

AN ABSTRACT OF THE THESIS OF

Justine Marie Gullaba for the degree of Master of Science in Animal Sciences presented on May 6, 2013

Title: Role of IL-8 and TIMP-2 on Term Canine Trophoblast Migration, Invasion, and Proliferation

Abstract Approved:

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Placental pathologies are not uncommon in dogs. For example, in primiparous bitches, the reported incidence of subinvolution of placental sites is 21% - 100%. Despite years of research in multiple species, the mechanisms regulating late gestation trophoblast behavior in the dog remain unexamined. Therefore, the objective of this study was to characterize normal *in vitro* term canine trophoblast (TCT) physiology, with respect to migration, invasion, and proliferation. In addition, the effects of interleukin-8 (IL-8) and tissue inhibitor of metalloproteinase-2 (TIMP-2) on trophoblast physiology were examined.

Following isolation of primary TCT, the cell suspension was seeded into the migration (wound-healing) assay, invasion (Matrigel) assay, and proliferation (MTT) assay. Cells were cultured under the same conditions at 37°C with 5% CO₂ for each

assay. Recombinant human IL-8 (200-08M, Peprotech, Rocky Hill, NJ, USA) was used at a concentration of 10 ng/ml for each assay. Recombinant human TIMP-2 (410-02, Peprotech) was used at a concentration of 0.5 µg/ml for each assay, respectively.

For the migration assay, cells were suspended in medium supplemented with 10% fetal bovine serum (10% FBS). Primary TCT (1000×10^3 cells/well) were cultured in 12-well tissue culture plates until a confluent monolayer was formed. A wound was added with a sterile pipette tip, the monolayer was rinsed, and 10% FBS with no factor (control), with IL-8 or TIMP-2 was then added. Photomicrographs of the wound were taken with phase-contrast microscopy (100X) after 8 h in culture and the area of the wound was measured using ImageJ v.1.34 software. The experiment was performed in triplicate (n=5 dogs).

The invasion assay was performed using Matrigel invasion chambers (#354480, BD Biosciences). Primary TCT (250×10^3) were suspended in protein-free medium (PFM) with no factor (control), with IL-8 or TIMP-2 and seeded onto Matrigel-coated filter membranes in the upper well. Chemoattractant (10% FBS) was placed in the lower well and the 24-well chambers were cultured for 22 h. Non-invading cells were removed from the filters that were then fixed and stained. Cells were counted under light microscopy (400X). The experiment was performed in triplicate (n=5 dogs). For the proliferation assay, cells were suspended in PFM.

For the proliferation assay (CGD-1, Sigma, St. Louis, MO, USA) primary TCT (100×10^3 cells/well) were suspended in PFM with no factor (control) or with IL-8 and cultured in 96-well tissue culture plate for 28 h. Cells were then incubated with MTT for

4 h, which was later replaced with 1-propanol and the plates were vigorously shaken. Absorbances were measured with a microplate reader at 570 nm and 690 nm (to remove background). Measurements were analyzed with SoftMax Pro (5.2 program SoftMax® Pro Data Acquisition & Analysis Software, Sunnyvale, CA). The experiment was performed in quadruplicate (n=4 dogs).

Statistical analysis of the data was performed using Repeated Measures ANOVA (migration), one-way ANOVA (invasion), or two-way ANOVA (proliferation) in PROC MIXED using SAS (Version 9.2, SAS Institute Inc., Cary, NC). For the migration assay, the mean of the control wound area at 0 h was set to 0% wound closure and the data were expressed as the percent wound closure of the control for each dog. For the invasion assay and proliferation assay, the mean of the controls was set to 100% and the data was expressed as the percentage of the control for each dog. Significance was defined as $P < 0.05$.

Analysis showed that IL-8 increased cell migration by 35% compared to the control ($P < 0.01$). TIMP-2 had no significant effect on cell migration ($P = 0.38$). There was no significant effect of IL-8 on cell invasion compared to the control ($P = 0.42$), whereas TIMP-2 decreased cell invasion by 57% compared to the control ($P < 0.05$). Also, for the proliferation assay IL-8 had no significant effect on cell proliferation ($P = 0.18$).

This study was the first to investigate term canine trophoblast physiology. Future studies should compare differences between normal term trophoblasts and those from cases of subinvolution of placental sites. It would also be of interest to compare differences in trophoblast physiology at different stages of gestation.

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Role of IL-8 and TIMP-2 on Term Canine Trophoblast Migration,
Invasion, and Proliferation

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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.

Justine Marie Gullaba, Author

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CONTRIBUTION OF AUTHORS

Dr. Michelle Kutzler was involved with the concept and design of experiments, and editing of this thesis. Dr. Alfred Menino, Jr. and Ms. Caitlin Donovan were involved with statistical analysis and data interpretation. Ms. Justine Gullaba was involved in conceptualizing the experimental design, data collection and interpretation, and thesis writing.

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CHAPTER I

INTRODUCTION

1.1 NORMAL TROPHOBLAST PHYSIOLOGY

1.1.1 Introduction

Trophoblasts are unique to mammalian reproduction. With the evolutionary divergence in vertebrates, there was a transition from oviparity (yolk-sac nutrition) to viviparity (nourishment through mother) with the emergence of the trophoblast (Moffett and Loke, 2006). Trophoblasts are specialized epithelial cells that form the chorion of the placenta. Trophoblasts serve endocrine, nutritive, and gas exchange functions between the mother and fetus. Originating from the trophoblast layer of the blastocyst, these are the first cells during embryologic development to differentiate. The trophoblast gives rise to trophoblast lineages that undergo migration, invasion, and proliferation, to aid in placental development.

1.1.2 Origin of the trophoblast

The origin of the trophoblast begins at the 32-cell blastocyst stage in mice (Pedersen et al., 1986) when external blastomeres give rise to the trophoblast (Pfeffer

and Pearton, 2012). In contrast, cattle trophoblast determination occurs at a later stage in pre-gastrulation embryos (Berg et al., 2011). In dogs, embryos enter the uterus between the 16-cell morula to early blastocyst stage (Andersen, 1927; Holst and Phemister, 1971). However, studies investigating the earliest origins of the trophoblast have not yet been conducted in the dog.

The trophoblast gives rise to two main lineages of trophoblasts: uninucleated cytotrophoblasts and multinucleated syncytiotrophoblasts (Thiede and Rudolph, 1961). Subsets within these two lineages vary greatly among species (Figure 1.1). In the canine placenta, syncytiotrophoblasts are in contact with the maternal blood vessels; absorptive cytotrophoblasts are present in the necrotic zone; and phagocytotic cytotrophoblasts line the marginal hematomas (Barrau et al., 1975). Since mitotic divisions can only occur in cytotrophoblasts, cytotrophoblasts are the primary trophoblast lineage that proliferates and differentiates to form syncytiotrophoblasts (Barrau et al., 1975). In the dog, both lineages have degradative and absorptive capabilities, although syncytiotrophoblasts have invasive capabilities whereas cytotrophoblasts do not (Barrau et al., 1975).

In the primate placenta, cytotrophoblasts differentiate into villous cytotrophoblasts and extravillous cytotrophoblasts (Bischof et al., 2000). Villous cytotrophoblasts proliferate and differentiate into syncytiotrophoblasts that cover placental villi. Extravillous cytotrophoblasts, located beneath the syncytiotrophoblasts, form a multilayer of motile and highly invasive cells. Extravillous cytotrophoblasts invade the endometrium and invade into the first third of the myometrium. Furthermore, extravillous cytotrophoblasts can differentiate into endovascular cytotrophoblasts

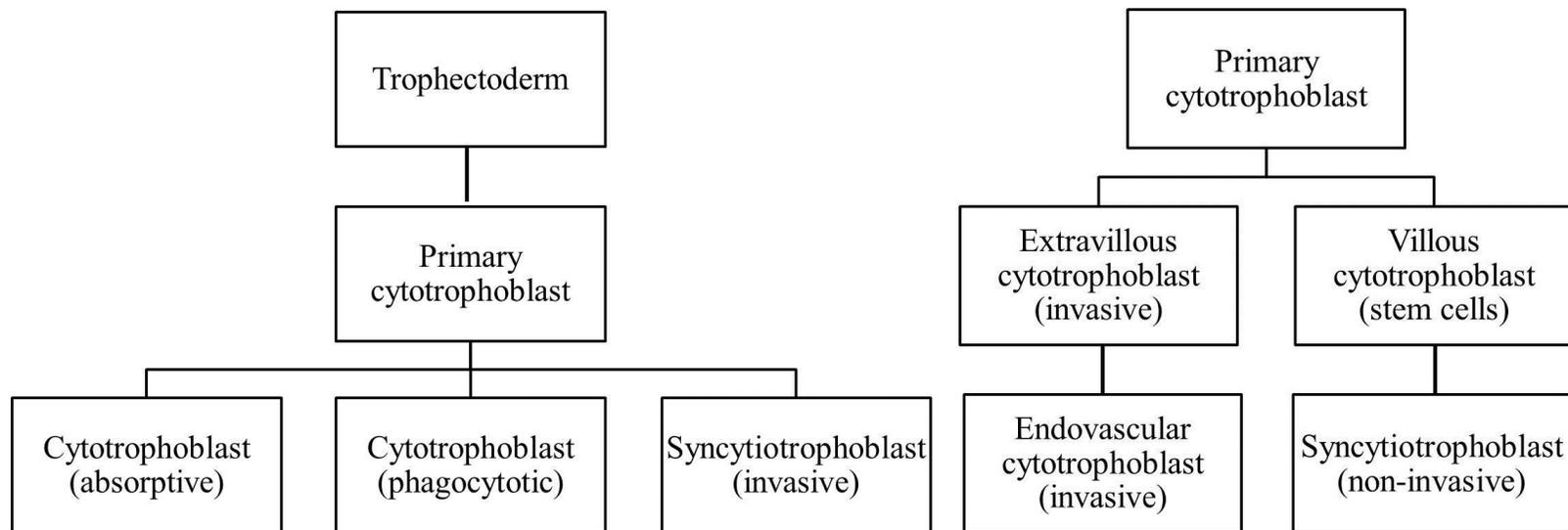


Figure 1.1. A schematic diagram showing the derivation of canine (left) and primate trophoblasts (right) and their cell lineages.

References: Andersen, 1927; Thiede and Rudolph, 1961; Wynn and Corbett, 1969; Holst and Phemister, 1971; Barrau et al., 1975; Bischof et al., 2000.

that migrate into and remodel the maternal spiral arteries, which allows increased maternal blood flow (Pennington et al., 2012).

1.1.3 Role of the trophoblast

Irrespective of species, trophoblasts migrate, invade, and proliferate to aid in subsequent placental development. Initially, trophoblasts appose the endometrial epithelium in an unstable adhesion (Hohn and Denker, 2002). Next, trophoblasts develop a stable adherence to the endometrial epithelium to form the feto-maternal interface (Staun-Ram and Shalev, 2005). Compared to rodents and equids, trophoblast apposition and adhesion in dogs are delayed. Canine blastocysts exist in a free-floating stage for 7 (Holst and Phemister, 1971) up to 16 days (Tietz and Seliger, 1967) after breeding, which is longer than rodents (3 days) and equids (7 days) (Austin, 1969). Finally, trophoblasts attach to the endometrial epithelium, which is characterized by trophoblast invasion that forms an intimate contact between the trophoblast and the endometrial epithelium (Gilbert, 2010). In dogs, trophoblast attachment of the blastocyst to the uterus occurs 21 days after breeding (Concannon et al., 1988). Invasion of trophoblasts into the endometrial epithelium involves extracellular matrix (ECM) transformation. Invasion of the endometrium by trophoblasts promotes firm attachment and anchoring of the placenta to the uterus (Hohn and Denker, 2002). Beginning as early as 13 days after breeding, canine syncytiotrophoblast invade the endometrium (Barrau et al., 1975). Syncytiotrophoblasts penetrate and become wedged between the maternal epithelial cells

and dislodge them from the basement membrane through phagocytosis (Barrau et al., 1975). Syncytiotrophoblasts invade deeper within the endometrium and into the base of the endometrial glands (crypts) by day 18. There are notable areas of necrosis (necrotic zones) that are lined by invading syncytiotrophoblasts and cytotrophoblasts (Barrau et al., 1975). By day 26 after breeding, syncytiotrophoblasts reach maximum invasion depth at the lacunae within the endometrium.

A unique characteristic of carnivore placentation are marginal hematomas that are present on each pole of the villous chorioallantois (Barrau et al, 1975). In these specialized areas, trophoblasts extensively erode the maternal endometrium and blood vessels, resulting in large pools of stagnant maternal blood. Cytotrophoblasts that enclose the hematomas engulf red blood cells to supply metabolites and iron to the fetus (Wynn and Corbett, 1969). Compared to dogs with an endotheliochorial placenta, primate trophoblasts of the hemochorial placenta are much more invasive, such that the embryo is fully embedded within the endometrium (Gilbert, 2010). Human extravillous cytotrophoblasts extensively invade through the endometrium and a third into the myometrium to be in direct contact with the maternal circulation (Hohn and Denker, 2002; Staun-Ram and Shalev, 2005). Hemochorial placentation differs from the marginal hematomas in carnivore endotheliochorial placentation because extravillous cytotrophoblasts come in direct contact with circulating red blood cells, where the red blood cells in the canine placenta are stagnant.

1.1.4 Conclusion

Trophoblasts are extra-embryonic epithelial cells originating from the trophoblast to form the chorion of the placenta. The trophoblast lineage varies greatly between species for each type of placentation. Depending upon their phenotype, trophoblasts migrate, invade, proliferate, absorb, and phagocytose.

1.2 *IN VITRO* METHODS FOR THE STUDY OF TROPHOBLAST PHYSIOLOGY

1.2.1 Summary

The use of *in vivo* models to study trophoblast physiology is uncommon because of complications that may occur during perturbation to the fetus and mother. *Ex vivo* studies on trophoblast physiology, with respect to migration, invasion, and proliferation, have been facilitated through the use of primary trophoblast cell culture, immortalized trophoblast cell lines, and *in vitro* assays.

1.2.2 Primary trophoblast cell culture

The development of a successful technique for extended *in vitro* primary cell culture of trophoblasts was first established by Thiede and Rudolph (1961) to study trophoblast physiology. Human chorionic villi were dissected from term placenta after

delivery (vaginal or c-section), washed, and subjected to a trypsin digestion until cells were isolated and plated. Freshly isolated trophoblasts are spherical then as they remain in culture flatten into a polygonal shape while adhering to the culture plate (Merviel et al., 1993) and grow into a cell monolayer to form cobblestone morphology (Tanaka et al., 1998). A mixed culture cell monolayer of cytotrophoblasts and syncytiotrophoblasts were observed *in vitro* with subsequent differentiation into fibroblasts at 37°C with 7% CO₂ atmosphere conditions (Thiede and Rudolph, 1961). Later studies modified the culture conditions to 37°C with 5% CO₂ atmosphere to give growth patterns of trophoblast *in vitro* that were similar to those described *in vivo*, including the development of filopodia-like projections, circular growths, and syncytial sprouts (Aladjem and Lueck, 1981). In addition to cell morphology, *in vitro* cultures can preserve *in vivo* phenotypic destiny, with primary trophoblasts undergoing differentiation into syncytiotrophoblasts and invasive cytotrophoblasts *in vitro* (Kilburn et al., 2000). Also, primary trophoblast cell cultures maintain the expression of *in vivo* trophoblast markers (ie. human chorionic gonadotropin (hCG), human placental lactogen (hPL), cytokeratins, vimentins, human leukocyte antigens (HLA-I)) (King et al., 2000).

Limitations to using primary trophoblast culture include: selecting a culture medium to optimize cell growth while controlling sterility, purity, and differentiation; as well as the challenge of preparing fresh cells for each experiment (Orendi et al., 2011). In particular, selecting tissue culture medium with sufficient amount of serum (e.g. fetal bovine serum) or serum-free supplement (e.g Nutridoma) is critical. Employing sterile tissue culture techniques is important to maintain cell viability and to avoid

contamination when working with primary trophoblast cultures. Another challenge is obtaining a pure trophoblast sample during isolation because even a small number of contaminating cells (e.g. stromal fibroblasts) may overgrow the comparatively slower proliferating trophoblasts when in culture for an extended time (Thiede and Rudolph, 1961; Stromberg et al., 1978). Studies have successfully characterized the purity of isolated trophoblast cells based on the expression of cytoskeletal proteins, mainly (e.g. cytokeratin 7, vimentin) (Pötgens et al., 2001).

1.2.3 Immortalized trophoblast lines

Immortalized trophoblast lines were developed to facilitate the investigation of trophoblasts and to avoid difficulties associated with maintaining primary cultures. Human immortalized trophoblast lines include choriocarcinoma cells (e.g. BeWo cells) or primary cytotrophoblasts (e.g. HTR-8/SVneo) (Orendi et al., 2011). The advantages of immortalized trophoblast lines are that they are readily available, can be placed in culture for extended periods without differentiation into another cell type, and are highly accessible because they can be repeatedly frozen and thawed for experiments (Kilburn et al., 2000). However, the comparisons between immortalized trophoblast lines to trophoblasts *in vivo* are more variable than with primary trophoblast culture (Table 1.1).

Table 1.1. A summary of trophoblast characteristics derived from primary culture and immortalized lines.

Trophoblast characteristic	Primary culture	Immortalized lines
Origin of cells	Primary placenta tissue	Primary placenta or choriocarcinoma
Availability of cells	Variable, dependent on tissue availability	Readily available, frozen until use
Length of culture time	Up to 10-14 days until differentiation	Unlimited
Recapitulate <i>in vivo</i> differentiation	Yes	No
Recapitulate <i>in vivo</i> migration	Yes	Yes
Recapitulate <i>in vivo</i> invasion	Yes	Yes
Recapitulate <i>in vivo</i> phenotype	Yes, express all trophoblast markers	No, express some trophoblast markers
References	Thiede and Rudolph, 1961; Aladjem and Lueck, 1981; King et al., 2000	Graham et al., 1993; Kilburn et al., 2000; King et al., 2000; Orendi et al., 2011

Studies have shown that not one of the established immortalized trophoblast lines retain all of the same phenotypic attributes and behaviors unique to trophoblasts *in vivo* (King et al., 2000). For example, HTR-8/SVneo is a commonly used immortalized trophoblast line derived from first-trimester human cytotrophoblasts (HTR) from villous explants that were transfected with Simian Virus (SV) 40 large T antigen and passaged about 8 times to result in a nontumorigenic cell line with an extended lifespan (Graham et al., 1993). HTR-8/SVneo shares some phenotypic features with primary cytotrophoblast cultures (including expression of cytokeratin, hCG, and type IV collagenase), but does not express the trophoblast-specific major histocompatibility protein, human leukocyte antigen type G (HLA-G) (Kilburn et al., 2000). Another limitation to HTR-8/SVneo cells is that unlike primary culture trophoblasts, they are unable to differentiate into the invasive extravillous cytotrophoblast phenotype. Scientists should exercise caution when selecting an immortalized cell line for studying trophoblast physiology, such that they consider the limitations of each cell line as these relate to their research goals (King et al., 2000).

1.2.4 Migration assays

Two-dimensional *in vitro* assays allow a simple way to observe cell migration. Three commonly used methods include: the scratch assay, stencil-based scratch assay, and the Boyden chamber assay (Table 1.2). Migration is defined as the two-

Table 1.2. A comparison of characteristics of three trophoblast migration assays.

Assay characteristic	Scratch assay	Stencil scratch assay	Boyden chamber assay
Chemotaxis	No	No	Yes
Measurement	Migration area	Migration area	Cell count, fluorescence
Live imaging	Yes	Yes	No
IF, IHC	Yes	Yes	Yes
Substrate	Plastic, glass or coated	Plastic, glass or coated	PC, PE membrane or coated
Direction of movement	Horizontal	Horizontal	Horizontal then vertical
Recapitulate <i>in vivo</i> migration	Collective sheet migration	Collective sheet migration	Single cell migration
Cell applicability	Adherent cells only	Adherent cells only	Adherent/suspension cells
References	Liang et al., 2007; Kramer et al., 2013	Liang et al., 2007; Kramer et al., 2013	Boyden, 1962; Kramer et al., 2013

IF- Immunofluorescence, IHC- Immunohistochemistry, PC- Polycarbonate, PE- polyethylenterephthalate

dimensional movement of cells on a substrate (e.g. extracellular matrix (ECM) fibers, plastic of the plate) (Kramer et al., 2013). Cell motility is characterized by a duality between collective and individual behaviors. On the one hand, the motility within the cell monolayer is long range, involving an orchestrated movement of many cells together. On the other hand, there are transient, active leader cells that destabilize the scratch border by “fingering” the border. The sides of the “fingers” have an actin belt that may regulate the mechanical signaling between the leader and the follower cells, supporting the hypothesis of migration where a mechanical communication between the cells occurs in response to the free plate surface (Poujade et al., 2007).

Two scratch assays are widely used in the study of trophoblast migration: the scratch (or wound-healing) assay and the stencil-based scratch assay. Both follow the same general procedure. Trophoblasts are plated onto a tissue culture plate and supplemented with a culture medium containing serum. The cells are incubated and allowed to grow into a confluent cell monolayer. A scratch is added into the monolayer, by inflicting the wound or using a stencil, and loosened cells are rinsed away and the culture medium is replaced for the remaining duration of the assay. Microphotographs of the wound are taken at regular incubation intervals to quantify the number of migrating cells as they close the wound (Liang et al., 2007).

There are several advantages of the scratch assay. First, it mimics the migration of cells *in vivo*, where filopodia-like extensions of the active leader cells develop to close the wound (Aladjem and Lueck, 1981). Second, this assay allows the study of the regulation of cell migration by cell interaction with the extracellular matrix (ECM) and

cell–cell interactions. Third, the scratch assay can be combined with time lapse microscopy to allow imaging of intracellular signaling events in micro-injection or gene transfection studies (Kramer et al., 2013). Lastly, the scratch assay is a simple, straightforward method to study cell migration *in vitro* that requires inexpensive supplies found in cell culture laboratories.

However, there are a number of limitations to the scratch assay. First, it takes a relatively longer time to perform the scratch assay compared to other methods due to the time it takes to develop a confluent monolayer in culture. Second, it does not utilize a chemical gradient to induce chemotaxis (e.g. Boyden chamber assay; Boyden, 1962). Despite these limitations, the *in vitro* scratch assay is still the method of choice for most investigators because it is a convenient and inexpensive method for analysis of cell migration *in vitro* (Liang et al., 2007).

Alternatively, other studies employ an injury-free wound-healing assay on epithelial cells (Poujade et al., 2007). Although inspired by the scratch assay, the stencil-based, or microfabrication-based, method is different because it focuses on the influence of the free surface and uncouples it from the other possible contributions such as cell damage and/or permeabilization. With the stencil-based method, the cleft between cells is produced by an elastic polycarbonate microstencil that is placed into a culture dish and cells are then plated into the same dish to adhere overnight. The flexible microstencil is then removed to leave a free space on the plate surface. The sudden release of available plate surface by removal of a stencil-based scratch on a confluent cell monolayer is sufficient to trigger cell motility (Poujade et al., 2007). The primary advantage of the

stencil-based method is that many identical clefts in the culture can be made, thereby increasing statistical power, while retaining the advantages of non-injury (Poujade et al., 2007). The disadvantages to this assay are: the cost of manufactured stencils, the inability to allow complete formation of a confluent cell monolayer because cells tend to adhere to the stencil, and increased setup time.

Another common assay to study trophoblast migration is the Boyden (transwell) chamber assay. Unlike the scratch and stencil-based scratch assays, which observe horizontal cell migration in response to mechanical perturbation, this assay observes vertical migration stimulated by a chemoattractant (i.e. medium containing a higher serum concentration) (Kramer et al., 2013). Boyden (1962) first studied an *in vitro* chemotaxis method with leukocytes using membrane filters, and the method is still commonly used for trophoblast studies. The Boyden chamber consists of an upper and lower well separated by a membrane filter with pores just slightly smaller than the cell size. The chemoattractant is placed in the lower well and cells are seeded into the upper well and after an incubation interval, the cells have migrated towards the chemoattractant. The number of migrating cells deposited at the underside of the filter is compared to the controls to determine the rate of migration. By eliminating the mechanical perturbation of the scratch assay, this technique can be used to elucidate factors that may promote or inhibit chemotaxis (Kramer et al., 2013). Disadvantages to the Boyden chamber assay are the cost of the chamber system including filter membranes as well as the effort required for optimization of the cell concentration and the chemoattractant.

1.2.5 Invasion assays

The processes of cell invasion are integral to the role of trophoblasts in placental development. Three-dimensional invasion assays, such as the Matrigel invasion assay, explant culture technique, and spheroid culture assay are examples of *in vitro* invasion assays used in studying trophoblast physiology (Table 1.3). Invasion will be defined as three-dimensional cell movement through a barrier involving restructuring and degradation of the ECM (Kramer et al., 2013).

The most common invasion assay used in trophoblast studies is the Matrigel invasion assay, a modified Boyden chamber assay with a coating of Matrigel on top of the membrane filter in the upper well. Matrigel is a reconstituted basement membrane isolated from the extracellular-rich Engelbreth-Holm-Swarm (EHS) mouse sarcoma. This reconstituted material contains laminin, collagen IV, entactin, heparin, proteoglycans, matrix metalloproteinases (MMPs), various growth factors and tissue plasminogen activator (BD Biosciences, 2011). Matrigel acts as an *in vitro* substitute for the ECM thus it contains similar components. Librach and associates (1991) first introduced the Matrigel invasion assay to study the invasive capacity of trophoblast in the presence of MMPs and serine activators and inhibitors. The procedure is the same as the Boyden chamber technique (Boyden, 1962), but includes thawing and rehydration of the Matrigel basement membrane before seeding cells into the upper well and adding the

Table 1.3. A comparison of characteristics of three trophoblast invasion assays.

Assay characteristic	Matrigel invasion assay	Villi explant culture	Spheroid invasion assay
Chemotaxis	Yes	No	No
Measurement	Number of cells	Invasion area	Invasion area
IF, IHC	Yes	Yes	Yes
Substrate	BME, collagen I	BME, collagen I, fibrin	BME, collagen I, fibrin
Direction of movement	Vertical	Any	Any
Recapitulate <i>in vivo</i> invasion	Invasion through matrix	Single/collective invasion	Single/collective invasion
Cell applicability	Adherent/ suspension cells	Heterotypic cell interaction	Heterotypic cell interaction
References	Librach et al., 1991; Genbacev, 1992; Kramer et al., 2013	Korff et al., 2004; Kramer et al., 2013	Korff et al., 2004; Kramer et al., 2013

IF- Immunofluorescence, IHC- Immunohistochemistry, BME- basement membrane extract.

chemoattractant to the lower well. There are several advantages to this versatile assay. It is commercially available, and it involves an easy, straightforward approach using chemotaxis to induce invasion, and it can be combined with other methods such as immunohistochemistry (IHC) and drug or factor studies. The disadvantages to the Matrigel invasion assay are: the great cost of the Matrigel-coated membrane filters, the variability when counting cells manually, and ensuring sterility when handling the assays to reduce cell death and decrease invasion. However, this is the most common invasion assay applied to trophoblasts, and it is the better method to study trophoblast physiology because of the ease of the assay and production of reliable, quantifiable results (Kramer et al., 2013).

Another common *in vitro* method is the use of explant culture of chorionic villi of the placenta. This method was first applied to trophoblasts by Genbacev and associates (1992) to study human first trimester extravillous cytotrophoblast differentiation and column formation. Explant culture allows the observation of cell column development, showing which cell columns are formed by the proliferation of villous cytotrophoblast stem cells to attach the placenta to the uterine wall (Enders, 1968). These cell columns supply the invasive extravillous cytotrophoblasts to invade the endometrium and remodel maternal vasculature in humans and rodents. The procedure begins with preparation of the gel with diluted agarose (collagen I) and Matrigel in culture medium and this is allowed to gel overnight in a 12-well tissue culture plate placed in the incubator. Meanwhile, tissue is collected from floating chorionic villi and cut into small pieces, which are then arranged on top of the prepared gel and flooded with culture medium to

undergo incubation and allow invasive projections to extend into the gel. The area of outgrowth from the explant is measured and IHC can be applied. Advantages to the explant culture are: it is able to preserve the architecture and behavior of trophoblasts observed *in vivo* as well as to maintain the structure of the placenta at anchorage sites (Korff et al., 2004; Kramer et al., 2013). Also, this method allows the study of cell differentiation, cell marker expression, and metabolic labeling using commercially available assay kits that are less costly than the Matrigel invasion assay. The main disadvantage of the explant culture is that the size of outgrowth varies greatly between cultures because the size of outgrowth depends upon the site where the chorionic villi were obtained and the site of origin where villous tips contact the gel surface (Korff et al., 2004). Another disadvantage of the explants culture is that the measurement of outgrowth is not precise due to the lack of uniform borders of the outgrowth area.

A third alternative method to study trophoblast invasion is to use the spheroid culture technique. Korff and associates (2004) first introduced this method to study growth, cell differentiation, and invasive capacity in first and third trimester human trophoblasts, although spheroid culture has been widely used in tumor and embryonic cells for cell differentiation studies and pharmaceutical applications. Spheroid culture for trophoblasts was developed because Matrigel invasion assays do not study differentiation and are limited in their application. Also explant culture of chorionic villi do not examine cell-cell interaction the way that spheroid culture for trophoblasts does, and the variation in cell types in the explant (trophoblasts, fibroblasts, lymphocytes) cause analysis of the effects to be difficult (Korff et al., 2004). The technique begins with the generation of

cytotrophoblast spheroids by trypsinization of a confluent cytotrophoblast monolayer to resuspend cells in a culture medium containing methylcellulose and then seeding into a nonadherent round-bottom 96-well plate for an incubation to allow growth of the spheroids into a defined size and cell number (Korff et al., 2004). The spheroids are embedded in a substrate gel of interest (collagen, laminin), transferred into a pre-warmed 24-well plate to polymerize, and then undergo incubation in a culture medium at standard tissue culture conditions. Invasion is then quantified by measuring the length of the invasive “sprouts” using imaging software (Korff et al., 2004). The advantages of this assay are: it is a straightforward approach that has the ability to study invasion as well as proliferation and differentiation, it provides a more precise measurement of behavior to give easily quantifiable data, and it can be combined with IHC and protein expression studies (Korff et al., 2004; Kramer et al., 2013). Although the disadvantages include: the extra time required to isolate cells and then form spheroids in culture, and mainly, the difficulty in producing uniformly sized spheroids, which may cause variability in measuring the invasive “sprouts”.

1.2.6 Proliferation assays

Several biological methods have been developed to measure proliferating cells (Table 1.4). Traditional methods are not efficient in quantifying large cell counts in the thousands or millions at several time points because of the long processing time and sample variation that occurs during washing steps. Radioactive isotopes can be automated

Table 1.4. A comparison of characteristics between trophoblast proliferation assays.

Assay characteristic	MTT assay	Dojindo assay
Type of assay	Rapid colorimetric assay	Rapid colorimetric assay
Substrate	MTT	WST-8
Incubation with substrate	4 hours	1 - 4 hours
Product	Water-insoluble formazan	Water-soluble formazan
Solubilization agent	Acid 1-propanol, DMSO	None required
Measurement	Optical density	Optical density
Recapitulate <i>in vivo</i> proliferation	Collective cell proliferation	Collective cell proliferation
Cell applicability	Adherent cells only	Adherent and suspension cells
References	Mosmann, 1983; Carmichael et al., 1987; Twentyman and Luscombe, 1987	Ishiyama et al., 1996; Dojindo, 2012

to quantify larger cell counts but still cannot be used to measure several assay points over time (Mosmann, 1983). However, colorimetric assays have the ability to rapidly measure large sample sizes with a high degree of precision due to their use of multiwell scanning spectrophotometers (ELISA plate readers). Although there are several rapid colorimetric assays available for measuring cell proliferation, the tetrazolium salt (MTT) assay is the most commonly used method and the Dojindo assay is one of the newest methods (Ishiyama et al., 1996).

The tetrazolium salt, (((3-)-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), MTT assay was first developed by Mosmann in 1983 to measure mammalian cell survival and proliferation. The assay depends upon the cellular reduction of water-soluble MTT by the mitochondrial dehydrogenase present in viable cells to form a water-insoluble blue formazan product that can be measured on a spectrophotometer (Carmichael et al., 1987; Twentyman and Luscombe, 1987). The optical density as measured on the spectrophotometer is proportional to the amount of formazan produced and therefore is proportional to the concentration of viable cells. Dead cells, cells without mitochondria (i.e. red blood cells) and tissue culture medium are unable to produce the formazan product, and thereby do not interfere with the optical density readings of the MTT assay (Carmichael et al., 1987).

There have been several variations of the MTT assay in order to optimize its ability to accurately detect and quantify the amount of living cells in a given sample. Optimization studies include: altering total cell counts or medium volumes per well, incubation time with MTT, and solubilizing agents used. When cells are plated in culture

medium with little to no serum, the greatest amount of formazan production is yielded (Twentyman and Luscombe, 1987). Isopropanol is the most effective solvent of the formazan crystals due to the complete solubilization with agitation or titration (Carmichael et al., 1987). Another common solvent is DMSO, although it is erosive to tissue culture plasticware and it only partially dissolves the formazan product. Ethanol has also been used as a solvent, but it forms a precipitate with serum proteins that interferes with the optical density readings (Twentyman and Luscombe, 1987).

Advantages to the MTT assay are: it has a straightforward setup, it is commercially available, and it provides rapid results. However, the disadvantage to the MTT assay is its sensitivity to culture medium remaining in the well before addition of the solvent. Even a small volume of culture medium can interfere with the optical density reading (Twentyman and Luscombe, 1987). Also, extra care must be taken during cell seeding to decrease variability. And, the commercial kit is costly. Although the MTT assay has few disadvantages, it is the most effective assay to measure cell viability and proliferation due to its ability to give consistent, accurate readings, and it can be easily compared to other studies.

In contrast to MTT, a commercially available rapid colorimetric assay (Cell Counting Kit-8 kit, Dojindo, Kumamoto, Japan) utilizing WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] is available as an alternative for cell counting. Generally, the procedure is similar to the MTT assay, but instead of using MTT as the substrate, WST-8 is used. WST-8 reacts similarly to MTT, in which it is reduced by mitochondrial dehydrogenases in viable cells

to produce an orange formazan product (Dojindo, 2012). The water-soluble formazan product is soluble in tissue culture medium, thus no solvent is required to dissolve the formazan product in this assay. Dojindo advertises that WST-8 is a suitable substrate because it is a non-toxic tetrazolium salt. Similar to the MTT assay, the amount of formazan produced is directly proportional to the number of living cells.

There are a number of advantages and disadvantages to the Dojindo assay. Similar to the MTT assay, the Dojindo assay provides rapid results. Although, the Dojindo assay is less expensive and requires less time to perform than the MTT (no solubilization step and shorter incubation with WST-8), it is also suitable for adherent as well as non-adherent cells whereas the MTT assay is only applicable to adherent cells. On the other hand, the Dojindo assay cross-reacts with some culture mediums by falsely undergoing color development. Also, the Dojindo assay can only accurately measure low cell concentrations and cannot accurately measure higher cell concentrations (Dojindo, 2012).

1.3 REGULATORS OF TROPHOBLAST MIGRATION, INVASION, AND PROLIFERATION

1.3.1 Summary

Trophoblasts interact with the endometrium through synchronized molecular and cellular events. Studies have identified factors involved in the regulation of trophoblast migration, invasion, and proliferation in carnivores including endopeptidases and their inhibitors, cytokines, and growth factors, hormones, and other factors (Kovacs and Ojeda, 2012).

1.3.2 Matrix metalloproteinases and their tissue inhibitors

Expression of matrix-metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) were detected in the canine trophoblast (Sahlfeld et al., 2012) and endometrium (Beceriklisoy et al., 2009). MMPs are identified as key enzymes in the degradation of the maternal endometrium, specifically MMP-2 and MMP-9 (Bischof et al., 2000). MMPs are zinc-dependent endopeptidases collectively capable of degrading all ECM components (Bischof et al., 2000). MMPs fall into five classes: (1) gelatinases (MMP-2 and MMP-9), (2) collagenases (MMP-1, 8, 13), (3) stromelysins (MMP-3, 7, 10, 11 and 12), (4) membrane-type metalloproteinases (MMP-14, 15, 16), and (5) non-classified MMPs of unknown function. Gelatinases MMP-2 and -9 are involved in ECM

degradation and the regulation of growth factors, cytokines and angiogenic factors that are important in trophoblast invasion (Bischof et al., 2000). Collagenases digest several types of collagen present in the ECM. Stromelysins digest collagens, laminin, fibronectin, elastin, proteoglycans and gelatin. Membrane-type MMPs activate MMP-2 at the cell surface to promote trophoblast invasion (Bischof et al., 2000). Gene expression of MMP-2 has been detected in canine trophoblasts and myometrial tissue (Beceriklisoy et al., 2007) and expression of MMP-9 detected in endometrial crypts and glands (Beceriklisoy et al., 2007). Both MMP-2 and MMP-9 have been detected in the pre-implantation embryo 10-12 days after mating (Schäfer-Somi et al., 2008). Around the time of implantation, mRNA expression of these MMPs is at its highest, suggesting that these endopeptidases are involved in the degradation of the endometrium required during implantation in canine pregnancy (Beceriklisoy et al., 2007). MMP activity, specifically MMP-1 and MMP-13, was also detected in feline trophoblasts (Walter and Schönkypl, 2006). It was proposed that these factors (with collagen I and IV, laminin and α -smooth muscle actin) balance ECM structure and degradation in the feline placental labyrinth for proper placental development and function (Walter and Schönkypl, 2006).

Tissue inhibitors of metalloproteinases (TIMPs) are the main inhibitors of MMPs. MMPs are inhibited by a 1:1 stoichiometric preferential binding by TIMPs (Bischof et al., 2000). *In vitro* studies suggest that successful implantation and placentation result from the balance between trophoblast secretion of MMPs and their inhibition by TIMPs. The MMP-TIMP interaction controls trophoblast invasion by restricting over or under-invasion into the uterus (Staun-Ram and Shalev, 2005). Immunohistochemical expression

of TIMP-2, MMP-2 and MMP-9 was detected in isolated canine trophoblast of term placenta (Sahlfeld et al., 2012). Fellows and company (2012) also observed a notable yet insignificant increase in TIMP-2 mRNA expression in canine placenta from pre-term to parturient stage, suggesting that as gestation progresses TIMP-2 inhibits trophoblast invasion.

1.3.3 Cytokines

The cytokine superfamily includes hematopoietic growth factors, interleukins (IL-1 β , IL-6, IL-8) and leukemia inhibitory factor (LIF), and inflammatory cytokine TNF- α , which are all involved in canine reproduction. Interleukins are known to play a major role in implantation by regulating MMP and adhesion activity (Van Mourik et al., 2009). A number of interleukins promote trophoblast invasive behavior. Trophoblast cells produce IL-1 β , and the IL-1 β receptor is present in uterine epithelial cells and trophoblasts. IL-1 β can stimulate MMP-9 activity in trophoblasts and expression in endometrial stromal cells for ECM degradation, thereby promoting trophoblast invasion for successful implantation in other species (Van Mourik et al., 2009). Also IL-6 and IL-8 were present at the fetomaternal interface. IL-6 stimulates MMP-2 and MMP-9 activity and enhances trophoblast invasion (Jovanović and Vićovac, 2009). IL-8 stimulates trophoblast viability, migration, invasion, and proliferation through the up-regulation of MMP-2, MMP-9, and integrins levels (Jovanović et al., 2010). Expression of IL-1 β , IL-6 and IL-8 in the pre-implantation embryos of dogs suggests that they contribute to trophoblast activity, in particular

invasive capacity, at implantation. IL-4 and IL-10 are known to down-regulate MMP-9 activity and therefore reduce trophoblast invasion (Van Mourik et al., 2009). IL-4 and IL-10 expression was found to be decreased at the placental sites during implantation to possibly regulate canine trophoblast invasion (Beceriklisoy et al., 2009).

Cytokine interaction with acute phase proteins has also been under investigation in dogs. An increase of plasma acute phase proteins (APPs) is known to occur during inflammation, infection, tissue injury, neoplastic growth or immunological disorders (Conner et al., 1988; Eckersall and Conner, 1988). APPs, such as C-reactive protein, haptoglobin, fibrinogen, ceruloplasmin, seromucoid, and glycoproteins, have been detected in the plasma of pregnant bitches 28 and 37 days after mating (Evans and Anderton, 1992). The rise in APPs was proposed to be involved with an immunological response by the maternal immune system during blastocyst hatching (Harvey, 1991). The presence of plasma APPs has been linked to an increase in cytokines IL-6 and TNF after inducing a localized inflammation in adult beagles (Yamashita et al., 1994). Further, a post-implantation increase of serum C-reactive protein was suggested to coincide with the inflammatory response at implantation (Concannon et al., 1996).

LIF and its receptor, LIFR, contribute to successful implantation in several species through ECM degradation (Arici et al., 1995; Salamonsen et al., 1997; Modric et al., 2000; Lei et al., 2004). LIF promotes uterine T helper cells to aid in embryo survival (Piccinini et al., 2002) and also prepares the uterus for blastocyst implantation (Passavant et al., 2000). LIF mRNA expression was detected in the primate pre-implantation embryo (Bischof et al., 2000) and canine pre-implantation embryos and uterus (Schäfer-Somi et

al., 2008). This suggests that LIF contributes to the breakdown of the ECM and regulates trophoblast invasion. Another study observed a significant rise in LIF mRNA expression in canine uterine tissue from pre-implantation to placentation stage (Schäfer-Somi et al., 2009), so it was concluded that LIF is important for the establishment of canine pregnancy. LIFR is also expressed in the pre-implantation uterus and blastocysts; therefore, it is proposed that the factor contributes to the feto-maternal interactions (Lei et al., 2004). Likewise, in another carnivore, the Western spotted skunk, LIF receptor β (LIFR β) mRNA was localized to syncytiotrophoblast and cytotrophoblast of post-implantation embryos. In the spotted skunk, increased LIF production is positively correlated with termination of embryonic diapause and preparation for implantation (Passavant et al., 2000).

TNF- α , now known as TNF, is an inflammatory cytokine present in trophoblasts and the endometrium (Staun-Ram and Shalev, 2005). *In vitro* studies showed that TNF increased trophoblastic urokinase-type plasminogen activator (uPA) secretion to promote ECM fibronectin degradation during implantation. This up-regulation of uPA increases MMP-9, which stimulates trophoblast invasiveness, but TNF decreases MMP-2 (Staun-Ram and Shalev, 2005). High levels of TNF are present in human pregnancy as part of the inflammatory response to implantation (Staun-Ram and Shalev, 2005). Immunolabelling of TNF was also detected during early canine pregnancy at the feto-maternal interface in trophoblast and endometrial stromal tissue and glands (Payan-Carreira et al., 2011). The presence of TNF suggests that it plays a role in implantation in dogs.

1.3.4. Growth factors

Insulin-like growth factors, IGF-I and IGF-II, are important metabolic and mitogenic factors promoting cell growth and metabolism. IGFs are peptide hormones in the same peptide family as relaxin and insulin, and are produced in the liver, bone and tissues under the influence of growth hormone. Circulating IGFs have endocrine and autocrine-paracrine effects on somatic growth and proliferation of various cells and tissues (Kovacs and Ojeda, 2012). IGF-II is known as a major fetal growth factor in several species (Monzani and Cohen, 2002), although IGF-I was only expressed at placental sites in the canine endometrium, suggesting an involvement in implantation (Beceriklisoy et al., 2009).

TGF- β inhibits trophoblast invasion and proliferation through stimulating TIMP secretion and decreasing MMP activation with a down-regulation of plasminogen activators in the plasminogen activator system (Salamonsen, 1999). TGF- β in conjunction with LIF mRNA expression is thought to inhibit gelatinolysis of the ECM at the time of implantation to balance the ECM-degradative properties of TNF- α and IL-6 (Mathialagan et al., 1992). TGF- β mRNA is expressed in the canine pre-implantation uterus. Therefore, the suggested role of TGF- β present in the canine pre-implantation uterus is regulation of ECM degradation and trophoblast invasion (Schäfer-Somi et al., 2008).

HGF is recognized as a growth-promoting peptide with a stimulatory effect in the growth of numerous tissues, organs, as well as tumor growth and metastasis (Kovacs and

Ojeda, 2012). Expression of HGF in the canine pre-implantation uterus and pre-implantation embryos (Schäfer-Somi et al., 2008) may suggest HGF regulation of endometrial degradation, tissue reorganization, and possibly trophoblast invasion (Nasu et al., 2000).

1.3.5. Hormones

Canine syncytiotrophoblast are the main source of relaxin (Tsutsui and Stewart, 1991). Relaxin is the only pregnancy-specific hormone in dogs (Concannon et al., 1988), however the role of relaxin and relaxin-like factor (RLF) in canine reproductive tissues is still unclear. Relaxin is a polypeptide homologue of insulin that, in conjunction with estrogen and progesterone, induces remodeling of connective tissues in the reproductive tract necessary to support and maintain pregnancy and parturition (Steinetz et al., 1989). The syncytiotrophoblast of the placenta has been identified as the main or sole source of relaxin in the pregnant bitch (Tsutsui and Stewart, 1991). Relaxin expression was localized in isolated term trophoblasts in dogs (Sahlfeld, personal communication). The endometrium does not express relaxin mRNA (Klonisch et al., 1999). Similarly, in felines the placenta is the main source of relaxin during pregnancy (Addiegio et al., 1987; Klonisch et al., 1999). Canine cytotrophoblasts surrounding maternal blood vessels also express RLF mRNA at early to mid-gestation (Klonisch et al., 2001). Differential expression of relaxin and RLF in the reproductive tissues suggests that these factors have a specific, localized function supported by cytokines and steroid hormones (Schäfer-

Somi, 2003). Further, canine mammary tumors also produce relaxin, which correlates with MMP-2 mRNA and cell invasion (Lamp et al., 2009). Thus, there is a possible interaction between relaxin, MMP-2 and trophoblast invasion in dogs.

Progesterone is essential for the establishment and maintenance of pregnancy in the bitch (Concannon et al., 1988) and in all mammals (Kovacs and Ojeda, 2012). Successful maintenance of pregnancy largely relies on progesterone because of its ability to block maternal cellular immune response (Kovacs and Ojeda, 2012). Inhibition of progesterone secretion leads to termination of pregnancy at any stage of gestation (Concannon et al. 1988). Abortion of the fetuses was observed in the bitch within 24 to 48 hours after ovariectomy, unless exogenous progesterone was administered, therefore in dogs it was proposed that the ovaries are the sole source of progesterone (Sokolowski, 1971). Expression of the progesterone receptor (PR) was also detected in canine uterine tissue at implantation (Schäfer-Somi et al., 2009). There was a significant decrease in PR expression and a significant rise in LIF expression in uterine tissue from pre-implantation to placentation stage (Schäfer-Somi et al., 2009). It was concluded that the decrease in PR levels contributes to the increase in LIF levels. It was also concluded that the embryo-driven preservation of PR mRNA expression at implantation and placentation sites, while expression is lost in the remainder of the uterus, may contribute to the establishment and maintenance of pregnancy (Schäfer-Somi et al., 2009). Progesterone receptor expression was detected in the pre-implantation uterus of the pregnant bitch (Schäfer-Somi et al., 2008).

Prolactin receptor (PRLr) expression was identified in canine fetomaternal tissue by Kowalewski and associates (2011). An increase in PRLr mRNA expression in canine was observed in the placental attachment sites following implantation. Immunohistochemistry and *in situ* hybridization localized expression to the trophoblast, proposing that PRL acts on the trophoblast PRLr during placentation and mediates trophoblast invasion (Kowalewski et al., 2011). In the Western spotted skunk, PRLr mRNA expression was localized in the uterine luminal and glandular epithelium as well as in the myometrium, where prolactin significantly increased uterine concentrations of LIF receptor type- β mRNA during pregnancy, suggesting a role in ending embryonic diapause and initiating embryonic development (Passavant et al., 2000).

1.3.6. Other important factors

Cyclooxygenases have recently been implicated in canine reproduction. Cyclooxygenase-2 (COX2) enzyme activates prostaglandin synthesis, thereby contributing to canine placental maturation during gestation and placental release at parturition. COX-2 mRNA expression is localized to the trophoblast surrounding the maternal vessels (Kowalewski et al., 2010) and in pre-implantation embryos (Schäfer-Somi et al., 2008), suggesting prostaglandins may regulate embryonic and endometrial cytokine activity. Similarly in the mink, COX-2 expression was present at sites of implantation at the fetomaternal interface, and it was proposed that endometrial COX-2 expression is regulated by the embryo and may contribute to implantation and

placentation in the mink (Song et al., 1998). Prostaglandins are unsaturated fatty acids that behave as locally active hormones (Drazner, 1987). Expression of prostaglandin-F₂ α (PGF₂ α) and prostaglandin-E₂ (PGE₂) has also been reported in the canine placental sites (Kowalewski et al., 2010). It is believed that trophoblasts are the main targets of these prostaglandins and are therefore controlled by prostaglandins. The mRNA expression of COX-1 in pre-implantation uterus was also detected (Schäfer-Somi et al., 2008), which leads to prostaglandin synthesis that may regulate embryonic and endometrial cytokine activity.

Peroxisome proliferator-activated receptor gamma (PPAR γ) is known to modulate biochemical and morphological placental trophoblast differentiation during implantation and placentation (Schaiff et al., 2000) by regulating steroidogenic capacity of reproductive tissues. PPAR γ is a nuclear hormone receptor of the PPAR family of transcription factors closely related to the steroid hormone receptors serving multiple roles in regulating reproductive function (Komar, 2005). PPAR γ mRNA expression has been localized in canine trophoblasts within the placenta (Kowalewski et al., 2011). The up-regulation of PPAR γ mRNA after implantation into mid-gestation (Kowalewski et al., 2011) suggests that this factor may be involved in trophoblast invasion. In the mink (*Mustela vison*), PPAR γ mRNA is also localized in the trophoblasts that directly contact the endometrium (Desmarais et al., 2007). Treatment with a prostaglandin, 15-deoxy-delta 12, 14 (15-d-PGJ₂) elicited an up-regulation in PPAR γ mRNA (Desmarais et al., 2007), suggesting that prostaglandin activity acts through the PPAR γ pathway. Also,

secretion of PGE2 was detected in mink embryos, although not during diapause (Desmarais et al., 2007).

CDs are immune cell molecules that act as cell markers on immune cells, B and T lymphocytes. T lymphocytes (T cells) of the immune system are divided into CD-4+ T-cells and CD-8+ T-cells that have both regulatory and suppressive effects. Detectable mRNA expression of CD-8 and not CD-4 was found in the early pregnancy uterus of the bitch (Schäfer-Somi et al., 2008), suggesting a possible maternal inflammatory response to embryo implantation.

1.4 ABNORMAL TROPHOBLAST INVASION AND PROLIFERATION

1.4.1 Summary

The depth of trophoblast invasion in the initiation of implantation and maintenance of pregnancy varies between species. Canine and feline trophoblasts invade into the maternal endometrium on a superficial level. In contrast, human and rodent trophoblasts are deeply invasive where they penetrate through the endometrium and into the myometrium to allow for endovascular remodeling during pregnancy (Hunkapiller and Fisher, 2008). Successful implantation requires synchronization of cellular and molecular interactions between trophoblasts and the maternal endometrium. Disruption of these interactions leads to development of placental pathologies from excessive or insufficient trophoblast proliferation and invasion. Complications in trophoblast

proliferation and invasion may not become apparent until later in pregnancy (Norwitz et al., 2001). Placenta-related pregnancy disorders affect about a third of human pregnancies (Wilcox et al., 1989). In the dog, pregnancy loss due to resorption was between 11-13% (Andersen and Simpson, 1973; Robertson et al., 1979). Embryo loss leading to stillbirth in dogs accounts for 2.2 to 4.5% incidence rate (Johnston and Raksil, 1987). Furthermore, peri-implantation failure accounts for nearly 80% of embryonic loss in farm animals (Roberts et al., 1990). The incidence, predisposing factors, etiopathophysiology, symptoms, diagnosis, and treatment of conditions caused from these pathologies will be discussed (Table 1.5).

1.4.2. Subinvolution of placental sites

Subinvolution of placental sites (SIPs) is a disorder of abnormal trophoblast invasion that causes a failure or delay of normal uterine involution in pregnant and postpartum bitches (Al-Bassum et al., 1981). The reported incidence of SIPs varies from 21% (20 out of 95) (Al Bassam et al., 1981) to 100% (9 out of 9) (Orfanou et al., 2009). SIPS is most commonly seen in primiparous bitches under 3 years old following a normal delivery (Orfanou et al., 2009). The retention of placenta in SIPs can in some cases associate with mild uterine inertia or the inability to produce effective contractions due to uterine dysfunction (Macrae, 1949). Predisposing factors to this placental retention include obesity, calcium-zinc deficiency, hypoglycemia, hypocalcemia, dystocia, uterine inertia, uterine torsion, and history of abortion (Mshelia and Chaudhari, 2001).

Table 1.5. A summary of conditions of abnormal and induced trophoblast physiology and placentation.

Conditions	Species Affected	Trophoblast Behavior	Depth of Invasion	References
Subinvolution of placental sites	<i>H. sapiens, C. domesticus</i>	↑ Invasion	Glandular layer	Al-Bassum et al., 1981
Choriocarcinoma	<i>H. sapiens, M. musculus, C. domesticus</i>	↑ Invasion & proliferation	Depends on species	Benirschke and Driscoll, 1967c; Soper, 2006
Retained placenta	<i>B. taurus, E. caballus, C. domesticus</i>	↑ Invasion	Uterine epithelium	Parer et al., 1968; Roberts, 1971; Coleman et al., 1985; Laven and Peters, 1996; Watts et al., 1997; Sevinga et al., 2004
Placenta accreta vera	<i>H. sapiens, primates</i>	↑ Invasion	Myometrium	Benirschke and Driscoll, 1967a; Miller et al., 1997
Placenta increta	<i>H. sapiens, primates</i>	↑↑ Invasion	Myometrium	Benirschke and Driscoll, 1967a
Placenta percreta	<i>H. sapiens, primates</i>	↑↑↑ Invasion	Serosa layer	Benirschke and Driscoll, 1967a
Hydatidiform mole	<i>H. sapiens</i>	↓ Differentiation	Myometrium	Benirschke and Driscoll, 1967b; Bracken et al., 1984
Placental site trophoblastic tumor	<i>H. sapiens</i>	↑ Proliferation	Myometrium	Bower et al., 1996
Early pregnancy loss	<i>All</i>	↓ Invasion	Uterine epithelium	Norwitz et al., 2001
Preeclampsia	<i>H. sapiens, primates, M. musculus</i>	↓ Invasion	Myometrium	Jauniaux et al., 2006; Zhou et al., 1997
Deciduoma	<i>M. musculus</i>	↑ Proliferation	N/A	Krehbiel, 1937

Subinvolution of the uteroplacental arteries of the placental bed was also described in humans (Andrew et al., 1989). Non-involuting vessels were present in 22 of the 25 cases where extravillous trophoblast invaded normally into the myometrium and replaced the endothelial cell lining of the vessel walls, leading to a failure in vessel involution resulting in postpartum hemorrhaging and increased maternal morbidity (Andrew et al., 1989).

The etiopathophysiology of SIPs is not clear. The evidence supports that this condition involves retained trophoblasts (Al-Bassum et al., 1981) and placenta tags at the site of implantation. The uterus continues to supply blood towards the placental tags resulting in the prolonged bleeding presented in SIPs. In normal bitches without SIPs, trophoblasts are evident in the loose connective tissue of the lamina propria two weeks postpartum (Al-Bassum et al., 1981). However in the bitches with SIPs, trophoblasts persist in the lamina propria and continue to invade abnormally deep into the glandular layer of the endometrium (Johnston et al., 2001). In some severe cases of SIPs, trophoblast invasion is deeper into the myometrium and can penetrate the serosal surface. SIPs causes a delay in normal uterine involution that is usually completed by 12 weeks postpartum (Yeager and Concannon, 1990).

The combination of excessive trophoblast invasion, damage to uterine vasculature due to this invasion, and failure of normal endometrial blood vessel thrombus formation causing secondary occlusion result in the symptoms associated with SIPs. Symptoms include a bright red sanguineous discharge ranging to severe hemorrhage from the vulva (Al-Bassam et al., 1981; Olson and Hosted, 1984). The discharge persists until after the

pups are weaned (up to 8 weeks postpartum) up until the next proestrus (Johnston et al., 2001). Cases of the severe form of SIPs with extensive blood loss are rare (Johnston et al., 2001). The chronic form of SIPs is more common with a prolonged discharge presenting in a healthy bitch (Johnston et al., 2001).

Presumptive diagnosis of SIPs is made on the basis of signalment and symptomology. Vaginal cytologic examination may reveal the presence of polynucleated, heavily vacuolated trophoblasts (Reberg et al., 1992; Sontas et al., 2011). However, Orfanou and coworkers have reported that the presence of trophoblasts in vaginal smears for up to 84 days postpartum is normal (2009), which coincides with normal uterine involution (Al Bassam et al., 1981). With transabdominal ultrasonography, the normal postpartum uterus is no longer distinguishable from the anestrus uterus by 15 weeks after whelping (Yeager and Concannon, 1990) therefore ultrasonography is not used to diagnose SIPs. Definitive diagnosis can only be made following uterine histopathology. Histologic diagnosis depends on extensive collagen sloughing on the placental site, with necrotic and hemorrhagic collagen masses, significant endometrial gland dilation, and/or presence of syncytiotrophoblast that invaded through the myometrium (Al-Bassum et al., 1981).

Treatment depends on the varying extent of hemorrhaging observed in the bitch. In a majority of the chronic cases, there is minimal discharge in an otherwise healthy bitch, which symptom resolves on its own (Schall et al., 1971; Johnston et al., 2001). Severe hemorrhaging requires surgery, commonly ovarian hysterectomy if the bitch is in good health and breeding is not an issue (Beck and McEntee, 1966). Other treatments

include hysterolaparotomy or partial removal (curettage) of the affected endometrium to preserve the reproductive ability of valuable breeding bitches. Methods to specifically treat hemorrhaging include ergonovine injection of an ergot alkaloid that may reduce bleeding in late gestation and promote uterine involution (Adams and Flowers, 1960), or blood transfusion in cases of severe blood loss (Mshelia and Chaudhari, 2001).

1.4.3. Placenta accreta

Placenta accreta is a serious condition of abnormal adherence of the placenta to the uterus (Benirschke and Driscoll, 1967a) resulting in a high rate of human maternal morbidity (60%) and mortality (7%) (Hudon et al., 1998). This condition often leads to failure of placenta separation and delivery and postpartum hemorrhaging (Benirschke and Driscoll, 1967a). Fetal complications are also increased due to difficulties during labor leading to pre-term delivery of small for gestational age babies, with 50-80% of deliveries requiring neonatal intensive care (Kent, 2009). The incidence varies from 1 in 540 to 1 in 70,000 deliveries, with an average incidence of approximately 1 in 7000 of human births (Breen et al., 1977). It should be noted that the reported incidence depends upon how the condition is defined and diagnosed, which varies greatly between studies.

Predisposing factors include history of a cesarean section or placenta previa, a condition in which the placenta forms in the lowest part of the uterus and occludes all or part of the cervix (Miller et al., 1997). Other risk factors include maternal age over 34 year, multiparity (> two children) or having multiple grandchildren, previous uterine

curettage, submucous myoma, Asherman's syndrome, smoking, chronic hypertension, and prior placenta accreta (Alanis et al., 2006; Sivan et al., 2010).

The etiopathophysiology of placenta accreta is unknown but the general physiology is agreed. Placenta accreta is an unusually firm adherence of placental (chorionic) villi to the uterus with an absence of the normally intervening decidua and fibrinoid layers of the uterine epithelium (Miller et al., 1997). Chorionic villi are trophoblastic projections formed during placentation, where fetal capillaries develop within these villi to carry fetal circulation. The placenta forms sinuses (lacunae) to surround the villi to carry the maternal circulation (Guyton and Hall, 2006). Placenta accreta has three variants: (1) placenta accreta vera, (2) placenta increta, and (3) placenta percreta. These variants are based on the depth of chorionic villi attachment and disease severity. In the most common form, placenta accreta vera, the placenta is attached (through chorionic villi) to the myometrium. Less common forms occur with placenta attachment extending into the myometrium in placenta increta, or through the entire myometrial layer in placenta percreta (Miller et al., 1997). In placenta percreta, the urinary bladder may become eroded by trophoblasts, greatly increasing maternal complications (Washecka and Behling, 2002). Placenta percreta, although rare, is the most severe form associated with excessive and possibly life-threatening hemorrhaging during and after pregnancy (Faranesh et al., 2007), and the symptoms of this can resemble the severe form of SIPs in dogs.

Two competing hypotheses of etiopathophysiology are supported: (1) placenta accreta involves an abnormal uteroplacental neovascularization related to trophoblast

invasion (Tseng et al., 2006) and/or (2) absent or defective uterine decidua (Earl et al., 1987). Immunohistochemistry identified an up-regulation of trophoblast-secreted growth, angiogenesis and invasion related factors in placenta accreta tissue (epidermal growth factor receptor (EGFR)) in syncytiotrophoblasts and up-regulation of vasculoendothelial growth factor (VEGF) and angiopoietin-2 (Ang-2) levels in human placental lysates. EGFR is known to be involved in regulating trophoblast migration (Fitzgerald et al., 2005). VEGF is a known angiogenic factor expressed in trophoblasts that regulate protein and hormone secretion (De Vries et al., 1992). Ang-2 plays a role in angiogenesis, and *in situ* hybridization studies detected Ang-2 mRNA in the cytotrophoblast of first trimester placenta, suggesting a role with trophoblast activity during early embryonic development (Wulff et al., 2002). Alternatively, it was determined that placenta accreta is not a condition of excessive trophoblast invasiveness or proliferation because immunohistochemical expression of trophoblast cell markers (CK-7, hCG, human placental lactogen (hPL) and pregnancy-specific β 1-glycoprotein (SP1)) in placenta accreta tissues were identical to those in the normal term placenta (Earl et al., 1987). It was concluded by Earl and associates (1987) that the absence of decidua was a prominent characteristic of placenta accreta.

Several diagnostic methods may be implemented to provide definitive placenta accreta diagnosis. The method of choice for presumptive diagnosis is through transabdominal ultrasonography (Alkazaleh et al., 2004), which can detect the condition as early as the first trimester (Alkazaleh et al., 2004). The gestational sac appears low-lying and attaches to the uterine scar, in which the myometrium appears thinner than

normal gestational sacs (Alkazaleh et al., 2004). Follow-up imaging in the second and third trimesters is recommended for verification. Second and third trimester placenta characteristics can be detected with three-dimensional Doppler ultrasonography, to be used in conjunction with antenatal diagnosis to detect distinctive placenta accreta architecture (numerous coherent vessels) (Shih et al., 2009). Magnetic resonance imaging (MRI) with intravenous gadolinium contrast dye can also be performed if the ultrasound findings are not definitive or if the placenta lies on the posterior uterine wall. Images detect abnormal findings, such as the placenta mass extending into the bladder, placental heterogeneity, abnormal placenta shape, absence of myometrium, or nodules within the placenta (Levine et al., 1997). Measuring elevated maternal serum biomarkers such as human chorionic gonadotropin and alpha fetoprotein is another technique for detection (Zelop et al., 1992). Definitive diagnosis is made histologically post-hysterectomy, where the chorionic villi are observed to be in direct contact with the myometrium and the decidua is absent (Benirschke and Driscoll, 1967a).

Recommended treatment for placenta accreta is immediate hysterectomy (also known as cesarean hysterectomy) after delivery without attempts to remove placenta to decrease maternal morbidity and mortality. However, this method leaves the woman sterile (Clark et al., 2008). Conservative management of placenta accreta through adjuvant methotrexate, selective arterial embolization, or intravascular balloon catheter can be effective and allow fertility preservation. Methotrexate has been proposed to speed up the postpartum involution of the placenta and therefore improve the success rate of conservative treatment (Oyelese and Smulian, 2006). There is a lack of evidence to

support the benefits of adjuvant methotrexate or selective arterial embolization treatments (Timmermans et al., 2007). Addition of a preoperative intravascular balloon catheter to treat occlusion, or arterial embolization aids in the reduction of blood loss, may be utilized. The purpose of insertion of intravascular balloon catheters is to decrease blood flow to the uterus and thereby decrease blood loss. Balloon catheters are placed either as proximal as the aorta (Salim et al., 2010) or distally within the internal iliac arteries (Dubois et al., 1997). Similarly, arterial embolization is minimally invasive, and it is performed by locally inducing an embolus (blood clot) with injection of polyvinyl alcohol (PVA) into the pelvic artery, causing fibroids (uterine growths of smooth muscle cells and fibrous connective tissue) within the circulation to stick to the vessel walls and form a clot. The blood supply is blocked and results in shrinking of the fibroids and reduction or complete cessation of symptoms (excessive uterine bleeding) (Johns Hopkins University, 2013).

1.4.4. Early pregnancy loss (miscarriage)

Miscarriage is the spontaneous loss of a pregnancy before the fetus has reached viability, including pregnancy losses from the time of conception until 24 weeks of gestation (Rai and Regan, 2006). Miscarriages can be sporadic (isolated losses) or recurrent (three or more consecutive losses) (Stirrat, 1990). The incidence of early pregnancy loss at post-implantation is high in humans, estimated at 25 to 40 percent (Wilcox et al., 1988), but occurs at less than 10% in laboratory rodents (Wilmot et al.,

1986) and other mammalian species (including primates) (Hassold, 1986). Prominent predisposing factors for miscarriage include advancing maternal and paternal age (both maternal and paternal age) with significant increase in risk after 35 years (De La Rochebrochard and Thonneau, 2002), and prior history of spontaneous abortion (Leridon, 1976; Regan et al., 1989; Fretts et al., 1995; Andersen et al., 2000). Recent studies have included obesity (Lashen et al., 2004) as well as maternal consumption of tobacco, maternal psychological problems, interval between pregnancies, previous use of the contraceptive pill (Risch et al., 1988; Dominguez et al., 1991) or paternal exposure to toxins (Lindbohm et al., 1991).

Genetic abnormalities contribute to a majority of early pregnancy losses (Simpson, 1980), estimating between 50-60% of all miscarriages are due to cytogenic abnormalities (trisomy, polyploidy, monosomy X). Other than genetic effects, there is often no known cause (Stirrat, 1990). With the complexity of early development, it is likely that other mechanisms during implantation and placentation contribute to early pregnancy loss (Norwitz et al., 2001; Jauniaux et al., 2006). In about two-thirds of early pregnancy failures, there is anatomical evidence of defective placentation (Burton et al., 1999). Mounting evidence supports that miscarriages are a placentation disorder of oxidative stress that result in the chorionic villous changes (Jauniaux et al., 2006). Studies support that abnormal changes in villous blood flow result from a thinner and fragmented trophoblast layer, reduced cytotrophoblast invasion into the maternal endometrium, and incomplete plugging of the lumen at the tips of the spiral arteries established soon after implantation (Hustin and Schaaps, 1987; Burton et al., 1999). These characteristics are

associated with the absence of spiral artery remodeling by trophoblasts and lead to a premature onset of the maternal circulation throughout the placenta. The presence of excessive maternal blood in the intervillous space throughout the placenta causes villous tissue to become trapped within large intervillous blood thrombi (blood clots), and also leads to O₂-mediated trophoblastic damage (oxidative degeneration), and increased apoptosis due to the abnormally high or rapidly fluctuating concentrations of O₂ harming the early villous tissue (Kokawa et al., 1998; Hempstock et al., 2003; Jauniaux et al., 2003). Ultimately, this results in placental degeneration with complete loss of syncytiotrophoblast function and detachment of the placenta from the uterine wall (Hustin and Schaaps, 1987). Further, there is also evidence that levels and profiles of circulating cytokines in the decidua are different in women who experience recurrent miscarriages (Jenkins et al., 2000; Makhseed et al., 2000; Baxter et al., 2001), but the mechanism between cytokines and invading trophoblasts is not understood.

Several symptoms characterize early pregnancy loss. A majority of pregnancy loss occurs in the first trimester before 12 weeks of gestation and less than 5% occur after detection of fetal heart activity (Brigham et al., 1999). Loss in the second trimester (12 to 24 weeks) occurs in below 4% of pregnancy outcomes (Ugwumadu et al., 2003). Early pregnancy loss can be categorized into two groups: (1) spontaneous abortions and (2) perinatal deaths. Both involve early pregnancy loss of the conceptus and both have overlapping etiopathophysiology and pre-disposing factors as mentioned above. The groups differ in time of expression, where spontaneous abortions are pre-viable losses or

stillbirths occurring during pregnancy, whereas perinatal death of the conceptus occurs soon after delivery (Benirschke and Driscoll, 1967a).

Diagnosis of early pregnancy loss can be detected through ultrasound. After initial ultrasound identification of pregnancy, ultrasonography will identify an absence of an identifiable pregnancy (fetal heartbeat, bone formation), in combination with a positive urine or serum human chorionic gonadotropin (HCG) pregnancy test. Miscarriage can be classified as early or late miscarriage (before or after 12 weeks). Mid-trimester loss seen in recurrent miscarriage has also been cited to be preceded by a diagnosis of cervical incompetence, based on a history of late miscarriage due to spontaneous rupture of membranes or painless cervical dilatation (Drakeley et al., 1998). Treatment of miscarriages includes: treatment of coexistent thrombophilic disorders, bacterial vaginosis infection, hyperinsulinaemia (insulin resistance), hyperprolactinaemia, and cerclage with prophylactic antibiotics (Rai and Regan, 2006).

1.4.5. Preeclampsia

Preeclampsia is a lesser dysfunction of shallow invasion of trophoblasts compared to miscarriage. In preeclampsia, trophoblast invasion is insufficient to completely convert the spiral arteries into low-resistance vessels (Jauniaux et al., 2006). This shallow trophoblast condition occurs predominantly in humans, although symptoms associated with preeclampsia (hypertension and/or proteinuria) have also spontaneously occurred in patas monkeys (Palmer et al., 1979) and guinea pigs (Seidl et al., 1979). Preeclampsia is

one of the most common diseases of human pregnancy, affecting 5–8% of pregnant women (Saftlas et al., 1990), accounting for nearly 18% maternal deaths in the United States (American College of Obstetricians and Gynecologists, 2004). Preeclampsia is also a leading cause of pre-term delivery and infants who are small for gestational age, increasing perinatal mortality by five-fold (Norwitz et al., 2001).

Predisposing factors for preeclampsia include maternal health disposition as well as personal and family history. Factors with significant association with preeclampsia include: maternal age, maternal birth weight/BMI, primiparity, multiple pregnancy, more than 10 years between pregnancies, underlying renal disease or chronic hypertension, mean arterial blood pressure, personal history of preeclampsia, family history of preeclampsia and family history of coronary heart disease (Sibai et al., 1995).

The cause of preeclampsia is unknown, though it is generally believed that the condition is associated with impaired, shallow extravillous interstitial trophoblast invasion and limited endovascular trophoblast invasion. Zhou and associates (1997) observed that preeclamptic extravillous trophoblast failed to express endothelial phenotypic markers (cadherins, VECAM-2, integrins) within uterine vessels. This failure to acquire the vascular phenotype of adhesion molecules impairs trophoblast invasion, resulting in high-resistance uterine arteries that cannot respond to the increasing fetal demands for blood flow (Zhou et al., 1997). The reduced blood supply to the fetus leads to fetal prematurity, fetal growth restriction, and stillbirth (Moffett and Loke, 2006). Another competing hypothesis suggests that preeclampsia is a multifactorial condition of the maladaptation and impaired invasion of uteroplacental arteries resulting from both

trophoblast dysfunction and abnormal expression of maternal uterine factors involved with uteroplacental arteries (Kaufmann et al., 2003).

Symptoms associated with shallow trophoblast invasion are presented as hypertension (blood pressure greater than 140 mmHg) or high diastolic blood pressure (greater than 90 mmHg), with elevated protein levels in urine (proteinuria) with greater than 300 mg to characterize the mild form of preeclampsia. Severe preeclampsia symptoms are more even higher levels of blood pressure and proteinuria, and sometimes organ dysfunction (American College of Obstetricians and Gynecologists, 2004).

Initial diagnosis of preeclampsia includes evaluation of medical history, physical examination, and laboratory testing (Wagner, 2004). A complete history should be taken to assess increased risk for preeclampsia, such as diabetes mellitus, hypertension, vascular and connective tissue disease, nephropathy, and antiphospholipid antibody syndrome. Physical exams measure blood pressure and fundal height because size less than dates may indicate intrauterine growth retardation sometimes associated with preeclampsia. Laboratory tests include a hepatic enzyme level, a platelet count, a serum creatinine level, and urine collection for protein levels (Wagner, 2004). Definitive diagnosis is made histologically after vaginal delivery or c-section, where limited trophoblast invasion is detected (Zhou et al., 1997).

Delivery of the placenta is the ultimate treatment for preeclampsia to fully resolve the condition (American College of Obstetricians and Gynecologists, 2004). Delivery may need to occur before a viable fetus can be delivered if the mother's life is threatened. To treat severe preeclampsia, magnesium sulfate is used to prevent eclamptic seizures

(Witlin and Sibai, 1998). Further treatments under investigation for prevention of preeclampsia include aspirin (Sibai, 2003), calcium (Atallah et al., 2004), and antioxidant supplementation (Chappell et al., 1999).

1.4.6. Hydatidiform mole

Hydatidiform mole, also referred to as molar pregnancy, is a disease of the trophoblast causing placental lesions. Morphologically, hydatidiform moles are abnormal placentas with swollen and vesicular villi formation, producing grape-like cysts (hydatidiforms) throughout the placenta, replacing the placental tissue. These masses are distorted villi formed into chains and clusters of vesicular structures filled with fluid and connected by branching stalks (Benirschke and Driscoll, 1967b). Histologically, there is hydropic expansion of the vilous stroma (edema, cystic degeneration) with abnormally actively hyperplastic trophoblast proliferation. The villous clusters cause swelling of the villous branches interconnecting the clusters, where increased trophoblast proliferation produces increased masses of syncytiotrophoblast and cytotrophoblasts (Benirschke and Driscoll, 1967b). The swollen villi often contain necrotic trophoblasts.

Hydatidiform moles are part of a group of gestational trophoblastic diseases (GTDs) to include choriocarcinoma and placental site trophoblastic tumors. GTDs are unique because they represent semiallografts, which are conditions derived from the fetus and expressed in the mother (Shih and Kurman, 2002). The relation between molar pregnancy and choriocarcinoma is unclear, where it may be confused whether they are

separate diseases or a continuation of the same disease. Hydatidiform mole is the major risk factor for choriocarcinoma (Bracken et al., 1984), where the incidence of molar pregnancy advancing into an invasive mole (choriocarcinoma) is 7 - 17% (Ichinoe, 1979), although both have distinct biological characteristics. The incidence of molar pregnancy in the United States is 1 in 923 pregnancies (Hayashi et al., 1982). Accurate rates of molar pregnancies are difficult to obtain because 86% of spontaneous abortions (miscarriages) were determined to be hydatidiform mole with triploidy cytogenetic makeup (Szulman et al., 1981).

Pre-disposing factors increasing the risk of molar pregnancy include women born outside North America, maternal age (greater than 30 years), and mothers who followed diets deficient in carotene (Berkowitz et al., 1985). Geographic distribution is a major factor, where incidences of molar pregnancy occur at much higher rate in Indonesia (11.5 per 1,000 deliveries) (Poeh and Djojopranoto, 1965) than the United States (1 per 923 deliveries) (Hayashi et al., 1982).

Hydatidiform mole is classified into two morphological categories: (1) complete (CHM) or (2) partial hydatidiform mole (PHM). In normal pregnancy, as the placenta develops from the trophoblast, the chorionic villi develop. Chorionic villi are the functional and structural region of the placenta that develops within the lacunar spaces among the syncytiotrophoblast (Kovacs and Ojeda, 2012). In molar pregnancy, chorionic villi are unable to undergo vascularization to develop into secondary or tertiary villi, rather, the villi become cystic and become filled with fluid, to form small grape-like hydatidiform structures (Soper, 2006; Kovacs and Ojeda, 2012). Complete molar

pregnancy is usually 46XX genetic composition and involves a conceptus where all chorionic villi are abnormal with cyst-like swelling of chorionic villi and fluid accumulation in the mesenchymal layer with absence of an embryo, umbilical cord, or amniotic membranes. Partial molar pregnancy is usually a triploidy chromosomal constitution and has some normal chorionic villi with fetal vasculature and presence of a fetus, umbilical cord, and amniotic membranes (Vassilakos et al., 1977).

The genetic etiopathophysiology of molar development is unclear, although some aspects are agreed. More than 80% of hydatidiform moles are female (Vassilakos et al., 1977), therefore the androgenetic 46XX karyotype contributing to complete molar pregnancy has three possible modes of fertilization: (1) dispermy, (2) diploid sperm, or (3) fertilization with a normal haploid spermatozoan that replicates in the ovum to produce 46XX diploidy (Bracken et al., 1984). Molar tissue has a 46, XX karyotype derived from the father that represents the reduplication of the sperm's haploid genome and the absence of the maternal chromosome complement. With this condition, the fetal development and vasculature is absent because the fetus resorbs before the circulatory system develops. Partial moles have predominantly triploidy chromosome constitution and are almost always dispermic (Bracken et al., 1984).

Symptoms of molar pregnancy include painless vaginal bleeding (spotting to profuse hemorrhage), uterine enlargement greater than expected for gestational age, hyperemesis, and hypertension (Soper, 2006). CHM are usually diagnosed in the first half of pregnancy via transabdominal ultrasonography in combination with increased hCG for gestational age.

Hydatidiform mole is diagnosed by transabdominal ultrasonography (Kohorn and Blackwell, 1968). Detection of major clinical signs include an enlarged uterus with absence of fetus and fetal heartbeat. Additionally, high levels of hCG in the blood or urine is indicative of the condition. Delivery of the placenta and histological examination (presence of enlarged, cyst-like villi with/without fetus) allows for definitive diagnosis. Before ultrasound was developed for diagnosis, moles were often spontaneously aborted, which led to extensive maternal hemorrhage (Benirschke and Driscoll, 1967b).

Treatment for CHM (complete hydatidiform mole) requires dilation and evacuation of the placenta. Hysterectomy and labor induction are not recommended because they promote increased blood loss and may predispose towards choriocarcinoma or placental site trophoblastic tumors following treatment (Soper, 2006).

1.4.7. Choriocarcinoma

Choriocarcinoma is another disease of the trophoblast that produces a highly malignant tumor arising from chorionic epithelium during pregnancy formed by excessive proliferation of syncytiotrophoblast and cytotrophoblast without chorionic villi (Soper, 2006). Nongestational choriocarcinoma occurs in which tumors can arise from gonads or extragonadal embryonic rest cells (Bracken et al., 1984). Pathologically, choriocarcinoma is characterized with malignant syncytiotrophoblasts with the absence of chorionic villi and where syncytiotrophoblasts have invaded through the endometrium and into the myometrium (Brewer et al., 1978), though invasion can also metastasize to

any site in the body (metastasis commonly described in the liver, brain and lung) (Bracken et al., 1984). Choriocarcinomas often develop early systemic metastases to the nervous system and lungs. Abnormal bleeding occurs at the metastatic sites. Choriocarcinoma has been described in humans (Novak, 1950), rodents (spontaneous choriocarcinoma) (Pirak et al., 1991) and dogs (primary gastric choriocarcinoma) (Poutahidis et al., 2008). Incidence rates of choriocarcinoma vary between countries where it is as low as 1 in 40,000 pregnancies in America (Hertig and Mansell, 1956) or 1 in 500 pregnancies in the Philippines (Acosta-Sison, 1964).

The main predisposing factor is hydatidiform mole, where in Japan 80% of moles progressed to choriocarcinoma (Tomoda, 1979). Other factors are Asian, African or Central American descent, advanced maternal age above 40 years, multiparity, and prior history of abortion (Bracken et al., 1984).

The etiopathophysiology of choriocarcinoma is not well described. However, evidence supports that the complete evacuation of molar pregnancy may lead to subsequent choriocarcinoma (Stone and Bagshawe, 1979). A study found that there was lower incidence of persistent trophoblast invasion when the pregnancy was removed through curettage or vacuum aspiration rather than hysterectomy or hysterotomy (Stone and Bagshawe, 1979). This hypothesis of excessive trophoblast invasion through the myometrium and even metastasizing into surrounding tissues, is similar to SIPs where the remaining placental tags after parturition lead to trophoblasts invading abnormally deep into the glandular tissue.

Symptoms of choriocarcinoma include vaginal bleeding occurring after pregnancy

and uterine subinvolution (Benirschke and Driscoll, 1967c). Also, spontaneous uterine rupture and invasion of adjacent tissues and organs are normal sequelae of this condition (Wilson et al., 1965). These symptoms may be comparable to SIPs in dogs.

Measurement of a serum hCG and exclusion of normal pregnancy are all that are required to diagnose choriocarcinoma because of the hCG-producing tumors distinct to the condition (Soper, 2006).

Treatments after diagnosis, include chemotherapy with primarily methotrexate (antimetabolite drug that inhibits proliferation of carcinoma cells) (Kim et al., 1986) or follow-up treatment of dactinomycin (antibiotic that inhibits/stops proliferation of carcinoma cells) (Limpongsanurak, 2001) in high risk molar pregnancies leads to a reduction of choriocarcinoma rates. These malignancies are highly responsive and it is often possible to cure them while preserving the woman's reproductive function (Soper et al., 1996). The majority of women (93%) are able to successfully conceive after chemotherapy (Hertz et al., 1964).

1.4.8. Placental Site Trophoblastic Tumor

Placental site trophoblastic tumors (PSTT) are rare tumors of the trophoblast. Histologically, this condition is presented as a cytotrophoblast proliferation and differentiation, relatively low hCG levels, little or no syncytiotrophoblast, and complete absence of chorionic villi within the tumor (Chang et al., 1999). PSTT can occur locally then spread via the lymphatics (Bower et al., 1996). Although PSTT occurs at a much

lower incidence (1.2% of pregnancies (Dunn, 1959)) than hydatidiform mole or gestational choriocarcinoma, PSTT can develop after any type of pregnancy (normal or molar pregnancy and abortion) (Soper, 2006) and has malignant potential (Twiggs et al., 1981).

Pre-disposing factors for PSTT are widely varied, though a common factor is an extended interval between the prior pregnancy and the presentation or diagnosis of PSTT. Intervals vary between 12 months to 15 years, with a median of 24 months, but this prolonged interval between pregnancy and diagnosis has been supported by several studies (Bower et al., 1996; Finkler et al., 1988; How et al., 1995).

Etiopathophysiology of PSTT is not well described, although a recent study supports the genetic origin of this condition (Oldt et al., 2002). The origin of PSTT has been localized to the extravillous trophoblast in a recent study of molecular genetics (Oldt et al., 2002). This study confirmed that the paternal contribution of the Y chromosome and paternal alleles contribute to PSTT, therefore it was concluded that PSTT has a trophoblast origin. Also, immunohistochemical studies detected a strong expression of HLA-G (human trophoblast marker) in extravillous trophoblast and fetal thymus to further suggest that PSTT is derived from extravillous trophoblast (Singer et al., 2002). Histologically, tumor cells of PSTT express the same markers and share a similar phenotype to extravillous trophoblasts in the implantation site, where their pattern of invasion into the myometrium in PSTT is similar to that of normal extravillous trophoblast (Shih and Kurman, 2002). These recent findings support the notion that

dysfunctional cytotrophoblast behavior may lead to maldevelopment of syncytiotrophoblast and chorionic villi in the placenta.

Presentation of this condition varies among individuals. Common symptoms of PSTT are abnormal vaginal bleeding with or without amenorrhea (loss of menstruation) (Bower et al., 1996).

Diagnosis can be performed with tumor biopsy, although the results are variable depending upon site of sample origin. With the biopsied tumor samples, PSTT shows a typical immunohistochemical staining of high proportion of hPL-positive cells and relatively few positive for hCG (Kurman et al., 1984). With very low hCG levels secreted by these tumors, hCG testing is not a reliable method of diagnosis (Gillespie et al., 2000).

Unlike choriocarcinomas, PSTT are not sensitive to chemotherapy. Since the tumors are usually confined to the uterus, hysterectomy is the preferred method of treatment (Soper, 2006). The cure rate for low-risk, non-metastatic PSTT approaches 100% (American College of Obstetricians and Gynecologists, 2004) with recurrence rates less than 5% (Mutch et al., 1990).

1.4.9. Other Conditions of the Placenta

A number of additional conditions of placentation occur in the literature, however retained placenta and decidualoma are two which will be discussed in brief.

Retained placenta has been described in bovine as the failure of placenta expulsion 12 hours postpartum (Roberts, 1971) and may result in systemic illness,

metritis, and infertility (Coleman et al., 1985). This condition has been described most commonly in bovine (Laven and Peters, 1996), as dogs (Watts et al., 1997), horses (Sevinga et al., 2004), and rhesus monkeys (Parer et al., 1968). In a study of nearly 7,400 calvings, 10% presented with retained placenta (Moller et al., 1967). Several predisposing factors including age, species, heredity, environment, hormones, and nutrition have been suggested as causes of RP (Hurley and Doane, 1989), although evidence is conflicting. The etiopathophysiology of retained placenta is unknown, however, the common physiology of this condition includes failure of the villi of the fetal cotyledons (fetal portion of the placenta) to separate from the crypts of the maternal caruncle (maternal portion of the placenta) at the placenta attachment sites (Roberts, 1971). Studies support a link between the decreased leukocyte chemotaxis towards the maternal cotyledon, where leukocytes obtained from cows with retained placenta were less able to recognize cotyledon tissue in a chemotaxis assay than leukocytes from normal cows (Gunnink, 1984). This study concluded that retained placenta is a condition resulting from a failure of maternal recognition of the fetus, resulting in a maternal immunological attack on the fetal placenta as a foreign body. The failure or placental expulsion may be comparable to placenta accreta in humans involving the abnormal adhesion of placenta to the uterine wall. Symptoms are presented during parturition, to include dystocia and metritis (Roberts, 1971). Diagnosis of retained placenta involves evaluating the prior history of metritis, endometritis and pyometra, which have been associated with retained placenta, as well as dystocia and abortion (Tennant and Peddicord, 1968). Treatment methods include monitoring of appetite, milk production,

and/or daily rectal temperature readings postpartum, intramuscular penicillin/streptomycin injection for temperatures exceeding 39°C. Treatment by the veterinarian was given for cows showing signs of systemic illness and parenteral antibiotics were given primarily before manual removal of placenta (Sandals et al., 1979).

Deciduoma is an induced condition characterized by a decidual response of massive decidual cell proliferation and development-induced mechanical stimulation to produce uterine progesterone in the absence of fertilized ova (De Feo, 1963). This endometrial response can be used as a tool to investigate factors involved in the decidual response to blastocyst implantation because it is histologically and hormonally similar to implantation (Krehbiel, 1937) and the subsequent decidualization (transformation of uterine mucosa into decidua) seen in humans and rodents with hemochorial placentation. Deciduoma has been described in rodents (Finn and Keen, 1963) and canines (Nomura, 1994). Shelesnyak (1952) was the first to observe that histamine injection into the uterine lumen initiated deciduoma in rats. Another study observed a significant initiation of deciduoma in response to intrauterine injections of oils, sulphated polysaccharides, agar, carrageenin, and heparin. These are involved in tissue formation, repair and remodeling (Finn and Keen, 1963). In a study by Nomura (1994), canine deciduoma could be induced by adding a silk suture into the left uterine lumen during the functional luteal stage (early to mid-diestrus) and not the regressive stage. It was proposed that deciduoma is dependent on the corpora lutea as seen in rodents. Therefore, it can be proposed that the corpora lutea is involved in decidualization and preparation of the uterus for implantation and pregnancy.

1.4.10. Conclusion

Conditions resulting from excessive trophoblast invasion occur in dogs (SIPs) and humans (placenta accreta, choriocarcinoma), where conditions resulting from insufficient trophoblast invasion (miscarriage, preeclampsia) or excessive trophoblast proliferation (hydatidiform mole, choriocarcinoma, PSTT) have been described in humans, rats and dogs. SIPs (canines) and choriocarcinoma (humans) as well as retained placenta (bovine, canine, equine, murine) and placenta accreta (humans) share similar symptomatic presentation and possibly similar etiopathophysiology, suggesting that these trophoblast conditions can be translated among species. Each condition can lead to maternal morbidity and mortality, although some are more life-threatening than others. Finding animal models or *in vitro* methods to investigate these pathologies is essential for developing preventative strategies.

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CHAPTER II

ROLE OF IL-8 AND TIMP-2 ON TERM CANINE TROPHOBLAST MIGRATION, INVASION, AND PROLIFERATION

2.1 INTRODUCTION

Complications during pregnancy and postpartum can be due to abnormal trophoblast behavior later in gestation. Successful release of the placenta requires synchronization of cellular and molecular interactions between trophoblasts and the maternal endometrium (Norwitz et al., 2001). Disruption of these interactions can lead to the development of placental pathologies, which can result from abnormal trophoblast migration, invasion, or proliferation. Excessive trophoblast invasion causes a failure or delay of normal uterine involution in pregnant and postpartum dogs (Al-Bassam et al., 1981), known as subinvolution of placental sites (SIPs). Choriocarcinoma is another condition of excessive trophoblast or tumor invasion that results in metastasis to any site in the body has been described in humans (Novak, 1950; Bracken et al., 1984), rodents (Pirak et al., 1991), and dogs (Poutahidis et al., 2008). Knowledge of normal late gestation canine trophoblast physiology will provide a basis for future research on these types of placenta-related disorders.

Matrix-metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are key regulators in signaling at the trophoblast-endometrial interface. The MMP-TIMP interaction controls trophoblast invasion by restricting over or under-invasion into the

endometrium (Staun-Ram and Shalev, 2005). MMPs are zinc-dependent endopeptidases important in the degradation of the maternal endometrium (Bischof et al., 2000). TIMP-2, which preferentially binds and inhibits MMP-2, is a 21.8 kDa protein that has been documented in the primate trophoblast and endometrium (Bischof et al, 2000). Gene expression of MMP-2 was localized in canine trophoblast and fetal blood vessels at the time of implantation and high levels of MMP-2 expression was observed in the endometrium and myometrium from pre-implantation to term (Beceriklisoy et al., 2007; Fellows et al., 2012). On the other hand, TIMP-2 mRNA expression had a notable yet insignificant increase from pre-term to parturient stage (Fellows et al., 2012), suggesting that TIMP-2 has a role at the end of gestation to restrict additional trophoblast invasion in dogs. However, the function of TIMP-2 in late gestation trophoblasts has not been evaluated.

Interleukins and growth factors of the cytokine family are dependent on MMP activity (Van Mourik et al., 2009). Interleukin-8 (IL-8/CXCL8), an 8 kDa inflammatory chemokine, mediates its effect through the IL-8 receptors, CXCR1 and CXCR2 (Leong et al., 1997). Human trophoblasts produce IL-8 (Shimoya et al., 1992) and CXCR1 (Hanna et al., 2006; Jovanović et al., 2010) and IL-8 stimulates first trimester human trophoblast proliferation in vitro (Hirota et al., 2009; Jovanović et al., 2010). In addition, IL-8 stimulates first trimester human trophoblast migration and invasion through up-regulation of MMP-2 (Jovanović et al., 2010). IL-8 is expressed in canine trophoblasts of the pre-implantation embryo (Schäfer-Somi et al., 2008), but its role in late gestation has not been described.

The objective of this study was to determine the effect of IL-8 and TIMP-2 on term canine trophoblast (TCT) migration, invasion, and proliferation. We hypothesized that TCT would respond similarly to term human trophoblasts in vitro, where IL-8 stimulates TCT migration, invasion, and proliferation, and TIMP-2 suppresses TCT migration and invasion.

2.2 MATERIALS AND METHODS

2.2.1 Reagents and factors

The cell isolation medium was prepared with 95% Dulbecco's Modified Eagle's Medium (DMEM) obtained from Gibco, Life Technologies (Grand Island, NY, USA) with 2.5% FBS (Gibco, Life Technologies), 1% Glutamine Plus obtained from Atlanta Biologicals (Lawrenceville, GA, USA), 1% Penicillin/Streptomycin (Life Technologies), and 0.5% Gentamycin (Life Technologies). The collagenase solution was prepared with 99.7% 1X phosphate buffer saline (PBS) (Gibco), 0.062% collagenase obtained from Sigma (St. Louis, MO, USA), 0.04% DNase (Sigma), 0.069% hyaluronidase (Sigma), and 0.1% bovine serum albumin (Sigma). The trypsin solution was prepared with 99.9% PBS (Gibco), 0.0069% trypsin (Sigma), 0.04% DNase (Sigma), and ethylenediaminetetraacetic acid (EDTA, Sigma).

The cell culture medium for the migration assay (medium A) consisted of 86.5% DMEM (Gibco, Life Technologies) with 1% HEPES (Life Technologies), 1% Glutamine

Plus (Atlanta Biologicals), 1% Penicillin/Streptomycin (Life Technologies), 0.5% Gentamycin (Life Technologies), and 10% FBS (Gibco).

The cell culture medium for the invasion assay and proliferation assay (medium B) consisted of 96.5% DMEM (Gibco) with 1% HEPES (Life Technologies), 1% Glutamine Plus (Atlanta Biologicals), 1% Penicillin/Streptomycin (Life Technologies), and 0.5% Gentamycin (Life Technologies). The chemoattractant for the invasion assay was the same as the cell culture medium with the addition of 10% FBS (Gibco). For the proliferation assay, MTT (((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/ml MTT in RPMI-1640 without phenol red) and 1-propanol (0.1 N HCl in anhydrous isopropanol) of the Cell Growth Determination Kit MTT-based were obtained from Sigma.

Recombinant human IL-8 was obtained from Peprotech (Rocky Hill, NJ, USA) and was used at a concentration of 10 ng/ml for each assay. Recombinant human TIMP-2 (Peprotech) was used at a concentration of 0.5 µg/ml for each assay, respectively.

2.2.2 Tissue collection

Term canine placentas were collected following elective C-section at the time of parturition (progesterone < 2.5 ng/ml) but prior to the onset of first stage labor (n=5 dogs). Following tissue collection, the villous chorioallantois was dissected away from adjacent placental tissues. Villous chorioallantois was minced and washed in cell

isolation medium by centrifugation to be further processed for primary trophoblast isolation.

2.2.3 Cell isolation and culture

Term canine trophoblasts (TCT) were isolated from the villous chorioallantois as previously described (Sahlfeld et al., 2012). Briefly, following serial collagenase and trypsin tissue digestions, cells were separated by Percoll density gradient centrifugation. The trophoblast-containing gradient layer was washed twice and resuspended in cell culture medium. The cell culture conditions were the same for all assays at 37 °C in 5% CO₂.

2.2.4 Migration assay

The migration (wound healing) assay was performed as previously described (Liang et al., 2007). Briefly, primary TCT (1000×10^3 cells/well) were suspended in 2 ml of medium A and cultured in 12-well tissue culture plates. Cells were grown to form a 70-80% confluent monolayer. A wound was added with a sterile 200 μ l pipette tip. The monolayer was rinsed with medium A to remove loosened cells. Medium A with no factor (control), with IL-8 or TIMP-2 was then added. Photomicrographs of the wound were taken with phase-contrast microscopy (100X magnification) after 8 h in culture

(Fig. 2.1). The area of the wound was measured using ImageJ v.1.34 software (Rasband, 2012). The experiment was performed in triplicate.

2.2.5 Invasion assay

An invasion (Matrigel) assay was performed as previously described (Librach et al., 1991) using Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA). Briefly, primary TCT (250×10^3 cells/well) were suspended in 0.5 ml medium B with no factor (control), with IL-8 or TIMP-2 and then cultured on Matrigel-coated filter membranes for 22 h. Non-invading cells were removed from the filter and the filters were then fixed and stained with DiffQuik (Harleco®, EMD Chemicals, Inc., Gibbstown, NJ, USA). Cells from ten randomly selected non-overlapping fields were counted under light microscopy (400X magnification). The experiment was performed in triplicate.

2.2.6 Proliferation assay

The proliferation (MTT) assay (Sigma), was performed as previously described (Carmichael et al., 1987). Primary TCT (100×10^3 cells/well) were suspended in 100 μ l medium B with no factor (control), with IL-8 or TIMP-2 and cultured in 96-well tissue culture plates for 28 h. MTT (10 μ l) was added to each well for 4 h. The MTT and medium B was replaced with 100 μ L 1-propanol and the plates were vigorously shaken for 5 minutes using a microplate reader (SpectraMax 190, Molecular Devices, LLC,

Sunnyvale, CA, USA), absorbances were measured at 570 nm and 690 nm.

Measurements were analyzed with SoftMax Pro 5.2 program SoftMax® Pro Data Acquisition & Analysis Software (Molecular Devices, LLC, Sunnyvale, CA). The experiment was performed in quadruplicate.

2.2.7 Statistical analysis

The data were analyzed statistically by Repeated Measures, One-Way or Two-Way ANOVA in PROC MIXED using SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA) for each assay, respectively. For the migration assay, the mean of the control wound area at 0 h was set to 0% wound closure and the data were expressed as the percent wound closure of the control for each dog. For the invasion assay and proliferation assay, the mean of the controls was set to 100% and the data was expressed as the percentage of the control for each dog. For the proliferation assay, measurements at 690 nm were subtracted from measurements at 570 nm to account for background. Data were expressed as mean \pm SEM and significance was defined as $P < 0.05$.

2.3 RESULTS

Representative fields for the migration assay are illustrated in Figure 2.1. For the migration assay, IL-8 increased cell migration by 35% compared to the control ($P=0.003$; Figure 2.2). TIMP-2 had no significant effect on cell migration ($P=0.38$; Figure 2.2).

Figure 2.1. Representative wells illustrating migration after 0 and 8 hours in culture for the control (C; no IL-8 or TIMP-2 treatment), IL-8-treated, and TIMP-2-treated primary term canine trophoblast cells (phase contrast;100X magnification).

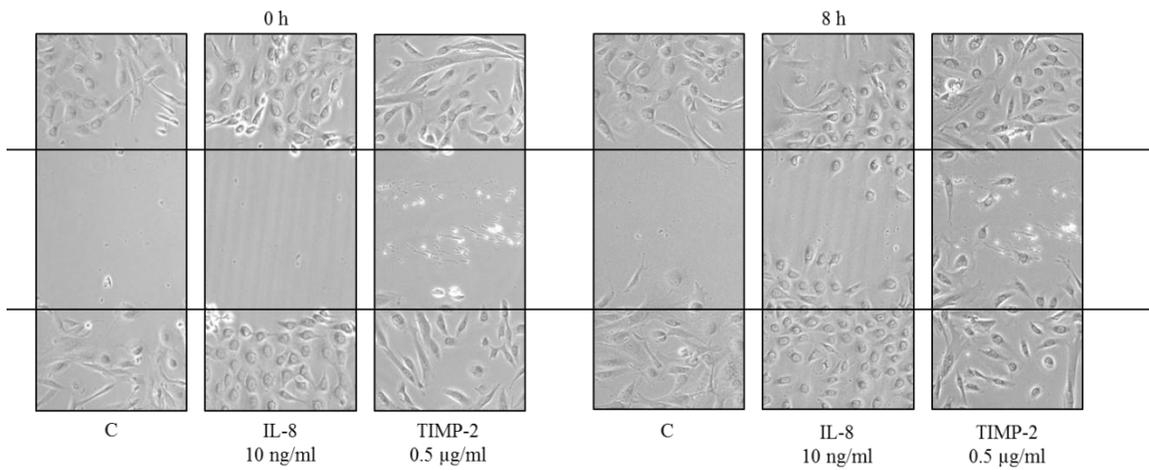
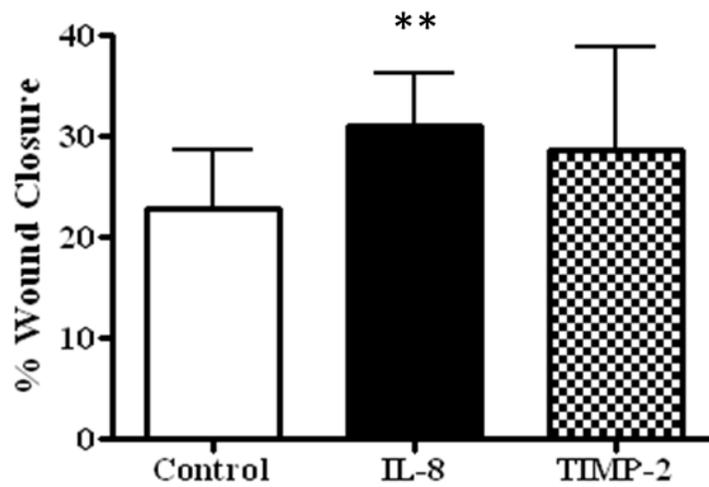


Figure 2.2. The effect of IL-8 and TIMP-2 on primary term canine trophoblast cell migration (mean \pm SEM). IL-8 increased cell migration by 35% compared to no treatment (C; $**P<0.01$). TIMP-2 had no significant effect on cell migration (wound closure) ($P=0.38$). Experiments were performed in triplicate ($n=5$ dogs).



Representative fields for the invasion assay are illustrated in Figure 2.3. There was no significant effect of IL-8 on cell invasion compared to the control ($P=0.42$; Figure 2. 4). On the other hand, TIMP-2 decreased cell invasion by 57% compared to the control ($P=0.03$; Figure 2.4). For the proliferation assay, IL-8 had no significant effect on cell proliferation ($P=0.18$; Figure 2.5).

2.4 DISCUSSION

In vitro canine trophoblast physiology has not been previously described.

Therefore, the objective of this study was to characterize normal *in vitro* term canine trophoblast (TCT) physiology using migration, invasion, and proliferation assays. In addition, the objective was to investigate the effect of IL-8 and TIMP-2 on these behaviors. Concentrations selected for IL-8 and TIMP-2 were based on dosages found to elicit maximum effects in previous studies with human trophoblasts (Librach et al., 1991; Jovanović et al., 2010).

In the current study, IL-8 increases TCT migration, which is similar to observations in first trimester human HTR-8/SVneo trophoblasts where IL-8 increases cell migration (Jovanović et al., 2010). Studies have also shown that IL-8 increases first trimester human trophoblast invasion and proliferation (Hanna et al., 2006; Jovanović et al., 2010). In addition, the application of IL-8-neutralizing antibodies reduces primary first trimester human trophoblast invasion (Hanna et al., 2006). However in the current study, IL-8 did not increase invasion and proliferation in primary TCT. Although TCT do retain invasive capacity, it is important to note that human trophoblasts do not retain any

Figure 2.3. Representative fields of cell invasion for control (A), IL-8-treated (10 ng/ml, B), and TIMP-2-treated primary term canine trophoblast cells (0.5 μ g/ml, C). Arrows indicate the trophoblast nuclei and arrowheads indicate the 8 μ m filter membrane pores (Diff-Quik staining; 400X magnification, scale bar=20 μ m).

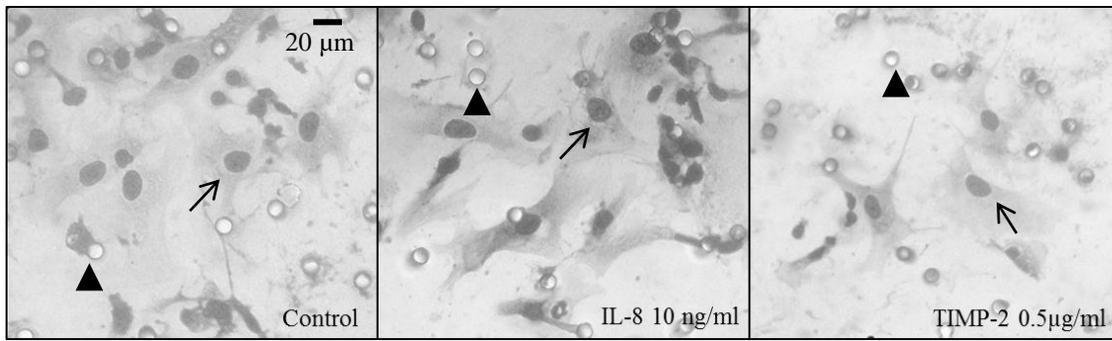


Figure 2.4. The effect of IL-8 and TIMP-2 on primary term canine trophoblast cell invasion (mean \pm SEM). There was no significant effect of IL-8 (10 ng/ml) on invasion compared to the no treatment (C; $P=0.42$). TIMP-2 (0.5 $\mu\text{g/ml}$) decreased cell invasion by 57% compared to the control ($*P<0.05$). Experiments were performed in triplicate (n=5 dogs).

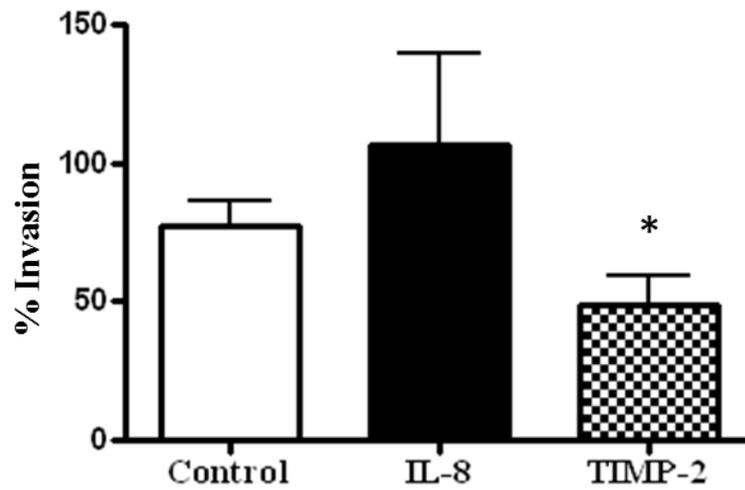
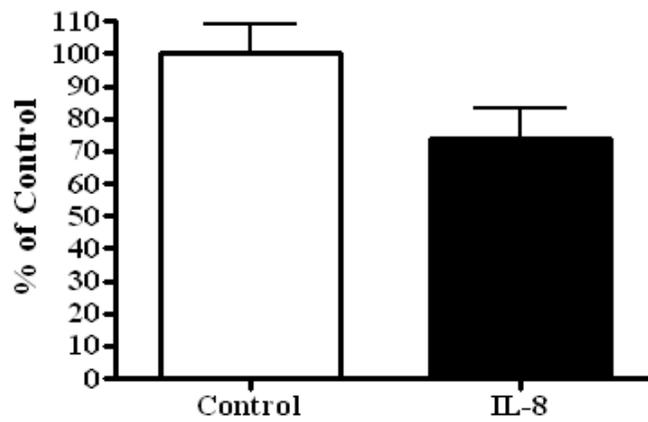


Figure 2.5. IL-8 (10 ng/ml) had no effect on mean \pm SEM primary term canine trophoblast proliferation compared to no treatment (C; P=0.18). Experiments were performed in quadruplicate (n=4 dogs).



invasive capacity at term (Librach et al., 1991). It is possible that TCT do not retain receptors for IL-8 at term. In humans, IL-8 mediates its effect through the CXCR1 and CXCR2 receptors (Tsui et al., 2004; Hanna et al., 2006). It is not known if a similar receptor exists in dogs or if it is temporally expressed during gestation. Investigations are needed to determine if canine CXCR1 and CXCR2 receptors exist.

Whereas the effects of IL-8 are dependent on MMP activity (Jovanović et al., 2010), MMP activity is regulated by TIMP in human trophoblast invasion (Librach et al., 1991; Zhang et al., 2012). Down-regulation of TIMP-2 mRNA increases migration and invasion of human first trimester HTR-8/SVneo trophoblasts (Zhang et al., 2012). The role of TIMP-2 in canine trophoblast migration and invasion has not been previously studied. Therefore, in the current study, it was shown that TIMP-2 decreases TCT invasion, but not migration. The role of TIMP-2 on cell migration in early gestation canine trophoblast should be investigated.

In conclusion, this is the first study to examine canine trophoblast physiology in vitro. The current study provides evidence that IL-8 promotes migration, whereas TIMP-2 decreases invasion in normal primary TCT. This information provides a basis for understanding placental pathologies that occur during the canine peripartum period.

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CHAPTER III

CONCLUSION AND FUTURE STUDIES

The studies contained within this thesis were the first to investigate term canine trophoblast (TCT) physiology *in vitro*. We found that IL-8 stimulates primary TCT migration, whereas TIMP-2 inhibits invasion. These findings provide a basis for future research on placental-related disorders resulting from a disruption of normal trophoblast proliferation or invasion, such as in cases of subinvolution of placental sites.

Future studies should investigate canine trophoblast differences in migration, invasion, and proliferation at different stages of gestation, especially during the period of apposition and implantation. Knowledge of early gestation canine trophoblast physiology would allow for direct comparison to other studies using human first trimester trophoblasts. Factors important in the signaling between the trophoblast and endometrium during implantation in humans (such as integrins α_1 , α_5 , and β_1 and extracellular matrix proteins) have not been investigated in dogs. It is expected that integrins and extracellular matrix proteins will promote invasion and migration, which is key to successful implantation and the establishment and maintenance of pregnancy.

Studies investigating the presence of the IL-8 receptors, CXCLR1 and CXCLR2, in TCT should also be pursued, to support modeling for human placental conditions. It is expected that dogs retain a form of the IL-8 receptors based on the IL-8-induced stimulation of cell migration presented in this research.

APPENDICES

APPENDIX A. SOLUTIONS AND REAGENTS

Cell isolation medium

The cell isolation medium (500 ml) was prepared with 475 ml DMEM (11965-092, Gibco, Life Technologies, Grand Island, NY) with 12.5 ml FBS (16000, Gibco, Life Technologies), 5 ml Glutamine Plus (B90210, Atlanta Biologicals, Lawrenceville, GA), 5 ml Penicillin/Streptomycin (15140-122, Life Technologies), and 2.5 ml Gentamycin (15750, Life Technologies).

Enzyme digestion solutions

The collagenase solution (100 ml) was prepared with 100 ml 1X DPBS (14109-144, Gibco), 0.062 g collagenase (C2674, Sigma, St. Louis, MO), 0.04 g DNase (DN25, Sigma), 0.069 g hyaluronidase (H3506, Sigma), and 0.10 g bovine serum albumin (A7906, Sigma).

The trypsin solution (100 ml) was prepared with 100 ml 1X DPBS (14109-144, Gibco), 0.0069 g trypsin (T8003, Sigma), 0.04 g DNase (DN25, Sigma), and 0.02 g EDTA (E5134, Sigma).

Percoll density gradient solutions

The Percoll density gradient consisted of 7 stock solutions: 90%, 70%, 60%, 50%, 40%, 30%, and 20% Percoll solutions. Each Percoll solution consisted of Percoll (17-0891-01, GE Healthcare Bio-Sciences AB, Pittsburgh, PA), 10X Hank's BSS without Phenol Red (14185, Invitrogen), 1X Hank's BSS without Phenol Red (14175,

Invitrogen), and 1X Hank's BSS with Phenol Red (14170, Invitrogen/Life Technologies, Grand Island, NY). The 1X Hank's BSS with and without Phenol Red was used to produce the discontinuous layers of the Percoll gradient, to aid in the separation of cells based on density. For 90% Percoll, 270 ml Percoll was combined with 30 ml 10X Hanks BSS without Phenol Red. For 70% Percoll, 35 ml 90% Percoll was combined with 10 ml 1X Hanks BSS without Phenol Red. For 60% Percoll, 30 ml 90% Percoll was combined with 15 ml 1X Hanks BSS with Phenol Red. For 50% Percoll, 25 ml 90% Percoll was combined with 20 ml 1X Hanks BSS without Phenol Red. For 40% Percoll, 20 ml 90% Percoll was combined with 25 ml 1X Hanks BSS with Phenol Red. For 30% Percoll, 15 ml 90% Percoll was combined with 30 ml 1X Hanks BSS without Phenol Red. For 20% Percoll, 10 ml 90% Percoll was combined with 35 ml 1X Hanks BSS with Phenol Red, tubes were stored at 4°C until use. After stock solutions were made, 6 ml of each solution was slowly layered in a 50 ml conical tube starting with 70% Percoll at the bottom, 60%, 50%, 40%, 30%, and up to 20% Percoll at the top. The Percoll gradient should be prepared the same day as the tissue collection, with 36 ml Percoll gradient for every 8 g of starting tissue.

Phosphate buffered saline (PBS) wash medium

PBS wash medium was made to perform washes during immunohistochemical studies, and is different from the 1 X DPBS used in the enzymes solutions above. PBS wash medium consisted of 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄·7H₂O, 2 g KH₂PO₄ (all from Sigma), diluted in 1 L H₂O. The PBS was then filtered through filter paper (0.45

μm , #HAWP02500, Millipore, Bedford, MA) and sterilized in the autoclave (Steam-It Dual-matic Sterilmatic, Market Forge, Everett, MA).

Bovine serum albumin (BSA) buffer solution

BSA buffer solution for the reconstitution of lyophilized IL-8 and TIMP-2. The 0.1% BSA buffer (10 ml) was prepared with 10 ml 1X DPBS (14109-144, Gibco) and 0.01 g BSA (A7906, Sigma).

Migration assay culture medium

For Experiment 1, the migration assay used both medium A and medium B to examine the effect of serum and serum-free medium on TCT cell culture and migration.

Medium A (500 ml) consisted of 482.5 ml DMEM (11995, Gibco, Life Technologies) with 5 ml Hepes (15630, Life Technologies), 5 ml Glutamine Plus (B90210, Atlanta Biologicals), 5 ml Penicillin/Streptomycin (15140-122, Life Technologies), 2.5 ml Gentamycin (15750, Life Technologies).

Medium B (500 ml) is the same as medium A with the addition of serum, so it consisted of 432.5 ml DMEM with 5 ml Hepes, 5 ml Glutamine Plus, 5 ml Penicillin/Streptomycin, 2.5 ml Gentamycin, and supplemented with 50 ml FBS (16000, Gibco, Life Technologies).

For Experiment 2, the migration assay only used medium B, because it was the best medium for TCT cell culture and cell migration.

Invasion assay culture medium

For Experiment 1, the invasion assay used mediums A, B, and C to determine the best medium and chemoattractant conditions to allow for maximal TCT invasion.

Mediums A and B were prepared as described above. Briefly, medium A consisted of 482.5 ml DMEM, 5 ml Hepes, 5 ml Glutamine Plus, 5 ml Penicillin/Streptomycin, and 2.5 ml Gentamycin. Medium B is the same as medium A with the addition of serum, so it was prepared with 432.5 ml DMEM, 5 ml Hepes, 5 ml Glutamine Plus, 5 ml Penicillin/Streptomycin, 2.5 ml Gentamycin, and 50 ml FBS.

Medium C was made with Nutridoma, a reduced protein alternative with <50 µg/ml for a 1% Nutridoma solution. Medium C (100 ml) consisted of 98 ml medium A supplemented with 2 ml Nutridoma (11011375001, Roche, Indianapolis, IN).

For Experiment 1, primary TCT were suspended in medium A (without serum) and seeded into the upper wells. The chemoattractant (medium B with serum or medium C reduced protein) was placed in the lower well. This gradient allows for maximal chemotaxis as the TCT degrade and invade the Matrigel from a lower serum or protein environment to the chemoattractant with a higher serum environment.

For Experiment 2, primary TCT were suspended in medium A with or without factor (IL-8 or TIMP-2) and the chemoattractant was medium B. These conditions allowed for the most number of invading cells using this assay.

Proliferation assay culture medium

For Experiment 1, the proliferation assay used both medium A and medium B, as described above to examine the effect of serum and serum-free medium on TCT proliferation. Primary TCT were cultured in medium A prepared with 482.5 ml DMEM with 5 ml HEPES, 5 ml Glutamine Plus, 5 ml Penicillin/Streptomycin, 2.5 ml Gentamycin. Primary TCT were also cultured in medium B, which is the same as medium A with the addition of serum, so it was prepared with 432.5 ml DMEM with 5 ml HEPES, 5 ml Glutamine Plus, 5 ml Penicillin/Streptomycin, 2.5 ml Gentamycin, and supplemented with 50 ml FBS. The proliferation assay kit MTT (CGD-1, Cell Growth Determination Kit MTT-based, Sigma, St. Louis, Missouri) consisted of 5 mg/ml MTT in RPMI-1640 without phenol red and 1-propanol (0.1 N HCl in anhydrous isopropanol).

Preparation of factors for Migration, Invasion, and Proliferation Assays

Recombinant canine and human IL-8 and recombinant human TIMP-2 factors and conversions are summarized in Tables A.1 through A.3. Concentrations selected for IL-8 and TIMP-2 were based on dosages found to elicit maximum effects in previous studies with human trophoblasts (Librach et al., 1991; Jovanović et al., 2010).

After determining the amount of IL-8 (ng/ml) and TIMP-2 ($\mu\text{g/ml}$) desired, stock solutions and dilutions of IL-8 and TIMP-2 for each experiment were summarized in Tables A.4 through A.9. For Experiment 2, human IL-8 was compared to canine IL-8 to determine which factor had the greater effect on TCT migration, and that factor would be used in Experiment 3.

Table A.1. Factors used for proliferation, migration, and invasion *in vitro* assays.

Factor	IL-8	IL-8	TIMP-2
Species	Canine	Human	Human
Migration assay	10, 100 ng/ml	10, 50, 100 ng/ml	0.05, 0.1, 0.5 µg/ml
Invasion assay	None	10, 50 ng/ml	0.5 µg/ml
Proliferation assay	None	10, 50 ng/ml	None
Catalog #	70009-DNAE	200-08M	410-02
Company	Sino Biological, Inc., Beijing, China	PeptoTech, Rocky Hill, NJ	PeptoTech, Rocky Hill, NJ

Table A.2. Unit conversion of IL-8 unit conversion from kDa to ng/ml.

IL-8 starting concentration (nM)	kDa to kg/mol	kg/mol to $\mu\text{g/L}$	$\mu\text{g/L}$ to $\mu\text{g/ml}$	$\mu\text{g/ml}$ to ng/ml
65 nM = 65 nmol/L	8 kDa = 8 kg/mol	$8 \text{ kg/mol} \times 65 \text{ nmol/L} = 520 \mu\text{g/L}$	$520 \mu\text{g/L} \times 10^{-3} \text{ L/ml} = 0.520 \mu\text{g/ml}$	$0.520 \mu\text{g/ml} \times 100 \text{ ng}/\mu\text{g} = 520 \text{ ng/ml}$
15 nM = 15 nmol/L	8 kDa = 8 kg/mol	$8 \text{ kg/mol} \times 15 \text{ nmol/L} = 120 \mu\text{g/L}$	$120 \mu\text{g/L} \times 10^{-3} \text{ L/ml} = 0.120 \mu\text{g/ml}$	$0.120 \mu\text{g/ml} \times 100 \text{ ng}/\mu\text{g} = 120 \text{ ng/ml}$
6 nM = 6 nmol/L	8 kDa = 8 kg/mol	$8 \text{ kg/mol} \times 6 \text{ nmol/L} = 48 \mu\text{g/L}$	$48 \mu\text{g/L} \times 10^{-3} \text{ L/ml} = 0.048 \mu\text{g/ml}$	$0.048 \mu\text{g/ml} \times 100 \text{ ng}/\mu\text{g} = 48 \text{ ng/ml}$
1 nM = 6 nmol/L	8 kDa = 8 kg/mol	$8 \text{ kg/mol} \times 1 \text{ nmol/L} = 8 \mu\text{g/L}$	$8 \mu\text{g/L} \times 10^{-3} \text{ L/ml} = 0.008 \mu\text{g/ml}$	$0.008 \mu\text{g/ml} \times 100 \text{ ng}/\mu\text{g} = 8 \text{ ng/ml}$

Table A.3. Unit conversion of TIMP-2 from kDa to $\mu\text{g/ml}$.

TIMP-2 starting concentration (nM)	kDa to kg/mol	kg/mol to $\mu\text{g/L}$	$\mu\text{g/L}$ to $\mu\text{g/ml}$
25 nM = 25 nmol/L	21.8 kDa = 21.8 kg/mol	$21.8 \text{ kg/mol} \times 25 \text{ nmol/L} = 545 \text{ } \mu\text{g/L}$	$545 \text{ } \mu\text{g/L} \times 10^{-3} \text{ L/ml} = 0.55 \text{ } \mu\text{g/ml}$
5 nM = 5 nmol/L	21.8 kDa = 21.8 kg/mol	$21.8 \text{ kg/mol} \times 5 \text{ nmol/L} = 109 \text{ } \mu\text{g/L}$	$109 \text{ } \mu\text{g/L} \times 10^{-3} \text{ L/ml} = 0.109 \text{ } \mu\text{g/ml}$
2.5 nM = 2.5 nmol/L	21.8 kDa = 21.8 kg/mol	$21.8 \text{ kg/mol} \times 2.5 \text{ nmol/L} = 55 \text{ } \mu\text{g/L}$	$55 \text{ } \mu\text{g/L} \times 10^{-3} \text{ L/ml} = 0.055 \text{ } \mu\text{g/ml}$

Table A.4. Preparation of IL-8 & TIMP-2 stock solutions.

Factor	Starting mass	Reconstitution medium	Final concentration
Canine IL-8	20 µg	200 µl sterile water	100 ng/µl
Human IL-8	25 µg	250 µl 0.1 % BSA	100 ng/µl
Human TIMP-2	10 µg	100 µl 0.1 % BSA	0.1µg/µl

Table A.5. Preparation of canine IL-8 dilutions for the migration assay.

IL-8 final concentration	Volume of canine IL-8	Volume of 10% FBS	Location & volume of aliquot
100 ng/ml	5 μ l of stock IL-8 (100 ng/ μ l)	5.0 ml	Migration assay test wells (1 ml/well)
10 ng/ml	0.5 ml of the above solution (100 ng/ml)	4.5 ml	Migration assay test wells (1 ml/well)

Table A.6. Preparation of human IL-8 dilutions for the migration assay.

IL-8 final concentration	Volume of human IL-8	Volume of 10% FBS	Location & volume of aliquot
100 ng/ml	10 μ l of stock IL-8 (100 ng/ μ l)	10 ml	Migration assay test wells (1 ml/well)
50 ng/ml	3.25 ml of the above solution (100 ng/ml)	3.25 ml	Migration assay test wells (1 ml/well)
10 ng/ml	2.0 ml of the above solution (50 ng/ml)	8.0 ml	Migration assay test wells (1 ml/well)

Table A.7. Preparation of human TIMP-2 dilutions for the migration assay.

TIMP-2 final concentration	Volume of human TIMP-2	Volume of 10% FBS	Location & volume of aliquot
0.5 µg/ml	32.5 µl of stock TIMP-2 (0.1 µg/µl)	6.5 ml	Migration assay test wells (0.5 or 1 ml/well)
0.1 µg/ml	1.0 ml of the above solution (0.5 µg/ml)	4.0 ml	Migration assay test wells (1 ml/well)
0.05 µg/ml	2.0 ml of the above solution (0.5 µg/ml)	2.0 ml	Migration assay test wells (1 ml/well)

Table A.8. Preparation of human IL-8 dilutions for the invasion assay.

IL-8 final concentration	Volume of human IL-8	Volume of PFM	Location & volume of aliquot
500 ng/ml	20 μ l of stock IL-8 (100 ng/ μ l)	2 ml	None, proceed to next dilution
100 ng/ml	0.6 ml of the above solution (500 ng/ml)	2.4 ml	None, proceed to next dilution
50 ng/ml	2.5 ml of the above solution (100 ng/ml)	2.5 ml	Invasion assay test wells (250 μ l/well)
10 ng/ml	0.6 ml of the above solution (100 ng/ml)	2.4 ml	Invasion assay test wells (250 μ l/well)

Table A.9. Recombinant human TIMP-2 dilutions for the invasion assay.

TIMP-2 final concentration	Volume of human TIMP-2	Volume of PFM	Location & volume of aliquot
0.5 µg/ml	10 µl of stock TIMP-2 (0.1µg/µl)	1.0 ml	Invasion assay test wells (250µl/well)

APPENDIX B. TERM CANINE TROPHOBLAST ISOLATION AND CULTURE

Summary

Using methods originally described Thiede and Rudolph (1961), scientists have isolated and cultured trophoblasts from freshly collected placentas in several species. Technical reviews of these methods for humans (Hunkapillar and Fisher, 2008) and dogs (Sahlfeld et al., 2012) have been published. The following techniques were modified from the work of Sahlfeld and coworkers (2012) for the purposes of the current thesis research.

Animals

Placenta was collected from pregnant bitches (n=21 dogs) that underwent elective c-section at term. The animals are described in Table B.1.

Tissue Collection

Canine placental tissue was collected following elective C-section with progesterone < 2.5 ng/ml at the time of pre-partum luteolysis (degradation of corpus luteum) but prior to labor onset. Following collection of the placenta, the villous chorioallantois was dissected free from the marginal hematoma, nonvillous chorioallantois, and connective tissues; and blood was also removed by blotting with a paper towel. Chorioallantois tissue was minced into 0.5-cm sections. The tissue was

placed in 50 ml conical tubes and suspended in ice-cold (4°C refrigeration) cell isolation medium (Appendix A) at 1:4 tissue to medium dilution.

Cell Isolation

Trophoblast isolation and culture was successfully developed by Thiede and Rudolph (1961), which they removed debris and undisaggregated tissue by a series of agitation and filtration steps until cells appeared loosened from the dissected placental villi and the cells were suspended in the surrounding cell isolation medium. In the current study, primary term canine trophoblast (TCT) isolation was performed as previously described (Sahlfeld et al., 2012), with modifications in enzyme solutions (1:4 rather than 1:6), addition of a third enzyme digestion (collagenase), and the volume of Percoll density gradient (1 tube:8 g tissue rather than 1 tube:6 g tissue, Appendix A). These modifications were made to conserve resources as lower concentrations of enzyme solutions and higher tissue concentrations in the Percoll density gradient were found to be just as effective for cell isolation.

Briefly, after collection, the chorioallantois was washed twice by centrifugation (1300 rpm for 5 min at 4°C) replacing with ice-cold medium after each wash. After the second wash, the medium was removed and warm (37°C) 0.062% collagenase solution (Appendix A) was added to the tissue at 1:4 dilution for the first enzyme digestion. The tubes of tissue and collagenase were then placed into a 37°C shaking incubator (175 rpm for up to 20 min) at <45° angle. After tissues have reached the desired amount of degradation (tissue fibers appear slightly eroded and loosened from the rest of the tissue

to making a “soupy” solution). The tubes of degraded tissues were placed upright in a bucket of ice for no longer than a minute to allow tissues to settle to the bottom, and supernatant was removed. Warm 0.0069% trypsin solution (Appendix A) was then added to the tissues at 1:4 dilution and were again placed in the shaking incubator (175 rpm for up to 10 min) for the second enzyme digestion. Once tissues have reached the desired amount of degradation (“soupy” appearance), the tubes were again placed in ice for the tissues to settle at the bottom. Although this time, after 1 min the loosened cells of interest are now suspended in the supernatant, which was collected and placed in new conical tubes up to 30 ml and the remainder of the tube was filled up to 45 ml with ice-cold medium. Tubes were then washed twice by centrifugation (1300 rpm for 8 min at 4°C), replacing with ice-cold medium after each wash, being careful to not disturb the cell pellet at the bottom. After the second wash, the medium was removed and warm collagenase solution (1 ml) was added to the cells and placed in the shaking incubator (150 rpm for up to 3 min) for the third enzyme digestion. Immediately, ice-cold medium was added to the tubes up to 45 ml and was washed once by centrifugation (1300 rpm for 8 min). The supernatant was removed and the cell pellet was suspended in ice-cold medium up to 25 ml. The cell suspension was then added to the top of the Percoll density gradient (Appendix A) and placed in long centrifugation (2500 rpm for 20 min at 4°C) to separate the cells according to their densities, so that the trophoblasts are isolated from other cells similar in size. The top two trophoblast-containing layers were collected between 30-36 ml of the tube, with medium collected just below 30 ml to ensure collection of isolated trophoblasts. Up to 25 ml of the collected trophoblast layer are

placed in a new conical tube, adding medium up to 45 ml and the cells was washed twice by centrifugation (1300 rpm for 8 min), replacing with medium A (Appendix A) after the first wash. After the second wash, medium A is aspirated and resuspended with more medium A up to 5 ml to count the cell concentration in a hemocytometer. After cell concentration is determined, the cells are suspended in PFM and aliquoted into each assay or placed in culture. An additional centrifugation step (1300 rpm for 5 min) is necessary to remove the supernatant and resuspend the cells in medium B (Appendix A) for immunohistochemistry, migration assay, and spontaneous migration experiments.

Cell Culture

For the original work by Thiede and Rudolph (19161), cells were plated on glass in 10 % fetal bovine serum medium with antibiotics and were placed in 37 °C with 7% CO₂ until trophoblasts attached to the glass to form monolayers. Recent studies utilize the standard tissue culture conditions of 37 °C with 7% CO₂, which has allowed for successful culture of trophoblast across species in humans (Hunkapillar and Fisher, 2008), dogs (Sahlfeld et al., 2012), and rodents (Rossant and Tamura-Lis, 1981). Cells were cultured at 37 °C with 5% CO₂ for this thesis work.

For immunohistochemistry, isolated primary TCT (1000X10³ cells/well) were suspended in medium B (2.5 ml/dish) and seeded onto glass coverslips that were placed inside 6- 33 mm² tissue culture dishes. Dishes were incubated and fed with new, warm (37°C) 10% FBS 24 h after seeding and every 48 h thereafter, until cells formed a 70-80% confluent monolayer after 10-14 days in culture.

For the migration assay (Appendix C), in Experiment 1 primary TCT (1000×10^3 cells/well) were suspended in medium A or medium B (2 ml/well) and plated into a 6-well tissue culture plate (BD Falcon, Franklin Lakes, NJ). For Experiment 2, primary TCT (1000×10^3 cells/well) were suspended in medium B (1 ml/well) with or without human IL-8 or canine IL-8 and plated into a 12-well tissue culture plate (BD Falcon). For Experiment 3, primary TCT (1000×10^3 cells/well) were suspended in medium B (1 ml/well) with or without IL-8 or TIMP-2 and plated into a 12-well tissue culture plate (BD Falcon). For all experiments, plates were then incubated and fed with fresh medium B until a confluent monolayer of cells was formed after 7-10 days in culture.

For the spontaneous migration assay, primary TCT (250×10^3 cells/well) were suspended in medium A or medium B (0.5 ml/well) and seeded onto 12- control filter membrane inserts in the upper wells. The chemoattractant (medium A or medium B) was placed into the lower wells (0.75 ml/well) and the 12-well tissue culture plates were incubated for 22 h in culture.

For the invasion assay, in Experiment 1 primary TCT (0.25, 2.5, 25, 50, 100, and 250×10^3 cells/well) were suspended in medium A or medium C (0.5 ml/well, Appendix A) and seeded onto 6- Matrigel-coated filter membranes in the upper wells. In Experiment 2, primary TCT (250×10^3 cells/well) in medium A with or without IL-8 or TIMP-2 were used. For both experiments, medium B was the chemoattractant (0.75 ml/well) and was placed into the lower wells and the 12-well tissue culture plates were then incubated for 22 h in culture.

For the proliferation assay, in Experiment 1 primary TCT (10×10^3 , 50×10^3 , 100×10^3 , 200×10^3 , and 500×10^3 cells/well) were suspended in medium A or medium B ($100 \mu\text{l/well}$) and seeded into a 96-well tissue culture plate. Plates were incubated for 24 h. In Experiment 2, primary TCT (20×10^3 , 50×10^3 , 100×10^3 , 200×10^3 , and 500×10^3 cells/well) in medium A with or without IL-8 were seeded. Plates were then incubated for 8, 32, 36, and 44 h in culture.

Appendix B. References

- Hunkapiller, N., Fisher, S., 2008. Placental remodeling of the uterine vasculature. *Methods Enzymol* 445, 281-302.
- Rossant, J., & Tamura-Lis, W., 1981. Effect of culture conditions on diploid to giant-cell transformation in postimplantation mouse trophoblast. *J Embryol Exp Morph* 62, 217-227.
- Sahlfeld, L., Hazzard, T., Kutzler, M., 2012. Cellular characteristics of cultured canine trophoblasts. *Reprod Domest Anim* 47, 161-164.
- Thiede, H., Rudolph, J., 1961. A method for obtaining monolayer cultures of human fetal cells from term placentas. In *Proceedings of the Society for Experimental Biology and Medicine*. Society for Experimental Biology and Medicine. New York, NY: Royal Society of Medicine 107, 565-569.

Table B.1. A summary of bitches that donated placenta for primary trophoblast isolation after elective c-section at term.

Dog	Samba	Gemma	Bugs Scott	Cher	Elvira	Sassy	Abby
Date collected	10/27/11	11/3/11	11/18/11	12/2/11	12/10/11	12/13/11	3/15/12
Breed	Australian Labradoodle	Bulldog	Clumber Spaniel	Rottweiler	Dandy Dinmont Terrier	Labradoodle	Mini Bull Terrier
Age (years)	4 1/2	2	3 1/2	6	2 1/2	1	3
Litter size	7	6	4	3	1	6	4
Parity	2	1	1	4	1	1	2
Source	WVC	WVC	WVC	WVC	GAC	WVC	WVC
Experiments	I	I	I	I	I	I	M, I

Source: GAC- Gresham Animal Clinic, OSU- Oregon State University Small Animal Clinic, WVC- Wilsonville Veterinary Clinic. Experiments: CC- Immunocytochemistry, M- Migration assay, I- Invasion assay, P- Proliferation assay.

Table B.1 (continued). A summary of bitches that donated placenta for primary trophoblast isolation after elective c-section at term.

Dog	Bella Seaberg	Rosie	Fancy	Cleo	Shiloh	Body	Mithril
Date collected	3/26/12	3/26/12	6/25/12	6/25/12	11/14/12	12/14/12	12/15/12
Breed	Bulldog	Labradoodle	Australian Labradoodle	Dachshund	Bulldog	Labradoodle	Bull Mastiff
Age (years)	2 ½	3	9	2	1	2	3
Litter size	4	12	4	4	1	12	4
Parity	1	3	2	2	1	1	1
Source	WVC	WVC	WVC	WVC	WVC	WVC	OSU
Experiments	M, I	M, I	CC, M, I	CC, M, I	M, I, P	M, I, P	M, I

Source: GAC- Gresham Animal Clinic, OSU- Oregon State University Small Animal Clinic, WVC- Wilsonville Veterinary Clinic. Experiments: CC- Immunocytochemistry, M- Migration assay, I- Invasion assay, P- Proliferation assay.

Table B.1 (continued). A summary of bitches that donated placenta for primary trophoblast isolation after elective c-section at term.

Dog	Tory	Force	Berry	CJ	Claire	Sophie	Pandora
Date collected	1/8/13	1/10/13	2/1/13	2/5/13	2/7/13	2/22/13	3/13/13
Breed	Bulldog	Yellow Labrador Retriever	Bulldog	Sharpei	Corgi	Bulldog	Bull Mastiff
Age (years)	2	3	2	2	5	5	6
Litter size	1	8	7	4	10	9	2
Parity	2	2	1	2	2	2	2
Source	OSU	WVC	WVC	WVC	WVC	WVC	WVC
Experiments	M, I, P	M, I, P	M, I, P	M, I, P	M, I, P	M, I, P	I, P

Source: GAC- Gresham Animal Clinic, OSU- Oregon State University Small Animal Clinic, WVC- Wilsonville Veterinary Clinic. Experiments: CC- Immunocytochemistry, M- Migration assay, I- Invasion assay, P- Proliferation assay.

APPENDIX C. MIGRATION ASSAY

Summary

The objective for using the migration assay, also known as a wound-healing or scratch assay, Experiment 1 first determined the best culture and migration medium (medium A or medium B, Appendix A). In addition, varying dosages of IL-8 and TIMP-2 (Appendix A, Table A.1) were added to observe their effect on TCT migration in Experiment 2.

Experiment 1

The migration assay was performed as previously described (Liang et al., 2007). Term canine trophoblasts (TCT; n=5 dogs) were suspended in medium A or medium B (2 ml/well) and seeded (1000×10^3 cells/well) in a 6-well tissue culture plate. The plate remained in culture at 37°C with 5% CO₂ until a monolayer of cells was formed after 7-10 days. When cultured in medium A, TCTs were unable to adhere to the coverslip and aggregate to form cell clusters and the lack of protein or serum supplement resulted in cell death after 7-10 days in culture. From then on, TCTs were cultured in 10% FBS to form a monolayer. After reaching 70-80% confluence, a scratch was added into the cell monolayer using sterile pipette tip and loosened cells were removed by rinsing the wound with warm (37°C) medium A and replacing with medium A or medium B (2 ml/well). Cells were then incubated for 24 h. The wound width was photographed with a digital camera (Fujifilm Fine AX300 14 MP Digital Camera with Fujinon 5x Wide Angle

Optical Zoom Lens, Tokyo, Japan) and measured with digital calipers (General® Tools Corp., New York City, NY) after 0, 8, 12 and 24 h in culture. The wound width at t=0 h served as the baseline at 0% wound closure. Migration assay experiments were performed in triplicate. Data was analyzed with Repeated Measures ANOVA (NCSS Statistical Software-8, NCSS LLC, Kaysville, Utah).

Experiment 2

Migration assays were performed to compare the effect of human IL-8 and canine IL-8 on primary TCT migration. Primary TCT (n=4 dogs) were plated in medium B at 1×10^6 cells/well into three 12-well tissue culture plates (1.25 ml/well). Cells were cultured at 37°C in 5% CO₂ until a 70-80% confluent cell monolayer was formed. After reaching 70-80% confluence, a scratch was added into the cell monolayer using sterile pipette tip and loosened cells were removed by rinsing and then replacing with 1 mL of medium B with or without recombinant canine IL-8 (0, 10, and 100 ng/mL) or with or without recombinant human IL-8 (0, 10, and 100 ng/mL). Cells were then incubated for 24 h and microphotographs were taken with a Nikon Eclipse *Ti* inverted microscope with attached digital camera (Nikon Instruments Inc., Melville, NY) after 8, 12, and 24 h in culture. Experiments were performed in triplicate. Data was analyzed with Repeated Measures ANOVA (NCSS LLC). Fixed effects of the model were treatment type (canine IL-8 vs. human IL-8), dosage (0, 10, and 100 ng/mL), and time after treatment (0, 8, 12, and 24 h).

Experiment 3

Migration experiments were performed using recombinant human IL-8 and human TIMP-2 to determine effect of IL-8 and TIMP-2. Primary isolated TCTs (n=5 dogs) were plated at 1×10^6 cells/well in medium B into three 12-well tissue culture plates (1.25 ml/well). Cells were cultured at 37°C in 5% CO₂ until a monolayer was formed. After reaching 70-80% confluence, a scratch was added into the cell monolayer using sterile pipette tip and loosened cells were removed by rinsing and then replacing with 1 mL of medium B with or without IL-8 (0, 10, 50, and 100 ng/mL), TIMP-2 (0, 0.05, 0.1, and 0.5 µg/mL). In addition, a combination of IL-8 (50 ng/mL) and TIMP-2 (0.5 µg/mL) were added to plates in a smaller subset of dogs (n=3 dogs). Cells were then incubated for 24 h and microphotographs were taken as stated in Experiment 1. Experiments were performed in triplicate. Data was analyzed with Repeated Measures ANOVA (NCSS LLC). Fixed effects of the model were treatment type (IL-8 vs. TIMP-2), dosage, and time after treatment (0, 8, 12, and 24 h).

Results

For Experiment 1, canine trophoblasts cultured in medium B in a 33 mm² culture dish grew to 70-80% confluence into a cell monolayer starting with approximately 100×10^3 cells/well. However, in medium A, trophoblasts remained at 5-10% confluence and cell death occurred within 7 days after plating. Based upon these results, the remaining experiments were all performed with TCTs cultured to 70-80% confluence using medium B. For the coverslips that were grown in medium B, at 8 h after the adding

the wound, there was no difference in wound closure between medium A and medium B ($p=0.48$), where the wound closure was $10.8\% \pm 1.9\%$ and $19.3\% \pm 8.2\%$ (mean \pm SEM).. After 12 h, there was also no difference in the percent wound closure between medium A or medium B ($p=0.21$), where the wound closure was $23.6\% \pm 6.5\%$ and $42.9\% \pm 7.8\%$, respectively. However, after 24 h wound closure was reduced in medium A compared to medium B ($p=0.05$), where the wound closure was $39.9\% \pm 16.7\%$ and $80.1\% \pm 7.8\%$, respectively.

For Experiment 2, there was no significant difference on cell migration between the treatment type X dosage interaction ($p=0.72$), and treatment type X time interaction ($p=0.97$). For Experiment 3, there was no difference between the dosage of IL-8 on migration ($p=0.56$), and no IL-8 X time interaction on migration ($p=0.95$). In the presence of TIMP-2, there was no difference between the dosage of TIMP-2 on migration ($p=1.00$), and no TIMP-2 X time interaction ($p=0.89$) on migration. For the Experiment 3 comparison, there was no TIMP-2 and IL-8 X time interaction ($p=0.91$) on migration.

Appendix C. References

- Jovanović, M., Stefanoska, I., Radojčić, L., Vicovac, L., 2010. Interleukin-8 (CXCL8) stimulates trophoblast cell migration and invasion by increasing levels of matrix metalloproteinase (MMP)2 and MMP9 and integrins $\alpha 5$ and $\beta 1$. *Reproduction* 139, 789-798.
- Liang, C., Park, A., Guan, J., 2007. *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2, 329-333.
- Librach, C., Werb, Z., Fitzgerald, M., Chiu, K., Corwin, N., Esteves, R., Fisher, S., 1991. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J Cell Biol* 113, 437-449.

Figure C.1. Representative fields of TCT migration for Experiment 1. After culturing to 70-80% confluence in medium A, a wound was added and the migration of cells with medium A or medium B (shown below) was observed at 0, 8, 12, and 24 h in culture (n= 5 dogs) and wound closure was measured.

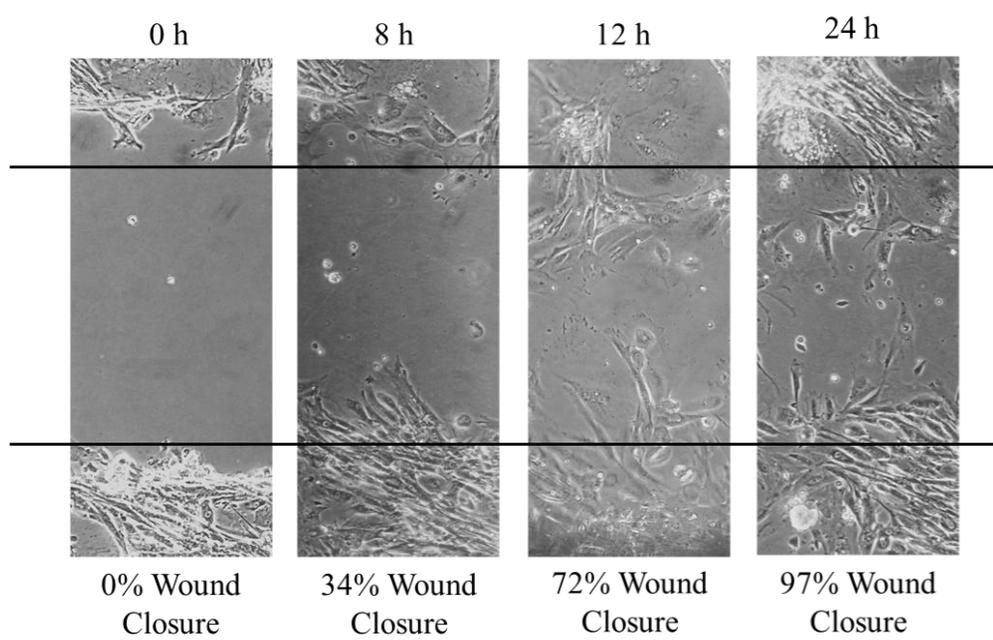


Figure C.2. A comparison of culture medium (medium A or medium B) on cell migration (n=5). At t= 24 h, 80% of the wound was closed with medium B media (10% FBS) whereas 40% of the wound was closed with medium A (protein-free medium, PFM) (p=0.0503).

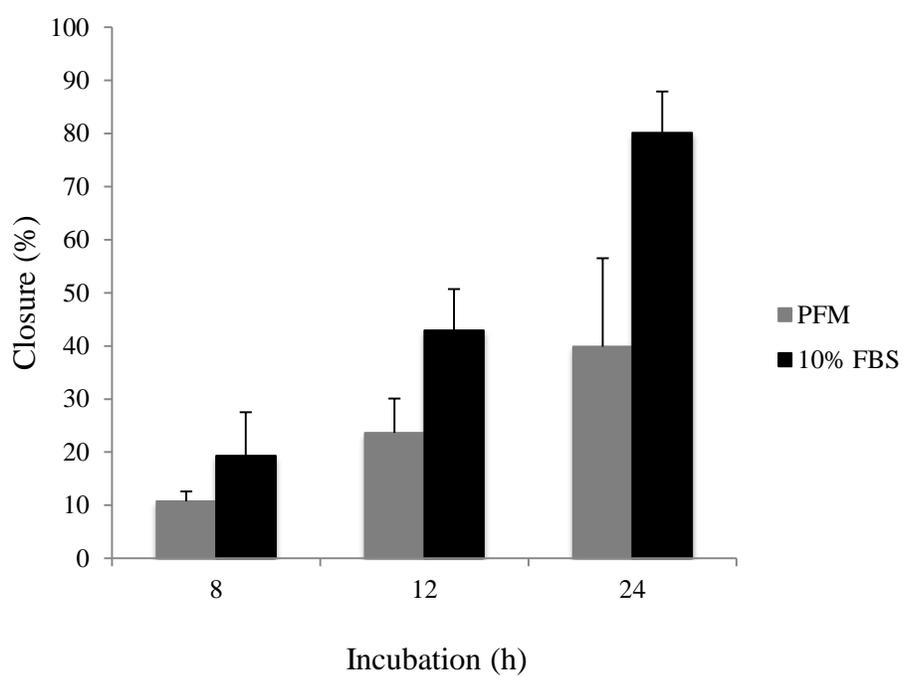


Figure C.3. A comparison of Sino Biologicals Inc. (Sino) canine recombinant IL-8 or PeptoTech (Pep) human recombinant IL-8 (0, 10, and 100 ng/mL) on cell migration (n=3 dogs). IL-8 did not increase wound closure ($p=0.72$). Data was expressed as percent wound closure of control at 0% baseline \pm SEM.

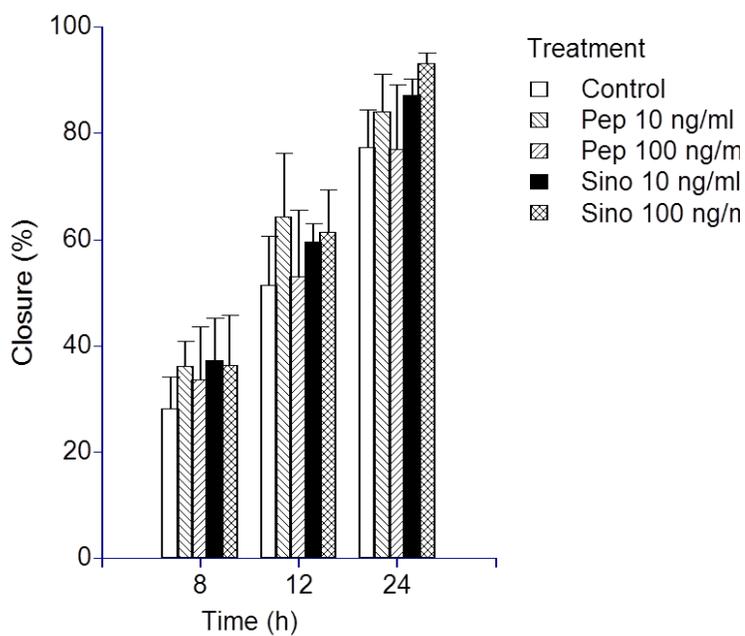


Figure C.4. The effect of human IL-8 (0, 10, and 100 ng/mL) on cell migration (n=5 dogs). IL-8 dosage had no effect on cell migration ($p=0.56$), also there was no IL-8 X time interaction ($p=0.95$). Data was expressed as percent wound closure of control at 0% baseline \pm SEM.

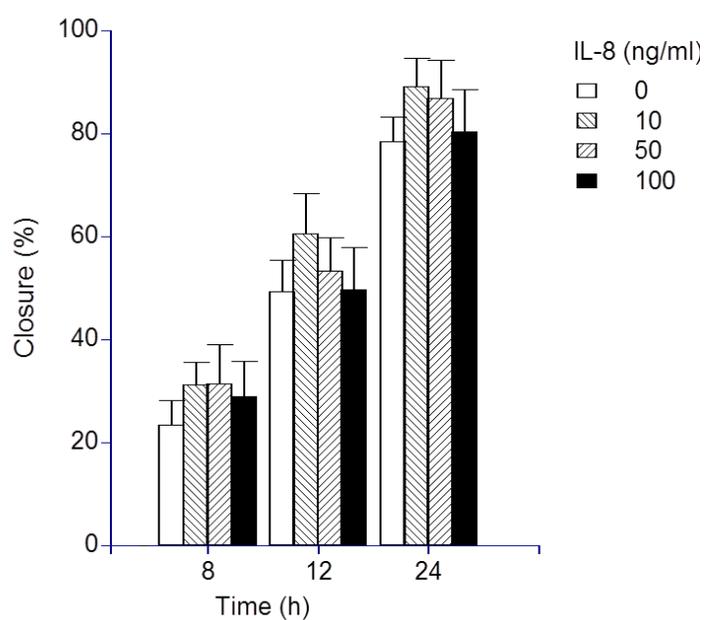


Figure C.5. TIMP-2 had no effect on cell migration at any of the dosages tested (0, 0.05, 0.1, and 0.5 $\mu\text{g/mL}$) ($p=1.00$) ($n=5$ dogs), also there was no TIMP-2 X time interaction ($p=0.89$). Data was expressed as percent wound closure of control at 0% baseline \pm SEM.

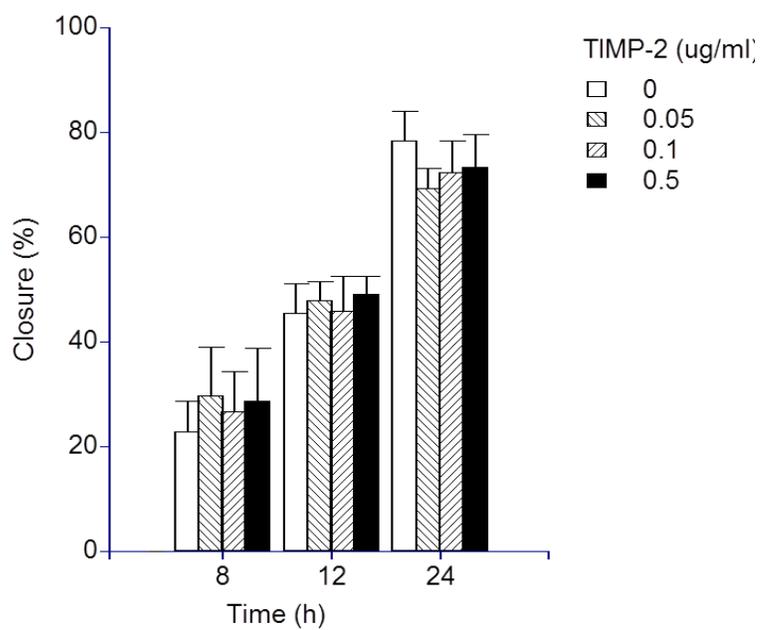
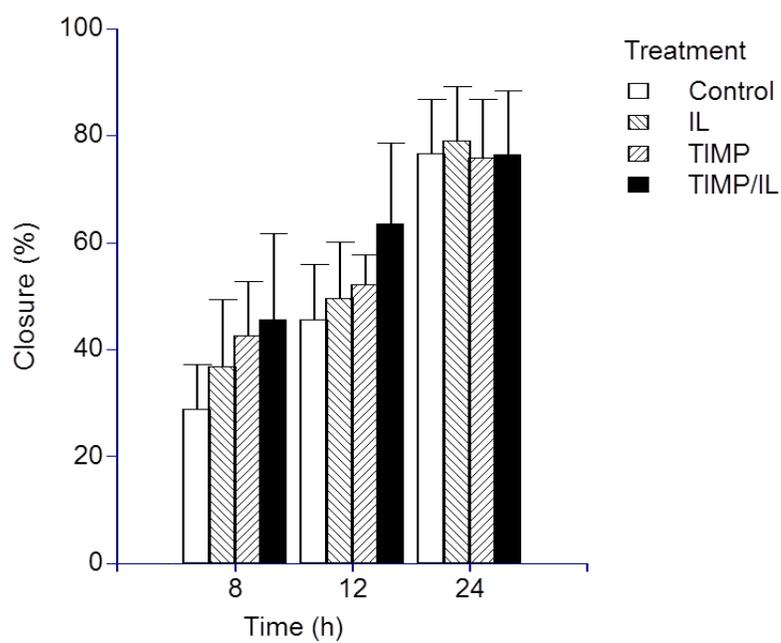


Figure C.6. The combinatorial effect of IL-8 & TIMP-2 on cell migration. There was no TIMP-2 and IL-8 X time interaction ($p=0.91$) on migration ($n=3$ dogs). Data was expressed as percent wound closure of control at 0% baseline \pm SEM.

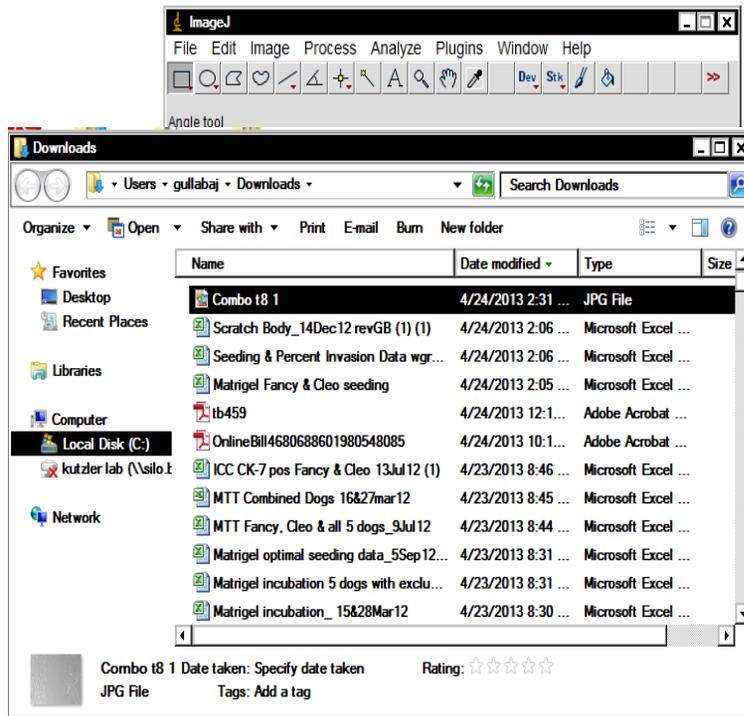


APPENDIX D. USE OF IMAGEJ PROGRAM FOR ANALYSIS

ImageJ is an image analysis program offered free by the National Institutes of Health, which was downloaded at <http://rsb.info.nih.gov/ij/>. This program was used to measure the wound area at each time point to calculate percent of wound closure. First, the program is started and the ImageJ Window appears on the desktop. To open a saved image file, on the Menu Bar under File, Open was selected to open the image file of choice. Alternatively, the image file can be dragged with a cursor to the Menu Bar. Next, the cursor can be used as a drawing tool to draw a perimeter around the denuded wound region. Then, the area of the wound is measured by selecting Analyze on the Menu Bar and Measure by typing Ctrl+M or just M. Measurement values will appear in a new data window to be recorded and to continue the percent wound closure calculation. Photo representation of steps taken for wound measurements are depicted in Figure D.1.

Figure D.1. A step-wise guide to using ImageJ to measure wound area for the migration assay.

Step 1



Step 2

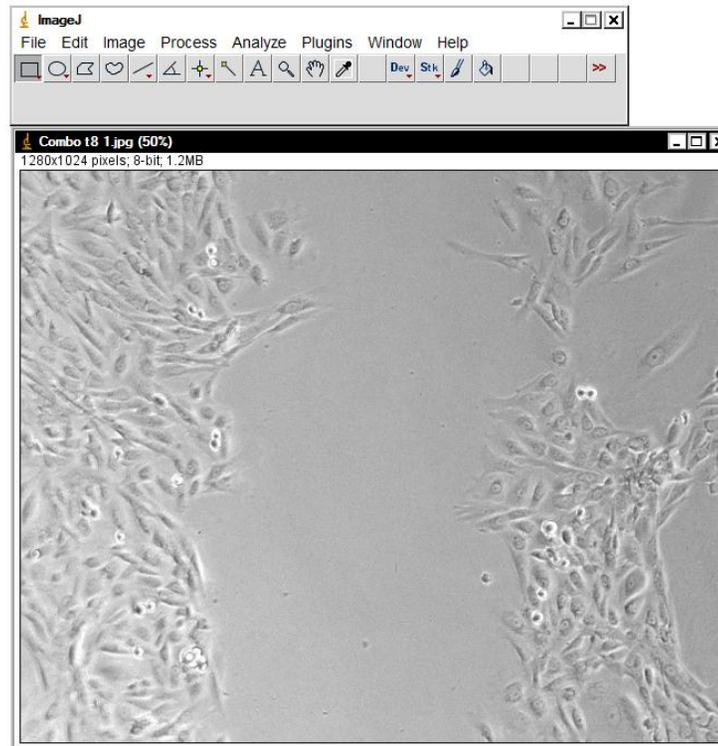
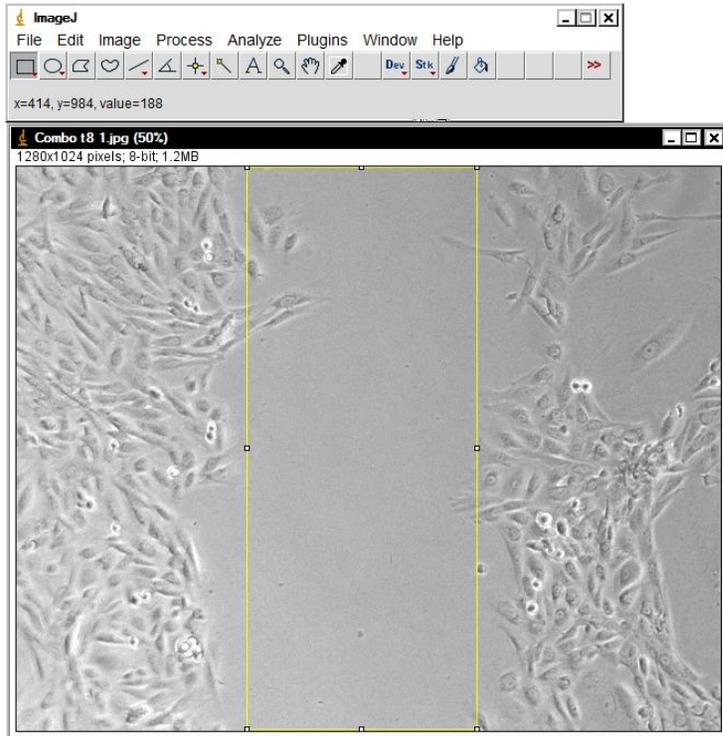


Figure D.1 (continued). A step-wise representation of wound measurements for the migration assay.

Step 3



Step 4

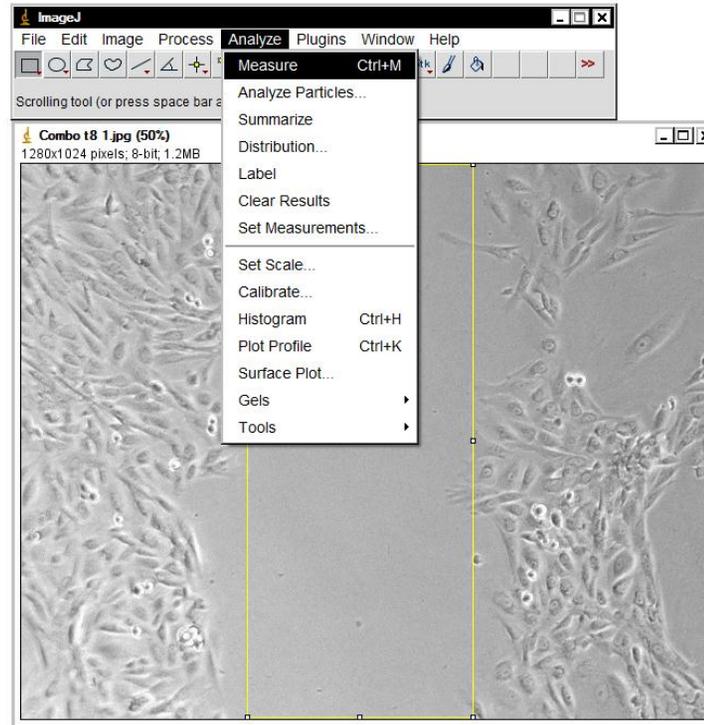
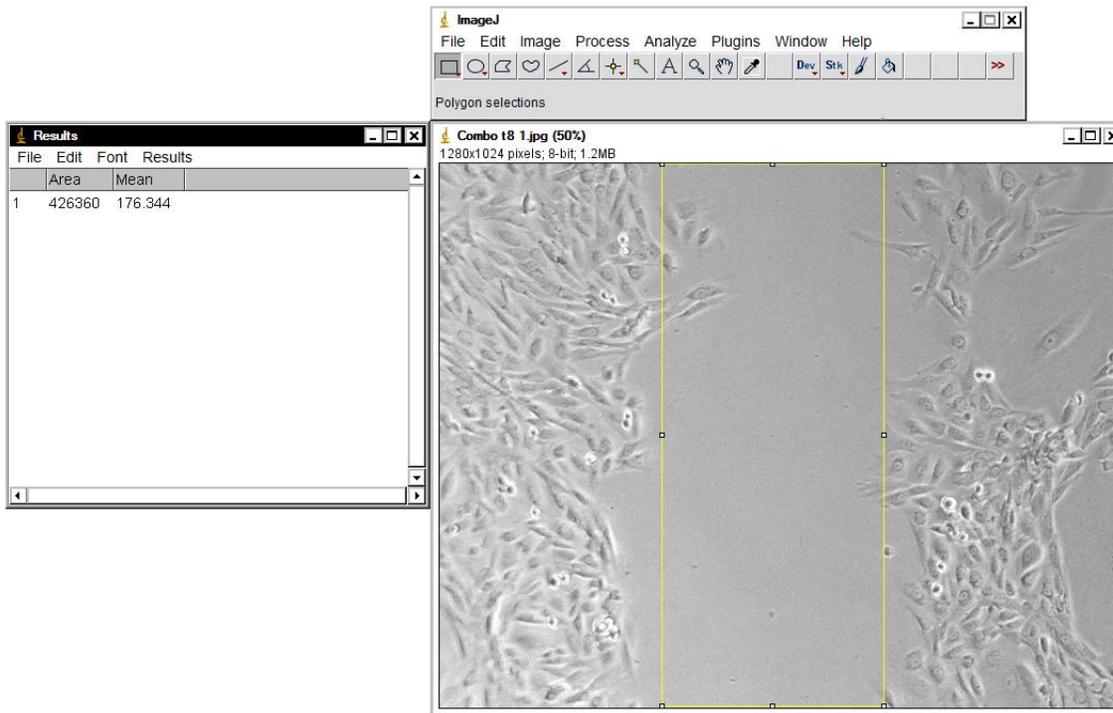


Figure D.1 (continued). A step-wise representation of wound measurements for the migration assay.

Step 5



APPENDIX E. IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed to verify trophoblast phenotype as previously described by Sahlfeld et al (2012). Expression of cytokeratin-7 (CK-7) was used for confirming canine trophoblast purity following isolation.

Briefly, primary term canine trophoblasts (TCT) from 2 dogs were seeded (1000×10^3 cells/well) on glass coverslips in a 6-well tissue culture plate. The effect of medium (medium A or medium B) on cell migration in 6-well tissue culture plates were examined. The migration assay was performed as previously described (Appendix C). The plate was cultured in medium B at 37°C with 5% CO₂ until a monolayer of cells was formed after 10-14 days. After reaching 70-80% confluence for a subset of coverslips, a scratch was added into the cell monolayer using sterile pipette tip and loosened cells were removed by rinsing the wound with warm (37°C) medium A and replacing with medium A or medium B (2 ml/well). Cells were then incubated for 24 h. The wound width was photographed with a digital camera (Fujifilm Fine AX300 14 MP Digital Camera with Fujinon 5x Wide Angle Optical Zoom Lens, Tokyo, Japan) and measured with digital calipers (General® Tools Corp., New York City, NY) after 0, 8, 12 and 24 h in culture. The wound width at t=0 h served as the baseline at 0% wound closure. Migration assay experiments were performed in triplicate. Data was analyzed with Repeated Measures ANOVA (NCSS Statistical Software-8, NCSS LLC, Kaysville, Utah).

Coverslips were fixed with 3 ml 70% methanol in water and culture dishes were placed in an air-tight container and stored at 4°C. Coverslips were washed with phosphate buffered saline wash medium (PBS, Appendix A), and treated with 5% donkey serum in PBS (blocking buffer) for 3 min at 20 °C to block non-specific secondary antibody binding. The coverslips were washed with PBS and then incubated overnight with the CK-7 primary antibody diluted in blocking buffer (1:20, p103620, DAKO, Carpinteria, CA) at 4 °C in a humidified chamber. Immunostaining specificity was verified by omission of the primary antibody. Following another wash with PBS, coverslips were incubated with the donkey anti-mouse Alexa Fluor 488 secondary antibody diluted in blocking buffer (1:1000, A21202, Life Technologies) for 2 h at 20 °C. Nuclei were detected with Hoechst 33342 (1:20,000, #H1399, Life Technologies) stain that was applied for 15 min at 20 °C. 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-F-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). CK-7 immunopositive cells were counted from 200 cells cultured on coverslips in this study. A cryopreserved primary TCT cell line 3&4 previously used in our laboratory as a positive control, was thawed, cultured in medium B on glass coverslips to 70-80% confluence, and fixed in 70% methanol.

Results

Irrespective of culture medium and wound placement, >94% of culture trophoblasts (Table E.1) were immunopositive for CK-7 (Figure E.1).

Appendix E. References

Sahlfeld, L., Hazzard, T., Kutzler, M., 2012. Cellular Characteristics of Cultured Canine Trophoblasts. *Reprod Domest Anim* 47, 161-164.

Figure E.1. Irrespective of culture medium (medium A or medium B), >95% of culture trophoblasts were immunopositive for CK-7. A) CK-7-positive trophoblasts in medium A and B) medium B (n= 2 dogs, 400X).

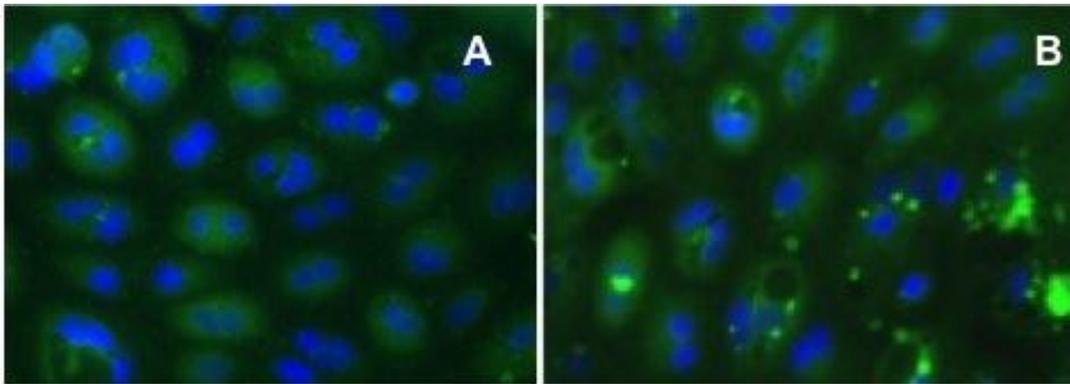


Table E.1. The effect of medium on CK-7 immunocytochemistry results of TCT with or without wound (n=2 dogs).

	% CK-7 Positive
Control Line 3&4, Medium B	99.50
Dog 1 No wound, Medium B	94.00
Dog 1 Wound, Medium A	97.00
Dog 1 Wound, Medium B	94.50
Dog 2 No wound, Medium B	97.50
Dog 2 Wound, Medium A	97.50
Dog 2 Wound, Medium B	96.50

APPENDIX F. SPONTANEOUS MIGRATION ASSAY

Summary

Cells travel towards a chemoattractant through a process chemotaxis. Chemotaxis mechanisms can be studied using a transwell chamber, commonly referred to as a Boyden chamber (Boyden, 1962). The objective of this appendix was to compare spontaneous migration of primary term canine trophoblasts (TCT) with that of a chemoattractant (medium B).

Spontaneous migration assay

A 24-well fitted plate is set up with 12 control filter membrane inserts (354578, BD Biosciences, Bedford, MA) using sterile forceps. The treatments were performed in triplicate as follows (upper well: lower well) with medium A (A) and medium B (B): 1) A:A, 2) A:B, 3) B:B, and 4) B:A. After primary TCTs were isolated, cells were suspended in medium A or medium B to make a final concentration of 250×10^3 cells/well. With forceps, inserts were pushed to the side of the well while adding 0.750 mL medium A or medium B to the lower chambers, then the control inserts were adjusted into the center of the wells containing the chemoattractant, ensuring that no air bubbles were trapped beneath the filters. Avoid bubbles by tipping the insert at a slight angle as it is lowered into the chemoattractant.

Immediately, 0.5 mL of the prepared cell suspensions was seeded onto the upper chambers. The 24-well plate with filters was then incubated for 22 hours in a tissue

culture incubator at 37°C with 5% CO₂ atmosphere. After the incubation stage, non-invading cells were gently removed with a cotton swab moistened with medium A, and this is repeated. Invading cells deposited at the underside of the filter were fixed and stained for 2 min with Solutions 1-3 of the DiffQuik Staining kit (Hemacolor, Merck, Darmstadt, Germany). With a sterile scalpel, filters were removed from their upper well chamber and placed bottom-side down onto a microscope slide with a drop of immersion oil used as mounting medium. Another small drop of immersion oil is placed on top of the stained filter and covered with a gridded glass coverslip. Invaded cells were counted at ten random, non-overlapping fields under light microscopy at 400X. Data was analyzed with one-way ANOVA and expressed as mean % migration of control \pm SEM.

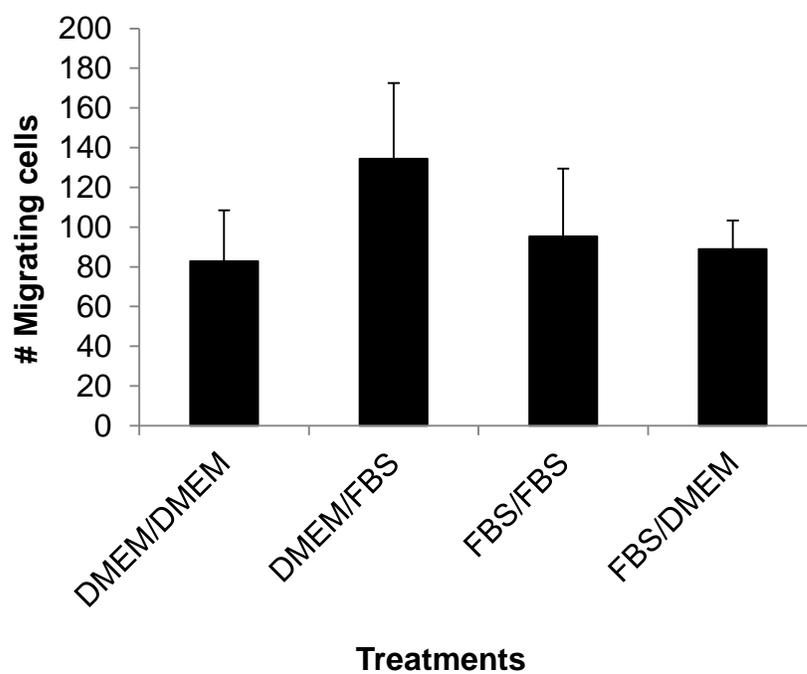
Results

The treatment of medium in the upper well and medium B in the lower well showed a tendency to attract more migrating cells than the other treatment conditions, although this increase was insignificant ($p > 0.05$; Figure F.1).

Appendix F. References

Boyden, S., 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J Exp Med* 115, 453-466.

Figure F.1. A comparison of various conditions on cell migration (n=3 dogs) in transwell chambers. The treatment of medium A (protein-free medium PFM) in the upper well and medium B (10% FBS) in the lower well showed more spontaneous migration than the other treatments (134 ± 7), although this was not significant ($p > 0.33$). Data were expressed as mean \pm SEM migrating cells per filter.



APPENDIX G. TIME-LAPSE PHOTOGRAPHY

Summary

Time-lapse photography is a useful tool to observe cell behavior while in culture (Liang et al., 2007). This method was applied to the migration assay using primary canine term trophoblasts (TCT) and the objective was to monitor wound closure during the incubation.

Preparation of time lapse apparatus

The tissue culture incubator was set to 37°C with 5% CO₂ on the day prior to taking time-lapse photographs of the wound (100X). The phase-contrast microscope (Model TMS-F, Nikon, Japan) was placed inside the incubator to ensure that the microscope maintained the same temperature as the incubator conditions throughout the length of the experiment.

The apparatus was consisted of a camera secured to the microscope and a circuit to limit the light exposure to the cells and control cell death due to extreme heat. The camera was a Cognex In-Sight 1403 (Natick, MA) and it should be noted that this camera is more of a computer. The camera was plugged into the network to allow off-site manipulation of the camera program regulate the pictures taken and then send these pictures to the computer as well as turn the microscope on and off to limit the amount of light on the cells. The camera was attached to an adapter that connected from the microscope to the c-mount with a 0.5x teleconverter (D50NLC, 0.5X c-mount for 38mm

port, Diagnostic Instruments, Inc., Sterling Heights, MI). The teleconverter was then attached to the photo port on the microscope

The lights were controlled by a home-built circuit that used a signal from the camera to switch a relay that could turn the power to the microscope on and off as if it were plugged or unplugged. A program was ran inside the camera to turn the microscope power on to take a picture, and then turn the power off and wait a set amount of time between pictures. Pictures were taken at 60 second intervals and the microscope light was switched on for two- 5 second blocks so that the light was on for 10 sec total per 60 sec block. The focus was adjusted periodically throughout the experiment to allow for maximal clarity of picture. The pictures were then sent to a computer on the same network as the camera via FTP for a total of 24 h in culture to allow complete closure of the wound. When the experiment was complete, all of the images that were sent to the computer were compiled using Windows Movie Maker to create a time-lapse. This was done by copying all the images into movie maker and then setting them to play back as a slide show at approximately 30 frames per second. This was then saved as a .wmv movie file. This experiment was performed 4 times with 4 dogs.

Results

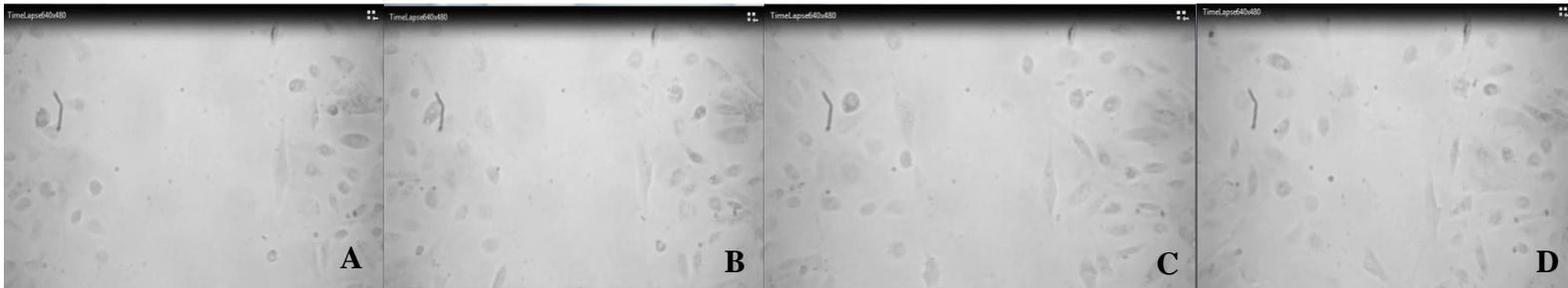
Using time-lapse photography to observe the cell behavior as the wound closed, it was evident that although cells along the periphery of the wound were proliferating, the cells displayed migratory behavior to close the wound with “leader cells” that would form filopodia-like extensions to reach across the freed surface of the plate to make cell

to cell interactions with the cells across the wound as the wound closed, previously described by Aladjem and Lueck (1981). This home-built time lapse apparatus was performed successfully, although adjustments such as light exposure and timing of pictures were made with 4 different dogs. For the first dog, the camera did not take pictures for the entire incubation duration and so all subsequent experiments the camera program was restarted to take pictures for 24 h. For the second dog, the camera would go out of focus. To adjust for this, we normalized the temperature of the entire apparatus by incubating the microscope with camera in the incubator for one day prior to taking photos for all subsequent experiments. For the third dog, we found that the microscope light would kill the cells due to the extreme heat, so a home-built switch was made to regulate the amount of light exposure. For the last dog, we adjusted the time intervals of pictures taken and found the most success with taking photos for once for every 60 seconds, with the light being on for 10 seconds for every 60 seconds. The images obtained from this time-lapse experiment reveal that the primary TCT undergo migration to close the wound but some proliferation may occur at the edge of the wound. Now that the protocol has been established, additional experiments to determine the extent to which proliferation contributes to wound closure.

Appendix G. References

- Aladjem, S., Lueck, J., 1981. Morphologic characteristics of the normal term human trophoblast maintained in prolonged in vitro cultures. *Brit J Obs Gyn* 88, 287-293.
- Liang, C., Park, A., Guan, J., 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2, 329-333.

Figure G.1. A representative set of 4 time-lapse photographs (A-D), which were merged to form a video of cells migrating to close the wound using the migration assay after 24 h in culture (n=1 dog).



APPENDIX H. INVASION ASSAY

Summary

The effects of cell seeding concentrations, chemoattractant, and incubation time on invasive behavior were determined for term canine trophoblasts (TCT) from 3 dogs using the Matrigel invasion assay (BDBioCoat Matrigel Invasion Chamber, #354480, BD Biosciences, Bedford, MA). Our studies utilized the invasion assay and applied IL-8 and TIMP-2 to observe their effect on canine trophoblast invasion, as seen within this thesis.

Experiment 1

The seeding concentrations compared were 0.25, 2.5, 25, 50, 100, and 250 X 10³ cells/well (n=9 dogs) in medium A (Appendix A). The chemoattractants tested were medium B and medium C (Appendix A) using 100 X 10³ cells/well (n=3 dogs). Lastly, the incubation times tested were 22 h and 46 h using 100 X 10³ cells/well (n=3 dogs). The invasion assay was performed as previously described (Librach et al., 1991). Briefly, the Matrigel and control (without Matrigel) filters (0.8 µm pores, polyethylene terephthalate) of the invasion assay are rehydrated with 0.5 mL warm (37°C) medium A to the lower and upper chamber of each well for at least 2 hours in a humidified tissue culture incubator at 37°C, 5% CO₂ atmosphere. After rehydration, the medium was carefully removed by pipetting and without disturbing the layer of Matrigel layer on the membrane. Primary culture cytotrophoblast cell suspensions (0.25, 2.5, 25, 50, 100, and 250 X 10³ cells/well) were prepared in medium A to make 0.5 mL cell suspensions to

observe the effect of seeding concentration on the percent of invading cells. Chemoattractant (medium B or medium C) was added at 0.750 mL to the lower chambers of the 24-well plate (BD Falcon) atmosphere to observe the effect of chemoattractant on the percent of invading cells. Immediately, 0.5 mL of the prepared primary cytotrophoblast cell suspensions was added to the upper chambers. One 24-well plate with inserts was incubated for 22 h or 46 h in a humidified tissue culture incubator at 37°C, 5% CO₂ atmosphere to observe the effect of incubation time on the percent of invading cells. Non-invading cells are removed with sterile cotton tipped swab moistened with medium A and apply gentle but firm pressure while moving the tip over the membrane surface. Each filter is scrubbed twice for complete removal. Bottom of the filters are then stained with DiffQuik (Harleco®, EMD Chemicals, Inc., Gibbstown, NJ) stain at 2 minutes per stain to quantify the number of invading cells under 400x light microscopy. The number of invading cells through filters with Matrigel is compared to the number of invading cells through filters without Matrigel (control) to calculate the percent invasion. Invasion assays are performed in triplicate. Data was analyzed with a one-way ANOVA and expressed as percent invasion \pm SEM.

Experiment 2

The seeding concentration used in the factor studies was 250×10^3 cells/well suspended in medium A in the upper well (Table F.1), and the chemoattractant was medium B in the lower well of the invasion chambers. For the factor studies, the procedure was performed as above, with one modification. The only modification was that primary TCT were suspended in 0.5 ml medium A with no factor (control), with IL-8

(10 or 50 ng/ml) or TIMP-2 (0.5 μ g/ml) and then cultured onto the Matrigel-coated filters for 22 h. The chemoattractant (medium B) was placed in the lower well. As above, the filters were then scrubbed to remove non-invading cells, and filters were then fixed and stained, and invading cells were counted under light microscopy (400X). The number of invading cells through filters with Matrigel is compared to the number of invading cells through filters without Matrigel (control) to calculate the percent invasion. Invasion assays are performed in triplicate (n=5 dogs).

Results

Percent invasion did not differ between seeding concentrations (p=0.36, n=9 dogs), although 250×10^3 cells/well were selected for future studies because it increased invasion by $62 \pm 22.11\%$, desirable when we undergo addition of stimulatory or inhibitory factors. Also, there was no difference in percent invasion between chemoattractants (p=0.06), although nearing significance. However, with 100×10^3 cells/well the percent invasion increased when incubation time was extended by 24 hours (p=0.28), although not significantly. There was no significant effect of IL-8 or TIMP-2 on invasion compared to the control (p=0.06), although nearing significance.

Summary

For the optimization studies, the effect of media (medium B or medium C) and incubation time (22 h or 46 h) was not significantly different (p=0.1661 and 0.2793, respectively). The cell seeding concentration of 250×10^3 cells/well was used for future

studies because it showed the least variability between dogs and a mean invasion of 60% at 22 h. For the factor studies, TIMP-2 decreased invasion ($p < 0.05$), whereas IL-8 had no effect on invasion ($p > 0.05$).

Appendix H. References

Librach, C., Werb, Z., Fitzgerald, M., Chiu, K., Corwin, N., Esteves, R., Fisher, S., 1991. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J Cell Biol* 113, 437-449.

Table H.1. Cell suspensions for the invasion assay for Experiment 2.

Starting cell concentration	Volume of suspension medium (medium A)	Location & volume of aliquot
250X10 ³ cells/ml	5 ml with or without IL-8 or TIMP-2	12 wells of matrigel-coated filters (0.5 ml/well)

Figure H.1. The effect of seeding concentration on primary TCT invasion. There was no effect of the seeding concentration on the percent invasion ($p>0.05$), however 250×10^3 cells/well elicited 62% invasion to allow for future studies using factors to promote or decrease invasion. Experiments were performed in triplicate ($n=9$ dogs). Data was expressed as percent invasion of control \pm SEM. Significance was defined as $p<0.05$.

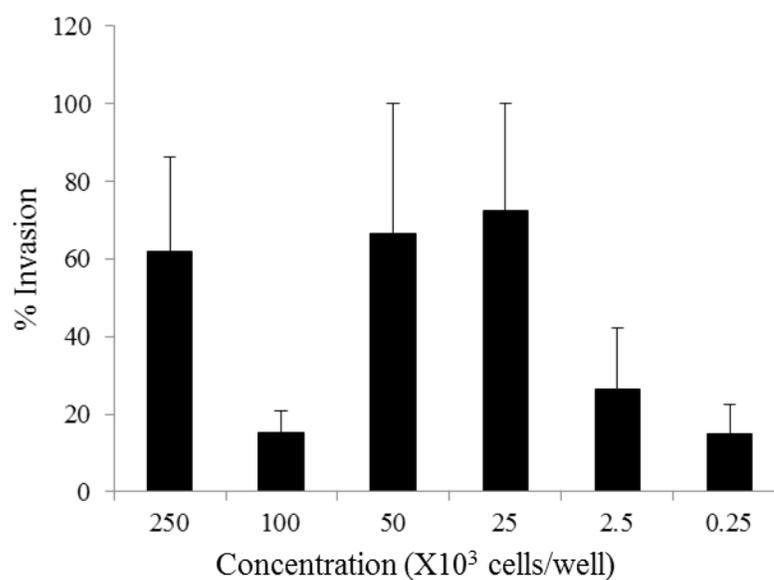


Figure H.2. The effect of chemoattractant on primary TCT invasion. There was no significant difference between the chemoattractants (medium B or medium C) on primary TCT (100×10^3 cells/well) invasion ($p > 0.05$) after 22 h in culture. Experiments were performed in triplicate ($n = 3$ dogs). Data was expressed as percent invasion of control \pm SEM. Significance was defined as $p < 0.05$.

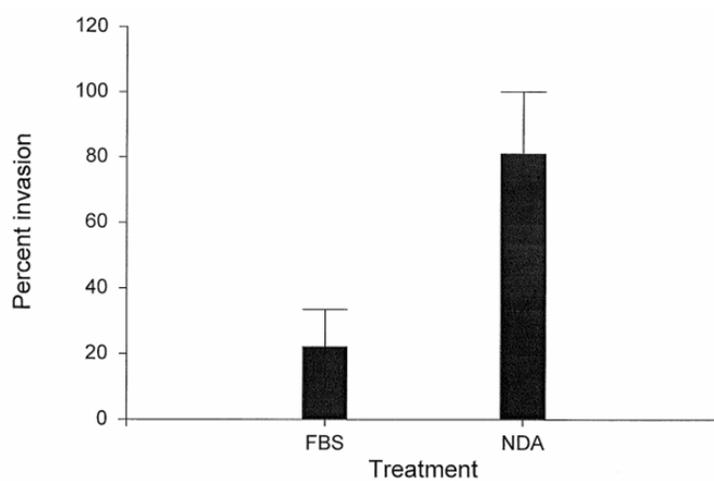


Figure H.3. The effect of incubation duration on primary TCT invasion. There was no significant effect of the length of incubation on TCT (100×10^3 cells/well) invasion after 22 and 46 h in culture ($p > 0.05$). Experiments were performed in triplicate ($n = 3$ dogs). Data was expressed as percent invasion of control \pm SEM. Significance was defined as $p < 0.05$.

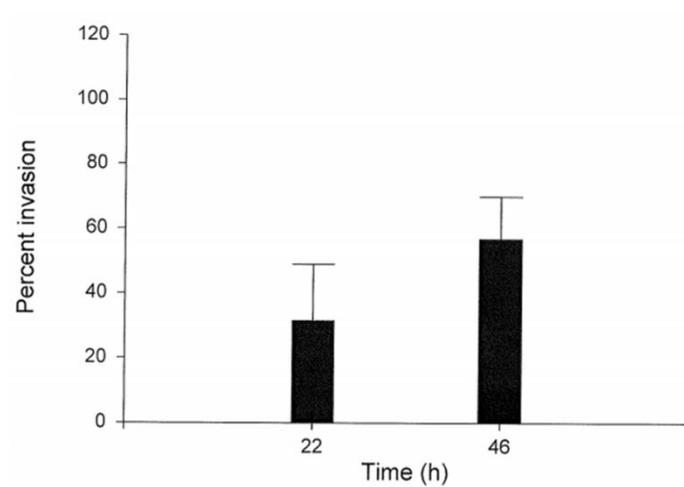
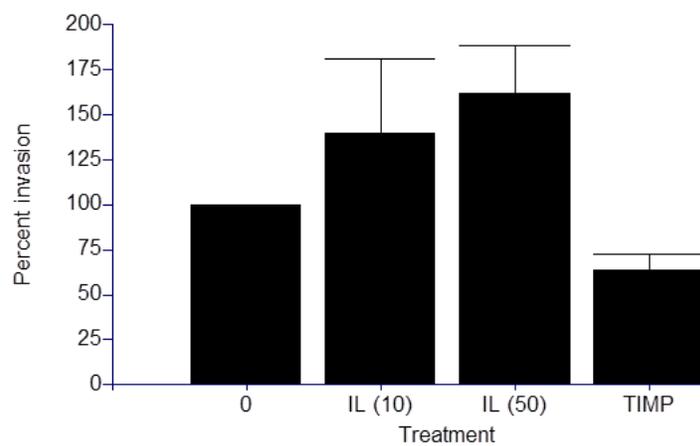


Figure H.4. The effect of IL-8 and TIMP-2 on primary TCT invasion. There was no significant effect of IL-8 (10 and 50 ng/ml) or TIMP-2 (0.5 $\mu\text{g/ml}$) on TCT (250×10^3 cells/well) invasion ($p > 0.05$) after 22 h in culture. Experiments were performed in triplicate ($n = 5$ dogs). Data was expressed as percent invasion of control \pm SEM. Significance was defined as $p < 0.05$.



APPENDIX I. PROLIFERATION ASSAY

Summary

The objectives for validating the MTT proliferation assay for term canine trophoblasts (TCT) included: 1) determining the optimal cell seeding concentrations, 2) comparing culture medium for cell proliferation; and 3) determining the IL-8 concentration most likely to effect canine trophoblast proliferation.

Experiment 1

Using a sterile 96-well microtiter plate, each column was seeded with a different cell concentration at 0, 10, 100, 1,000, 10,000, 100,000, 200,000-cells/well and incubated overnight in medium A or medium B (Appendix A). Briefly, after trophoblast cell isolation, cells were suspended in 100 μ l of medium (medium A or medium B) in varying concentrations (0, 1×10^3 , 10×10^3 , 50×10^3 , 100×10^3 , 200×10^3 cells/well and seeded in a 96-well plate.

Experiment 2

Proceeding from the optimization studies, factors were then added to the MTT assay to observe their effect on TCT. After primary trophoblast cell isolation, 100×10^3 cells/well were suspended in 100 μ l of medium A. Five 96-well tissue culture plates were prepared to study cell proliferation: 1) standard plate at t=4 h, 2) factor plate at t=32 h, 3)

factor plate at t=36 h, 4) factor plate at t=36 h, 5) factor plate at t=48 h. Experiments were performed in quadruplicate in 5 dogs.

Standard plate

Only $\frac{1}{4}$ of a 96-well plate were used to determine the standard curve. For the standard plate, cells were suspended in 100 μ l medium A to yield varying cell concentrations (0, 20×10^3 , 50×10^3 , 100×10^3 , 200×10^3 , and 500×10^3 cells/well) and seeded into Lanes 1-6 and Rows A-D of a 96-well plate. Immediately after cell seeding, 10 μ l MTT (5 mg/ml) was added to each well. The plate was then incubated at 37°C with 5% CO₂ for 4 h. After the incubation period, old medium was aspirated and replaced with 100 μ l 1-propanol per well and the plate was shaken on a rotator (Model #3520, Orbit Shaker, Lab-line Instruments, Inc., Melrose Park, IL) for 5 min until formazan salts were fully solubilized. If crystals were still visible, pipetting up and down (trituration) was required to dissolve the formazan. Keep in mind that plates must be read on a plate reader within 1 hour of adding the 1-propanol solvent. Absorbance was measured at 570 nm using a Microplate reader (LKB) and absorbance measured at 690 nm was subtracted as background.

Factor plates

The factors plates were developed the same as the standard plate, with modifications. Briefly, Only $\frac{1}{4}$ of a 96-well plate were used for factor experiments. For the factor plate, cells were suspended in 100 μ l medium A (10×10^3 cells/well) and seeded into Lanes 1-6 and Rows A-D of a 96-well plate. Lane 4 contained 100×10^3 cells/well

with 50 ng/ml IL-8 and Lane 5 contained 100×10^3 with 10 ng/ml IL-8. Three- 96-well plates were seeded in this manner to account for the different time points. Cells were allowed to adhere overnight then incubated an additional 8, 12, or 24 h to make the time points at $t = 32, 36, 48$ h after cell seeding in a tissue culture incubator at 37°C with 5% CO_2 . At the end of each given incubation ($t= 32, 36, 48$ h), $10 \mu\text{l}$ of MTT (5 mg/ml) was added to each well and incubated for 4 h at 37°C with 5% CO_2 . The formazan solubilization and measurements were performed as previously stated in the optimization experiments. The cell suspensions for the proliferation assay and invasion assay is described below.

Suspensions and dilutions

Cell suspensions and factor dilutions were performed as described in Tables I.1 and I.2.

Results

No protein precipitate was observed in the wells, which may interfere with absorbance readings. Data was analyzed with Two-way ANOVA. For the optimization studies, there was a cell seeding effect ($p < 0.001$), but there was no observed difference in proliferation between medium A and B ($p = 0.96$) and no seeding and medium interaction ($p = 0.91$). The optimal seeding concentration was determined to be 100×10^3 cells/well because the remaining concentrations elicited too low of an absorbance to allow for future studies that would stimulate or inhibit proliferation. For the factor studies, medium

A was used for the TCT cell suspensions. IL-8 and time had no effect on proliferation ($p=0.96$ and $p=0.49$, respectively) and there was no IL-8 and time interaction ($p=0.87$). A linear regression test was performed to convert optical density measurements into corresponding cell concentration, which the r value= 0.66. It was determined that at 20, 50, 100, 200X10³ cells/well allow accurate measurement of cell proliferation and 500X10³ cells/well is beyond the assay's sensitivity.

Summary

There was no significant difference between using medium A or medium B. From 0 to 1,000 cells/well the OD is virtually undetectable, therefore future experiments will be continue with higher seeding concentrations of 10X10³ cells/well and above.

Appendix I. References

Carmichael, J., DeGraff, W., Gazdar, A., Minna, J., Mitchell, J., 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 47, 936-942.
Cell Growth Determination Kit MTT Based, 2011. Catalog # CGD-1, Sigma, St. Louis, MO.

Table I.1. Cell suspensions for the proliferation assay for Experiment 2.

Starting cell concentration	Volume of medium A	Location & volume of aliquot	Final cell count
5000X10 ³ cells/ml	Suspended in 10 ml	a) Lane 1 of standard & factor plates (100 µl)	a) 500X10 ³ cells/well
5000X10 ³ cells/ml	1.5X remaining cell suspension volume	a) Lane 2 of standard & factor plates (100 µl) b) Lane 4 & Lane 5 of factor plates (50 µl) + PFM with or without IL-8 (50 µl) c) Lanes 2 - 5 of dose response plates	a) 200X10 ³ cells/well b) 100X10 ³ cells/well c) 100X10 ³ cells/well
2000X10 ³ cells/ml	1X remaining cell suspension volume	a) Lane 3 of standard & factor plates (100 µl) b) Lanes 1 of dose response plates (100 µl)	a) 100X10 ³ cells/well b) 100X10 ³ cells/well
500 X10 ³ cells/ml	1X remaining cell suspension volume	a) Lane 4 of standard plate (100 µl) b) Lane 5 of the standard plate (20 µl) + PFM (80 µl)	a) 50X10 ³ cells/well b) 10X10 ³ cells/well

Table I.2. Recombinant human IL-8 dilutions for Experiment 2 of the proliferation assay.

IL-8 concentration	Volume of human IL-8	Volume of medium A	Location & volume of aliquot
500 ng/ml	20 µl of stock IL-8 (100 ng/µl)	2 ml	Lane 5 dose response plate (50 µl/well)
100 ng/ml	0.6 ml of the above solution (500 ng/ml)	2.4 ml	Lane 4 dose response plate (50 µl/well)
50 ng/ml	2.5 ml of the above solution (100 ng/ml)	2.5 ml	Lane 3 dose response plate (50 µl/well) Lane 4 of factor plate (50 µl/well) Invasion assay test wells (250 µl/well)
10 ng/ml	0.6 ml of the above solution (100 ng/ml)	2.4 ml	Lane 2 of dose response plate (50 µl/well) Lane 5 of factor plate (50 µl/well) Invasion assay test wells (250 µl/well)

Figure I.1. A comparison of optical density (OD) using varying seeding concentrations and culture medium. There was a cell seeding effect ($p < 0.001$), but there was no observed difference in proliferation between medium A (protein-free) and medium B (10% FBS) and no interaction effect ($p > 0.05$, respectively). Experiments were performed in quadruplicate ($n = 5$ dogs). Mean \pm SEM absorbance positively corresponds to cellular proliferation using the MTT assay. Significance was defined as $p < 0.05$.

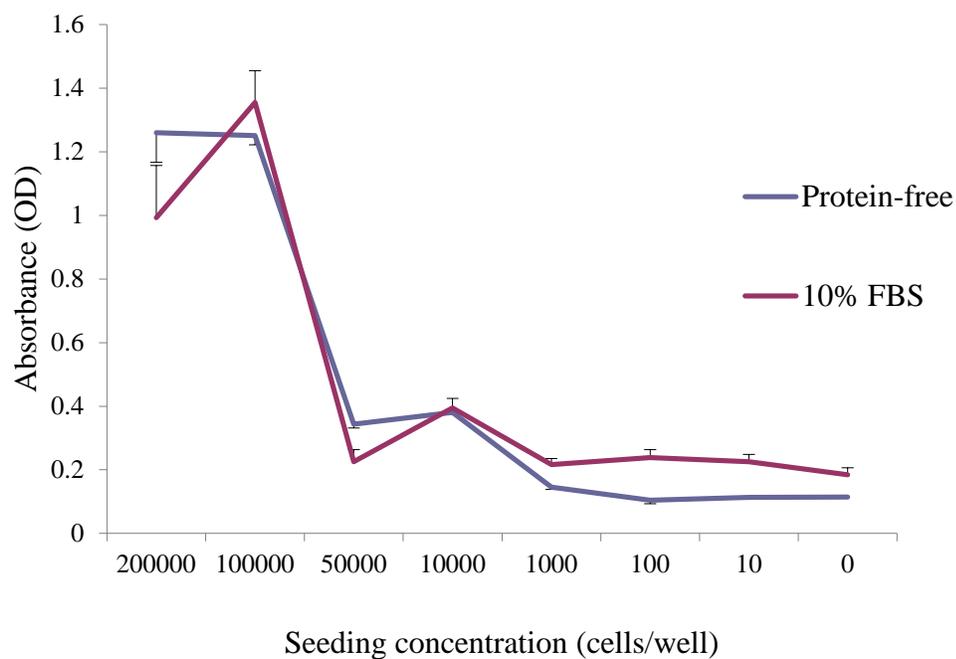


Figure I.2. A conversion of OD to cell concentration using the MTT assay. Linear regression determined that OD corresponds positively to the increasing cell proliferation (r value=0.66). A comparison of optical density using varying trophoblast seeding concentrations and culture medium. Experiments were performed in quadruplicate (n=5 dogs). Mean \pm SEM absorbance positively corresponds to cellular proliferation using the MTT assay. Significance was defined as $p < 0.05$.

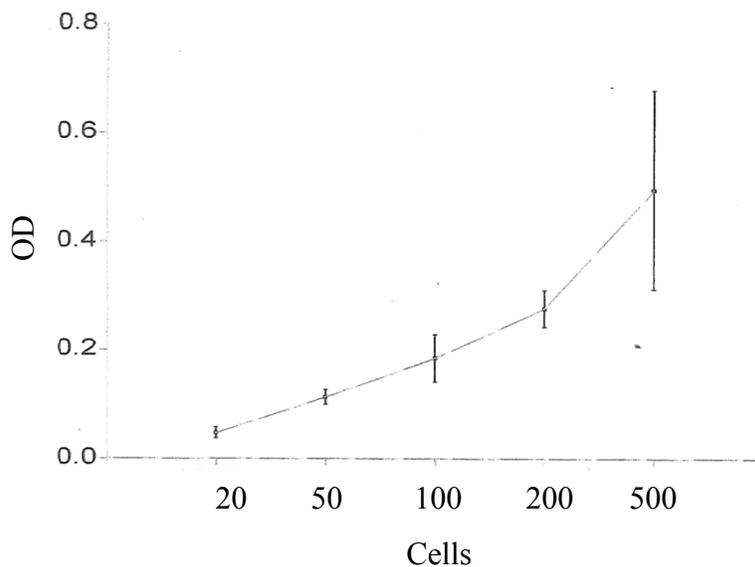
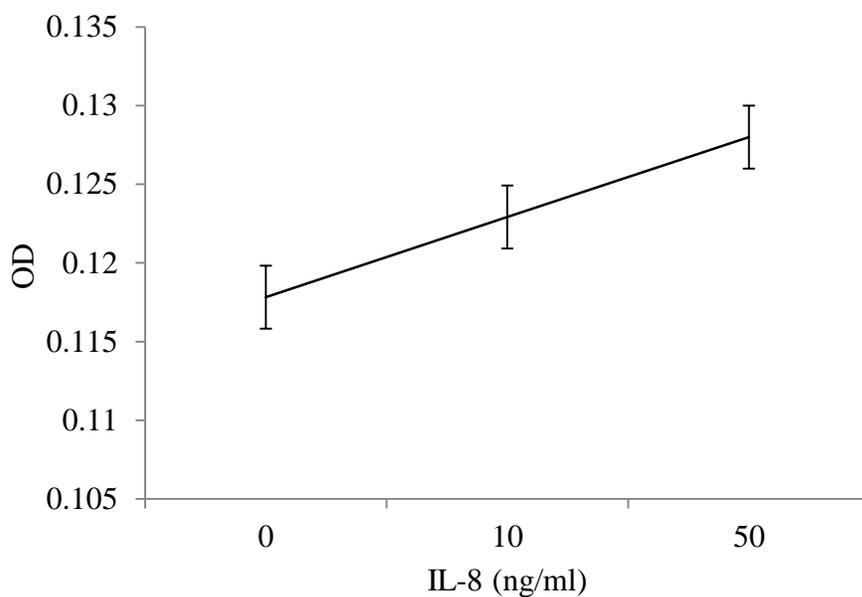


Figure I.3. The effect of IL-8 on primary TCT proliferation using the MTT assay. IL-8 and time had no effect on proliferation ($p=0.96$ and $p=0.49$, respectively) and there was no IL-8 and time interaction ($p=0.87$). The effect of IL-8 at $t=32$ h is shown below. Experiments were performed in quadruplicate ($n=5$ dogs). Mean \pm SEM absorbance positively corresponds to cellular proliferation. Significance was defined as $p<0.05$.



APPENDIX J. COMPARISON OF TWO PROLIFERATION ASSAYS

Summary

A comparison between the MTT and Dojindo proliferation assays was performed to examine which test had a better sensitivity and precision for measuring term canine trophoblasts (TCT) proliferation *in vitro* in the presence of IL-8.

History

According to Mosmann, the creator of the MTT assay, stock MTT (M2128, Sigma, Appendix A) was dissolved in phosphate buffered saline (PBS) at 5 mg/ml and then filtered to sterilize and remove insoluble MTT remnants (1983). Mouse lymphoma cells were seeded into a 96-well plate in 100 μ L RPMI medium supplemented with 5-10% fetal bovine serum (FBS). The stock MTT was added into each well at 10 μ L MTT per 100 μ L medium and cells were incubated with MTT for 4 hours at 37°C, 6% CO₂ atmosphere. Then 100 μ L acid-isopropanol (0.04 N HCl in isopropanol, Appendix A) was added to the wells and mixed to thoroughly dissolve the dark blue crystal produced and allowed to rest at room temperature for a few minutes. Plates were then read within an hour of adding the acid-isopropanol on an ELIZA reader at 570 nm with a reference wavelength of 630 nm. MTT served as the most useful reagent when several tetrazolium salts were incubated with cells for preliminary tests (Mosmann, 1983), due to the production of a dark blue formazan crystal product.

Importantly, studies by Mosmann found that the various cell types examined - mitogen stimulated T and B cells, myeloma, T lymphoma, and tumor cell lines - were able to reduce MTT to produce formazan suggesting that the MTT assay is widely applicable in the proliferation and survivability of various cell types. Shortly thereafter, Carmichael and associates found that significant modification of experimental conditions was required to optimize the application for drug sensitivity, or chemosensitivity, tests (1987) in Chinese hamster lung fibroblasts and human lung cancer cell lines. Although, Mosmann established the first protocol for the MTT proliferation assay (1983) in measuring live mammalian cells. Mainly, the issue of completely dissolving the blue formazan product was examined by using different solvents. Carmichael and associates followed the method established by Mosmann (1983), but used DMSO to achieve full solubilization of formazan in non-adherent cells and mineral oil in adherent cells. Absorbances were read at 540nm and 570nm for DMSO solvents and mineral oil, respectively.

Other cell viability and proliferation assays utilized various tetrazolium salts as substrates to add to the cells. XTT, sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate, formed a water-soluble, highly colored formazan product when cleaved by metabolically-active, viable cells. The water-soluble formazan product can be fully dissolved in tissue culture medium, thus a solvent is not needed, thereby reducing the assay time and cell handling and allowing for comparable assay sensitivity. Although, the reduction of XTT was

incomplete in their various murine cell lines, requiring an addition of electron coupling agents such as phenazine methosulfate (PMS) or menadione (MEN) (Roehm et al., 1991).

Recently, Dojindo has presented an alternative proliferation assay to the MTT assay, with the claim that Dojindo's Cell Counting Kit 8 (CCK-8, Dojindo Molecular Technologies, Rockville, MD) is a sensitive colorimetric assay for the determination of viable cells using a non-toxic substrate, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. The company proposes that their proliferation assay is faster (requiring no solubilization step) and more sensitive than the MTT assay and WST-8 has longer stability and is non-toxic to cells in culture, whereas MTT is toxic and causes cell death within 24 h in culture (Dojindo, 2012).

Factor Plates

The proliferation was performed as previously described (Carmichael et al., 1987; Dojindo, 2012). After overnight incubation to allow cell adherence to the plate, IL-8 (10 and 50 ng/ml) were then added to the Dojindo assay to observe their effect on TCT. After primary trophoblast cell isolation, 1×10^6 cells/well were suspended in 100 μ l of medium A. Five 96-well tissue culture plates were prepared to study cell proliferation: 1) standard plate at t=4 h, 2) factor plate at t=32 h, 3) factor plate at t=36 h, 4) factor plate at t=36 h, 5) factor plate at t=48 h, as stated in Appendix G. In addition, an extra plate was prepared as a IL-8 dose response with 0, 10, 50, 100 ng/ml using 100×10^3 cells/well. Experiments were performed in quadruplicate in 5 dogs.

Comparison Studies

The dose response plates were developed for the MTT and Dojindo assay the same way as the standard plate, with modifications. Briefly, $\frac{1}{4}$ of a 96-well plate were used for dose response experiments with or without 0, 10, 50, 100, 500 ng/ml. TCTs were suspended in 100 μ l medium A (100×10^3 cells/well) and seeded into Lanes 1-5 and Rows A-D of a 96-well plate. Lane 1 contained 100×10^3 cells/well with 0 ng/ml IL-8, lane 2 contained cells with 10 ng/ml, lane 3 contained cells with 50 ng/ml, lane 4 contained cells with 100 ng/ml, lane 5 contained PFM only. Cells were allowed to adhere overnight then incubated an additional 8, 12, or 24 h to make the time points at $t = 32, 36, 48$ h after cell seeding in a tissue culture incubator at 37°C with 5% CO_2 . At the end of each given incubation ($t = 32, 36, 48$ h), 10 μ l of MTT or WST-8 was added to each well and incubated. The formazan is then solubilized with 1-propanol for 5 min shaking vigorously. No formazan solubilization step is required for the Dojindo assay. Absorbance is measured at 570 nm for the MTT assay or 450 nm for the Dojindo assay using a Microplate reader (LKB). Background absorbance was measured at 690 nm. Experiments are performed in quadruplicate ($n=3$ dogs) to obtain optical density levels and cell concentrations are determined using a Linear Regression analysis.

Results

No protein precipitate was observed in the wells, which may interfere with absorbance readings. Data was analyzed with Two-way ANOVA. For the Dojindo factor studies, IL-8 and time had no effect on proliferation ($p=0.86$ and $p=0.88$, respectively)

and there was no IL-8 and time interaction ($p=1.0$). A linear regression test was performed to convert optical density measurements into corresponding cell concentration (r value= 0.76). It was determined that at 20, 50, 100, 200×10^3 cells/well allow accurate measurement of cell proliferation and 500×10^3 cells/well is beyond the assay's sensitivity. For the dose response comparison studies, there was an assay effect on cell proliferation ($p<0.001$), however there was no IL-8 dose response effect or assay and IL-8 ($p=0.88$ and $p=0.72$, respectively).

Summary

There was a significant difference between using the MTT assay and Dojindo assay. It was observed that Dojindo has a higher sensitivity in measuring cell proliferation compared to MTT ($p<0.001$), due to the higher OD measurements and less variation of viable cell counts between dogs ($n=3$ dogs). For the Dojindo assay, it was determined that between 20 - 200×10^3 cells/well the assay gives accurate readings of proliferation. Lastly, there was no effect of IL-8 ($p=0.88$) or interaction between assay and IL-8 ($p=0.72$).

Appendix J. References

- Carmichael, J., DeGraff, W., Gazdar, A., Minna, J., Mitchell, J., 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 47, 936-942.
- Dojindo, 2012. Cell counting kit-8: cell proliferation and cytotoxicity assay. Technical manual. Retrieved on March 30, 2013 from http://www.dojindo.com/TechnicalManual/Manual_CK04.pdf.

Figure J.1. A conversion of OD to cell concentration using the Dojindo assay. Linear regression determined that OD corresponds positively to the increasing TCT proliferation (r value=0.76). A comparison of optical density using varying TCT seeding concentrations and culture medium. Experiments were performed in quadruplicate (n=3 dogs). Mean \pm SEM absorbance positively corresponds to cellular proliferation. Significance was defined as $p < 0.05$.

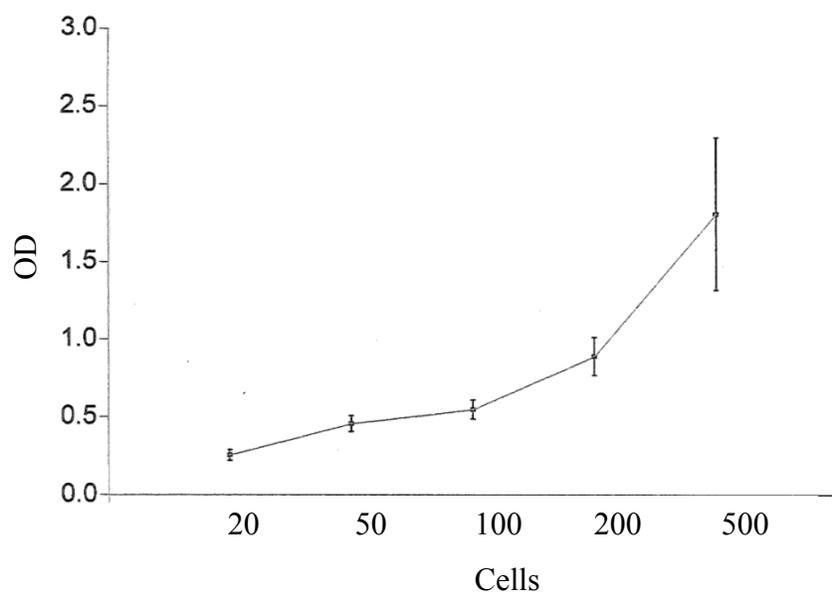


Figure J.2. The effect of IL-8 on primary TCT proliferation using the Dojindo assay. IL-8 and time had no effect on proliferation ($p=0.86$ and $p=0.88$, respectively) and there was no IL-8 and time interaction ($p=1.0$). Experiments were performed in quadruplicate ($n=3$ dogs). Mean \pm SEM absorbance positively corresponds to cellular proliferation. Significance was defined as $p<0.05$.

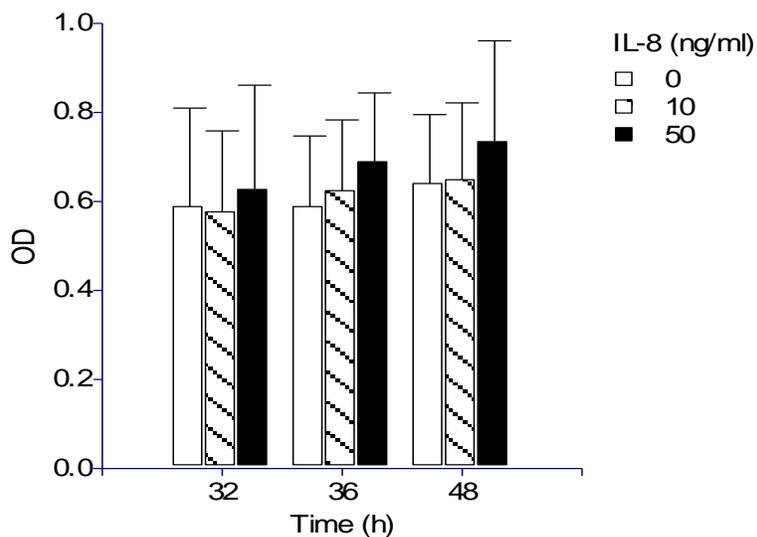
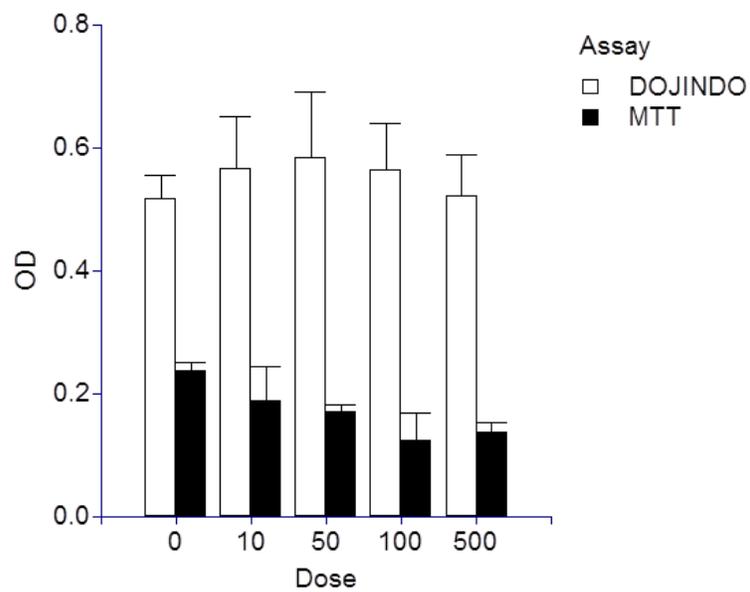


Figure J.3. The effect of IL-8 on primary TCT proliferation using the MTT and Dojindo assays. There was an assay effect ($p < 0.001$), although IL-8 and IL-8 and assay interaction had no effect on proliferation ($p = 0.86$ and $p = 0.72$, respectively). Experiments were performed in quadruplicate ($n = 3$ dogs). Mean \pm SEM absorbance positively corresponds to cellular proliferation. Significance was defined as $p < 0.05$.



APPENDIX K. IMMUNOSTAINING OF MATRIGEL INVASION FILTERS

The immunostaining of the Matrigel-coated filter membranes was performed as previously described (Musch et al., 2007; Sahlfeld et al., 2012) to validate the trophoblast identity of invading cells in the invasion assay. Cells were isolated from term canine placentas (n=1 dog) and seeded into the Matrigel invasion assay and incubated for 22 h. Filters were fixed with 70% methanol (35 ml methanol with 15 ml sterile water) and washed three times. Using forceps to hold the upper well chamber with filter membrane intact, the filter was gently rinsed by adding PBS (dropwise from transfer pipet, Appendix A) indirectly onto the filter to prevent washing off cells. The filter was then tipped into the waste container to drain excess PBS. After three washes, the filter was removed from the upper well chamber using a scalpel and placed bottom-side up where the invading cells were deposited into a Parafilm® lined 33 mm² culture dish.

A blocking buffer (1:20 dilution) was prepared with 180 µl donkey serum in 3420 ml sterile, filtered PBS to make 3600 µl. A drop (approximately 25 µl) of blocking buffer was added to form a dome on each filter and incubated for 3 minutes. Filters were then again washed three times by adding 25 µL PBS onto the filter for 3 minutes at room temperature. The primary antibody of cytokeratin-7 (CK-7, Dako, p103620) was prepared in blocking buffer at a 1:200 dilution and 25 µL was applied to each filter. The filters were placed into humidified chambers created by placing PBS-moistened kimwipes in the bottom of a plastic box for overnight incubation at 4°C. Filters were then washed again three times with PBS as earlier described.

The secondary antibody was prepared by diluting a donkey anti-mouse Alexa Fluor 488 (Life Technologies, A21202) at 1:1000 in blocking buffer. Secondary antibody (25 μ L) was added to form a dome on each filter and coverslips were wrapped in foil and place in dark, humidified box to incubate at room temperature for 2 hours. After the incubation, filters were again washed 3 times for 3 minutes each with PBS. To stain nuclei, 25 μ L of Hoechst 3342 nuclear stain (H1399, Invitrogen, Carlsbad, CA) was prepared (1:20,000) and added for a 15 minute incubation at room temperature for 15 minutes 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 magnification on a Leica DM 4000B microscope. Digital images were captured using a mounted digital camera (QImaging QICAM 12-bit, QIC-F-M-12-C, QImaging, Surrey, BC) with image capture software (QCapturePro, QImaging, Surrey, BC).

Results

There was too much Hoechst (blue) background fluorescence to allow for accurate counting of Hoechst-positive nuclei and CK-7-positive cells.

Appendix K. References

- Musch, M., Walsh-Reitz, M., Kosovsky, M., 2007. Preparation of BD Falcon™ Cell Culture Inserts for Confocal Indirect Immunofluorescence: Fixation and Staining of Caco-2/bbe (C2) Cells with Various Dyes. BD Biosciences Technical Bulletin #459.
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APPENDIX L. ABSTRACTS AND POSTER

Summary

I was fortunate to have the opportunity to present my research at regional, national and international conferences throughout my graduate career.

Abstract presented at the 2012 Northwest Reproductive Sciences Symposium (NWRSS) in Portland, OR and the 2012 International Symposium on Canine and Feline Reproduction (ISCFR) in Whistler, British Columbia, Canada.

Determining seeding concentrations for in vitro canine cytotrophoblast invasion studies.

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INTRODUCTION: Cytotrophoblasts are specialized epithelial cells of the placenta that proliferate and invade the endometrium at implantation. After formation of a chorioallantois villous labyrinth in early pregnancy, canine cytotrophoblasts continue to advance into the maternal endometrium and remodel the endometrial tissues throughout gestation (1). Little is known about the mechanisms that regulate these processes in dogs, much less the mechanisms that go awry when pathologic cytotrophoblast conditions occur (e.g., subinvolution of placental sites). In humans and rodents, cytotrophoblast invasion has been studied in vitro using Matrigel invasion assays (2,3). To yield the desired 50% cell invasion, research in these species has shown that the optimal seeding concentration for Matrigel invasion assays is 250×10^3 cells/ml (4). The objective of the current study was to determine the optimal seeding concentration of canine cytotrophoblasts for Matrigel invasion assays. Based upon the previously mentioned research in humans, we hypothesized that 250×10^3 cells/ml would yield the desired 50% cell invasion.

METHODS: Canine chorioallantois tissue was collected without the marginal hematoma following elective term C-sections (n=3) and cytotrophoblasts were isolated as described by Sahlfell and coworkers (5). Briefly, cytotrophoblasts were isolated using collagenase and trypsin digestions with Percoll density gradient centrifugation. Four seeding concentrations were compared (0.25, 2.5, 25 and 250×10^3 cells/ml). Cytotrophoblasts were cultured for 22 hours at 37°C, 5% CO₂ in a protein-free media using the Matrigel invasion assay (8µm pore diameter, polyethylene terephthalate, BD Falcon) using 5% fetal bovine serum as a chemoattractant. Cytotrophoblasts were grown on inserts without Matrigel and treated in the same manner to serve as controls. Cells were stained with Diff Quik® (Harleco, Inc.), and all invading cells were counted at 400X magnification. Percent invasion was determined for each of the conditions by dividing the number of cytotrophoblasts that invaded through the Matrigel by the number of cytotrophoblasts that invade through the control inserts without Matrigel. Results were summarized as mean ± SEM and significance was defined as $p < 0.05$.

RESULTS: Percent invasion did not differ significantly between the four seeding concentrations (Figure 1). However, the concentration that was closest to the desired 50% cell invasion was 250 X 10³ cells/ml.

CONCLUSION: Canine cytotrophoblast invasion is an unexplored field. This study demonstrated that canine cytotrophoblast invasion can be investigated in vitro using Matrigel invasion assays. Our results suggest that 250 X 10³ cells/ml is the seeding concentration that would yield approximately 50% cell invasion.

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- (4) Lei T, Hohn HP, Behr R et al. Influences of extracellular matrix and of conditioned media on differentiation and invasiveness of trophoblast stem cells. *Placenta* 2007;14-21.
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Figure 1. Influence of seeding concentration on cell invasion. Four cell concentrations (0.25, 2.5, 25 and 250 X 10³ cells/ml) were seeded onto Matrigel inserts. After a 22-hour incubation, the invading trophoblasts were counted to determine mean±SEM percent invasion.

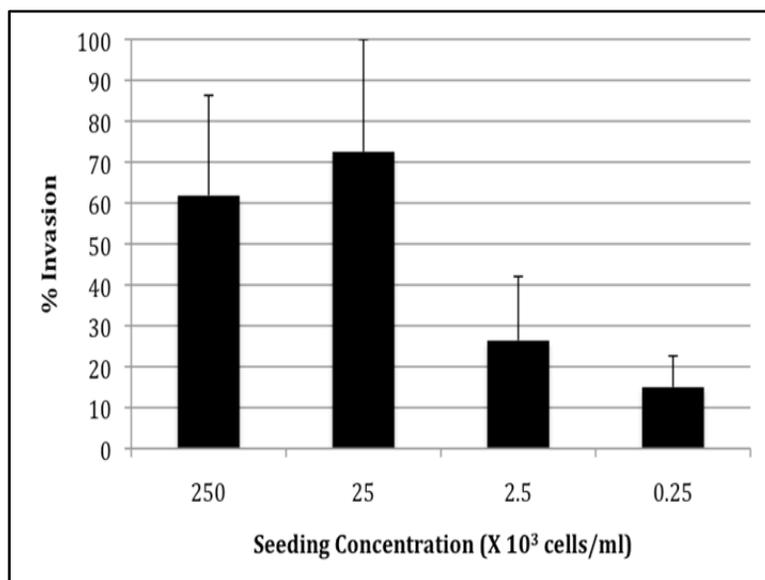
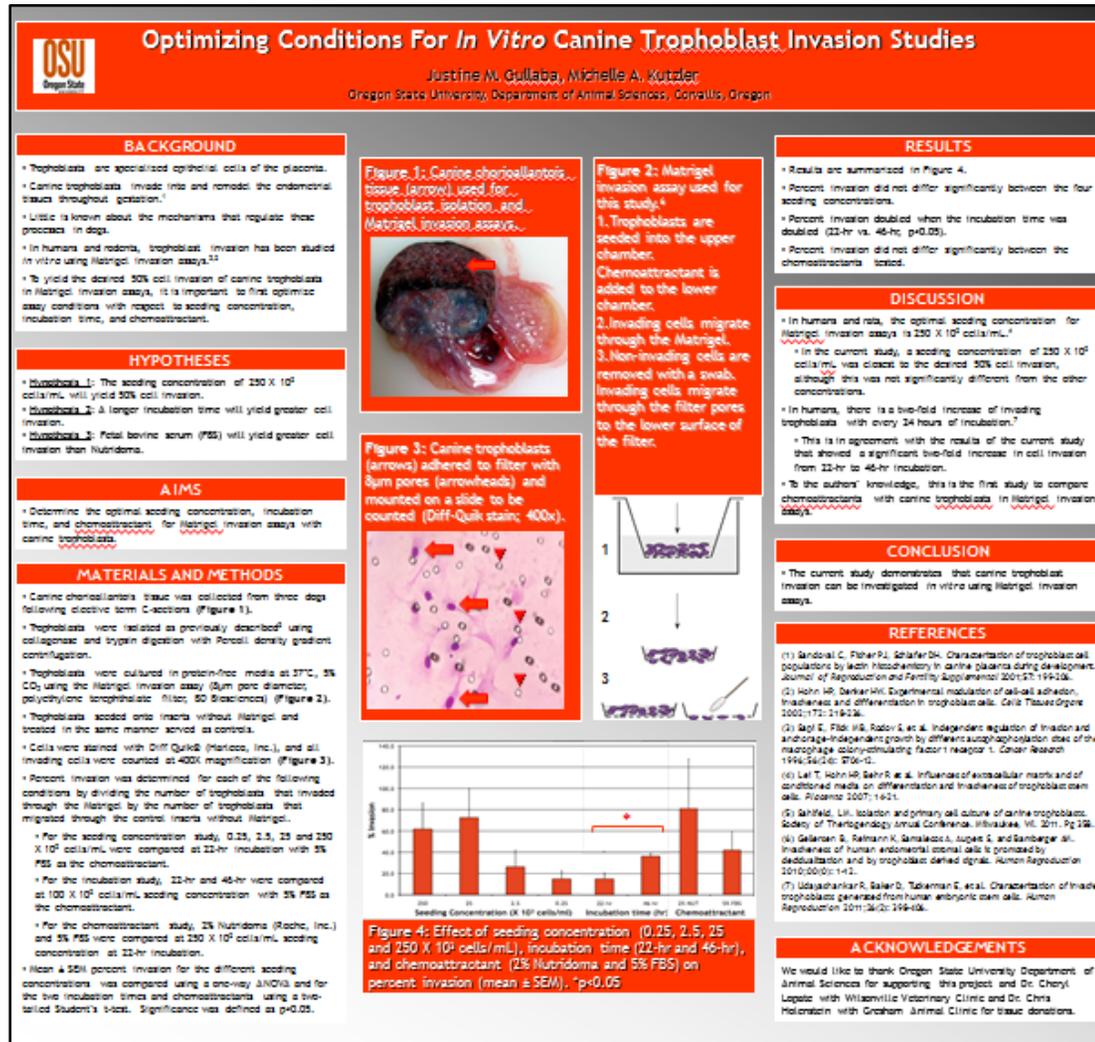


Figure L.1. Poster presented at the 2012 NWRSS in Portland, OR and the 2012 ISCFR in Whistler, British Columbia, Canada.



Abstract presented at the 2013 Society for Theriogenology Annual Conference in
Louisville, KY.

Utilizing a Wound Healing Assay to Study Canine Trophoblast Physiology In Vitro

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Introduction The wound healing assay is used to study a cell's ability to proliferate and migrate in culture [1]. Interleukin-8 (IL8) and tissue inhibitor of metalloproteinase-2 (TIMP2) stimulate and inhibit, respectively, term human trophoblast proliferation and migration in vitro [2, 3]. We hypothesized that term canine trophoblasts (TCTs) would respond similarly. The objectives were to compare wound healing rates in TCTs at increasing concentrations of IL8 and TIMP2.

Methods Chorioallantois was collected from 4 dogs following term elective C-sections. TCTs were isolated from the chorioallantois as previously described [4] and seeded (1×10^6 cells/well) in 24-well plates in DME/H-21 medium (Gibco, #11965, Life Technologies, Grand Island, NY) supplemented with 10% FBS (Atlanta Biologicals, L11061, Lawrenceville, GA). TCTs were cultured at 37 °C with 5% CO₂ until confluent. Cell proliferation and migration was studied using the wound healing assay as previously described [1]. The effects of recombinant human IL8 (#200-08M, Peprotech, Rocky Hill, NJ) and recombinant human TIMP2 (#410-02, Peprotech) were evaluated at 0, 10, 50, 100 ng/ml or 0, 0.05, 0.1, 0.5 µg/ml, respectively. The wound was photographed at 100X phase-contrast magnification with a digital camera (Fujifilm Fine AX300 14 MP, Tokyo, Japan) at 0, 8, and 12 hours of incubation. The wound area was measured using ImageJ v.1.34 software (<http://imagej.nih.gov/ij/>). The wound width at t=0 h was defined as 0% wound closure. Experiments were performed in triplicates. Data was analyzed by Repeated Measures ANOVA using SAS (Version 9.2, SAS Institute Inc., Cary, NC) in PROC MIXED. Results were summarized as mean±SEM % wound closure. Significance was defined as $p < 0.05$.

Results Compared to controls, wound healing rates were promoted with IL8 (10 and 50 ng/ml) at t=8 hr ($p=0.0172$ and 0.0046 , respectively) and 12 hr incubation ($p=0.0133$ and 0.0005 , respectively) and were reduced with TIMP2 (0.5 µg/ml) at t=8 hr ($p=0.0097$) (Figure 1).

Discussion An understanding of normal TCT physiology will provide a basis for research on abnormal TCT conditions (e.g. placental retention or subinvolution of placental sites). Further research is needed to determine if differences in wound healing result from effects on migration, proliferation or both.

References

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- [2] Jovanovic M, et al. *Reproduction* 2010;139:789-98.
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Figure 1. Effect of IL8 (open bars) or TIMP2 (solid bars) on wound healing in TCT (*p<0.05).

