AN ABSTRACT OF THE THESIS OF

Ruben Mendoza for the degree of Master of Science in Animal Science presented on June 12, 2013.

Title: Identification of the Plasminogen Activator Inhibitor Produced by the Day 14 Bovine Embryo

Approved:

---

Alfred R. Menino, Jr.

Day 12-14 bovine embryos produce urokinase-type plasminogen activator (uPA) and an uPA-plasminogen activator inhibitor (PAI) complex. The type of PAI, -1 or -2, in the complex has not been determined therefore the objective of this research was to identify the PAI produced by the Day 14 bovine embryo. In experiment 1, Western blot analysis was used to determine the presence of PAI-1 and/or PAI-2 in the Day 14 bovine embryo. In experiment 2, a caseinolytic-agar gel immunoneutralization assay was used to determine the concentration of PA in pooled Day 14 embryo-conditioned medium incubated with primary antibodies directed against PAI-1 or PAI-2 previously used in the Western blotting. Western blot analysis detected two PAI-1

bands in embryos (61 and 76 kD) and only the high molecular mass form in conditioned medium (75 kD). Likewise, Western blotting detected two PAI-2 bands in embryos (52 and 66 kD) and a high molecular mass form in conditioned medium (61 kD). In the immunoneutralization assay, PA activity was greater ($P< 0.05$) in conditioned medium following treatment with anti-PAI-1 or anti-PAI-2 compared to treatment with non-specific immunoglobulins. These data suggest Day 14 bovine embryos produce both PAI-1 and PAI-2.

# IDENTIFICATION OF THE PLASMINOGEN ACTIVATOR INHIBITOR PRODUCED BY THE DAY 14 BOVINE EMBRYO

by

Ruben Mendoza

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Presented June 12, 2013

Commencement June 2014

Master of Science thesis of Ruben Mendoza presented June 12, 2013.

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.

---

Ruben Mendoza, Author

ACKNOWLEDGMENTS

The amount of people that need to be thanked for the completion of this thesis and my M.S. degree is monumental. I would first like to thank my major professor, Dr. Alfred Menino, Jr. His guidance, friendship, advice, professionalism, great patience, and comical relief made this experience enjoyable beyond words! I am very thankful of my committee members, Drs. Jim Males, Barbara Taylor, and Phillip Brownell for taking the time to review this thesis and to partake in my defense.

A big thank you to my lab colleagues; the always dangerous and famous "Team Repro": Adrienne, Jenny, Katie, Keely, and Nicole. Without you, the lab just wouldn't be the same and fun as I showed you guys the latest in YouTube video humor. Also, thank you for all your help with flushing cows, injections, among many other things. Your friendships will always be something very special to me and I am very thankful for them. A sincere thank you goes to all of the staff and other grad students in the department of Animal and Rangeland Sciences who made this experience enjoyable.

I would also like to thank God for guiding me in this direction and for allowing me to grow as an individual through this experience. Nothing would ever be possible without him.

To my parents who have always believed in me no matter how crazy the task at hand may seem. They have always been a very strong foundation and support system for my siblings and I. None of this would be possible if it wasn't for them and the sacrifices and pain they have endured to give us a better life. When we came to

the U.S. from Mexico, I wanted to turn right around because I thought a snow covered Boardman seemed more like the purgatory than a powdered winter wonderland. However, they made the best of it and taught me how to always make the best of a lousy situation because the outcome will be worth it if the direction is right and you know how to fight. With phrases like "echale ganas" and "dios te bendiga," they have always been there for me.

To my grandfather, "Buelo," for instilling the passion for animals, especially cattle, in me at a very young age. The first cow that I ever called my own was a gift from him when I was only three years old and that cow went on seventeen years without skipping a calving season. Memories of him make me proud of where I came from and where I am going with my passion for cattle. To my uncle Artemio, who really demonstrated the value of a good education by completing a Doctoral degree at New Mexico State University in Las Cruses, NM. His legacy and kind personality will always be in my heart. To all of my family back in Mexico and those who have found a new home here in the U.S. thank you for being such hardworking people and blazing the way so that I could accomplish my education.

I would like to extend my sincere gratitude to Dr. Dale Weber for his friendship, guidance, good life discussions, dinners, and occasional yard work at his house. I may very well say that the yard work provided me with a good outlet to clear my mind from school and other things. It's good to never forget the value of physical labor.

A very warm thank you to Kent and Nancy Searles for their friendship and financial support during this chapter in my life. Crabbing on the Oregon coast adds to some great experiences in life. To Dr. Greg Garcia "Doc" and family for taking me into to their home and teaching me more about cattle reproduction, life values, and how it's always the little things that matter. To Dave Boor and the pirate wrestling club for always committing to excellence and teaching the value of hard work as well as being a good citizen.

Last, but not least I would like to thank all of the good people that I have had the privilege of meeting here at Oregon State University. To all the folks at the C.A.M.P and E.O.P programs for all of their friendships, financial, and academic support.

As I begin the next chapter in my life, I go with greatly acquired knowledge, hopes, and dreams. I am very thankful for all of the friendships I have made here at Oregon State University, including meeting my girlfriend and partner in crime, Marisol Cardoza. I look forward to making new great memories in different places of the world and enjoying every second of it.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

Page

LIST OF FIGURES

LIST OF TABLES

# Introduction

Plasminogen activators (PA) are serine proteases that convert the extracellular proenzyme plasminogen into the active serine enzyme plasmin (Danø et al., 1985). PA are associated with a variety of physiological processes including fibrinolysis, ovulation, embryo implantation, and tumor invasion (Yi-xun et al., 2004). There are two major forms of PA; urokinase-type PA (uPA) and tissue-type PA (tPA). Tissue-type PA (70 kD) is the principal PA involved in clot lysis whereas uPA (50 kD) is involved in cell migration. Embryonic production of PA has been identified in a variety of species, including rodents (Strickland et al., 1976), swine (Fazlebas et al., 1983), cattle (Menino and Williams, 1987), and sheep (Bartlett and Menino, 1993). In cattle embryos, PA production has been observed during blastoceolic expansion and hatching (Menino and Williams, 1987), and is positively correlated with embryonic size, developmental stage and cell number (Kaaekuahiwi and Menino, 1990). Berg and Menino (1992) suggested that Day 12-14 (where Day 0= onset of estrus) bovine embryos secrete uPA.

Cattle embryos may also produce PA inhibitors (PAI) that appear by Days 12-14 of embryonic development (Dyk and Menino, 1991). There are two principal PAI; PAI-1 and PAI-2. PAI-1 (50 kD) is the principal PAI present in plasma and has a higher affinity for tPA (Egbert et al., 1995). PAI-1 is often found associated with the extracellular matrix (ECM) protein vitronectin, which stabilizes it in an active conformation. In humans, PAI-2 is produced by the placenta and plasma levels increase as gestation progresses (Astedt et al., 1998). PAI-2 is also synthesized by monocytic cell lines under conditions promoting cellular differentiation. PAI-2 occurs

in two molecular forms: low (47 kD) and high (60 kD) molecular weight PAI-2 (LMW and HMW, respectively) (Egbert et al., 1995). LMW PAI-2 is intracellular and non-glycosylated and HMW PAI-2 is secreted and glycosylated. PAI-2 also has a greater affinity for uPA than for tPA (Egbert et al., 1995). Dyk and Menino (1991) observed two plasminogen-dependent caseinolytic proteases in zymographs of Day 12-14 bovine embryos and conditioned medium with molecular masses of 41.5-47.0 kD and 86.1-92.2 kD. Based on size, the lower and higher molecular mass forms were considered as an uPA and either a tPA or PA-PAI complex, respectively. Berg and Menino (1992) confirmed the presence of uPA in Day 12-14 bovine embryos using antibodies and amiloride, a specific inhibitor of uPA. In fact, all PA activity in the Day 12-14 bovine embryo was amiloride-sensitive suggesting the high molecular mass form observed by Dyk and Menino (1991) was indeed an uPA-PAI complex. The identity of the bovine PAI remained undetermined. Bartlet and Menino (1993) conducted a similar series of experiments with ovine embryos and also observed low and high molecular mass forms of plasminogen-dependent caseinolytic activity in zymographs of Day 7, 9, and 11embryos and conditioned medium. Likewise, all PA activity during this period of development was amiloride-sensitive and due to uPA. Bartlet and Menino (1993) also observed changes in PA activity when conditioned medium was treated with a panel of antibodies in an immunoneutralization assay; specifically, an increase in PA activity when conditioned medium was treated with a PAI-2 antibody. These data suggest the presence of PAI-2 in ovine embryo conditioned medium and the likelihood of an uPA-PAI-2 complex constituting the

plasminogen-dependent caseinolytic higher molecular mass form observed by zymography.

The presence of PAI-2 in ovine and bovine embryos during this period of development would be somewhat similar to the human where PAI-2 is detected during pregnancy (Astedt et al., 1998). In humans, placental production of PAI-2 is so great it is easily quantified in the plasma. In fact, PAI-2 could be used for pregnancy diagnosis in humans but the earlier appearance and greater abundance of human chorionic gonadotropin (hCG) has made the latter the more applicable tool. However, in cattle, a diagnostic method for early pregnancy detection with precision and accuracy similar to hCG in humans has not been identified. Identification of such a method would have significant application to beef and dairy cattle production by providing an easy to use kit, similar to over-the counter hCG kits used in human pregnancy detection. If PAI-2 is indeed produced by bovine embryos, the concentration would need to be high enough to be detected in the plasma in order for developing any application towards pregnancy diagnosis. Therefore, the objective of this research was to identify the PAI produced by the Day 14 bovine embryo.

# Review of the Literature

## The Plasminogen/Plasmin System

The successful activation of the fibrinolytic system is dependent upon the conversion of the plasma zymogen, plasminogen, to the serine protease plasmin (Castellino and Ploplis, 2005). A variety of enzymatic cascades are involved in achieving extracellular matrix (ECM) degradation for a variety of cells and tissues within an organism. The primary *in vivo* function of plasmin is to regulate vascular potency by degrading fibrin-containing thrombi. Plasmin is responsible for a number of physiological processes including, but not limited to degradation of protein barriers, and therefore mediating cell migration such as in embryogenesis, wound healing, angiogenisis, trophoblast implantation, ovulation and fetal development. It is also involved in pathological processes, including tumor growth, metastasis dissemination, acute and chronic inflammation, preeclampsia, and intrauterine growth retardation (Egbert et al., 1995). To complete this process there are intermediate proteins that play a role in making the conversion. Plasminogen activators (PA), including tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are among these proteins. There are also four arginine-specific serine protease inhibitors (arg-serpins) that are of particular relevance to the PA-plasmin system (Vassalli et al., 1991).

The liver is the primary organ that synthesizes plasminogen (Castellino and Ploplis, 2005). However, other organs are also sources of plasminogen. These organs include the kidneys, brain, testis, heart, lungs, uterus, spleen, adrenal glands, thymus,

and the gastrointestinal tract (Castellino and Ploplis, 2005). Human plasminogen is synthesized as an 810-amino acid polypeptide. The mature form of this protein is 791-amino acids, due to cleavage of a 19-amino acid leader polypeptide. The mature form of the protein has a molecular mass of 92 kD (Liu, 2004). Human plasminogen consists of a 77-amino acid residue activation peptide, five consecutive kringle domains, a cleavage activation loop, and the functional serine protease domain. The kringle domains are of great importance and are responsible for facilitating the binding of plasminogen to large substrates such as fibrinogen, bacterial proteins, mammalian cell surfaces, and small molecule ligands (Castellino and Ploplis, 2005). In order for human plasminogen to be converted to plasmin, the cleavage of Arg561-Val562 has to occur. This cleavage results in the specific generation of Glutamic acid1-Pm (Glu1-Pm), which contains an N-terminal heavy chain of 561 amino acids and a disulfide-linked carboxy-terminal light chain of 230 amino acids (Castellino and Ploplis, 2005). The catalytic triad consists of His603, Asp646 and Ser741, and is found in the light chain. The existence of another protelytic reaction that has physiological relevance is the Glu1-Pm and Lys78-Pm-catalyzed hydrolysis of the N-terminal 77 amino acids of Glu-1Pg or Glu1-Pm, converting Glu1-Pg to Lys78-Pg and Glu1-Pm to Lys78-Pm (Ohisson et al., 1993). The human plasminogen gene has been mapped to chromosome 6q26-6q27 (Leonardsson et al., 1995). The gene is 52 kb and consists of 19 exons and 18 introns. A 57 bp signal sequence and the remaining coding sequence (2,373 nucleotides) comprise the mature protein (Castellino and Ploplis, 2005).

Plasminogen has many receptors in a broad variety of eukaryotic and prokaryotic cells (Miles et al., 2005). Plasminogen receptors are present on monocytes, monocytoid cells, macrophages, endothelial cells, fibroblasts, platelets, adrenal medullary cells, and carcinoma cells among others. The broad distribution of the plasminogen receptors among all of these cell types states the importance of their functions. The plasminogen capacity for most cells is relatively high, ranging from 37,000 sites per platelet to millions on endothelial cells. For this reason, no single molecule is expected to account for the entire complement of plasminogen binding sites on a cell (Miles et al., 2005). The molecular identity of plasminogen receptors is cell-type specific. There are various subsets of cell-surface plasminogen binding proteins that are synthesized. Some of these proteins are synthesized with carboxyl-terminal lysines and promote plasminogen activation. Others, bind plasminogen, but do not cause plasminogen activation (Miles et al., 2005). These among other proteins are involved in composing the plasminogen receptor system in cells.

## Plasminogen Activators

Plasminogen activators (PA) are serine proteases that convert the zymogen, plasminogen to the enzyme, plasmin by limited proteolysis (Danø et al.,1985). There are two main types of PA; tissue type PA (tPA) and urokinase type PA (uPA). These two types of PA are structurally similar but are functionally and immunologically different. Both uPA and tPA are capable of converting close to 200 mg/l of plasma zymogen plasmingen to plasmin (Medcalf, 1998). Plasminogen activation results from the specific cleavage of the Arg560-Val561 peptide bond (Danø et al., 1985).

### *Urokinase-type Plasminogen Activator*

uPA was originally isolated from urine in vertebrates (Danø et al., 1985). The role that uPA plays within the system is most notable during periods when extracellular matrix is breaking down related to activities such as tissue remodeling and tumor invasion (Sier et al., 1993). Other than urine, uPA has been found in small amounts within plasma and seminal plasma (Danø et al., 1985). uPA is a50 kD glycoprotein. Endogenously secreted and externally added uPA binds to its specific receptor; uPAR, which has a molecular mass in the range of 55-60 kD (Baker et al., 1990). uPAR is found on a variety of cells including tumor cells and many others. uPA exists in two forms, single chain (sc-uPA) and two chain (tc-uPA). The active form of uPA is the tc-uPA, and the conversion from sc-uPA to tc-uPA is catalyzed by plasmin (Danø et al., 1985). The tc-uPA form contains a heavy chain or B-chain, which has a molecular mass of 30 kD and a light chain, or A-chain, which has a molecular mass of 20 kD. Both of these forms are linked by a disulfide bond (Baker et al., 1990). The A-chain contains a single kringle domain, near the carboxyl terminal, and has an epidermal growth factor-like domain at the amino terminal which comes before the kringle domain. The B-chain contains the serine protease domain, where His204, Asp255, and Ser356 compromise the catalytic triad.

### *Tissue-type Plasminogen Activator*

The other form of PA is tissue-type PA (tPA). tPA has a much broader presence than uPA. tPA (molecular mass of 70 kD) was first extracted from tissues and is believed to have originated from endothelial cells. tPA has been identified in saliva, plasma, tears, seminal plasma, uterine fluid, urine, and in varying amounts in all tissues in both eukaryotic and prokaryotic organisms. tPA was the first protease to have been identified in murine oocytes and it was found to also play a role in oocyte migration in the oviduct (Sappino et al., 1989). tPA, like uPA can be found in two forms, single chain (sc-tPA) and two chain (tc-tPA). The two chain form is the proteolytically active PA. As with uPA, conversion of sc-tPA to tc-tPA is catalyzed by plasmin. It is also catalyzed by tissue kallikrein and blood coagulation factor Xa. Tc-tPA contains a heavy chain, also called the A-chain, (40 kD) and a light chain, also called the B-chain, (30 kD). At the amino terminus, the A-chain contains an epidermal growth factor-like domain, a fibronectin-like finger domain, and two kringles. The fibronectin-like finger domain and the second kringle domain are responsible for the high affinity tPA has for fibrin. The serine protease domain is located on the B-chain, and the catalytic triad of His322, Asp 371, and Ser478 is homologous to the active sites found in other serine proteases.

## Plasminogen Activator Inhibitors

Similar to uPA and tPA the existence of their inhibitors is also in variable levels within a balance. This regulation is important because overproduction of plasmin could potentially have damaging effects, and this is crucial to the normal process of non-invasive cell function. Plasminogen activator inhibitors (PAI) belong to the super family of serine protease inhibitors (SERPINS). SERPINS are globular proteins, which contain 3 beta-sheets and 9 alpha-helices. SERPINS also have an exposed peptide loop, the reactive center loop (RCL), which is of decisive importance for the inhibitory mechanism (Dupont et al., 2009). Two principal inhibitors regulate PA activity: PAI-1(SERPINE1) and -2 (SERPINB2). There are other similar inhibitors such as SERPINA1, SERPINE2, SERPINA7, SERPINC1, and SERPING1, but are in less existence throughout eukaryotic and prokaryotic organisms than PAI-1 and PAI-2 (Silverman et al., 2001). PAI-1 is regarded as the inhibitor for tPA whereas PAI-2 is considered the main inhibitor for uPA. Earlier on in the discovery of these inhibitors PAI-1 was termed as circulating PAI and PAI-2 was termed as the intracellular PAI. Protein C has also been referred to as PAI-3 (Binder et al., 2002).

### *Plasminogen Activator Inhibitor-1*

Plasminogen activator inhibitor 1is also known as SERPINE1 by more modern nomenclature on the basis of phylogenetic relationships it has with other SERPINS (Binder et al., 2002). Although, PAI-1 is the major inhibitor for tPA, it is also a potent inhibitor of uPA in several lineages of cells. tPA is typically responsible for intravascular plasminogen activation, and its activity is regulated by the presence

of fibrin, whereas uPA is typically the major PA in migrating cells. For these two reasons PAI-1 is capable of inhibiting not only intravascular fibrinolysis, but also cell-associated proteolysis. This inhibitor serves as the primary regulator of plasminogen activation *in vivo*. PAI-1 is a single chain glycoprotein and has a molecular mass of 47 kDa. Early on, PAI-1 was known as the "fast acting" inhibitor, the endothelial cell inhibitor and the beta-migrating endothelial inhibitor. The first form of human PAI-1 purified to apparent homogeneity was in 1984 (Dellas and Loskutoff, 2005). PAI-1 binds in a stoichiometric manner to PA with rapid and irreversible inhibition which has led many to describe PAI-1 as a "suicide inhibitor" (Mehta and Shapiro, 2008). It is known that hepatocytes, endothelial cells, adipocytes, and megakaryocytes are all able to synthesize and secrete PAI-1 into the circulation (Mehta and Shapiro, 2008). The mature, secreted form of this inhibitor is composed of 379 amino acids and contains 13% carbohydrate. This glycoprotein contains multiple methionine residues and lacks cysteine residues. The lack of cysteine residues and hence disulfide bonds may in turn account for its instability in solution. The reactive center of the inhibitor is Arg346-Met347 and is contained within the exposed so-called strained loop region at the carboxy terminus of the molecule and serves as the pseudosubstrate for the target serine protease. Several latent stages of PAI-1 have also been observed, which means that it is abundant in several conformations other than the active and inactive ones. The active form of PAI-1 has the ability to spontaneously convert over to the latent form with a half-life of 1 hr. The latent form can also be converted into the active form by treatment with denaturants, negatively charged phospholipids, or vitronectin, although the third

reaction is relatively slow compared to the others (Binder et al., 2002). As far as it is known, PAI-1 is the only SERPIN that can reversibly switch between the active and latent conformational states. The interaction that PAI-1 has with heparin and vitronectin is something to consider when discussing activity of the plasmin system. In plasma or in the extracellular matrix, PAI-1 is stabilized by vitronectin. In solution, vitronectin bound PAI-1 is approximately twice as stable as the unbound state of PAI-1, and on the extracellular matrix the half-life is reported as less than 24h. Most of the PAI-1 found in an organism appears to be in whole blood and a very small amount tends to be found in normal fresh plasma. Out of these concentrations the majority appears to be in the latent form and contained within platelets. Platelets contain vitronectin, which can potentially function to reactivate latent platelet PAI-1. The binding affinity of PAI-1 for heparin is of very high affinity. This glucosaminoglycan-binding property of PAI-1 serves as an additional mechanism to anchor PAI-1 to the matrix. However, the functional activity of PAI-1 is not influenced by heparin (Hekman and Loskutoff, 1988).

The human PAI-1 gene is 12.2 kb and is composed of nine exons and eight introns. The PAI-1 gene is located on the long arm of chromosome seven. Two different transcripts result in mRNA of different lengths in their untranslated regions that appear to be formed by alternative polyadenylation. The 3' end of the larger transcript contains an AT-rich sequence homologous to the sequences implicated in the control of mRNA stability and may contribute to the differential regulation of PAI-1 protein expression. The 5' region has been extensively looked at and the transcription initiation site has been identified as well as consensus TATAA sequence

for RNA polymerase binding. Within the PAI-1 structural gene and flanking DNA there are two types of repetitive DNA elements. There are 12 Alu elements and 5 repeats of a long poly (Pur) element. 4G/5G and G/A polymorphisms are also present in the promoter. The Alu-Pur elements may represent a subset of the more abundant Alu family of repetitive sequence elements (Binder et al., 2002).

PAI-1 has many functions and many places where it can be detected within an organism. For this reason it could often be used as a diagnostic measure in a variety of medical issues. Such use exists in the case of vascular diseases, metabolic diseases, homeostatic disorders, and more. It has been shown that increased levels of PAI-1 are positively correlated with the risk of developing coronary artery disease. It has also been correlated to a certain extent with the risk of sclerosis, restenosis, myocardial infarction, and deep vein thrombosis. For these reasons it seems to be apparent that PAI-1 is a key molecule in thrombic vascular disease (Kohler and Grant, 2000). High PAI-1 levels in the blood also tend to correlate with obesity. Weight reduction by either significant dieting or by surgical procedures has shown to have positive effects on the reduction of PAI-1 levels in the blood of human adults. Along with the disorders already mentioned, PAI-1 seems to be heavily involved in acute and chronic inflammatory lung disorders and renal diseases. These are only some of the clinical implications that PAI-1 has brought to modern medicine (Kohler and Grant, 2000).

### *Plasminogen Activator Inhibitor-2*

PAI-2, like PAI-1, belongs to the family of serine protease inhibitors and is known as SERPINB2. PAI-2 is an efficient inhibitor of u-PA and tc-tPA, but is slower than PAI-1. PAI-2 is a very slow inhibitor of sc-tPA when compared to PAI-1, which makes it very unlikely PAI-2 is even an inhibitor of sc-tPA *in vivo*. Another observation contributing to this theory is fibrin-bound tPA appears to be protected from fibrin inhibition by PAI-2 (Egbert, 1998). PAI-2 has been found to occur in two molecular forms, low and high molecular weights (LMW and HMW, respectively). LMW PAI-2 is estimated to be approximately 42 kD and HMW PAI-2 is approximately 60 kD. The two forms of PAI-2 have distinct differences in their cellular localizations. LMW PAI-2 is intracellular and non-glycosylated whereas HMW PAI-2 is glycosylated and secreted (Astedt et al., 1998). PAI-2 is known to play roles in such processes as pregnancy, skin growth and maintenance, inflammation, wound healing, tumor invasion, and metastasis. Kawano et al. (1968) first reported the presence of a urokinase inhibitor within the placenta. Conditioned medium harvested from cultured human placental tissue inhibited PA activity (Kawano et al., 1968). Astedt (1988) reported that PAI-2 was localized to the trophoblastic epithelium. The first pure form of PAI-2 was reported to have been obtained from human placenta, however, human U937 monocyte-like cells stimulated with phorbol ester also produce PAI-2**.** When initially extracted, the purified form of the protein appeared to be the non-glycosylated LMW form (Egbert et al., 1995). A total of 15 human placentas were used to obtain 1mg of purified PAI-2 (Astedt et al., 1995).

The amino acid sequence of PAI-2 secures the protein as a member of the SERPIN super family of proteins. The 1,900 bp cDNA of human PAI-2 encodes a protein of 415 amino acids, having a predicted unglycosylated MW of 46,543, which approximates the 47 kD cytosolic form of PAI-2. The gene structure of PAI-2 is one that led to many elusive conclusions early on in the study of the protein. In humans, the gene is located on chromosome 18q21.3 and the chromosomal region of the protein is relatively well-characterized and contains a SERPIN cluster (Ye et al., 1989). The PAI-2 gene contains 8 exons and 7 introns and its transcription initiation site is contained between 22- 25 bp downstream of a TATAAAA consensus sequence. To date, the sequence of 2,000 bp of the 5' flanking region of the 65-bp first exon, as well as the restriction map of 8,000 bp surrounding the transcription initiation site are known. The PAI-2 promoter contains two Alu repeat sequences that are between -1390 and -1100 and between -870 and -580.

## Embryonic Development in Mammals

Mammalian early embryonic development is composed of many cellular and physiological changes. The changes that occur from fertilization to the hatched blastocyst stage are monumental in the development of the animal. The basic outline of the pattern that mammalian embryogenesis follows is important to understand any roles the plasmin system has on early development. Embryo development begins during fertilization, when the male and female gametes fuse to form the zygote or one-celled embryo. Soon after its formation, the zygote undergoes cleavage and generates two daughter cells or blastomeres to form the two-cell embryo. Each of the

two-cell stage blastomeres possesses the ability to develop into a completely intact individual. Identical twins for example, form from blastomeres of a two cell embryo that divide independently to form two separate embryos. Following the two-cell embryo stage, cleavage divisions continue to give rise to the four-, eight-, and 16-celled embryos culminating in a ball of cells called the morula. During the morula stage, the outer cells begin to compact more than the inner cells in the center. The inner cells develop gap junctions that allow for intracellular communication, whereas the cells on the outside develop tight junctions that allow them to adhere to other cells. When the tight junctions form, fluid begins to accumulate on the inside of the embryo. This stage of development is the early blastocyst, and it consists of an inner cell mass (ICM), a single layer of cells called the trophectoderm, and the fluid-filled cavity known as the blastocele. The ICM will eventually give rise to the embryo and the ectodermal cells will give rise to one of the fetal membranes, the chorion (Senger, 2005).

As divisions continue, more fluid builds up in the blastocele causing pressure to develop within the blastocyst. The blastocyst produces proteolytic enzymes that degrade the zona pellucida, and in combination with the increasing fluid, cause the embryo to hatch out of the zona. This stage is referred to as the hatching blastocyst stage. Once hatching is complete, the embryo is now in a free-floating state within the confines of the uterine lumen. At this point, the hatched blastocyst is now dependent entirely on the uterine environment for survival. A healthy viable embryo is dependent upon adequate luteal function, proper progesterone synthesis, and the responsiveness of the uterus towards progesterone. Time relative to stage of

development varies among the different species. For example, the bovine embryo follows a time line for a 2-cell, 4-cell, 8-cell, morula, blastocyst, and hatched blastocyst of 1, 1.5, 3, 4-7, 7-12, and 9-11 d, respectively. The human embryo on the other hand takes 1, 2, 3, 4, 5, and 5-6 d, respectively, to reach the corresponding stages. During this time, the uterine location of the embryo also varies across species. The cow and the human both have similar profiles in terms of when the embryo descends into certain portions of the reproductive tract; 96-120 h and 72-96 h, respectively. The sow and the mare on the other hand, are different in terms of when the embryo descends into the uterus from the oviduct; 2 d and 5 d, respectively. These two species lower the embryo down to the uterus at the 8-cell stage, whereas the others don't allow this until the morula stage (Senger, 2005).

Following hatching, the blastocyst will undergo a dramatic growth phase. In the cow, at Day 14 the blastocyst is approximately 3-4 mm in diameter and is in an elongated state. During the next four days, the conceptus will grow to be approximately 250 mm in length and by Day 18 of gestation will occupy space in both of the uterine horns. The blastocyst of the ewe is quite similar to that of the cow in its development. Other species such as the pig are much more exaggerated in embryonic growth. On Day 10 of gestation the pig embryo is a sphere measuring 2 mm in diameter but by Day 12 it measures close to 200 mm in length. The embryo elongates approximately 4 to 8 mm per hr. At that rate it gets to be close to 1000 mm in length by Day 16 (Senger, 2005).

Within mammalian embryos there are two different ways the process of embryo implantation can be classified. They could either be in a group where the pre-

attachment period within the uterus is very long, or they could be in a group where the blastocyst implants soon after hatching. In some species including humans and other primates, rabbits, and rodents the blastocyst implants very soon after hatching. Cattle and most other domestic species are included in the first group. During this elongated pre-implantation period, the extraembryonic membranes are formed by a folding process that generates the amnion, chorion, and the allantochorion. In species where the blastocyst implants soon, the extraembryonic membranes develop after implantation or attachment. This also depends on the type of placentation the particular animal possesses. Cattle, for example, have cotyledonary placentation, dogs and cats have zonary placentation, horses and pigs have a diffuse placentation, and humans, as well as other primates and rodents have discoid placentation. The extended interval between hatching and attachment in bovine embryos allows for non-surgical collection of hatched blastocysts at 14d post-fertilization. This is very important, especially when studying the embryo at later stages of development for certain molecular markers and other experiments (Senger, 2005).

## The Plasminogen System in Reproduction

### *Male Reproduction*

The plasminogen/plasmin system is not only present within the female reproductive system, but it is also present within the male reproductive system in significant amounts. Several studies have shown different levels of uPA, tPA, and PAI-1 within seminal plasma, spermatozoa, and different sections of the male

reproductive organs. It has also been shown to play many roles in fertilization and sperm motility. Xunbin et al. (1997) demonstrated uPA has a positive effect on sperm motility in sub-fertile humans. They concluded that close to 30% of patients with fertility complications suffered from low sperm motility. By adding uPA they were able to see a significant increase in the motility of sperm samples. Through this they suggested the possibility exists for male contraception using an uPA inhibitor available to the human body that can suppress sperm motility. Other reports have demonstrated presence of tPA, uPA, and PAI-1 in monkey Sertoli cells (Yi-xun et al., 1995). Zhang et al. (1997) observed tPA and uPA in significant amounts within rhesus monkey epididymis. Through in situ hybridization, Zhang et al. (1997) demonstrated high levels of tPA and PAI-1 mRNA in the epithelial cells of the caput region of the epididymis in the adult rhesus monkey. They were able to demonstrate some levels of expression for uPA, however levels were nowhere as high as compared to tPA and PAI-1. In comparison to adults, no detectable amounts of mRNA could be found within the epididymis of infant rhesus monkeys. For this reason, Zhang et al. (1997) evaluated levels of LH and FSH within the epididymis. They found that LH up-regulated the activity of uPA and tPA, whereas FSH was not detectable and had no effect on gene expression in rhesus monkey epididymis. Gunnarson et al. (1999) reported through in situ hybridization significant levels of tPA, uPA, uPAR, PAI-1, and PAI-2 mRNA in human seminiferous epithelium. Strongest signals were obtained from tPA, uPA, and PAI-1. PAI-2 had widespread signals throughout certain sections of the testis, including the testicular tubules and interstitial tissue. Weak expression of PAI-2 was considered as such because it was

not consistently detected in all specimens used for the study. Huarte et al. (1987) reported that mouse testicular sperm contained low levels of uPA, whereas ejaculated sperm had high levels. They were able to show high levels of uPA mRNA in the vas deferens and seminal vesicles. The majority of uPA in all tissues examined was located in the epithelium and vas deferens. Hence, the high levels of uPA in ejaculated spermatozoa were acquired by passage through the efferent duct system.

### *Female Reproduction*

The plasminogen/plasmin system is involved in several events in female reproduction, including ovulation, fertilization and embryogenesis among others. Events using proteolytic degradation, tissue remodeling, and cell migration depend on the PA system.

## Ovulation

In mammals, ovulation is triggered by the pre-ovulatory surge of LH, which results in rupture of the mature follicle and liberation of the oocyte. This is a controlled process that is tightly regulated and repeated during each estrous or menstrual cycle. The process entails cell proliferation, cell apoptosis, oocyte maturation, detachment of cumulus-oocyte complexes, and ovulation (Yi-xun et al., 2004). In each of these physiological events, proper extracellular proteolysis has to occur and it has to happen in a manner that will maintain the integrity of the ovary. The extracellular matrix (ECM) of the ovary is a structural component that maintains its organizational integrity. Proteolysis of the ECM requires the PA system due to the

amount of cell migration and wound healing processes (Irving-Rodgers et al., 2006). Therefore, the plasminogen/plasmin system provides a specific controlled way of proteolytic activity. Tissue-type PA and PAI-1 have been shown to have the most impact in the process and uPA, u-PAR, and PAI-2 have been detected in lesser amounts, with PAI-2 in only minute amounts. Mouse granulosa cells express mainly tPA. Rat, mouse, rhesus monkey, and human oocytes have the ability to synthesize t-PA. Levels of PAI-1 in the mouse ovary have been shown to correlate with levels of tPA. During the pre-ovulatory period there is a high transient expression of tPA and PAI-1 activity which are important for ovulation to occur.

## Fertilization

As discussed before, the plasminogen/plasmin system has large roles in both male and female gametes. Therefore, it should make sense that this system should also have a role in the actual event of fertilization. Several authors have reported the PA system plays a significant role in the fertilization process of mammals. Mondejar et al. (2012) conducted experiments using bovine and porcine oocytes to detect plasminogen and PA as well as to clarify the role of the plasminogen/plasmin system in mammalian fertilization. Plasminogen and, therefore, plasmin were secreted at steady rates within the oviductual fluid of both species. When plasminogen and plasmin were added to IVF medium, sperm penetration rates were affected. When both plasminogen and plasmin were added to IVF medium, sperm penetration rates decreased, therefore significantly decreasing rates of polyspermy. In their experiments, Mondejar et al. (2012) showed significant levels of both tPA and uPA as

well as PAI-1 within the fertilization process in bovine and porcine oocytes. These parameters are of significant importance to prove the role of the plasminogen/plasmin system in fertilization, as well as how they could improve the success of IVF procedures.

## Early Embryonic Development

The PA system is expressed in great abundance during early embryo development in several species including rodents, rabbits, swine, cattle, and sheep.

### *Rodents*

Mice and rats are some of the most commonly used research models for the plasmin/plasminogen system. Strickland et al. (1976) reported that PA is present as early as developmental day 6 in mouse embryos cultured *in vitro*. They found following the initial detection of PA, that cultured blastocysts produced PA in a biphasic time course, with the maximum amount of PA present at day 8. PA production by the trophoblast cells occurred during the first phase and was proposed to be involved in embryo implantation.

PA detection within the embryo has shown to be a particular method of determining its presence. Zhang et al. (1997) reported the detection of PA based on gene expression and enzymatic activities within the pre-implantation embryo. Tissue-type PA mRNA was present in rat oocytes and two-cell embryos. Urokinase-type PA mRNA was first detected in two-cell rat embryos and was present through the blastocyst stage. The PA present in higher levels within the pre-implantation embryo

was uPA. This further supported previous findings of PA being present in early embryo development around the time of implantation.

Rat embryos developing *in vitro* express less PA when compared to *in vivo* developing embryos. Both uPA and tPA are expressed in rat embryos throughout the *in vivo* and *in vitro* pre-implantation period. Urokinase-type PA was localized mainly in the cell cytoplasm whereas tPA was detected mainly on the cell surface and in the perivitelline space. Aflalo et al. (2005) reported that both *in vitro* and *in vivo* developing blastocysts have tPA and uPA localized in the trophectoderm cells. When compared to the earlier stages of development, blastocysts had significantly less uPA content.

### ***Bovine***

Several studies have quantified the PA system in bovine embryos. Menino and Williams (1987) evaluated plasminogen activation in the early bovine embryo. Using a casienolytic agar-gel assay they measured plasminogen activator and plasmin levels in culture media harvested from embryos at different incubation periods. Percentages of embryos developing to the initiating hatching blastocyst, hatched blastocyst, attached blastocyst, and attached blastocyts with trophoblastic outgrowth stages were not significantly different between the different levels of plasminogen. However initiation and completion of hatching accelerated as plasminogen concentration increased in the culture media. PA activity was consistently low for the first 48-72 h of culture but, increased sharply and plateaued after 120 h.

Kaaekuahiwi and Menino (1990) further explained the relationship between PA production and bovine embryo development *in vitro*. Total PA production was positively correlated to embryonic size, developmental stage, and cell number. They also found it was weakly correlated to zona pellucida thickness. Hatched embryos produced more total PA than those failing to hatch. These data suggested as embryonic size and cell number increased and development progressed, bovine embryos liberated more PA.

Dyk and Menino (1991) suggested Day 12-14 bovine blastocysts produced uPA and a high MW form that was either a novel t PA or a PA inhibitor in complex with the uPA. Blastocysts contained and secreted light and heavy forms of PA. Intact blastocysts and trophoblast produced both forms of PA but PA were not detected in embryonic disks. Whiteside et al. (2001) suggested that uPA is produced by pre-implantation bovine embryos. They assayed conditioned medium collected at different days of development for uPA activity using a chromogenic assay, and detected uPA in all samples.

There is evidence of PA being present in developing bovine embryos as well as developing oocytes. Rekkas et al. (2002) reported tPA in extracts of bovine oocyte cortical granules, and that its activity significantly varies with the duration of oocyte *in vitro* maturation. These findings suggest tPA activity of oocyte origin may have a role in maturation or fertilization of bovine oocytes. It may also play a role in post-fertilization events such as those present in the cortical reaction and formation of the zona block to polyspermy.

### *Ovine*

Bartlett and Menino (1993) used embryo culture media recovered from different embryonic stages to identify the type of PA produced by pre-attachment ovine blastocysts. Days 7, 9 and 11 embryos were cultured to equivalent gestational day (EGD) 15 and conditioned medium was recovered at 24-hr intervals. PA activity in the conditioned medium was quantified using a caseinolytic agar gel assay, one-dimensional SDS-PAGE and zymography. Identification of the type of PA produced was performed by pooling the blastocysts and conducting an immunoprecipitation experiment. No differences were observed over the 8 d of culture in PA activities in conditioned medium recovered from Day 7 embryos. PA activities in conditioned medium recovered from Day 9 embryos did not differ from EGD 10-14 but were less than EGD 15. Although PA activities in conditioned medium recovered from Day 11 embryos were greater on EGD 14 and 15, no significant differences were observed because of high variation in PA production by the different embryos. Two plasminogen-dependent lytic zones were revealed by zymographic analysis of the conditioned medium. The lower molecular mass lytic zone (50 kD) was present from EGD 7-15 and the higher molecular mass lytic zone (82 kD) was present from EGD 13-15. Bartlett and Menino (1993) suggested the ovine PA produced during EGD 7-15 was uPA and the heavier MW form was due to a uPA-PAI complex.

### *Swine*

The pig blastocyst is unique when it comes to its different developmental stages. It undergoes drastic changes in morphology following day 10.5 of pregnancy.

At this stage the blastocyst will elongate from a small sphere approximately 10mm in diameter, to a long thread-like form which continues to grow to 1m in length prior to uterine attachment at day 18. Flazlebas et al. (1983) showed that swine blastocysts produce PA. Blastocysts between 10 and 16 d of gestation were collected and PA secreted into the medium was quantified. PA was greater in Day 12 blastocysts, which is considered to be the early elongation phase. Flazlebas et al. (1983) concluded the swine blastocyst has the ability to generate plasmin within the uterine lumen. Mullins et al. (1980) suggested the swine endometrium secretes a plasmin inhibitor into the uterine lumen. In pregnant animals, the amount of plasmin inhibitory activity rose 7-fold between Day 10.5, when the embryo was spherical, and Day 12, during the filamentous stage.

## Embryonic Implantation

Embryonic implantation is one of the most important factors in achieving a successful pregnancy in all species. This process involves a heavy series of events involving tissue remodeling. The plasminogen/plasmin system makes a very strong presence during this event. In most mammals, during the transition of the morula to the blastocyst the embryo enters the uterus. At that point in time it is sustained by oxygen and a well composed supply of metabolic substrates in uterine secretions. During mammalian embryonic implantation, the embryo attaches to the surface of the endometrium. The embryonic trophoblast traverses the uterine epithelium anchoring the uterine connective tissue. In humans for example, the embryo adheres to the endometrium at around 6-7 d post ovulation. When the embryo and the endometrial

adhesion molecules that are present have access to each other, the embryonic trophoblast cells will attach to the endometrial epithelial cells. There are several families of adhesion molecules present during this time, including the integrins, tastin/trophinin complexes, heparin sulfate proteoglycans, cadherins, lectin/glycan interactions and glycan/glycan interactions. During the invasion process the dam and early fetus have a fine balance between conflicting biological needs. The invading cells secrete collagenases and express PAI-1 while losing integrins associated with basement membrane interactions and gaining integrins that interact specifically with fibronectin and type 1 collagen. At this point the outer layer of trophoblast cells will fuse to form a multinucleated syncytiotrophoblast layer that will cover the columns of invading cells. The layer will proliferate very rapidly and will form numerous processes including the chorionic villi, which is the layer that surrounds the embryo and extraembryonic membranes.

## Pregnancy and the Placenta

In humans, the placenta is one of the tissues where PAI-2 is strongly secreted. During pregnancy and parturition there are significant changes in coagulation and fibrinolysis (Zhao-Yuan et., al 1999). The placenta is a very important source of PAI-1, u-PA, t-PA, and most notably PAI-2. Levels of PAI-2 are known to increase dramatically during gestation in humans. The main reason behind the changes in the coagulation and fibrinolytic system comes from the need to change the uterine environment to maintain a pregnancy and support placentation. Fetal growth and development requires an adequate supply of maternal blood. Maternal blood is

delivered into the intervillous spaces of the primate placenta and this is a complex process associated with uterine-fetal tissue remodeling and vascularization. The conversion of spiral arterioles into uteroplacental arterioles by trophoblast invasion during the earlier stages of pregnancy is one of the most essential steps in the proper establishment of an adequate choriodecidual blood flow in primate placentation (Meekins et al., 1994). Trophoblast implantation, vascular remodeling and the maintenance of intervillous blood flow depends on the regulated production of PA and PAI. Plasmin generation by the precisely balanced expression of PA and PAI in the trophoblast and other maternal tissues leads to the degradation of the collagenous and non-collagenous components of the extracellular matrix within the placenta (Mackay et al.,1990). Zhao-Yuan et al. (1999) reported strong PAI-2 expression in extravillous trophoblast cells of the basal plate and septae and in trophoblasts of the chorionic plate in both the monkey and human placenta. During pregnancy, concentration of PAI-2 antigen increases from below detection limits (<5 ng/ml) to 250 ng/ml at term (Egbert et al., 1995). Endothelial cell-related PAI-1 as well as tPA and uPA have also been reported to increase notably during pregnancy. Pregnancy plasma contains mainly the HMW form of PAI-2, but small quantities of the LMW form have also been reported. The umbilical cord contains mainly the LMW form and the amniotic fluid is known to contain equal amounts of both HMW and LMW species. PAI-2 antigen levels in amniotic fluid have been shown to increase from 70 ng/ml in the first trimester to170 ng/ml in the third trimester. At term, PAI-2 concentrations have been shown to remain elevated for a few days postpartum. Levels of PAI-1 however, drop down to their normal level within a few hours after labor.

The slow decrease in PAI-2 is believed due to remnants of decidual or placental tissue attached to the endometrium.

When certain complications occur in pregnancy, such as preeclampsia or intrauterine growth retardation, PAI-1 and -2 as well as tPA and uPA levels are affected. The main changes associated with these two complications are increased PAI-1 and tPA and decreased PAI-2 and uPA compared to normal pregnancies. PA and PAI further vary depending on the combination of complications that occur. For example, if only intrauterine growth retardation occurs and not preeclampsia, PAI-2 levels are decreased whereas levels of PAI-1 are similar to a normal pregnancy. On the other hand, if only preeclampsia occurs and intrauterine growth retardation is absent, levels of PAI-2 remain the same as in a normal pregnancy and levels of PAI-1 are increased. For this reason, elevated levels of PAI-1 are regarded as a good marker for preeclampsia (Egbert et al., 1995). In an earlier study, Roes et al. (2002) reported a more in-depth version examining the significance of preeclampsia on the plasmin system. They reported subjects with preeclampsia had significantly higher levels of t-PA and unchanged uPA plasma levels. They also reported levels of PAI-2 were lower and PAI-1 was not changed. When levels of these proteins were quantified in arterial and venous umbilical cord plasma, PAI-1 was significantly higher, which is in agreement with the previous study, and uPA levels were lower.

# MATERIALS AND METHODS

## *Embryo Collection and Culture*

Cross-bred beef cows maintained at the OSU Beef Cattle center were estrous synchronized with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse®, Pfizer, Inc., New York, NY) and superovulated using porcine follicle stimulating hormone (pFSH; Folltropin®-V, Bioniche, Belleville, ON). Two 25-mg injections of $PGF_{2\alpha}$ were administered intramuscularly 12 d apart (Day 0= first $PGF_2\alpha$ injection) and twice daily intramuscular injections of pFSH were given on days 10, 11, 12, and 13. A third injection of $PGF_{2\alpha}$ (12.5-mg) was administered on day 13 in the a.m. Estrous detection was initiated 12 h after the 12.5 mg dose of $PGF_{2\alpha}$. Upon being confirmed in standing estrus, cows were artificially inseminated at 0, 12, and 24 h after estrus onset with one straw of frozen bull semen. Two sires were used at different intervals to allow for improved fertilization rates. The first service of artificial insemination was accompanied by a 100 µg intramuscular injection of gonadotropin releasing hormone (GnRH; Fertagyl®, Intervet Inc., Roseland, NJ). Cows not observed in estrus were artificially inseminated 24 h after the initial heat check and injected with GnRH. Fourteen d after the onset of estrus (Day 0 = day of estrus onset), hatched blastocysts were non-surgically collected from the uterus. The uterus was thoroughly flushed via gravity medium flow using DPBS containing 2 ml/L heat-treated fetal cow serum (HTFCS) and 10 ml/L antibiotic/antimycotic solution (Sigma Aldrich, Co., St. Louis, MO). Blastocysts were recovered by filtering the flush medium through an

appropriate sized embryo filter and scanning the filtered medium in an embryonic collection dish using a dissecting microscope at 10X magnification.

Embryos were recovered from the search dishes using sterile Pasteur pipettes and washed three times in wash drops containing Hams F-12 (Sigma Aldrich, Co.) with 0.15% BSA (EMD Biosciences, Inc., Darmstadt, Germany). Embryos were either cultured or snap-frozen and stored in liquid nitrogen for future PAI-1 and PAI-2 analysis. Embryos were cultured in 50µl microdrops of Hams F-12 with 0.15% BSA under paraffin oil in a humidified atmosphere of 5% $CO_2$ in air at 39°C. At 24-h intervals, approximately 40-45 µl of conditioned medium were recovered and frozen at -20 °C until further analysis. Culture continued until embryos failed to maintain expanded blastocoels and collapsed. At the conclusion of the culture, embryos were placed in cryotubes containing 50µl of DPBS and snap-frozen and stored in liquid nitrogen until further analysis. Animals utilized in this experiment were cared for in accordance to the Institutional Animal Care and Use Committee of Oregon State University.

### *Western Blotting of PAI-1 and PAI-2*

In experiment 1, Western blotting was used to identify the type of PAI present in Day 14 bovine embryos. A discontinuous polyacrylamide gel consisting of a 4% stacking gel and a 10% running gel was used for the identification of both proteins. Day 14 embryos were removed from the liquid $N_2$, thawed for a maximum of five min, and briefly viewed with a dissecting microscope. Once thawed, an equivalent amount of SDS-sample buffer (0.5M Tris-HCL, glycerol, 10% SDS, and 0.5%

bromophenol blue) containing 5% β-mercaptoethanol was added to the volume of embryo and medium contents. Embryos were homogenized by repeated aspiration with a micropipetter. Recombinant human PAI-1 (rhPAI-1) (EMD Biosciences, Inc.) and recombinant human PAI-2 (rhPAI-2) (Abcam®, Cambridge, MA) were used as standards for each of the respective experiments. RhPAI-1 standards were loaded at 10, 50, 100, and 200 ng and rhPAI-2 standards were loaded at 5, 10, 50, and 100 ng. Kaleidoscope Precision Plus Protein Standards (Bio-Rad Laboratories, Inc; MW: 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kD) were used to calculate molecular masses. Samples and standards were boiled at 95° C for 5 min to denature all proteins and allowed to cool before loading into the stacking gel. Day 14 embryos and conditioned and non-conditioned medium, PAI-1 and PAI-2 standards and kaleidoscope protein standards were loaded into castellated wells at volumes of 35, 30, and 10 µl, respectively. Electrophoresis was performed using a mini trans-blot electrophoretic transfer cell chamber (Mini-PROTEAN®II Cell; Bio-Rad Laboratories, Inc.) at 200V for 40 min in electrode buffer (1.0 M Tris base, glycine, and SDS; pH 8.3). Upon completion of electrophoresis, the polyacrylamide gel was equilibrated by submerging in electrode transfer buffer (25mM Tris, 192 mM glycine, 20% methanol) for 30 min. Proteins were transferred electrophoretically from the gel to a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Inc.) at 100V for 1 h. Following transfer, the nitrocellulose membrane was blocked in 3% BSA in TTBS (1.0 M Tris HCL, 1.5 M NaCl, 0.1% Tween-20; pH 7.5) for 90 min. The nitrocellulose membrane was washed with 1X TTBS and changed 3X within 30 min. The membrane was incubated with primary antibody for the respective protein being

investigated for a total of 2 h. PAI-1 was detected using rabbit anti-human SERPINE1 IgG and rabbit anti-human PAI-1 IgG (Sigma Aldrich, Co. and Santa Cruz Biotechnology, Inc., Dallas, TX, respectively). PAI-2 was detected using goat anti-human PAI-2 IgG (American Diagnostica Inc., Stamford, CT). The nitrocellulose membrane was washed with 1X TTBS, changed 3X within 30 min and incubated with a secondary detection antibody for 1.5 h. The secondary antibody used to detect both primary PAI-1 antibodies was goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Aldrich, Co.). A monoclonal anti-goat/sheep IgG conjugated to alkaline phosphatase (Sigma Aldrich, Co.) was used as the secondary antibody for PAI-2. The nitrocellulose membrane was washed with 1X TTBS and changed 3X within 30 min. At the completion of the antibody incubations, the Western blot was developed using an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Inc.). For both PAI-1 and PAI-2, Western blots were conducted where the primary antibody was replaced with rabbit and goat non-specific IgG, respectively (Sigma Aldrich, Co.). This was performed to prove the bands appearing as PAI-1 or PAI-2 were specific and not a result of non-specific binding of the first antibody. Band migration distance was measured and relative mobilities (Rm) of the protein and PAI standards, and embryonic and media bands were calculated. Molecular masses of the PAI standards and embryonic and media bands were determined from equation of the line calculations of log molecular mass vs. Rm. Images of the blots were captured using a Gel Logic 212 PRO (Carestream Molecular Imaging, 4 Science Park New Haven, CT).

### *Plasminogen Activator Assay*

Plasminogen activator levels in the conditioned medium were determined using a caseinolytic agarose gel assay with urokinase as the standard (Menino and Williams, 1987). The casein-agarose solution was prepared by using the protocol described by Bjerrum et al. (1975). Two% nonfat dry milk was dissolved in a caseinolytic gel assay buffer (0.038 M tris, 10 M glycine, and .195 g/l $CaCl_2 \cdot 2H_2O$) and heated to 60° C. An equal volume of 2% agarose (Sigma Aldrich, Co.) was dissolved in water by boiling, cooled to 60°C and combined with the milk solution. Fifteen ml of the casein-agar solution were poured into rectangular plates (85 x 65 x 2 mm) and allowed to solidify for 15 min at room temperature. Once the casein-agar gel solution solidified, 4 mm diameter wells were cut into each plate. For the standard curve, 45 µl of 120 µg/ml human plasminogen (hPgn) (Sigma Aldrich, Co.) were combined with 30 µl of 0, 0.01, 0.05, 0.1, 0.5, 1.0, or 5.0 milliunits/ml (mU/ml) urokinase (UK). For the media samples, 15 µl of 120 µg/ml hPgn were combined with 10 µl of conditioned medium. UK standards and conditioned medium containing hPgn were incubated for 15 min in a humidified atmosphere of 5% $CO_2$ in air at 39°C. After completion of the incubation period, 20 µl of the UK standards or conditioned media were pipetted into wells in the casein-agar gel plates and incubated at room temperature for 24 h. UK standards were run in triplicate. Plates were fixed for 15 min with 3% acetic acid and rinsed in tap water. Ring diameters (mm) of the developed lytic zones were measured using a digital electronic caliper. PA activity in conditioned medium was determined from equation of the line calculations of log [UK+1] vs. ring diameters.

### *Immunoneutralization Assay*

Identification of the type of PAI produced by the Day 14 bovine embryo was determined by using an immunoneutralization assay. Rabbit and goat antibodies used for Western blotting were used to detect the presence of PAI-1 and PAI-2 in conditioned medium. Rabbit and goat non-specific immunoglobulins (NSIgG; Sigma Aldrich, Co.) served as controls. Ten µl of primary antibody, NSIgG, or Ham's F-12 in 0.15 % BSA were combined with 10 µl of pooled Day 14 conditioned medium and incubated in a humidified atmosphere of 5% $CO_2$ in air at 39°C for 15 min. At the completion of the incubation period, 30 µl of 120 µg/ml hPgn were added to the samples and incubated in 5% $CO_2$ in air at 39°C for 15 min. Two 20-µl aliquots from each sample were pipetted into wells in the casein-agar gel plates as described previously for the PA assay. Plates were incubated at room temperature for 24 h, diameters of the lytic zones were measured and PA activity calculated as described previously.

### *Statistical Analysis*

Differences in PA activities in the conditioned medium in the immunoneutralization assay were analyzed using one-way analysis of variance (ANOVA) where IgG-type was the main effect If a significant difference was found in the ANOVA, differences between means were determined using Fisher's least significant differences procedure. All analyses were performed using the Number Cruncher Statistical System software program (NCSS, version 2007, J. Hintze, Kaysville, UT, USA).

# RESULTS

## *Embryo Collection and Culture*

A total of 25 Day 14 embryos of normal morphology, including spherical and elongate forms (Figures 1 and 2), were recovered from 5 cows for a recovery rate of five embryos per cow. Fourteen embryos were directly frozen in liquid $N_2$ at -196°C. Eleven embryos were cultured in Ham's F-12 with 0.15% BSA (Figure 3) for as long as the blastocoele remained fully expanded. At 24-h intervals, 35-45 µl of conditioned medium were from each microdrop and frozen at -20°C. An equal volume of fresh medium was restored to each microdrop. Cultured embryos were frozen in liquid $N_2$ at the first indication of blastocoel collapse. Embryos and conditioned medium were randomly used throughout the Western blotting experiments. An average of 180 µl of conditioned medium was recovered from the cultured embryos over a period varying from 6-24 days.

## *Western Blotting of PAI-1 and PAI-2*

Western blot analyses of Day 14 bovine embryos, PAI-1 and -2 standards, and conditioned and non-conditioned media were conducted using a variety of primary antibodies due to variation in sensitivity and specificity in bovine antigen detection. Rabbit anti-human PAI-1 IgG obtained from Santa Cruz Biotechnology detected two bands in embryos, a strongly staining band at 76 kD and a lighter staining one at 61 kD (Table 1; Figure 4). Rabbit anti-human PAI-1 IgG obtained from Sigma Aldrich, Co. also detected two bands in embryos, both strongly staining, at similar molecular

masses (76 and 61 kD); the lighter band represented by a doublet (Table 1; Figure 4). No bands were detected in non-conditioned medium whereas a band at 75 kD was detected in conditioned medium (Table 1; Figure 4). Both antibodies detected the rhPAI-1 standard. The molecular mass of the standard derived from equation of the line calculations of log molecular mass vs. relative mobility for molecular mass markers electrophoresed in a collateral lane was 48 kD (Table 1; Figure 4). The limit of detection for both antibodies resided between 50-100 ng of rhPAI-1. When the primary antibody was replaced with rabbit non-specific IgG, no positive staining was observed in lanes containing embryos, conditioned medium or rhPAI-1 (Figure 5).

Goat anti-human PAI-2 IgG obtained from American Diagnostica, Inc. detected two bands in embryos with molecular masses of 66 and 52 kD (Table 1; Figure 6). A faint staining band was detected in conditioned medium at 61 kD but a similar band was not observed in non-conditioned medium (Table 1; Figure 6). The molecular mass of the rhPAI-2 standard derived from equation of the line calculations of log molecular mass vs. relative mobility for molecular mass markers was 57 kD (Table 1; Figure 6). The limit of detection for goat anti-human PAI-2 IgG approximated 5 ng of rhPAI-2 (Figure 6). When the goat anti-human PAI-2 primary antibody was replaced with goat non-specific IgG, no bands were detected in lanes containing embryos, conditioned medium or rhPAI-2 (Figure 7).

### *Immunoneutralization Assay*

The immunoneutralization assay served as an indirect method for detection of PAI in embryo-conditioned medium. Antibodies detecting bovine PAI-1 and -2 in Western blotting were used in the immunoneutralization assay. PA activity in conditioned medium incubated with 0.2 mg/ml rabbit anti-human PAI-1 IgG obtained from Sigma Aldrich, Co. was greater ($P<0.05$) than in conditioned medium incubated with 0.2 mg/ml rabbit non-specific IgG (Figure 8). However, PA activity in conditioned medium incubated with 0.2 mg/ml rabbit anti-human PAI-1 IgG obtained from Santa Cruz Biotechnology did not differ ($P>0.10$) from conditioned medium incubated with 0.2 mg/ml rabbit non-specific IgG but was less ($P<0.05$) than PA activity in conditioned medium incubated with 0.2 mg/ml rabbit anti-human PAI-1 IgG obtained from Sigma Aldrich, Co. (Figure 8). No differences ($P>0.10$) in PA activities were observed in conditioned medium incubated with 1.0 mg/ml rabbit anti-human PAI-1 IgG obtained from Sigma Aldrich, Co. and conditioned medium incubated with 1.0 mg/ml rabbit non-specific IgG (Figure 8).

PA activity in conditioned medium incubated with 0.2 mg/ml goat anti-human PAI-2 IgG was greater ($P<0.05$) than in conditioned medium incubated with 0.2 mg/ml goat non-specific IgG (Figure 9). Similar to results observed with 1.0 mg/ml rabbit anti-human PAI-1 IgG, no differences ($P>0.10$) in PA activities were observed in conditioned medium incubated with 1.0 mg/ml goat anti-human PAI-2 IgG and conditioned medium incubated with 1.0 mg/ml goat non-specific IgG (Figure 9).

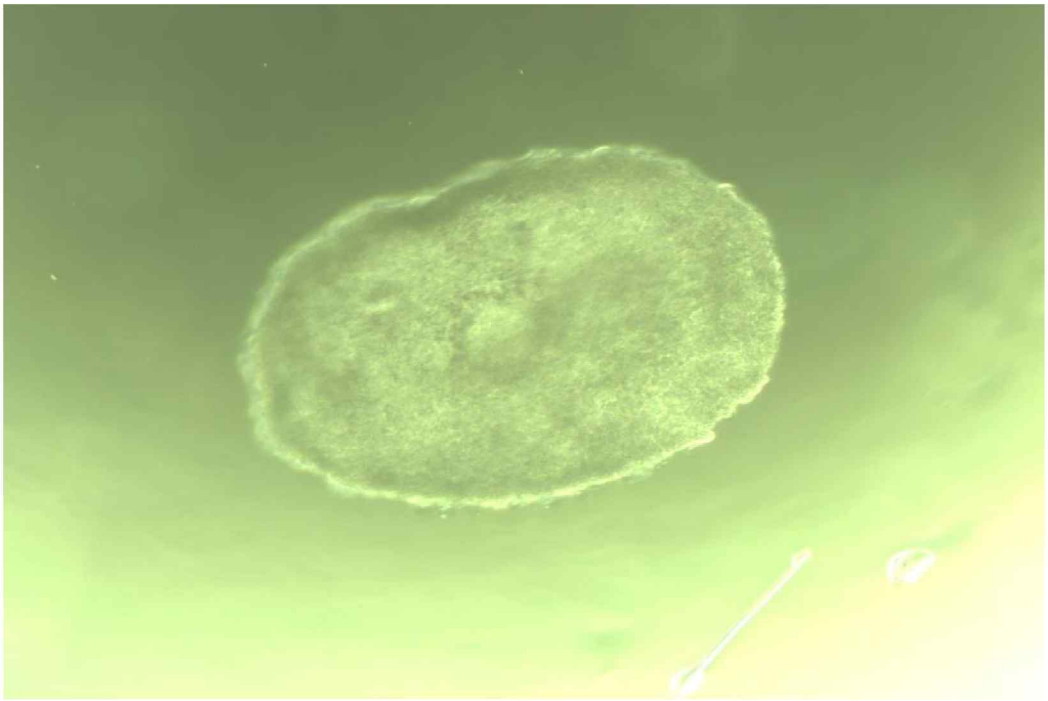

Figure 1. Bovine hatched blastocyst collected 14 d after onset of estrus (Embryo = 2.5 mm in diameter)

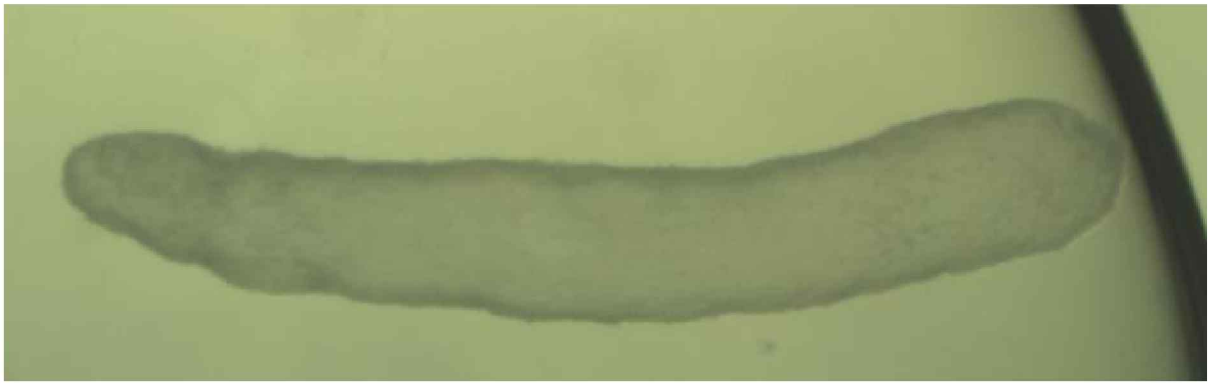

Figure 2. Elongated hatched bovine blastocyst collected 14 d after onset of estrus. (Embryo = 4.5 x 0.5 mm in size)

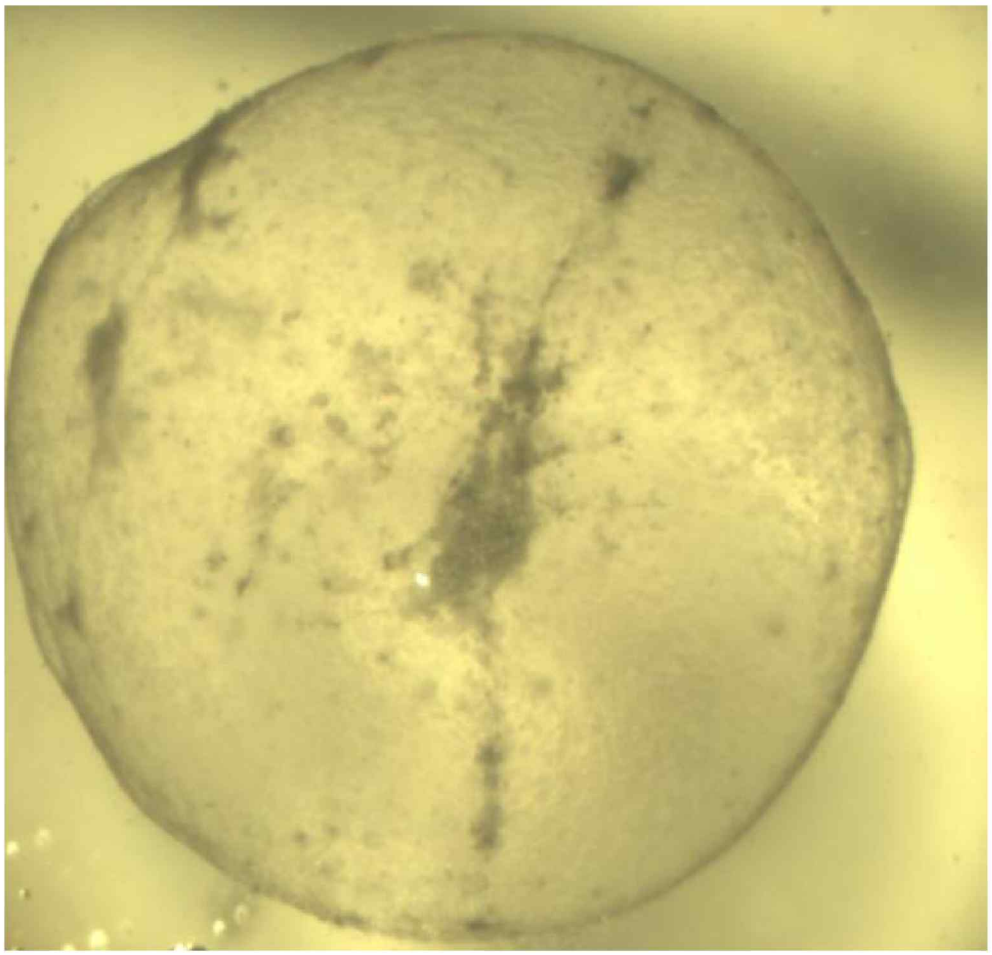

Figure 3. Hatched bovine blastocyst recovered 14 d after onset of estrus and cultured in Hams F-12 in 0.15% BSA for 24 d. (Embryo = 6.5 mm in diameter)

Table 1. Molecular masses[a] of presumptive PAI in Day 14 bovine embryos and conditioned medium and rhPAI-1 and -2 standards.

| Source | PAI-1 | PAI-2 |
|---|---|---|
| Embryos | 75.6 ± 0.1 | 66.2 ± 2.4 |
| | 61.3 ± 2.6 | 52.0 ± 1.8 |
| Medium | 75.4 ± 0.3 | 61.2 ± 3.9 |
| Standards | 48.4 ± 5.4 | 56.9 ± 4.0 |

[a]Data presented are means ± SE kD.

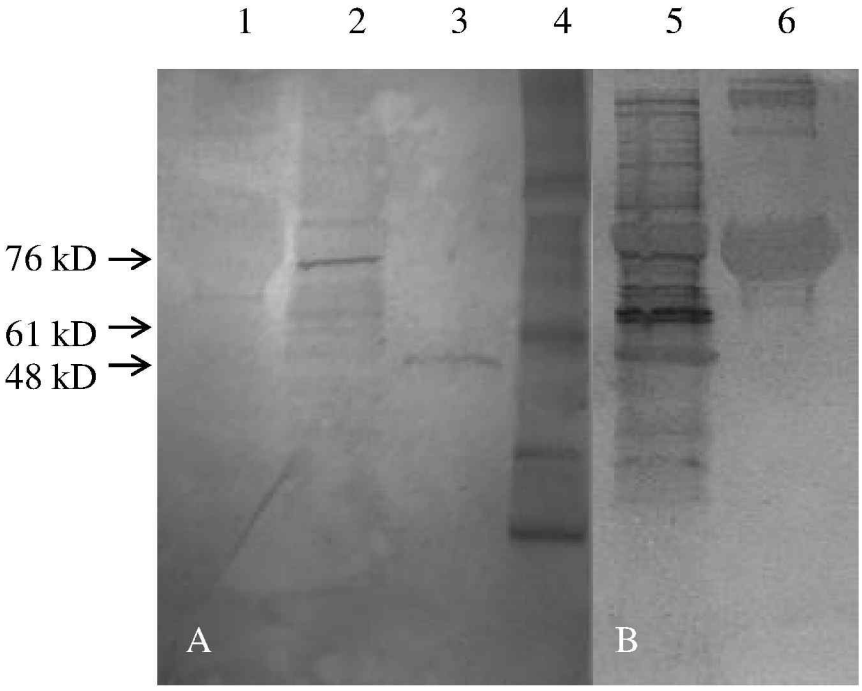


Figure 4. Western blot analysis for PAI-1 in Day 14 bovine embryos and culture medium. Blots A and B were treated with rabbit anti-hPAI-1 IgG obtained from Santa Cruz Biotechnology and Sigma Aldrich, Co., respectively. Lanes are non-conditioned medium (1 and 6), embryo (2 and 5), 100 ng rhPAI-1 (3) and molecular mass markers (4).

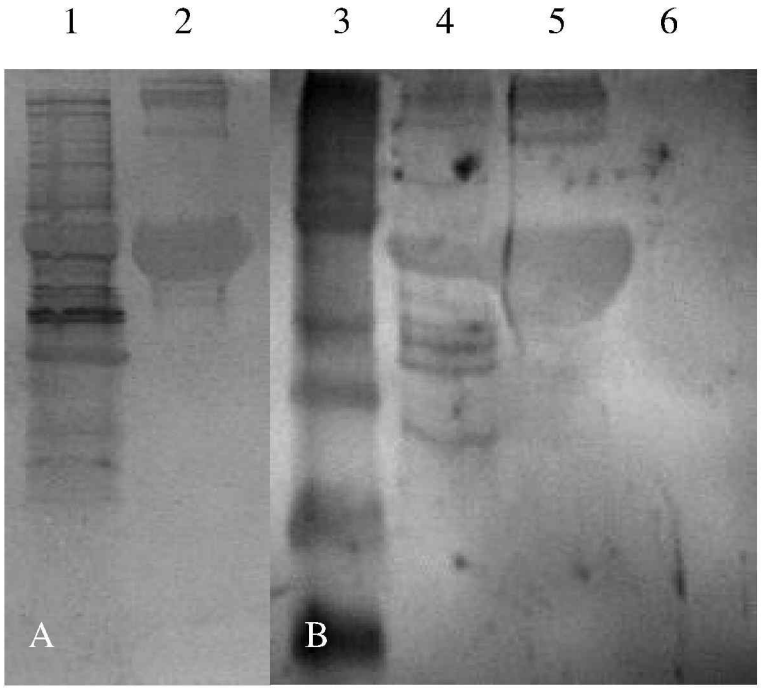


Figure 5. Western blot analysis for PAI-1 in Day 14 bovine embryos and culture medium. Blot A is depicted in Figure 4 and was treated with rabbit anti-hPAI-1 IgG as the first antibody. In place of rabbit anti-hPAI-1 IgG, blot B was treated with rabbit non-specific IgG as the first antibody. Lanes are embryo (1 and 4), non-conditioned medium (2 and 5), molecular mass markers (3) and 100 ng rhPAI-1 (6).

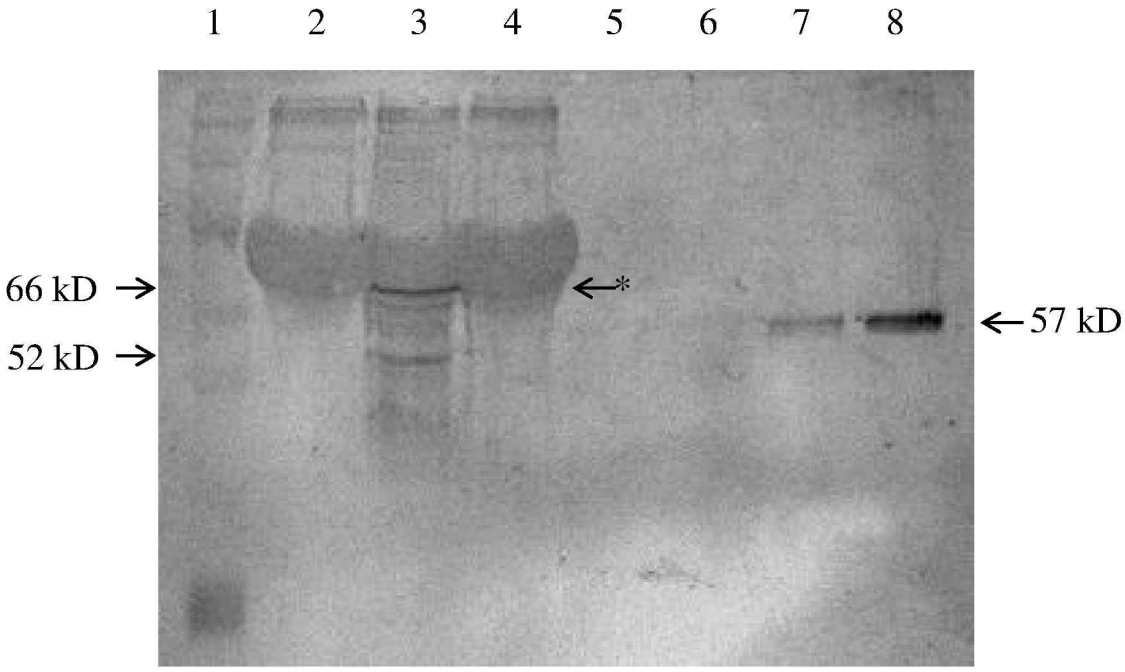


Figure 6. Western blot analysis for PAI-2 in Day 14 bovine embryos and culture medium. Blot was treated with goat anti-hPAI-2 IgG as the first antibody. Lanes are molecular mass markers (1), non-conditioned medium (2), embryo (3), conditioned medium (4), and 1 (5), 5 (6), 10 (7) and 50 (8) ng rhPAI-2. Arrows on the left and right indicate molecular masses of proteins in the embryo and the rhPAI-2 standards, respectively. Asterisked arrow indicates molecular mass of protein in the conditioned medium (61 kD).

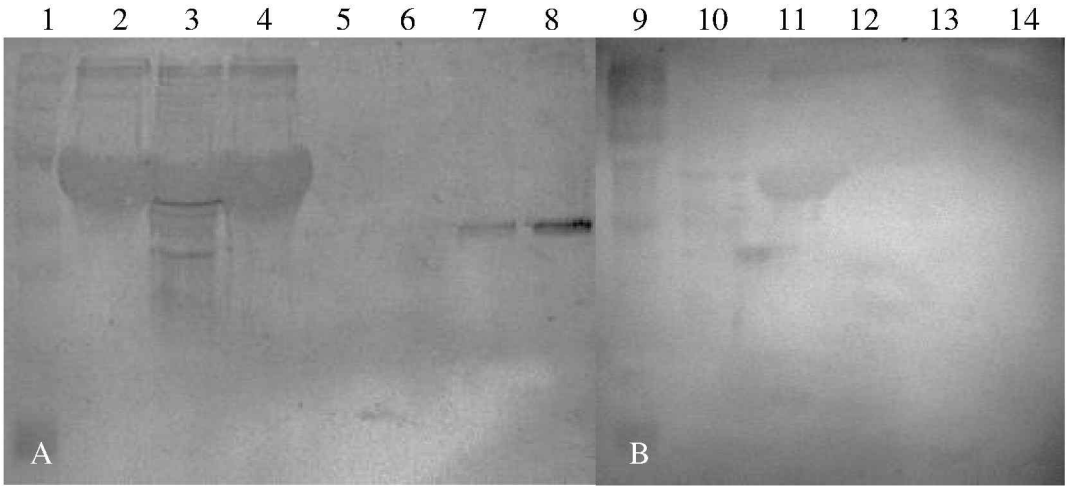


Figure 7. Western blot analysis for PAI-2 in Day 14 bovine embryos and culture medium. Blot A is depicted in Figure 6 and was treated with goat anti-hPAI-2 IgG as the first antibody. In place of goat anti-hPAI-2 IgG, blot B was treated with goat non-specific IgG as the first antibody. Lanes are molecular mass markers (1 and 9), non-conditioned medium (2 and 11), embryo (3 and 10), conditioned medium (4), and 1 (5), 5 (6 and 12), 10 (7 and 13) and 50 (8 and 14) ng rhPAI-2.

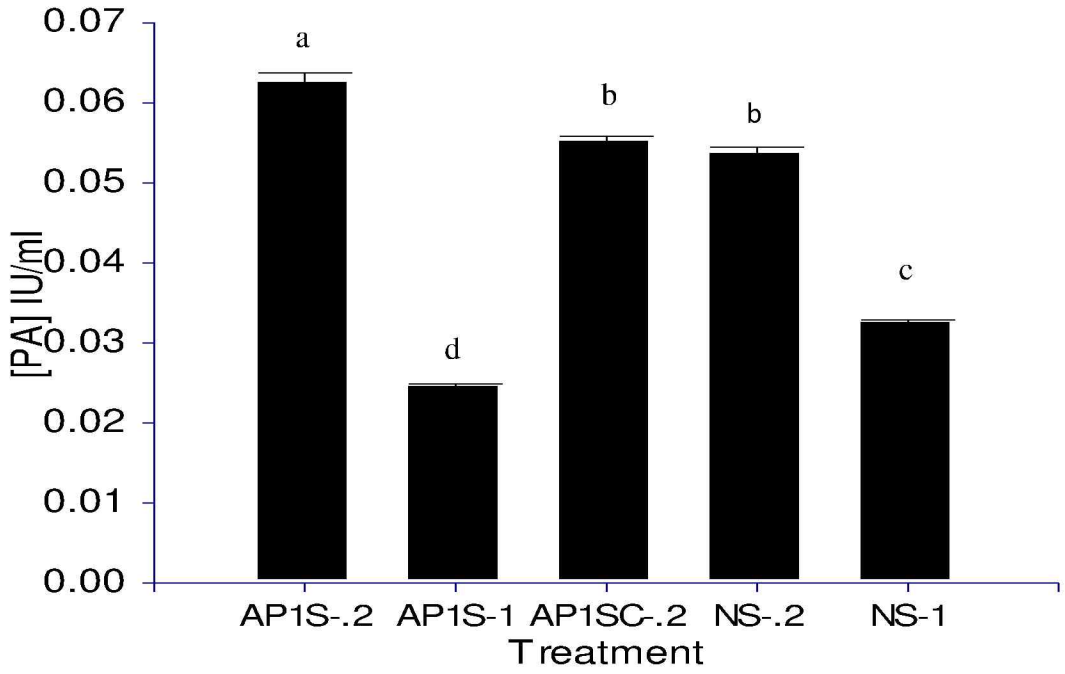


Figure 8. Plasminogen activator activities in pooled conditioned medium recovered from Day 14 bovine embryos and treated with 0.2 or 1.0 mg/ml rabbit anti-hPAI-1 IgG (AP1S-.2 and AP1S-1, respectively) obtained from Sigma Aldrich Co., 0.2 mg/ml rabbit anti-hPAI-1(AP1SC-.2) obtained from Santa Cruz Biotechnology, Inc., or 0.2 or 1.0 mg/ml non-specific IgG (NS-.2 and NS-1, respectively).
[a, b, c, d] Means without common superscripts differ ($P < 0.05$).

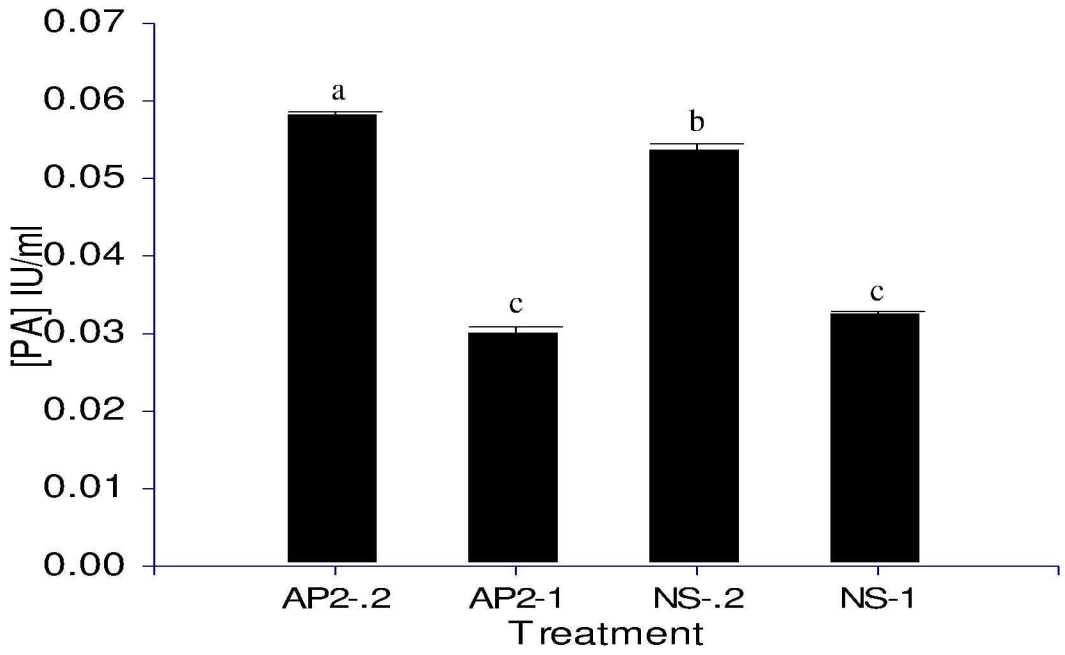


Figure 9. Plasminogen activator activities in pooled conditioned medium recovered from Day 14 bovine embryos and treated with 0.2 or 1.0 mg/ml goat anti-human PAI-2 IgG (AP2-.2 and AP2-1, respectively) or 0.2 or 1.0 mg/ml non-specific IgG (NS-.2 and NS-1, respectively).
[a, b, c] Means without common superscripts differ ($P<0.05$).

# DISCUSSION

Both rabbit antibodies used in the present study for Western blot analysis for PAI-1 detected two molecular mass forms in the embryo (61and 76 kD) and the high molecular mass form in conditioned medium. The PAI-1standard used in blots in this study was a recombinant human molecule with a mass of 43 kD (EMD Biosciences, Inc.) however endogenous PAI-1 has a molecular mass of 50 kD due to glycosylation (Dellas et al., 2005). Equation of the line calculations generated from our log molecular mass vs. Rm plots assigned the PAI-1 standard a mass of 48 kD, 5 kD or approximately 10% greater than the actual mass. Therefore, calculations from our plots appear to be overestimating molecular mass. Nevertheless, the lower molecular mass form may be the native PAI-1 molecule. The 76 kD form is in all likelihood a uPA-PAI-1 complex as its mass approximates that assigned to the high molecular mass plasminogen-dependent caseinolytic protease observed by (Dyk and Menino, 1991).

Western blot analysis of PAI-2 using goat anti-human PAI-2 primary antibody from American Diagnostica, Inc. detected two PAI-2 bands in the embryo (66 kD and 52 kD), closely corresponding to the HMW and LMW PAI-2 forms observed endogenously (Astedt et al., 1998). It is very likely the embryo has both of these molecules because the LMW PAI-2 is intracellular and non-glycosylated whereas the HMW is the glycosylated, secreted form (Astedt et al., 1998). The HMW species appears to have more significant staining than the 52 kD band suggesting the glycosylated form of PAI-2 is in greater abundance. The HMW mass PAI-2 band also

was observed in embryo-conditioned medium further confirming its identity as the secreted glycosylated form.

When primary antibodies were replaced with non-specific IgG, no positive signals were observed in embryos or conditioned medium. Likewise when non-conditioned medium was probed with primary antibodies to PAI-1 and -2 no positive signals were observed. The shadows seen on the blots are due to BSA which was a component of the culture medium and the medium used to pick up and store the embryos.

Results of the immunoneutralization assay confirm the presence of both PAI-1 and -2 in conditioned medium. The increase in activity when conditioned medium was treated with antibodies to PAI-1 and -2 suggests the antibodies prevent PAI from interacting with PA, thereby allowing development of greater activity. Bartlett and Menino (1993) used a similar assay and reported Day 7, 9, and 11 ovine embryos produced uPA (50 kD) and likely PAI-2 that enters in a complex with uPA (82 kD). When NSIgG was used in place of primary antibodies comparably lower PA activities were generated. IgG used at 1mg/ml seemed to have a non-specific effect on reducing PA activity compared to 0.2 mg/ml. This may have been due to a total protein effect where the greater amount of protein as IgG competed with the casein for PA-directed proteolysis.

Little is known about the role of PAI-1 and -2 in early development. Bartlett and Menino (1993) in sheep, like Dyk and Menino (1991) with cattle, have identified similar PAI molecules produced during the post-hatching, early embryo elongation phase of development. Research in our laboratory has shown that PAI-1 inhibits

cellular outgrowth from the inner cell mass and development of extraembryonic endoderm, hence it may function to regulate cell migration events in the early embryo. Alternatively these inhibitors may have some role in limiting extracellular proteolysis to control the degree of penetration between the trophectoderm and the endometrium during embryo attachment. Kawata et al. (1996) suggested PAI-2 may have a role in protecting the Day 15 mouse embryo from possible protease attack in the amniotic fluid. They further suggested PAI-2 may play a role in structurally maintaining and protecting the surfaces of certain developing organs in the embryo.

The plasminogen/plasmin system is involved in several aspects of mammalian reproduction such as spermatogenesis, ovulation, fertilization, embryonic development, implantation, and pregnancy. The results of the present study suggest both PAI-1 and PAI-2 are present in Day 14 bovine embryos. Pregnancy diagnosis methods for cattle would be greatly advanced if PAI levels reached detectable concentrations in the plasma. Current blood-based pregnancy diagnostic kits for cattle are sometimes not a feasible option due to variability in the accuracy of detecting an actual pregnancy. Although the current study suggests PAI-1 is secreted by the embryo, it would probably not be the best choice because of reasonably high concentrations already present in the plasma. PAI-2 however, as observed in the human by Astedt et al. (1998), would only be secreted by the embryo and placenta and, if produced in high enough amounts, could serve as the basis for a diagnostic assay for pregnancy detection. PAI-2 would also be a good candidate if it was secreted by the embryo earlier in gestation compared to other methods not useful until Day 30 of pregnancy. Although, the current study demonstrates PAI-1 and -2 are

produced by Day 14 bovine embryos, further research is needed to determine if either of these proteins would be feasible to develop as diagnostic assays for pregnancy detection in cattle.

## BIBLIOGRAPHY

Aflalo, E.D., Sod-Moriah, U.A., Potashnik, G., Har-Vardi, I. 2005. Expression of Plasminogen Activators in Preimplantation Rat Embryos Developed *in vivo* and *in vitro. Reproductive Biology and Enocrinology* 3: 7-15.

Arcellana-Panlilio, M.Y., Schultz, G.A. 1993. Analysis of Messenger RNA. *Methods in Enzymology* 225: 303-321.

Astedt, B., Lindoff, C., Lecander, I. 1998. Significance of Plasmingoen Activator Inhibitor of Placental Type (PAI-2) in Pregnancy. *Seminars in Thrombosis and Hemostasis* 24: 431-435.

Baker, M.S., Bleakley, P., Woodrow, G.C. 1990. Inhibition of Cancer Cell Urokinase Plasminogen Activator by its Specific Inhibitor PAI-2 and Subsequent Effects on Extracellular Matrix Degradation. *Cancer Res.* 50: 4676-4684.

Bartlett, S.E., Menino, A.R. 1993. Partial Characterization of the Plasminogen Activator Produced by Ovine Embryos in Vitro. *Biology of Reproduction* 49: 381-386.

Berg, D.A., Menino, A.R. 1992. Bovine Embryos Produce a Urokinase-Type Plasminogen Activator.*Molecular Reproduction and Development* 31:14-19.

Binder, B.R., Christ, G., Gruber, F., Crubic, N., Hufnagl, P., Krebs, M., Mihaly, J., Prager, G.W. 2002. Plasminogen Activator Inhibitor 1: Physiological and Pathophysiological Roles. *News Physiol Sci* 17: 56-61.

Bjerrum, m.D., Ramlau, J., Clemmesen, I., Agnete Ingild, Bøg-Hansen, T.C. 1975. An Artefact in Quantitative Immunoelectrophoresis of Spectrin Caused by Proteolytic Activity in Antibody Preparations. *Scandinavian Journal of Immunology.* 4:81-88.

Chomczynski, P., Sacchi, N. 1987. Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Analytical Biochemistry.* 162: 156-159.

Castellino, F.J., Ploplis, V.A. 2005. Structure and Function of the Plasminogen/Plasmin System. *Thromb Haemost* 93: 647-654.

Cochran, B.J., Guanawardhana, L.P., Vine, K.L., Lee, J.A., Lobov, S., Ranson, M. 2009. The CD-Loop of PAI-2 (SERPINB2) is Redundant in the Targeting, Inhibition, and Clearance of Cell Surface u-PA Activity. *BMC Biotechnology* 9.

Croucher, D. R., Saunders, D.N., Stillfried, G.E., Ranson, M. 2007. A Structural Basis for Differential Cell Signaling by PAI-1 and PAI-2 in Breast Cancer Cells. *Biochem. J.* 408: 203-210.

Danø, K., Andreason, P.A., Grøndahl-Hanson, J., Kristensen, P., Nielson, L.S., Skriver, L. 1985. Plasminogen Activators, Tissue Degradation, and Cancer. Adv. Cancer Res. 44:139-266.

Dellas, C., Loskutoff, D.J.2005, Historical Analysis of PAI-1 from its Discovery to its Potential Role in Cell Motility and Disease. *Thromb Haemost* 93:631-640.

Devin, J.K., Johnson, J.E., Eren, M., Gleaves, L.A., Bradham, W.S., Bloodworth, J.R., Vaughan, D.E. 2007. Transgenic Over-expression of Plasminogen Activator Inhibitor-1 Promotes the Development of Polycystic Ovarian Changes in Female Mice. *J. Molecular Endocrinology* 39: 9-16.

Dupont, D.M., Madsen, J.P., Kristensen, T., Bodker, J.S., Blouse, G.E., Wind, T., Andreason, P.A. 2009. Biochemical Properties of Plasminogen Activator Inhibitor-1. *Frontiers in Bioscience* 14:1337-1361.

Dyk, A.R., Menino, A.R. 1991. Electrophoretic Characterization of the Plasminogen Activator Produced by Bovine Blastocyts. *J. Reprod. Fert.* 93: 483-489.

Egbert, K.O. K., Baker, M.S., Bunn, C.L. 1995. Biological and Clinical Aspects of Plasminogen Activator Inhibitor Type 2. *Blood J.* 86: 4007-4024.

Egbert, K.O. K., Thang, C.T., Gudinchet, A., Hauert, J., Nicoloso, G., Genton, C., Welti, H., Bechmann, F. 1987. Fibrinolysis in Pregnancy: A Study of Plasminogen Activator Inhibitors. *Blood J.* 69: 460-466.

Fazleabas, A.T., Geisert, R.D., Bazer, F.W., Roberts, R.M., 1983. Relationship Between Release of Plasminogen Activator and Estrogen by Blastocysts and Secretion of Plasmin Inhibitor by Uterine Endometrium in the Pregnant Pig. *Biology of Reproduction* 29: 225-238.

Gunnarson, M., Lecander, I., Abrahamsson, P.A. 1999. Factors Affecting Plasminogen Activator System in Human Testis, as Demonstrated by In-situ Hybridization and Immunohistochemistry. *Molecular Human Reproduction* 5: 934-940.

Hekman, C.M., Loskutoff, D.J., 1988. Kinetic Analysis of the Interactions Between Plasminogen Activator 1 and Both Urokinase and Tissue Plasmiogen Activator. *Arch. Biochem Biophys* 262:199-210.

Hintze,J.2007. NCSS 2007. NCSS, LLC. Kaysville, Utah, USA. www.ncss.com

Huarte, J., Belin, D., Bosco, D., Sappino, A.P., Vassalli, J.D. 1987. Plasminogen Activator and Mouse Spermatozoa: Urokinase Synthesis in the Male Genital Tract and Binding of the Enzyme to the Sperm Cell Surface. *J. Cell Biology* 104: 1281-1289.

Irving-Rodgers, H.F., Catanzariti, K.D., Aspden, W.J., D'occhio, M.J., Rodgers, R.J., 2006. Remodeling of the Etracellular Matrix at Ovulation of the Bovine Ovarian Follicle. *Molecular Reproduction and Development* 73:1292-1302.

Kaaekuahiwi, M.A., Menino, A.R. 1990. Relationship between Plasminogen Activator Production and Bovine Embryo Development in Vitro. *J. Anim Sci.* 68: 2009-2014.

Kawano, T., Morimoto, K., Uemura, Y. 1968. Urokinase Inhibitor in Human Placenta. *Nature*.217: 253-254.

Kawata, Y., Mimuro, J., Kaneko, M., Shimada, K., Sakata, Y. 1996. Expression of Plasminogen Activator Inhibitor 2in the Adult and Embryonic Mouse Tissues. *Thromb Haemost* 76:569-576.

Kohler, H.P., Grant, P.J. 2000. Plasminogen-Activator Inhibitor Type 1 and Coronary Artery Disease. *N. Eng J. Medicine* 342: 1792-1801.

Leonardsson, G., Peng, X.R., Liu, K., Nordstrom, L., Carmeliet, P., Mulligan, R., Collen, D., Ny, T. 1995. Ovulation Efficiency is Reduced in Mice that Lack Plasminogen Activator Gene Function: Functional Redundancy Among Physiological Plasminogen Activators. *J. of Physiology* 92: 12446-12450.

Mackay, A.R., Corbitt, R.H., Hartzler, J.L.1990. Basement Membrane Type IV Collagen Degradation: Evidence for the Involvement of a Proteolytic Cascade Independent of Metalloproteinases. *Cancer Research* 50:5997-6001.

Meekins, J.W., Pijnenborg, R., Hanssens, M., McFadyen, I.R., van Asshe, A. 1994. A Study of Placental bed Spiral Arteries and Trophoblast Invasion in Normal and Severe Pre-eclamptic Pregnancies. *Br J. Obstet Gynaecol* 101:669-674

Medcalf, R.L., Van den Berg, E., Schleuning, W.-D. 1988. Glucocorticoid-Modulated Gene Expression of Tissue-and Urinary-Type Plasminogen Activator and Plasminogen Activator Inhibitor 1 and 2. *J. Cell Biology* 106: 971-978.

Mehta, R., Shapiro, A.D. 2008. Plasminogen Activator Inhibitor Type 1 Deficiency. *Haemophilia* 14: 1255-1260.

Menino, A.R., Williams, J.S. 1987. Activation of Plasminogen by the Early Bovine Embryo. *Biology of Reproduction* 36:1289-1295.

Miles, L.A., Hawley, S.B., Biak, N., Andronicos, N.M., Castellino, F.J., Parmer, R.J. 2005. Plasminogen Receptors: The Sine Qua Non of Cell Surface Plasminogen Activation.*Frontiers in Bioscience* 10: 1754-1762.

Mondejar, I., Grullon, L.A., Garcia-Vasquez, F.A., Romar, R., Coy, P. 2012. Fertilization Outcome Could be Regulated by Binding of Oviductal Plasminogen to Oocytes and by Releasing of Plasminogen Activators During Interplay Between Gametes. *Fertility and Sterility* 97: 453-461.

Mullins, D. E.,Bazer, F. W. and Roberts, R. M. 1980.Secretion of a Progesterone Induced Inhibitor of Plasminogen Activator by the Porcine Uterus. *Cell* 20: 865-872.

Ohlsson, M., Leonardsso, X.C. J., Feng, P., Ny, T. 1993. Transcriptional Regulation of the Rat Tissue Type Plasminogen Activator Gene: Localization of DNA Elements and Nuclear Factors Mediating Constitutive and Cyclic AMP-Induced Expression. *Mol. Cell Biol.* 13: 266-275.

Rekkas, A., Constantino., Besenfelder, Urban., Havlicek, Vitezslav., Vainas, Emmanuel., Havlicek, Vitezslav.2002. Plasminogen Activator Activity in Cortical Granules of Bovine Oocytes During *In-Vitro* Maturation. *Theriogenology* 57: 1897-1905.

Roes, E.M., Sweep, C.G. F., Thomas, C.M., Zusterzeel, P.L., Moespot, A.G., Peters, W.H. Steegers, E.A. 2002. Levels of Plasminogen Activators and their Inhibitors in Maternal and Umbilical Cord Plasma in Severe Preeclampsia. *Am. J. Obstet. Gynecol.* 187: 1019-1025.

Sappino, A.P., Huarte, J., Belin, D., Vassalli, J.D. 1989. Plasminogen Activators in Tissue Remodeling and Invasion: mRNA Localization in Mouse Ovaries and Implanting Embryos. *J. Cell Biology* 109: 2471-2479.

Schafer, B.M., Maier, K., Eickoff, U., Todd, R.F., Kramer, M.D. 1994. Plasminogen Activation in Healing human Wounds. *A. J. of Pathology* 144: 1269-1280.

Senger, Phillip L. *Pathways to Pregnancy and Parturition.* Pullman: Current Conceptions, 2005.

Sier, C.F., Verspaget, H.W., Griffioen, G., Ganesh, S., Vloedgraven, H.J., Lamers, C.B. 1993. Plasminogen Acrivators in Normal Tissue and Carcinomas of the Human Oesophagus and Stomach. *Gut* 34: 80-85.

Silverman, G.A., Bird, P.I., Carrell, R.W., Church, F.C., Coughlin, P.B., Gettins, P.G.W., Irving, J.A., Lomas, D.A., Luke,C.J., Moyer, R.W., Pemberton, P.A., O'Donnell, E.R., Salvesen, G.S., Travis, J., Whisstock, J.C. 2001. The Serpins are an Expanding Superfamily of Structurally Similar but Functionally Diverse Proteins. *J. of Biol. Chem.* 276:33293-33296.

Strickland, S., Reich, E.,Sherman, MI.1976. Plasminogen Activator in Early Embryogenesis: Enzyme Production by Trophoblast and Parietal Endoderm. *Cell.* 9:231-240.

Vassalli, J.D., Sappino, A.P., Belin, D. 1991.The Plasminogen Activator/Plasmin System. *J. Clin. Invest.* 88: 1067-1072.

Whitside, J. E., Kan, M., Jackson, M.M., Thompson, J.G., McNaughton, C., Herington, C.A., Harvey, B.M. 2001. Urokinase-type Plasminogen Activator (uPA) and Matrix Metalloproteinase-9 (MMP-9) Expression and Activity During Early Embryo Development in the Cow. *Anatomy and Embryology.* 204:477-483.

Xunbin, H., Wenjia, X., Chengliang, X., Dunzhen, X., Jiyun, S., Jieling, Z. 1997. Studies on the Relationship Between Urokinase Plasminogen Activator (uPA) and Human Sperm Motility. *J. of Tongji* 17: 213-217.

Ye, R.D., Ahern, S.M., Le Beau, M.M., Lebo, R.V., Sadler, J.E. 1989. Structure of the Gene for Human Plasminogen Activator Inhibitor 2. The Nearest Mammalian Homologue of Chicken Ovalbumin. *Biology and Chemistry* 264:5495-5502.

Yi-xun, L., Ya-xiong, C., Fa-wu, S., Qiang, F.1995. Studies on the Role of Plasminogen Activators and Plasminogen Activator Inhibitor Type-1 in Rat Corpus Luteum of Pregnancy. *Biology of Reproduction* 53: 1131-1138.

Yi-xun, L. 2004. Plasminogen Activator/Plasmingoen Activator Inhibitors in Ovarian Physiology. *Frontiers in Bioscience* 9: 3356-3373.

Zhang, T., Guo, C.X., Hu, Z.H., Lui, Y.X. 1997. Localization of Plasminogen Activator and Inhibitor, LH and Androgen Receptors and Inhibin Subunits in Monkey Epididymis. *Molecular Human Reproduction* 3: 945-952.

Zhao-Yuan, H., Yi-Xun, L., Kui, L., K., Byrne, S., Tor, N., Quing, F., Ockleford, C. 1999. Expression of Tissue Type and Urokinase Type Plasmingen Activators as Well as Plasminogen Activator Inhibitor Type-1 and Type-2 in Human and Rhesus Monkey Placenta. *J. Anat.* 194: 183-195.

# APPENDIX

**Domestic Animal Reproduction Laboratory**
**Department of Animal and Rangeland Sciences**
**Oregon State University**

**R. Mendoza**
**June, 2013**

***Western Blot Gel Electrophoresis Protocol***

1. Make sure ice container for electrophoresis is frozen first and foremost
2. Gel Casting:
    - Cast discontinuous polyacrylamide gel on casting stand
        - Cast 10% resolving gel for 45 min
            - Add $ddH_2O$ to top of gel
            - Remove $ddH_2O$ with blotting paper before pouring stacking gel
        - Cast 4% stacking gel for 35min
            - Wash wells with $ddH_2O$ and dry well before adding samples and standards.
3. Assemble gel electrophoresis stand and submerge in gel running buffer
4. Perform electrophoresis with samples and standards @ 200 V for 35 min
5. Prepare blotting pad:
    - Soak sponge pads, filter papers, and nitrocellulose membrane in transfer buffer for at least ½ hr prior to transfer.
6. Upon completion of gel electrophoresis equilibrate gel in transfer buffer for 30 min.
7. Cassette Assembly:
    a. Lay black side of cassette down and perform the fallowing steps in THIS EXACT ORDER:
        1. Lay down one of the soaked sponge fiber pads
        2. Lay down one of the sheets of filter paper

(Soak again with transfer buffer)

3. Lay down gel (Soak again with transfer buffer)
4. Lay down nitrocellulose (shiny side up; ensure there are no bubbles by rolling a glass Pasteur pipette over the nitrocellulose membrane, then soak with transfer buffer)
5. Lay down second 2nd filter paper and soak with transfer buffer
6. Lay down 2nd sponge fiber pad and soak with transfer buffer

b. Close cassette and insert it into electrode module so that the black side of the cassette is facing the black side of the module

c. Set whole module into buffer tank and place ice tray in tank next to module. Fill to the top of module with transfer buffer

- Note: If only 1 liter of transfer buffer is made, the buffer used to equilibrate and soak the gel and pads may have to be reused

d. Place entire tank over mixer plate and use a small stir bar to keep the temperature in the tank equilibrated

e. Perform transfer electrophoresis at 100V for 1 h. Check the buffer change after each run to make sure nothing has changed.

**Antibody work procedures:**

1. Block nitrocellulose membrane for 1hr in 1X TTBS (Tris Buffered Saline) +3% BSA (a total volume of 20 ml covers entire membrane)
2. Wash nitrocellulose membrane for 30 min in 1X TTBS with 3 changes
3. Add 1st antibody suspended in 1X TTBS+0.5 % BSA for 2 h

4. Wash nitrocellulose membrane for 30 min in 1X TTBS with 3 changes
5. Add 2$^{nd}$ antibody suspended in 1X TTBS+0.5% BSA and for a minimum of 1.5 h
6. Wash nitrocellulose membrane for 30 min in 1X TTBS with 3 changes
7. Add alkaline phosphatase conjugate color development solution (Bio-Rad Alkaline Phosphatase conjugate substrate kit (Cat #: 170-6432))
8. Add dd$H_20$ to stop reaction if needed (wash for 10 min with 1 change)
9. Air dry nitrocellulose membrane
10. Use image software to obtain image of fully developed nitrocellulose membrane

## *Immunoneutralization assay protocol*

(Caseinolytic Assay)

1. Create Urokinase Standards:
   a. 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0 IU/ml
   b. Make 300 µl volumes of each standard
      i. For example, 10 IU stock = 300µl Urokinase, 5 IU= 150 µl Urokinase + 150 µl Ham's F-12+0.15% BSA, etc.
   c. Label microcentrifuge tubes and refrigerate at 4°C when done making standards (good for 1-2 weeks)
   d. Create all sample standards as needed and in volumes needed
2. Plate set up:
   a. Calculate the number of plates you needed based on the number of samples and standards that will be used. Run all samples in triplicate.
      i. Example: For a standard curve assay that consists of eight standards and each sample is run in triplicate, you need 3 plates
3. Make casein agar gel
   a. Each plate holds 15ml, so for 3 plates you will need at least 45ml. It is always good to make extra to make up for evaporated materials. Make 15ml extra when 45 ml are needed exactly.
4. Pour plates:
   a. The casein-agar melt is a 50:50 mix of 2% agarose (Sigma Aldrich) and 2% skim milk.
      i. Example: 60 ml= 30 ml agarose+30 ml skim milk

b. Using a 25 ml plastic disposable serological pipette, dispense 15 ml into each plate and allow to solidify for 15 minutes

c. Using suction and an appropriate hole punch, make holes in the center of each well and make the holes as perfectly round and central as possible

5. While the gel is solidifying make human plasminogen stock

a. For this assay, a final concentration of 120 µg/ml is needed

- The stock concentration comes in 1.2 mg/ml. Calculate as follows:

$$C_1V_1 = C_2V_2$$

- Dilute with H-F12+0.15% BSA

i. For example, 400µl final volume = 360µl diluent + 40µl of 1.2 mg/ml human plasminogen stock.

- *Note:* Add plasminogen to diluent and not in reverse order

b. Label 500 µl microcentrifuge tubes with their respective standard or sample name and add 45µl of human plasminogen to each tube, then add 30µl of respective standard or sample to each tube and mix.

c. Iincubate samples for 15 min @ 39°C in 5% $CO_2$

d. When sample incubation time is completed, pipette 20µl to each well created in the agar gel plates

6. Place lids on plates without completely covering and without touching the casein-agar gel. Incubate plates at room temperature for 24 hrs.

7. At 24 hrs fix plates with 3% acetic acid for 15 min. Rinse acetic acid solution

from plates using a gentle flow of tap water and measure lytic zone ring diameters in mm using an electronic digital caliper.

**Reagents:**

**1.** Caseinolytic Assay Buffer:

a.

| **Reagent:** | **Molarity** | **g/L** |
|---|---|---|
| $CaCl_2*2H_2O$ | .0013 | 0.195 |
| Glycine | .10 | 7.51 |
| TRIS | .038 | 4.6 |

**2.** Dissolve 2g of non-fat dry milk in 100 ml of assay buffer (2%)

a. Dissolve 2g of agarose (always use agarose from Sigma for routine use) in 100 ml of $ddH_2O$ (2%)

**3.** Heat agarose solution to boiling. Cover Erlenmeyer flask with a small glass evaporating dish to prevent excessive evaporation. Allow heating to continue 20 sec after boiling has initiated and allow the glass plate to rattle as an indicator.

**4.** Combine 2% solutions.

a. Bring both solutions to 60°C and combine

- Note[1]: add agarose to skim milk as it stirs on a warmer plate. DO NOT add skim milk to agarose.
- Note[2]: DO NOT stir to the point where the solution creates bubbles because they will ruin the integrity of the plate when solidified.

- Note[3]: Be quick with the solution and pour onto plate using a 25 ml serological plastic pipette.

***mRNA extraction procedure from Day14 Bovine embryo***

1. Add to frozen embryos:
    a. 2:1 volume of equal mixture of water-saturated phenol and Sevag's solution (24:1 chloroform-isoamyl alcohol)
    b. For example, if embryo volume is 100 µL, add 200 µL volume of phenol (100 µL):Sevag's solution (100 µL)
    c. 10 µg of *Escherichia coli* rRNA at 2.5 µg/µl
2. Vortex for 15 sec 2X
3. Spin in microcentrifuge for 5-10 min at 14,000 g to separate phases
4. Transfer aqueous phase of supernatant to 1.6 ml microcentrifuge tube and add 2X's volume of Sevag's solution to re-extract
5. Vortex for 15 sec 2X
6. Spin in microcentrifuge for 5-10 min at 14,000 g to separate phases
7. Remove aqueous phase and transfer to 1.6 ml microcentrifuge tube
8. Add 0.1X 3M sodium acetate (mix with pipetter) and 2.5X of cold 95% ethanol
9. Allow solution to precipitate overnight at -20°C
10. Centrifuge precipitate @ 14,000g for 30 min
11. Wash pellet in 70% ethanol
12. Spin in microcentrifuge for 15 min at 10,000 g
13. Air dry resulting pellet

14. When pellet is completely dry, add 10µl of autoclaved $ddH_2O$, heat at 70°C for 10 min, quick spin and freeze at -80 °C.

***Reverse Transcription-Polymerase Chain Reaction Protocol***

A. Reverse transcription reaction

1. To 500 µL sterile tube add:
    a. 1µL oligo (dT) $_{12\text{-}18}$ (500µg/ml) (Source: Invitrogen by life Technologies Co.)
    b. 1µg embryo mRNA (can be total RNA)
    c. 10 µL Sterile $ddH_2O$ to make a total of 12 µL
        i. Heat mixture to 70°C for 10 min
        ii. Quick chill on ice
        iii. Quick spin (microcentrifuge @ top speed for 30-60 sec)
2. To the reaction tube, add:
    a. 4 µL 5X RT buffer (supplied in kit: 250 mM Tris-HCl. pH 8.3 at 42°C; 300mM KCl;15mM $MgCl_2$)
    b. 2µL 0.1m DTT (supplied in kit)
    c. 1µL dNTP stock (10mM each dATP, dCTP, dTTP, dGTP @ neutral pH)
        i. Mix contents by gentle vortexing/ collect mixture by brief centrifugation
        ii. Incubate mixture @42°C for 1-2 h

iii. Incubate mixture @ 95°C for 10 min (this is done to denature the enzyme/stop rxn)

iv. Add 30 µL autoclaved dd$H_2O$ to bring to 50µL volume

v. Store cDNA @ -20°C

B. Polymerase Chain Reaction (for a 50µL reaction volume use a 500µL centrifuge tube.

a. 5.0 µL 10X PCR buffer (100m*M* Tris-HCl, pH 8.3;500m*M* KCL; 1mg/ml BSA)

b. 5.0 µL 15m*M* $MgCl_2$

c. 1.0 µL 10m*M* dNTPs stock (see RT protocol)

d. 0.2 µL Taq DNA Polymerase

e. 1.0 µL (p mol) of each primer (made up as 100 µ*M* stock)

f. 5.0 µL Reversed-transcribed product

g. Water to (31.8µL) 50 µL volume

h. Mix contents; overlay with equal content of paraffin oil (light mineral oil)

- Negative control = water
- Positive control = cDNA that gives a positive signal = ovarian cDNA
- Place in Thermocycler programmed to give the following temperature profile:
    - 4 min at 94°C
    - Denaturation for 30 sec to 1 min at 94°C, annealing for 1 to 2 min at 55°C to 72°C, and extension for 2 min at 72°C
    - Repeat second step for 25 to 40 cycles
    - Incubate for 7 min at 72°C

- Soak at 4°C

PCR Buffer Prep:

| Stock Concentration: | Volume Stock: Final | Concentration in 1.0 ml |
|---|---|---|
| 1.0 *M* Tris-HCl, pH 8.3 | 100µL | 100m*M* |
| 1.0 *M* KCL | 500µL | 500m*M* |
| 10 mg/ml BSA | 100µL | 1mg/ml |
| Add $ddH_2O$ | 300µL | 1.0 ml |

Note: Buffer stored at -20°C

***PCR Product Gel Electrophoresis:***

- 2% Agarose gel
  - 80 ml of 2% agarose gel solution
    - 1.6 g agarose powder for routine use (Sigma Aldrich)
    - 1.6 ml (50X) TAE
    - 78.4 ml $ddH_2O$
- 50X TAE (Tris-Acetate) Electrophoresis Gel Buffer
  - Working solution:
    - 1X:0.04M Tris-acetate
    - 0.001M EDTA
  - Concentrated Stock Solution (50X): 1L
    - 242 g Tris Base
    - 57.1 ml glacial (straight from the glass jug) acetic acid
    - 100 ml 0.05M EDTA (pH 8.0)

- 1X TAE Gel buffer: 1L
  - 980 ml $ddH_2O$
  - 20 ml 50X TAE

Step by Step Procedure:

  - Note: Make sure all materials being used are completely clean and rinsed in $ddH_2O$ before use

- Set up casting stand
  - Set up casting stand in between two black rubber blocks
- Mix gel ingredients
  - Pour $ddH_2O$ into beaker and add a stir bar to it
  - Set it over plate warmer and begin mixing (stir and make sure no air bubbles are forming)
  - Add agarose powder
  - Add 50XTAE
  - Heat up to boiling point in microwave by setting the microwave to high and heating for approximately 5 min
  - Make sure all agarose powder is homogenized and cool solution down to $60^0$ C
  - Pour on casting stand and set in appropriate comb
  - Allow gel to solidify for approximately 20-25 min
- Meanwhile the gel solidifies thaw samples that are going to be used
- Mix samples with respective volumes
  - Molecular weight markers:

        - 2 µl Gel loading buffer+2 µl Molecular weight ladder+8 µl $ddH_2O$
    - Samples:
        - 2 µl Gel loading buffer+10 µl sample (make sure no oil goes in and that the sample is pure)
- Once gel has solidified, carefully load samples using the p10 and make sure to not puncture through the bottom of the gel. Also, DO NOT allow for any bubbles to form.
- Set casting stand into buffer tank and fill the tank until the buffer level is at least ¼ inch above the surface of the gel
- Connect electrical supply and set to 100V. Allow electrophoresis to continue for 50 minor until the leading marker is close to the edge of the gel
- Once electrophoresis has been completed, stain the gel with SYBR Green stain
    - DO NOT expose SYBR Green stock to the ambient light!!!!
    - Thaw stock from freezer and mix with 1X TAE Gel buffer
        - 1:10,000 dilution : i.e., 150 ml stain solution
            - 15 µl Stock
            - 150 ml 1X TAE Gel buffer
    - Allow to stain for approximately 15 min in a dark place while mixing on small mixer (cabinet or drawer)
    - Once stain is completed dip entire gel in $ddH_2O$
    - Photograph the gel in GEL LOGIC 212 PRO computer camera system under the UV Trans and SYBR Green settings