AN ABSTRACT OF THE THESIS OF

Elizabeth Jane Fellows for the degree of Master of Science in Animal Sciences presented on August 30, 2012

Title: Gene Expression in the Peripartum Canine Placenta

Abstract Approved:

Michelle A. Kutzler

This research investigated gene expression in the canine placenta during the peripartum period. Previous studies have recognized molecular changes that occur in the placenta around the time of placental release in other species, but no study has looked at gene expression in the late gestation canine placenta. Of particular significance for this thesis work is the groundwork laid for future studies modeling placental abnormalities in dogs (e.g. subinvolution of placental sites) and humans (e.g. preeclampsia, placenta accreta). Despite years of research in multiple species, the exact mechanisms and processes regulating trophoblast invasion and placental release remain unclear. Therefore, the specific objective of this research was to characterize gene expression changes that occur during the peripartum period in the dog using microarray and real-time RT-PCR. Following total RNA isolation, the microarray analysis was performed by hybridizing total RNA to the Canine 2.0 Array (Affymetrix, Santa Clara, CA). Microarray analysis was carried out using the limma and affy packages through the Bioconductor software in the R statistical environment. Differential expression was defined as $p \leq 0.05$, FDR $p \leq 0.10$ and a log fold change of ≥ 1.2. Following cDNA synthesis, real-time RT-PCR was performed using TaqMan primer and probes that were pre-made and pre-optimized for

canine tissues (Applied Biosystems, Carlsbad, CA). Microarray analysis showed differential expression in 18 genes between pre-term and pre-labor sample groups, 38 genes that were differentially expressed between pre-term and parturient samples and no genes that were differentially expressed between pre-labor and parturient samples. Microarray analysis led to the identification of several candidate genes for closer investigation using real-time RT-PCR. These genes included MMP-1, MMP-2, MMP-9, TIMP-2, VEGF-A, Flt-1, CD44, DAG-1, IL-6 and CXCL10. All of these genes have been linked to trophoblast invasion or regression or placental release in a number of species including humans, cattle and rodents. Using real-time RT-PCR, there was a significant difference in MMP-9 mRNA expression in pre-term samples compared to pre-labor and parturient samples ($p<0.05$). However, there was no significant difference in mRNA expression of MMP-2, TIMP-2, VEGF-A, Flt-1 CD44, DAG-1, IL-6 or CXCL10. Future studies may focus on additional candidate genes identified by microarray that play a role in tissue remodeling at the end of canine gestation such as IL-8, EPHX2, PI3 and SERPINE1.

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APPROVED:

Major Professor representing Animal Sciences

Head of the Department of Animal and Rangeland Sciences

Dean of the Graduate School

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.

Elizabeth Jane Fellows, Author

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CONTRIBUTIONS OF AUTHORS

Dr. Timothy Hazzard assisted in the collection of placentas and edited the manuscript. Lauren Watson assisted in the analysis of microarray data and edited the manuscript. Justine assisted in the isolation of trophoblasts and edited the manuscript.

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CHAPTER I

INTRODUCTION

1.1 GENETIC REGULATION OF TROPHOBLAST MIGRATION AND INVASION

1.1.1 Significance

Determining the mechanisms of trophoblast invasion in various placental types is crucial for a better understanding of pathologic trophoblast processes and conditions. Clarifying the regulatory mechanisms of normal trophoblast invasion will allow a better understanding of abnormal trophoblast invasion. The etiologies of pathologic conditions involving shallow trophoblast invasion such as preeclampsia in humans (Brosens et al., 1972; Pijnenborg et al., 1991) and uninhibited trophoblast invasion such as placenta accreta in humans (Davidson, 1944; Khong and Robertson, 1987) or subinvolution of placental sites (SIPs) in dogs (Al-Bassam et al., 1981; Fernández et al., 1998) remain elusive.

1.1.2 Summary

Trophoblast invasion is crucial for anchoring the embryo to the uterus (Gao et al., 2001) and is essential for the successful development of the placenta in deciduate species. A general placental classification distinguishes between deciduate and adeciduate types. In the adeciduate placental type, the endometrium is left intact at parturition. This type of placenta is seen in larger domestic animals (e.g. ruminants, pigs, horses). The deciduate placental type is seen in primates and rodents, with a modified form seen in carnivores (Roberts, 1986). In this type of placenta, the endometrium is lost at parturition. In

primates and rodents, deeper layers of the endometrium are lost, whereas this loss is more superficial in dogs (Jainudeen and Hafez, 1993).

In addition to mechanical disruption and penetration of the endometrium, trophoblast invasion is an active process involving secretion of multiple factors (Fisher et al., 1985). Within the placenta, the trophoblast is the only cell with the ability to degrade the extracellular matrix within the endometrium (Pijnenborg et al., 1980; Fisher et al., 1985). Many studies compare trophoblast invasion to tumor invasion. However, unlike tumor invasion, trophoblast invasion is regulated by time and space (Graham and Lala, 1991; Bischof et al., 2000). A number of autocrine and paracrine factors regulate trophoblast invasion including matrix metalloproteinases, tissue inhibitors of metalloproteinases, components of the extracellular matrix, and cytokines (Bischof et al., 2000; Bischof et al., 2002).

1.1.3 Matrix Metalloproteinases and their Tissue Inhibitors

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are crucial for the process of cell invasion. Matrix metalloproteinases are a family of over 20 zinc dependent proteases that can degrade the extracellular matrix (ECM) (McCawley and Matrisian, 2001). This family includes gelatinases (MMP-2, MMP-9), which degrade collagen type IV, a major component of the basement membrane. Other members of this family include collagenases (MMP-1, MMP-8, MMP-13), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11, MMP-12) and membrane-bound metalloproteinases (MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP) (Matrisian, 1990; Bischof and Campana, 2000). Membrane-bound metalloproteinases are important for the activation of latent MMPs such as pro-MMP-2 (Sato et al., 1994; Bjørn et al., 2000).

Support for a role of MMPs in trophoblast invasion comes from the localization of these factors in trophoblasts of several species: sheep (Salamonsen et al., 1995; Riley et al., 2000), pigs (Menino et al., 1997), cows (Hashizume 2007) and goats (Uekita et al., 2004). In the horse, MMPs are involved in chorionic girdle trophoblast invasion

(Vagnoni et al., 1995). In humans, both mRNA and protein expression of MMP-9 (Hurskainen et al., 1996; Huppertz et al., 1998; Xu et al., 2000) and MMP-14 have been reported in isolated first trimester trophoblasts (Huppertz et al., 1998; Xu et al., 2000). In addition, MMP-3 is also expressed in invasive human trophoblasts (Huppertz et al., 1998; Husslein et al., 2009) and MMP-7 (Vettraino et al., 1996) is present in intermediate trophoblasts and the decidua suggesting both of these MMPs influence trophoblast invasion of the spiral arteries.

Matrix metalloproteinases, specifically MMP-2 and MMP-9, have been identified as rate-limiting enzymes for trophoblast invasion in mice (Behrendtsen et al., 1992; Alexander et al., 1996), non-human primates (Gao et al., 2001; Wang et al., 2001) and humans (Librach et al., 1991; Staun-Ram et al., 2004). In murine trophoblasts, MMP-2 and MMP-9 mRNA expression show major spatial and temporal changes during implantation (Das et al., 1997). Furthermore, MMP-2 has been localized to specialized cell membrane extensions known as “invadiopodia”, involved with degradation of the extracellular matrix (Monsky et al., 1993). In vivo and in vitro studies have shown that MMP-2 (Isaka et al., 2003; Staun-Ram et al., 2004) and MMP-9 (Librach et al., 1991; Morgan et al., 1998; Luo et al., 2011) are critical for trophoblast invasion in humans; whereas MMP-2, MMP-9, and MMP-14 (MT1-MMP) are crucial for trophoblast invasion in rhesus monkeys (Wang et al. 2001).

Tissue inhibitors of metalloproteinases (TIMPs) are involved with the regulation of trophoblast invasion through MMP inhibition. The activity of MMPs, specifically MMP-2 and MMP-9, is regulated at the transcriptional level by proenzyme activation as well as by TIMP-1 and TIMP-2 (Matrisian, 1990; Woessner, 1991). The TIMP family consists of four members: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. Within the placenta, TIMP-1 has been identified in both trophoblasts and fibroblasts, while TIMP-2, TIMP-3 and TIMP-4 are primarily secreted by trophoblasts (Riley et al., 1999). TIMP-1 has the ability to inhibit most MMPs (Gao et al., 2001; Goldman et al., 2003) and its expression is non-cell specific, suggesting its role is to protect the trophoblast from degradation by its own MMPs (Gao et al., 2001). TIMP-2 is most likely of maternal origin and inhibits

invasion of first trimester human trophoblasts (Librach et al., 1991), possibly through regulation of MMP-2 (Itoh et al., 1998).

The mRNA expression of TIMP-3 has been shown to correlate with MMP-9 during early gestation at the peak of trophoblast invasion (Bass et al., 1997). A balance between MMP-9 and TIMP-3 activity is proposed to regulate the depth of trophoblast invasion in monkeys (Wang et al., 2001) and rodents (Das et al., 1997). In rhesus monkeys, TIMP-1, TIMP-2 and TIMP-3 mRNA expression is thought to be crucial for regulation of trophoblast invasion (Wang et al., 2001). Major spatiotemporal changes in TIMP-3 mRNA expression also occur during murine implantation (Das et al., 1997). In humans, both mRNA and protein expression of MMP-9, TIMP-1, TIMP-2 and TIMP-3 have been localized to early gestation cytotrophoblasts (Hurskainen et al., 1996; Huppertz et al., 1998; Xu et al., 2000).

1.1.4 KiSS-1 and its receptor KiSS-1R

The product of the KiSS-1 gene and its receptor, KiSS-1R have been isolated at high levels from the human placenta (Ohtaki et al., 2001) and from trophoblast giant cells of the rat placenta (Terao et al., 2004). Prior to its localization in the placenta, KiSS-1 was found to suppress MMP-9 expression in fibrosarcoma cells through reduced NK-κB binding to the MMP-9 promoter (Yan et al., 2001), suggesting a role of KiSS-1 in cell invasion. The expression of KiSS-1 and KiSS-1R have been found to be parallel, suggesting an autocrine mechanism of activation.

There is still debate over the temporal expression of KiSS-1 and KiSS-1R in the placenta. In the rat, mRNA expression of KiSS-1 and KiSS-1R is seen in the trophoblast giant cells on embryonic day 12.5. After embryonic day 12.5, its expression gradually decreases until term (Terao et al., 2004). KiSS-1 and KiSS-1R have been found to be expressed at significantly higher levels in first trimester human trophoblasts than term cells using semi-quantiative RT-PCR and DNA microarray (Bilban et al., 2004), suggesting their role is more important in early pregnancy. However, in another study,

using real-time RT-PCR, KiSS-1R mRNA levels were higher in first trimester human trophoblasts compared to term, while KiSS-1 mRNA levels did not significantly change during gestation (Janneau et al., 2002). The decline of KiSS-1 and KiSS-1R expression from early gestation to term reported in multiple studies seems counterintuitive, but perhaps the role of KiSS-1 in early gestation is to regulate the depth of trophoblast invasion.

Despite differences in temporal expression of KiSS-1 and KiSS-1R, the evidence suggests it may play a significant role in the inhibition of trophoblast invasion. Kisspeptin-10 (Kp-10), a product of the KiSS-1 gene, has been shown to inhibit trophoblast migration in vitro presumably by inhibiting protease activity of MMP-2 (Bilban et al., 2004). Furthermore, the invasion ability of trophoblasts transfected with the KiSS-1 gene is significantly lower than non-transfected cells and controls (Zhang et al., 2011). In addition, activation of the KiSS-1 receptor inhibits cell invasion and addition of KiSS-1 peptide to cells transfected with the receptor inhibits their invasion (Stafford et al., 2002).

1.1.5 Vascular Endothelial Growth Factor-A and its Receptor (Flt-1)

Vascular endothelial growth factor subtype A (VEGF-A) and its receptor (Flt-1) are expressed in human trophoblasts (Shore et al., 1997; Lash et al., 1999; Lash et al., 2003; Lash et al., 2010). Following the binding of VEGF-A, Flt-1 initiates a JNK signaling cascade (Shore et al., 1997). VEGF acts as a chemoattractant for first trimester human trophoblasts but not term trophoblasts, which suggests trophoblasts change their responsiveness to VEGF throughout gestation (Lash et al., 2003).

Treatment of trophoblasts with exogenous VEGF increases trophoblast motility but not invasion (Lash et al., 1999), suggesting that VEGF may play a role in the initiation of cell invasion, but lacks the protease activity needed to carry out the necessary degradation to complete the invasion process. In addition, levels of soluble Flt-1 secreted by human cytotrophoblasts increase with gestational age, suggesting a limit on the

bioavailability of placental VEGF-A as pregnancy progresses (Lash et al., 2010). In human umbilical vein endothelial cells, VEGF has been shown to stimulate the expression of Flt-1 and MMP-1 (Sato et al., 2000). Additionally, VEGF treatment of human smooth muscle cells has also been shown to increase mRNA expression of MMP-1, -3 and -9 as well as enhance invasion of these cells in vitro (Wang and Keiser, 1998).

1.1.6 Interleukins

Members of the interleukin family have been implicated in the regulation of trophoblast invasion in humans and non-human primates through the regulation of MMP expression (as reviewed by Bischof et al., 2000). Immunolocalization of IL-1α, IL-1β and IL-6 in cells of the conceptus and at the implantation site in rhesus monkeys (Sengupta et al., 2003) supports a role for interleukins in the implantation process. Both interleukin-1β (IL- 1β) (Librach et al., 1994) and IL-1α (Meisser et al., 1999b; Gonzalez et al., 2001) increase MMP-9 activity. Interleukin-6 (IL-6) may also be involved with the regulation of trophoblast invasion by stimulating the activation of MMP-2 and MMP-9, possibly through an increased secretion of leptin by cytotrophoblasts (Meisser et al., 1999a).

Interleukin-1β (IL-1β) up-regulates MMP-2, MMP-9, MT1-MMP, MT2-MMP and urokinase plasminogen activator (uPA) mRNA in first trimester trophoblasts (Karmakar and Das, 2002). In immortalized human trophoblast cell lines ED_{27}, ED_{31}, and $ED_{77,}$ expression of MMP-9 is stimulated by IL-1β (Morgan et al., 1998). In addition, recombinant IL-1β treatment of trophoblasts increases MMP-3 mRNA expression (Husslein et al., 2009). IL-1β has also been shown to stimulate invasion in vitro (Librach et al., 1994).

It has been suggested that IL-1β acts in both an autocrine and paracrine manner to influence proteases during pregnancy and may initiate a cascade that activates MT-MMPs, which then activate other MMPs (Karmakar and Das, 2002). Alternatively, it has been suggested that IL-1β directly promotes trophoblast motility using the uPA/PAI system. IL-1β induces the secretion of uPA, PAI-1 (plasminogen activator inhibitor

(PAI)-1 and PAI-2 by trophoblasts, influencing trophoblast invasion. In addition, up-regulation of IL-1 receptor (IL-1R1) expression during extravillous trophoblast differentiation suggests IL-1R1 may also affect invasion of the trophoblasts (Prutsch et al., 2012).

Interleukin-8 (IL-8) as well as its receptor CXCR1 are expressed in the immortalized first trimester trophoblast cell line HTR-8/SVneo and isolated cytotrophoblasts (Jovanović et al., 2010). Invasion assays and zymography have shown that treatment of trophoblasts with IL-8 stimulates migration and invasion as well as increases proMMP-2 and MMP-9 levels. In addition, α_5 and β_1 integrins have been linked to an increase in trophoblast invasion following IL-8 treatment (Jovanović et al., 2010). Therefore, the role of IL-8 in trophoblast invasion may be to stimulate expression of MMP-2, MMP-9 and integrins.

Incubation of the human placental choriocarcinoma cell line (JEG-3) with interleukin 15 (IL-15) causes a significant increase in MMP-1 concentration. This suggests a role for IL-15 in cell invasion through the regulation of MMP-1 production by trophoblasts. Furthermore, treatment of trophoblasts with IL-15 results in a significant increase in invasion and migration in vitro (Zygmunt et al., 1998).

Trophoblasts express interleukin 10 (IL-10) receptor mRNA and IL-10 synthesis by trophoblasts is inversely related to MMP-9 secretion. Furthermore, treatment of trophoblasts in vitro with IL-10 decreases MMP-9 mRNA levels. This has lead to the hypothesis that IL-10 is an autocrine regulator of trophoblast invasion. IL-10 likely plays a role in regulating the MMP/TIMP balance in order to affect trophoblast invasion (Roth and Fisher, 1999). It has been proposed that IL-10 suppresses invasion through MMP-9 inhibition without affecting TIMP-3 expression and this regulation appears to occur at the transcriptional level (Roth and Fisher, 1999).

1.1.7 Integrins

The ability of trophoblasts to invade may be related to their temporal and spatial regulation of integrin expression (Damsky et al., 1994; Zhou et al., 1997). Late gestation cytotrophoblasts show a decreased ability to invade and are unable to up-regulate α1β1 integrin complexes, suggesting a role for these integrins in invasion (Damsky et al., 1994). Extravillous α6 integrin immunopositive cytotrophoblasts are invasive, secrete large amounts of gelatinase, and appear to switch integrin expression thus changing their phenotype (Bischof et al., 1995). Furthermore, blocking expression of α5 or β1 integrin subunits almost completely inhibits migration of the trophoblast in vitro, supporting the notion that these subunits are important for trophoblast migration (Irving and Lala, 1995).

Spatial regulation of αVβ3 integrin has been reported on differentiating human cytotrophoblasts suggesting involvement with invasion of first trimester trophoblasts (Zhou et al., 1997) through localization with MMP-2 at the cell surface (Brooks et al., 1996). Expression of αVβ3 integrin is increased in cytotrophoblasts that have invaded the uterine wall in vivo. In addition, anti-αVβ3 antibody reduces in vitro invasion in a matrigel by 75% (Zhou et al., 1997).

1.1.8 Urokinase-type Plasminogen Activator and Plasminogen

Plasminogen activators (PAs) and their inhibitors (PAIs) have been proposed to contribute to the balance of proteolytic activities during matrix turnover (Vassalli et al., 1991). As reviewed by Vassalli and colleagues (1991) plasminogen activators are serine proteases whose preferred substrate is plasminogen (Vassalli et al., 1991). The urokinase-type plasminogen activator (uPA) and plasminogen system has been proposed to be involved with trophoblast migration and invasion in the rhesus monkey (Feng et al., 2000) and human (Liu et al., 2003). It has been proposed that plasminogen activators and their inhibitors regulate ECM degradation, while modifying migration and invasion of extravillous trophoblasts during early pregnancy in the rhesus monkey (Feng et al.,

2000). This idea is supported by the observation that in vitro migration of human trophoblast cell lines is stimulated through interaction of uPA with its receptor, uPAR (Liu et al., 2003).

Further support comes from the expression of the uPA gene in invasive trophoblasts (Feng et al., 2000) and their ability to secrete PAI-1 and PAI-2 (Zini et al., 1992). In addition, PAI-1 mRNA is only expressed in extravillous trophoblasts attached or close to cells at the maternal-fetal interface, suggesting the expression of PAI-1 is related to invasion and regulation of invasion depth (Feng et al., 2000). Term trophoblasts express fewer urokinase receptors compared to first trimester trophoblasts, further supporting a role for these receptors in placentation (Zini et al., 1992).

1.1.9 Other Important Factors

There are many other factors that may play a role in trophoblast invasion. Endothelin-1 stimulates proliferation and invasion of first trimester trophoblasts in vitro and may act in an autocrine or paracrine manner (Cervar et al., 1996). Insulin-like growth factor- II (IGF-II) and IGF binding protein-1 (IGFBP-1) have been localized to the decidual-trophoblast interface (Irwin et al., 1999). IGF-II (Hamilton et al., 1998; McKinnon et al., 2001) and IGFBP-1 (Irving and Lala, 1995) enhance migration of extravillous trophoblasts in vitro. IGF-II presumably stimulates migration through the activation of the IGF type 2 receptor and the mitogen-activated protein kinase (MAPK) pathway (McKinnon et al., 2001). It has also been suggested that IGF-II is trophoblast-derived and acts in an autocrine manner while IGFBP-1 originates in the decidua, acts in a paracrine manner to stimulate trophoblast migration (Hamilton et al., 1998). Studies also demonstrate that the stimulatory effect of IGFBP-1 on migration depends on integrin expression (Irving and Lala, 1995), specifically α_5 integrin expression (Hamilton et al., 1998).

Cadherins may also be important in the development of an invasive phenotype in trophoblasts. E-cadherin immunopositive staining is reduced in areas where invasion is

occurring in human first trimester placental tissue (Zhou et al., 1997). Trophoblasts treated with an anti-E-cadherin antibody in vitro demonstrated increased invasion, while anti-VE-cadherin antibody reduced invasion. This suggests VE-cadherin plays a role in facilitating trophoblast invasion (Zhou et al., 1997).

Leptin and its receptor have been identified in the human placenta and are thought to be involved with invasion by regulating MMP activity and secretion. Using an ELISA, leptin treatment increases the secretion of immunoreactive MMP-2 in cultured trophoblasts, but not MMP-9 (Castellucci et al., 2000). Using zymography, treatment of trophoblasts with leptin increases MMP-9 activity, but not MMP-2 (Castellucci et al., 2000). This study suggests that leptin can regulate factors identified to be crucial for trophoblast invasion. Leptin is secreted by cytotrophoblasts (Masuzaki et al., 1997), which is stimulated by interleukin-6 (IL-6) (Meisser et al., 1999a).

Angiotensin II treatment of trophoblasts induces plasminogen inhibitor type 1 (PAI-1) mRNA expression and appears to decrease invasion through activation of the PAI-1 gene (Xia et al., 2002). Transforming growth factor (TGF)-β down-regulates mRNA levels of MMP-9, MT1-MMP, MT2-MMP and urokinase-type plasminogen activator (uPA) while inducing the expression of TIMP-1 and TIMP-2 in first trimester trophoblasts (Karmakar and Das, 2002). Together, this suggests that TGF- β has an inhibitory effect on trophoblast migration. Transforming growth factor-β isoforms 1, 2 and 3 inhibit trophoblast invasion in vitro presumably by lowering protease activity. This is supported by evidence that trophoblast secretion of MMP-9 and uPA is significantly reduced following treatment (Lash et al., 2005). The isoform TGF-β2 has also been shown to increase integrin expression and reduce trophoblast migration (Irving and Lala, 1995).

Hepatocyte growth factor activator inhibitor-1 (HAI-1) is a serine protease inhibitor that is essential for maintaining the integrity of the basement membrane, apparently by regulating protease activities during placental development (Fan et al., 2007). Mice lacking HAI-1 tend to have a smaller than normal trophoblast layer and die in utero due to placental labyrinth defects. The reportedly compact morphology seen in

HAI-1 knock-out mice suggests that trophoblasts with this genotype fail to migrate properly (Fan et al., 2007).

Tumor necrosis factor-alpha (TNFα) significantly increases total gelatinase activity, specifically MMP-9 activity (Meisser et al., 1999b). TNFα is also thought to inhibit invasion through up-regulation of PAI-1 in vitro (Bauer et al., 2004). Peroxisome proliferator-activated receptor gamma (PPARγ) is expressed in invading trophoblasts and has been proposed to be involved with trophoblast invasion in the mink (Desmarais et al., 2007) and human (Fournier et al., 2007). CXCL10 has been shown to act as a chemoattractant for trophoblasts in vitro and may influence invasion in vivo (Dominguez et al., 2008). Leukemia inhibitory factor (LIF) has been immunolocalized to implantation sites in the rhesus monkey endometrium and conceptus (Sengupta et al., 2003). In the immortalized human trophoblast cell line HTR-8/SVneo, LIF is thought to stimulate trophoblast invasion through PGE_2 production and PGE_2 receptor expression (Horita et al., 2007). Leukemia inhibitory factor (LIF) and epidermal growth factor (EGF) stimulate production of uPA and MMP-9 activity in murine blastocyst outgrowths in culture (Harvey et al., 1995).

1.1.10 Conclusion

Trophoblast invasion is a process that is regulated both spatially and temporally in transcription and translation. The regulation of trophoblast invasion is likely due to the integrated actions of multiple factors. Discovering the link between these factors and more clearly defining their role may finally elucidate the regulatory mechanism of this process.

1.2 GENETIC REGULATION OF TROPHOBLAST REGRESSION AND PLACENTAL RELEASE AT PARTURITION

1.2.1 Significance

A better understanding of the factors that regulate trophoblast regression, placental release, and tissue remodeling in normal parturition is crucial for the study of placental abnormalities in the peripartum period. Retention of placental membranes in cows is thought to be due to a failure of collagen breakdown following parturition (Eiler and Hopkins, 1992). Changes in protease activity such as matrix metalloproteinases (MMPs) may be related to pathologic conditions like retention of placental membranes in cattle (Maj and Kankofer, 1997). The balance of MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) may also be associated with pre-term rupture of fetal membranes in humans (Athayde et al., 1998; Parry and Strauss III, 1998). However, before the mechanisms behind these conditions can be determined a clear understanding of the molecular factors in placental release needs to be achieved.

1.2.2 Summary

The initiation of placental release is likely controlled by a variety of endocrine, inflammatory, and mechanical factors. Multiple factors have been proposed to regulate the expression of genes involved in placental release (Gibb et al., 2006) and these factors vary from species to species (Smith, 2007). Despite years of research however, the exact genetic regulation of placental release and trophoblast regression remains elusive.

1.2.3 Matrix Metalloproteases and their Tissue Inhibitors

Several species rely on the degradation of the extracellular matrix for the detachment and separation of placental membranes at parturition, including the cow (Eiler and Hopkins, 1993; Walter and Boos, 2001), rat (Lei et al., 1996) and human (Xu et al., 2002). A balance between MMPs and TIMPs are thought to help modulate the release of the placenta following labor (Parry and Strauss III, 1998). Degradation of collagen when fetal membranes rupture in normal term parturition is also thought to rely on the balance of MMPs and TIMPs (Parry and Strauss III, 1998).

In humans, separation of the placenta from the endometrium and loss of placental mass may rely upon MMP-1 (collagenase-1) and MMP-3 expression (Vettraino et al., 1996). In addition, a rise in MMP-9 mRNA expression (Xu et al., 2002) and enzymatic activity (Tsatas et al., 1999) in the human placenta is associated with the onset of labor suggesting MMP-9 facilitates the separation of placental membranes during human parturition (Tsatas et al., 1999; Xu et al., 2002). MMP-9 activity may be triggered by tumor necrosis factor (TNF)-α or interleukin (IL)-1 (Vadillo-Ortega et al., 1995). Evidence suggests MMP-1 activation may rely on increased expression of both MMP-3 and MMP-9 in order to degrade ECM components during placental release (Bryant-Greenwood and Yamamoto, 1995).

MMP-9 mRNA and protein increase in the human placenta with the onset of labor (Vadillo-Ortega et al., 1995); whereas MMP-9, MMP-3, and MMP-2 gene expression is increased in tissues collected following natural delivery compared to Cesarean section. ProMMP-9 and proMMP-2 activity also significantly increases in the human placenta following spontaneous delivery (Bryant-Greenwood and Yamamoto, 1995).

Reports are contradictory in regard to the expression of TIMPs during labor. Goldman and colleagues (2003) describe a decrease in TIMP-1 expression in the human placenta during labor (Goldman et al., 2003); whereas Bryant-Greenwood and Yamamoto (1995) found an increase in TIMP-1 mRNA (Bryant-Greenwood and Yamamoto, 1995). Secretion of TIMPs into the human amniotic fluid decreases with the onset of labor

(Riley et al., 1999), while MMP-9 (Vadillo-Ortega et al., 1996; Athayde et al., 1998) and MMP-3 (Park et al., 2003) concentrations in amniotic fluid increase. Conversely, serum levels of TIMP-1 increase at labor and significantly higher levels of TIMP-1 through the post-partum period suggest that it may play a role in uterine involution and remodeling (Clark et al., 1994).

Matrix metalloproteinases and TIMPs are also thought to play a role in placental separation at parturition in cattle (Maj and Kankofer, 1997; Walter and Boos, 2001; Takagi et al., 2007; Dilly et al., 2011), goats (Uekita et al., 2004) and sheep (Vagnoni et al., 1998). Specifically, MMP-14 (MT1-MMP) (Dilly et al., 2011), MMP-2 and TIMP-2 (Takagi et al., 2007; Dilly et al., 2011) expression are involved with placental release during bovine parturition. In the cow (Walter and Boos, 2001) and goat (Uekita et al., 2004), a decrease in TIMP-2 expression near the end of gestation may be essential for placental release. Furthermore, MMP-2, MMP-9 and TIMP-1 protein expression in the ovine placenta during the last third of gestation suggest these factors may be also involved in sheep placental release (Vagnoni et al., 1998).

1.2.4 KiSS-1 and its Receptor KiSS-1R

The exact role of KiSS-1 and its receptor KiSS-1R at the end of gestation remains unclear and contradictory results have been reported. Placental KiSS-1 mRNA expression reportedly increases in placentas collected after term vaginal delivery compared to those following term Cesearean sections (Torricelli et al., 2008). However, others suggest the anti-invasive effect of KiSS-1 and KiSS-1R is only present in the first trimester (Bilban et al., 2004).

1.2.5 Other Important Factors

Toll-like receptor 8 (TLR8), nuclear factor of kappa light polypeptide gene enhancer in B-cell 1 (NFKB1), inhibin, VEGF-A, Flt-1 and sFlt-1 are up-regulated in

human placentas collected following spontaneous vaginal delivery compared to those collected following elective c-section (Cindrova-Davies et al., 2007; Lee et al., 2010). Leptin and COX-2 protein have also been shown to increase in human placentas collected following spontaneous vaginal delivery compared to those collected following elective c-section (Cindrova-Davies et al., 2007). Furthermore COX-2 is primarily seen in the trophoblasts and the increase in placental COX-2 is associated with NF-κB activation, suggesting a role for this pathway in placental release (Cindrova-Davies et al., 2007).

CD44 (hyaluronan receptor) is expressed at the end of gestation in basal plate cytotrophoblasts and decidual cells, which may be important in human placental release (Marzioni et al., 2009). Major histocompatibility antigen expression in bovine trophoblasts peaks just prior to parturition (Davies et al., 2000). This suggests MHC Class I antigen may play a role in placental separation, possibly through maternal immunological recognition (Davies et al., 2004). Tissue plasminogen activator is also reported to increase in human placenta collected following spontaneous natural delivery compared to elective Cesarean section (Bryant-Greenwood and Yamamoto, 1995). At the end of gestation in humans, endometrial relaxin acts on trophoblasts to increase tPA, MMP-1, and MMP-3 at the transcriptional and translational level (Qin et al., 1997a; Qin et al., 1997b).

1.2.6 Conclusion

A number of molecular factors are involved in the regulation of trophoblast regression, placental release, and tissue remodeling in the peripartum period. Most of these systems include proteases (e.g. metalloproteinases). Dysregulation of these pathways can result in abnormal trophoblast regression and/or placental retention following parturition.

1.3 GENES INVOLVED IN CARNIVORE IMPLANTATION, PLACENTAL DEVELOPMENT AND PARTURITION

1.3.1 Summary

Placentation in most carnivores like the dog is endotheliochorial (Sandoval et al., 2001; Johnston et al., 2001). This placental type is invasive, but the degree of invasiveness is relatively shallow compared to other invasive placental type species. Canine syncytiotrophoblasts and cytotrophoblasts invade the maternal endothelium and endometrial glands early in gestation. These cells form "cuffs" around the maternal blood vessels and form the placental labyrinth (Barrau et al., 1975; Sandoval et al., 2001. Little is known regarding the regulation of trophoblast invasion in carnivores. Molecular studies have identified factors in the canine uterus recognized as important for implantation in other species, but few have looked specifically at placental gene expression and none have examined the placental transcriptome.

1.3.2 Canine Uterine and Placental Gene Expression

Matrix metalloproteinase (MMP)-2 expression has been detected in canine trophoblasts and myometrium (Beceriklisoy et al., 2007); whereas MMP-9 expression has been detected in endometrial crypts and glands (Beceriklisoy et al., 2007). In addition, both MMP-2 and MMP-9 have been detected in the pre-implantation embryo 10-12 days after mating (Schäfer-Somi et al., 2008). Furthermore, the highest expression of MMP-2 and MMP-9 previously reported is around the time of implantation (Beceriklisoy et al., 2007), suggesting that these proteases may be involved in this process. Peroxisome proliferator-activated receptor gamma (PPARγ) mRNA is expressed in the canine placenta and localized to trophoblasts (Kowalewski et al., 2011). The up-regulation of PPARγ mRNA after implantation and through mid-gestation (Kowalewski et al., 2011), suggests it too may play a role in trophoblast invasion.

As previously reported by Kowalewski and coworkers (2010), components of the prostaglandin system are temporally expressed in the canine uteroplacental unit (Table 1.1). Trophoblasts may be the primary target for PGF2α and PGE2 (Kowalewski et al., 2010). Cycloooxygenase (COX-2, also known as PTGS2) expression is localized to trophoblasts surrounding maternal vessels (Kowalewski et al., 2010). These factors may contribute to canine placental maturation and release at parturition.

Table 1.1. A summary of previously reported prostaglandin components genetically expressed in the canine uteroplacental unit through the first half of gestation (Kowalewski et al., 2010) PGR: Prostaglandin receptor, PTGS2: Cyclooxygenase (COX-2), PGFS: Prostaglandin F2α synthase, PTGFR: Prostaglandin F Receptor, PTGES: Prostaglandin E2 synthase, PTGER2: Prostaglandin E Receptor 2, PTGER4: Prostaglandin E Receptor 4

Prostaglandin	**Pre-implantation**	**Post-implantation**	**Mid-gestation**	**Prepartal luteolysis**
PGR	High	Moderate	Moderate	Moderate
PTGS2	Low	Low	Low	High
PGFS	Low	Moderate	High	Moderate
PTGFR	High	Low	Low	High
PTGES	Low	Moderate	High	High
PTGER2	High	Moderate	Moderate	High
PTGER4	High	Moderate	Low	Low

The placenta has been identified as the main (if not sole) source of relaxin in the dog (Tsutsui and Stewart, 1991). Canine cytotrophoblasts also express relaxin-like factor mRNA during early and mid-gestation (Klonisch et al., 2001). The expression of relaxin-like factor at the end of gestation has not been studied. The canine endometrium does not express relaxin mRNA (Klonisch et al., 1999a). However, canine mammary tumors can produce relaxin, whose expression correlates with MMP-2 mRNA expression and cell invasion (Lamp et al., 2009) suggesting a role for relaxin in trophoblast cell invasion. This suggests a possible correlation between relaxin, MMP-2 and trophoblast invasion in dogs.

The expression of other factors has been identified in canine pre-implantation embryos. Pre-implantation embryos express mRNA for interleukin (IL)-1β, interleukin (IL)-8, hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF), COX-2 and tumor necrosis factor (TNF)-α (Schäfer-Somi et al., 2008). Interleukin-1β (Librach et al., 1994), interleukin-8 (Jovanović et al., 2010), leukemia inhibitory factor (Sengupta et al., 2003) and TNFα (Meisser et al., 1999b) have all been proposed to influence trophoblast invasion in other species. A strong expression of COX-2 is thought to indicate activation of prostaglandin synthesis, which may be important in the regulation of embryonic and endometrial cytokines. The expression of TNFα and IL-6 is thought to be important in regulating the balance of tissue remodeling during canine implantation (Schäfer-Somi et al., 2008).

Expression of CD-8, COX-1, transforming growth factor (TGF)-β, HGF, insulin-like growth factor (IGF)-1, IL-4, IL-10 and LIF have been reported in canine endometrium at placental sites (Beceriklisoy et al., 2009). IL-10 expression decreases at implantation in placental sites, which may facilitate trophoblast invasion (Beceriklisoy et al., 2009). Furthermore, IGF-1 is only expressed at placental sites and may also be important for implantation (Beceriklisoy et al., 2009).

1.3.3 Placental Gene and Protein Expression in Other Carnivores

The feline placenta is also endotheliochorial (Miglino et al., 2006) and similar to the dog, it is the main source of relaxin during pregnancy (Addiego et al., 1987; Klonisch et al., 1999b). Matrix metalloproteinase activity (specifically MMP-1 and MMP-13) has also been identified in feline trophoblasts (Walter and Schönkypl, 2006). Immunohistochemistry experiments have shown that MMP-2 is expressed in maternal connective tissue surrounding endometrial glands, decidual cells in the junctional zone and giant cells of the placental labyrinth (Walter and Schönkypl, 2006), while MMP-9 was not detected. The latent forms of MMP-2 and MMP-9 are the most common, with very low levels of active enzymes present in the feline placenta during late gestation (Walter and Schönkypl, 2006). Furthermore, collagen types I and III in the feline placenta decrease throughout gestation suggesting a role for matrix degrading enzymes in this species (Walter and Schönkypl, 2006).

In the mink (*Mustela vison*), peroxisome proliferator-activated receptor gamma (PPARγ) mRNA is expressed in the placenta following implantation and its expression is specific to the trophoblasts in contact with the endometrium (Desmarais et al., 2007). PPARγ ligands may also be involved with the regulation of implantation and maintenance of the placenta (Desmarais et al., 2007). In an in vitro study, PPARγ mRNA expression was significantly up-regulated following treatment of 15-deoxy-delta 12, 14 prostaglandin (15-d-PGJ2) (Desmarais et al., 2007). This suggests that 15-d-PGJ2 acts through the PPARγ pathway. Mink embryos also secrete PGE2, but embryos in diapause do not (Lopes et al., 2006). COX-2 protein expression has been localized to the sites of embryo attachment and invasion in the mink (Song et al., 1998) and there is a corresponding increase in PGE synthase in the same location. It has been proposed that this regulates VEGF expression at this location (Lopes et al., 2006). Vascular endothelial growth factor protein expression has also been localized to invasive mink trophoblasts. In addition, Flt-1 expression has been noted to increase in the uterus around implantation along with other VEGF isoforms (Lopes et al., 2003).

1.3.4 Conclusion

Although relatively shallow compared to primates and rodents, canine trophoblast invasion is significantly more extensive and complex as compared to other domestic animals. Limited information is available regarding the ontogenic molecular changes that occur in the carnivore placenta, particularly at the end of gestation. Detailed investigations are needed in order to better understand differences and similarities between carnivore trophoblast invasion and that of other domestic animals.

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CHAPTER II

GENE EXPRESSION IN PRE-TERM, PRE-LABOR, AND PARTURIENT CANINE PLACENTA

2.1 INTRODUCTION

The completion of parturition involves the delivery of the placenta. Complications arise when the placenta is not properly delivered within a defined time following delivery of the fetus. Two common placental abnormalities in the dog are placental retention and subinvolution of placental sites (SIPs), which involve pathologic trophoblast invasion. Placental retention increases the risk of the bitch developing postpartum uterine infections and metritis (Wallace, 1994). In addition, placental retention can be life threatening if the bitch becomes very ill (e.g. fever, anorexia, depression). The mechanisms behind placental retention remain unknown.

Subinvolution of placental sites (SIPS) is characterized by abnormally deep invasion of the trophoblasts into the myometrium following delivery of the placenta (Al-Bassam et al., 1981), resulting in a failure or delay in uterine involution (Beck and McEntee, 1966). Instead of degenerating after parturition, trophoblasts persist within the endometrium, preventing endometrial blood vessels from developing thromboses, resulting in persistent bleeding (Al-Bassam et al., 1981). In addition, trophoblasts may on rare occasions erode through the serosa resulting in peritonitis (Beck and McEntee, 1966). The factors involved with SIPs are unknown.

Many molecular factors are involved in trophoblast invasion as well as placental release during parturition. Some of these factors include matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPs), vascular endothelial growth factor (VEGF)-A, VEGF-A receptor (Flt-1) and the KiSS-1 receptor (KiSS-1R) (Lei et al., 1996; Maj and

Kankofer, 1997; Lash et al., 1999; Xu et al., 2002; Zhang et al., 2011). MMPs and TIMPs regulate normal trophoblast invasion and migration in humans (Librach et al., 1999). MMP-2 and MMP-9 are likely responsible for conditions of abnormal placentation in humans (Zhang et al., 2011). KiSS-1 and KiSS-1R may also play a role in the regulation of human trophoblast invasion (Zhang et al., 2011), while VEGF-A may be important in the initiation of trophoblast invasion (Lash et al., 1999).

Detachment and release of the placenta at parturition in the human (Xu et al., 2002), cow (Walter and Boos, 2001) and rat (Lei et al., 1996) relies on enzymatic degradation of the extracellular matrix likely by MMP activity. Disorders affecting the regulation of MMPs have been proposed to contribute to placental retention in the cow (Maj and Kankofer, 1997; Walter and Boos, 2001). Knowledge of the factors involved in normal placental release during parturition and pathologic placental conditions during the postpartum period in the dog is lacking. To the authors' knowledge, gene expression of VEGF-A, Flt-1, KiSS-1R, MMP-2, MMP-9 and TIMP-2 in the canine placenta have not been previously published. The aim of this study was to determine whether MMP-2, MMP-9, TIMP-2, VEGF-A, Flt-1 and KiSS-1R are expressed in the canine placenta. Once established, the objective was to compare expression patterns during late gestation in the bitch (pre-term), around the initiation of labor (pre-labor) and after delivery (parturient).

2.2 MATERIALS AND METHODS

2.2.1 Tissue Collection

Canine chorioallantoic tissue was collected from various breeds following elective pregnancy termination by ovariohysterectomy at 61 ± 1 day past the LH surge (late gestation; n=4), elective C-section at 64 ± 1 day past the LH surge prior to first stage labor (pre-labor; n=4), and natural delivery (parturient; n=3). Prior to being flash frozen

in liquid nitrogen, the marginal hematoma was dissected away and any excess tissue and blood were removed. Tissue was then stored at -80°C until analyzed.

2.2.2 RNA Isolation

Total RNA was isolated using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA concentration and purity was determined using a spectrophotometer (NanoPhotometer, IMPLEN, Munich, Germany). Total RNA samples were submitted to the Center for Genome Research and Biocomputing (CGRB) at Oregon State University to measure RNA quality. RNA integrity numbers (RIN) were obtained for each sample using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

2.2.3 TaqMan Real-Time RT-PCR

RNA was reverse transcribed to cDNA using oligo dT as a primer with the SuperScript III First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen). Concentrations of the cDNA samples were measured using the DNA setting on a spectrophotometer (NanoPhotometer, IMPLEN) and then diluted 10-fold for RT-PCR. TaqMan primers and probes of proprietary sequences developed for canine MMP-2, MMP-9, TIMP-2, VEGF-A, Flt-1 and KiSS-1R (Table 2.1) by the company were used for real-time RT-PCR on the ABI 7300 RT-PCR machine (Applied Biosystems, Carlsbad, CA). Default cycling conditions were used (Table 2.2). Gene expression was normalized to mRNA expression of hypoxanthine-phosphoribosyl transferase 1 (HPRT-1) based upon its success in other canine studies (Brinkhof et al., 2006; Etschmann et al., 2006; Schlotter et al., 2009) and its stable expression in this study (Ct mean ± SEM; 32.7 ± 0.52). The first parturient sample collected was used as a calibrator sample. All RT-PCR reactions were performed in triplicate with a final

reaction volume of 20 µl each. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.2.4 Statistical Analysis

Statistical analysis was performed using a one-way ANOVA with Bonferroni corrections to make comparisons between the three groups. Statistical significance was calculated using the Stata 12.0 software (StataCorp LP, College Station, TX). Data were expressed as mean ± standard deviation (SD). Significance was defined as $p<0.05$.

Table 2.1. TaqMan Primers and Probes used for real-time RT-PCR

Gene Name	Assay ID	RefSeq	Exon Boundary	Assay Location	Amplicon Length
MMP-2	Cf02623423_m1	XM_535300.2	3 to 4	520	58
MMP-9	Cf02621845_m1	NM_001003219.1	12 to 13	2015	59
TIMP-2	Cf02623335_m1	NM_001003082.1	1 to 2	234	81
VEGF-A	Cf02623453_m1	NM_001003175.1	7 to 8	906	62
Flt-1	Cf02696454_g1	XM_534520.2	26 to 27	3489	116
KiSS-1R	Cf02715933_u1	XM_850105.1	3 to 3	635	141
HPRT-1	Cf026262558_m1	NM_001003357.1	8 to 9	605	129

Table 2.2. Real-time RT-PCR cycling conditions

Stage	**Temperature (°C) and Time (Mins)**	**Replications**
Stage 1	50.0°C for 2:00	1
Stage 2	95.0°C for 10:00	1
Stage 3	95.0°C for 0:15 then 60.0°C for 1:00	40

2.3 RESULTS

All RNA samples used in the synthesis of cDNA for real-time RT-PCR studies were determined to be of good quality based on a RIN > 7.0. MMP-9 mRNA expression was significantly higher in pre-term samples compared to pre-labor and parturient samples (Table 2.3). Although not statistically significant ($p=0.21$), there is a similar apparent decrease in expression of VEGF-A prior to parturition (Table 2.3). There was no significant difference in mRNA expression between the samples for MMP-2, TIMP-2 or Flt-1 (Table 2.3). Based on Ct values of greater than 38 following a protocol with 10 ng of starting cDNA, KiSS-1R was not expressed in any of the placentas in this study (data not shown).

Table 2.3. Relative quantitation (mean ± SD) of placental gene expression at three time points prior to parturition. *p<0.05

	Pre-term (n=4)	Pre-labor (n=4)	Parturient (n=3)
MMP-9	15.2 ± 4.30*	2.16 ± 1.23	4.27 ± 2.32
MMP-2	0.62 ± 0.19	1.57 ± 0.89	1.34 ± 1.0
TIMP-2	0.08 ± 0.04	0.37 ± 0.26	9.31 ± 14.4
VEGF-A	0.98 ± 0.54	0.40 ± 0.30	0.71 ± 0.26
Flt-1	0.99 ± 0.25	0.64 ± 0.30	0.77 ± 0.26

2.4 DISCUSSION

Molecular changes in the canine placenta in the periparturient period have not been previously described in the literature. Therefore the aim of this study was to characterize changes in the expression of genes known in other species to regulate trophoblast invasion from late gestation to immediately following parturition. In the current study, MMP-9 mRNA expression was significantly higher in pre-term tissues compared to pre-labor and parturient tissues. MMPs have been shown to play an essential role in the regulation of trophoblast invasion in humans (Librach et al., 1991), so these factors may also be important in the abnormal regulation of trophoblast invasion in SIPs.

Previous research has also described the involvement of MMPs in placental release at parturition in cattle and humans. Walter and Boos (2001) suggested that MMP-2 and MMP-9 are important in the detachment of placental membranes at parturition in the cow. A similar role at the end of pregnancy for MMP-9 has been proposed in the human placenta by Xu and coworkers, who demonstrated an increase in MMP-9 at labor, but no change in MMP-2 (Xu et al., 2002). MMP-9 has been proposed to be crucial in the degradation of the extracellular matrix at the end of gestation for successful detachment of placental membranes in the human (Xu et al., 2002). In the present study, MMP-9 mRNA expression was significantly increased during late gestation and then decreased around the initiation of labor suggesting that while it may play a role during this period in the dog, this role may be different than that reported in humans and cows.

The relationship of these factors in canine placental retention remains unknown. Maj and Kankofer (1997) suggested that a lack of active MMP-2 in maternal and fetal parts of retained placenta could contribute to the improper release of fetal membranes at the time of parturition. Lack of MMP inhibition has been demonstrated to be important in placental release. Walter and Boos (2001) reported a decrease in TIMP-2 expression at parturition in cows and suggested this decrease was required for normal placental release. The decrease in TIMP-2 in their study differs from what was found in the current study as TIMP-2 mRNA expression was not significantly different between the three study

groups. This suggests the role of TIMP-2 in the dog may differ slightly from that in the cow. However, the relationship of all these factors to canine placental retention will require further study.

VEGF-A and associated receptor, Flt-1, are important in initiating trophoblast invasion in humans (Lash et al., 1999). In addition, Flt-1 placental mRNA increased in women who experienced labor compared to those that did not go through labor (Lee et al., 2010). Based on these studies, we expected to see a change in VEGF-A and Flt-1 mRNA expression at the end of gestation in dogs, but this was not the case. In the current study, VEGF-A and Flt-1 were not significantly different between the periparturient groups. One possibility for the different expression patterns in the dog compared to the human at the end of gestation may be the difference in placental types. The human placenta is more invasive than the canine placenta, which may necessitate a lesser role of VEGF-A and Flt-1 in the dog at the time of parturition.

Previous studies in humans suggest the primary role of KiSS-1 and its receptor KiSS-1R occurs during the first trimester of pregnancy where it acts to suppress trophoblast invasion by inhibiting protease activity of factors such as MMP-2 (Bilban et al., 2004). In addition, KiSS-1 placental mRNA increased in women who experienced labor compared to those that did not go through labor (Torricelli et al., 2008). Studies in vitro have shown that KiSS-1 transfection of human trophoblast cells decreases cell invasion (Zhang et al., 2011) and an up-regulation has been reported in women with abnormal placentation such as preeclampsia (Zhang et al., 2011). The results of this study led to the conclusion that there was no expression of KiSS-1R mRNA in any of the canine tissues based upon a cut-off for relevant expression that was chosen according to the upper limit of the possible detection range (40 cycles) following a starting cDNA amount of 10 ng (Goni et al., 2009). The receptor does not appear to play a role or be present in these tissues at this time in the dog. Further studies are needed to investigate the KiSS-1 gene itself as well as expression of KiSS-1R earlier in canine gestation.

In summary, MMP-9 mRNA expression was significantly different in the pre-term groups compared to pre-labor and parturient groups. While the increase in MMP-2

mRNA expression prior to labor was not statistically significant it warrants recognition due to the relevance to previous studies in other species. The results of this study suggest that parturition influences the expression of MMP-9 in the dog, which is similar to what has been reported in humans and cattle. However, further studies are needed to determine whether changes in mRNA expression of MMP-2, MMP-9, TIMP-2, VEGF-A, Flt-1 and KiSS-1R play a role in canine placental abnormalities such as retained placental membranes and excessive trophoblast invasion (SIPs) in dogs.

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CHAPTER III

INVESTIGATING THE CANINE PLACENTAL TRANSCRIPTOME IN THE PERIPARTUM PERIOD

3.1 INTRODUCTION

In the dog, physical interactions between maternal and fetal tissues are restricted to an equatorial belt surrounding the fetus in the canine zonary endotheliochorial placenta (Anderson, 1969; Barrau et al., 1975; Sandoval et al., 2001; Burton et al., 2006). Normal canine trophoblast invasion during implantation is relatively shallow compared to other deciduate placental species (e.g. primates, rodents) (Carter et al., 2006). Canine syncytiotrophoblasts and cytotrophoblasts invade the maternal endothelium and endometrial glands early in gestation. These cells form "cuffs" around the maternal blood vessels and form the labyrinth (Barrau et al., 1975; Sandoval et al., 2001). With the exception of the marginal hematoma that forms by mid-gestation (Barrau et al., 1975; Sandoval et al., 2001), canine trophoblasts do not come into direct contact with maternal blood (Carter et al., 2006).

Despite years of research using a variety of species, the regulatory mechanisms behind trophoblast invasion at implantation and placental release at parturition remain largely unknown. Many studies have identified different factors involved within these critical periods of pregnancy including matrix metalloproteinases, interleukins, and growth factors. Aberrent trophoblast invasion and retained placental membranes are seen in many species (Al-Bassam et al., 1981; Brosens et al., 1972; Fernández et al., 1998; Maj and Kankofer, 1997; Pijnenborg et al., 1991). In humans, pathologically shallow trophoblast invasion results in preeclampsia (Brosens et al., 1972; Pijnenborg et al., 1991), while uninhibited trophoblast invasion results in placenta accreta (Davidson, 1944; Khong and Robertson, 1987). Uninhibited trophoblast invasion and failure of trophoblast

regression at parturition (subinvolution of placental sites (SIPs)) also occur in dogs (Al-Bassam et al., 1981; Fernández et al., 1998). Before conditions of pathologic trophoblast invasion and retention can be properly investigated, the mechanisms involved in normal trophoblast invasion and release must be studied. Therefore, the objective of this research was to characterize global gene expression changes in the peripartum period, using a canine model.

3.2 MATERIALS AND METHODS

3.2.1 Tissue Collection

Canine chorioallantoic tissue was collected following elective pregnancy termination by ovariohysterectomy at 61 ± 1 day past the LH surge (pre-term; n=4), following elective C-section at 64 ± 1 day past the LH surge prior to first stage labor (pre-labor; n=4), and natural delivery (parturient; n=4). Upon tissue collection, the marginal hematoma was dissected away and any blood or excess tissue (nonvillous chorioallantois, amnion and umbilical cord) were removed. Tissues were flash frozen in liquid nitrogen and stored at -80°C until RNA was isolated.

3.2.2 RNA Isolation

Total RNA was isolated using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA concentration and purity was determined using a spectrophotometer (NanoPhotometer, IMPLEN, Munich, Germany). RNA integrity numbers (RIN) were obtained for each sample using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only RNA samples with a RIN >7.0 were used for microarray analysis.

3.2.3 Microarray Analysis

Microarray was performed at the Center for Genome Research and Biocomputing Core Laboratory (CGRB) at Oregon State University using the standard protocol for the facility. Briefly, labeled target amplified antisense RNA (aRNA) was prepared from 200 ng of total RNA using the MessageAMP Premier Amplification Kit (Life Technologies, Grand Island, NY). Samples were in vitro transcribed (IVT) and incubated for 16 hours. Following incubation, 10 μg of aRNA from each sample was hybridized to an Affymetrix Canine Genome 2.0 Array (Affymetrix, Santa Clara, CA) for 18 hours. All samples were hybridized in one day in two sets. Washing, staining and scanning was performed using the GeneChip Expression Wash, Stain and Scan Manual for Cartridge Arrays (Affymetrix) using the GeneChip Command Console Software (AGCC) software with a Fluidics Station 450 (Affymetrix) and a GeneChip Scanner 3000 with an autoloader (Affymetrix). Image processing and data extraction was performed using AGCC Software Version 3.0 (Affymetrix).

3.2.4 Statistical Analysis

Overall gene expression changes between groups were averaged and plotted in scatterplots using ArrayStar (DNASTAR, Madison, WI). In order to graph these comparisons the average expression levels of the sample groups were plotted on the x- and y-axes. Linear correlation coefficients were calculated within the ArrayStar program.

Pre-processing, normalization of the microarray data as well as differential expression statistics was carried out in the open-source statistical program known as R (www.R-project.org) using the Bioconductor software package. Quantile normalization was carried out using robust multichip average (RMA) (Irizarry et al., 2003). Linear modeling and differential gene expression using an empirical Bayes analysis was calculated using the Bioconductor software along with the limma and affy software packages (Smyth, 2004) in R. This involves calculating a moderated t-statistic, which

draws inferences from all genes in the set to shrink the standard errors toward a common value. This provides more degrees of freedom and more stable standard error estimates (Smyth, 2004). A false discovery rate adjusted (FDR) p value was used to adjust for multiple testing and false positives (Benjamini and Yekutieli, 1995). Differential expression was defined by a p-value ≤ 0.05, FDR adjusted p value ≤ 0.1 and a log fold change ≥ 1.2 based on parameters used in previous studies (Sitras et al., 2009; Hoegh et al., 2010; Lian et al., 2010). Gene ontology was explored using ArrayStar (DNASTAR, Madison, WI).

3.3 RESULTS

Global gene expression profiles between pre-term, pre-labor and parturient canine chorioallantois tissue were compared. Initially for microarray analysis the parturient group had a sample size of four. One sample from the parturient group was eliminated based on the knowledge that the litter died from Canine Herpes Virus resulting in a sample size of three parturient dogs for microarray analysis. High correlation coefficients within and between sample groups indicated good concordance within the study groups, but minimal overall variation between groups (mean correlation coefficient = 0.985). This suggests there is little overall variation in global gene expression between the three sample groups (Figure 3.1).

Microarray analysis demonstrated 18 genes that were differentially expressed between pre-term and pre-labor sample groups (Table 3.1) and 38 genes that were differentially expressed between pre-term and parturient samples (Table 3.2). There were no differentially expressed genes between pre-labor and parturient sample groups (Table 3.3). There were 12 genes that were up-regulated and 20 down-regulated genes between pre-term samples compared to parturient samples (Figure 3.2). Between pre-term and pre-labor groups, there were 8 up-regulated genes and 4 down-regulated genes. In addition there were 6 differentially expressed genes whose expression changed in the same direction between pre-term and pre-labor as well as pre-term to parturient (Figure

3.2). The most represented biological processes included proteolysis, maintenance of location, cell adhesion, stem cell division, hormone catabolic processing, peptide hormone processing, protein metabolic process, morphogenesis of a branching structure and collagen catabolic process (Table 3.4).

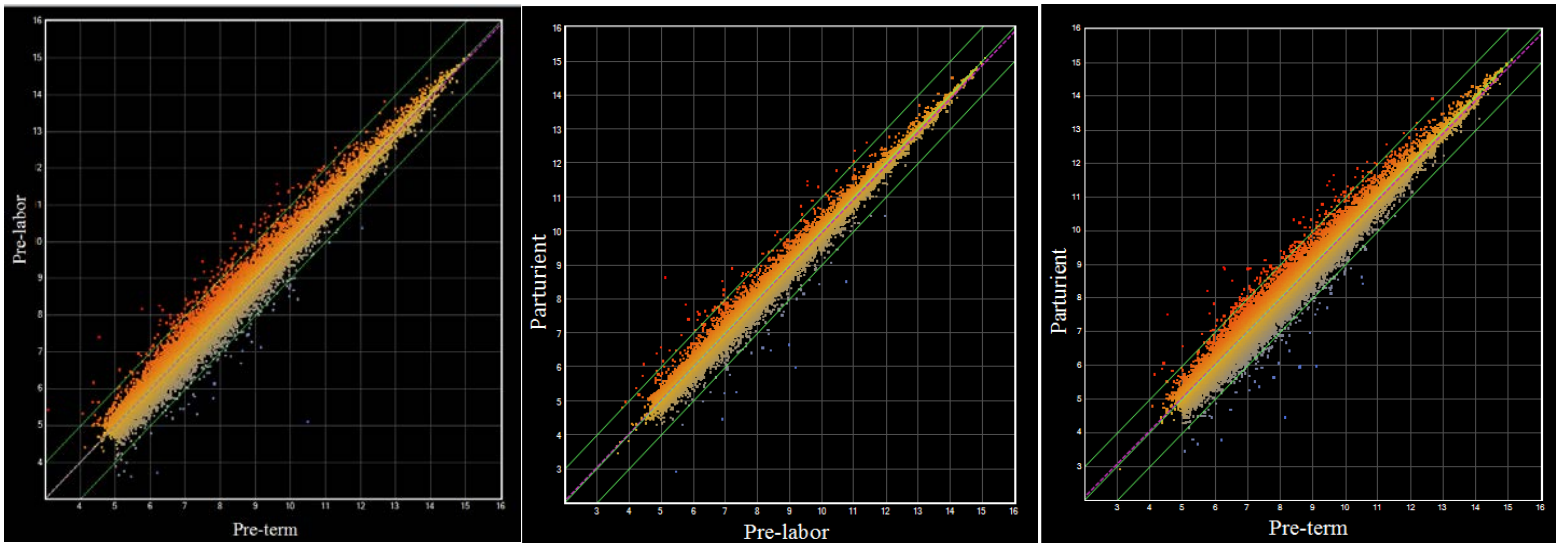

Figure 3.1. Correlation of overall gene expression profiles between sample groups (r=0.985).

Table 3.1. Top differentially expressed genes between pre-term and pre-labor sample groups ($p \leq 0.05$, FDR adjusted $p \leq 0.1$, $logFC \geq 1.2$) (continued on next page)

Gene	**Gene Name**	**logFC**	**P.Value**	**FDR adj.P.Val**
MXD1	MAX dimerization protein 1	-1.45	2.95E-05	0.08
SERINC5	serine incorporator 5	-1.33	6.40E-05	0.08
LOC607225	hypothetical protein	1.40	9.12E-05	0.08
HBD	hemoglobin, delta	1.40	9.13E-05	0.08
PI3	peptidase inhibitor 3, skin-derived	-1.81	0.0001	0.10
LOC475901	hypothetical protein	1.50	0.0002	0.10
SLC26A11	solute carrier family 26, member 11	-1.20	0.0002	0.10
FTSJD2	FtsJ methyltransferase domain containing 2	-1.65	0.0003	0.10
ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	1.23	0.0003	0.10
RBP7	retinol binding protein 7, cellular	1.90	0.0004	0.10

Table 3.1. Top differentially expressed genes between pre-term and pre-labor sample groups ($p \leq 0.05$, FDR adjusted $p \leq 0.1$, $logFC \geq 1.2$) (continued from previous page)

Gene	Gene Name	logFC	P.Value	FDR adj.P.Val
EPHX2	epoxide hydrolase 2, cytoplasmic	1.28	0.001	0.10
CD44	CD44 molecule (Indian blood group)	-1.20	0.001	0.10
POU1F1	POU class 1 homeobox 1	1.50	0.001	0.10
NR4A1	nuclear receptor subfamily 4, group A, member 1	1.75	0.001	0.10
ULK4	unc-51-like kinase 4 (C. elegans)	1.20	0.001	0.10
ERRFI1	ERBB receptor feedback inhibitor 1	-1.30	0.001	0.10
SLC37A2	solute carrier family 37 (glycerol-3-phosphate transporter), member 2	-1.40	0.002	0.10
MFI2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	-1.27	0.002	0.10

Table 3.2. Top differentially expressed genes between pre-term and parturient sample groups ($p \leq 0.05$, FDR adjusted $p \leq 0.1$, $logFC \geq 1.2$) (continued on next page)

Gene	Gene Name	logFC	P.Value	FDR adj.P.Val
BDH2	3-hydroxybutyrate dehydrogenase, type 2	1.20	1.06E-06	0.02
CIRBP	cold inducible RNA binding protein	1.55	3.32E-06	0.02
DAG1	dystroglycan 1 (dystrophin-associated glycoprotein 1)	1.47	5.58E-06	0.02
EGLN3	egl nine homolog 3 (C. elegans)	-1.31	1.78E-05	0.02
CXCL10	chemokine (C-X-C motif) ligand 10	1.84	1.89E-05	0.02
NDC80	NDC80 homolog, kinetochore complex component (S. cerevisiae)	1.25	1.93E-05	0.02
RBP7	retinol binding protein 7, cellular	2.62	5.30E-05	0.03
CELA1	chymotrypsin-like elastase family, member 1	1.35	0.0001	0.04
HSP70	heat shock protein 70	-1.92	0.0002	0.05

Table 3.2. Top differentially expressed genes between pre-term and parturient sample groups ($p \leq 0.05$, FDR adjusted $p \leq 0.1$, $logFC \geq 1.2$) (continued on next page)

Gene	Gene Name	logFC	P.Value	FDR adj.P.Val
BPHL	biphenyl hydrolase-like (serine hydrolase)	1.50	0.0002	0.05
MXD1	MAX dimerization protein 1	-1.21	0.0002	0.05
SERINC5	serine incorporator 5	-1.21	0.0002	0.05
ERRFI1	ERBB receptor feedback inhibitor 1	-1.53	0.0005	0.06
ART3	ADP-ribosyltransferase 3	-1.33	0.0005	0.06
FTSJD2	FtsJ methyltransferase domain containing 2	-1.60	0.001	0.06
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	-1.28	0.001	0.07
LIPG	lipase, endothelial	-1.62	0.001	0.07
TEX10	testis expressed 10	1.30	0.002	0.09
DEPDC1	DEP domain containing 1	1.20	0.002	0.09

Table 3.2. Top differentially expressed genes between pre-term and parturient sample groups ($p \leq 0.05$, FDR adjusted $p \leq 0.1$, $logFC \geq 1.2$) (continued on next page)

Gene	**Gene Name**	**logFC**	**P.Value**	**FDR adj.P.Val**
SPAM1	sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	1.80	0.002	0.09
SORT1	sortilin 1	-1.20	0.002	0.09
MFI2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	-1.36	0.002	0.09
DEGS2	degenerative spermatocyte homolog 2, lipid desaturase (Drosophila)	-1.20	0.002	0.10
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	-2.99	0.003	0.10
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-1.50	0.003	0.10
DIO3	deiodinase, iodothyronine, type III	-1.26	0.004	0.10
SOCS1	suppressor of cytokine signaling 1	-1.21	0.004	0.10
FBN2	fibrillin 2	1.85	0.004	0.10

Table 3.2. Top differentially expressed genes between pre-term and parturient sample groups ($p \leq 0.05$, FDR adjusted $p \leq 0.1$, $logFC \geq 1.2$) (continued from previous pages)

Gene	Gene Name	logFC	P.Value	FDR adj.P.Val
NRN1	neuritin 1	2.10	0.004	0.10
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1	-1.20	0.004	0.10
HRH3	histamine receptor H3	-1.22	0.005	0.10
ALDH4A1	aldehyde dehydrogenase 4 family, member A1	-1.28	0.005	0.10
TUFT1	tuftelin 1	-1.20	0.005	0.10
SSTR2	somatostatin receptor 2	-1.30	0.005	0.10
COCH	coagulation factor C homolog, cochlin (Limulus polyphemus)	-1.82	0.005	0.10
MARK2	MAP/microtubule affinity-regulating kinase 2	-1.30	0.005	0.10
PHLDA1	pleckstrin homology-like domain, family A, member 1	-1.20	0.005	0.10
IL6	interleukin 6 (interferon, beta 2)	-1.72	0.006	0.10

Table 3.3. Top genes reported from pre-labor to parturient samples (continued on next page)

Gene	Gene Name	logFC	P.Value	FDR adj.P.Val
CIRBP	cold inducible RNA-binding protein	1.03	1.37E-05	0.15
ARRDC3	arrestin domain containing 3	-0.82	2.38E-05	0.19
BDH2	3-hydroxybutyrate dehydrogenase, type 2	0.85	2.82E-05	0.19
RCOR3	REST corepressor 3	-0.55	9.46E-05	0.45
ALKBH3	alkB, alkylation repair homolog 3 (E. coli)	0.40	0.0001	0.47
LYRM5	LYR motif containing 5	0.75	0.0001	0.47
LOC609334	similar to leiomodin-2-like	-0.78	0.0001	0.47
NFIL3	nuclear factor, interleukin 3 regulated	-0.87	0.0002	0.47
ZBTB38	zinc finger and BTB domain containing 38	0.50	0.0002	0.47
PUS7L	pseudouridylate synthase 7 homolog (S. cerevisiae)-like	-1.14	0.0002	0.47

Table 3.3. Top genes reported from pre-labor to parturient samples (continued from previous page)

Gene	Gene Name	logFC	P.Value	FDR adj.P.Val
GADD45B	growth arrest and DNA-damage-inducible, beta	-1.38	0.0002	0.47
NFIL3	nuclear factor, interleukin 3 regulated	-0.81	0.0002	0.47
DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9	0.41	0.0002	0.50
IFI44L	interferon-induced protein 44-like	0.94	0.0002	0.50
LOC612530	hypothetical protein LOC612530	0.61	0.0003	0.56
HSP70	heat shock protein 70	-1.93	0.0003	0.56
HABP4	hyaluronan binding protein 4	-0.39	0.0003	0.56
XRCC6BP1	XRCC6 binding protein 1	0.48	0.0004	0.60

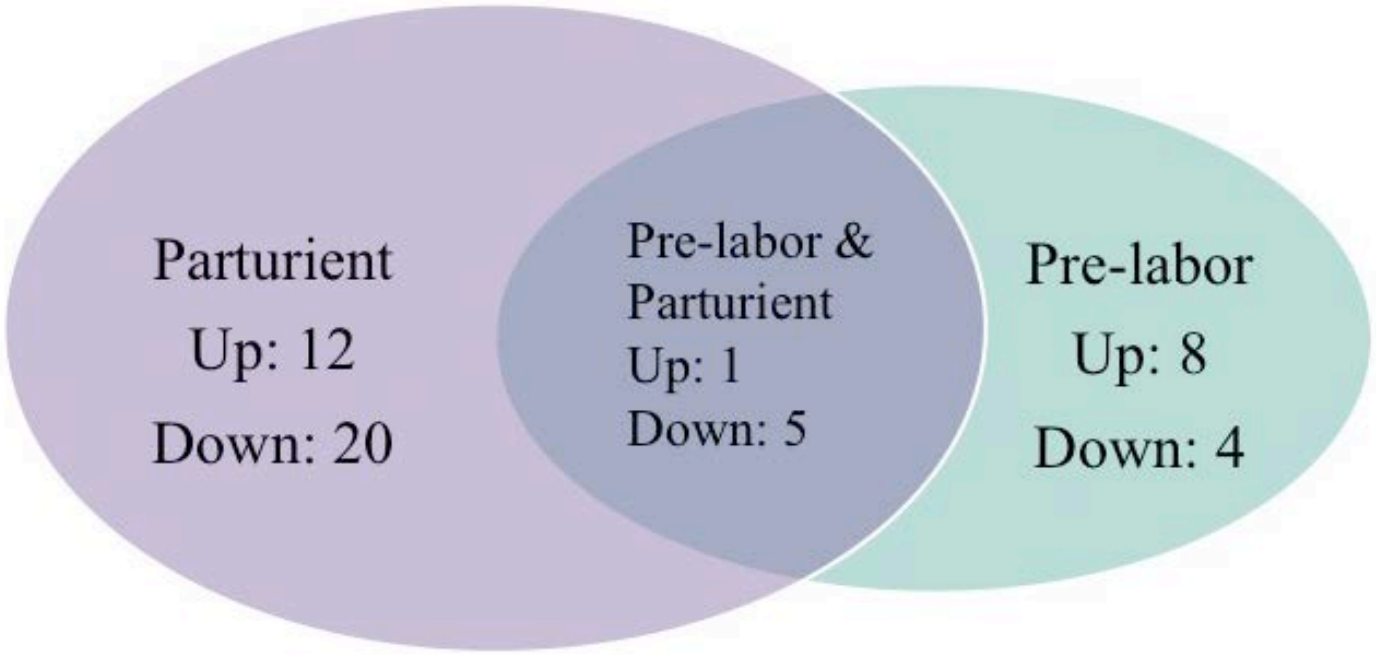

Figure 3.2. Venn Diagram demonstrating down-regulation and up-regulation of genes among sample groups compared to pre-term.

Table 3.4. Most over-represented biological processes with the most differentially expressed genes based on 95% significance according to an ANOVA test

Biological Process	**P-value**
Proteolysis	0.004
Maintenance of Location	0.006
Stem Cell Division	0.019
Cell Adhesion	0.02
Hormone Catabolic Process	0.031
Peptide Hormone Processing	0.031
Protein Metabolic Process	0.044
Morphogenesis of a Branching Structure	0.047
Collagen Catabolic Process	0.048

3.4 DISCUSSION

Overall, knowledge is lacking regarding molecular changes in the canine placenta around the time of parturition. To the authors' knowledge, this is the first report of gene expression in the canine placenta using microarray analysis at any gestational age. While previous studies have looked at gene expression in the canine uterus and uteroplacental tissues (Klonisch et al., 2001; Beceriklisoy et al., 2007; Schäfer-Somi et al., 2008; Schäfer-Somi et al., 2009; Kowalewski et al., 2010; Kanca et al., 2011; Kowalewski et al., 2011), no studies have focused on the chorioallantois tissue of the placenta at the end of gestation.

The current study of the canine placental transcriptome demonstrates little global variation between sample groups in the peripartum period. Nevertheless, there were many genes involved in trophoblast invasion and placental release that were differentially expressed between peripartal groups. This is consistent with what has been found in a similar microarray study of the human placenta (Vu et al., 2008; Lee et al., 2010).

The most represented biological processes included proteolysis, cell adhesion, hormone catabolism, collagen catabolic process and protein metabolic processes. The overrepresentation of these processes in the differential gene expression studies may be evidence for late gestation tissue remodeling in the dog. Degradation of extracellular matrix proteins and breakdown of collagen is considered important for the rupture of fetal membranes in humans (Parry and Strauss III, 1998) and placental release in cattle (Eiler and Hopkins, 1993; Walter and Boos, 2001). Therefore, the overrepresentation of these processes in the current study supports a role for the differentially expressed genes in both tissue remodeling at the time of labor and possibly rupture of fetal membranes at canine parturition. This placental transcriptome analysis has lead to the identification of several candidate genes for future study including IL-6, MMP-1, DAG-1, CXCL10 and CD44.

The receptor for hyaluronan, CD44 (Aruffo et al., 1990; Goshen et al., 1996) may be important in trophoblast invasion as well as tissue remodeling in late gestation in the

human (Marzioni et al., 2009). The migration of human melanoma cells has been correlated to a high expression of CD44 protein (Thomas et al., 1993) suggesting a role in migration of other cell types. Hyaluronan is internalized upon binding to CD44 and degraded within the cell. This receptor-mediated degradation of hyaluronan is thought to be important in the process of macrophage cell migration (Culty et al., 1992).

CD44 protein expression has been identified on human trophoblast cells. In addition, invasive extravillous trophoblast cells express an alternatively spliced form of CD44 known as CD44v7-8. It has been proposed that invading trophoblasts use alternative splicing to become invasive. Furthermore, CD44 isoform CD44v7-8 expression may be involved in placenta accreta (Goshen et al., 1996) a condition of aberrant trophoblast invasion in the human (Davidson, 1944; Khong and Robertson, 1987). Others suggest expression of CD44 during the second half of pregnancy plays a role during late gestation tissue remodeling (Marzioni et al., 2009). In microarray analysis, CD44 was down-regulated from pre-term to pre-labor. The expression of CD44 in late gestation in the dog supports a role for CD44 in late gestation tissue remodeling. The decline in expression around labor seen in this study may suggest that CD44 is needed during late gestation (61 days of gestation), but not at the initiation of labor.

The expression of DAG-1 was up-regulated between pre-term and parturient samples in microarray analysis. The mammalian DAG-1 gene encodes for α and β subunits of the dystrophin-glycoprotein complex. Human dystroglycan is a laminin receptor thought to function in the stabilization of the extracellular matrix in skeletal muscle, but it is thought to play a similar role in membrane stability in other cell-types as well (Ibraghimov-Beskrovnaya et al., 1993). Dystroglycan is required for the development of Reichert's membrane during murine development. Furthermore, mice null for DAG-1 display fatal disruptions to this membrane (Williamson et al., 1997).

Recently, both α- and β-dystroglycan has been identified in human placental tissue and trophoblast cell lines. It has been proposed that dystroglycan may have an inhibitory effect on trophoblast invasion as the invasive first-trimester trophoblast cell line HTRSV40 does not express α-dystroglycan. In addition, first-trimester human

placental samples express lower levels compared to term placental samples (Street et al., 2012). The increase in DAG-1 observed in the current study around parturition leads to the speculation that an increase in DAG-1 mRNA expression at canine parturition may be important for maintaining membrane integrity during placental release.

Interleukin-6 (IL-6) has been implicated in trophoblast invasion and its expression has been noted to change depending on the occurrence of labor. IL-6 stimulates invasion of human cytotrophoblast cells in vitro (Jovanoić and Vićovac, 2009) and may be involved with the regulation of trophoblast invasion by stimulating MMP-2 and MMP-9 activation (Meisser et al., 1999). An increased production of IL-6 by placental cell cultures is associated with normal term labor in humans (Steinborn et al., 1999). Furthermore, the expression of TNFα and IL-6 in the pre-implantation canine embryo is thought to be important in regulating the balance of tissue remodeling during implantation (Schäfer-Somi et al., 2008). Based on previous studies we expected to see a distinct expression pattern around the time of canine parturition. Interleukin-6 (IL-6) was down-regulated from pre-term to parturient in microarray analysis. This difference in the present study compared to what has been previously been reported in humans may suggest IL-6 plays a different role in the canine placenta.

Degradation of the extracellular matrix (ECM) by matrix metalloproteinases is thought to be essential not only for trophoblast invasion, but also the separation of placental membranes at parturition in a number of species (Walter and Boos, 2001; Xu et al., 2002). Matrix metalloproteinase (MMP)-1 or collagenase-1 is a member of the MMP family (Matrisan, 1990). Separation of the placenta from the uterine wall and loss of placental mass may rely on expression of MMPs such as MMP-1 and MMP-3 in the human (Vettraino et al., 1996). In addition, an increased expression of MMP-9 and MMP-3 mRNA in the human choriondecidua is proposed to activate MMP-1 in order to degrade ECM components during labor (Bryant-Greenwood and Yamamoto, 1995). MMP-1 was significantly expressed between pre-term and parturient samples suggesting that tissue remodeling in the canine placenta may be occurring around the time of parturition in the dog. MMP activity has also been noted in the feline placenta suggesting

MMP expression may play an important role in the endotheliochorial placental type (Walter and Schönkypl, 2006).

Chemokines play an important role during implantation. CXCL10 acts as a chemoattractant for the trophoblast cell line JEG-3 (Dominguez et al., 2008). CXCL10 has been identified in the caprine endometrium while its receptor CXCR3 mRNA and protein is expressed by caprine trophoblast cells (Nagaoka et al., 2003; Imakawa et al., 2006). It has been proposed that CXCL10 produced by the endometrium in the goat may stimulate adhesion and invasion of the trophoblast to the uterus by interacting with its receptor CXCR3 found on the trophoblast (Nagaoka et al., 2003; Imakawa et al., 2006).

Chemokines such as CXCL10 are considered important molecules in the regulation of normal and pathological parturition (Gomez-Lopez et al., 2010). CXCL10 is a chemokine that was once known as interferon-γ-inducible protein 10 (IP-10) and is induced by factors such as interleukin-1 and tumor necrosis factor-α in multiple cell types (Dominguez et al., 2008). It is thought that in humans, specific chemokines, including CXCL10 are primarily produced by the choriodecidua and amnion early in labor resulting in the recruitment of various leukocyte populations who along with local cells secrete cytokines and MMPs. This results in the cascade of events that lead to parturition (Gomez-Lopez et al., 2010). The up-regulation of CXCL10 in the current study between pre-term and parturient samples suggests the chemokine CXCL10 in the dog may play a role in the initiation of parturition, similar to that reported in humans.

In conclusion, this is the first published report of a canine placental gene expression profile during the peripartum period. This placental transcriptome analysis presents a number of candidate genes that warrant further study to better understand the molecular changes that occur at the end of gestation in the bitch. Characterizing gene expression changes at the end of gestation is the first step in better understanding the regulation of trophoblast invasion and regression. Further study is needed to investigate global gene expression earlier in canine gestation as well as compare gene expression of normal placentas to pathologic placental tissue

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CHAPTER IV

CONCLUSION AND FUTURE STUDIES

We have demonstrated that canine chorioallantois tissue expresses CD-44, CXCL10, DAG-1, Flt-1, HPRT-1, IL-6, MMP-1, MMP-2, MMP-9, TIMP-2, and VEGF-A during the peripartum period using technologies such as real-time RT-PCR and microarray analysis. These genes are of interest in human studies related to abnormal trophoblast invasion (Nishizawa et al., 2007; Sitras et al., 2009; Winn et al., 2009; Lian et al., 2010; Centlow et al., 2011; Mayor-Lynn et al., 2011; Zhang et al., 2011) and placental release (Vettraino et al., 1996; Xu et al., 2002; Torricelli et al., 2008; Street et al., 2012). This is the first report on global gene expression in the canine placenta using microarray analysis.

Throughout pregnancy, the placenta is vital in the transport of nutrients to and removal of waste from the developing fetus, as well as synthesizing substances necessary for pregnancy maintenance (Johnston et al., 2001). The tissues studied in this research represent a time period when the placenta transitions from this vital role to the expulsion during parturition. Despite the significant physiological changes that are occurring within the peripartum canine placenta, overall gene expression changes were minimal. Results on peripartum placental gene expression in human studies vary. Similar to our findings, several report minimal overall global gene expression variation (Sitras et al., 2008; Vu et al., 2008); while others report significantly different gene expression profiles (Cindrova-Davies et al., 2007; Lee et al., 2010) in tissues from normal term spontaneous labor compared to those obtained by term elective Cesearean section. Although the overall gene expression changes in the canine placenta were much smaller than those reported in human placental transriptome studies (Cindrova-Davies et al., 2007; Lee et al., 2010), the differential expression reported was between similar genes such as Flt-1 (Cindrova-

Davies et al., 2007; Lee et al., 2010), SERPINE1 (Lee et al. 2010), CIRBP (Cindrova-Davies et al., 2007) and MMP-1 (Vu et al., 2008).

It is possible the difference between this research and other human microarray studies that showed greater global gene expression variability may represent the uniqueness of the modified deciduate and endotheliochorial placenta of the dog. Future studies may focus on determining if this lack of variability is characteristic to other carnivores (similar kind of placenta) or other domestic species (having a non-deciduate, epitheliochorial placenta). In addition, future studies should focus on other canine reproductive tissues at this time period (e.g., endometrium) to better capture gene expression changes that mirror the physiologic changes known to occur at parturition.

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APPENDICES

APPENDIX A: TOTAL RNA ISOLATION FROM CANINE CHORIOALLANTOIS TISSUE AND ISOLATED CANINE TROPHOBLASTS

Cleaning and Preparatory Steps

Prior to RNA isolation, the work space was cleaned meticulously to prevent RNA degradation from RNases present in the environment. The day before the isolation was to take place fresh blotter paper was laid down in the fume hood and the surrounding area. On the day of the isolation, further cleaning and preparation took place. The first step was to turn on the microcentrifuge and set it to 4°C to insure that this temperature was reached at the appropriate time in the protocol. The fume hood and any surfaces that tubes containing RNA sample may come into contact with were cleaned using a kim-wipe sprayed with RNase Zap (BIOHIT, Inc., Cat. No. 724000, Neptune, NJ), In addition, the hand-held homogenizer handle was wiped with RNase Zap (BIOHIT, Inc). A 2% Absolve solution (PerkinElmer Inc. Waltham MA) was prepared in a labeled 50 ml conical tube. Another labeled 50 ml conical tube was filled with NanoPure water and the homogenizer blades were cleaned using 2% Absolve (PerkinElmer Inc.) followed by a wash in NanoPure water.

RNA Isolation

Total RNA was isolated from frozen chorioallantois tissue using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Approximately 100 mg of frozen canine chorioallantois tissue was sliced off previously flash frozen specimens using a razor blade, placed in a labeled 50 ml conical tube, and then stored at -80°C if not used immediately. One reagent within the RNA Purification Kit (Wash Buffer II) was prepared prior to the first isolation using the kit. In the vial provided by the kit, 60 ml of 100% ethanol was added to Wash Buffer II and the buffer was stored at room temperature.

The following procedure was carried out under the fume hood due to the use of TRIzol Reagent provided with the kit, which contains phenol and guanidine isothiocyanate. In the 50 ml conical tube containing the tissue sample, 1 ml of TRIzol

Reagent (Invitrogen, Carlsbad, CA) was added, and then the sample was homogenized. Following a 5 minute incubation at room temperature, 0.2 ml of chloroform was added to the lysate. The tube was shaken vigorously by hand for 15 seconds, but not vortexed based on the manufacturers instructions. The sample was then transferred to a microcentrifuge tube where it was incubated at room temperature for 3 minutes.

Total RNA was isolated from isolated trophoblasts using the same TRIzol Plus RNA Purification Kit. Cells that had previously been isolated and frozen were removed from the liquid nitrogen tank and thawed on ice. Once thawed, the vials were centrifuged, the supernatant was pipetted off and 1 ml of TRIzol was added to the vial. The cells were then lysed in the TRIzol by pipetting up and down. The cells and TRIzol reagent were then pipetted into a fresh 1.5 ml RNase-free microcentrifuge tube. The lysates were incubated in the TRIzol at room temperature for 5 minutes. Following the incubation, 0.2 ml of chloroform was added to the tube. The tube was shaken vigorously by hand for 15 seconds and then incubated at room temperature for 3 minutes.

Irrespective of sample type (chorioallantois tissue or isolated trophoblasts), the sample was then centrifuged at 12,000 x g for 15 minutes at 4°C. After centrifugation, the mixture separated into an organic phase (lower, red phenol-chloroform portion), a white interphase (containing DNA) and an upper, clear phase (containing RNA). After centrifuging, 400 µl of the clear RNA containing phase was transferred to a new RNase-free microcentrifuge tube.

A final ethanol concentration of 35% was achieved by adding 400 µl of 70% ethanol to the tube. The tube was then mixed by vortexing and inverted to disperse any visible precipitate that may form after the addition of the ethanol. After the tube was inverted, 400 µl of the sample was transferred to a spin cartridge with a collection tube provided by the kit. The spin cartridge was then centrifuged at 12,000 x g for 15 seconds at room temperature. The effluent located in the collection tube after centrifuging was discarded and the spin cartridge was reinserted into the collection tube. This step was repeated until the entire sample was processed through the spin cartridge.

After the last processing step, the effluent was discarded and 700 µl of Wash Buffer I (provided by the kit) was added to the spin cartridge. The spin cartridge was then centrifuged at 12,000 x g for 15 seconds at room temperature. The effluent in the collection tube was then discarded as previously described and the spin cartridge was reinserted into a new collection tube. Wash Buffer II provided with the kit (500 µl) was then added to the spin cartridge. The sample was centrifuged at 12,000 x g for 15 seconds at room temperature and the effluent was discarded as described previously. The step requiring the addition of the Wash Buffer II was repeated once. The sample was then centrifuged at 12,000 x g for 15 seconds at room temperature and the spin cartridge was placed in the same collection tube. The sample was then centrifuged again at 12,000 x g for 1 minute at room temperature to dry the membrane with the attached RNA. After this centrifugation step, the collection tube was discarded and the spin cartridge was inserted into a recovery tube provided by the kit.

Once in the recovery tube, 100 µl of RNase-free water was added to the spin cartridge and the sample was incubated at room temperature for 1 minute. The spin cartridge was then centrifuged with the recovery tube at 12,000 x g for 2 minutes at room temperature. After the centrifugation, 100 µl of RNase-free water was added again, the sample was incubated and centrifuged as just described. This was repeated until the final volume of the eluate was 300 µl.

Determination of RNA Concentration and Purity

Following RNA isolation, the RNA concentration and purity of the sample was determined using a NanoPhotometer, Version 2.1 (IMPLEN, Munich, Germany). Concentration was determined using the concentration measurement through the RNA setting on the NanoPhotometer (IMPLEN). Purity was determined using the $A_{260/280}$ ratio where a ratio of 1.8-2.0 indicated a pure RNA sample (IMPLEN, 2012).

Briefly, the clear cover was removed from the machine and the black cuvette was removed from its location. The NanoPhotometer was powered on and allowed to heat up and self-calibrate. Once it was calibrated the black cuvette was returned to its holder. A

kim-wipe was used to gently wipe the surface of the cuvette. On the menu screen “Option 1” or “LabelGuard Applications” was selected. On the next screen, “RNA” was selected. On the third screen, “Lid Factor: 10” was highlighted and the button labeled “OK” was pushed to select the lid factor. RNase-free water was used to blank the NanoPhotometer. Approximately 3.5 µl of RNase free water was pipetted on the well of the cuvette. The lid labeled “Factor 10” was placed over the water on the cuvette. The NanoPhotometer was “blanked” for further measurements by pressing the blue button on the keypad labeled “Blank”. A kimwipe was used to gently wipe the surface of the cuvette. In order to measure absorbance, 3.5 µl of the sample to be measured was carefully pipetted on to the well on the cuvette surface. The lid labeled “Factor 10” was again placed on the cuvette, covering the sample. To run the sample, the button on the keypad labeled “Sample” was pressed. Sample concentration as well as absorbance was measured and appeared on the screen. If the sample was too concentrated for Lid Factor 10 an image appeared in the upper left hand corner of the NanoPhotometer screen with a 10→50 image indicating the sample should be rerun with the lid labeled “Factor 50”. Once the measurement was satisfactory the button on the keypad labeled “Options” was pressed and “2” or “Print” was clicked in order to print the results. A kim-wipe was then used to wipe the cuvette before the machine was powered off. The RNA concentrations determined for placental tissue and isolated trophoblasts are provided in Tables A.1 and A.2, respectively.

Determination of RNA Integrity

Following determination of RNA concentration, a 2 µl aliquot of sample was pipetted into a 0.5 ml microcentrifuge tube to determine RNA integrity on the Agilent 2100 Bioanalyzer (Agilent Techonolgies, Santa Clara, CA). The samples were stored at -80°C until twelve samples had been prepared and they were submitted as a batch to the Center for Genome Research and Biocomputing (CGRB) at Oregon State University. During the RNA isolation procedure, samples were eluted in water so they were submitted to the CGRB suspended in water. Concentrations of samples to be submitted

needed to be between 25-500 ng/ µl. Some samples were too concentrated, so they were diluted and submitted as 10 µl aliquots.

Prior to submitting samples to the CGRB, an online account was set up through the staff at the CGRB core lab. Samples were submitted to the CGRB through the online submission form at http://www.cgrb.oregonstate.edu. Once the samples were submitted using the online submission form, the samples were taken to the CGRB core lab located in Room 3012 of the Agricultural Life Sciences Building for analysis. A labeled test tube rack was placed in the -80°C freezer. Staff from the CGRB ran the samples on the Agilent 2100 and emailed the results when they were complete. The software produces an electropherogram that displays the size distribution of fragments of each RNA sample and allows a visual inspection of the RNA quality as well as a RNA integrity number (RIN) (Figure A.1).

Appendix A. References

IMPLEN, 2012. Nucleic Acids Estimation of Purity. [WWW Document]. URL http://implen.com/nanophotometer/applications.php.

Table A.1. RNA Concentration, $A_{260/280}$ ratio and RNA integrity number (RIN) for chorioallantois samples. R1-R3: first-third placenta in right uterine horn from uterine body, respectively, #1-3: tissue replicates used in Appendix F

Sample	**RNA concentration**	**$A_{260/280}$**	**RIN**
681 R1	304 ng/µl	1.969	9.60
405 R1 #1	424 ng/µl	1.970	9.60
405 R1 #2	345 ng/µl	1.986	9.50
405 R1 #3	391 ng/µl	1.988	10
638 R3 #1	415 ng/µl	1.973	9.60
638 R3 #2	499 ng/µl	1.986	10
638 R3 #3	448 ng/µl	1.989	10
311 R2 #1	56.4 ng/µl	1.932	9.5
311 R2 #2	416 ng/µl	2.004	9.9
311 R2 #3	404 ng/µl	2.004	9.7
Twix	245 ng/µl	1.971	9.70
Gracie	348 ng/µl	2.016	9.30
Piper	268 ng/µl	2.003	8.90
Samba	367 ng/µl	2.007	9.70
Gemima	328 ng/µl	1.983	9.80
Gidget	409 ng/µl	1.990	9.50
Cher	232 ng/µl	1.997	9.60
Amber	236 ng/µl	2.010	9.8

Table A.2. RNA Concentration, $A_{260/280}$ ratio and RNA integrity number (RIN) for isolated trophoblasts

Sample	**RNA concentration**	**$A_{260/280}$**	**RIN**
BS1118	226 ng/µl	2.014	9.9
S123	136 ng/µl	2.012	8.8
Abb315	81.6 ng/µl	1.943	9.8
Cleo624	669 ng/µl	1.940	9.3
Fan624	527 ng/µl	1.966	9.2

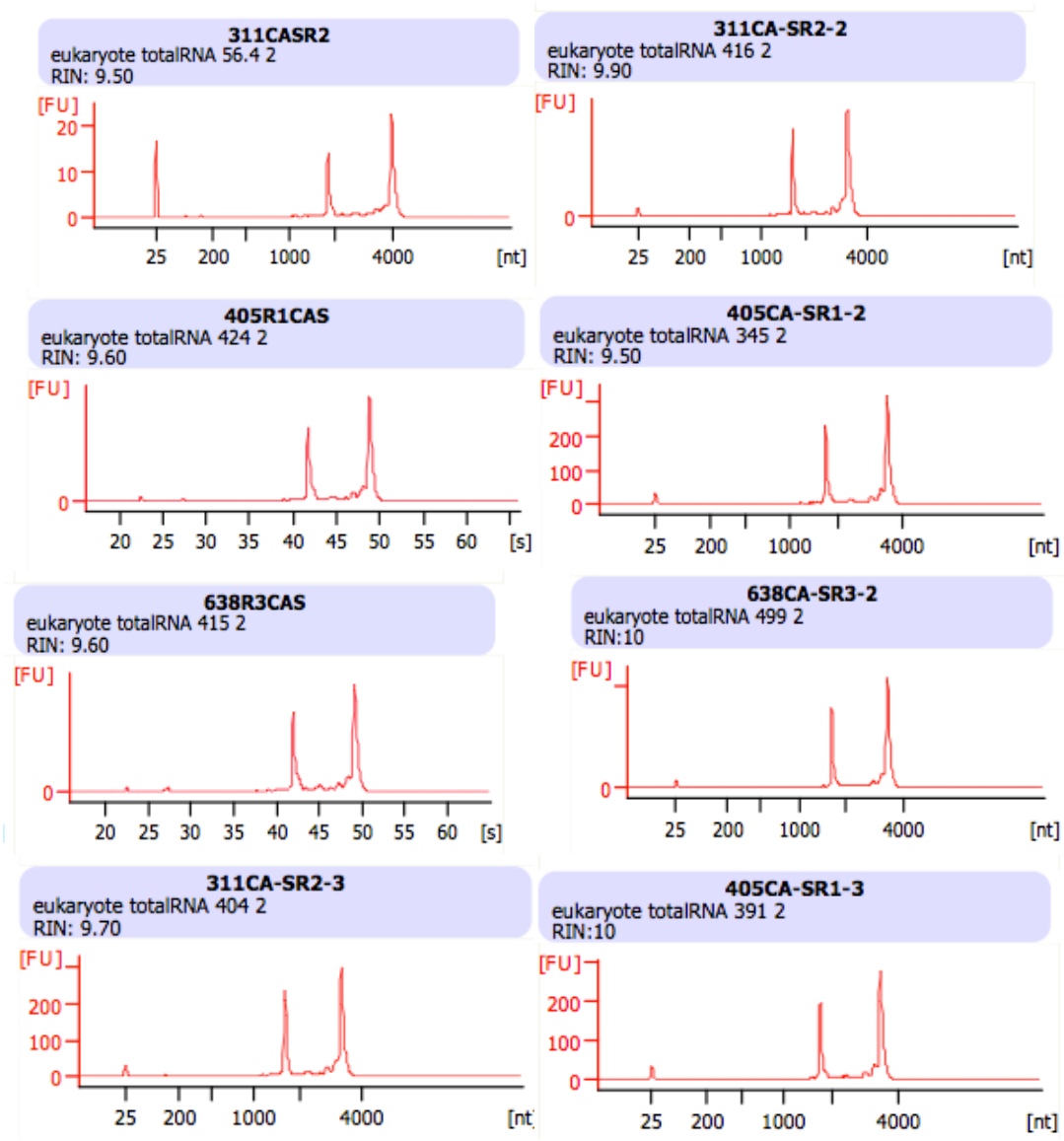

Figure A.1. Agilent 2100 Bioanalyzer results.

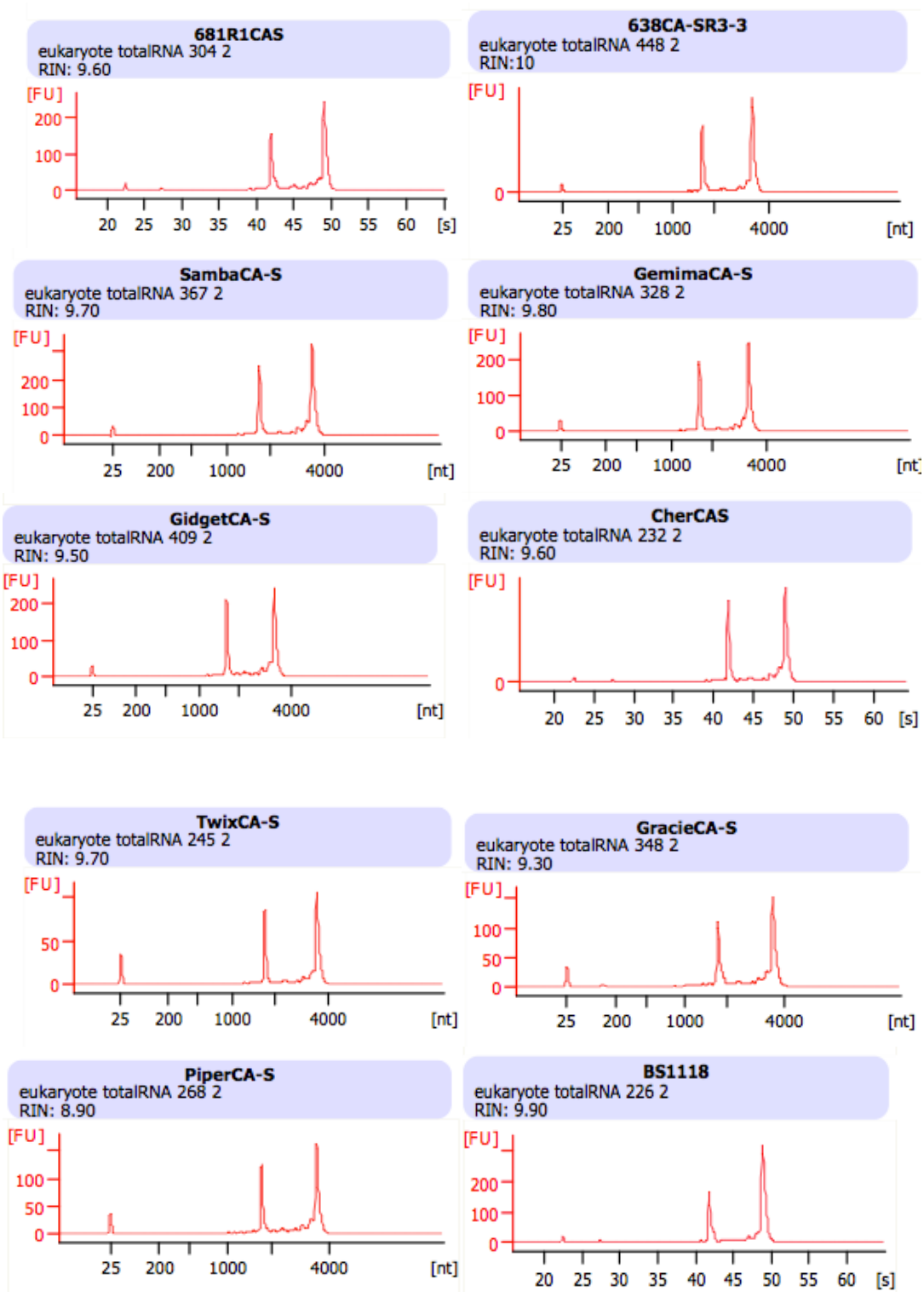

Figure A.1. Agilent 2100 Bioanalyzer results (continued).

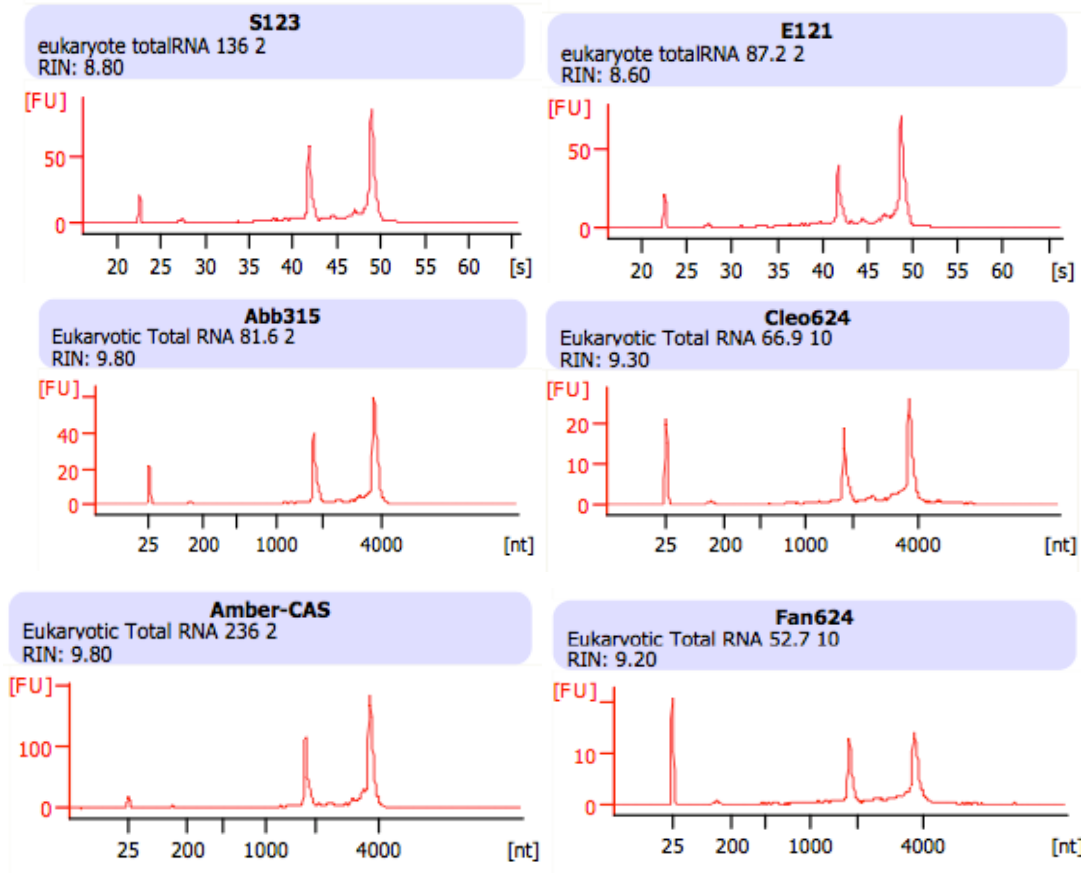

Figure A.1. Agilent 2100 Bioanalyzer Results (continued).

APPENDIX B: CDNA SYNTHESIS USING THE SUPERSCRIPT III SYSTEM

Synthesis of cDNA was performed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, Cat No. 18080-051) according to the manufacturer's instructions. Prior to starting the cDNA synthesis, the thermal cycler was set up to the protocol; 65°C for 5 minutes, 50°C for 50 minutes, then 85°C for 5 minutes followed by 37° C for 20 minutes. This protocol was saved in the thermal cycler in Withycombe 315 under the username "ejfellows"; protocol "cdnalf". First, 0.2 ml microcentrifuge tubes were labeled to correspond to the appropriate RNA sample to be reverse transcribed. The amount of RNA sample needed to be up to 5 µg of RNA for successful reverse transcription. The amount of RNA was calculated by multiplying the concentration (µg/ml) by 8 because 8 µl of sample was removed to fulfill the kit protocol.

The RNA samples and kit reagents were thawed on ice. Once thawed, the samples and reagents were briefly mixed by flicking. The samples were then briefly centrifuged and 8 µl of RNA sample was pipetted in the appropriately labeled tube. Next, 1 µl of 50 µM oligo(dT) primer and 1 µl of 10 mM dNTP mix (both provided by the kit) were added to the sample tube. The tube was then placed into the thermal cycler for the first step of the "cdnalf" protocol (65°C for 5 minutes). After the 5 minutes were up, the thermal cycler was paused and the tube was removed from the thermal cycler. The sample was placed on ice for 3 minutes. During this time, a cDNA synthesis mix was prepared in a separate tube containing 2 µl of 10X RT Buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNase OUT and 1 µl of SuperScript III RT to get a final volume of 10 µl per reaction. This tube was mixed by flicking and centrifuged briefly. After the sample sat on ice for 3 minutes, 10 µl of the cDNA synthesis mix was added to the RNA/primer mixture tube. The tube was then mixed gently by flicking and the reaction was collected by brief centrifugation. The sample was returned to the thermal cycler to resume the "cdnalf" protocol by incubating at 50°C for 50 minutes and then 85°C for 5 minutes. After the 5 minutes, the protocol was again paused and the sample was removed from the thermal cycler. The sample was chilled on ice for 1 minute and after the 1 minute was up 1 µl of RNase H, provided by the kit was pipetted into the tube. RNase H is an

endoribonuclease that specifically degrades the RNA strand of the RNA:DNA hybrid in order to create 5' phosphate- terminated oligoribonucleotides and single-stranded DNA (Invitrogen, 2012). The sample was then returned to the thermal cycler where the protocol was resumed and the sample incubated for 20 minutes at 37°C. The cDNA concentration was measured using the DNA setting on the NanoPhotometer (IMPLEN, Munich, Germany), as previously described in Appendix A. Once concentration had been determined, a 2 µl sample was pipetted into a tube of 98 µl of RNase-free water for future real-time RT-PCR experiments. The cDNA sample was then stored at -20°C until further analysis.

Appendix B. References

Invitrogen, 2012. Ribonuclease H. [WWW Document] URL http://tools.invitrogen.com/content/sfs/manuals/18021014.pdf.

APPENDIX C: SELECTION OF A REFERENCE GENE

Reference genes or housekeeping genes are an important part of measuring relative gene expression using real-time RT-PCR. An effective reference gene should be one whose expression levels do not significantly differ between different conditions or gestation age. Initially, glyceraldhyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA were selected based on previous studies that identified these two genes as effective housekeeping genes in other canine tissues. GAPDH was identified as a good reference gene in tissues that included the canine corpus luteum, uteroplacental samples (Kowalewski et al., 2011), kidney (Rao et al., 2003) and endometrium (Kida et al., 2010). Several canine studies have used 18S rRNA as a reference gene in other canine tissues including canine hemangiosarcoma (Tamburini et al., 2010), corpus luteum and uteroplacental tissues (Kowalewski et al., 2011).

Following the first experiment, 18S rRNA expression appeared to be stable, however GAPDH did not amplify. After consultation with technical support at Applied Biosystems (Vivian Cullen, personal communication), GAPDH was eliminated from further study. Additional review of the literature identified a problem with the use of 18S rRNA. The cDNA synthesis protocol used relies on oligo dT as a primer, which binds to the poly-A tail of mRNA to synthesize cDNA. Since 18S rRNA does not have a poly-A tail, its use in this protocol could result in unreliable synthesis of the gene (Vivian Cullen, personal communication). Therefore, this gene was eliminated from the studies as a reference gene.

Hypoxanthine-phosphoribosyl transferase 1 (HPRT-1) was then selected for investigation based upon its success in other canine studies (Brinkhof et al., 2006; Etschmann et al., 2006; Schlotter et al., 2009). HPRT-1 was stably expressed in all chorioallantois tissues within sample groups and between sample groups as determined by Ct values (Ct mean ± SEM; 32.02 ± 0.24) as well as an inter-assay and intra-assay coefficient of variation of 3.86% and 3.60% respectively. Coefficients of variation of

<5% indicate little variation between a normalized measure of distribution (Gerd Bobe, personal communication).

Appendix C. References

Brinkhof, B., Spee, B., Rothuizen, J., Penning, L., 2006. Development and evaluation of canine reference genes for accurate quantification of gene expression. Anal Biochem 356, 36-43.

Etschmann, B., Wilcken, S., Stoevesand K., von der Schulenburg, A., Sterner-Kock, A., 2006. Selection of reference genes for quantitative real-time PCR analysis in canine mammary tumors using the GeNorm algorithm. Vet Pathol 43, 934-942.

Kida, K., Maezono, Y., Kawate, N., Inaba, T., Hatoya, S., Tamada, H., 2010. Epidermal growth factor, transforming growth factor-α, and epidermal growth factor receptor expression and localization in the canine endometrium during the estrous cycle and in bitches with pyometra. Theriogenology 73, 36-47.

Kowalewksi, M., Meyer, A., Hoffmann, B., Aslan, S., Boos, A., 2011. Expression and functional implications of peroxisome profliferator-activated receptor gamma (PPARγ) in canine reproductive tissues during normal pregnancy and parturition and at antiprogestin induced abortion. Theriogenology 75, 877-886.

Rao, V., Lees, G., Kashtan, C., Nemori, R., Singh, R., Meehan, D., Rodgers, K., Berridge, B., Bhattacharya, G., Cosgrove, D., 2003. Increased expression of MMP-2, MMP-9 (type IV collagenases/gelatinases), and MT1-MMP in canine X-linked Alport syndrome (XLAS). Kidney Int 63, 1736-1748.

Schlotter, Y., Veenhof, E., Brinkhof, B., Rutten, V., Spee, B., Willemse, T., Penning, L., 2009. A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs and dogs with atopic dermatitis. Vet Immunol Immunopathol 129, 115-118.

Tamburini, B., Phang, T., Fosmire, S., Scott, M., Trapp, S., Duckett, M., Robinson, S., Slanksy, J., Sharkey, L., Cutter, G., Wojciezyn, J., Bellgrau, D., Gemmill, R., Hunter, L., Modiano, J., 2010. Gene expression profiling identifies inflammation and angiogenesis as distinguishing features of canine hamangiosarcoma. BMC Cancer 10, 619. doi: 10.1186/1471-2407-10-619.

APPENDIX D: PROTOCOL FOR REAL-TIME RT-PCR FOR RELATIVE QUANTITATION OF GENE EXPRESSION IN CANINE CHORIOALLANTOIS TISSUE

Setting up the Plate and Calculations

In order to visualize the 96-well plate, the wells were diagrammed and color-coded indicating which samples would be placed in what wells. The cDNA samples and reagents were thawed on ice. Once thawed, the cDNA samples and reagents were mixed by flicking and collected by brief centrifugation. The concentration of each cDNA sample was measured using the NanoPhotometer (Appendix A). The amount of cDNA sample needed in each well to provide 10 ng of cDNA per well was calculated by dividing 10 by the concentration of cDNA. Then the amount of water needed in each well was calculated by subtracting the amount of sample needed from 9 (Table D.1). For each sample run in triplicate, a corresponding "no template control (NTC)" was also run in triplicate (Table D.2). The NTC samples contained the master mix, TaqMan primer assays and water, but no cDNA sample. The purpose of NTC wells was to control for contamination in the wells containing sample and act as negative controls.

Table D.1. Examples of how to determine the volume of cDNA template needed for each reaction

Samples	**Concentration**	**Volume of Sample for 10 ng cDNA**	**Water needed (µl)**
Example 1	14.8 ng/µl	0.68 µl	8.32
Example 2	19.6 ng/µl	0.51 µl	8.49
Example 3	17.4 ng/µl	0.57 µl	8.43

Table D.2. Sample table of no template control (NTC) and corresponding sample calculations. The total volume per well was always 20 µl

Sample	**Master Mix Volume**	**Water**	**cDNA**
NTC4052MMP9	11 µl per well	9 µl per well	0 µl per well
405-2 MMP9	11 µl per well	8.32 µl per well	0.68 µl per well
NTC464053 MMP9	11 µl per well	9 µl per well	0 µl per well
405-3 MMP9	11 µl per well	8.49 µl per well	0.51 µl per well

Preparing the Master Mix and Triplicates

Each reaction contained 10 µl of the TaqMan Universial PCR Master Mix (Applied Biosystems, Carlsbad, CA) and 1 µl of the TaqMan Gene Assay (Applied Biosystems). The TaqMan gene assays used were primer and probes that were pre-made and pre-optimized for canine tissues (Table D.3). A "master" master mix was prepared for each gene assay and endogenous control in a 1.5 ml microcentrifuge tube according to how many reactions were to be run (Table D.4). Each 1.5 ml microcentrifuge tube containing master mix stock was vortexed and centrifuged briefly. The reagent stocks for each cDNA and NTC sample to be run in triplicate were calculated (Table D.5) and combined in labeled 1.5 ml microcentrifuge tubes. Each tube was then mixed by inversion and flicking. The tubes were centrifuged briefly and kept on ice. Once all samples were ready, 20 µl of each triplicate was pipetted into the appropriate wells on a 96-well plate. Once all the wells contained 20 µl of the reaction an optical cover slip was placed over the top. The cover slip was sealed to the plate by running a fingernail and a pen along the edges to prevent evaporation during the experiment. The plate was then placed on ice until it was ready to be run in the RT-PCR machine. Prior to placing the plate into the machine the plate was carried on ice to Dr. Gerd Bobe's Laboratory in the Linus Pauling Building to be run on a centrifuge that could hold plates. The plate was centrifuged as maximum speed for about 20 seconds and then the plate was observed to make sure all samples were in the bottom of the wells. The plate was then placed back on ice.

Table D.3. TaqMan Primer and Probe Assays (continued on the next page)

Gene	Assay ID	RefSeq	Exon Boundary	Assay Location	Amplicon Length
MMP-2	Cf02623423_m1	XM_535300.2	3 to 4	520	58
MMP-9	Cf02621845_m1	NM_001003219.1	12 to 13	2015	59
TIMP-2	Cf02623335_m1	NM_001003082.1	1 to 2	234	81
VEGF-A	Cf02623453_m1	NM_001003175.1	7 to 8	906	62
Flt-1	Cf02696454_g1	XM_534520.2	26 to 27	3489	116
KiSS-1R	Cf02715933_u1	XM_850105.1	3 to 3	635	141
CD44	Cf02628940_m1	XM_533152.2	3-4	552	76
CXCL10	Cf-26225529_m1	NM_001010949.1	2-3	187	69

Table D.3. TaqMan Primer and Probe Assays (continued from previous page)

Gene	**Assay ID**	**RefSeq**	**Exon Boundary**	**Assay Location**	**Amplicon Length**
DAG-1	Cf03023355_m1	NM_001033992.1	2-3	505	93
IL-6	Cf02624153_m1	NM_001003301.1	4-5	516	66
MMP-1	Cf02651000_g1	XM_546546.2	2-3	383	63
HPRT-1	Cf026262558_m1	NM_001003357.1	8 to 9	605	129
18S rRNA	Hs99999901_s1	X03205.1	1 to 1	609	187

Table D.4. Example of a gene specific “master” master mix preparation where n is the number of reactions. N +4 was found to be an appropriate amount to allot for pipetting error

Reagent	**Volume per Reaction**	**n+4**
TaqMan Universal PCR Master Mix	10 µl	220 µl
MMP9 TaqMan Assay	1 µl	20 µl

Table D.5. Example of Triplicate Stocks. An n+0.25 was found to be effective at allotting for pipetting error

Sample	**Master Mix (n+0.25)**	**Water (n+0.25)**	**cDNA Sample (n+0.25)**
NTC4052MMP9	35.75 µl	29.25 µl	0 µl
405-2 MMP9	35.75 µl	27.04 µl	2.21 µl
NTC4053MMP9	35.75 µl	29.25 µl	0 µl
405-3 MMP9	35.75 µl	27.60 µl	1.66 µl

Creating A Relative Quantification (RQ) Plate Document

The computer program SDS Software 1.3.1 (Applied Biosystems, Carlsbad, CA) was used to perform relative quantification using the 7300 RT-PCR Machine (Applied Biosystems). The computer program was started by selecting Start>Programs>Applied Biosystems 7300>Applied Biosystems SDS software on the computer hooked up to the 7300 RT-PCR machine (Applied Biosystems). To create a new document, "File" on the tool bar was selected and "New" was selected from the drop down menu in order to start a new document. In the assay drop-down list of the "New Document Wizard", "Relative Quantification (ddCt) Plate" was selected. The default settings for container and template (96-Well Clear and Blank Document) were accepted. A name was then entered in the Default Plate Name Field and "Next" was clicked. Detectors including MMP-9, MMP-2, TIMP-2, Flt-1, VEGF-A, KiSS-1R, CD44, IL-6, CXCL10, MMP-1 and DAG-1 were selected to be added to the plate document when needed. These detectors were assigned as the targets. This was repeated for HPRT-1, which was designated as the endogenous control (ENDO) (see Appendix C). The detectors were added to the plate document by clicking "Add" and then clicking "Next". Detectors and tasks for each well were then specified by clicking a well or group of wells. Once a well was selected a detector was clicked on to add it to the well and a task (target or endo) was assigned to the well by clicking the drop down menu "Task Column". To finalize the detectors and tasks, "Use" was clicked followed by "Finish". Sample names were added by opening the "Well Inspector". The wells were clicked to select the replicate wells and the sample name was then typed in. This was repeated until all sample names and passive reference dyes for each well were specified. The information was then verified in each well using the "Setup Tab" located at the top of the screen.

Specifying Thermal Cycling Conditions and Starting the Run

The "Instrument Tab" was then selected to verify the thermal cycling conditions. The default PCR thermal cycling conditions were used (Table D.6) and 20 µl sample volume was entered. Once this was verified, "File" was selected at the tool bar and the

RQ Plate Document was saved by clicking “Save As”. The plate was then loaded into the instrument and “Start” was selected to begin the run. Each run took about 1 hour and 41 minutes. If the run was successful a message popped up that said “Run Successful” at the end of the 1 hour and 41 minute time period. At this time the plate was removed and discarded.

Table D.6. Real-time RT-PCR default cycling conditions

Stage	**Temperature and Time**	**Replications**
Stage 1	50.0°C for 2 min	1
Stage 2	95.0°C for 10 min	1
Stage 3	95.0°C for 15 sec then 60.0°C for 1 min	40

Analyzing Data in an RQ Study

A new RQ study document was created by selecting "File" and "New". In the assay drop-down list of the New Document Wizard, Relative Quantification (ddCt) study was selected. The default settings for container and template (96-Well Clear and Blank Document) were accepted. The name of the plate was then entered into the Plate Name field and "Next" was selected. RQ plates to be analyzed were then added to the study by clicking "Add Plates". The plates to be added were selected and opened by clicking "open" followed by "finish". The file was then saved under the name of the samples on the plate. The RQ detector grid allowed detectors to be selected that were associated with the study. Each detector, color, detector name, threshold value, auto Ct and baseline are shown in the RQ detector grid. In the RQ Sample Grid, the samples associated with the selected detectors are displayed. This grid has two subtabs called Sample Summary and Well Information that provide numerical results of the RQ calculations. The RQ Results Panel contains three results-based tabs Plate, Amplification Plot and Gene Expression to allow more visualization of the results.

Configuring Analysis Settings

To configure analysis settings, "Analysis" was selected from the tool bar and "Analysis Settings" was selected from the drop-down menu. In the detector drop-down menu "All" was selected. The SDS software automatically generated baseline and threshold values for each well when "Auto Ct" was selected. The Calibrator sample was selected (usually this was the first sample collected of the group run on the plate, but overall during the analysis the calibrator was the first parturient sample collected known as "Piper"). The endogenous control detector was selected as HPRT-1 and RQ Min/Max Confidence Level was selected at 95%. To finish the analysis "OK & Reanalyze" was selected. After the analysis, the RQ Manual that came with the 7300 Machine (Applied Biosystems) was used to verify visually that the baseline and threshold were called correctly for each detector.

Exporting RQ Study Data

The data was exported into a .csv file for analysis in Microsoft Excel (Microsoft, Redmond, WA) and Stata 12.0 (StataCorp LP, College Station, TX). To export the data "File" was selected from the tool bar, "Export" was selected from the drop-down menu and "Results" was then selected. Both Sample Summary data and Well Information data was selected for export. The .csv file was then saved for further analysis.

Analyzing RT-PCR Data

Following exportation from the SDS Software 1.3.1, the .csv files containing exported results were saved into Microsoft Excel (Microsoft, Redmond, WA) spreadsheets for analysis. First, Ct values from NTC samples were observed to ensure they were not less than 37. The Ct value negatively correlates to the amount of gene present. All NTC samples on plates used for analysis were listed as "undetermined", which indicates no amplification was measured during the run in the RT-PCR machine. Any NTC triplicates that were consistently less than 35 were suspect of contamination or faulty Master Mix. Average Ct values for the genes of interest and endogenous controls were calculated for the three triplicates of each sample using Microsoft Excel (Microsoft, Redmond, WA). The Ct values of the genes of interest were normalized to the controls by calculating the ΔCt. The ΔCt was calculated by subtracting the Average Housekeeping Gene Ct (HPRT-1) from the Average Gene Ct. The first parturient sample (Piper) collected was selected as the control and calibrator for the sake of the relative expression calculation. The ΔΔCt was calculated by subtracting the ΔCt of the first parturient sample collected (Piper) from each sample ΔCt. Relative expression of the genes of interest was calculated using the standard $2^{-\Delta\Delta Ct}$ method. This was achieved by setting the sample "Piper" to one by calculating RQ = $2^{-\Delta\Delta Ct}$ for each sample including "Piper".

APPENDIX E: RT VERSUS NO-RT CONTROL EXPERIMENT

All assays used for RT-PCR studies were pre-made, pre-optimized TaqMan Primer and Probe Gene Assays (Applied Biosystems, Santa Clara, CA). However, two of the assays (Flt-1 and MMP-1) might detect genomic DNA or one assay (KiSS-1R) might detect unspliced transcripts based on where the primers annealed to the gene (Cullen, 2009). In order to confirm that amplification achieved with these assays was not due to the presence of genomic DNA, an RT (+RT) versus no RT (–RT) experiment was performed (Cullen 2009). In this experiment, several representative RNA samples were run on a plate after being reverse transcribed to cDNA (Table E.1) alongside samples that had not been reverse transcribed (Figure E.1). MMP-9 was used as the tester gene because we had previously shown that it was expressed in the tissues.

After running the samples in the standard two-step protocol described in Appendix D, +RT samples and –RT samples should have Ct values that are at least 6-7 Ct values apart. Any difference in Ct value between +RT and –RT samples less than 6 corresponds to contribution from genomic DNA of greater than 1% (Cullen 2009). The –RT samples run in this experiment did not amplify at all as indicated by an "undetermined" Ct value (Table D.2.) indicating there was no contribution from genomic DNA.

Appendix E. References

Cullen, V., 2009. "Introduction to Real-Time PCR". Applied Biosystems. PowerPoint Presentation.

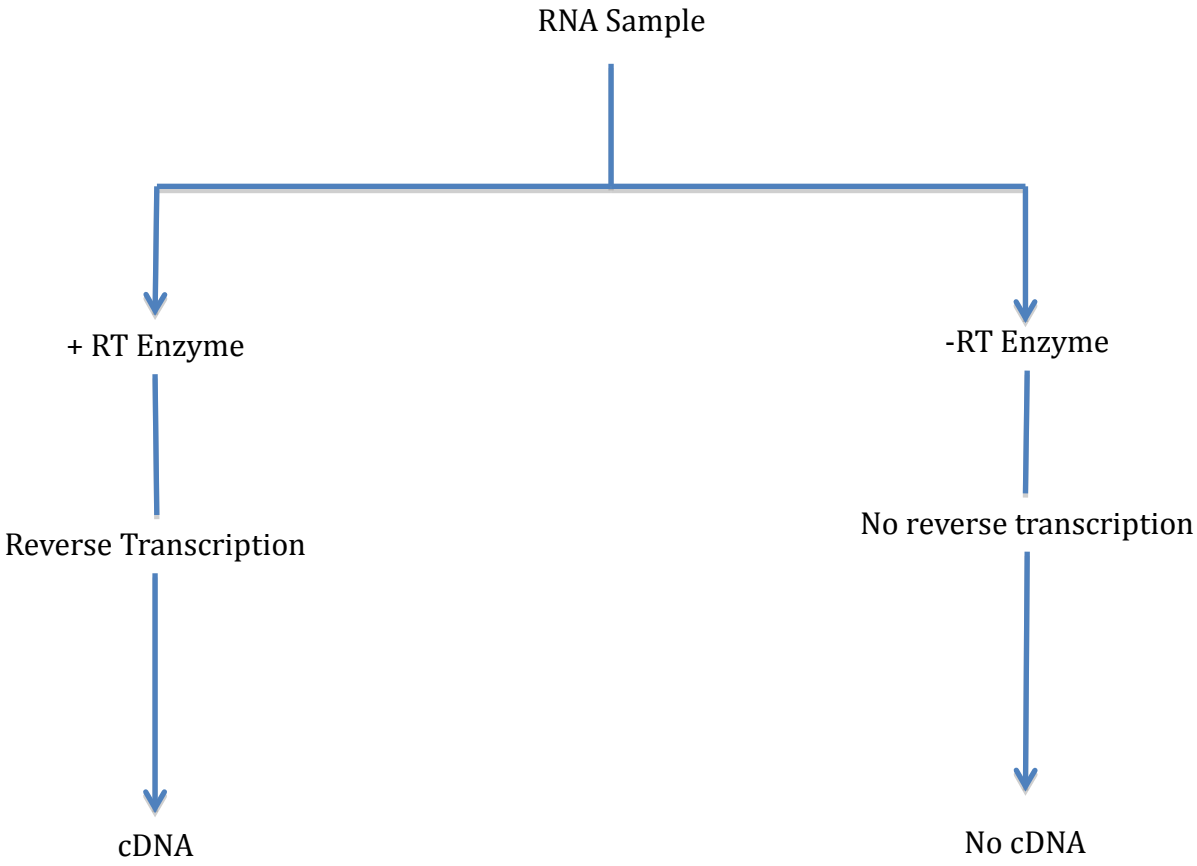

Figure E.1. Flow chart for RT (+RT) versus no RT (–RT) experiment.

Table E.1. 96-well plate set up for RT (+RT) versus no RT (–RT) experiment

681 MMP9 +RT	681 MMP9 +RT	681 MMP9 +RT	405 MMP9 +RT	405 MMP9 +RT	405 MMP9 +RT	311 MMP9 +RT	311 MMP9 +RT	311 MMP9 +RT	638 MMP9 +RT	638 MMP9 +RT	638 MMP9 +RT
681 MMP9 –RT	681 MMP9 –RT	681 MMP9 –RT	405 MMP9 –RT	405 MMP9 –RT	405 MMP9 –RT	311 MMP9 –RT	311 MMP9 –RT	311 MMP9 –RT	638 MMP9 –RT	638 MMP9 –RT	638 MMP9 –RT
									Gemima MMP9 +RT	Gemima MMP9 +RT	Gemima MMP9 +RT
									Gemima MMP9 –RT	Gemima MMP9 –RT	Gemima MMP9 –RT
Gidget MMP9 +RT	Gidget MMP9 +RT	Gidget MMP9 +RT	Piper MMP9 +RT	Piper MMP9 +RT	Piper MMP9 +RT	Gracie MMP9 +RT	Gracie MMP9 +RT	Gracie MMP9 +RT	Samba MMP9 +RT	Samba MMP9 +RT	Samba MMP9 +RT
Gidget MMP9 –RT	Gidget MMP9 –RT	Gidget MMP9 –RT	Piper MMP9 –RT	Piper MMP9 –RT	Piper MMP9 –RT	Gracie MMP9 –RT	Gracie MMP9 –RT	Gracie MMP9 –RT	Samba MMP9 –RT	Samba MMP9 –RT	Samba MMP9 –RT

Table E.2. Results from RT versus no RT experiments using MMP9 as a detector. All no RT reactions had undetermined Ct values

Sample	Ct	Ct std err	Avg Ct	Sample	Ct	Ct std err	Avg Ct
681 MMP9 RT	32.138	0.039	32.18	Gemima MMP9 RT	31.884	0.163	32.067
681 MMP9 RT	32.257	0.039	32.18	Gemima MMP9 RT	32.391	0.163	32.067
681 MMP9 RT	32.143	0.039	32.18	Gemima MMP9 RT	31.925	0.163	32.067
405 MMP9 RT	32.598	0.126	32.83	Gidget MMP9 RT	35.258	0.19	35.066
405 MMP9 RT	33.03	0.126	32.83	Gidget MMP9 RT	35.255	0.19	35.066
405 MMP9 RT	32.861	0.126	32.83	Gidget MMP9 RT	34.686	0.19	35.066
311 MMP9 RT	35.45	0.16	35.746	Piper MMP9 RT	32.521	0.224	32.966
311 MMP9 RT	35.788	0.16	35.746	Piper MMP9 RT	33.147	0.224	32.966
311 MMP9 RT	36	0.16	35.746	Piper MMP9 RT	33.229	0.224	32.966
638 MMP9 RT	33.141	0.224	32.936	Gracie MMP9 RT	30.156	0.169	29.978
638 MMP9 RT	33.178	0.224	32.936	Gracie MMP9 RT	30.138	0.169	29.978
638 MMP9 RT	32.489	0.224	32.936	Gracie MMP9 RT	29.639	0.169	29.978

APPENDIX F: REAL-TIME RT-PCR REPLICATES OF CANINE CHORIOALLANTOIS SAMPLES

To the author's knowledge, no other studies have been published on gene expression in the canine chorioallantois. Due to the lack of knowledge of gene expression in this tissue, it was not known whether the location of the tissue used for RNA isolation within a single chorioallantois tissue sample would have an affect on gene expression. Therefore, an experiment was performed to determine if there were any differences in gene expression between tissue samples collected from different locations within the same chorioallantois tissue sample. Chorioallantois tissue was used from three pre-term samples (311, 405 and 638). Total RNA was isolated, RNA concentrations were determined, RNA integrity was verified, total RNA was reverse transcribed to cDNA, and real-time RT-PCR was performed as described in the previous appendices. The gene used for this experiment was MMP-9 because it had previously been shown to be expressed in all of these samples. A one-way ANOVA with bonferroni corrections was performed to determine if there were any differences in Ct values between the replicates using the Stata 12.0 software (StataCorp LP, College Stations, TX). Significance was set at $p < 0.05$. There was no significant difference ($p > 0.05$) between Ct values of the replicates of the same sample (Table F.1.).

Table F.1. Results for Replicates

Sample	**Replicate #1 Ct Value (Mean ± SD)**	**Replicate #2 Ct Value (Mean ± SD)**	**Replicate #3 Ct Value (Mean ± SD)**
405 R1	33.36 ± 0.16	34.01 ± 0.16	34.45 ± 0.19
638 R3	31.89 ± 0.16	34.21 ± 0.26	31.91 ± 0.26
311 R2	35.319 ± 0.09	29.74 ± 0.08	32.03 ± 0.08

APPENDIX G: MICROARRAY ANALYSIS PROTOCOL

Sample Preparation

Total RNA was isolated and RNA concentration, purity and integrity were measured as previously described in Appendix A. All samples submitted for microarray analysis had an $A_{260/280}$ ratio of between 1.8-2.0 and a RNA integrity number (RIN) of >7.0. Total RNA samples were submitted to the Center for Genome Research and Biocomputing (CGRB) at Oregon State University in 20 µl aliquots to ensure a minimum of 200 ng of RNA.

Microarray Protocol

All total RNA samples were run on microarray at the CGRB using their standard protocol for Affymetrix chips. Briefly, labeled target aRNA was prepared from 200 ng of total RNA using the MessageAMP Premier Amplification Kit (Life Technologies, Grand Island, NY). Samples were IVT incubated for 16 hours and 10 µg of aRNA from each sample was hybridized for 18 hours to an Affymetrix Canine 2.0 Array (Affymetrix, Santa Clara, CA). Samples were hybridized in one day in two sets. Washing, staining and scanning was performed using the GeneChip Expression Wash, Stain and Scan Manual for Cartridge Arrays (Affymetrix) using the AGCC software with a Fluidics Station 450 (Affymetrix) along with a GeneChip Scanner 3000 with autoloader (Affymetrix). Image processing and data extraction was performed using AGCC Software version 3.0 (Affymetrix).

Microarray Analysis Protocol

Differential gene expression was calculated using the Bioconductor software with the limma and affy packages (Smyth, 2004) in the open-source statistical program known as R. In order to perform statistical analysis on microarray data the R statistical program was downloaded from www.R-project.org. Microarray data was received from the CGRB core lab on a CD and data files were then downloaded on to the computer. All .CEL files

were then saved in a separate folder on the desktop titled “CASCEL”. To perform analysis the program R was then opened and the limma and affy packages were downloaded from www.bioconductor.org using the R commands:

>source(http://bioconductor.org/biocLite.R)

>biocLite(limma) and >biocLite(“affy”)

The limma library and affy software packages were then loaded into R using the commands:

>library(limma) and >library(affy)

The directory the R program read the data from was then changed by selecting File>Change dir… The directory was changed to the file that contained the .CEL files (C:/Users/fellowel/Desktop/CASCEL). The R program was directed to read the raw data using the command:

>myrawdata<-ReadAffy().

Pre-processing and normalization of the microarray data was carried out using robust multichip average (RMA) for quantile normalization (Irizarry et al., 2003) within R. The normalization using RMA was achieved with the command:

>eset<-rma(myrawdata).

Limma works by fitting a linear regression model to all of the genes and treating the data as a linear model. This way an analysis can be performed similar to an ANOVA. In order to perform this analysis a matrix was created within R using the following command:

>design<-model.matrix(~-1+factor(c(1,1,1,1,2,2,2,2,3,3,3,3,4,4,4,4)))

Column names and sample group names were assigned using the command:

>colnames(design)<-c(“parturient”, “prelabor”, “preterm”)

A linear model was fit to the data using the command:

>fit<-lmFit(eset, design)

Pairwise comparisons were made between sample groups using a contrast matrix created by the following commands:

>contrast.matrix<-makeContrasts(prelabor-parturient, preterm-prelabor, preterm-parturient, levels=design)

>fit2<-contrasts.fit(fit, contrast.matrix)

>fit2<-eBayes(fit2)

The top differentially expressed genes were exported into Microsoft Excel (Microsoft, Redmond, WA) for the comparison using the commands:

>write.table(topTable(fit2, coef=1, adjust="fdr", sort.by="B", number=50000), file"limma_complete.xls", row.names=F, sep="\t")

>write.table(topTable(fit2, coef=1, adjust="fdr", sort.by="B", number=50000), file"limma_complete2.xls", row.names=F, sep="\t")

>write.table(topTable(fit2, coef=1, adjust="fdr", sort.by="B", number=50000), file"limma_complete3.xls", row.names=F, sep="\t")

A p-value, log fold change and a p value adjusted for a false discovery rate (FDR) was calculated for each gene. Linear modeling and differential gene expression using an empirical Bayes analysis used in this microarray study involves calculating a moderated t-statistic, which draws inferences from all genes in the set to shrink the standard errors toward a common value. This provides more degrees of freedom and more stable standard error estimates (Smyth, 2004). A false discovery rate (FDR) is a commonly used p value that is adjusted for studies such as microarray that involve multiple testing. The FDR is the proportion of incorrect rejections among all rejections of a tested hypothesis (Benjamini and Yekutieli, 1995).

Following exportation into Excel, the spreadsheet only contained probe IDs and no gene names or symbols. A "Master Key" Excel spreadsheet that was exported from ArrayStar (DNASTAR, Madison, WI) contained all information on each gene probe and was copied into each Excel spreadsheet. Probe IDs for each gene were then matched with the appropriate gene name and symbol by entering the formula: =VLOOKUP(A2, 'Master Key'!A:C,2,FALSE) into the top cell of the "Gene Symbol" column of the exported spreadsheet. Then all gene symbols and probe IDs were matched for their appropriate gene name in the "Master Key" excel spreadsheet with the following formula: =VLOOKUP(A2,'Master Key"!A:C,3 FALSE). There are a certain number of probes that are present on the Canine 2.0 Chip (Affymetrix) that do not have a gene name

or symbol associated with them. For the purposes of this study, these were deleted from the Excel spreadsheet. Significance was defined as $p \leq 0.05$, FDR adjusted p value ≤ 0.10 and a log fold change greater ≥ 1.2 based on parameters used in previous studies (Sitras et al., 2009; Hoegh et al., 2010; Lian et al., 2010). Genes were first sorted based on p value and any that did not meet criteria were deleted. Genes that were statistically significant and had a log fold change of less than 1.2 were then eliminated to further condense the gene table. A Venn diagram was also created to visualize the genes that were up-regulated and down-regulated between sample groups using Microsoft Powerpoint (Microsoft, Redmond, WA).

Gene ontology was explored using ArrayStar (DNASTAR). ArrayStar was accessed through the CGRB website at timeslots reserved prior to analysis. Once ArrayStar was opened, the files to be analyzed were loaded by selecting File>Open Project and selecting the project file. To look at differentially expressed genes Data>Show Gene Table was selected from the toolbar. In order to identify differentially expressed genes Filter>Filter All was selected from the toolbar to open the filter dialog box. The (-) button was clicked in the dialog box on the second filter row so that only one filter row remained. Statistics was then selected from the first drop down menu in the dialog box's filter row. To compare between the three sample groups, all three groups were selected for analysis and an ANOVA was then selected for analysis with 95% significance. The search button was then clicked to search for genes that were significant. Next, Gene Set in All Views was selected from the tool bar and genes were sorted by selecting the column header Gene Ontology Biological Process. Next, the toolbar option Data>Show Gene Ontology was selected. Clicking the p-value column header after opening the Gene Ontology View sorted the Gene Ontology Tree by p-value.

Appendix G. References

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APPENDIX H EXPRESSION OF SELECTED CANDIDATE GENES IN CANINE CHORIOALLANTOIS TISSUE USING REAL-TIME RT-PCR

Canine chorioallantoic tissue was collected following ovariohysterectomy at 61 ± 1 day past the LH surge (pre-term; n=4), elective C-section at 64 ± 1 day past the LH surge prior to first stage labor (pre-labor; n=4), and natural delivery (parturient; n=4). Following collection, the marginal hematoma was dissected away and any blood or excess tissue (nonvillous chorioallantois, amnion and umbilical cord) were removed. Tissues were flash frozen in liquid nitrogen and stored at -80°C until RNA was isolated.

Total RNA was isolated and RNA concentration, purity and integrity were measured as previously described in Appendix A. Microarray analysis was performed as described in Appendix G. Real-time RT-PCR was carried out to validate microarray results as described in Appendix D following cDNA synthesis (Appendix B). Of particular interest were genes previously shown to be involved in trophoblast invasion or parturition in other species. Five genes were chosen for real-time RT-PCR validation including DAG-1, CD44, CXCL10, MMP-1 and IL-6 based on their differential expression (p value ≤ 0.05, FDR adjusted p value ≤ 0.10 and a log fold change ≥ 1.2).

Statistical analysis was performed using a one-way ANOVA with Bonferroni corrections to make comparisons between the three groups. Statistical significance was calculated using the Stata 12.0 software (StataCorp LP, College Station, TX). Data was expressed as mean ± standard deviation (SD). Significance was defined as $p<0.05$. There was no significant difference in expression of any of the five candidate genes between the three samples (Table H.1).

Table H.1. Relative quantitation (mean ± SD) of placental gene expression at three time points prior to parturition

Gene	**Pre-term (n=4)**	**Pre-labor (n=4)**	**Parturient (n=4)**
CD44	5.19 ± 3.66	1.92 ± 0.16	1.31 ± 0.34
IL-6	2.87 ± 1.28	2.47 ± 0.44	2.84 ± 3.03
CXCL10	6.51 ± 4.72	3.93 ± 1.54	1.12 ± 14.4
MMP-1	1.00 ± 1.04	20.44 ± 39.8	0.71 ± 0.34
DAG-1	0.91 ± 0.63	4.86 ± 8.21	1.95 ± 2.10

APPENDIX I: GENE EXPRESSION PROFILES OF CANINE CHORIOALLANTOIS TISSUE COMPARED TO ISOLATED CANINE TROPHOBLASTS

Tissue Collection and RNA Isolation

Chorioallantois tissue was collected as previously described for the pre-labor sample group (n=4) and isolated trophoblast cells from four dogs were used according to a protocol used in the lab. Total RNA was isolated and quality of RNA was determined as described in Appendix A. Total RNA samples containing a minimum of 200 ng were submitted to the Center for Genome Research and Biocomputing (CGRB) at Oregon State University for microarray (Appendix G).

Microarray Analysis

Microarray analysis was carried out as previously described (Appendix G). All R commands were the same as described in Appendix G except for the commands for setting up the comparisons. A comparison was made between the pre-labor chorioallantois tissue samples and the isolated trophoblast samples using the command to specify column names:

```
>colnames(design)<-c( “prelabor”, “cells”)
```

A linear model was then fit to the data using the command:

```
>fit<-lmFit(eset, design)
```

Pairwise comparisons were made between the two sample groups using a contrast matrix created by the following commands:

```
>contrast.matrix<-makeContrasts(prelabor-cells, levels=design)
>fit2<-contrasts.fit(fit, contrast.matrix)
>fit2<-eBayes(fit2)
```

All subsequent exportation of results, preprocessing and analysis was performed as described in Appendix G.

Results

Overall global variation of gene expression between the isolated trophoblast cells and pre-labor chorioallantois tissue was larger than that seen between the different chorioallantois tissue samples (Figure H.1). There were 1150 genes that were differentially expressed between isolated canine trophoblast cells compared to pre-labor chorioallantois tissue based on a p value ≤ 0.05, an FDR adjusted p value ≤ 0.05 and a log-fold change of >1.2 (Table H.1). In addition, there were 576 genes that were down-regulated in the chorioallantois tissue compared to isolated trophoblast cells and 574 up-regulated genes in the tissue samples compared to the isolated cell samples. Interestingly, several genes already examined in this thesis including, MMP-2, MMP-1, TIMP-2, FLT-1, CD44, IL-6 and CXCL10 were differentially expressed between isolated trophoblast cells and chorioallantois tissue.

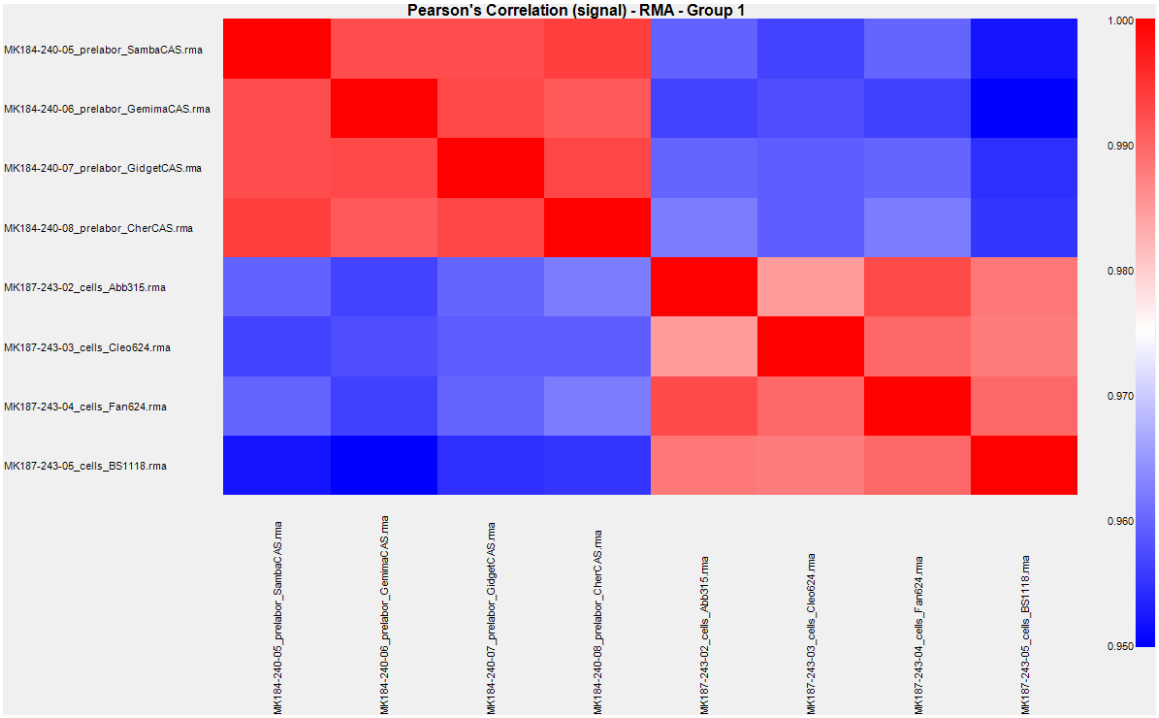

Figure I.1. Correlation coefficients of gene expression profiles from each pre-labor placental tissue and pre-labor isolated trophoblasts. Red indicates 1.0, whereas blue indicates 0.950.

Table H.1. Top differentially expressed genes between trophoblasts and chorioallantois tissue

Gene	Name	logFC	P.Value	adj.P.Val
HSD3B1	hydroxy-Δ-5-steroid dehydrogenase, 3 β- and steroid Δ-isomerase 1	3.80	1.25E-16	5.36E-12
CD320	CD320 molecule	4.35	1.16E-15	1.66E-11
TMEM45A	transmembrane protein 45A	4.18	3.64E-15	3.13E-11
CP	ceruloplasmin (ferroxidase)	2.86	5.67E-15	3.231E-11
BMP4	bone morphogenetic protein 4	2.52	1.19E-14	4.66E-11
TSPAN5	tetraspanin 5	3.35	1.58E-14	5.68E-11
TMEM108	transmembrane protein 108	3.71	1.79E-14	5.94E-11
FST	follistatin	4.46	1.94E-14	5.95E-11
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	4.54	2.18E-14	6.10E-11
SLC35F2	solute carrier family 35, member F2	5.22	2.37E-14	6.10E-11
SCD	stearoyl-CoA desaturase (Δ-9-desaturase)	4.64	3.59E-14	8.59E-11
SLC5A6	solute carrier family 5 (Na^+-dependent vitamin transporter) member 6	4.05	3.80E-14	8.61E-11
NAPEPLD	N-acyl phosphatidylethanolamine phospholipase D	3.24	4.24E-14	9.13E-11
BMP10	bone morphogenetic protein 10	5.26	5.87E-14	1.10E-10
LPGAT1	lysophosphatidylglycerol acyltransferase 1	4.30	1.02E-13	1.75E-10
CSNK2A2	casein kinase 2, α prime polypeptide	2.48	1.40E-13	2.13E-10
SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal	4.97	1.44E-13	2.13E-10
BACE1	β-site APP-cleaving enzyme 1	4.69	1.55E-13	2.15E-10

TFRC	transferrin receptor (p90, CD71)	4.05	1.72E-13	2.24E-10
CADM1	cell adhesion molecule 1	2.53	1.85E-13	2.34E-10
BBOX1	butyrobetaine (γ), 2-oxoglutarate dioxygenase (γ-butyrobetaine hydroxylase) 1	4.58	2.12E-13	2.55E-10
PGF	placental growth factor	3.83	2.38E-13	2.63E-10
EDNRB	endothelin receptor type B	2.43	2.39E-13	2.63E-10
UBE2A	ubiquitin-conjugating enzyme E2A (RAD6 homolog)	1.64	2.44E-13	2.63E-10
TIMP2	TIMP metallopeptidase inhibitor 2	2.58	3.16E-13	3.17E-10
WIPI1	WD repeat domain, phosphoinositide interacting 1	2.85	3.18E-13	3.17E-10
LASS5	LAG1 homolog, ceramide synthase 5	3.25	4.92E-13	4.41E-10
AZGP1	α-2-glycoprotein 1, Zn^{++}-binding	5.19	5.31E-13	4.66E-10
CYB5R1	NAD(P)H:quinone oxidoreductase type 3, polypeptide A2	2.29	6.08E-13	5.03E-10
FZD7	frizzled homolog 7 (Drosophila)	2.96	6.79E-13	5.41E-10
SLC16A7	solute carrier family 16, member 7 (monocarboxylic acid transporter 2)	3.61	7.54E-13	5.90E-10
MAN1C1	mannosidase, α, class 1C, member 1	4.04	8.10E-13	6.00E-10
NFE2L3	nuclear factor (erythroid-derived 2)-like 3	4.36	8.31E-13	6.00E-10
OSBPL2	oxysterol binding protein-like 2	2.74	1.47E-12	9.30E-10
AACS	acetoacetyl-CoA synthetase	2.74	1.54E-12	9.62E-10
SLC4A10	solute carrier family 4, sodium bicarbonate transporter, member 10	4.80	1.68E-12	1.02E-09
CAPNS2	calpain, small subunit 2	3.78	1.77E-12	1.06E-09
SEC14L2	SEC14-like 2 (*S. cerevisiae*)	3.78	1.97E-12	1.14E-09
ADRB2	adrenergic, β-2-, receptor, surface	3.51	2.29E-12	1.27E-09
PLA2G16	phospholipase A2, group XVI	3.20	2.33E-12	1.27E-09
MSI2	musashi homolog 2 (Drosophila)	2.05	2.79E-12	1.46E-09
LDOC1	leucine zipper, down-regulated in cancer 1	2.92	2.82E-12	1.46E-09

EGFLAM	EGF-like, fibronectin type III and laminin G domains	2.29	3.02E-12	1.55E-09
ELOVL4	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	3.50	3.12E-12	1.58E-09
PRDX6	peroxiredoxin 6	1.92	3.71E-12	1.83E-09
LIFR	leukemia inhibitory factor receptor α	3.63	3.74E-12	1.83E-09
SLC31A1	solute carrier family 31 (Cu^{++} transporters), member 1	2.87	4.02E-12	1.92E-09
REPS2	RALBP1 associated Eps domain containing 2	1.99	4.06E-12	1.92E-09
RHPN2	rhophilin, Rho GTPase binding protein 2	3.33	4.47E-12	2.09E-09
ABHD2	abhydrolase domain containing 2	2.95	5.18E-12	2.35E-09
HPSE	heparanase	4.15	5.67E-12	2.54E-09
ATXN2	ataxin 2	1.94	6.43E-12	2.80E-09
MFSD2A	major facilitator superfamily domain containing 2A	4.84	7.34E-12	3.15E-09
KATNA1	katanin p60 (ATPase-containing) subunit A 1	2.80	8.37E-12	3.44E-09
SPATS2	spermatogenesis associated, serine-rich 2	-1.38	9.46E-12	3.77E-09
GXYLT2	glucoside xylosyltransferase 2	2.69	1.03E-11	4.01E-09
TFAP2A	transcription factor AP-2 α (activating enhancer binding protein 2 α)	3.00	1.36E-11	5.00E-09
SQLE	squalene epoxidase	2.08	1.36E-11	5.00E-09
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	1.82	1.39E-11	5.06E-09
GNA12	guanine nucleotide binding protein (G protein) α 12	3.72	1.40E-11	5.07E-09
HHIPL2	HHIP-like 2	5.60	1.48E-11	5.19E-09
SERINC5	serine incorporator 5	4.11	1.49E-11	5.19E-09
TMEM164	transmembrane protein 164	2.46	1.96E-11	6.60E-09
DQX1	DEAQ box RNA-dependent ATPase 1	1.92	2.24E-11	7.30E-09
RNF180	ring finger protein 180	3.50	2.32E-11	7.46E-09
DUSP4	dual specificity phosphatase 4	2.62	2.39E-11	7.55E-09

TGFBR3	transforming growth factor, β receptor III	2.33	2.51E-11	7.76E-09
SLC7A2	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	4.84	2.53E-11	7.76E-09
FAR2	fatty acyl CoA reductase 2	2.04	2.58E-11	7.83E-09
BPGM	2,3-bisphosphoglycerate mutase	2.62	2.64E-11	7.89E-09
STAU2	staufen, RNA binding protein, homolog 2 (Drosophila)	2.54	2.73E-11	8.10E-09
SLC37A2	solute carrier family 37 (glycerol-3-phosphate transporter), member 2	5.34	2.82E-11	8.32E-09
RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5	2.98	2.94E-11	8.56E-09
MCFD2	multiple coagulation factor deficiency 2	1.62	2.94E-11	8.56E-09
WIPI2	WD repeat domain, phosphoinositide interacting 2	1.30	3.28E-11	9.23E-09
ALPL	alkaline phosphatase, liver/bone/kidney	4.31	3.47E-11	9.62E-09
MSX2	msh homeobox 2	3.02	3.51E-11	9.62E-09
GCNT4	glucosaminyl (N-acetyl) transferase 4, core 2	3.62	3.59E-11	9.71E-09
DCLK3	doublecortin-like kinase 3	2.43	3.63E-11	9.75E-09
LRP2	low density lipoprotein receptor-related protein 2	5.39	3.65E-11	9.76E-09
FANCE	Fanconi anemia, complementation group E	3.23	3.90E-11	1.04E-08
CASR	Ca^{++} -sensing receptor	5.54	4.43E-11	1.17E-08
ADIPOR2	adiponectin receptor 2	1.92	4.45E-11	1.17E-08
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	2.17	4.47E-11	1.17E-08
PRCP	prolylcarboxypeptidase (angiotensinase C)	2.75	4.50E-11	1.17E-08
GOLGA2	golgin A2	1.78	4.59E-11	1.18E-08
ST8SIA1	ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase 1	3.51	4.84E-11	1.22E-08
PPM1A	protein phosphatase, Mg2+/Mn2+ dependent, 1A	3.25	4.96E-11	1.22E-08
GPR87	G protein-coupled receptor 87	2.72	4.98E-11	1.22E-08

SEMA6D	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	2.20	5.06E-11	1.23E-08
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	2.90	5.19E-11	1.25E-08
PTHLH	parathyroid hormone-like hormone	3.74	5.19E-11	1.25E-08
GPRC5B	G protein-coupled receptor, family C, group 5, member B	3.18	5.33E-11	1.26E-08
PRKCD	protein kinase C, Δ	2.42	5.34E-11	1.26E-08
CD164	CD164 molecule, sialomucin	2.00	5.72E-11	1.34E-08
CCDC6	coiled-coil domain containing 6	1.43	5.83E-11	1.36E-08
PPAP2B	phosphatidic acid phosphatase type 2B	2.45	6.01E-11	1.39E-08
IL4I1	interleukin 4 induced 1	4.03	6.09E-11	1.40E-08
GRB14	growth factor receptor-bound protein 14	2.37	6.15E-11	1.40E-08
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	3.52	6.41E-11	1.44E-08
CTSA	cathepsin A	3.29	6.65E-11	1.49E-08
FGF20	fibroblast growth factor 20	4.04	7.28E-11	1.59E-08
GAS2	growth arrest-specific 2	3.80	7.86E-11	1.69E-08
NPPC	natriuretic peptide precursor C	5.14	7.90E-11	1.69E-08
FGFBP1	fibroblast growth factor binding protein 1	4.93	8.31E-11	1.76E-08
MPP6	membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)	2.66	8.67E-11	1.81E-08
SLC19A2	solute carrier family 19 (thiamine transporter), member 2	3.20	8.78E-11	1.83E-08
XIAP	X-linked inhibitor of apoptosis	1.93	9.33E-11	1.93E-08
SC4MOL	sterol-C4-methyl oxidase-like	1.93	1.06E-10	2.11E-08
TIMP3	TIMP metallopeptidase inhibitor 3	3.37	1.09E-10	2.15E-08
SFRP2	secreted frizzled-related protein 2	5.11	1.12E-10	2.20E-08
SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	2.43	1.17E-10	2.28E-08

PPARD	peroxisome proliferator-activated receptor Δ	2.40	1.19E-10	2.29E-08
YPEL1	yippee-like 1 (Drosophila)	2.05	1.20E-10	2.30E-08
PLA2G7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	3.71	1.27E-10	2.40E-08
LBH	limb bud and heart development homolog (mouse)	3.03	1.35E-10	2.52E-08
DNAJC1	DnaJ (Hsp40) homolog, subfamily C, member 1	1.91	1.42E-10	2.63E-08
MANBA	mannosidase, βA, lysosomal	2.64	1.45E-10	2.66E-08
TP53INP1	tumor protein p53 inducible nuclear protein 1	2.09	1.49E-10	2.72E-08
KREMEN1	kringle containing transmembrane protein 1	3.12	1.51E-10	2.75E-08
EXT1	exostosin 1	1.64	1.54E-10	2.77E-08
cOR2AV3	olfactory receptor family 2 subfamily AV-like	1.58	1.57E-10	2.80E-08
CMAS	cytidine monophosphate N-acetylneuraminic acid synthetase	2.01	1.81E-10	3.12E-08
BPGM, LOC610322	2,3-bisphosphoglycerate mutase; hypothetical protein LOC610322	2.48	1.87E-10	3.21E-08
STXBP2	syntaxin binding protein 2	2.17	1.93E-10	3.25E-08
NCOR1	nuclear receptor corepressor 1	2.31	2.02E-10	3.36E-08
NRP1	neuropilin 1	-1.68	2.11E-10	3.47E-08
SLC38A2	solute carrier family 38, member 2	2.32	2.11E-10	3.47E-08
CDH5	cadherin 5, type 2 (vascular endothelium)	-1.33	2.13E-10	3.49E-08
SERAC1	serine active site containing 1	2.02	2.18E-10	3.55E-08
HVCN1	H^+ voltage-gated channel 1	1.99	2.40E-10	3.87E-08
SLC45A4	solute carrier family 45, member 4	2.66	2.41E-10	3.88E-08
DHRS13	dehydrogenase/reductase (SDR family) member 13	2.87	2.48E-10	3.94E-08
ZNF281	Zn^{++} finger protein 281	1.44	2.51E-10	3.96E-08
FERMT2	fermitin family member 2	1.44	2.52E-10	3.96E-08

H2AFY2	H2A histone family, member Y2	4.30	2.55E-10	3.97E-08
GCOM1	GRINL1A complex locus	-1.76	2.58E-10	3.99E-08
KHK	ketohexokinase (fructokinase)	1.93	2.59E-10	4.00E-08
MFI2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	4.35	2.71E-10	4.16E-08
MXD1	MAX dimerization protein 1	2.79	2.78E-10	4.25E-08
SH3BGRL3	SH3 domain binding glutamic acid-rich protein like 3	2.16	2.80E-10	4.26E-08
DLX5	distal-less homeobox 5	3.42	3.02E-10	4.54E-08
JUNB	jun B proto-oncogene	-2.48	3.10E-10	4.63E-08
CHCHD6	coiled-coil-helix-coiled-coil-helix domain containing 6	1.50	3.10E-10	4.63E-08
ECSCR	endothelial cell-specific chemotaxis regulator	-1.90	3.23E-10	4.79E-08
PIP5K1B	phosphatidylinositol-4-phosphate 5-kinase, type I, β	2.95	3.25E-10	4.79E-08
MDM2	Mdm2 p53 binding protein homolog (mouse)	-2.13	3.36E-10	4.91E-08
EBPL	emopamil binding protein-like	-1.40	3.36E-10	4.91E-08
SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	3.02	3.67E-10	5.26E-08
SDC2	syndecan 2	2.71	3.98E-10	5.61E-08
TRIM36	tripartite motif-containing 36	3.28	4.19E-10	5.87E-08
NTRK3	neurotrophic tyrosine kinase, receptor, type 3	3.30	4.20E-10	5.87E-08
KLHDC5	kelch domain containing 5	2.71	4.34E-10	6.03E-08
GM2A	GM2 ganglioside activator	1.89	4.37E-10	6.03E-08
MAP7	microtubule-associated protein 7	1.97	4.40E-10	6.05E-08
GALNT11	UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 11 (GalNAc-T11)	3.07	4.51E-10	6.18E-08
FGD4	FYVE, RhoGEF and PH domain containing 4	1.66	4.55E-10	6.22E-08

STIM1	stromal interaction molecule 1	3.23	4.69E-10	6.35E-08
PIGN	phosphatidylinositol glycan anchor biosynthesis, class N	-1.24	4.70E-10	6.35E-08
CCDC34	coiled-coil domain containing 34	2.04	4.72E-10	6.35E-08
NSDHL	NAD(P) dependent steroid dehydrogenase-like	1.81	4.74E-10	6.35E-08
CHORDC1, LOC609430	cysteine and histidine-rich domain (CHORD)-containing 1; similar to 3-β-hydroxysteroid-Δ(8),Δ(7)-isomerase (Cholestenol Δ-isomerase) (Δ8-Δ7 sterol isomerase) (D8-D7 sterol isomerase) (Emopamil-binding protein)	2.13	4.84E-10	6.45E-08
TMEM47	transmembrane protein 47	-1.88	4.99E-10	6.59E-08
ZNF532	Zn^{++} finger protein 532	2.09	4.99E-10	6.59E-08
ZC3H12C	Zn^{++} finger CCCH-type containing 12C	1.98	5.11E-10	6.70E-08
UXS1	UDP-glucuronate decarboxylase 1	2.66	5.20E-10	6.80E-08
CES1	carboxylesterase 1	4.41	5.27E-10	6.86E-08
SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	2.06	5.36E-10	6.94E-08
PECAM1	platelet/endothelial cell adhesion molecule	-1.96	5.40E-10	6.98E-08
GRN	granulin	1.56	5.57E-10	7.18E-08
FDPS	farnesyl diphosphate synthase	1.18	5.61E-10	7.20E-08
SLC22A23	solute carrier family 22, member 23	3.03	5.68E-10	7.28E-08
GPC4	glypican 4	2.39	5.83E-10	7.43E-08
NAA11	N(α)-acetyltransferase 11, NatA catalytic subunit	1.27	5.85E-10	7.43E-08
STT3B	STT3, subunit of the oligosaccharyltransferase complex, homolog B (*S. cerevisiae*)	2.17	6.13E-10	7.72E-08
ANGPT2	angiopoietin 2	3.35	6.15E-10	7.72E-08
NOS1	nitric oxide synthase 1 (neuronal)	3.05	6.26E-10	7.81E-08

MITF	microphthalmia-associated transcription factor	2.30	6.61E-10	8.18E-08
AK3L1	adenylate kinase 3-like 1	-1.40	6.64E-10	8.19E-08
NDRG3	NDRG family member 3	1.39	6.81E-10	8.38E-08
MANF	mesencephalic astrocyte-derived neurotrophic factor	1.69	6.93E-10	8.47E-08
GPR155	G protein-coupled receptor 155	2.42	7.02E-10	8.53E-08
RBBP6	retinoblastoma binding protein 6	2.39	7.04E-10	8.53E-08
UBXN11	UBX domain protein 11	2.24	7.19E-10	8.65E-08
UTRN	utrophin	-1.62	7.40E-10	8.85E-08
NEK7	NIMA (never in mitosis gene a)-related kinase 7	1.86	7.84E-10	9.22E-08
PPM1K	protein phosphatase, Mg2+/Mn2+ dependent, 1K	1.76	7.94E-10	9.31E-08
CD19	CD19 molecule	1.40	7.96E-10	9.31E-08
SVIP	small VCP/p97-interacting protein	2.24	8.10E-10	9.45E-08
GSK3B	glycogen synthase kinase 3 β	1.74	8.22E-10	9.53E-08
SLC26A6	solute carrier family 26, member 6	2.46	8.29E-10	9.56E-08
CFI	complement factor I	-1.85	8.39E-10	9.65E-08
ALDH1L2	aldehyde dehydrogenase 1 family, member L2	-1.25	8.51E-10	9.74E-08
RECK	reversion-inducing-cysteine-rich protein with kazal motifs	2.46	8.89E-10	1.01E-07
ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	2.12	9.42E-10	1.05E-07
IER2	immediate early response 2	-2.55	9.55E-10	1.07E-07
COL4A3BP	collagen, type IV, α 3 (Goodpasture antigen) binding protein	1.41	9.80E-10	1.08E-07
ERBB2IP	erbb2 interacting protein	1.57	1.01E-09	1.10E-07
GGH	γ-glutamyl hydrolase (conjugase, folylpoly γglutamyl hydrolase)	2.58	1.03E-09	1.12E-07
JMY	junction mediating and regulatory protein, p53 cofactor	1.71	1.08E-09	1.18E-07
RGS1	regulator of G-protein signaling 1	-2.92	1.09E-09	1.18E-07

OSGIN2	oxidative stress induced growth inhibitor family member 2	2.59	1.15E-09	1.23E-07
CANX	calnexin	1.29	1.17E-09	1.25E-07
RALGPS2	Ral GEF with PH domain and SH3 binding motif 2	1.42	1.18E-09	1.25E-07
TRAM2	translocation associated membrane protein 2	1.54	1.20E-09	1.27E-07
TGFBR1	transforming growth factor, β receptor 1	2.34	1.22E-09	1.28E-07
FDFT1	farnesyl-diphosphate farnesyltransferase 1	2.23	1.25E-09	1.30E-07
SAR1A	SAR1 homolog A (S. cerevisiae)	1.86	1.30E-09	1.35E-07
ANK3	ankyrin 3, node of Ranvier (ankyrin G)	1.52	1.32E-09	1.35E-07
IREB2	Fe^{++}-responsive element binding protein 2	1.23	1.33E-09	1.36E-07
LPAR1	lysophosphatidic acid receptor 1	1.44	1.35E-09	1.37E-07
GATA3	GATA binding protein 3	1.91	1.35E-09	1.37E-07
SNTA1	syntrophin, α 1 (dystrophin-associated protein A1, 59kDa, acidic component)	2.76	1.35E-09	1.37E-07
PTPRF	protein tyrosine phosphatase, receptor type, F	2.00	1.39E-09	1.39E-07
CRB3	crumbs homolog 3 (Drosophila)	1.60	1.39E-09	1.39E-07
GPR157	G protein-coupled receptor 157	3.13	1.46E-09	1.45E-07
PNPLA8	patatin-like phospholipase domain containing 8	1.25	1.46E-09	1.45E-07
LGMN	legumain	1.32	1.48E-09	1.46E-07
FLNB	filamin B, β	2.05	1.50E-09	1.48E-07
SPP2	secreted phosphoprotein 2, 24kDa	3.98	1.60E-09	1.55E-07
ZBTB10	Zn^{++} finger and BTB domain containing 10	1.46	1.66E-09	1.60E-07
S100P	S100 Ca^{++} binding protein P	1.31	1.70E-09	1.62E-07
SC5DL	sterol-C5-desaturase (ERG3 Δ-5-desaturase homolog, S. cerevisiae)-like	2.37	1.72E-09	1.63E-07
OVOL2	ovo-like 2 (Drosophila)	1.34	1.77E-09	1.67E-07
KLHL5	kelch-like 5 (Drosophila)	-1.55	1.80E-09	1.69E-07

APIP	APAF1 interacting protein	1.31	1.85E-09	1.72E-07
TJP1	tight junction protein 1 (zona occludens 1)	-1.16	1.95E-09	1.80E-07
HSF2	heat shock transcription factor 2	-1.16	1.97E-09	1.82E-07
MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4	1.66	2.00E-09	1.84E-07
PMM1	phosphomannomutase 1	1.44	2.04E-09	1.87E-07
GUCY1A3	guanylate cyclase 1, soluble, α 3	-1.67	2.12E-09	1.93E-07
ABR	active BCR-related gene	1.78	2.17E-09	1.97E-07
SGCG	sarcoglycan, γ (35kDa dystrophin-associated glycoprotein)	3.19	2.18E-09	1.97E-07
MGST1	microsomal glutathione S-transferase 1	1.85	2.19E-09	1.98E-07
SDAD1	SDA1 domain containing 1	1.59	2.27E-09	2.03E-07
ICAM2	intercellular adhesion molecule 2	-1.68	2.36E-09	2.07E-07
NNT	nicotinamide nucleotide transhydrogenase	-1.42	2.36E-09	2.07E-07
NAT10	N-acetyltransferase 10 (GCN5-related)	1.59	2.54E-09	2.20E-07
TGFBR2	transforming growth factor, β receptor II (70/80kDa)	2.62	2.56E-09	2.21E-07
CCDC113	coiled-coil domain containing 113	2.22	2.57E-09	2.22E-07
GRHL2	grainyhead-like 2 (Drosophila)	1.89	2.60E-09	2.24E-07
CDS1	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1	1.59	2.62E-09	2.25E-07
TACC1	transforming, acidic coiled-coil containing protein 1	-1.31	2.63E-09	2.25E-07
IRF2	interferon regulatory factor 2	1.31	2.63E-09	2.25E-07
AMIGO2	adhesion molecule with Ig-like domain 2	2.20	2.64E-09	2.25E-07
EIF2AK2	eukaryotic translation initiation factor 2-α kinase 2	1.60	2.65E-09	2.25E-07
TF	transferrin	-1.49	2.66E-09	2.256E-07
USHBP1	Usher syndrome 1C binding protein 1	-1.45	2.67E-09	2.26E-07
CMTM7	CKLF-like MARVEL transmembrane domain containing 7	2.16	2.72E-09	2.30E-07

CHSY1	chondroitin sulfate synthase 1	-1.94	2.76E-09	2.33E-07
C1GALT1	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-β-galactosyltransferase, 1	2.48	2.78E-09	2.34E-07
SPTLC3	serine palmitoyltransferase, long chain base subunit 3	2.60	2.79E-09	2.34E-07
IRX3	iroquois homeobox 3	2.29	3.01E-09	2.49E-07
SNTB2	syntrophin, β 2 (dystrophin-associated protein A1, 59kDa, basic component 2)	-1.32	3.07E-09	2.51E-07
PLBD1	phospholipase B domain containing 1	1.77	3.11E-09	2.53E-07
PPIC	peptidylprolyl isomerase C (cyclophilin C)	-1.61	3.12E-09	2.53E-07
CTSH	cathepsin H	1.92	3.13E-09	2.54E-07
TMEM126A	transmembrane protein 126A	-1.24	3.18E-09	2.58E-07
SASH1	SAM and SH3 domain containing 1	2.19	3.22E-09	2.60E-07
ANO6	anoctamin 6	2.08	3.23E-09	2.61E-07
TRAK1	trafficking protein, kinesin binding 1	1.35	3.36E-09	2.69E-07
DDAH2	dimethylarginine dimethylaminohydrolase 2	-1.34	3.36E-09	2.69E-07
ATP1B1	ATPase, Na+/K+ transporting, β1 polypeptide	1.41	3.49E-09	2.76E-07
RCN2	reticulocalbin 2, EF-hand Ca^{++} binding domain	1.54	3.56E-09	2.81E-07
CLIC2	chloride intracellular channel 2	-1.75	3.57E-09	2.81E-07
FBLN2	fibulin 2	-1.55	3.61E-09	2.83E-07
RABGAP1L	RAB GTPase activating protein 1-like	1.27	3.65E-09	2.86E-07
SLC18A2	Solute carrier family 18 (vesicular monoamine), member 2	-1.38	3.68E-09	2.87E-07
SSFA2	sperm specific antigen 2	1.87	3.72E-09	2.89E-07
CD2AP	CD2-associated protein	2.20	3.73E-09	2.89E-07
INSIG1	insulin induced gene 1	2.40	3.73E-09	2.89E-07
CDCP1	CUB domain containing protein 1	2.17	3.90E-09	3.01E-07
TUSC3	tumor suppressor candidate 3	1.50	4.02E-09	3.07E-07

EMP1	epithelial membrane protein 1	-2.07	4.13E-09	3.12E-07
LYZ	lysozyme	-2.02	4.19E-09	3.16E-07
KANK3	KN motif and ankyrin repeat domains 3	-1.82	4.23E-09	3.18E-07
GNPAT	glyceronephosphate O-acyltransferase	-1.46	4.24E-09	3.18E-07
AP2A1	adaptor-related protein complex 2, α1 subunit	2.75	4.28E-09	3.21E-07
SNX27	sorting nexin family member 27	1.69	4.33E-09	3.24E-07
HAGH	hydroxyacylglutathione hydrolase	1.63	4.37E-09	3.26E-07
IGF2R	insulin-like growth factor 2 receptor	-1.28	4.43E-09	3.28E-07
GRAMD1B	GRAM domain containing 1B	2.05	4.50E-09	3.31E-07
ITGAV	integrin, αV (vitronectin receptor, α polypeptide, antigen CD51)	1.67	4.53E-09	3.33E-07
LPCAT2	lysophosphatidylcholine acyltransferase 2	2.16	4.84E-09	3.51E-07
IVNS1ABP	influenza virus NS1A binding protein	-1.35	4.86E-09	3.52E-07
FGFR2	fibroblast growth factor receptor 2	1.63	5.00E-09	3.59E-07
CCBL2	cysteine conjugate-βlyase 2	2.04	5.19E-09	3.70E-07
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	4.42	5.26E-09	3.74E-07
NFIB	nuclear factor I/B	-1.47	5.33E-09	3.77E-07
TEAD1	TEA domain family member 1 (SV40 transcriptional enhancer factor)	1.48	5.37E-09	3.79E-07
SGPL1	sphingosine-1-phosphate lyase 1	1.75	5.49E-09	3.86E-07
ASS1	argininosuccinate synthase 1	2.31	5.52E-09	3.87E-07
ZNF185	Zn^{++} finger protein 185 (LIM domain)	1.44	5.65E-09	3.95E-07
C6	complement component 6	-1.69	5.82E-09	4.04E-07
ITPK1	inositol 1,3,4-triphosphate 5/6 kinase	1.68	5.94E-09	4.09E-07
WWC1	WW and C2 domain containing 1	1.59	5.97E-09	4.10E-07
DAPK1	death-associated protein kinase 1	2.31	6.00E-09	4.12E-07

BRP44L	brain protein 44-like	1.30	6.19E-09	4.22E-07
GRIK4	glutamate receptor, ionotropic, kainate 4	1.67	6.81E-09	4.59E-07
LMO1	LIM domain only 1 (rhombotin 1)	1.95	6.83E-09	4.60E-07
ERO1LB	ERO1-like β (*S. cerevisiae*)	1.92	7.23E-09	4.81E-07
FBXO7	F-box protein 7	2.47	7.52E-09	4.96E-07
MYST4	MYST histone acetyltransferase (monocytic leukemia) 4	1.96	7.66E-09	5.04E-07
TUFT1	tuftelin 1	1.47	8.05E-09	5.22E-07
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	-1.72	8.18E-09	5.28E-07
FRMD3	FERM domain containing 3	1.40	8.26E-09	5.32E-07
SLC6A8	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	3.40	8.28E-09	5.32E-07
SMAD2	SMAD family member 2	1.39	8.35E-09	5.35E-07
LRRC70	leucine rich repeat containing 70	-1.90	8.74E-09	5.55E-07
GPLD1	glycosylphosphatidylinositol specific phospholipase D1	4.11	8.92E-09	5.64E-07
RASSF6	Ras association (RalGDS/AF-6) domain family member 6	2.53	9.09E-09	5.72E-07
CASD1	CAS1 domain containing 1	-1.40	9.19E-09	5.75E-07
SPECC1	sperm antigen with calponin homology and coiled-coil domains 1	1.60	9.26E-09	5.79E-07
NOSTRIN	nitric oxide synthase trafficker	-1.62	9.33E-09	5.82E-07
KRTCAP3	keratinocyte associated protein 3	1.50	9.42E-09	5.86E-07
TSPAN12	tetraspanin 12	-1.55	9.79E-09	6.04E-07
ERRFI1	ERBB receptor feedback inhibitor 1	3.17	9.81E-09	6.04E-07
GRAMD3	GRAM domain containing 3	1.96	9.92E-09	6.09E-07
KLHL20	kelch-like 20 (Drosophila)	1.62	1.01E-08	6.19E-07
NMI	N-myc (and STAT) interactor	-1.41	1.03E-08	6.26E-07
ORAI2	ORAI Ca^{++} release-activated Ca^{++} modulator 2	1.78	1.05E-08	6.38E-07

COLEC12	collectin sub-family member 12	-1.75	1.06E-08	6.38E-07
EIF4EBP2	eukaryotic translation initiation factor 4E binding protein 2	1.46	1.09E-08	6.57E-07
GJA5	gap junction protein, α 5, 40kDa	-1.68	1.10E-08	6.60E-07
ISM1	isthmin 1 homolog (zebrafish)	2.59	1.12E-08	6.70E-07
BTBD3	BTB (POZ) domain containing 3	1.68	1.14E-08	6.76E-07
VAMP8	vesicle-associated membrane protein 8 (endobrevin)	2.31	1.14E-08	6.77E-07
ZNF564	Zn^{++} finger protein 564	-1.35	1.14E-08	6.77E-07
RNF138	ring finger protein 138	-1.77	1.20E-08	7.11E-07
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	2.72	1.21E-08	7.16E-07
CISD1	CDGSH Fe^{++} sulfur domain 1	-1.37	1.23E-08	7.24E-07
HIBADH	3-hydroxyisobutyrate dehydrogenase	-1.23	1.24E-08	7.27E-07
SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	1.63	1.29E-08	7.51E-07
PNPLA3	patatin-like phospholipase domain containing 3	3.68	1.38E-08	7.96E-07
CYTH1	cytohesin 1	1.66	1.40E-08	8.06E-07
LIPA	lipase A, lysosomal acid, cholesterol esterase	3.74	1.43E-08	8.15E-07
RILPL1	Rab interacting lysosomal protein-like 1	1.52	1.46E-08	8.27E-07
NAP1L5	nucleosome assembly protein 1-like 5	1.55	1.46E-08	8.30E-07
DCLK2	doublecortin-like kinase 2	3.09	1.49E-08	8.42E-07
PSIP1	PC4 and SFRS1 interacting protein 1	-1.28	1.54E-08	8.66E-07
ZSWIM1	Zn^{++} finger, SWIM-type containing 1	1.65	1.58E-08	8.83E-07
NAP1L1	nucleosome assembly protein 1-like 1	-1.30	1.61E-08	8.96E-07
SF1	splicing factor 1	1.37	1.63E-08	9.3E-07
DST	dystonin	-1.68	1.66E-08	9.15E-07
EIF2B2	eukaryotic translation initiation factor 2B, subunit 2 β, 39kDa	1.47	1.68E-08	9.23E-07

LIMS1	LIM and senescent cell antigen-like domains 1	1.26	1.69E-08	9.25E-07
LCLAT1	lysocardiolipin acyltransferase 1	1.72	1.71E-08	9.28E-07
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	1.73	1.75E-08	9.44E-07
SOAT1	sterol O-acyltransferase 1	1.62	1.75E-08	9.44E-07
PREB	prolactin regulatory element binding	1.53	1.77E-08	9.50E-07
SHE	Src homology 2 domain containing E	-1.48	1.80E-08	9.63E-07
PCYT2	phosphate cytidylyltransferase 2, ethanolamine	1.33	1.83E-08	9.77E-07
SFXN1	sideroflexin 1	2.11	1.83E-08	9.77E-07
PCP2	Purkinje cell protein 2	2.64	1.84E-08	9.81E-07
SREBF2	sterol regulatory element binding transcription factor 2	2.36	1.85E-08	9.81E-07
FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	-2.10	1.86E-08	9.84E-07
DENND4C	DENN/MADD domain containing 4C	2.75	1.86E-08	9.87E-07
SH3TC2	SH3 domain and tetratricopeptide repeats 2	1.67	1.89E-08	9.93E-07
TM7SF2	transmembrane 7 superfamily member 2	2.41	1.95E-08	1.02E-06
TPCN1	two pore segment channel 1	1.81	1.95E-08	1.02E-06
ARHGEF26	Rho guanine nucleotide exchange factor (GEF) 26	2.28	2.08E-08	1.07E-06
PLEKHA6	pleckstrin homology domain containing, family A member 6	1.48	2.14E-08	1.09E-06
ZDHHC2	Zn^{++} finger, DHHC-type containing 2	2.08	2.14E-08	1.09E-06
MYO1B	myosin IB	-1.83	2.21E-08	1.12E-06
UBE2V1	ubiquitin-conjugating enzyme E2 variant 1	1.26	2.22E-08	1.12E-06
PTPN14	protein tyrosine phosphatase, non-receptor type 14	2.23	2.27E-08	1.14E-06
IDS	iduronate 2-sulfatase	1.82	2.28E-08	1.14E-06
LRRC8D	leucine rich repeat containing 8 family, member D	1.35	2.29E-08	1.14E-06
CSRP2BP	CSRP2 binding protein	1.58	2.30E-08	1.14E-06

ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)	-1.68	2.33E-08	1.15E-06
CAMSAP1L1	calmodulin regulated spectrin-associated protein 1-like 1	1.44	2.36E-08	1.16E-06
FNBP1L	formin binding protein 1-like	-1.45	2.45E-08	1.20E-06
MYL6B	myosin, light chain 6B, alkali, smooth muscle and non-muscle	1.29	2.45E-08	1.20E-06
LONRF1	LON peptidase N-terminal domain and ring finger 1	1.93	2.53E-08	1.23E-06
MYH9	myosin, heavy chain 9, non-muscle	-2.05	2.60E-08	1.26E-06
SCHIP1	schwannomin interacting protein 1	1.46	2.69E-08	1.30E-06
KIF14	kinesin family member 14	2.51	2.70E-08	1.30E-06
ARHGEF5	Rho guanine nucleotide exchange factor (GEF) 5	1.50	2.73E-08	1.31E-06
AAK1	AP2 associated kinase 1	1.37	2.76E-08	1.32E-06
ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila)	-1.66	2.77E-08	1.32E-06
DSE	dermatan sulfate epimerase	-1.37	2.77E-08	1.32E-06
ANKRD55	ankyrin repeat domain 55	1.40	2.81E-08	1.33E-06
PRDX4	peroxiredoxin 4	-1.36	2.82E-08	1.34E-06
PAPOLG	poly(A) polymerase γ	2.48	2.82E-08	1.34E-06
LZTS2	leucine zipper, putative tumor suppressor 2	1.39	2.91E-08	1.36E-06
ZNF608	Zn^{++} finger protein 608	-1.80	2.94E-08	1.37E-06
LUM	lumican	-1.93	2.94E-08	1.37E-06
ZNF292	Zn^{++} finger protein 292	-1.44	3.01E-08	1.40E-06
USP53	ubiquitin specific peptidase 53	-1.52	3.06E-08	1.41E-06
ERO1L	ERO1-like (S. cerevisiae)	1.47	3.12E-08	1.43E-06
DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	1.50	3.14E-08	1.44E-06
FLVCR1	feline leukemia virus subgroup C cellular receptor 1	1.22	3.17E-08	1.45E-06
PON2	paraoxonase 2	1.24	3.17E-08	1.45E-06

JAM2	junctional adhesion molecule 2	-1.64	3.21E-08	1.46E-06
PMP22	peripheral myelin protein 22	-1.78	3.22E-08	1.46E-06
LZTS1	leucine zipper, putative tumor suppressor 1	3.13	3.22E-08	1.46E-06
BCL2	B-cell CLL/lymphoma 2	2.30	3.24E-08	1.46E-06
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	1.68	3.31E-08	1.49E-06
FGL1	fibrinogen-like 1	-1.90	3.34E-08	1.49E-06
SLC25A40	Solute carrier family 25, member 40	-1.42	3.40E-08	1.52E-06
NAALAD2	N-acetylated α-linked acidic dipeptidase 2	-1.64	3.49E-08	1.55E-06
CEP120	centrosomal protein 120kDa	-1.31	3.54E-08	1.57E-06
CMTM8	CKLF-like MARVEL transmembrane domain containing 8	2.36	3.57E-08	1.58E-06
AZIN1	antizyme inhibitor 1	1.26	3.59E-08	1.59E-06
GRINA	glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)	2.57	3.62E-08	1.60E-06
MEF2C	myocyte enhancer factor 2C	-1.69	3.68E-08	1.61E-06
SMARCD1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1	1.24	3.71E-08	1.62E-06
SLC25A38	solute carrier family 25, member 38	1.26	3.80E-08	1.65E-06
SYNE2	spectrin repeat containing, nuclear envelope 2	-1.88	3.93E-08	1.70E-06
FXYD6	FXYD domain containing ion transport regulator 6	-1.65	3.95E-08	1.71E-06
EPHA3	EPH receptor A3	1.67	3.98E-08	1.72E-06
ARHGAP18	Rho GTPase activating protein 18	2.09	4.02E-08	1.73E-06
KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	-1.24	4.03E-08	1.73E-06
TPST2	tyrosylprotein sulfotransferase 2	-1.25	4.06E-08	1.74E-06
PROM1	prominin 1	-2.21	4.07E-08	1.74E-06

ARMC2	armadillo repeat containing 2	2.24	4.20E-08	1.79E-06
SPDEF	SAM pointed domain containing ets transcription factor	1.36	4.23E-08	1.81E-06
DUSP6	dual specificity phosphatase 6	-1.40	4.27E-08	1.81E-06
SLC9A2	solute carrier family 9 (sodium/hydrogen exchanger), member 2	2.76	4.28E-08	1.81E-06
DPF3	D4, Zn^{++} and double PHD fingers, family 3	1.51	4.28E-08	1.81E-06
ROBO2	roundabout, axon guidance receptor, homolog 2 (Drosophila)	-1.63	4.32E-08	1.82E-06
PRNP	prion protein	-1.38	4.40E-08	1.84E-06
BCHE	butyrylcholinesterase	-2.50	4.40E-08	1.84E-06
LETM2	Leucine zipper-EF-hand containing transmembrane protein 2	-1.61	4.47E-08	1.86E-06
CD164L2	CD164 sialomucin-like 2	1.84	4.49E-08	1.87E-06
SULF2	sulfatase 2	-1.69	4.53E-08	1.88E-06
RALGAPA2	Ral GTPase activating protein, α subunit 2 (catalytic)	1.41	4.55E-08	1.89E-06
DCN	decorin	-1.26	4.57E-08	1.89E-06
SPATS2L	spermatogenesis associated, serine-rich 2-like	1.35	4.73E-08	1.95E-06
MECOM	MDS1 and EVI1 complex locus	-1.58	4.86E-08	1.99E-06
USP6NL	USP6 N-terminal like	1.59	4.95E-08	2.02E-06
NPTN	neuroplastin	-1.22	4.99E-08	2.03E-06
KAT2B	K(lysine) acetyltransferase 2B	1.88	4.99E-08	2.03E-06
ART3	ADP-ribosyltransferase 3	2.74	5.01E-08	2.03E-06
TCF4	transcription factor 4	-1.36	5.02E-08	2.04E-06
RSAD1	radical S-adenosyl methionine domain containing 1	-1.35	5.04E-08	2.04E-06
CDR2	cerebellar degeneration-related protein 2, 62kDa	1.80	5.21E-08	2.09E-06
HSF1	heat shock transcription factor 1	1.27	5.24E-08	2.10E-06
ZBTB20	Zn^{++} finger and BTB domain containing 20	-1.44	5.24E-08	2.10E-06

ROD1	ROD1 regulator of differentiation 1 (S. pombe)	1.27	5.46E-08	2.18E-06
BIRC2	baculoviral IAP repeat-containing 2	-1.29	5.61E-08	2.22E-06
NFIL3	nuclear factor, interleukin 3 regulated	-2.07	5.74E-08	2.26E-06
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	2.65	5.79E-08	2.28E-06
SMC5	structural maintenance of chromosomes 5	-1.91	5.83E-08	2.29E-06
GPR161	G protein-coupled receptor 161	2.38	5.96E-08	2.32E-06
CTSK	cathepsin K	1.98	5.97E-08	2.33E-06
UNC13D	unc-13 homolog D (*C. elegans*)	2.42	6.15E-08	2.38E-06
LYPD6	LY6/PLAUR domain containing 6	1.90	6.26E-08	2.41E-06
WBP2	WW domain binding protein 2	1.39	6.30E-08	2.43E-06
PLA2G10	phospholipase A2, group X	2.11	6.30E-08	2.43E-06
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	2.29	6.32E-08	2.43E-06
PTPRB, PTPRR	protein tyrosine phosphatase, receptor type, B; protein tyrosine phosphatase, receptor type, R	-1.73	6.34E-08	2.44E-06
LRRC8C	leucine rich repeat containing 8 family, member C	-1.91	6.37E-08	2.44E-06
C1R	complement component 1, r subcomponent	-1.82	6.40E-08	2.44E-06
TFPI2	tissue factor pathway inhibitor 2	1.56	6.41E-08	2.44E-06
TMEM135	transmembrane protein 135	1.48	6.43E-08	2.45E-06
ARAP3	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3	-1.25	6.61E-08	2.51E-06
RENBP	renin binding protein	1.67	6.71E-08	2.54E-06
FAHD1	fumarylacetoacetate hydrolase domain containing 1	1.56	6.76E-08	2.56E-06
PHLDB2	pleckstrin homology-like domain, family B, member 2	2.19	6.80E-08	2.56E-06
LMBRD2	LMBR1 domain containing 2	1.34	6.86E-08	2.57E-06
CAV1	caveolin 1, caveolae protein, 22kDa	-1.63	6.95E-08	2.60E-06

ALAD	aminolevulinate dehydratase	2.05	7.14E-08	2.65E-06
GJC1	gap junction protein, γ 1, 45kDa	-1.66	7.17E-08	2.66E-06
HORMAD1	HORMA domain containing 1	3.21	7.38E-08	2.72E-06
ADD3	adducin 3 (γ)	-1.47	7.60E-08	2.79E-06
SLC35A1	solute carrier family 35 (CMP-sialic acid transporter), member A1	-1.36	7.72E-08	2.82E-06
APOA2	apolipoprotein A-II	1.42	7.92E-08	2.88E-06
MAP3K1	mitogen-activated protein kinase kinase kinase 1	1.45	8.05E-08	2.92E-06
DEGS2	degenerative spermatocyte homolog 2, lipid desaturase (Drosophila)	2.40	8.17E-08	2.95E-06
EGLN3	egl nine homolog 3 (C. elegans)	1.90	8.29E-08	2.98E-06
ANXA6	annexin A6	-1.62	8.33E-08	2.99E-06
TTPA	tocopherol (α) transfer protein	2.69	8.42E-08	3.01E-06
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1	1.48	8.54E-08	3.04E-06
ROGDI	rogdi homolog (Drosophila)	1.36	8.82E-08	3.14E-06
PCSK5	proprotein convertase subtilisin/kexin type 5	2.01	8.84E-08	3.14E-06
TRIM13	tripartite motif-containing 13	1.37	8.98E-08	3.18E-06
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	-1.40	9.10E-08	3.21E-06
DHCR7	7-dehydrocholesterol reductase	2.15	9.20E-08	3.24E-06
SIRPA	signal-regulatory protein α	2.62	9.25E-08	3.25E-06
FSD1L	fibronectin type III and SPRY domain containing 1-like	-1.46	9.29E-08	3.27E-06
CARHSP1	Ca^{++} regulated heat stable protein 1, 24kDa	1.70	9.44E-08	3.30E-06
P2RY14	purinergic receptor P2Y, G-protein coupled, 14	1.64	9.78E-08	3.41E-06
NHLRC1	NHL repeat containing 1	1.42	9.82E-08	3.42E-06
CPE	carboxypeptidase E	-1.56	1.00E-07	3.47E-06

CCL3	chemokine (C-C motif) ligand 3	-3.23	1.00E-07	3.48E-06
F2RL2	coagulation factor II (thrombin) receptor-like 2	-1.64	1.01E-07	3.50E-06
MNDA	myeloid cell nuclear differentiation antigen	-1.54	1.02E-07	3.52E-06
F2R	coagulation factor II (thrombin) receptor	-1.21	1.03E-07	3.53E-06
DEPDC6	DEP domain containing 6	1.70	1.04E-07	3.56E-06
MAP3K4	mitogen-activated protein kinase kinase kinase 4	-1.33	1.04E-07	3.56E-06
ADPRHL1	ADP-ribosylhydrolase like 1	2.27	1.04E-07	3.57E-06
ILKAP	integrin-linked kinase-associated serine/threonine phosphatase	1.77	1.05E-07	3.57E-06
RAP2A	RAP2A, member of RAS oncogene family	1.62	1.06E-07	3.60E-06
EDEM1	ER degradation enhancer, mannosidase α-like 1	1.56	1.08E-07	3.66E-06
GNS	glucosamine (N-acetyl)-6-sulfatase	2.10	1.08E-07	3.68E-06
CPEB2	cytoplasmic polyadenylation element binding protein 2	1.80	1.09E-07	3.70E-06
TMC4	transmembrane channel-like 4	1.99	1.12E-07	3.77E-06
TBX3	T-box 3	1.32	1.16E-07	3.87E-06
NEURL2	neuralized homolog 2 (Drosophila)	1.62	1.19E-07	3.94E-06
NR1H4	nuclear receptor subfamily 1, group H, member 4	-1.76	1.20E-07	3.96E-06
NPR2	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)	-1.27	1.22E-07	4.00E-06
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	-1.47	1.28E-07	4.18E-06
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	1.27	1.29E-07	4.20E-06
CHRM4	cholinergic receptor, muscarinic 4	2.06	1.32E-07	4.28E-06
YIPF6	Yip1 domain family, member 6	1.24	1.34E-07	4.34E-06
PPAPDC3	phosphatidic acid phosphatase type 2 domain containing 3	-1.63	1.36E-07	4.39E-06
GAB1	GRB2-associated binding protein 1	2.06	1.38E-07	4.41E-06

P4HA2	prolyl 4-hydroxylase, α polypeptide II	-1.34	1.38E-07	4.41E-06
GLT8D2	glycosyltransferase 8 domain containing 2	-1.62	1.38E-07	4.42E-06
TWF1	twinfilin, actin-binding protein, homolog 1 (Drosophila)	1.31	1.43E-07	4.55E-06
FADS1	fatty acid desaturase 1	2.52	1.44E-07	4.57E-06
CLEC1A	C-type lectin domain family 1, member A	-1.70	1.46E-07	4.60E-06
RLN	relaxin 1	1.36	1.46E-07	4.61E-06
SEMA4D	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	1.86	1.51E-07	4.72E-06
TEC	tec protein tyrosine kinase	1.90	1.51E-07	4.72E-06
CEP170	centrosomal protein 170kDa	-1.35	1.52E-07	4.76E-06
EGR1	early growth response 1	-4.31	1.54E-07	4.79E-06
MAP3K5	mitogen-activated protein kinase kinase kinase 5	1.55	1.54E-07	4.80E-06
RFK	riboflavin kinase	1.66	1.55E-07	4.82E-06
FKBP9	FK506 binding protein 9, 63 kDa	-1.33	1.59E-07	4.88E-06
LAMC1	laminin, γ1 (formerly LAMB2)	1.38	1.63E-07	4.99E-06
ATF3	activating transcription factor 3	-3.85	1.63E-07	4.99E-06
DUSP10	dual specificity phosphatase 10	2.31	1.67E-07	5.09E-06
DUSP1	dual specificity phosphatase 1	-2.00	1.67E-07	5.10E-06
HIGD1B	HIG1 hypoxia inducible domain family, member 1B	-1.51	1.67E-07	5.10E-06
FARP2	FERM, RhoGEF and pleckstrin domain protein 2	1.74	1.68E-07	5.12E-06
TBX4	T-box 4	-1.33	1.68E-07	5.12E-06
ATP10A	ATPase, class V, type 10A	-1.36	1.70E-07	5.16E-06
DISC1	disrupted in schizophrenia 1	2.67	1.71E-07	5.16E-06
GLIPR1L2	GLI pathogenesis-related 1 like 2	1.42	1.71E-07	5.16E-06

CDK16	cyclin-dependent kinase 16	1.22	1.73E-07	5.19E-06
ALDH3A1	aldehyde dehydrogenase 3 family, member A1	2.64	1.73E-07	5.19E-06
TLE1	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	-1.79	1.78E-07	5.33E-06
RND3	Rho family GTPase 3	-2.05	1.78E-07	5.33E-06
MPDZ	multiple PDZ domain protein	-1.93	1.84E-07	5.46E-06
FOLR2	folate receptor 2 (fetal)	-1.66	1.84E-07	5.46E-06
PIK3C2A	phosphoinositide-3-kinase, class 2, α polypeptide	-1.36	1.86E-07	5.50E-06
LCN2	lipocalin 2	1.21	1.86E-07	5.50E-06
RASGRP3	RAS guanyl releasing protein 3 (Ca^{++} and DAG-regulated)	-2.09	1.88E-07	5.53E-06
ZEB1	Zn^{++} finger E-box binding homeobox 1	-1.76	1.89E-07	5.54E-06
RASSF8	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	-2.09	1.94E-07	5.68E-06
PCDH9	protocadherin 9	1.35	1.95E-07	5.69E-06
C3	complement component 3	-1.26	1.95E-07	5.70E-06
PTPRG	protein tyrosine phosphatase, receptor type, G	-1.34	1.96E-07	5.70E-06
LAMA4	laminin, α 4	-1.59	1.96E-07	5.70E-06
IRX2	iroquois homeobox 2	1.23	1.99E-07	5.76E-06
CALU	calumenin	1.32	2.00E-07	5.78E-06
LACTB2	lactamase, β 2	1.24	2.00E-07	5.80E-06
TACC2	transforming, acidic coiled-coil containing protein 2	1.21	2.04E-07	5.86E-06
41156	septin 4	-1.37	2.05E-07	5.88E-06
PLEKHA3	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 3	-1.38	2.05E-07	5.88E-06
PLK2	polo-like kinase 2	-2.19	2.06E-07	5.90E-06
SNX16	sorting nexin 16	-1.21	2.08E-07	5.94E-06

BAZ1A	bromodomain adjacent to Zn^{++} finger domain, 1A	-1.36	2.12E-07	6.06E-06
MEGF9	multiple EGF-like-domains 9	1.90	2.13E-07	6.07E-06
BAZ2B	bromodomain adjacent to Zn^{++} finger domain, 2B	-1.40	2.16E-07	6.13E-06
COL5A2	collagen, type V, α 2	-1.79	2.20E-07	6.21E-06
LIN7A	lin-7 homolog A (*C. elegans*)	1.27	2.23E-07	6.29E-06
FBXL5	F-box and leucine-rich repeat protein 5	-1.26	2.25E-07	6.33E-06
CD40	CD40 molecule, TNF receptor superfamily member 5	-1.26	2.25E-07	6.33E-06
CUBN	cubilin (intrinsic factor-cobalamin receptor)	-1.68	2.30E-07	6.44E-06
ACSF2	acyl-CoA synthetase family member 2	-1.29	2.31E-07	6.47E-06
CTSS	cathepsin S	-1.61	2.36E-07	6.57E-06
ZFP106	Zn^{++} finger protein 106 homolog (mouse)	1.48	2.39E-07	6.64E-06
SPRED1	sprouty-related, EVH1 domain containing 1	1.65	2.44E-07	6.73E-06
IRS2	insulin receptor substrate 2	1.48	2.49E-07	6.84E-06
SKAP2	src kinase associated phosphoprotein 2	-1.26	2.50E-07	6.84E-06
FADS1, LOC612278	fatty acid desaturase 1, similar to fatty acid desaturase 1	2.12	2.53E-07	6.93E-06
SLC30A3	solute carrier family 30 (Zn^{++} transporter), member 3	1.73	2.53E-07	6.93E-06
IGF1	insulin-like growth factor 1 (somatomedin C)	3.11	2.56E-07	6.98E-06
FBXO30	F-box protein 30	-1.68	2.58E-07	7.03E-06
BCL2L1	BCL2-like 1	1.66	2.64E-07	7.17E-06
PIBF1	progesterone immunomodulatory binding factor 1	-1.65	2.65E-07	7.18E-06
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	-1.81	2.66E-07	7.21E-06
DYSF	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	-1.67	2.67E-07	7.22E-06
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	1.64	2.70E-07	7.28E-06

ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	2.24	2.73E-07	7.34E-06
PITX2	paired-like homeodomain 2	-1.76	2.73E-07	7.34E-06
RAB3IP	RAB3A interacting protein (rabin3)	-1.28	2.73E-07	7.34E-06
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	-1.30	2.77E-07	7.40E-06
GTF2H5	general transcription factor IIH, polypeptide 5	1.27	2.79E-07	7.44E-06
RGS2	regulator of G-protein signaling 2, 24kDa	-1.87	2.84E-07	7.55E-06
OLFML2B	olfactomedin-like 2B	2.09	2.89E-07	7.62E-06
ZNF521	Zn^{++} finger protein 521	-1.34	2.89E-07	7.62E-06
FGL2	fibrinogen-like 2	-3.19	2.91E-07	7.67E-06
DENND5A	DENN/MADD domain containing 5A	-1.30	2.99E-07	7.84E-06
SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	-1.23	3.00E-07	7.86E-06
PPP1R11	protein phosphatase 1, regulatory (inhibitor) subunit 11	1.66	3.06E-07	8.00E-06
IER3	immediate early response 3	-1.50	3.08E-07	8.02E-06
NR2F1	nuclear receptor subfamily 2, group F, member 1	-1.33	3.15E-07	8.14E-06
NFKBIZ	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, zeta	-2.09	3.17E-07	8.18E-06
RAD21L1	RAD21-like 1 (*S. pombe*)	1.43	3.19E-07	8.21E-06
XAB2	XPA binding protein 2	1.66	3.19E-07	8.21E-06
EPHA6	EPH receptor A6	1.23	3.25E-07	8.34E-06
FAP	fibroblast activation protein, α	-2.60	3.26E-07	8.34E-06
PCTP	phosphatidylcholine transfer protein	-1.35	3.26E-07	8.34E-06
HECTD2	HECT domain containing 2	-1.34	3.32E-07	8.45E-06
HOMER1	homer homolog 1 (Drosophila)	1.75	3.35E-07	8.53E-06
PROCR	protein C receptor, endothelial	-1.74	3.39E-07	8.59E-06

ACACA	acetyl-CoA carboxylase α	1.25	3.47E-07	8.77E-06
ANAPC11	anaphase promoting complex subunit 11	1.62	3.6E-07	9.10E-06
HLA-DQA1	major histocompatibility complex, class II, DQ α1	-2.30	3.78E-07	9.42E-06
WIPF1	WAS/WASL interacting protein family, member 1	1.50	3.82E-07	9.50E-06
ITCH	itchy E3 ubiquitin protein ligase homolog (mouse)	1.61	3.83E-07	9.52E-06
HSD17B6	hydroxysteroid (17-β) dehydrogenase 6 homolog (mouse)	1.28	3.84E-07	9.52E-06
PLEKHA1	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	2.17	3.85E-07	9.54E-06
ZNF609	Zn^{++} finger protein 609	1.59	3.87E-07	9.58E-06
STXBP5	syntaxin binding protein 5 (tomosyn)	1.32	3.98E-07	9.82E-06
EPHB1	EPH receptor B1	1.75	4.07E-07	9.99E-06
SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1	2.42	4.09E-07	1.00E-05
PDE7A	phosphodiesterase 7A	1.48	4.09E-07	1.00E-05
ESM1	endothelial cell-specific molecule 1	-1.95	4.16E-07	1.02E-05
IL8	interleukin 8	-4.29	4.16E-07	1.02E-05
SAMD9L	sterile α motif domain containing 9-like	-1.34	4.26E-07	1.03E-05
B4GALT6	UDP-Gal:β GlcNAc β1,4- galactosyltransferase, polypeptide 6	1.65	4.28E-07	1.04E-05
HMCN1	hemicentin 1	-2.01	4.36E-07	1.05E-05
TMPRSS11E	transmembrane protease, serine 11E	2.51	4.41E-07	1.06E-05
FAT1	FAT tumor suppressor homolog 1 (Drosophila)	1.41	4.41E-07	1.06E-05
TPD52	tumor protein D52	1.53	4.45E-07	1.07E-05
STX5	syntaxin 5	1.22	4.49E-07	1.08E-05
CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	2.31	4.50E-07	1.08E-05
PRR15	proline rich 15	1.53	4.50E-07	1.08E-05

COL5A1	collagen, type V, α 1	-1.66	4.59E-07	1.09E-05
RASIP1	Ras interacting protein 1	-1.33	4.62E-07	1.10E-05
TTLL12	Tubulin tyrosine ligase-like family, member 12	-1.41	4.64E-07	1.10E-05
ALDH4A1	aldehyde dehydrogenase 4 family, member A1	2.93	4.65E-07	1.10E-05
PGM2L1	phosphoglucomutase 2-like 1	-1.64	4.68E-07	1.11E-05
TET1	tet oncogene 1	1.76	4.76E-07	1.12E-05
EMCN	endomucin	-2.25	4.89E-07	1.15E-05
PDGFRA	platelet-derived growth factor receptor, α polypeptide	1.39	4.93E-07	1.16E-05
ATP11C	ATPase, class VI, type 11C	1.36	4.94E-07	1.16E-05
ENTPD3	ectonucleoside triphosphate diphosphohydrolase 3	1.59	4.95E-07	1.16E-05
A2M	α-2-macroglobulin	-1.56	4.96E-07	1.16E-05
HRH3	histamine receptor H3	2.80	5.01E-07	1.17E-05
DDA1	DET1 and DDB1 associated 1	1.42	5.05E-07	1.18E-05
RIC8B	resistance to inhibitors of cholinesterase 8 homolog B (*C. elegans*)	-1.26	5.08E-07	1.18E-05
TSPAN13	tetraspanin 13	-1.85	5.12E-07	1.19E-05
SENP6	SUMO1/sentrin specific peptidase 6	-1.37	5.12E-07	1.19E-05
SIPA1L1	signal-induced proliferation-associated 1 like 1	1.56	5.12E-07	1.19E-05
NUP153	nucleoporin 153kDa	1.53	5.25E-07	1.21E-05
FSTL1	follistatin-like 1	-1.44	5.38E-07	1.23E-05
SLC25A33	solute carrier family 25, member 33	-1.55	5.45E-07	1.24E-05
HTR1A	5-hydroxytryptamine (serotonin) receptor 1A	1.52	5.61E-07	1.27E-05
SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member 1	-2.12	5.78E-07	1.30E-05
LGR4	leucine-rich repeat-containing G protein-coupled receptor 4	1.51	6.11E-07	1.36E-05
GPR146	G protein-coupled receptor 146	1.46	6.12E-07	1.36E-05

PRKACB	protein kinase, cAMP-dependent, catalytic, β	1.84	6.12E-07	1.36E-05
ATP6V0D1	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	1.23	6.12E-07	1.36E-05
CCL4	chemokine (C-C motif) ligand 4	-4.04	6.12E-07	1.36E-05
UBXN4	UBX domain protein 4	1.21	6.17E-07	1.37E-05
WDR44	WD repeat domain 44	-1.21	6.22E-07	1.37E-05
SOX9	SRY (sex determining region Y)-box 9	2.87	6.22E-07	1.37E-05
KIDINS220	kinase D-interacting substrate, 220kDa	1.44	6.23E-07	1.38E-05
CHMP2B	chromatin modifying protein 2B	1.36	6.26E-07	1.38E-05
TMEM49	transmembrane protein 49	-1.53	6.30E-07	1.39E-05
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	-1.41	6.55E-07	1.43E-05
NRN1	neuritin 1	5.24	6.62E-07	1.44E-05
ZNF704	Zn^{++} finger protein 704	2.13	6.67E-07	1.45E-05
MIPOL1	mirror-image polydactyly 1	-1.22	6.78E-07	1.48E-05
HSD17B11	hydroxysteroid (17-β) dehydrogenase 11	-1.51	6.87E-07	1.49E-05
PPP2R3A	protein phosphatase 2, regulatory subunit B, α	1.46	7.00E-07	1.51E-05
MYCT1	myc target 1	-1.70	7.05E-07	1.52E-05
SALL4	sal-like 4 (Drosophila)	1.31	7.08E-07	1.52E-05
TPST1	tyrosylprotein sulfotransferase 1	-1.35	7.10E-07	1.53E-05
PTGES	prostaglandin E synthase	1.57	7.18E-07	1.54E-05
FAIM	Fas apoptotic inhibitory molecule	-1.26	7.40E-07	1.58E-05
AVPI1	arginine vasopressin-induced 1	1.54	7.53E-07	1.59E-05
PRKCDBP	protein kinase C, Δ binding protein	1.83	7.62E-07	1.61E-05
RND1	Rho family GTPase 1	-1.61	7.63E-07	1.61E-05

ARHGAP12	Rho GTPase activating protein 12	1.26	7.69E-07	1.62E-05
LIX1L	Lix1 homolog (mouse)-like	-1.26	7.71E-07	1.62E-05
ZNF395	Zn^{++} finger protein 395	1.52	8.02E-07	1.68E-05
RGS12	regulator of G-protein signaling 12	1.36	8.10E-07	1.69E-05
FOS	FBJ murine osteosarcoma viral oncogene homolog	-4.61	8.24E-07	1.71E-05
IFT80	intraflagellar transport 80 homolog (Chlamydomonas)	-1.31	8.27E-07	1.71E-05
COL4A5	collagen, type IV, α 5	-2.13	8.42E-07	1.74E-05
GUCY1B3	guanylate cyclase 1, soluble, β 3	-1.34	8.44E-07	1.74E-05
RBP1	retinol binding protein 1, cellular	1.57	8.45E-07	1.74E-05
EBF1	early B-cell factor 1	-1.61	8.58E-07	1.76E-05
ADA	adenosine deaminase	-1.27	8.69E-07	1.77E-05
SLC2A13	solute carrier family 2 (facilitated glucose transporter), member 13	-1.58	8.83E-07	1.79E-05
KLHL7	kelch-like 7 (Drosophila)	-1.44	9.29E-07	1.87E-05
PALMD	palmdelphin	-2.01	9.40E-07	1.89E-05
FBN1	fibrillin 1	-1.99	9.42E-07	1.89E-05
PCM1	pericentriolar material 1	-1.21	9.47E-07	1.90E-05
NEIL2	nei endonuclease VIII-like 2 (E. coli)	1.38	9.52E-07	1.90E-05
SHROOM1	shroom family member 1	1.27	9.57E-07	1.91E-05
FANK1	fibronectin type III and ankyrin repeat domains 1	1.21	9.60E-07	1.92E-05
ACADS	acyl-CoA dehydrogenase, C-2 to C-3 short chain	2.05	9.70E-07	1.93E-05
SMAD1	SMAD family member 1	-1.42	9.74E-07	1.94E-05
CAV2	caveolin 2	-1.33	9.75E-07	1.94E-05
ATP9A	ATPase, class II, type 9A	1.68	1.01E-06	1.98E-05
MLANA	melan-A	2.71	1.02E-06	2.01E-05

CLIC4	chloride intracellular channel 4	-1.28	1.02E-06	2.01E-05
TNS1	tensin 1	1.65	1.03E-06	2.02E-05
MERTK	c-mer proto-oncogene tyrosine kinase	-1.72	1.03E-06	2.02E-05
FEM1B	fem-1 homolog b (C. elegans)	-1.37	1.04E-06	2.03E-05
ELL2	elongation factor, RNA polymerase II, 2	1.32	1.04E-06	2.03E-05
PRKD1	protein kinase D1	-1.45	1.04E-06	2.03E-05
C1S	complement component 1, s subcomponent	-1.74	1.05E-06	2.05E-05
PCDHGA10	protocadherin γ subfamily A, 10	1.66	1.07E-06	2.07E-05
TMEFF1	transmembrane protein with EGF-like and two follistatin-like domains 1	-1.64	1.07E-06	2.08E-05
SIRT1	sirtuin 1	-1.22	1.11E-06	2.13E-05
CD38	CD38 molecule	1.54	1.12E-06	2.15E-05
ARHGAP42	Rho GTPase activating protein 42	-1.26	1.12E-06	2.15E-05
UNC5B	unc-5 homolog B (C. elegans)	1.62	1.15E-06	2.20E-05
SSX2IP	synovial sarcoma, X breakpoint 2 interacting protein	1.97	1.16E-06	2.21E-05
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	-1.72	1.16E-06	2.22E-05
PTGES2	prostaglandin E synthase 2	1.46	1.16E-06	2.22E-05
KLF6	Kruppel-like factor 6	-1.48	1.18E-06	2.24E-05
DOCK8	dedicator of cytokinesis 8	1.38	1.18E-06	2.25E-05
CCDC63	coiled-coil domain containing 63	2.02	1.18E-06	2.25E-05
SLC1A3	solute carrier family 1 (glial high affinity glutamate transporter), member 3	1.64	1.20E-06	2.28E-05
BMP5	bone morphogenetic protein 5	-1.41	1.24E-06	2.34E-05
PLXNB1	plexin B1	1.37	1.29E-06	2.40E-05
PDE4D	phosphodiesterase 4D, cAMP-specific	-1.24	1.30E-06	2.42E-05
TTC39B	tetratricopeptide repeat domain 39B	-1.35	1.32E-06	2.45E-05

PDZK1	PDZ domain containing 1	2.03	1.34E-06	2.48E-05
CD274	CD274 molecule	2.01	1.34E-06	2.48E-05
OSBPL3	oxysterol binding protein-like 3	-1.37	1.35E-06	2.50E-05
DTX2	deltex homolog 2 (Drosophila)	1.65	1.35E-06	2.50E-05
IL33	interleukin 33	-2.91	1.37E-06	2.53E-05
PSTPIP2	proline-serine-threonine phosphatase interacting protein 2	1.31	1.39E-06	2.55E-05
EPHA1	EPH receptor A1	1.58	1.43E-06	2.60E-05
SH2B1	SH2B adaptor protein 1	1.85	1.44E-06	2.62E-05
DPP4	dipeptidyl-peptidase 4	1.59	1.44E-06	2.63E-05
UNC93B1	unc-93 homolog B1 (C. elegans)	1.25	1.45E-06	2.63E-05
ARMCX3	armadillo repeat containing, X-linked 3	-1.29	1.47E-06	2.67E-05
SERPINB11	serpin peptidase inhibitor, clade B (ovalbumin), member 11 (gene/pseudogene)	2.18	1.49E-06	2.69E-05
NSUN7	NOP2/Sun domain family, member 7	2.37	1.51E-06	2.72E-05
SLC27A3	solute carrier family 27 (fatty acid transporter), member 3	-1.29	1.52E-06	2.75E-05
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	2.01	1.52E-06	2.75E-05
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	-1.43	1.54E-06	2.78E-05
BBS7	Bardet-Biedl syndrome 7	-1.27	1.57E-06	2.81E-05
CD109	CD109 molecule	-1.37	1.57E-06	2.81E-05
ANKRD11	ankyrin repeat domain 11	1.50	1.57E-06	2.81E-05
MAN1A1	mannosidase, α, class 1A, member 1	-1.57	1.60E-06	2.86E-05
ENG	endoglin	-1.43	1.63E-06	2.90E-05
PSMB8	proteasome (prosome, macropain) subunit, β type, 8 (large multifunctional peptidase 7)	-1.24	1.63E-06	2.90E-05
ELOVL5	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2,	-1.39	1.67E-06	2.95E-05

	SUR4/Elo3-like, yeast)			
CTTN	cortactin	-1.46	1.68E-06	2.97E-05
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	-2.25	1.68E-06	2.97E-05
CCL2	chemokine (C-C motif) ligand 2	-2.58	1.71E-06	3.00 E-05
PTPRB	protein tyrosine phosphatase, receptor type, B	-1.90	1.71E-06	3.00E-05
ATP2C1	ATPase, Ca^{++} transporting, type 2C, member 1	1.58	1.71E-06	3.00E-05
CHN2	chimerin (chimaerin) 2	-1.42	1.72E-06	3.01E-05
GUF1	GUF1 GTPase homolog (S. cerevisiae)	-1.34	1.73E-06	3.03E-05
GLOD5	glyoxalase domain containing 5	1.43	1.73E-06	3.03E-05
PARP8	poly (ADP-ribose) polymerase family, member 8	-1.81	1.79E-06	3.11E-05
CLSTN1	calsyntenin 1	-1.68	1.81E-06	3.13E-05
SLC6A4	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	2.20	1.82E-06	3.15E-05
CAB39	Ca^{++} binding protein 39	1.51	1.86E-06	3.20E-05
HLA-DRB1	MHC class II DLA DRB1 β chain	-1.80	1.86E-06	3.20E-05
TTC3	tetratricopeptide repeat domain 3	-1.28	1.88E-06	3.24E-05
CCRL2	chemokine (C-C motif) receptor-like 2	-1.42	2.03E-06	3.44E-05
ABCA8	ATP-binding cassette, sub-family A (ABC1), member 8	-1.67	2.07E-06	3.50E-05
RBMS3	RNA binding motif, single stranded interacting protein 3	-1.69	2.09E-06	3.52E-05
GPR125	G protein-coupled receptor 125	1.72	2.10E-06	3.53E-05
PRLR	prolactin receptor	1.70	2.12E-06	3.56E-05
FAM96B	family with sequence similarity 96, member B	1.24	2.15E-06	3.59E-05
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (γ)	-1.47	2.18E-06	3.65E-05
TCEA3	transcription elongation factor A (SII), 3	1.22	2.19E-06	3.66E-05
PRELP	proline/arginine-rich end leucine-rich repeat protein	-1.56	2.19E-06	3.66E-05

CLINT1	clathrin interactor 1	1.45	2.22E-06	3.69E-05
ARMCX1	armadillo repeat containing, X-linked 1	-1.43	2.23E-06	3.70E-05
DENND2D	DENN/MADD domain containing 2D	1.32	2.24E-06	3.72E-05
SPARCL1	SPARC-like 1 (hevin)	-1.97	2.28E-06	3.76E-05
CSTB	cystatin B (stefin B)	1.27	2.28E-06	3.77E-05
ZHX1	Zn^{++} fingers and homeoboxes 1	-1.79	2.36E-06	3.87E-05
ACSS3	acyl-CoA synthetase short-chain family member 3	1.25	2.40E-06	3.92E-05
ZCCHC11	Zn^{++} finger, CCHC domain containing 11	-1.41	2.43E-06	3.95E-05
ARHGAP25	Rho GTPase activating protein 25	1.74	2.43E-06	3.95E-05
N4BP2L1	NEDD4 binding protein 2-like 1	-1.30	2.44E-06	3.98E-05
P4HA3	prolyl 4-hydroxylase, α polypeptide III	-2.16	2.50E-06	4.04E-05
S100A1	S100 Ca^{++} binding protein A1	2.95	2.50E-06	4.04E-05
TM4SF18	transmembrane 4 L six family member 18	-1.52	2.55E-06	4.10E-05
EML1	echinoderm microtubule associated protein like 1	-1.38	2.57E-06	4.12E-05
RAB22A	RAB22A, member RAS oncogene family	1.32	2.59E-06	4.14E-05
RHOG	ras homolog gene family, member G (rho G)	1.52	2.62E-06	4.18E-05
CH25H	cholesterol 25-hydroxylase	-2.95	2.84E-06	4.48E-05
THBS2	thrombospondin 2	-1.42	2.90E-06	4.55E-05
TAOK2	TAO kinase 2	1.37	2.91E-06	4.55E-05
ANKRD29	ankyrin repeat domain 29	-1.42	2.91E-06	4.55E-05
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	-1.77	2.93E-06	4.58E-05
KARS	lysyl-tRNA synthetase	-1.16	2.93E-06	4.58E-05
SYNE1	spectrin repeat containing, nuclear envelope 1	-1.67	3.06E-06	4.75E-05
VCAM1	vascular cell adhesion molecule 1	-2.01	3.10E-06	4.80E-05

BACE2, LOC478419	β-site APP-cleaving enzyme 2, similar to Protein FAM3B precursor (Cytokine-like protein 2-21)	-1.52	3.16E-06	4.87E-05
OSTF1	osteoclast stimulating factor 1	1.24	3.17E-06	4.88E-05
SLC46A3	solute carrier family 46, member 3	-1.26	3.17E-06	4.88E-05
GOLIM4	golgi integral membrane protein 4	-1.44	3.25E-06	4.98E-05
GADD45B	growth arrest and DNA-damage-inducible, β	-2.04	3.30E-06	5.03E-05
GJA1	gap junction protein, α 1, 43kDa	-1.97	3.32E-06	5.05E-05
PRPS2	phosphoribosyl pyrophosphate synthetase 2	-1.58	3.33E-06	5.06E-05
CXCR4	chemokine (C-X-C motif) receptor 4	-1.69	3.41E-06	5.15E-05
FOXO1	forkhead box O1	1.32	3.44E-06	5.19E-05
TMPO	thymopoietin	-1.27	3.56E-06	5.33E-05
OCA2	oculocutaneous albinism II	1.72	3.58E-06	5.37E-05
UGGT2	UDP-glucose glycoprotein glucosyltransferase 2	-1.47	3.63E-06	5.42E-05
EGFR	epidermal growth factor receptor	1.63	3.78E-06	5.58E-05
CREM	cAMP responsive element modulator	-1.29	3.80E-06	5.62E-05
SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	1.56	3.81E-06	5.62E-05
PON3	paraoxonase 3	-1.80	3.86E-06	5.68E-05
PRRG1	proline rich Gla (G-carboxyglutamic acid) 1	1.21	3.91E-06	5.74E-05
SUSD1	sushi domain containing 1	-1.37	3.94E-06	5.77E-05
SLC15A1	solute carrier family 15 (oligopeptide transporter), member 1	-1.72	4.03E-06	5.86E-05
FRY	furry homolog (Drosophila)	-1.32	4.09E-06	5.93E-05
UST	uronyl-2-sulfotransferase	-1.22	4.33E-06	6.19E-05
SVEP1	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	1.41	4.52E-06	6.38E-05
EPSTI1	epithelial stromal interaction 1 (breast)	-1.33	4.55E-06	6.42E-05

VEGFC	vascular endothelial growth factor C	-1.49	4.57E-06	6.43E-05
NCOA7	nuclear receptor coactivator 7	-1.46	4.67E-06	6.54E-05
PLD3	phospholipase D family, member 3	2.65	4.70E-06	6.57E-05
PQLC3	PQ loop repeat containing 3	-1.57	4.70E-06	6.57E-05
NR1D2	nuclear receptor subfamily 1, group D, member 2	-1.36	4.72E-06	6.58E-05
RAB31	RAB31, member RAS oncogene family	-1.27	4.72E-06	6.58E-05
DAPL1	death associated protein-like 1	1.90	4.75E-06	6.61E-05
SLMAP	sarcolemma associated protein	1.34	4.80E-06	6.66E-05
RRAD	Ras-related associated with diabetes	-1.57	4.83E-06	6.69E-05
KIFC3	kinesin family member C3	2.08	4.85E-06	6.71E-05
USP33	ubiquitin specific peptidase 33	1.48	4.86E-06	6.72E-05
ZSWIM6	Zn^{++} finger, SWIM-type containing 6	1.54	4.88E-06	6.75E-05
NOC3L	nucleolar complex associated 3 homolog (S. cerevisiae)	1.51	4.98E-06	6.85E-05
SLC31A2	solute carrier family 31 (Cu^{++} transporters), member 2	1.39	5.09E-06	6.96E-05
RAB25	RAB25, member RAS oncogene family	1.36	5.12E-06	7.00E-05
B4GALT1	UDP-Gal:βGlcNAc β 1,4- galactosyltransferase, polypeptide 1	1.36	5.20E-06	7.08E-05
AGPAT4	1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, Δ)	-1.76	5.23E-06	7.11E-05
STK25	serine/threonine-protein kinase 25	1.57	5.31E-06	7.17E-05
SLTM	SAFB-like, transcription modulator	-1.21	5.32E-06	7.18E-05
SDR16C5	short chain dehydrogenase/reductase family 16C, member 5	1.35	5.33E-06	7.19E-05
PEG3	paternally expressed 3	-1.42	5.40E-06	7.27E-05
FICD	FIC domain containing	1.53	5.40E-06	7.27E-05
STARD6	StAR-related lipid transfer (START) domain containing 6	-1.22	5.45E-06	7.32E-05

SNX25	sorting nexin 25	-1.34	5.54E-06	7.41E-05
ISG20	interferon stimulated exonuclease gene 20kDa	1.45	5.60E-06	7.48E-05
AMPD3	adenosine monophosphate deaminase 3	1.41	5.68E-06	7.56E-05
LGALS12	lectin, galactoside-binding, soluble, 12	1.23	5.76E-06	7.64E-05
ASB9	ankyrin repeat and SOCS box-containing 9	-1.27	5.84E-06	7.73E-05
PKD2L1	polycystic kidney disease 2-like 1	2.35	5.84E-06	7.74E-05
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	-1.69	5.97E-06	7.86E-05
RBM47	RNA binding motif protein 47	1.42	6.09E-06	7.99E-05
NBEA	neurobeachin	-1.29	6.10E-06	7.99E-05
FMO2	flavin containing monooxygenase 2 (non-functional)	1.80	6.13E-06	8.03E-05
RBM11	RNA binding motif protein 11	1.38	6.25E-06	8.14E-05
RRAGD	Ras-related GTP binding D	2.31	6.42E-06	8.30E-05
DLA-DRA	MHC class II DR α chain	-1.87	6.483E-06	8.36E-05
BICD1	bicaudal D homolog 1 (Drosophila)	1.65	6.50E-06	8.38E-05
RCL1	RNA terminal phosphate cyclase-like 1	1.35	6.53E-06	8.41E-05
GPR116	G protein-coupled receptor 116	-1.36	6.58E-06	8.46E-05
SPATA6	spermatogenesis associated 6	-1.46	6.77E-06	8.63E-05
GAS6	growth arrest-specific 6	-1.63	6.85E-06	8.72E-05
CTSL2	cathepsin L2	1.70	6.88E-06	8.75E-05
CD200	CD200 molecule	-1.32	6.99E-06	8.87E-05
TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2	-1.30	7.05E-06	8.94E-05
DCAF4	DDB1 and CUL4 associated factor 4	1.55	7.08E-06	8.97E-05
HERC5	hect domain and RLD 5	2.14	7.18E-06	9.06E-05

RGS10	regulator of G-protein signaling 10	-1.22	7.35E-06	9.20E-05
TRA2A	transformer 2 α homolog (Drosophila)	-1.21	7.38E-06	9.23E-05
FGF9	fibroblast growth factor 9 (glia-activating factor)	2.01	7.52E-06	9.36E-05
PPP2R2B	protein phosphatase 2, regulatory subunit B, β	1.50	7.61E-06	9.45E-05
AP1AR	adaptor-related protein complex 1 associated regulatory protein	-1.32	7.68E-06	9.51E-05
FOLR1	folate receptor 1 (adult)	2.02	7.78E-06	9.61E-05
HIP1	huntingtin interacting protein 1	-1.21	7.83E-06	9.66E-05
NCSTN	nicastrin	1.67	7.90E-06	9.72E-05
MBOAT2	membrane bound O-acyltransferase domain containing 2	-1.49	7.98E-06	9.80E-05
MLF2	myeloid leukemia factor 2	1.21	8.57E-06	0.0001
TDRD6	tudor domain containing 6	1.42	8.65E-06	0.0001
PPP4R1	protein phosphatase 4, regulatory subunit 1	-1.31	8.67E-06	0.0001
FKBP7	FK506 binding protein 7	-1.43	8.81E-06	0.0001
GOLM1	golgi membrane protein 1	-2.06	8.96E-06	0.0001
WARS	tryptophanyl-tRNA synthetase	-1.82	9.21E-06	0.0001
GPER	G protein-coupled estrogen receptor 1	1.35	9.23E-06	0.0001
NPNT	nephronectin	2.29	9.69E-06	0.0001
SNRNP40	small nuclear ribonucleoprotein 40kDa (U5)	-1.33	9.90E-06	0.0001
GPATCH1	G patch domain containing 1	1.32	9.92E-06	0.0001
EGF	epidermal growth factor	2.96	9.96E-06	0.0001
LIPH	lipase, member H	1.71	9.98E-06	0.0001
HMOX1	heme oxygenase (decycling) 1	2.19	9.98E-06	0.0001
GATA2	GATA binding protein 2	1.55	1.03E-05	0.0001
RALBP1	ralA binding protein 1	1.22	1.05E-05	0.0001

SNCAIP	synuclein, α interacting protein	-1.28	1.07E-05	0.0001
ST8SIA6	ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase 6	1.41	1.10E-05	0.0001
NAMPT	Nicotinamide phosphoribosyltransferase	1.53	1.10E-05	0.0001
SDC4	syndecan 4	1.41	1.12E-05	0.0001
MAP2	microtubule-associated protein 2	-1.26	1.13E-05	0.0001
ARL15	ADP-ribosylation factor-like 15	-1.22	1.15E-05	0.0001
SLC16A10	solute carrier family 16, member 10 (aromatic amino acid transporter)	-1.46	1.18E-05	0.0001
HNMT	histamine N-methyltransferase	-1.26	1.19E-05	0.0001
SP1	Sp1 transcription factor	1.32	1.25E-05	0.0001
LAMB1	laminin, β 1	1.76	1.26E-05	0.0001
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	-1.24	1.29E-05	0.0001
TPM4	tropomyosin 4	-1.80	1.32E-05	0.0002
LATS1	LATS, large tumor suppressor, homolog 1 (Drosophila)	1.37	1.32E-05	0.0002
DUSP7	dual specificity phosphatase 7	1.73	1.34E-05	0.0002
SLC6A2	solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	1.44	1.38E-05	0.0002
HOXD10	homeobox D10	-1.27	1.38E-05	0.0002
BIN2	bridging integrator 2	1.85	1.39E-05	0.0002
SERPINF1	serpin peptidase inhibitor, clade F (α-2 antiplasmin, pigment epithelium derived factor), member 1	-1.42	1.43E-05	0.0002
NUP50	nucleoporin 50kDa	1.30	1.47E-05	0.0002
TSPAN2	tetraspanin 2	-1.76	1.54E-05	0.0002
PHYHIP	phytanoyl-CoA 2-hydroxylase interacting protein	3.26	1.56E-05	0.0002
KDM6B	lysine (K)-specific demethylase 6B	1.52	1.65E-05	0.0002
GPD1	glycerol-3-phosphate dehydrogenase 1 (soluble)	2.19	1.65E-05	0.0002

DOCK9	dedicator of cytokinesis 9	-1.23	1.68E-05	0.0002
PTPN6	protein tyrosine phosphatase, non-receptor type 6	1.22	1.70E-05	0.0002
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	-1.37	1.71E-05	0.0002
CFH	complement factor H	-1.82	1.71E-05	0.0002
IL20RA	interleukin 20 receptor, α	-1.28	1.72E-05	0.0002
STK17B	serine/threonine kinase 17b	-1.22	1.72E-05	0.0002
CERK	ceramide kinase	1.40	1.74E-05	0.0002
LMCD1	LIM and cysteine-rich domains 1	-1.33	1.75E-05	0.0002
TEX12	testis expressed 12	1.22	1.75E-05	0.0002
USP5	ubiquitin specific peptidase 5 (isopeptidase T)	1.40	1.75E-05	0.0002
COL12A1	collagen, type XII, α 1	-1.30	1.90E-05	0.0002
CDH6	cadherin 6, type 2, K-cadherin (fetal kidney)	-1.51	1.96E-05	0.0002
APOL5	apolipoprotein L, 5	-1.36	1.98E-05	0.0002
SLC44A3	solute carrier family 44, member 3	1.40	2.01E-05	0.0002
FAR1	fatty acyl CoA reductase 1	-1.21	2.02E-05	0.0002
TMEM156	transmembrane protein 156	-1.31	2.02E-05	0.0002
TRIB2	tribbles homolog 2 (Drosophila)	-1.21	2.08E-05	0.0002
FKBP8	FK506 binding protein 8, 38kDa	1.45	2.09E-05	0.0002
CEP192	centrosomal protein 192kDa	-1.21	2.12E-05	0.0002
ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial	-1.22	2.12E-05	0.0002
TIMP1	TIMP metallopeptidase inhibitor 1	-1.32	2.18E-05	0.0002
IFT81	intraflagellar transport 81 homolog (Chlamydomonas)	-1.26	2.25E-05	0.0002
NDE1	nudE nuclear distribution gene E homolog 1 (A. nidulans)	1.25	2.28E-05	0.0002
MCAM	melanoma cell adhesion molecule	-1.36	2.32E-05	0.0002

PIM1	pim-1 oncogene	1.25	2.50E-05	0.0003
CYR61	cysteine-rich, angiogenic inducer, 61	-2.69	2.52E-05	0.0003
YTHDC1	YTH domain containing 1	1.55	2.53E-05	0.0003
UHRF2	ubiquitin-like with PHD and ring finger domains 2	-1.38	2.54E-05	0.0003
ST3GAL5	ST3 β-galactoside α-2,3-sialyltransferase 5	-1.62	2.55E-05	0.0003
FTSJD2	FtsJ methyltransferase domain containing 2	1.74	2.60E-05	0.0003
ACSL5	acyl-CoA synthetase long-chain family member 5	-1.58	2.64E-05	0.0003
VANGL1	vang-like 1 (van gogh, Drosophila)	1.47	2.65E-05	0.0003
ICA1	islet cell autoantigen 1, 69kDa	-1.38	2.66E-05	0.0003
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	-1.23	2.67E-05	0.0003
ZNF182	Zn^{++} finger protein 182	-1.30	2.68E-05	0.0003
CXCR7	chemokine (C-X-C motif) receptor 7	-1.40	2.72E-05	0.0003
ABHD3	abhydrolase domain containing 3	-1.55	2.75E-05	0.0003
CIRBP	cold inducible RNA binding protein	-1.23	2.78E-05	0.0003
FGF13	fibroblast growth factor 13	-1.47	2.84E-05	0.0003
BMX	BMX non-receptor tyrosine kinase	-1.24	2.85E-05	0.0003
HBEGF	heparin-binding EGF-like growth factor	-1.45	2.93E-05	0.0003
CD93	CD93 molecule	-1.24	2.97E-05	0.0003
ADSS	adenylosuccinate synthase	-1.44	2.97E-05	0.0003
ACE2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	-1.98	2.99E-05	0.0003
NTN4	netrin 4	-2.13	3.00E-05	0.0003
MTHFD1	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	-1.27	3.00E-05	0.0003
ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif, 6	-1.88	3.21E-05	0.0003

EXOC5	exocyst complex component 5	-1.23	3.40E-05	0.0003
DPT	dermatopontin	-1.92	3.45E-05	0.0003
PKM2	pyruvate kinase, muscle	-1.48	3.51E-05	0.0003
ODZ3	odz, odd Oz/ten-m homolog 3 (Drosophila)	1.32	3.58E-05	0.0003
USP11	ubiquitin specific peptidase 11	1.30	3.61E-05	0.0003
ADCY4	adenylate cyclase 4	-1.25	3.63E-05	0.0003
MRC1	mannose receptor, C type 1	-1.42	3.65E-05	0.0003
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	2.71	3.73E-05	0.0003
ANXA3	annexin A3	-1.24	3.76E-05	0.0003
LAMA3	laminin, α 3	-2.16	3.78E-05	0.0003
SMOC2	SPARC related modular Ca^{++} binding 2	-1.31	3.85E-05	0.0004
CCL8	chemokine (C-C motif) ligand 8	-1.73	3.90E-05	0.0004
FBLN1	fibulin 1	1.35	3.92E-05	0.0004
ZYX	zyxin	1.33	3.94E-05	0.0004
SCMH1	sex comb on midleg homolog 1 (Drosophila)	1.57	4.16E-05	0.0004
WLS	wntless homolog (Drosophila)	-1.26	4.26E-05	0.0004
STMN1	stathmin 1	-1.35	4.30E-05	0.0004
AP1M2	adaptor-related protein complex 1, mu 2 subunit	1.38	4.36E-05	0.0004
RAB27B	RAB27B, member RAS oncogene family	2.03	4.44E-05	0.0004
WASF1	WAS protein family, member 1	-1.27	4.53E-05	0.0004
MTMR7	myotubularin related protein 7	1.63	4.56E-05	0.0004
DIO3	deiodinase, iodothyronine, type III	-1.35	4.63E-05	0.0004
TLR4	toll-like receptor 4	-1.45	4.72E-05	0.0004
ASPA	aspartoacylase	-1.25	4.77E-05	0.0004

CALCRL	calcitonin receptor-like	-1.49	4.78E-05	0.0004
RREB1	ras responsive element binding protein 1	1.37	5.00E-05	0.0004
CAPN1	calpain 1, (mu/I) large subunit	-1.67	5.10E-05	0.0004
TUBA4A	tubulin, α 4a	1.88	5.12E-05	0.0004
IL1A	interleukin 1, α	-1.87	5.32E-05	0.0005
RASSF9	Ras association (RalGDS/AF-6) domain family (N-terminal) member 9	-1.45	5.40E-05	0.0005
SLC29A1	solute carrier family 29 (nucleoside transporters), member 1	1.23	5.85E-05	0.0005
ITIH3	inter-α (globulin) inhibitor H3	-1.64	5.92E-05	0.0005
C1QTNF1	C1q and tumor necrosis factor related protein 1	3.18	6.05E-05	0.0005
PTPRE	protein tyrosine phosphatase, receptor type, E	1.84	6.08E-05	0.0005
SBSN	suprabasin	-3.41	6.24E-05	0.0005
GPRC5A	G protein-coupled receptor, family C, group 5, member A	1.56	6.48E-05	0.0005
HES6	hairy and enhancer of split 6 (Drosophila)	1.94	6.70E-05	0.0005
APBB1	amyloid β (A4) precursor protein-binding, family B, member 1 (Fe65)	1.40	6.80E-05	0.0006
ABCA5	ATP-binding cassette, sub-family A (ABC1), member 5	-1.30	6.93E-05	0.0006
LARP6	La ribonucleoprotein domain family, member 6	-1.26	6.97E-05	0.0006
CCBE1	collagen and Ca^{++} binding EGF domains 1	1.45	7.08E-05	0.0006
ARHGAP8	Rho GTPase activating protein 8	1.50	7.34E-05	0.0006
CD14	CD14 molecule	-1.33	7.35E-05	0.0006
AGTRAP	angiotensin II receptor-associated protein	1.63	7.57E-05	0.0006
GLUL	glutamate-ammonia ligase	1.53	7.66E-05	0.0006
WFS1	Wolfram syndrome 1 (wolframin)	1.46	7.89E-05	0.0006
MATN2	matrilin 2	-1.88	8.17E-05	0.0006
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	-1.36	8.18E-05	0.0006

IL6	interleukin 6 (interferon, β 2)	-2.29	8.23E-05	0.0006
ITGB4	integrin, β4	1.26	8.48E-05	0.0007
EML2	echinoderm microtubule associated protein like 2	1.54	8.65E-05	0.0007
SLC47A1	solute carrier family 47, member 1	1.44	8.72E-05	0.0007
MADD	MAP-kinase activating death domain	1.23	9.02E-05	0.0007
TGFB2	transforming growth factor, β 2	-1.66	9.19E-05	0.0007
CRYM	crystallin, mu	2.23	9.23E-05	0.0007
TMEM178	transmembrane protein 178	-2.02	9.31E-05	0.0007
HOXA5	homeobox A5	-1.26	9.39E-05	0.0007
CFB	complement factor B	-1.67	9.49E-05	0.0007
STK39	serine threonine kinase 39	1.34	9.53E-05	0.0007
CD34	CD34 molecule	-1.36	9.55E-05	0.0007
IL18	interleukin 18 (interferon-γ-inducing factor)	1.32	9.58E-05	0.0007
RBP7	retinol binding protein 7, cellular	-1.97	9.59E-05	0.0007
POSTN	periostin, osteoblast specific factor	-1.73	9.86E-05	0.0007
GSTT1	glutathione S-transferase theta 1	-1.22	0.0001	0.0008
ZNF300	Zn^{++} finger protein 300	-1.45	0.0001	0.0008
UBA1	ubiquitin-like modifier activating enzyme 1	1.24	0.0001	0.0008
PLXNA2	plexin A2	-1.34	0.0001	0.0008
NKX3-1	NK3 homeobox 1	1.43	0.0001	0.0008
FIBIN	fin bud initiation factor homolog (zebrafish)	1.31	0.0001	0.0008
FGD6	FYVE, RhoGEF and PH domain containing 6	1.26	0.0001	0.0008
KLHDC1	kelch domain containing 1	-1.24	0.0001	0.0008
TMEM100	transmembrane protein 100	-1.21	0.0001	0.0008

FBXW10	F-box and WD repeat domain containing 10	-1.73	0.0001	0.0008
SRSF7	serine/arginine-rich splicing factor 7	-1.81	0.0001	0.0008
JUN	jun proto-oncogene	-3.12	0.0001	0.0008
TRIM3	tripartite motif-containing 3	1.51	0.0001	0.0009
DLGAP1	discs, large (Drosophila) homolog-associated protein 1	1.42	0.0001	0.0009
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	-1.39	0.0001	0.0009
PARP2	poly (ADP-ribose) polymerase 2	-1.29	0.0001	0.0009
CLDN10	claudin 10	-1.88	0.0001	0.0009
BIRC3	baculoviral IAP repeat-containing 3	-1.56	0.0001	0.0009
NR4A1	nuclear receptor subfamily 4, group A, member 1	-2.17	0.0001	0.0009
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	-1.65	0.0001	0.0010
SAA1	serum amyloid A1	-1.94	0.0001	0.0010
CA4	carbonic anhydrase IV	-1.89	0.0001	0.0010
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	-1.93	0.0001	0.0010
PHLDA1	pleckstrin homology-like domain, family A, member 1	1.63	0.0001	0.0010
BFAR	bifunctional apoptosis regulator	1.25	0.0001	0.0010
ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6	-1.40	0.0002	0.0010
HSP70	heat shock protein 70	-2.18	0.0002	0.0010
ADAM10	ADAM metallopeptidase domain 10	1.27	0.0002	0.0010
FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	-1.30	0.0002	0.0010
ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	-1.23	0.0002	0.0011
NAPA	N-ethylmaleimide-sensitive factor attachment protein, α	1.50	0.0002	0.0011

APOC1	apolipoprotein C-I	-1.51	0.0002	0.0011
CXCL10	chemokine (C-X-C motif) ligand 10	-1.73	0.0002	0.0011
FEZ2	fasciculation and elongation protein zeta 2 (zygin II)	-1.26	0.0002	0.0011
FKBP4	FK506 binding protein 4, 59kDa	1.24	0.0002	0.0011
PLAUR	plasminogen activator, urokinase receptor	-1.29	0.0002	0.0011
UBL5	ubiquitin-like 5	1.29	0.0002	0.0011
IQGAP2	IQ motif containing GTPase activating protein 2	-1.22	0.0002	0.0012
ASPN	asporin	-1.45	0.0002	0.0012
VPS13D	vacuolar protein sorting 13 homolog D (S. cerevisiae)	1.46	0.0002	0.0012
TMTC1	transmembrane and tetratricopeptide repeat containing 1	-1.24	0.0002	0.0012
CCNL1	cyclin L1	-1.94	0.0002	0.0013
AVPR1A	arginine vasopressin receptor 1A	-1.27	0.0002	0.0013
COMP	cartilage oligomeric matrix protein	-2.30	0.0002	0.0013
MAP3K8	mitogen-activated protein kinase kinase kinase 8	-1.31	0.00021	0.0014
NNAT	neuronatin	-1.44	0.0002	0.0014
CASC5	cancer susceptibility candidate 5	-1.28	0.0002	0.0014
MATR3	matrin 3	-1.22	0.0002	0.0014
WNT5B	wingless-type MMTV integration site family, member 5B	1.58	0.0002	0.0014
EHMT2	euchromatic histone-lysine N-methyltransferase 2	1.45	0.0002	0.0015
SGIP1	SH3-domain GRB2-like (endophilin) interacting protein 1	-1.79	0.0003	0.0016
CHMP4C	chromatin modifying protein 4C	-1.26	0.0003	0.0016
DDX26B	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26B	-1.31	0.0003	0.0016
CPXM1	carboxypeptidase X (M14 family), member 1	2.42	0.0003	0.0016
PDGFRL	platelet-derived growth factor receptor-like	-1.21	0.0003	0.0017

NXPH2	neurexophilin 2	-1.41	0.0003	0.0017
PDE6H	phosphodiesterase 6H, cGMP-specific, cone, γ	-1.25	0.0003	0.0017
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-1.66	0.0003	0.0017
MARK2	MAP/microtubule affinity-regulating kinase 2	2.25	0.0003	0.0018
ACP5	acid phosphatase 5, tartrate resistant	-1.29	0.0003	0.0018
THBS1	thrombospondin 1	-1.40	0.0003	0.0019
CD200R1	CD200 receptor 1	-1.46	0.0003	0.0019
ATR	ataxia telangiectasia and Rad3 related	-1.26	0.0003	0.0019
EDNRA	endothelin receptor type A	-1.24	0.0003	0.0019
PDK4	pyruvate dehydrogenase kinase, isozyme 4	-1.34	0.0003	0.0020
LRRK2	leucine-rich repeat kinase 2	-1.37	0.0003	0.0020
ICAM1	intercellular adhesion molecule 1	-1.49	0.0003	0.0020
MYOM1	myomesin 1, 185kDa	-1.28	0.0003	0.0020
B3GNT4	UDP-GlcNAc:β Gal β-1,3-N-acetylglucosaminyltransferase 4	1.27	0.00032	0.0021
PRKCSH	protein kinase C substrate 80K-H	1.40	0.0004	0.0021
CTSD	cathepsin D	1.71	0.0004	0.0022
MLL3	myeloid/lymphoid or mixed-lineage leukemia 3	1.25	0.0004	0.0022
P4HA1	prolyl 4-hydroxylase, α polypeptide I	-1.22	0.0004	0.0023
SLC39A14	solute carrier family 39 (Zn^{++} transporter), member 14	-1.24	0.0004	0.0023
POLE2	polymerase (DNA directed), epsilon 2 (p59 subunit)	-1.24	0.0004	0.0023
LAPTM5	lysosomal protein transmembrane 5	-1.55	0.0004	0.0023
THBS4	thrombospondin 4	-3.42	0.0004	0.0023
GDI1	GDP dissociation inhibitor 1	1.25	0.0004	0.0023

BCAT1	branched chain amino-acid transaminase 1, cytosolic	-1.59	0.0004	0.0025
MLLT1	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 1	2.33	0.0004	0.0025
COL8A1	collagen, type VIII, α 1	-1.22	0.0005	0.0026
NRG1	neuregulin 1	1.80	0.0005	0.0026
CXCL14	chemokine (C-X-C motif) ligand 14	-1.48	0.0005	0.0027
ELOVL2	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2	-1.25	0.0005	0.0027
TMOD1	tropomodulin 1	-1.22	0.0005	0.0028
BAIAP2	BAI1-associated protein 2	1.38	0.0005	0.0029
GTSF1	gametocyte specific factor 1	1.59	0.0005	0.0029
GNA14	guanine nucleotide binding protein (G protein), α 14	1.22	0.0006	0.0031
ILDR2	immunoglobulin-like domain containing receptor 2	-1.40	0.0006	0.0031
AASS	aminoadipate-semialdehyde synthase	1.95	0.0007	0.0034
OMD	osteomodulin	-1.21	0.0007	0.0035
RAB38	RAB38, member RAS oncogene family	1.38	0.0007	0.0035
NPY	neuropeptide Y	4.00	0.0007	0.0035
SSBP2	single-stranded DNA binding protein 2	-1.38	0.0007	0.0035
KCNS3	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3	2.20	0.0007	0.0036
ANKRD45	ankyrin repeat domain 45	1.22	0.0007	0.0036
SGMS2	sphingomyelin synthase 2	1.36	0.0007	0.0036
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, β	-1.29	0.0007	0.0037
CDH13	cadherin 13, H-cadherin (heart)	-1.49	0.0007	0.0037
AKT1S1	AKT1 substrate 1 (proline-rich)	1.90	0.0007	0.0038
PNLIPRP2	pancreatic lipase-related protein 2	2.08	0.0008	0.0039

IDO1	indoleamine 2,3-dioxygenase 1	-1.98	0.0008	0.0039
GLCCI1	glucocorticoid induced transcript 1	1.94	0.0008	0.0039
KIRREL	kin of IRRE like (Drosophila)	1.24	0.0008	0.0040
EDN1	endothelin 1	-1.69	0.0008	0.0040
PAQR5	progestin and adipoQ receptor family member V	1.21	0.0008	0.0041
ZBTB16	Zn^{++} finger and BTB domain containing 16	-1.25	0.0008	0.0042
PDE9A	phosphodiesterase 9A	-1.48	0.0009	0.0044
CD44	CD44 molecule (Indian blood group)	1.23	0.0009	0.0047
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1	-1.23	0.0010	0.0048
PTBP2	polypyrimidine tract binding protein 2	-1.37	0.0010	0.0050
DUSP5	dual specificity phosphatase 5	-1.35	0.0010	0.0051
HTR1D	5-hydroxytryptamine (serotonin) receptor 1D	1.46	0.0010	0.0051
ACPL2	acid phosphatase-like 2	-1.65	0.0011	0.0052
CLPTM1	cleft lip and palate associated transmembrane protein 1	1.28	0.0011	0.0053
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	1.22	0.0011	0.0055
ACOXL	acyl-CoA oxidase-like	-1.37	0.0012	0.0057
LPHN3	latrophilin 3	-1.92	0.0012	0.0057
ITGB6	integrin, β 6	1.73	0.0012	0.0058
ISG15	ISG15 ubiquitin-like modifier	-1.46	0.0012	0.0058
HNRNPUL1	heterogeneous nuclear ribonucleoprotein U-like 1	1.31	0.0013	0.0059
COCH	coagulation factor C homolog, cochlin (Limulus polyphemus)	-1.91	0.0013	0.0061
BPIL3	bactericidal/permeability-increasing protein-like 3	2.86	0.0013	0.0062
DNAH17	dynein, axonemal, heavy chain 17	-1.36	0.0014	0.0063
TGFB3	transforming growth factor, β 3	-1.37	0.0014	0.0063

KRT7	keratin 7	1.56	0.0014	0.0065
PLAT	plasminogen activator, tissue	-1.36	0.0015	0.0069
UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	-1.45	0.0016	0.0071
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	-2.70	0.0016	0.0072
PDGFD	platelet derived growth factor D	-1.45	0.0017	0.0076
NET1	neuroepithelial cell transforming 1	1.55	0.0018	0.0081
SCCPDH	saccharopine dehydrogenase (putative)	1.29	0.0019	0.0085
ST14	suppression of tumorigenicity 14 (colon carcinoma)	1.62	0.0020	0.0088
MAOB	monoamine oxidase B	-1.31	0.0021	0.0090
PTGDR	prostaglandin D2 receptor (DP)	-1.38	0.0021	0.0091
MFAP5	microfibrillar associated protein 5	-2.15	0.0021	0.0092
PAPPA2	pappalysin 2	1.27	0.0022	0.0093
OLFML3	olfactomedin-like 3	1.34	0.0022	0.0094
MYO1E	myosin IE	1.23	0.0022	0.0095
TAF1D	TATA box binding protein (TBP)-associated factor, RNA polymerase I, D, 41kDa	-1.21	0.0023	0.0098
LAPTM4B	lysosomal protein transmembrane 4 β	-1.33	0.0023	0.0098
STOM	stomatin	-1.21	0.0026	0.0108
SPINK5	serine peptidase inhibitor, Kazal type 5	-1.68	0.0026	0.0109
GP1BA	glycoprotein Ib	-1.44	0.0030	0.0122
SMPDL3A	sphingomyelin phosphodiesterase, acid-like 3A	-1.60	0.0030	0.0123
SELE	selectin E	-1.78	0.0031	0.0125
LARP7	La ribonucleoprotein domain family, member 7	-1.38	0.0035	0.0137
ANO3	anoctamin 3	-1.79	0.0037	0.0139

RBP4	retinol binding protein 4, plasma	-1.32	0.0042	0.0160
GADD45G	growth arrest and DNA-damage-inducible, γ	-1.21	0.0043	0.0163
MAGED2	melanoma antigen family D, 2	-1.47	0.0044	0.0166
BAI3	brain-specific angiogenesis inhibitor 3	-1.32	0.0045	0.0170
CLEC1B	C-type lectin domain family 1, member B	-1.22	0.0048	0.0180
TIA1	TIA1 cytotoxic granule-associated RNA binding protein	-1.22	0.0049	0.0181
CALB1	calbindin 1, 28kDa	1.56	0.0051	0.0187
MFGE8	milk fat globule-EGF factor 8 protein	1.58	0.0052	0.0190
FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)	1.79	0.0058	0.0208
GDA	guanine deaminase	-1.30	0.0063	0.0225
MATN3	matrilin 3	2.22	0.0064	0.0226
SLC2A9	solute carrier family 2 (facilitated glucose transporter), member 9	-1.81	0.0092	0.0307
FBN2	fibrillin 2	-1.24	0.0107	0.0346
ANKRD32	ankyrin repeat domain 32	-1.26	0.0112	0.0360
FABP5	fatty acid binding protein 5 (psoriasis-associated)	1.54	0.0118	0.0375
AK3L1, LOC475238, LOC609761, LOC611161, LOC611170	adenylate kinase 3-like 1; similar to ZK84.1; similar to Nascent polypeptide-associated complex α subunit, muscle-specific form (α-NAC, muscle-specific form); hypothetical protein LOC611161; similar to CG13648-PA	1.25	0.0126	0.0396
DKK1	dickkopf homolog 1 (*Xenopus laevis*)	1.70	0.0128	0.0399
IGSF10	immunoglobulin superfamily, member 10	-1.61	0.0153	0.0463
CA2	carbonic anhydrase II	1.52	0.0171	0.0508
ULK4	unc-51-like kinase 4 (*C. elegans*)	-1.22	0.0176	0.0521

APPENDIX J: ABSTRACTS AND PRESENTATIONS

Appendix J.1 Summary

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

Appendix J.2 Accepted Abstract to the 2011 Annual Northwest Reproductive Sciences Symposium (Corvallis, OR)

Comparison of RNA Yield Using Two Commercial Kits

Elizabeth Fellows, Timothy Hazzard, Michelle Kutzler
Department of Animal Science, Oregon State University

Introduction: A wide variety of RNA isolation kits are commercially available. Several investigators have reported that the most effective method for isolating RNA utilizes TRIzol (Invitrogen, Carlsbad, CA) followed by oligo (dt) cellulose spin columns.[1,2] However, there have been no published studies comparing RNA yield and purity using only TRIzol or oligo (dt) cellulose spin columns. Therefore, we sought to compare the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) that isolates total RNA using phenol and guanidine isothiocyanate to the Fast Track 2.0 Kit that isolates mRNA using oligo (dt) cellulose. Both kits use silica based spin columns. We hypothesized that 1) the TRIzol Plus RNA Purification Kit would result in a greater amount of RNA isolated and 2) that the Fast Track 2.0 Kit would result in increased purity of the RNA sample as determined by the $A_{260/280}$ ratio.

Methods: Tissues from late gestation canine fetuses were flash frozen in liquid nitrogen. Following the Fast Track Kit's instructions, RNA was isolated from 1000 mg of lung, liver and chorioallantois. Following the TRIzol Plus RNA Purification Kit's instructions, RNA was isolated from 100 mg of each type of tissue. A nanophotometer (IMPLEN, Munich, Germany) was used to determine the A_{260} measurement, which was then used to determine RNA concentration. The RNA concentration was then used to determine the amount of isolated RNA. The A_{260}/A_{280} ratio was used to determine RNA purity.

Results: Preliminary results indicate the purity of RNA isolated from each protocol was not different, but the RNA yield was higher with the TRIzol Plus RNA Purification Kit (Table 1).

Discussion: A significant limitation of the Fast Track 2.0 Kit is that it requires ten times the amount of tissue (1000 mg versus 100 mg) compared to the TRIzol Plus RNA Purification Kit. However, the Fast Track 2.0 Kit has the advantage of yielding an enriched mRNA sample; whereas the TRIzol Plus RNA Purification Kit yields total RNA, of which approximately 1-5% is mRNA. The decision regarding what kit to use should be based on the amount of tissue available and what type of RNA is needed for the experiment.

Table 1. RNA amount (μg) and purity ($A_{260/280}$ ratio) from fetal canine tissues isolated using two commercially-available kits.

	TRIzol Plus RNA Purification Kit		FastTrack 2.0 Kit	
Tissue	Total RNA Yield (μg)	Purity	mRNA Yield (μg)	Purity
Liver	109.20	1.947	18.96	2.097
Lung	80.40	1.985	7.02	1.973
Chorioallantois	130.50	1.957	41.40	2.025

References:

[1] Honscha KU, Schirmer A, Reischauer A, Schoon HA, Einspanier A, Gabel. Expression of ABC-transport proteins in canine mammary cancer: consequences for chemotherapy. *Reprod Dom Anim.* 2009. 44 (Suppl. 2): 218-223.
[2] Wang HB. Wang QG, Zhang XY, Gu XF, Wang SB, Wu SB, Li H. Microarray analysis of genes differentially expressed in the liver of lean and fat chickens. *Animal.* 2010. 4(4): 513-522.

Appendix J.3 2011 Annual Northwest Reproductive Sciences Symposium Poster

Comparison of RNA Yield Using Two Commercial Kits

Elizabeth Fellows, Timothy Hazzard, Michelle Kutzler

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

BACKGROUND

Microarray analysis has emerged as a useful tool to examine gene expression. The ultimate goal of my graduate research is to document ontogenic changes in canine placental gene expression over gestation. Microarray analysis requires the isolation of RNA. In order to do this effectively, different methods of RNA isolation must be considered.

A wide variety of RNA isolation kits are commercially available. Several investigators have reported that the most effective method for isolating RNA utilizes TRIzol (Invitrogen, Carlsbad, CA) followed by oligo (dt) cellulose spin columns.[1,4] However, there have been no published studies comparing RNA yield and purity using only TRIzol or oligo (dt) cellulose spin columns.

Therefore, for the purposes of this study, we sought to compare the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) that isolates total RNA using phenol and guanidine isothiocyanate to the Fast Track 2.0 Kit (Invitrogen, Carlsbad, CA) that isolates mRNA using oligo (dt) cellulose. Both kits utilize silica based spin columns.

HYPOTHESES

- That the TRIzol Plus RNA Purification Kit would yield a greater amount of RNA.
- That the Fast Track 2.0 Kit would result in increased purity of the RNA sample as determined by the $A_{260/280}$ ratio.

MATERIALS AND METHODS

- Tissues from late gestation canine placentas (Figure 1) and fetuses were flash frozen in liquid nitrogen.
- Following the Fast Track Kit's instructions, RNA was isolated from 1000 mg of lung, liver and chorioallantois (Figure 2).
- Following the TRIzol Plus RNA Purification Kit's instructions, RNA was isolated from 100 mg of each type of tissue (Figure 3).
- A nanophotometer (IMPLEN, Munich, Germany) was used to determine the A_{260} measurement, which was then used to calculate RNA concentration.
- The RNA concentration was then used to determine the amount of isolated RNA.
- The A_{260}/A_{280} ratio was used to determine RNA purity.

Figure 1. Canine fetus and placenta near term. Chor = chorioallantois, All = allantois, Am = amnion.

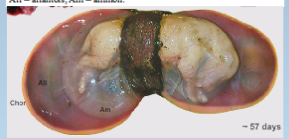

Figure 2. Fast Track 2.0 Kit (Invitrogen) procedure Flow Chart

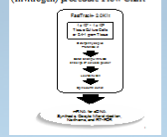

Figure 3. TRIzol Plus RNA Purification Kit (Invitrogen) Procedure Flow Chart

Table 1. RNA amount (µg) and purity ($A_{260/280}$ ratio) from fetal canine tissues isolated using two commercially-available kits.

	TRIzol Plus RNA Purification Kit		FastTrack 2.0 Kit	
Tissue	Total RNA Yield (µg)	Purity	mRNA Yield (µg)	Purity
Liver	109.20	1.947	18.96	2.097
Lung	80.40	1.985	7.02	1.973
Chorioallantois	130.50	1.957	41.40	2.025

RESULTS

Preliminary results indicate the purity of RNA isolated from each protocol was not different, but the RNA yield was higher with the TRIzol Plus RNA Purification Kit (Table 1). The chorioallantois produced the highest RNA yield using both kits compared to the other tissue samples.

DISCUSSION

A significant limitation of the Fast Track 2.0 Kit is that it requires ten times the amount of tissue (1000 mg versus 100 mg) compared to the TRIzol Plus RNA Purification Kit. However, the Fast Track 2.0 Kit has the advantage of yielding an enriched mRNA sample; whereas the TRIzol Plus RNA Purification Kit yields total RNA, of which approximately 1-5% is mRNA.

The decision regarding what kit to use should be based on the amount of tissue available and what type of RNA is needed for the experiment.

Based on the results of this study, I plan on using the TRIzol Plus RNA Purification kit for future RNA isolations. The TRIzol Plus RNA Purification kit requires less tissue, takes less time to perform and provides adequate RNA for my research purposes.

FUTURE STUDIES

In order to properly carry out further study of isolated RNA, the integrity of RNA must be determined. Integrity of RNA can be determined by the RNA integrity number (RIN), which can be measured using the Agilent 2100 Bioanalyzer[2].

Microarray analysis using Affymetrix GeneChip Canine 2.0 Array will be used on canine placental samples from different gestation ages to identify candidate genes that show similar ontogeny to other species, namely human.

These analyses can be done at the Center for Genome and Biocomputing Research at Oregon State University

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[2] Schroeder A, Mueller O, Stocker S, Salowsky R., Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol. 2006. 7:3 doi:10.1186/1471-2199-7-3
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Appendix J.4 Presented at the 2012 Annual Northwest Reproductive Sciences Symposium (Beaverton, OR) and the 7th International Symposium on Canine and Feline Reproduction (Whistler, BC, Canada)

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in pre-term, pre-labor and parturient canine placentas.

Elizabeth J. Fellows, Timothy M. Hazzard, Michelle A. Kutzler
Department of Animal Science, Oregon State University, Corvallis, OR USA 97330
fellowel@onid.orst.edu

INTRODUCTION: Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are postulated to be involved in trophoblast differentiation, migration and invasion during primate and ruminant placentation (1-3, 5). It has also been proposed that MMPs may contribute to rentention of placental membranes in the cow (3, 5). Previous studies have reported MMP-2 and MMP-9 expression in the canine uterus and pre-implantation embryo (4, 6), but no studies have examined MMP expression in the canine placenta. Abnormal expression of these factors at the end of canine gestation may contribute to post-parturient trophoblast persistence as in the case of subinvolution of placental sites (SIPS). The expression of TIMP-2 has not been described in the canine reproductive tract. Therefore, the aim of this study was to examine mRNA expression of MMP-2, MMP-9 and TIMP-2 in canine chorioallantois tissue at pre-term, pre-labor and during parturition. The hypothesis was that expression of MMP-2, MMP-9 and TIMP-2 would be lower in parturient canine chorioallantois tissues compared to pre-labor and pre-term tissues.

METHODS: Following ovariohysterectomy at 61±1 days past the LH surge (pre-term; n=4), chorioallantois tissue was collected without the marginal hematoma and flash frozen in liquid nitrogen. Chorioallantois tissue was collected in the same manner following elective C-section at 64±1 days past the LH surge prior to first stage labor (pre-labor; n=3) and following natural delivery (parturient; n=3). Total RNA was isolated using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Quantitative RT-PCR was performed using primers and probes developed for canine MMP-2, MMP-9 and TIMP-2 (Applied Biosystems, Carlsbad, CA). Gene expression was normalized to 18S rRNA expression and the first parturient sample collected was used as a calibrator. Relative expression was calculated using the relative quantitation ($2^{-\Delta\Delta Ct}$) method. Statisical analysis was performed using a one-way ANOVA with Bonferroni corrections using Stata 12.0 software (StataCorp LP, College Station, TX). Significance was defined as $p<0.05$.

RESULTS: MMP-9 and MMP-2 expression was higher in pre-labor samples compared to pre-term and parturient samples (Figure 1). There was no significant difference between TIMP-2 expression among groups.

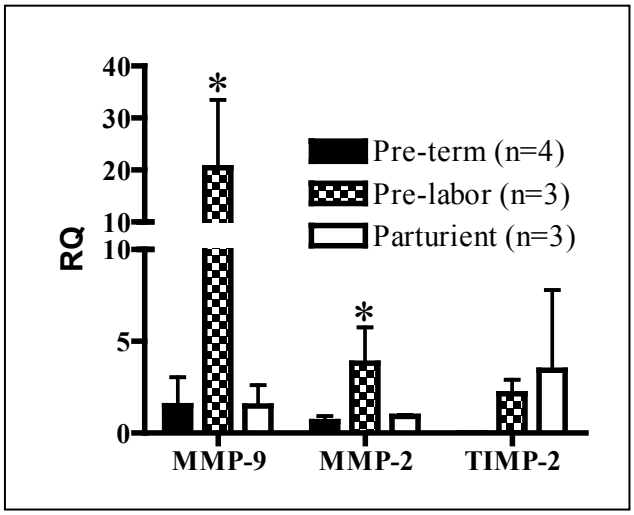

Figure 1. Relative Quantitation (RQ) of MMP-9, MMP-2 and TIMP-2 in pre-term, pre-labor and parturient samples. MMP-9 and MMP-2 expression was higher in pre-labor samples compared to pre-term and parturient samples. *$p<0.05$

CONCLUSION: This is the first investigation to report MMP-2, MMP-9 and TIMP-2 expression in pre-term, pre-labor and parturient canine chorioallantois. The mRNA expression of MMP-2 and MMP-9 may related to the onset of labor. This differs from the human where MMP-2 and MMP-9 mRNA expression gradually decrease with approaching parturition (1). TIMP-2 mRNA expression appears to be constitutive at the end of gestation. This is similar to what has been reported in cattle (3).

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Appendix J.5 2012 Annual Northwest Reproductive Sciences Symposium Poster

Gene Expression in Pre-term, Pre-labor and Parturient Canine Placenta

Elizabeth J. Fellows, Timothy M. Hazzard, Michelle A. Kutzler
Department of Animal Sciences, Oregon State University, Corvallis, Oregon

BACKGROUND

- Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are involved in trophoblast migration and invasion in primate and ruminants (1-2).
- In humans, MMP-2 and MMP-9 mRNA expression gradually decreases with approaching parturition (1).
- In cattle, TIMP-2 mRNA expression appears to be constitutive at the end of gestation (2).
- Vascular endothelial growth factor A (VEGF-A) and its receptor Flt-1 have been studied extensively in primate placentas, where they have been proposed to play a role in trophoblast invasion (3).
- In addition, placental Flt-1 expression has been shown to increase at term in humans (4).
- Previous studies have reported MMP-2 and MMP-9 expression in the canine uterus and pre-implantation embryo (5), but no studies have examined MMP expression in the canine placenta.
- The expression of TIMP-2 has not been described in the canine reproductive tract.
- In addition, no studies have evaluated the expression of VEGF-A and Flt-1 in the canine placenta around the time of parturition.
- Abnormal expression of these factors at the end of canine gestation may contribute to placental retention or subinvolution of placental sites (SIPS).

OBJECTIVE

- The objective of this study was to examine mRNA expression of MMP-2, MMP-9, TIMP-2, VEGF-A and Flt-1 in canine chorioallantois tissue at pre-term, pre-labor and during parturition.

HYPOTHESIS

- The hypothesis was that expression of MMP-2, MMP-9 ,TIMP-2, VEGF-A and Flt-1 mRNA expression would be lower in parturient canine chorioallantois tissues compared to pre-labor and pre-term tissues.

MATERIALS AND METHODS

- Following ovariohysterectomy at 61±1 days past the LH surge (pre-term; n=4), chorioallantois tissue was collected without the marginal hematoma and flash frozen in liquid nitrogen.
- Chorioallantois tissue was collected in the same manner following elective C-section at 64±1 days past the LH surge prior to first stage labor (pre-labor; n=3) and following natural delivery (parturient; n=3).
- Total RNA was isolated using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.
- Quantitative RT-PCR was performed using primers and probes developed for canine MMP-2, MMP-9, TIMP-2, VEGF-A, and Flt-1 (Applied Biosystems, Carlsbad, CA).
- Relative expression was calculated using the relative quantitation ($2^{-\Delta\Delta Ct}$) method.
- Statistical analyses were performed with a Student's *t* test to make comparisons between groups using Excel (Microsoft, Redmond, WA) and a one-way ANOVA with Bonferroni corrections to make comparisons among groups using Stata 12.0 software (StataCorp LP, College Station, TX).
- Significance was defined as $p<0.05$.

RESULTS

- MMP-9 and MMP-2 mRNA expression was higher in pre-labor samples compared to pre-term and parturient samples (**Figure 1**).
- There was no significant difference between TIMP-2 mRNA expression among groups.
- Flt-1 mRNA expression was higher in pre-labor and parturient placentas when compared to pre-term placentas.
- However, there were no differences between groups in VEGF-A mRNA expression.

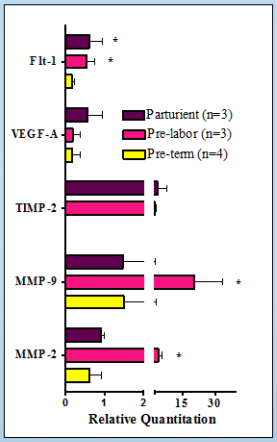

Figure 1. Relative gene expression of MMP-9, MMP-2, TIMP-2, VEGF-A and Flt-1 between the three sample groups. *p<0.05

DISCUSSION

- This is the first report of MMP-2, MMP-9, TIMP-2 VEGF-A, and Flt-1 expression in pre-term, pre-labor and parturient canine chorioallantois.
- The mRNA expression of MMP-2 and MMP-9 may be related to the onset of labor.
- In dogs, MMP-9 and MMP-2 mRNA expression was higher in pre-labor samples compared to pre-term and parturient samples, which differs from the human where MMP-2 and MMP-9 mRNA expression gradually decrease with approaching parturition (1).
- Similar to what has been reported in cattle (2), canine TIMP-2 mRNA expression does not appear to have a distinct pattern of expression.
- Like TIMP-2, VEGF-A mRNA is constitutively expressed from late gestation through parturition in the dog.
- However, its receptor, Flt-1, increases in the canine placenta at the time of parturition, similar to what has been reported in humans (3).
- Flt-1 has been reported to be directly up-regulated by hypoxic conditions (4), so Flt-1 may up-regulated following parturition in the dog as a result of the hypoxic conditions that occur during whelping.

FUTURE STUDIES

- Placental abnormalities in the dog are not well understood.
- It has been proposed that MMPs may contribute to retention of placental membranes in the cow (2) and further research is needed to see if this is also true with dogs.
- In addition, studies of the expression of these factors in bitches diagnosed with SIPs will help elucidate a role for these factors in this condition.
- Molecular changes in the canine placenta throughout gestation have not previously been described.
- Therefore, future studies will involve comparing late gestation with earlier gestational time points.

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ACKNOWLEDGEMENTS

- Dr. Cheryl Lopate & Wilsonville Veterinary Clinic
- Heartland Humane Society
- Charlie Dubois & Vivian Cullen, ABI

Appendix J.6 2012 7th International Symposium on Canine and Feline Reproduction Presentation

Gene Expression in Pre-term, Pre-labor & Parturient Canine Placenta

Elizabeth Fellows

Department of Animal & Rangeland Sciences
Oregon State University
7th International Symposium on Canine & Feline Reproduction
Whistler, British Columbia, Canada July 2012

Background

- Completion of parturition involves delivery of the placenta
- Complications arise when the placenta is not delivered within a particular time-frame
- Two common placental abnormalities in the dog are placental retention & subinvolution of placental sites (SIPs)

Molecular Factors & Parturition

- Many molecular factors are involved in trophoblast invasion as well as placental release during parturition
  - Matrix metalloproteinases (MMPs)
  - Tissue inhibitors of matrix metalloproteinases (TIMPs)
  - Vascular endothelial growth factor (VEGF)-A & VEGF-A receptor (Flt-1)
  - Kisspeptin & its receptor (KiSS-1R)

Matrix Metalloproteinases (MMPs) & their Tissue Inhibitors (TIMPs)

- MMPs & TIMPs
  - Regulate normal trophoblast invasion & migration in humans
    - MMP-2 & MMP-9 are likely responsible for conditions of abnormal placentation in humans
  - Play a role at the time of parturition in humans, cows & rodents
    - MMP-2 & MMP-9 play a role in placental retention in cattle

Lei et al. 1996; Maj & Kankofer 1997; Librach et al. 1999: Xu et al. 2002; Zhang et al. 2011

KiSS-1R

- Plays a role in the regulation of trophoblast invasion in humans
  - Shown to inhibit trophoblast invasion *in vitro* presumably by inhibition of MMP activity
- Shown previously to increase at labor in humans

Torricelli et al. 2008; Zhang et al. 2011

VEGF-A & Flt-1

- Flt-1 is the receptor for VEGF in the human placenta
  - VEGF-A acts in the initiation of trophoblast invasion & motility

Shore et al. 1997; Lash et al. 1999

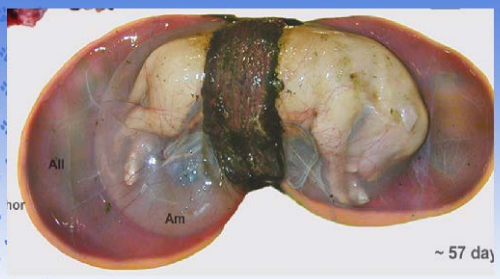
Objective

- Determine whether MMP-2, MMP-9, TIMP-2, VEGF-A, Flt-1 & KiSS-1R are expressed in the canine placenta
- Compare expression patterns during late gestation in the bitch, around the initiation of labor & after delivery

Methods-Sample Collection

- Pre-term (n=4) chorioallantois tissue was collected following ovariohysterectomy at 61±1 day past the LH surge
- Pre-labor (n=4) chorioallantois tissue was collected following elective C-section at 64±1 day past the LH surge
- Parturient (n=3) chorioallantois tissue was collected following vaginal delivery

Methods



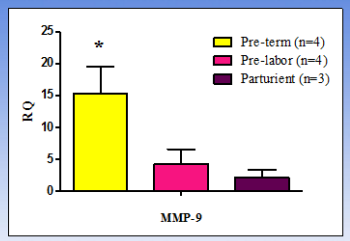
Methods

- Tissue was flash frozen in LN2 & stored at -80°C until processing
- Total RNA was isolated using the TRIzol Plus RNA Purification Kit (Invitrogen)
  - RNA integrity was confirmed with a 2100 Agilent Bioanalyzer
- Real-Time RT-PCR was performed using TaqMan primers & probes (Applied Biosystems) developed for dogs

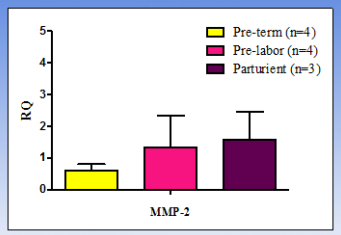
Methods

- Gene expression was normalized to HPRT-1 mRNA expression
  - 1st parturient sample collected was used as a calibrator
- Relative quantitation (RQ) or $2^{-\Delta\Delta Ct}$ method
- Statistical analysis was performed using a one-way ANOVA with Bonferroni corrections

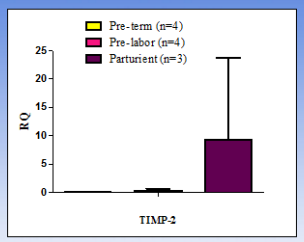
Results: MMP-9



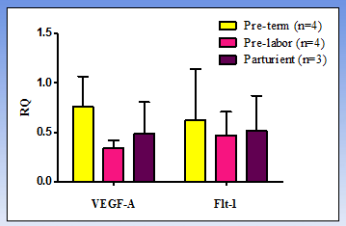
Results: MMP-2



Results: TIMP-2

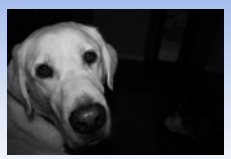


Results: VEGF-A & Flt-1



Results: KiSS-1R

- No expression of KiSS-1R was detected in the tissues studied
  - Based off of Ct values of greater than 38



Discussion

- Degradation of the extracellular matrix is crucial for successful placental separation in the cow, rat & human
- Gene expression of MMP-9 mRNA may be related to the onset of labor in the dog
  - MMP-9 has been reported to increase with the onset of labor in humans

Lei et al. 1996; Walter & Boos 2001; Xu et al. 2002

Discussion

- No significant difference in MMP-2 mRNA expression
  - Slight apparent increase with the onset of labor
- No significant difference in TIMP-2 mRNA expression
  - Differs from that reported in the cow where a decrease in TIMP-2 is proposed to be essential in placental separation

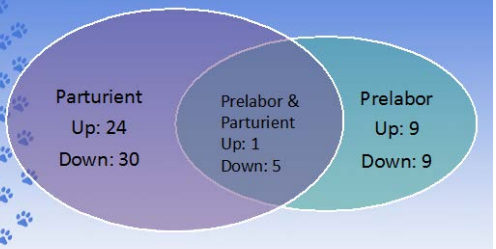
Walter & Boos, 2001

Discussion

- No significant difference in VEGF-A or Flt-1
  - A slight apparent decrease in VEGF-A at the onset of labor
  - Flt-1 has been shown to increase at labor in humans
    - The difference in expression between dogs & humans may be explained by the differences in placental type

Lee et al. 2010

Current Studies: Microarray



Acknowledgements

- Dr. Michelle Kutzler
- Dr. Tim Hazzard
- Members of the Theriogenology Lab
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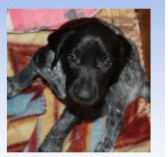


Appendix J.7 Presented at the 2012 Annual Conference for the Society for Theriogenology (Baltimore, MD)

Expression of vascular endothelial growth factor A and its receptor Flt-1 in canine placenta

Elizabeth J. Fellows, Timothy M. Hazzard, Michelle A. Kutzler
Department of Animal Science, Oregon State University, Corvallis, OR USA 97330

INTRODUCTION: Vascular endothelial growth factor A (VEGFA) and its receptor Flt-1 have been studied extensively in primate placentas, where they have been proposed to play a role in trophoblast invasion (1). In addition, placental Flt-1 mRNA expression has been shown to increase at term in humans (2). No studies have been performed on the expression of these factors in the canine placenta at the time of parturition. Dysregulation of these factors could, for example, contribute to abnormal post-partum involution of the placental sites (subinvolution of placental sites). Therefore, the aim of this study was to examine VEGFA and Flt-1 mRNA expression in canine chorio-allantoic tissue at pre-term, pre-labor and during parturition. The hypothesis was that expression of VEGFA and Flt-1 will be greater in term (pre-labor or parturient) than in pre-term bitches.
METHODS: Following ovariohysterectomy at 61±1 days past the LH surge (pre-term; n=4), chorio-allantoic tissue was collected without the marginal hematoma and flash frozen in LN2. Chorio-allantoic tissue was collected in the same manner following elective Caesarean section at 64±1 days past the LH surge prior to first stage labor (pre-labor; n=3) and following natural delivery (parturient; n=3). Total RNA was isolated using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Quantitative RT-PCR was performed using primers and probes developed for canine VEGFA and Flt-1 (Applied Biosystems, Carlsbad, CA). Gene expression was normalized to 18S rRNA expression and the first parturient sample collected was used as a calibrator. Relative expression was calculated using the relative quantitation ($2^{-\Delta\Delta Ct}$) method. Statistical analysis was performed using a Student's *t* test in Excel (Microsoft, Redmond, WA). Significance was defined as $p<0.05$.
RESULTS: Flt-1 expression was higher in tissues collected from pre-labor and parturient bitches than in tissues from pre-term bitches. However, there were no differences between groups in VEGFA expression.
CONCLUSION: Based on these results, VEGFA mRNA is constitutively expressed during late gestation and parturition in the dog. Its receptor, Flt-1 increases in the canine chorio-allantois at the time of parturition, similar to what has been reported in primates (2). Flt-1 has been reported to be directly up-regulated by hypoxic conditions (2), suggesting that placental hypoxia associated with parturition may lead to the observed up-regulation of Flt-1 during whelping.
REFERENCES:

1. Sgambati E, Marini M, Zappoli Thyrion GD, et al. VEGF expression in the placenta from pregnancies complicated by hypertensive disorders. *BJOG.* 2004; 111: 564-570.
2. Lee KJ, Shim SH, Kang KM, et al. Global gene expression changes induced in the human placenta during labor. *Placenta.* 2010; 31(8): 698-704.

Appendix J.8. Presentation for the 2012 Annual Conference for the Society for Theriogenology (Baltimore, MD)

Gene Expression in the Peripartum Canine Placenta

Elizabeth Fellows

Department of Animal & Rangeland Sciences
Oregon State University

Background

- Completion of parturition involves delivery of the placenta
- Complications arise when the placenta is not delivered within a particular timeframe
- Two common placental abnormalities in the dog are placental retention & subinvolution of placental sites

Placental Release

- Many molecular factors are involved in trophoblast invasion as well as tissue remodeling during placental release
  - Matrix metalloproteinases (MMPs)
  - Tissue inhibitors of matrix metalloproteinases (TIMPs)
  - Vascular endothelial growth factor (VEGF)-A
  - VEGF-A receptor (Flt-1)

Matrix Metalloproteinases (MMPs)

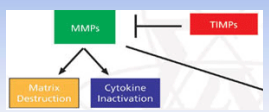
- Regulate normal trophoblast invasion & migration in humans
  - MMP-2 & MMP-9 are likely responsible for conditions of abnormal placentation in humans
- Play a role at the time of parturition in humans, cows & rodents
  - MMP-2 & MMP-9 play a role in placental retention in cattle

Lei et al. 1996; Maj & Kankofer 1997; Librach et al. 1999: Xu et al. 2002; Zhang et al. 2011

Tissue Inhibitors of Metalloproteinases (TIMPs)

- TIMPs
  - Inhibit MMP activity
  - A decrease in TIMP-2 in bovine placenta is important for placental release at parturition

Walter and Boos, 2001

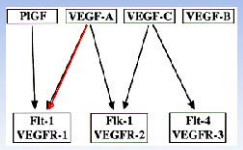


pathway-slides-and/the-mmp-cytokine-connection.html

VEGF-A & Flt-1

- Flt-1 is the receptor for VEGF-A in the human placenta
  - VEGF-A acts in the initiation of trophoblast invasion & motility

Shore et al. 1997; Lash et al. 1999



h.tp://www.bioscience.org/1998/v3/e/isner/3.htm

Hypotheses

- Hypothesis 1:
  - Expression of MMP-9, MMP-2, VEGF-A & Flt-1 mRNA would be higher following natural delivery compared to pre-term & pre-labor groups, whereas TIMP-2 expression would be lower in parturient tissues
- Hypothesis 2:
  - Global gene expression changes in the peripartum canine placenta would demonstrate changes in expression of genes involved in placental release in other species

Objectives

- Compare expression patterns of MMP-2, MMP-9, TIMP-2, VEGF-A & Flt-1 in the peripartum canine placenta using real-time RT-PCR
- Compare global gene expression patterns in the peripartum canine placenta using microarray & identify candidate genes for future studies

Methods

- Sample collection
  - Pre-term (n=4) chorioallantois tissue was collected following ovariohysterectomy at 61±1 day past the LH surge
  - Pre-labor (n=4) chorioallantois tissue was collected following elective C-section at 64±1 day past the LH surge
  - Parturient (n=3) chorioallantois tissue was collected following vaginal delivery

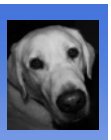
Methods

- Sample collection
  - Tissue was flash frozen in LN2 & stored at -80°C until processing
  - Total RNA was isolated using the TRIzol Plus RNA Purification Kit (Invitrogen)
  - RNA integrity was confirmed with a 2100 Agilent Bioanalyzer

Methods: Hypothesis 1

- Real-time RT-PCR was performed using TaqMan primers & probes (Applied Biosystems) developed for dogs
- Gene expression was normalized to HPRT-1 mRNA expression
  - 1st parturient sample collected was used as a calibrator

Methods: Hypothesis 2



- RNA was hybridized to the Canine 2.0 Array (Affymetrix)
- Pre-processing & normalization of the microarray data using robust multichip average (RMA) in Bioconductor Software using limma & affy packages

Irizarry et al., 2003

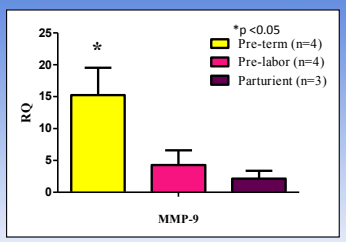
Statistical Analysis

- Real-time RT-PCR
  - Relative quantitation (**RQ**) or $2^{-\Delta\Delta Ct}$ method
  - One-way ANOVA with Bonferroni corrections
- Microarray analysis
  - Bioconductor software with the limma & affy packages in the R statistical program using a false discovery rate (FDR) adjusted p value ≤ 0.1 with a log fold change ≥ 1.2

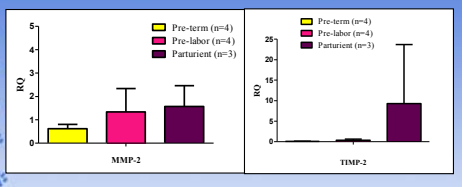
Smyth, 2004, Sitras et al., 2009; Hoegh et al., 2010; Lian et al., 2010

- Significance defined as $p \leq 0.05$

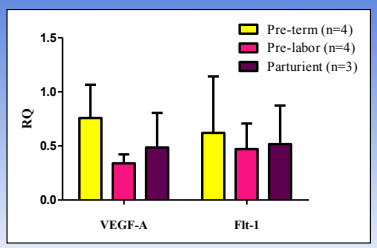
Hypothesis 1: Results



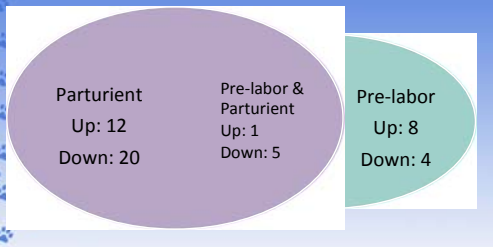
Hypothesis 1: Results



Hypothesis 1: Results



Hypothesis 2: Results



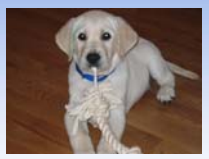
Discussion

- Gene expression of MMP-9 mRNA may be related to the onset of labor in the dog
  - Degradation of the extracellular matrix is crucial for successful placental separation in the cow, rat & human
  - MMP-9 has been reported to increase with the onset of labor in humans

Lei et al. 1996; Walter & Boos 2001; Xu et al. 2002

Discussion

- First report describing the canine placental transcriptome using microarray
  - Identified several candidate genes that warrant further study
    - CD44
    - DAG-1
    - MMP-1
    - CXCL10
    - IL-6



Acknowledgements

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- Dr. Gerd Bobe
- Dr. Yanming Di
- Anne-Marie Girard