody that recognizes an unknown human trophoblast protein (Hsi and Yeh, 1986). Maldonado-Esruada and colleagues (2004) compared CK-7 and GB25 in trophoblasts from isolated first trimester human placentas and concluded that CK-7 was superior for identifying trophoblasts. In addition, this antibody is not commercially available, which prevents further comparisons.

HLA-G (human leukocyte antigen) is a class I major histocompatibility complex (MHC) antigen. It is expressed by trophoblasts so it is commonly used as a marker (Ellis et al., 1986; Blaschitz et al., 2005; Nagamatsu et al., 2004; James et al., 2006). HLA-G has four membrane-bound (G1, G2, G3, and G4) and three soluble (G5, G6, G7) isoforms (Ishitani and Geraghty, 1992; Paul et al., 2000). The key role of HLA-G is in modulating cytokine secretion to control trophoblast invasion (Le Bouteiller et al., 2003). It has been suggested that HLA-G plays a role in preventing the mother’s immune system from rejecting trophoblasts (Sargent, 2005). Preeclamptic trophoblasts of term placentas have reduced HLA-G expression compared to control pregnancies (Goldman-Wohl et al., 2000). Unlike HLA-G, CK-7 is constitutively expressed in trophoblasts, irrespective of stage of gestation or placental disease state.

In humans, relaxin is produced by cytotrophoblasts and syncytiotrophoblasts of the decidua basalis (Sakbun et al., 1990; Hansell et al., 1991), where it is an autocrine and paracrine hormone at the maternal-fetal interface (Bryant-Greenwood et al., 2005). Relaxin upregulates vascular gelatinase activity by inhibiting tissue inhibitors of matrix metalloproteinases and collagen expression (Jeyabalan et al., 2003). In early human pregnancy, uteroplacental blood flow is also affected by relaxin (Jauniaux et al., 1994). Low serum relaxin is associated with pregnancy complications in humans (MacLennan et al., 1986) and several species (Stewart and Stabenfeldt, 1985; Stewart
et al., 1992; Steinetz et al., 1996). In women, human chorionic gonadotrophin is a
stimulus for relaxin production during pregnancy (Davison et al., 2004).

Human chorionic gonadotropin (hCG) can be detected at nidation and peaks at
7 to 12 weeks gestation in humans before dwindling to low levels (Cole, 2009).
Immunohistochemical experiments in human first trimester placentas demonstrate that
hCG is produced mainly by syncytiotrophoblasts (Guibourdenche et al., 2010;
Handschuh et al., 2007). Some functions of hCG include advancement of
angiogenesis, trophoblast differentiation, decidualization and immune cell regulation
(Cole, 2010). Changes in hCG production or function could be related to the
development of preeclampsia (Norris et al., 2011).

1.3.2 Mediators of Human Trophoblast Invasion

Matrix metalloproteinases (MMPs), especially MMP2 and MMP9, act during
trophoblast invasion and parturition to remodel the extracellular matrix (Demir-
Weusten et al., 2007; Dimo et al., 2011). MMP2 and MMP9 have been the focus of
many preeclampsia investigations because they degrade type IV collagen, which is an
important component of the endometrial basement membrane (Köhrmann et al., 2009).
MMPs are tightly regulated by tissue inhibitors of matrix metalloproteinases (TIMPs)
(Demir-Weusten et al., 2007; Palei et al., 2008). TIMP1 and TIMP2 are the major
inhibitors of MMP9 and MMP2 (Palei et al., 2008). TIMP3 also inhibits MMP9
(Schultz and Edwards, 1997). It is debatable however whether TIMP3 functions
purely as a regulator of MMP9 activity or whether TIMP3 contributes to other aspects
of the decidualization process (Schultz and Edwards, 1997).

MMPs have been studied extensively in the human placenta (Staun-Ram et al.,
2004; Demir-Weusten et al., 2007; Palei et al., 2008; Dimo et al., 2011). MMP2 and
MMP9 are both expressed by human trophoblasts (Bischof et al., 1991; Shokry et al.,
2009) and MMP9 is responsible for trophoblast invasion (Librach et al., 1991). As
such, MMPs and their inhibitors are thought to play a role in the shallow trophoblast
invasion of preeclampsia (Lockwood et al., 2008; Palei et al., 2008; Shokry et al., 2009).

Angiogenesis requires pro-angiogenic factors, like vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) (Tjwa et al., 2003). VEGF binds to the VEGF receptor-1 (VEGFR-1), also called fms-like tyrosine kinase (flt-1), and VEGFR-2. PIGF only binds to the flt-1 (Tjwa et al., 2003). The placenta is a rich source of VEGF and PIGF (Ahmed et al., 1995; Khaliq et al., 1996). VEGF, PIGF and the flt-1 receptor have been shown to be key components in regulating trophoblast regulation, growth and differentiation in the first trimester human placenta (Ahmed et al., 1995; Crocker et al., 2001). Trophoblasts also secrete soluble flt-1 (sflt-1), which acts as an VEGF and PIGF antagonist (Clark et al., 1998; Maynard et al., 2003). There is evidence for increased placental expression of sflt-1 and reduced free/bioactive VEGF and PIGF in preeclampsia (Maynard et al., 2003; Tsatsaris et al., 2003; Chung et al., 2004).

Peroxisome proliferator-activated receptor gamma (PPARγ) regulates trophoblast proliferation and invasion (Parast et al., 2009). Placental abnormalities include reduced spongiotrophoblasts, expanded giant cell layers and small labyrinth development (Barak et al., 1999; Kubota et al., 1999). Due to these placental abnormalities, PPARγ-null mice embryos die during midgestation (Barak et al., 1999; Kubota et al., 1999). Placental protein 13 (PP-13, also known as galectin-13) was isolated by Bohn and colleagues in 1983 (Bohn et al., 1983; Visegrády et al., 2001; Burger et al., 2004). PP-13 binds to proteins between the placenta and endometrium (extracellular matrix), and acts in placental implantation and remodeling of maternal uterine arteries (Spencer et al., 2007). Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is expressed by villous cytotrophoblasts, not syncytiotrophoblasts (Pötgens et al., 2003) and has been used as a marker (Pötgens et al., 2003). Glucose-regulated protein 78 (GRP78; an endoplasmic reticulum stress protein) and pregnancy-associated plasma protein A (PAPP-A) are other marker used for identifying
trophoblasts (Laverrière et al., 2009; Cowans and Spencer, 2007; Spencer et al., 2007; Spencer, Cowans, and Nicolaides, 2008; Spencer, Cowans, Molina, et al., 2008).

1.4 CANINE PLACENTA

1.4.1 Gross and Histologic Description

During canine pregnancy, implantation occurs 16 to 18 days after the LH surge (Johnston et al., 2001). The placenta forms a zonary band (about 2.5 to 7.5 cm in width) around the uterine lumen (Johnston et al., 2001). Knobs of trophoblastic syncytium form in the uterine luminal epithelium as trophoblast invasion continues deeper into the endometrium (Barrau et al., 1975). The dog has an endotheliochorial placenta, which means the trophoblasts are adjacent to the uterine vessel endothelium (Johnston et al., 2001). As the syncytium spreads around maternal vessels, the trophoblasts create lacunae, which resemble the intervillous spaces within the primate placenta (Barrau et al., 1975; Ahokas and McKinney, 2009).

1.4.2 Selected Markers for Canine Trophoblasts

The placenta is the major contributor to the serum relaxin levels in the pregnant bitch. The trophoblast syncytium, formed after the onset of endotheliochorial placentation, has been identified as the source for relaxin in the canine placenta (Steinetz et al., 1989, Klonisch et al., 1999). Klonisch and colleagues (1999) found that immunohistochemical staining of canine uteroplacental tissue at day 30 of gestation positively immunostained for relaxin.

Using gelatin zymography, Beceriklisoy and colleagues (2007) found higher MMP2 activity in the endometrium and myometrium of pregnant dogs from 5-30 days after mating compared to nonpregnant dogs. With respect to MMP9, these investigators found increased activity in the endometrium and myometrium at 20-30
days after mating compared to nonpregnant dogs (Beceriklisoy et al., 2007). However, at 15-19 days after mating, MMP9 activity in the endometrium was lower compared to the nonpregnant dogs but higher in the myometrium compared to the nonpregnant dogs (Beceriklisoy et al., 2007). The highest MMP2 and MMP9 levels were reached around the time of implantation (Beceriklisoy et al., 2007).
1.5 REFERENCES


CHAPTER II

CELLULAR CHARACTERISTICS OF CULTURED CANINE TROPHOBLASTS

2.1 INTRODUCTION

Placental dysfunction caused by shallow trophoblast invasion is a serious complication of human pregnancies and a major cause of maternal morbidity, mortality, and premature delivery. Preeclampsia is an example of a gestational disease in humans that results from shallow trophoblast invasion (Brosens and Renaer, 1972; Brosens, Robertson, and Dixon, 1972; Naicker et al., 2003; Huppertz, 2008). Despite decades of research into the etiology of preeclampsia, the underlying cause of this disease (shallow trophoblast invasion) remains poorly understood (Cox et al., 2009). Current laboratory animal models (mouse, rat, guinea pig) have not been useful for evaluating targeted therapeutic intervention for shallow trophoblast invasion (Carter, 2007).

Unlike the hemochorial placenta of primates and rodents, the canine placenta is endotheliochorial, which is a naturally-occurring shallow trophoblast invasion form of placentation. Due to this feature, the canine placenta may be an important model to investigate the cellular and molecular processes involved in pathologic shallow trophoblast invasion in humans. In addition, healthy canine placental tissue at all stages of gestation can be obtained from animal shelter population control programs.

As in the hemochorial placenta, trophoblasts invade the uterine epithelium and endometrial stroma (decidua) in endotheliochorial placentation (Stoffel et al., 1998). However, in hemochorial placentas, trophoblasts continue to invade deeper into the myometrium (De Wolf et al., 1980; Pijnenborg et al., 1983). In rodents, the hemochorial placenta does not develop until nearly halfway through gestation, while in bats, the definitive hemochorial placenta is preceded by an endotheliochorial
placenta (Enders and Carter, 2004). Another feature of both hemochorial and endotheliochorial placentation is that transformation of the endometrium (decidualization) may play a role in the regulation of trophoblast invasion. However, in the endotheliochorial placenta, only those trophoblasts that line the marginal hematoma (a hematophagous zone) have direct contact with maternal blood (Barrau et al., 1975), which is in contrast to the hemochorial placenta.

There are many factors that have been attributed to regulating trophoblast invasion in humans, but those regulating trophoblast invasion in dogs are not as well described. Matrix metalloproteinases (MMPs) play a crucial role in trophoblast implantation and invasion in other species (Salamonsen, 1999). Of these, MMP2 and MMP9 are involved in the degradation of the extracellular matrix and cell migration by human trophoblasts (Shokry et al., 2009). Chu and colleagues (2002) showed that MMPs were associated with endometrial remodeling in dogs. With respect to the canine uterus and placenta, Beceriklisoy and coworkers (2007) showed that MMP2 and MMP9 expression peaked at the time of implantation.

To establish a baseline for future studies using the endotheliochorial placenta model, an understanding of in vitro canine trophoblast cellular characteristics needs to be established. The objective of this research was to describe cellular characteristics in isolated canine trophoblasts from fresh and cryopreserved placental tissues. We hypothesized that canine trophoblasts would express MMP2 and MMP9 in a pattern similar to results reported in human trophoblasts. In addition, we hypothesized that cryopreserved canine placental tissue could be used for trophoblast cell isolation instead of relying on the availability of fresh placental tissue.

2.2 MATERIALS AND METHODS

2.2.1 Cell Isolation and Culture
Following Oregon State University Institutional Animal Care and Use Committee approval, one placenta was collected from each dog (n=8) and the marginal hematoma was removed from the chorioallantois. Trophoblasts were isolated from the chorioallantois using serial collagenase and trypsin digestions followed by Percoll density gradient centrifugation, as previously described for human placentas by Hunkapiller (Hunkapiller and Fisher, 2008) with modifications. The filtering steps described by Hunkapiller were not necessary for yielding highly enriched cultures of canine trophoblasts so these steps were omitted to reduce the possibility of bacterial contamination during primary cell culture. Culture media for canine trophoblasts consisted of 50% EBM®-2 Basal Medium (#CC-3156, Lonza, Walkersville, MD) with EGM®-2 SingleQuots (#CC-4176, Lonza) and 50% DMEM (#11995, Life Technologies, Grand Island, NY) with 2% Nutridoma (#11011375001, Roche, Indianapolis, IN), 1% Hepes (#15630, Life Technologies)/Glutamine Plus (#B90210, Atlanta Biologicals)/Penicillin/Streptomycin (#15140-122, Life Technologies), and 0.1% Gentamycin (#15750, Life Technologies). Cells were cultured on 22 mm² coverslips at 37 ºC with 5% CO₂ an average of 8 days at which time the coverslips were fixed in 70% methanol.

2.2.2 Immunocytochemistry

Trophoblasts can be positively identified using immunostaining for cytokeratin-7 (CK-7) expression (Blaschitz et al., 2000; Pavlov et al., 2003; Nagamatsu et al., 2004). Therefore, CK-7 was used to confirm canine trophoblast purity following isolation. Cellular expression of CK-7, active MMP2 and active MMP9 was determined by fluorescent immunocytochemistry. Briefly, after washing with phosphate buffered saline (PBS), coverslips were treated with 5% goat or donkey serum in PBS (blocking buffer) for 3 min at 20 ºC to block non-specific secondary antibody binding. The coverslips were washed with PBS and then incubated with the primary antibody (Table 1) diluted in blocking buffer overnight at 4 ºC in a humidified
chamber. Specificity of immunostaining was verified by omission of the primary antibody. Following another wash with PBS, coverslips were incubated with their respective secondary antibody (Table 1) diluted in blocking buffer for 2 h at 20 ºC. Nuclei were detected with Hoechst 33342 (1:20,000, #H1399, Life Technologies) applied for 15 min at 20 ºC. Finally, the coverslips were washed with PBS and mounted using Molecular Probes Prolong® Antifade Kit (#p7481, Life Technologies). Images were captured on a Leica DM4000B microscope with a QImaging QICAM 12-bit (#QIC-M-12-C, QImaging, Surrey, BC) digital camera and QCapture PRO (QImaging, Surrey, BC) image capture software. Images were merged using Adobe Photoshop CS2 (Adobe, San Jose, CA).

2.2.3 Cryopreservation

Based upon work performed by Huppertz and associates (Huppertz et al., 2011), methods for cryopreserving human placental tissue prior to trophoblast isolation were applied to canine placental tissue. Briefly, 0.5 cm X 0.5 cm X 0.1 cm pieces of chorioallantois were incubated with 10% dimethyl sulfoxide in fetal calf serum at 20 ºC for 15 min before being divided into 3 groups (CONTROL, DIRECT, GRADUAL). The control tissue was not cryopreserved (CONTROL). The remainder of the tissue pieces were loaded into cryovials and frozen by plunging directly into liquid nitrogen cooling from 20 ºC to -196 ºC (DIRECT) or frozen in a Mr. Frosty (#5100-0001, Thermo Scientific, Rochester, NY) at -80 ºC freezer for 24 h prior to plunging into liquid nitrogen (GRADUAL). Following freezing, cells were thawed by submerging into a 37 ºC water bath for 3 min. Trophoblasts were isolated and cultured as described above with one million cells/ml plated per 25 cm² flask. Flasks were cultured in duplicate for each dog. After eight days in culture, cells were lifted with trypsin (TrypLE™ Express, #12605, Life Technologies) and counted on a hemocytometer.
Table 1. Antibodies used for immunocytochemistry

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>CK-7</th>
<th>MMP-2</th>
<th>MMP-9</th>
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<tr>
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<td>Mouse</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Dilution</td>
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<td>1:100</td>
<td>1:100</td>
</tr>
<tr>
<td>Catalog #</td>
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<td>MS-806-P0</td>
<td>RB-1539-P0</td>
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<tr>
<td>Source</td>
<td>DAKO</td>
<td>Thermo Scientific</td>
<td>Thermo Scientific</td>
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<tr>
<td>Secondary Antibody</td>
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<td>Alexa Fluor 488</td>
<td>Texas Red</td>
</tr>
<tr>
<td>Species</td>
<td>Donkey</td>
<td>Donkey</td>
<td>Goat</td>
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<td>1:100</td>
<td>1:100</td>
</tr>
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<td>Life Technologies</td>
<td>Life Technologies</td>
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</tr>
</tbody>
</table>
2.2.4 Data Analysis

After counting all the nuclei in multiple fields, the number of CK-7, MMP2 and MMP9 positive cells were counted to determine the percentage positive cells. The mean±SD percentage of MMP2 and MMP9 positive cells were compared using Student $t$ test. To determine the effect of placental tissue cryopreservation on post-thaw cell isolation and culture, the total number of cells after 8 days in culture was compared between cryopreservation groups. The mean±SEM cell count for each cryopreservation group were compared using the Kruskal-Wallis test. Statistical analysis was completed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Significance was defined at $p<0.05$.

2.3 RESULTS

Cellular morphology of isolated canine placental cells was polygonal and epithelial-like. Cells grew in a cobblestone pattern. More than 97% of the canine placental cells expressed CK-7, confirming that this cell isolation method yielded a highly enriched culture of trophoblasts (Figure 1A). Cytokeratin-7 expression was localized to the cytoplasm. More cultured canine trophoblasts expressed MMP9 ($54.7±3.4\%$) compared to MMP2 ($40.3±1.8\%$) ($p=0.02$). Although both MMPs were immunolocalized to the cytoplasm, MMP2 (Figure 1B) was found in large coalescing granules, whereas MMP9 was more diffusely expressed (Figure 1C). Cellular proliferation following primary culture of cryogenically preserved canine placental tissues was not influenced by freezing rate (CONTROL $54.3±24.9 \times 10^6$, DIRECT $41.5±12.0 \times 10^6$, GRADUAL $41.5±13.6 \times 10^6$; $p=0.37$).
Figure 1 (opposite page). Immunoexpression of cytokeratin-7 (A), MMP2 (B) and MMP9 (C) in cultured canine trophoblasts.
2.4 DISCUSSION

This is the first report to describe isolation and culture of canine trophoblasts. Canine trophoblast cells were easily isolated following the methods previously described for humans (Hunkapiller et al., 2008). However, the protocol was adjusted in order to accommodate the zonary type of placenta. The marginal hematoma was removed from the chorioallantois and the filtering steps described by Hunkapiller and colleagues (2008) were omitted in order to reduce the possibility of bacterial contamination.

Cellular morphology of the isolated canine placental cells was similar to that of human cultured trophoblasts (Stromberg et al., 1978; Logothetou-Rella et al., 1989; Bax et al., 1989). However, cellular morphology is not a dependable method for distinguishing cell type (epithelial versus fibroblast) in monolayer culture of the placenta (Thiede, 1960). Cytokeratin-7 expression, on the other hand, is commonly used to distinguish cell type in primary placenta cell culture, as well as determine the level of purity of trophoblast isolates (Blaschitz et al., 2000; Nagamatsu et al., 2004; Pötgens et al., 2001; Maldonado-Estrada et al., 2004). In the current study, more than 97% of canine placental cells expressed CK-7, confirming that this cell isolation method yielded a highly enriched culture of trophoblasts.

Matrix metalloproteinases are matrix degradation enzymes, but not all MMPs are equally important for trophoblast invasion (Bischof and Campana, 2000). Chu and colleagues (2002) as well as Beceriklisoy and colleagues (2007) found that both MMP2 and MMP9 are essential during endometrial remodeling and placental development in canids. The invasive capacity of trophoblasts has been directly linked to their ability to express and produce matrix metalloproteinases (Bischof et al., 1995; Staun-Ram et al., 2004). Trophoblasts from early gestation canine placentas (15-19 days after mating) express significantly more MMP2 than MMP9 (Beceriklisoy et al., 2007). In the current study, trophoblasts from late gestation canine placentas express significantly more MMP9 compared to MMP2. This occurrence is consistent with
results from human trophoblasts where MMP2 is predominately expressed in early gestation (Ioannidis et al., 2010) while MMP9 is dominant in late gestation (Shimonovitz et al., 1994).

Using immunohistochemistry, MMP2 and MMP9 are prominently expressed in human trophoblasts (Shokry et al., 2009; Demir-Weusten et al., 2007). In isolated canine trophoblasts, MMP2 and MMP9 are also prominently expressed. However, the fluorescent immunocytochemistry expression pattern in the cytoplasm differed in canine trophoblasts such that MMP2 was concentrated in large coalescing granules whereas MMP9 was more diffusely expressed throughout the cell. A similar staining pattern was reported in human trophoblasts for MMP2 and MMP9 (Erices et al., 2011).

A major disadvantage in cell culture studies using placental tissues is the insufficient availability of fresh tissue. Cryogenically stored placental tissue can serve as a resource for primary cell culture in humans (Huppertz et al., 2011). In the current study, canine placental tissues were cryopreserved using two different freezing rates. The cryopreserved tissues were then thawed and processed alongside fresh canine placentas to compare trophoblast proliferation rates. Cryopreservation of canine placental tissue prior to primary cell culture had no effect on cell proliferation (p=0.37), which is supported by the work of Colleoni and colleagues (2012) who demonstrated that the cryopreservation of human placental tissue had no deleterious effects on mitochondrial coupling and function when compared to fresh placenta.

In conclusion, canine trophoblasts can be isolated using methods similar for human trophoblasts and their purity can be verified using CK-7. In addition, canine trophoblasts can be isolated from fresh or cryopreserved placental tissue. Similar to human, canine trophoblasts express both MMP2 and MMP9 and late gestation canine trophoblasts express more MMP9 than MMP2. This information has established a baseline for future studies using the canine endotheliochorial placenta model for disorders of shallow trophoblast invasion in humans.
2.5 REFERENCES


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Cryopreservation of placental biopsies for mitochondrial respiratory analysis. Placenta 33, 122-123.


Staun-Ram, E., Goldman, S., Gabarin, D., Shalev, E., 2004. Expression and


CHAPTER 3

CONCLUSION AND FUTURE STUDIES

We have demonstrated that canine trophoblasts can be isolated and proliferate in culture. In addition, we found that isolated canine trophoblasts positively expressed cytokeratin-7, MMP2, MMP9, TIMP2, relaxin, and VEGF in a similar pattern to preeclamptic human trophoblasts. We also demonstrated that after exposing canine placenta to a cryoprotectant and testing two freezing rates, we found no difference in cell proliferation in culture compared to the control. This research is relevant to public health because an understanding of cellular protein expression in canine trophoblasts and provides a basis for future research reveal mechanisms regulating shallow trophoblast invasion.

The use of invasion chambers with Matrigel has been used to study trophoblast invasion, whereas the “scratch wound healing” assay has been used to study trophoblast migration. Exposure to interleukin-6 or tumor necrosis factor-α during these in vitro physiology experiments should increase or decrease trophoblast invasion and migration, respectively. These additional experiments would add to the foundation of knowledge presented within this thesis and further our understanding of factors regulating shallow trophoblast invasion in dogs.
APPENDICES
APPENDIX A: CANINE TROPHOBLAST ISOLATION AND CULTURE

A protocol for isolating human trophoblasts from the chorioallantois using serial collagenase and trypsin digestions followed by Percoll density gradient centrifugation was previously described by Hunkapiller and Fisher (2008). This protocol was followed with some modifications for isolating canine trophoblasts. Term canine placentas were collected following hysterotomy either during an ovariohysterectomy (n=3) or during c-section (n=2). The extra-chorionic membranes (amnion and allantoic) and marginal hematoma were dissected from the villous chorioallantois. Placental tissue was blotted to remove blood clots and then cut into 0.5 cm pieces with a scalpel blade. Up to 5 g of placental tissue was placed into a sterile 50 mL conical tube and then six times the weight (volume) of wash media (Table A.1) was added. Samples were placed on ice for transport to the laboratory and then centrifuged at 365Xg for 5 minutes at 4°C. From this point on, all of the procedures were carried out under strict aseptic conditions. The supernatant was removed without disturbing the pellet. This “washing step” was repeated once. Next, a collagenase solution (Table A.2) was added to the tubes at six times the tissue weight (volume) of tissue and the suspension was incubated at 37°C in a horizontal shaker at a speed correlated to complete agitation of the tissue for approximately 10 minutes until the tissue shows obvious signs of breakdown. Samples were then placed on ice at an angle to allow the tissue to settle at the bottom. The supernatant containing syncytiotrophoblasts was aspirated and discarded. Then, a trypsin solution (Table A.2) was added at six times the tissue weight (volume) and the mixture was incubated at 37°C in a horizontal shaker at speed correlated to complete agitation of the tissue for approximately 10 minutes until the tissue shows obvious signs of breakdown. Samples were placed on ice at an angle to allow the tissue to settle at the bottom. The supernatant was collected and transferred into a new conical tube with an equal volume of wash media and 5 mL of fetal calf serum (FCS) to halt enzymatic digestion. Next, samples were centrifuged at 365Xg for 8 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in a pre-warmed collagenase
solution using 1 mL per gram of starting tissue and incubated at 37°C in a horizontal shaker at speed correlated to complete agitation of the tissue for approximately 3 minutes until the tissue shows obvious signs of breakdown. Then, wash media was added to the solution up to 45 mL and centrifuged at 365Xg for 8 minutes at 4°C. The supernatant was discarded and the cell pellet was then resuspended in 4 mL of cell culture media (Table A.3) and added to the top of a Percoll gradient (20-70%) (Table A.4). The samples were centrifuged at 1573Xg for 25 minutes at 4°C. The top two bands of the Percoll gradient were collected and this solution was combined with up to 45 mL of wash media. Samples were centrifuged at 365Xg for 8 minutes at 4°C and this “washing step” was repeated twice with the last containing 10 mL of cell culture media instead of wash media. The supernatant was aspirated and discarded and the cell pellet was resuspended in cell culture media and distributed on 25 cm² flasks and 22 mm² coverslips.

Cells were cultured at 37°C with 5% CO₂ in cell culture media that was replenished within the first 24 hours and then every 48 hours until they reached 70-80% confluence at which time coverslips were fixed in 70% methanol at 4°C and the cells from flasks were either passaged or cryopreserved. Flasks were passaged by pre-warming trypsin-LE solution (TrypLE™ Express, #12605, Life Technologies) and Dulbecco’s phosphate buffered saline (DPBS) without calcium and magnesium to 37°C. The spent medium was removed from the flask and discarded. The cell surface was carefully washed to avoid damaging the monolayer with 5 mL of DPBS, which was then removed and discarded. Trypsin-LE solution (2.5 mL) was added to the flask and distributed evenly over the monolayer by gently rotation. Flasks were incubated at 37°C and observed at 5 minute intervals until cells detached. Flasks were firmly tapped to dislodge cells remaining cells. Cell culture media (10 ml) was added to each flask then the flasks were tilted in all directions to thoroughly rinse. The cell suspension was transferred to a 15 mL sterile conical tube, which was then centrifuged for 10 minutes at 100Xg. After removing from the centrifuge, the supernatant was discarded and cells suspended with 12.5 mL of media. With this cell
suspension, an additional 12.5 mL of media was added to each 75 cm² flask (total volume = 25 mL) and the flasks were incubated at 37°C with 5% CO₂ until they reached 70-80% confluence.

When passaged cells were cryopreservation, all of the previously described procedures for passaging were followed with the following modifications. The cell surface was washed with 12.5 mL of DPBS and 5 mL of trypsin-LE solution was used. After removing from the centrifuge, the supernatant was discarded until there was 0.7 mL of media remaining in the conical tube with the cell pellet. The cell pellet was resuspended in the remaining media and combined with an equal volume of freezing media (50% fetal calf serum, 30% cell culture media, 20% DMSO), which was then transferred into a 2 mL cryogenic vial. Vials were put into a Mr. Frosty (#5100-0001, Thermo Scientific, Rochester, NY) at room temperature and then the apparatus was placed in a –80°C freezer overnight. Vials were removed from the Mr. Frosty the following day and directly submerged into a liquid nitrogen (–196°C).

References

Table A.1. Wash Media

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<tr>
<th>Ingredients</th>
<th>Volume (mL)</th>
<th>Amount (%)</th>
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<tbody>
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<td>DMEM High Glucose 1X (Gibco, 11965-092)</td>
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<td>Fetal Bovine Serum (Lonza, CC-4101A)</td>
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<tr>
<td>Glutamine Plus (Atlanta Biologicals, B90210)</td>
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<tr>
<td>Penicillin/Streptomycin (Life Technologies, 11292)</td>
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<tr>
<td>Gentamycin (50 mg/ml) (Invitrogen, 15750)</td>
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### Table A.2. Prepared Enzymatic Digestion Solutions

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<td>EDTA (Sigma, E-5134)</td>
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Table A.3. Cell Culture Media

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<td>R3-IGF-1 (Lonza, CC-4115A)</td>
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<td>0.05</td>
</tr>
</tbody>
</table>
Table A.4. Percoll Centrifugation Solution

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percoll Stock Solutions</th>
<th>90% Percoll*</th>
<th>Hank’s 1X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percoll (GE Healthcare Bio-sciences AB, 17-0891-01)</td>
<td>70%</td>
<td>35 mL</td>
<td>10 mL (w/o Phenol Red)</td>
</tr>
<tr>
<td>10X Hank’s BSS without Phenol Red (Invitrogen, 14185)</td>
<td>60%</td>
<td>30 mL</td>
<td>15 mL (with Phenol Red)</td>
</tr>
<tr>
<td>1X Hank’s BSS without Phenol Red (Invitrogen, 14175)</td>
<td>50%</td>
<td>25 mL</td>
<td>20 mL (w/o Phenol Red)</td>
</tr>
<tr>
<td>1X Hank’s BSS with Phenol Red (Invitrogen, 14170)</td>
<td>40%</td>
<td>20 mL</td>
<td>25 mL (with Phenol Red)</td>
</tr>
<tr>
<td><strong>Percoll Stock Solutions</strong></td>
<td>30%</td>
<td>15 mL</td>
<td>30 mL (w/o Phenol Red)</td>
</tr>
<tr>
<td><strong>90% Percoll</strong> (270 mL Percoll + 30 mL 10X Hanks BSS without Phenol Red)</td>
<td>20%</td>
<td>10 mL</td>
<td>35 mL (with Phenol Red)</td>
</tr>
</tbody>
</table>

*90% Percoll (270 mL Percoll + 30 mL 10X Hanks BSS without Phenol Red)
Appendix A.1 Bacteriology

Over the course of hundreds of primary cell cultures, 48 culture flasks or plates were suspected of being contaminated with bacteria. The criteria for culturing was discolored media (orange or yellow) indicative of a pH change, or the presence of cellular debris floating in the media indicative of cell death. Flasks or plates were submitted to the Oregon State University Veterinary Diagnostic Laboratory for aerobic bacterial culture. Of the 48 culture flasks or plates tested, there were 23 that were positive for bacteria, with *Enterococcus* sp. most commonly isolated (15/23, 65%). However, it is important to note that there have been no suspicious flasks or plates since 10/19/2010 (Figure A.1.1).

![Bar chart showing the number of flasks/plates infected with different bacterial species and those with no bacteria isolated.](image)

Figure A.1.1. Number of flasks/plates infected with bacteria.
APPENDIX B: CANINE TROPHOBLAST IMMUNOCYTOCHEMISTRY

Expression of cytokeratin-7 (CK-7), matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9), tissue inhibitor of metalloproteinase 2 (TIMP2), relaxin, VEGF, and S100A4 was determined in cultured canine trophoblasts using immunocytochemistry. The objective of this experiment was to determine if these proteins that are expressed in human trophoblasts are also expressed in dogs. Briefly, trophoblasts from five dogs were grown on coverslips to 70-80% confluency and then fixed in 70% methanol. Coverslips were then washed three times in PBS to remove residual methanol and transferred to a new Parafilm® lined 22 mm² culture dish. After washing, non-specific secondary antibody binding was blocked for 3 minutes at room temperature with 350 µl blocking buffer containing either 5% serum goat or donkey depending upon the secondary antibody used. Blocking buffer was removed by aspiration and coverslips were washed for 3 minutes in PBS. After which, 350 µl primary antibody diluted in blocking buffer was pipetted onto the coverslip for over-night incubation at 4ºC in a humidified chamber. This incubation time and temperature was selected to promote specific staining (R&D Systems, 2012). Specificity of immunostaining was verified by omission of the primary antibody (negative control). Published immunocytochemistry results from human trophoblasts using the same antibodies served as positive controls.

After three washes with PBS for 3 minutes, 350 µl of secondary antibody diluted in blocking buffer was added for 2 hour incubation at room temperature in a dark humidified chamber to reduce dimming the fluorescence and prevent the slides from drying out. Following three additional washes with PBS for 3 minutes, 350 µl of diluted (1:20,000) Hoechst 33342 nuclear stain (#H1399, Invitrogen, Carlsbad, CA) was added for a 15 minute incubation at room temperature in a dark humidified chamber. Finally, the coverslips were washed twice with PBS for 3 minutes and then mounted onto glass microscope slides using Molecular Probes Prolong antifade kit (#p7481, Invitrogen, Carlsbad, CA). Specific fluorescence staining was visualized at
100X-1000X magnifications on a Leica DM4000B microscope. Digital images were
captured using a digital camera (QImaging QICAM 12-bit, #QIC-F-M-12-C,
QImaging, Surrey, BC) with image capture software (QCapturePro, QImaging,
Surrey, BC). When examining slides, cells were first visualized using the A4 (blue)
fluorescent filter to identify nuclei. Then, depending upon the secondary antibody
used, the cells were visualized using the L5 (green) fluorescent filter or the TX2 (red)
fluorescent filter. To capture images, the QCapturePro program on the computer is
opened on the desktop and the “Camera” icon is pressed to open a dialog box in which
“Preview” is clicked. The 00.500.000 setting was used to control for exposure time
between slides. This setting was determined following preliminary studies to
determine the amount of autofluorescence in cultured canine trophoblasts as well as
non-specific fluorescence in the negative controls. Images were captured from all
three fluorescent filters at this exposure time for all slides. Because of the software,
pictures are initially captured in black and white, requiring the microscoper to input
the appropriate color by clicking on the “Process” tab, then the “Color Channel” tab,
and finally choosing the color (R, B, or G). When this is finished, the microscoper
must select “OK” and then go to the “Edit” tab and hover over “Convert to…” . Next,
choose “RB24”, then click “Convert”. Each image file was saved as an individual
picture and then images were merged using Adobe Photoshop CS2 (Adobe, San Jose,
CA). To merge pictures using this software, first open all the pictures needing to be
merged. If needed, color, brightness, and contrast can be adjusted at this time (e.g.
click on “Adjustments” in the “Images” tab “Auto Color Levels”). Next, open the
“Channels” box by going to the “Window” tab and then the “Channel” tab. Select one
picture using “ctrl-A” and then go to the “Edit” tab and click “Copy”. Next, click on a
different picture to select it and go to the “Edit” tab and click “Paste”. Finally, click
on the “Layers” tab in the “Channels” box and double click on “Layer 1” and change
the color setting to either R, B, or G (e.g. color not in the picture).

Isolated canine trophoblasts were stained with CK-7, MMP2, MMP9, TIMP2,
VEGF, relaxin, and S100A4 (Table B.1). Isolated primary canine trophoblasts stained
positive for cytokeratin-7 (Figure B.1A) and negative for S100A4 (data not shown). Using immunocytochemistry, cultured primary canine trophoblasts expressed MMP2, MMP9, TIMP2, VEGF and relaxin (Figure B.1B-F). Passaged cells stained positive for S100A4 (Figure B.1G), indicating that these cells were no longer trophoblasts (Table B.2). Negative controls (primary antibodies omitted) displayed weak autofluorescence (Table B.3), which was accounted for by adjusting the exposure time.

References

Figure B.1 (opposite page). Immunoexpression of cytokeratin-7 (A), MMP2 (B), MMP9 (C), TIMP2 (D), relaxin (E), VEGF (F), S100A4 (G) and negative control (H) in cultured canine trophoblasts.
Table B.1. Antibodies tested on isolated canine trophoblasts in primary culture.

<table>
<thead>
<tr>
<th>Dog ID</th>
<th>Date</th>
<th>1st Primary Antibody (Dilution)</th>
<th>2nd Primary Antibody (Dilution)</th>
<th>Special Modifications</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susie</td>
<td>1-11-11</td>
<td>CK-7 (1:250)</td>
<td>S100A4 (1:250)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 3+, S100A4 negative</td>
</tr>
<tr>
<td>Molly</td>
<td>1-11-11</td>
<td>CK-7 (1:250)</td>
<td>S100A4 (1:250)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 3+, S100A4 negative</td>
</tr>
<tr>
<td>Molly</td>
<td>1-11-11</td>
<td>CK-7 (1:250)</td>
<td>S100A4 (1:250)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 3+, S100A4 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>1-13-11</td>
<td>CK-7 (1:250)</td>
<td>S100A4 (1:125)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 3+, S100A4 negative</td>
</tr>
<tr>
<td>Molly</td>
<td>1-13-11</td>
<td>CK-7 (1:250)</td>
<td>S100A4 (1:125)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 3+, S100A4 negative</td>
</tr>
<tr>
<td>Molly</td>
<td>1-13-11</td>
<td>CK-7 (1:250)</td>
<td>S100A4 (1:125)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 2+, S100A4 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>1-18-11</td>
<td>S100A4 (1:150)</td>
<td>None</td>
<td>None</td>
<td>S100A4 negative</td>
</tr>
<tr>
<td>Janie</td>
<td>1-22-11</td>
<td>None</td>
<td>None</td>
<td>No primary</td>
<td>Autofluorescence detected, staining intensity 1+</td>
</tr>
<tr>
<td>Sample</td>
<td>Date</td>
<td>Antibody 1 (Dilution)</td>
<td>Antibody 2 (Dilution)</td>
<td>Blocking Buffer</td>
<td>Result Description</td>
</tr>
<tr>
<td>--------</td>
<td>--------</td>
<td>------------------------</td>
<td>------------------------</td>
<td>----------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Janie</td>
<td>1-22-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:200)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 2+</td>
</tr>
<tr>
<td>Janie</td>
<td>1-22-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:200)</td>
<td>No blocking buffer</td>
<td>CK-7 positive, staining intensity 2+</td>
</tr>
<tr>
<td>Janie</td>
<td>1-22-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:200)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 2+</td>
</tr>
<tr>
<td>Janie</td>
<td>1-22-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:200)</td>
<td>No secondary antibody</td>
<td>Autofluorescence detected, staining intensity 1+</td>
</tr>
<tr>
<td>Janie</td>
<td>1-22-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:200)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 1+, S100A4 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>1-22-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:200)</td>
<td>No Hoechst</td>
<td>CK-7 positive, staining intensity 1+, S100A4 negative</td>
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<td>CK-7 (1:200)</td>
<td>S100A4 (1:200)</td>
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<td>CK-7 positive, staining intensity 1+, S100A4 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>1-22-11</td>
<td>S100A4 (1:200)</td>
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<td>None</td>
<td>S100A4 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>1-22-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:200)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 1+, S100A4 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>1-22-11</td>
<td>CK-7 (1:400)</td>
<td>S100A4 (1:400)</td>
<td>Lower dilutions</td>
<td>CK-7 positive, staining intensity 1+, S100A4 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>1-22-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:200)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 2+, S100A4 negative</td>
</tr>
<tr>
<td>Name</td>
<td>Date</td>
<td>Antibody</td>
<td>Staining</td>
<td>Staining</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>-----------</td>
<td>----------</td>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Molly</td>
<td>7-14-11</td>
<td>CK-7 (1:200)</td>
<td>None</td>
<td>None</td>
<td>CK-7 positive, staining intensity &lt;1</td>
</tr>
<tr>
<td>Susie</td>
<td>7-27-11</td>
<td>CK-7 (1:100)</td>
<td>S100A4 (1:100)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 3+, S100A4 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>7-27-11</td>
<td>CK-7 (1:100)</td>
<td>S100A4 (1:100)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 2+, S100A4 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>11-29-11</td>
<td>MMP2 (1:100)</td>
<td>S100A4 (1:100)</td>
<td>None</td>
<td>MMP2 positive, staining intensity 3+</td>
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<tr>
<td>Janie</td>
<td>11-29-11</td>
<td>MMP2 (1:100)</td>
<td>S100A4 (1:100)</td>
<td>None</td>
<td>MMP2 positive, staining intensity 1+, S100A4 positive, staining intensity 1+</td>
</tr>
<tr>
<td>Susie</td>
<td>11-29-11</td>
<td>CK-7 (1:100)</td>
<td>MMP9 (1:100)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 2+, MMP9 positive, staining intensity 3+</td>
</tr>
<tr>
<td>Janie</td>
<td>11-29-11</td>
<td>CK-7 (1:100)</td>
<td>MMP9 (1:100)</td>
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<td>CK-7 positive, staining intensity 2+, MMP9 positive, staining intensity 3+</td>
</tr>
<tr>
<td>Elvira</td>
<td>12-19-11</td>
<td>MMP2 (1:100)</td>
<td>None</td>
<td>None</td>
<td>MMP2 positive, staining intensity 3+</td>
</tr>
<tr>
<td>Sassy</td>
<td>12-19-11</td>
<td>MMP2 (1:100)</td>
<td>None</td>
<td>None</td>
<td>MMP2 positive, staining intensity 2+</td>
</tr>
<tr>
<td>Elvira</td>
<td>12-19-11</td>
<td>MMP9 (1:100)</td>
<td>None</td>
<td>None</td>
<td>MMP9 positive, staining intensity 2+</td>
</tr>
<tr>
<td>Name</td>
<td>Date</td>
<td>Testant</td>
<td>Concentration</td>
<td>Staining</td>
<td>Result</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>---------</td>
<td>---------------</td>
<td>----------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>Sassy</td>
<td>12-19-11</td>
<td>MMP9</td>
<td>(1:100)</td>
<td>None</td>
<td>MMP9 positive, staining intensity 2+</td>
</tr>
<tr>
<td>Elvira</td>
<td>12-19-11</td>
<td>CK-7</td>
<td>(1:100)</td>
<td>None</td>
<td>CK-7 positive, staining intensity 2+</td>
</tr>
<tr>
<td>Sassy</td>
<td>12-19-11</td>
<td>CK-7</td>
<td>(1:100)</td>
<td>None</td>
<td>CK-7 positive, staining intensity &lt;1</td>
</tr>
<tr>
<td>Elvira</td>
<td>12-19-11</td>
<td>CK-7</td>
<td>(1:100)</td>
<td>MMP9</td>
<td>CK-7 positive, staining intensity 2+, MMP9 positive, staining intensity 3+</td>
</tr>
<tr>
<td>Sassy</td>
<td>12-19-11</td>
<td>CK-7</td>
<td>(1:100)</td>
<td>MMP9</td>
<td>CK-7 positive, staining intensity 2+, MMP9 positive, staining intensity 3+</td>
</tr>
<tr>
<td>Elvira</td>
<td>12-19-11</td>
<td>VEGF</td>
<td>(1:100)</td>
<td>None</td>
<td>VEGF positive, staining intensity 2+</td>
</tr>
<tr>
<td>Sassy</td>
<td>12-19-11</td>
<td>VEGF</td>
<td>(1:100)</td>
<td>None</td>
<td>VEGF positive, staining intensity 2+</td>
</tr>
<tr>
<td>Elvira</td>
<td>5-4-12</td>
<td>Relaxin</td>
<td>(1:200)</td>
<td>None</td>
<td>Relaxin positive, staining intensity 3+</td>
</tr>
<tr>
<td>Susie</td>
<td>5-4-12</td>
<td>Relaxin</td>
<td>(1:20)</td>
<td>None</td>
<td>Relaxin positive, staining intensity 3+</td>
</tr>
<tr>
<td>Janie</td>
<td>5-4-12</td>
<td>Relaxin</td>
<td>(1:100)</td>
<td>None</td>
<td>Relaxin positive, staining intensity 2+</td>
</tr>
<tr>
<td>Janie</td>
<td>5-4-12</td>
<td>TIMP2</td>
<td>(1:100)</td>
<td>None</td>
<td>TIMP2 positive, staining intensity 2+</td>
</tr>
</tbody>
</table>
Table B.2. Non-primary culture/passaged placental cells.

<table>
<thead>
<tr>
<th>Dog ID</th>
<th>Date</th>
<th>1st Primary Antibody (Dilution)</th>
<th>2nd Primary Antibody (Dilution)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susie</td>
<td>7-6-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:100)</td>
<td>S100A4 positive, staining intensity 1+, CK-7 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>7-6-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:100)</td>
<td>S100A4 positive, staining intensity 1+, CK-7 negative</td>
</tr>
<tr>
<td>Molly</td>
<td>7-6-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:100)</td>
<td>S100A4 positive, staining intensity 1+, CK-7 negative</td>
</tr>
<tr>
<td>Molly</td>
<td>7-6-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:100)</td>
<td>S100A4 positive, staining intensity 1+, CK-7 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>7-7-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:100)</td>
<td>S100A4 positive, staining intensity &lt;1, CK-7 negative</td>
</tr>
<tr>
<td>Susie</td>
<td>7-7-11</td>
<td>CK-7 (1:200)</td>
<td>S100A4 (1:100)</td>
<td>S100A4 positive, staining intensity &lt;1, CK-7 negative</td>
</tr>
<tr>
<td>Molly</td>
<td>7-11-11</td>
<td>S100A4 (1:100)</td>
<td>None</td>
<td>S100A4 positive, staining intensity 1+</td>
</tr>
<tr>
<td>Molly</td>
<td>7-11-11</td>
<td>CK-7 (1:100)</td>
<td>None</td>
<td>CK-7 negative</td>
</tr>
<tr>
<td>Molly</td>
<td>7-11-11</td>
<td>CK-7 (1:50)</td>
<td>None</td>
<td>CK-7 negative</td>
</tr>
<tr>
<td>Molly</td>
<td>7-11-11</td>
<td>CK-7 (1:10)</td>
<td>None</td>
<td>CK-7 negative</td>
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</table>
Table B.3. Test for autofluorescence.

<table>
<thead>
<tr>
<th>Date</th>
<th>1st Primary Antibody (Dilution)</th>
<th>Special Modifications</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-28-11</td>
<td>None</td>
<td>Exposure times:</td>
<td>Trophoblasts autofluorescing in both green and red filters</td>
</tr>
<tr>
<td></td>
<td></td>
<td>00.200.000 sec for blue, 00.500.000 sec for green, 00.500.000 for red.</td>
<td></td>
</tr>
<tr>
<td>1-28-11</td>
<td>CK-7 (1:10 µl)</td>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>1-28-11</td>
<td>S100A4 (1:10 µl)</td>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>1-28-11</td>
<td>None</td>
<td>Mounting media/anti fade (1:10 µl)</td>
<td>Green filter autofluorescing specks</td>
</tr>
<tr>
<td>1-28-11</td>
<td>None</td>
<td>Unfiltered PBS</td>
<td>All filters autofluorescing specks</td>
</tr>
<tr>
<td>1-28-11</td>
<td>None</td>
<td>Hoechst</td>
<td>Blue filter autofluorescing specks</td>
</tr>
</tbody>
</table>
APPENDIX C: CANINE FIBROBLAST CULTURE AND IMMUNOCYTOCHEMISTRY

The expression of S100A4, vimentin and S100 was tested on fibroblasts cultured on coverslips using immunocytochemistry. Purchased canine fibroblasts were originally obtained from normal tracheal tissue from a one year old female golden Labrador retriever dog (ATCC; #CRL-6244™). The cells were received in a 1 mL vial, which was thawed by gentle agitation in a 37°C water bath for 2 minutes. To reduce the possibility of bacterial contamination from the water bath, the O-ring and cap were kept out of the water during thawing. After removing the vial from the water bath, it was decontaminated by spraying it with 70% isopropyl alcohol. From this point on, all of the procedures were carried out under strict aseptic conditions. The contents of the vials were transferred to a 15 mL conical tube containing 8 mL ATCC-formulated Dulbecco’s Modified Eagle’s Medium (DMEM) and the vial was rinsed with an additional 1 mL of DMEM and then added to the 15 mL conical tube. The conical tube was centrifuged for 5 minutes at 168Xg and 9 mL of supernatant was discarded. The cell pellet was resuspended with 9 mL DMEM and 7.5 mL of the cell suspension was transferred to a 25cm² culture flask with the remainder transferred onto one 22mm² coverslip. Cells were cultured at 37°C with 5% CO₂ in cell culture media that was replenished within the first 24 hours and then every 48 hours until they reached 70-80% confluence at which time the coverslip was fixed in 70% methanol at 4°C and the cells from flasks were either passaged or cryopreserved. Cells were seeded to multiple coverslips after each passage.

Immunocytochemistry was performed on cultured canine fibroblasts with antibodies directed against CK-7, S100, S100A4, and vimentin (Table C.1). None of the canine fibroblasts examined expressed CK-7 or S100 (data not shown). Cultured canine fibroblasts expressed vimentin and S100A4 (Figure C.1), with the latter having greater staining intensity.
Table C.1. Antibodies tested on canine fibroblasts

<table>
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Figure C.1. Canine fibroblast immunocytochemistry for vimentin (A) and S100A4 (B).
APPENDIX D: CANINE PLACENTAL IMMUNOHISTOCHEMISTRY

Briefly, four canine placentas were fixed in 10% buffered formalin and paraffin-embedded. Serial 4-5 µm sections were cut from paraffin blocks and mounted on positively charged slides. Slides were deparaffinized in xylene and rehydrated in a graded ethanol series (95%, 100%, 80%) to distilled water. Depending upon the antigen, microwave or enzymatic pretreatment was used for antigen retrieval. Antibodies and antigen retrieval tested on paraffin-embedded canine placenta are summarized in Table D.1. For TIMP2 and S100A4, microwave pretreatment for antigen retrieval was accomplished with a pressure cooker (Viking Ware tender cooker) (5 pounds of pressure) heated until pressure was achieved and then treated under pressure for 10 minutes in modified citrate buffer, pH 6.1(#S169984, DAKO Target Retrieval Solution) followed by 20 minutes of cooling at room temperature to expose epitopes. For CK-7, MMP2 and relaxin, proteinase K was used as antigen retrieval. For MMP9, no antigen retrieval was used. Immunohistochemical staining was performed using an automatic stainer (DAKO Autostainer Universal Staining System). Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide for 5 minutes. After rinsing with TBS-Tween 20 (washing buffer), the sections were incubated with DAKO serum free protein block (#X0909, DAKO) for 10 minutes at room temperature to prevent nonspecific binding reactions. Buffer was blown off by the autostainer. Subsequently, primary antibody diluted in an Antibody Diluent with a background reducing component (#S3022, DAKO) was applied to the slide and incubated at room temperature for 30 minutes followed by two rinses in TBS-Tween 20. A secondary antibody with horse radish peroxidase (HRP)-conjugated dextrane polymer (MaxPoly-One™, MaxVision Biosciences Inc.) was used to enhance the signal. Finally, sections were washed with PBS and peroxidase activity was detected with VECTOR NovaRED Peroxidase Substrate Kit (#SK-4800, Vector Laboratories) for 5 minutes at room temperature. The sections were washed twice in distilled water and counterstained in hematoxylin histological staining reagent (diluted 1:3 in water, #S3302, DAKO) applied for 5 minutes. The slides were then rinsed with water,
buffered with TBS-Tween 20 and rinsed with water. Finally, the tissue was dehydrated in a graded ethanol series (95%, 95%, 100%, 100%) to xylene and mounted with Richard-Allan Scientific Cytoseal XYL (#8312-4, Thermo Scientific). Negative controls included replacement of primary antibody with DAKO universal negative control rabbit (#N1699, DAKO) and mouse (#N1698, DAKO). All placental sections were examined for the presence of specific staining on a Leica DMRB microscope at 20X or 40X magnification and a Nikon Coolpix 950 digital camera was used to capture images.

CK-7, MMP9, TIMP2 and relaxin was expressed in trophoblasts along the villous margin with MMP9, TIMP2 and relaxin extending towards the basement membrane (Figure D.1). S100A4 was minimally expressed in the basement membrane (Figure D.1). MMP2 was strongly expressed within the basement membrane (Figure D.1).
Figure D.1 (opposite page). Immunohistochemical staining (40X) of paraffin-embedded canine chorioallantois with (A) CK-7; (B) S100A4; (C) MMP2; (D) MMP9; (E) TIMP2; (F) Relaxin; (G) Negative Control Mouse; (H) Negative Control Rabbit.
Table D.1. Antibodies and antigen retrieval tested on paraffin-embedded canine placenta.

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<td>1:200</td>
<td>EDTA, pH 8.0</td>
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APPENDIX E: CRYOPRESERVATION OF CANINE PLACENTAL TISSUE

A major disadvantage in cell culture studies using placental tissues is the insufficient availability of fresh tissue. Cryogenically stored placental tissue can serve as a resource for primary cell culture in humans (Huppertz et al., 2011). Canine placental tissues (n=3) were cryopreserved using two different freezing rates. Chorioallantois (0.5 cm pieces) was pre-incubated with 10% DMSO in fetal calf serum at room temperature for 15 minutes. To determine the effect of cryopreservation on post thaw trophoblast isolation, samples were divided into 3 groups (CONTROL, DIRECT, GRADUAL). The control tissue was not cryopreserved. The remainder of the tissue pieces were loaded into cryovials and either plunged directly into liquid nitrogen (DIRECT) or put into a -80°C freezer in a Mr. Frosty (controlled freezing container) for 24 hours prior to plunging into liquid nitrogen (GRADUAL). The direct cooling rate was from 20°C to -196°C at -200°C/seconds. The gradual freezing cooling rate was a two-step process from 20°C to -80°C at -1°C/minutes then -80°C to -196°C at -116°C/second. Following freezing, cryovials were thawed by submersion into a 37°C water bath for 3 minutes. Immediately after thawing, trophoblasts were isolated and cultured as described in appendix 4.1 with one million cells/ml plated per 25 cm² flask. Flasks were cultured in duplicate for each dog. After eight days in culture, cells were lifted with a trypsin-LE solution (TrypLE™ Express, #12605, Life Technologies) and cell concentration was determined using a hemocytometer. The results are summarized in chapter II.

References

APPENDIX F: ABSTRACTS AND PRESENTATIONS

Appendix F.1 Summary

Throughout my graduate career I have been privileged to present my research at regional, national and international conferences.
Appendix F.2 Presented at the 2011 Annual Northwest Reproductive Sciences Symposium (Corvallis, OR) and the 2011 Annual Conference for the Society for Theriogenology (Milwaukee, WI)

**Isolation and Primary Cell Culture of Canine Trophoblasts**

Laura Sahlfeld, Timothy Hazzard, Michelle Kutzler

Department of Animal Sciences, Oregon State University, Corvallis, OR, USA

**Introduction**

Preeclampsia is a problem that affects 5-7% of human pregnancies and decades of in vitro research in this area has been unsuccessful to learn how to prevent it. In preeclampsia, trophoblasts shallowly invade the endometrial endothelium (Pijnenborg and Hanssens, 2010). This defective trophoblast invasion is detrimental to human pregnancies but represents normal endotheliochorial placentation in dogs. The objective of this research was to establish canine trophoblast cell lines to study in vitro trophoblast invasion and migration as a model for preeclampsia in humans. Cytokeratin-7 is a type II cytokeratin that positively labels human trophoblasts (Pavlov et al., 2003; Handschuh et al., 2009). For this experiment, we hypothesized that cultured canine trophoblasts would also be positive for cytokeratin-7.

**Methods**

Placentas was removed via hysterotomy from four beagles at 61±1 days from the LH surge (term=65 days). Following methods previously described for isolating human trophoblasts (Hunkapiller and Fisher, 2008), trophoblasts were isolated using collagenase and trypsin with Percoll density gradient centrifugation. Cells were then cultured in DMEM media (#829415, Gibco-Invitrogen, Carlsbad, CA) at 38°C with 5% CO₂ and grown to 70% confluency on coverslips. Cells were fixed in 70% methanol and expression of cytokeratin-7 (#p103620, DAKO, Carpinteria, CA) was confirmed using fluorescent immunohistochemistry (Alexa Flour 488, #A21202,
Invitrogen, Carlsbad, CA). Hoescht 33342 (#H1399, Invitrogen, Carlsbad, CA) was used to count cells.

**Results**

Cellular morphology was consistent with that of trophoblasts forming round cells in a cobblestone pattern. Occasionally, spherical syncytium of trophoblast occurred. More than 80% of the cells cultured expressed cytokeratin-7.

**Discussion**

Canine trophoblasts express cytokeratin-7 similar to human trophoblasts. Future in vitro studies using these cell lines will focus on characterizing canine trophoblast invasion and migration.

**Keywords:** Canine, cytokeratin-7, immunohistochemistry, preeclampsia, trophoblast

**References:**


Isolation and Primary Cell Culture of Canine Trophoblasts
Laura Sahlfeld, Timothy Urozza, Michelle Kutzki
Department of Animal Science, Oregon State University, Corvallis, Oregon

BACKGROUND
- The objective of this study was to isolate trophoblasts from canine placenta using methods published for culturing trophoblasts from human placentas.
- The trophoblasts isolated were used to investigate the potential of these cells for future research.

HYPOTHESIS AND OBJECTIVES
- The hypothesis was that trophoblasts isolated from canine placentas could be used as a model system to study the biological processes of trophoblasts.
- The objective was to culture trophoblasts from canine placentas and analyze their characteristics.

MATERIALS AND METHODS
- Placentas were removed from pregnant females at necropsy and were transported to the laboratory within 24 hours.
- Placental tissue was washed and minced and then cultured in DMEM supplemented with 10% FBS.
- Cells were grown to confluence and then harvested for further analysis.

RESULTS
- Trophoblasts were successfully isolated and cultured from canine placentas.
- The isolated trophoblasts showed typical morphological characteristics of trophoblasts, including cell rounding and the formation of multinucleated giant cells.

CONCLUSIONS
- Trophoblasts isolated from canine placentas can be used as a model system for studying trophoblastic biology.
- Further research is needed to understand the potential applications of these cells in reproductive biology.

REFERENCES

Appendix F.4 2011 Society for Theriogenology Presentation

**Isolation & Primary Cell Culture of Canine Trophoblasts**

**Introduction**

- **Preeclampsia**
  - Affects 5-7% of human pregnancies
  - 3rd leading cause of maternal mortality in U.S.
    - Accounts for 20% of pregnancy related deaths

**Placental development**

- Normal human placental development
  - Trophoblasts invade the endometrial epithelium & differentiate into an inner layer of mononucleated cytotrophoblasts & an outer layer of multinucleated syncytiotrophoblasts
  - Invade the tight spiral arteries within the endometrium & remodel them into wide, flaccid uteroplacental vessels
    - Allows for increased blood flow to the placenta

- Placental development in preeclampsia
  - Trophoblasts shallowly invade the endometrium & insufficiently remodel spiral arteries
    - Maternal blood flow to the placenta is reduced

**Overall Goal**

- The overall goal of my graduate research is to isolate canine trophoblasts & describe their cellular characteristics in comparison with those characteristics reported for human trophoblasts from normal & preeclamptic placentas
Objective & Hypothesis
- The objective of this current study was to isolate trophoblasts from canine placentas using methods published for isolating trophoblasts from human placentas.
- We hypothesized that both syncytiotrophoblasts & cytotrophoblasts could be isolated from canine placentas.

Materials & Methods
- Following hysterotomy, we removed one placenta from three beagles at 61±1 days from the LH surge (term=65 days).
- Following delivery, we obtained one term placenta from three dogs.

Materials & Methods
- Trophoblasts were isolated using collagenase & trypsin digestion with Percoll density gradient centrifugation.

Materials & Methods
- Cells were cultured in DMEM at 38°C with 5% CO₂ & grown to 70% confluency in flasks & on coverslips.
- Cells grown on coverslips were fixed in 70% methanol.

Results
- Cellular morphology was consistent with that of cytotrophoblasts.
- Round cells in a cobblestone pattern.
- Occasionally, spherical syncytium of trophoblasts developed.
Materials & Methods

- Cytokeratin-7 is a type II cytokeratin that is expressed by human trophoblasts.
- We performed fluorescent immunohistochemistry on our cultured cells for cytokeratin expression as well as S100A4, which is a protein specific to fibroblasts.
- We confirmed S100A4 expression in cultured canine fibroblasts.

Results

- More than 97% of the cells expressed cytokeratin-7, confirming that this cell isolation method yields a highly enriched culture of canine trophoblasts.

Conclusion

- This is the first report describing canine trophoblast isolation for cell culture.
- Future studies will continue to compare immuno expression to a variety of proteins expressed in normal & preeclamptic human trophoblasts.

Acknowledgements

- OSU, Department of Animal Sciences
- Dr. Michelle Kutzler
- Dr. Timothy Hazzard
- Shaundra Epperson
Appendix F.5 Presented at the 2011 Annual Conference for the International Society for Cryobiology (Corvallis, OR)

Cryogenic Preservation of Canine Placental Tissue
*Laura Sahlfeld, Timothy Hazzard, Michelle Kutzler
Department of Animal Sciences, Oregon State University, Corvallis, OR, USA

Introduction
Primary cell culture studies are often hindered by the lack and convience of fresh samples. Cryogenically stored tissue has the potential to be used for future studies. The objective of this research was to evaluate whether canine placental tissue that was cryopreserved can be successfully used for primary cell culture in the future. For this experiment, we hypothesized that placenta cryopreserved using a slow freezing rate would yield cells with a higher rate of proliferation than a fast freezing rate.

Methods
One placenta was collected following delivery from two dogs. Following methods recently published for using cryoprotectants on human placental tissue (Huppertz et al., 2011), 0.5 cm pieces of chorioallantois were pre-incubated with 10% Me2SO in fetal calf serum at room temperature for 15 minutes before being loaded into cryovials and either 1) plunged directly into liquid nitrogen (FAST) or 2) put into a -80°C freezer in a Mr. Frosty for 24 hours prior to plunging into liquid nitrogen (SLOW). Cytotrophoblasts (CYTO) and syncytiotrophoblasts (SYN) were isolated using collagenase and trypsin with Percoll density gradient centrifugation as previously described for isolating canine trophoblasts (Sahlfeld et al., 2011). Cells were then cultured in duplicate in DMEM media (#829415, Gibco-Invitrogen, Carlsbad, CA) at 38°C with 5% CO2 and grown to 70-80% confluency. Cells were passaged twice and counted after each passage using a hemocytometer. Average cell count per day was determined by dividing the number of cells contained within each flask by the number of days in culture.

Results
Cellular proliferation following primary culture of cryogenically preserved canine placental tissues was not influenced by freezing rate. The average daily cell counts for all four groups (CYTO-SLOW, CYTO-FAST, SYN-SLOW, SYN-FAST) ranged from 4.060-8.750 million trophoblasts.

Discussion
Future studies using these cell lines will focus on characterizing canine trophoblast invasion as part of our laboratory’s focus on pre-eclampsia.

References
Appendix F.6 2011 International Society for Cryobiology Presentation

Introduction

- Normal human placental development
  - Trophoblasts invade the endometrium & differentiate into an inner layer of cytotrophoblasts & an outer layer of syncytiotrophoblasts
  - Trophoblasts invade the tight spiral arteries & remodel them into wide, cavernous, flaccid uteroplacental vessels
    - Allows for increased blood flow

- Placental development in preeclampsia
  - Trophoblasts shallowly invade the endometrium & insufficiently remodel spiral arteries
    - Maternal blood flow to the placenta is reduced

- Overall Goal
  - The overall goal of my graduate research is to isolate, describe, proliferate & immortalize canine trophoblasts so that these cells can be used for future experiments to develop a novel model to study preeclampsia
Objective

Because canine placental tissue is not always available at the most opportune times for my research, the objective of this research was to evaluate whether canine placental tissue that was cryopreserved can be successfully used for primary cell culture in the future.

Hypothesis

We hypothesized that placenta cryopreserved using a (two-step) slow freezing rate would yield cells with a higher rate of proliferation than a (one-step) fast freezing rate. The basis for this hypothesis was previous work by Huppertz.

Materials & Methods

One term placenta was collected from each of 3 dogs.

0.5 cm pieces of chorionic villi were pre-incubated with 10% MeSO in fetal calf serum at room temperature for 15 min before being loaded into cryovials.

Materials & Methods

Cryovials were divided into 3 groups:

1. Control: not cryopreserved but directly cultured
2. FAST: cooling from 20°C to -196°C at -200°C/sec
3. SLOW: cooling from 20°C to -80°C at -1°C/min then -80°C to -196°C at -116°C/sec

Following freezing, FAST & SLOW vials were thawed by submerging into a 37°C water bath for 3 min.

Materials & Methods

Trophoblasts were isolated from placental pieces using collagenase & trypsin with Percoll density gradient centrifugation as described by Hunkapiller and colleagues in 2008 for human tissues.
**Materials & Methods**

- Cells were cultured in DMEM @ 38°C in 5% CO₂ & grown to 70% confluency in flasks & on coverslips
  - Cells grown on coverslips were fixed in 70% methanol

- Cells were passaged twice & counted after each passage using a hemocytometer
- Average cell counts per day were determined by dividing the number of cells contained within each flask by the number of days in culture
- Groups were compared by Kruskal-Wallis test
- Significance was defined as p<0.05

**Results**

- The average daily cell counts for all groups were not significantly different (p=0.4159)

**Conclusion**

- Cellular proliferation following primary culture of cryogenically preserved canine placental tissues was not influenced by freezing rate

**Acknowledgements**

- OSU, Department of Animal Sciences
- Dr. Michelle Kutzler
- Dr. Timothy Hazzard
- Shandra Epperson
Who Let The Dogs In: A Canine Trophoblast Invasion Model For Preeclampsia

Laura Sahlfeld Department of Animal Science, Oregon State University, Corvallis, OR 97331, USA

Preeclampsia is a pregnancy-specific syndrome that affects 2-8% of pregnant women worldwide. It is the third leading cause of maternal mortality in the United States, accounting for 20% of maternal deaths, for which the only known cure is delivery of the placenta. Preeclampsia results from abnormal cytotrophoblast invasion of the endometrium and myometrium that morphologically is described as shallow. This superficial trophoblast invasion results in insufficient remodeling of the spiral arteries and hypoperfusion of the human placenta. Despite intensive investigation for more than 50 years; the causes of preeclampsia are largely unknown and the effectiveness of current models has been limited. An effective animal model is crucial to understanding the underlying causes of preeclampsia. To our knowledge, no existing model demonstrates the shallow trophoblast invasion observed in preeclampsia. A larger non-rodent animal model could be more easily manipulated to demonstrate changes in morphology, histochemistry, and gene expression throughout pregnancy.

Compared to other domestic animal models (e.g. sheep, pig) used to study human pregnancy-related disorders, the canine placenta is significantly more invasive therefore more like the human model. It is important to note that the morphologic and histologic similarities between normal canine trophoblast invasion and that of the preeclamptic trophoblast invasion are striking. In both types of placentation, cytotrophoblasts invade the endometrium (uterine epithelium) and the endometrial stroma (decidua) but do not completely invade the myometrium. Another feature of both forms of placentation is that transformation of the endometrium (decidualization) may play a role in regulation of trophoblast invasion. We believe that the canine model will be a useful improvement over the current efforts to investigate
preeclampsia and other disorders of shallow trophoblast invasion. The long-range goal of our laboratory’s research is that with a canine model, new treatments (and possibly preventions) for the underlying cause of preeclampsia (e.g. shallow trophoblast invasion) can be developed. The central hypothesis to this approach is that canine trophoblasts display several cellular and molecular similarities to human preeclamptic trophoblasts. Our laboratory is currently working to identify time points during pregnancy when canine trophoblasts exhibit invasive properties. To do this, fresh placental tissue is collected from pregnant dogs, cytotrophoblasts are isolated and in vitro invasive properties are compared.

Canine pregnancy length is 65±1 days from the onset of the surge in luteinizing hormone (LH) and is not influenced by breed or litter size. Canine pregnancy can be divided into three stages: preimplantation (from day 0 to day 20), embryonic and placental development (day 20 to day 45), and fetal and placental maturation (day 45 to day 65). Our laboratory is comparing changes in trophoblast cellular behavior and gene expression at the beginning (48% of pregnancy; 31±1 days past the LH surge; n=8) and end (68% of pregnancy; 44±1 days past the LH surge; n=8), of trophoblast invasion in canine pregnancy as well as at two later time points, 88% of pregnancy (57±1 days past the LH surge; n=8) and at the end of pregnancy (65±1 days past the LH surge; n=8), which are important for comparison to published human studies where placentas are obtained at similar gestational ages. Most human studies of preeclampsia use placentas collected from the second and third trimester after delivery or C-section. Since matrix metalloproteinases (MMPs) are essential for the penetrative ability of human cytotrophoblasts in vitro and in vivo, the role of MMP2 and MMP9 in cultured canine trophoblasts and in culture media as well as from maternal serum concentrations throughout pregnancy are being investigated. In addition, MMP2 and MMP9 gene expression is being examined using RT-PCR at each of these time points during pregnancy from both whole placental tissues and isolated cytotrophoblasts.
We believe that investigation into these processes will advance scientific knowledge in the field of abnormal placentation with respect to trophoblast invasion and specifically relating to preeclampsia. Ultimately this line of research may lead to the discovery of new genes important in the regulation of trophoblast invasion and a novel therapeutic or preventive treatment. In addition, determining the gene involved with canine trophoblast invasion may identify causes of infertility relating to pregnancy loss and placental/trophoblast retention (e.g., subinvolution of placental sites).
Appendix F.8 2012 Northwest Reproductive Sciences Symposium Presentation

Background

1. Placental dysfunction caused by shallow trophoblast invasion is a serious complication of human pregnancies & a major cause of maternal morbidity, mortality & premature delivery.

2. Preeclampsia is an example of a gestational disease in humans that results from shallow trophoblast invasion.

Background

1. Despite decades of research into the etiology of preeclampsia, the underlying cause of this disease (shallow trophoblast invasion) remains poorly understood.

2. Current laboratory animal models (mouse, rat, guinea pig) have not been useful for evaluating targeted therapeutic intervention for shallow trophoblast invasion.

Background

1. Unlike the hemochorial placenta of primates & rodents, the canine placenta is endetheliocorial, which is a naturally-occurring shallow trophoblast invasion form of placenta.

2. Due to this feature, the canine placenta may be an important model to investigate the cellular & molecular processes involved in pathologic shallow trophoblast invasion in humans.

Background

1. As in the hemochorial placenta, canine trophoblasts invade the uterine epithelium & endometrial stroma (decidua) in endetheliocorial placentation.

   a. However, in hemochorial placentas, trophoblasts continue to invade deeper into the myometrium.

Background

1. Another feature of both hemochorial & endetheliocorial placentation is the transformation of the endometrium (decidualization) may play a role in the regulation of trophoblast invasion.

   a. However, in the endetheliocorial placenta, only those trophoblasts that line the marginal hematoma (a hematothrophius zone) have direct contact with maternal blood, which is in contrast to the hemochorial placenta.
### Background

1. There are many factors that regulate trophoblast invasion in humans, but those regulating trophoblast invasion in dogs are not as well described.
2. Matrix metalloproteinases (MMPs) play a crucial role in trophoblast implantation & invasion in other species.

### Overall Objective

1. To establish an endotheliocorial placenta model for studying disorders of shallow trophoblast invasion.

### Hypothesis

1. We hypothesized that canine trophoblasts would express matrix metalloproteinases and their inhibitors in a pattern similar to human trophoblasts.

### Objective

1. The objective of this research was to describe cellular & molecular expression of MMP2, MMP9 & TIMP2 in canine trophoblasts.

### Methods

1. Placentas were collected from dogs at pre-term (hysterectomy), pre-labor (C-section) & post-parturition.
2. Placental tissue was either flash frozen in LN2, fixed in 10% formalin or dissociated.
   - Trophoblasts were isolated using collagenase & trypsin digestion with Percoll density gradient centrifugation.

### Methods-ICC

1. Cellular expression of CK7, MMP2 & MMP9 was determined by fluorescent immunocytochemistry on fixed dissociated cells.
2. Briefly, coverslips were treated with 5% goat or donkey serum in PBS (blocking buffer) to block non-specific secondary antibody binding.
3. Specificity of immunostaining was verified by omission of the primary antibody.
4. Nuclei were detected with Hoechst 33342 (1:20,000).
### Methods-IHC

- Placentas were paraffin-embedded for immunohistochemistry
- Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide
- Sections were incubated with DAKO serum free protein block to prevent nonspecific binding reactions
- Primary antibodies were diluted in Antibody Diluent with background reducing component
- A secondary antibody with horse radish peroxidase (HRP)-conjugated dextrane polymer (MaxPolyOne™, MaxVision Biosciences Inc.) was used to enhance the signal
- Peroxidase activity was detected with VECTOR NovaRED Peroxidase Substrate Kit & counterstained in hematoxylin histological staining reagent
- Negative controls included replacement of primary antibody with DAKO universal negative control rabbit/mouse

### Methods-RNA & cDNA

- RNA isolation
  - TRIzol RNA Plus Purification Kit (Invitrogen)
- RNA concentration
  - Nanodrop spectrophotometer (IMPLEN)
- RNA integrity
  - Agilent 2100 Bioanalyzer (CGRB)
- cDNA synthesis
  - SuperScript III System (Invitrogen)

### Methods-RTPCR

- TaqMan probes & primers validated by manufacturer for canine MMP2, MMP9 & TIMP2
- 18S rRNA used as a reference gene
- 7300 Real Time PCR Machine (Applied Biosystems)

### Data Analyses

- **t-ICC**
  - Students t test (Excel)
- **RTPCR**
  - Relative expression was calculated using the standard 2^ΔΔCt method
  - One-way ANOVA with Bonferroni corrections (Stata 12.0 software, StataCorp LP)
- Significance was defined as $P<0.05$
**Results-ICC**

- More cultured canine trophoblasts expressed MMP-9 (54.7±3.4%) compared to MMP2 (40.3±1.8%) (p<0.02)
- Although both MMPs were immunolocalized to the cytoplasm, MMP2 was found in large coalescing granules, whereas MMP9 was more diffusely expressed.

**Results-RTPCR**

- MMP9 & MMP2 expression was higher in pre-labor samples compared to pre-term & parturient samples.
- There was no significant difference between TIMP2 expression among groups.

**Discussion-ICC**

- Human trophoblasts are positively identified using immunostaining for CK7 expression.
  - This is also true for canine trophoblasts.
In the current study, trophoblasts from late gestation canine placentas express significantly more MMP9 compared to MMP2.

- Trophoblasts from early gestation canine placentas (15-10 days after mating) express significantly more MMP2 than MMP9.

- This occurrence is consistent with results from human trophoblasts where MMP2 is predominately expressed in early gestation while MMP9 is dominant in late gestation.

MMP9 & MMP2 mRNA expression was higher in pre-labor samples compared to pre-term & parturient samples.

- This differs from the human where MMP2 & MMP9 mRNA expression gradually decrease with approaching parturition.

TIMP2 mRNA expression appears to be constantly expressed at the end of canine gestation.

- This is similar to what has been reported in cattle.

Studies Underway
Matrix Metalloproteinase Expression in Cultured Canine Trophoblasts
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INTRODUCTION. The factors regulating trophoblast invasion into the canine decidua are not well described. Matrix metalloproteinases play a crucial role in trophoblast implantation and invasion in many species (Salamonsen, 1999). Of these, MMP-2 and -9 are involved in the degradation of the extracellular matrix and cell migration. Trophoblast expression of MMP-2 and -9 has been demonstrated in normal and abnormal human placentas (Shokry, 2009). To establish a baseline for future studies investigating placental disorders in dogs, the objective of this research was to determine MMP-2 and -9 expression in cultured canine trophoblasts. We hypothesized that cultured canine trophoblasts would express MMP-2 and -9.

METHODS. Following methods previously described (Sahlfeld et al., 2011), trophoblasts were isolated from three canine placentas using collagenase and trypsin with Percoll density gradient centrifugation. Cells were then cultured in DMEM media (#829415, Gibco-Invitrogen, Carlsbad, CA) at 38°C with 5% CO₂ and grown to 70% confluency on coverslips. Cells were fixed in 70% methanol and expression of MMP-2 (#MS806P0, clone Ab4, Neomarkers, Freemont, CA) and MMP-9 (#RB1539P0, clone Ab9, Neomarkers, Freemont, CA) was confirmed using fluorescent immunohistochemistry (Alexa Flour 488, #A21202, Invitrogen, Carlsbad, CA; Texas Red, #T2767, Invitrogen, Carlsbad, CA). Both MMP antibodies had been used previously for immunohistochemistry in the canine uterus (Kanca et al., 2011). Expression of cytokeratin-7 (#p103620, DAKO, Carpinteria, CA) was to confirm cell type. Hoescht 33342 (#H1399, Invitrogen, Carlsbad, CA) was used to count cells. The average percentage of MMP positive cells for multiple fields was determined for each placenta and reported as the mean±SEM MMP-2 and MMP-9 percent positive. MMP-
2 and MMP-9 percent positive cells were compared using a Students t test. The staining intensity and stain localization within MMP positive cells was also noted.

RESULTS. More cultured canine trophoblasts expressed MMP-9 (54.7±3.4%) compared to MMP-2 (40.3±1.8) (p=0.02). However, MMP-2 was more intensely expressed within cells compared to MMP-9 (Figure F.9.1). Although both MMPs were immunolocalized to the cytoplasm, MMP-2 was found in large vesicles, whereas MMP-9 was more diffusely expressed.

DISCUSSION. In trophoblasts from normal human pregnancies, MMP-2 and MMP-9 are expressed at a similar intensity and frequency (75% and 78.5%, respectively) (Shokry, 2009). However, it was found that MMP-9 expression was reduced to 15% in trophoblasts from pregnancies complicated with preeclampsia (e.g., those having shallow trophoblast invasion) (Shokry, 2009). The canine endotheliochorial placenta is a naturally-occurring shallowly invasive placenta. The lower frequency of MMP positive cells reported in the present study with canine trophoblasts and in the previous study (Shokry, 2009) with preeclamptic human trophoblasts could be related to their limited ability to deeply invade the decidua. Activated MMP-2 can activate proMMP-9 (Erices et al., 2011) but the reverse has not been shown. This may explain why more canine trophoblasts expressed MMP-9 compared to MMP-2. Previous research has demonstrated that the staining pattern in human trophoblasts for MMP-9 is diffuse; whereas the staining pattern for MMP-2 is granular (Fridman et al., 1995). Similar results were found in canine trophoblasts. In addition, we have shown that MMP-2 is more intensely expressed within canine cultured trophoblasts than MMP-9. Future studies using canine placental tissues will investigate the mechanism and significance of this expression, as well as determine if the addition of MMP-2 in culture can induce greater MMP-9 expression.

References:
Sahlfeld, L., Hazzard, T., Kutzler, M., 2011. Isolation and primary cell culture of

Figure F.9.1 MMP-2 (left) and MMP-9 (right) fluorescent immunostaining in cultured canine trophoblasts.