AN ANALYSIS OF ASPARTIC PEPTIDASES EXPRESSED BY TROPHOBLASTS AND PLACENTA OF EVEN-TOED UNGULATES

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Bhanu Prakash V.L. Telugu

Dr. Jonathan A. Green, Thesis supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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presented by Bhanu Prakash, V.L. Telugu, a candidate for the degree of doctor of philosophy, and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Jonathan A. Green

Dr. R. Michael Roberts

Dr. Randall S. Prather

Dr. Steven R. Van Doren

Dr. Dennis B. Lubahn

Dedicated to..

My Mother and Father

… for their invaluable love and support.

….for ingraining moral values and providing necessary eduction.

My Wife

…for her unconditional love and devotion to me.

…for her incessant support.

My Sister and Brother

…for being there for me always.

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Bhanu Prakash Telugu

Dr. Jonathan A. Green, Dissertation Supervisor

ABSTRACT

The studies discussed in this dissertation are focused on characterizing proteolytic enzymes known as aspartic peptidases (AP) that are expressed by the extraembryonic membranes of cattle, pigs and related ungulates. Most of the APs that were investigated in these experiments are members of the pregnancy- associated glycoprotein (PAG) gene family. In one chapter, however, an AP expressed by porcine embryos was partially characterized, but it remains unclear if the enzyme responsible for the activity belongs to the PAG group or to another AP grouping.

The Pregnancy Associated Glycoproteins (PAGs) represent a multigene family of trophoblast expressed proteins, found exclusively in the placenta of even-toed (hooved) mammals (*Artiodactyla* order). The ruminant PAGs can be classified into *ancient* and *modern* PAGs based on their genetic relationships with one another. However, the distinctions between these two groups are not limited to their phylogeny alone. There are clear differences in terms of purported enzymatic activity as well as transcriptional regulation of expression. Such differences in expression are evident in their distribution pattern, time of induction, and relative abundance of message. Many of the modern PAGs are predicted to be enzymatically inactive due to mutations in and around the catalytic center – particularly in the normally conserved sequences (F/I-D-T-G-S/T) surrounding

two aspartic acids known to be involved in the catalytic mechanism. In contrast, most of the ancient PAGs of ruminants and swine have all the hallmarks of typical APs.

Of the large number of PAGs discovered, the majority of them are expressed in ruminant ungulates such as cattle, sheep, etc. In cattle, multiple members of the PAG gene family have been cloned and the expressed sequences have been well characterized. However, the exact number of functional genes and the organization of their regulatory regions remained largely unknown. From our analysis of the bovine genome, which was recorded in chapter –II, we found 18 distinct PAG genes and 14 pseudogenes. Based on our preliminary analysis of the proximal promoter regions [500 base pairs (bp) upstream of the translational start point (TSP)], we found that this region is highly conserved in all the bovine PAG (boPAG) genes that were examined and, therefore, this region likely contains crucial regulatory elements of these genes. We also found that, within this critical region there are pockets of conserved sites that have consensus recognition sequences for transcription factors (TFs). Interestingly, we noted a disproportionate skewing of the number of such unique regions towards modern boPAGs, relative to ancient boPAGs. Presumably, these regions likely influence the observed differences in expression between ancient and modern boPAGs. We also gathered evidence by Real-time PCR and global analysis of expressed ESTs that confirm that boPAG-2 is the most abundant of all boPAGs. We also showed experimentally that Ets-2 and c-Rel related TFs have a potential role in the regulation of the boPAG-2 gene.

Although ancient boPAGs have retained all the hallmarks of proteolytically active APs, such activity has not been determined. In chapter –III, we documented the results from experiments on two ancient boPAG paralogs, boPAG -2 and -12. We studied

boPAG-2 because it is the most abundant of ancient PAGs, and we included its closest paralog boPAG-12 for comparison. We found that both boPAGs -2 and -12 had proteolytic activity, and that their activity was optimal under acidic pH conditions. We also illustrated differences in proteolytic activity and specificity towards substrates and in their relative affinity towards an AP inhibitor (pepstatin A).

Because the two studied ancient boPAGs displayed proteolytic activity, we sought to determine if the PAGs expressed in other species, especially pig, will have similar activity. The porcine PAGs (poPAGs) constitute a simple grouping with only two distinct PAG genes. One of the two PAGs, poPAG-2 contains an intact catalytic center and, based on molecular modeling studies, was predicted to be an active peptidase. In our studies described in chapter –IV, we found that indeed, poPAG-2 is an active AP. We also found that it followed the characteristics of traditional APs, such as having acidic pH optima, and being inhibited by pepstatinA. In comparison to the two bovine paralogs, boPAG -2 and -12 that were studied in chapter-III, poPAG-2 is a more robust enzyme.

The growing embryo actively secretes several molecules to fulfill a number of important biological roles. In chapter -V, we presented data that shows that the porcine embryo actively secretes one or more acid peptidases into the surrounding medium. We demonstrated that the AP activity can be readily measured in the medium conditioned by the culture of porcine embryos either individually or in pools for variable lengths of time. We also observed that such activity seemed to correlate with stage and quality of embryos (assessed morphologically) *in vitro.* We, therefore, believe that this proteolytic activity potentially could serve as a marker for developmental competence of the embryos.

ABBREVIATIONS

AP	Aspartic peptidase
PAG	Pregnancy-associated glycoprotein
bp	base pair
TSP	Translational start point
boPAG	bovine Pregnancy-associated glycoprotein
ovPAG	ovine Pregnancy-associated glycoprotein
caPAG	caprine Pregnancy-associated glycoprotein
poPAG	porcine Pregnancy-associated glycoprotein
pepF	pepsinogen F
TF	transcription factor
ICM	inner cell mass
LE	luminal epithelium
dpc	days post conception
TR	trophoblast
BNC	binucleated cell
MNC	mononuclear trophoblast cell
TNC	trinucleated cell
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$

ZP	zona pellucida
IG	integrin
ECM	extra cellular matrix
MMPs	matrix metalloproteinases
SPP-1	osteopontin
IGF	insulin like growth factor
IGFBP	insulin like growth factor binding protein
EGF	epidermal growth factor
TGF	transforming growth factor
Upa	urokinase type plasminogen activator
PA	plasminogen activator
α_2M	apha -2 macroglobulin
UPTI	urokinase type plasminogen inhibitor
TKDP	trophoblast kunitz domain protein
PSPB	pregnancy specific protein B
MYA	million years ago
UTR	untranslated region
CDS	coding sequence
EST	expressed sequence tag

TE	transposable element
ORF	open reading frame
dS	degree of synonymous changes per synonymous sites
YHWAG	tyrosine 3- monooxygenase / tryptophan- 5 - monoxygenase activation protein / gamma polypeptide
Q-PCR	quantitative real- time polymerase chain reaction
FRET	fluorescence resonance energy transfer
pBP	porcine basic protein
APA	acid peptidase activity
SP1	pregnancy specific β- 1 glycoprotein
mTBM	modified tris buffered medium
IVF	in vitro fertilization
BSA	bovine serum albumin
PBS	phosphate buffered saline
DMSO	dimethyl sulphoxide
STD	standard deviation
CV	coefficient of variation
ANOVA	analysis of variance

Chapter –I

Literature Review

Embryo and early conceptus development

Upon fertilization of the oocyte, a series of cell divisions occur that result in the formation of a mass of cells (the morula) encased within a glycoprotein coat, the zona pellucida (Machaty et al., 1998b). The morula is soon transformed into a hollow ball of cells, the blastocyst, which represents the first morphologically obvious cell differentiation event in early mammalian development. At this stage, the embryo consists of an 'inner cell mass', or 'ICM', and an outer sphere of cells, the trophoblast, surrounding a fluid filled cavity (blastocoelic cavity) (Figure 1). In some species (e.g. mouse, human) the ICM is appropriately named since it is literally internal, being covered by polar trophoblast on the one side, while the other side is exposed to the blastocoele fluid. However in other species (e.g. swine and ruminant ungulates), the term 'inner cell mass' is somewhat of a misnomer since these cells are actually contiguous with the trophoblast. Instead the analogous structure is named as germ disc or embryonic disc. This is because, once the blastocyst escapes from the zona pelucida the trophoblast cells overlying the ICM quickly degenerates exposing the ICM to the extraembryonic environment (Fléchon et al., 2004).

In regard to cell lineage specification, the trophoblast cells of the blastocyst contribute exclusively to the extraembryonic membranes, while the ICM develops into the embryo/fetus, but also contributes to the components of the yolk sac, amnion and

allantois (Fujimori et al., 2003; Hardy et al., 1989; Piotrowska et al., 2001; Piotrowska and Zernicka-Goetz, 2002).

Immediately after blastocyst formation, endoderm cells (arising from the ICM adjacent to the blastocoele) begin to spread along the inner surface of the trophoblast until they eventually line the blastocoelic cavity (Figure 1). This two layered structure (the bilaminar omphalopleure) creates the yolk sac cavity bound by extraembryonic endoderm and trophoblast (except in the region of the embryonic disc). Later in development, mesoderm in most species arising from ingression of embryonic ectoderm through the primitive streak of the embryonic disk begins to spread between the embryonic ectoderm and embryonic endoderm and between the trophectoderm and the extraembryonic endoderm (Figure 1). Outside the embryonic disk, the mesoderm/trophoblast combination comprises the 'chorion' or 'somatopleure'. The mesoderm + extraembryonic endoderm combination comprises the yolk sac 'splanchnopleure'. As these different tissues are formed, a space becomes created between them; this is the 'exocoelom' (Mossman, 1987).

The mesodermal tissue associated with the yolk sac endoderm soon develops a blood supply, thus establishing the first efficient maternal fetal exchange system: the yolk sac placenta. When the yolk sac is in contact with trophoblast, the structure is referred to as the 'trilaminar omphalopleure' or 'choriovitelline' membrane (Figure 1) (Jollie, 1990; Mossman, 1987). Later in development, the allantois, arising initially as splanchnopleure (in most species) grows as a projection into the exocoelom until it comes into contact with the chorion. Vascularization of the allantois to create the umbilical vessels results in the establishment of the chorioallantoic placenta (Figure 1). In many eutherian mammals,

the expanding allantois displaces the yolk sac from the trophoblast. Therefore, the yolk sac placenta formed early in pregnancy is soon superseded by the establishment of a chorioallantoic placenta. In some mammals (e.g. humans), a true yolk sac placenta is never formed. In others (e.g. rodents and lagomorphs), the yolk sac placenta persists until term. In these species, there is either a partial or complete inversion of cell layers such that the yolk sac endoderm actually forms an absorptive epithelium that faces the maternal tissues. This arrangement is often referred to as an "inverted yolk sac placenta" (Jollie, 1990).

Classification of Placental Membranes

Placentas have been categorized in a number of ways. Most of the classification systems are based on morphological characteristics such as the appearance of the extraembryonic tissues and associated organs, placental shape, or the type and number of cell layers between the maternal and fetal circulations (Carter and Enders, 2004; Leiser and Kaufmann, 1994; Steven and Morris, 1975). The three principal means of classification are based on:

I. Classification based on feto-maternal interdigitation

II. The gross shape and distribution of the chorionic tissues most intimately interacting with the uterine tissues.

III. The number of cell types separating the maternal and fetal circulations.

I. Classification of placenta based on the type of feto-maternal interdigitation

Folded, lamellar, trabecular, villous and labyrinthine are different terms used to describe the conformations of interacting tissues at the feto-maternal interface (Figure 2). The simplest form of association is the 'folded' type and perhaps the most extreme is the 'labyrinthine' form. A folded type of arrangement is seen in the placentation of swine, where the chorionic surface closely apposes the primary and secondary folds (later in pregnancy) of the uterus (King, 1993b). When the folds of the interacting surface are further drawn out, the result is a 'lamellate' organization, which is observed in carnivores such as the domestic cat. In this model, the maternal and fetal tissues form a series of tall, closely packed sheets (Leiser and Kobb, 1993). In the 'trabeculate' design, which is observed in some primates, the folds are incomplete and engage in secondary branching (King, 1993a). In line of growing complexity, a 'villous' configuration is observed in the placenta of humans (Wooding and Flint, 1994). In this type, the fetal tissue initiates a complex branching process that culminates in a three dimensional tree like structure with numerous slender villi. Finally, the 'labyrinthine' pattern is observed in the placenta of rodents. It consists of a complex three dimensional meshwork of vascularized fetal trophoblast creating cavities through which maternal blood flows (Pijnenborg et al., 1981). This arrangement has been described as being analogous to the "substance and pores of a sponge" (Kaufmann and Burton, 1994).

II. Classification based on placental shape:

There are four general placental types that have been established based on the *shape* of the placenta. These will be summarized briefly here.

A. Diffuse Placenta.

Examples of the diffuse placenta can be observed in the pig and horse (King, 1993b). In swine, the placenta is completely non-invasive. The chorionic villi are not localized to a particular region and are instead distributed nearly over the entire surface of the uterine lumen (Figure 3). The closely packed and convoluted chorionic villi yield an extensive surface area to facilitate movement of diffusible molecules between the maternal and fetal circulations and participate in the uptake of nutritional secretions from uterine glands. The equine placenta, while still considered diffuse, is distinct from the sow placenta in that it possesses localized regions of contact known as 'microcotyledons' and an invasive trophoblast population known as chorionic girdle cells that, upon migration into the uterine endometrium, can form transitory structures known as 'endometrial cups' (King, 1993b; Steven, 1982).

B. Cotyledonary Placenta.

Ruminant ungulates possess a 'cotyledonary' placenta (Steven and Morris, 1975). The cotyledons are vascularized villous trophoblasts that intercalate into aglandular structures in the uterine endometrium known as 'caruncles' (Figure 3). The fetal cotyledons begin to associate with maternal caruncles early in gestation and interdigitation of these tissues is well underway by Day 35 in ewes and Day 45 in cattle.

Together, the combined unit of cotyledons and caruncles is referred to as a "placentome" (King, 1993b).

C. Zonary Placenta.

Numerous species, including carnivores, possess a 'zonary' placenta. This type consists of a band of chorion surrounding the middle of the fetus (Figure 3). This zone of chorion forming the most intimate contact with the maternal uterus is the basis for the name of this placenta type (Bjorkman, 1973; Carter and Enders, 2004; Wooding and Flint, 1994).

D. Discoid Placenta.

Higher primates and rodents possess a placenta that is characterized by one or more distinct discs comprised of localized regions of fetal chorion that interface with uterine tissues, thus the descriptive name 'discoid' (Figure 3) (Carter and Enders, 2004; King, 1993a; Pijnenborg et al., 1981; Wooding and Flint, 1994).

III. Placental classification based on the number of intact cell layers at the fetal-maternal interface:

This system was first proposed by O. Grosser in 1909 and 1927 (Grosser, 1927, 1909) and is described and referenced by Mossman (Mossman, 1987), Steven (Steven and Morris, 1975) and Amoroso (Amoroso, 1952b). It is generally considered one of the most useful and instructive methods for functionally describing placental types. In this

system, the extraembryonic membranes are classified within one of four broadly descriptive headings: hemochorial, endotheliochorial, syndesmochorial, and epitheliochorial (Figure 4). Grosser's system has since been amended by the replacement of 'syndesmochorial' with the more descriptive heading, synepitheliochorial, which more accurately describes the type of placenta present in ruminant ungulates (Wooding, 1992).

A. The **epitheliochorial** placenta is the least invasive type of placenta wherein both the luminal epithelium (LE) of the uterine endometrium and the epithelium of the chorionic villi remain intact. In the sow there is no erosion of the LE. In horses, there is only moderate and transient invasion of the LE and uterine stroma by a subset of trophoblasts (chorionic girdle trophoblasts; described later). In these species, the uterine epithelium remains intact and there are six cell layers separating the maternal and fetal circulations.

B. The **endotheliochorial** placenta is a widespread type and is represented in most carnivores, insectivores, some bats and numerous other phylogenetic orders (Carter and Enders, 2004; Mess and Carter, 2006). It is similar to the hemochorial placenta in that its invading trophoblasts breach the uterine epithelium and stroma. However, the trophoblasts do not directly contact maternal blood. Rather, they become associated with the endothelium of the maternal capillary network. Therefore, species with an endotheliochorial placenta have four cell populations between the fetal and maternal circulations.

C. The **hemochorial** placenta of rodents, lagomorphs (rabbits and hares), higher primates and several other groupings is a highly invasive entity in which the trophoblasts of the chorion penetrate the maternal uterine epithelium, the underlying connective tissue

(stroma), and the endothelium of the maternal vasculature to establish direct contact with maternal blood. There are only three cell types between the fetal and maternal blood: fetal trophoblast, fetal connective tissue or interstitium, and the fetal endothelial cells comprising the fetal capillaries. A further distinquishing characteristic within this grouping is in the number of trophoblast cell layers present. The hemochorial placenta can be further designated hemomonochorial, hemodichorial or hemotrichorial depending on whether there are one, two or three layers of trophoblast present, respectively (Enders, 1965; Enders and Blankenship, 1999; Enders and Welsh, 1993; Pijnenborg et al., 1981).

D. The **synepitheliochorial** placenta of ruminant ungulates is derived from the epitheliochorial type. These animals possess specialized binucleated trophoblast cells that fuse with the uterine epithelium (described later). In some animals in the *Ruminantia* suborder (e.g. domestic cattle), this fusion event results in the production of short lived trinucleated cells. In others (e.g. sheep and goats), continued migration and fusion of the binucleated trophoblasts can form an extensive fetal-maternal syncytial cell layer. These animals have five to six cell types between the fetal and maternal circulatory systems, depending on whether or not a syncytium is present (King, 1993b; Steven and Morris, 1975).

Epitheliochorial Placentation of Swine (*Sus scrofa*)

Perhaps the most basic and simplest chorioallantoic placentation of eutherian mammals exists in swine. It, therefore, often serves as a model for comparison when studying placentation in other eutherian mammals. Porcine blastocysts escape their zonae early in the second week of pregnancy and begin to enlarge. By 10 days post-conception (dpc) they form spherical vesicles of 8-10 mm in diameter (Figure 5a) (King, 1993b; Perry, 1981). During this stage, the conceptuses are distributed evenly within the uterine lumen by active peristaltic movements of the myometrium, which fade gradually as the blastocyst becomes fixed in position (Keye, 1923). On about day 11-12 of the ~114 day gestation period, the embryos begin to elongate. This elongation process results in elongated slender conceptuses that can reach up to a length of 1 meter, or longer (Figure 5a) (Anderson, 1978; Heuser and Streeter, 1929; Patten, 1948; Perry, 1981). They form highly convoluted structures that occupy a relatively small portion within the uterine horn. The elongated blastocysts now make contact with neighboring conceptuses. This distribution seems to be a function of contact with neighboring conceptuses, since, the length of the conceptus tends to be larger in pregnancies with fewer conceptuses (Perry, 1981). On average there must be at least four viable conceptuses present in order for the pregnancy to proceed much beyond the third week. In swine, conceptus-derived estrogen is essential to change the course of secretion of the antiluteolytic compound, prostaglandin ($PGF_{2\alpha}$), from an endocrine pattern (into the maternal circulation) to an exocrine secretory pattern (into the uterine lumen). The result is that this luteolysin fails to gain access to the corpora lutea to cause their demise (Bazer et al., 1984a; Bazer et al.,

1982; Bazer and Thatcher, 1977; Dhindsa and Dziuk, 1968; Geisert et al., 1982b; Perry et al., 1973; Perry et al., 1976).

The rapid increase in length of the pig conceptus to form a convoluted thread by d12 is accompanied by only a minor concomitant increase in DNA or RNA synthesis or cell division, which suggests that the initial extensive elongation is mainly due to cellular rearrangements (Geisert et al., 1982a; Mattson et al., 1990). The attachment of conceptuses to the endometrium is initiated at this stage and is usually accompanied by hyperemia of the uterus (Perry and Rowlands, 1962). The attachment is underway by d18 with a transient loss of microvilli on the trophectoderm, presumably to facilitate closer association between the maternal and fetal layers (Keys and King, 1990). Once the union between the chorion and the uterine epithelial cells is achieved, the chorionic microvilli quickly reform and interdigitate with microvilli on uterine epithelial cells (Crombie, 1972; Dantzer, 1985; Hasselager, 1985).

These noticeable external developments correspond with marked reorganization internally. The mesodermal primordium originates as ectoderm erupting from the primitive streak that spreads between the endoderm and ectoderm separating them. This mesoderm contributes to both embryonic and extraembryonic mesenchyme. Immediately following the generation of the extraembryonic mesoderm, a cavity forms as mesoderm begins lining both the extraembryonic endoderm and ectoderm, respectively. This cavity is referred to as ‘extraembryonic coelom’ or ‘exocoele’ (Figure 6). The mesoderm associated with the overlying trophoblast forms the ‘somatopluere’ or chorion. The chorion adjacent to the embryonic disc develops evaginations known as amniotic folds that grow and subsequently converge over the embryonic disc, forming the amniotic sac

around the fetus. It eventually loses contact with the chorion (Figure 6). The embryonic disc at this stage transforms from a saucer shaped structure into a tubular form and is contiguous with the developing yolk sac on the ventral side. The endoderm of the yolk sac together with the mesoderm overlying it, constitute the bilaminar splanchnopluere which eventually reaches the chorion and establishes contact with it. The mesoderm of the yolk sac becomes vascularized forming a choriovitelline placenta that is transient. The expanding exocoelom separates the yolk sac from chorion and by d20 it becomes inconspicuous (Figure 6).

In most mammals, an evagination from the posterior of the hind gut develops into a structure that is called an 'allantois'. It is an endodermal derivative and is lined by mesoderm. The growing allantois begins to fill the entire exocoelom and subsequently associates with chorion, forming the chorioallantois (Figure 6). The mesoderm of the allantois eventually becomes vascularized establishing the definitive pig placenta.

The trophoblasts of the porcine placenta form neither a syncytium with the maternal epithelium (as for ruminant ungulates; see below) nor is there any compelling evidence of trophoblasts migrating or invading into the endometrium (Dantzer, 1985). An intact uterine epithelium persists throughout gestation and, therefore, the porcine placenta is an epitheliochorial type (Figure 7).

The area of contact between the placenta and the endometrium reaches a maximum at midgestation at which point it declines until term (Dantzer and Nielsen, 1984). Initial associations with the uterine epithelium begin adjacent to the embryo and spread toward the extreme ends of the conceptus. During the fourth week, the chorion evolves villi that penetrate into troughs within the endometrium to increase the intimacy

and complexity of association (Michael et al., 1985; Wigmore and Strickland, 1985). The fetal and maternal villi become narrower on the sides and tips as development proceeds (Steven, 1983). The villi are lined by extensive vascular networks that progressively indent them, resulting in an effective decline of interhemal distance to as little as 2 μm as opposed to 20-100 μm at the base of the villi (Figure 7b &c) (Steven, 1983; Wooding and Flint, 1994). The net result is a considerable increase in the surface area and a minimal barrier for diffusion.

The chorionic membranes adjacent to the mouths of the uterine glands become corrugated, forming specialized regions for nutrient uptake called 'areolae' (Figure 5b & 5c) (Amoroso, 1952b). These areolae serve to locally enhance the surface area for absorption of glandular secretions (King et al., 1982). They are lined by specialized trophectodermal cells that actively uptake secretions of the glands which are important for fetal growth. Although the placenta is fully formed by day 30, the areolae continue to increase in number and size throughout gestation (Figure 5c) (Dantzer et al., 1981; Friess et al., 1981). By term, around 7000 evenly spaced areolae are observed per fetus. These areolae constitute about 10% of the total absorptive surface by mid gestation, almost tripling in area until term (Brambel, 1933). The areolae represent an important structural modification for nutrient acquisition in swine and other species, such as the horse, with epitheliochorial placentas. These species rely heavily on histotrophic nutrition, especially for macromolecules and metals, such as iron, that cannot easily traverse the interhemal membrane (Baker and Morgan, 1970; Gitlin et al., 1964). The secretions of the uterine glands are rich in these macromolecules and are comparable to an "enriched embryo culture medium" (Bazer et al., 1978). In species such as lagomorphs and humans with

hemochorial placentas, the chorion comes in direct contact with maternal blood and actively takes up macromolecules by means of receptors and coated pits present on their cell surface (Bright et al., 1994; Malassine et al., 1987; Whyte, 1980). For example, they have receptors for transferrin on their apical surface which conjugates with circulating transferrin- iron complexes, subsequently internalizing it and releasing iron inside the cells (Faulk and Galbraith, 1979; Turkewitz and Harrison, 1989; Wada et al., 1979). In swine, the lack of direct access to maternal blood is circumvented by copious secretion of nutrients by the uterine glands throughout pregnancy. For instance, the iron complexed in a specialized acid phosphatase glycoprotein called 'uteroferrin', is secreted in large quantities (up to 1 gm can be recovered on d60) via uterine glands from d35 until d105 of gestation (Basha et al., 1979; Buhi et al., 1982; Chen et al., 1973; Chen et al., 1975; Murray et al., 1972; Palludan et al., 1969; Roberts and Bazer, 1980). According to the accepted model, secreted uteroferrin is subsequently picked up by the chorioallantois. The uteroferrin in the allantoic sac (Bazer et al., 1975) then transfers the iron to fetal transferrin via a low molecular weight intermediary. This fetal transferrin then distributes iron to the developing fetus (Buhi et al., 1982).

Synepitheliochorial placentation in ruminants (*Bos taurus, Ovis aries, Capra hircus, etc*):

Grosser described the ruminant placenta as 'syndesmochorial' with the assumption that the uterine epithelium was lost, leading to a direct apposition of trophoblast with maternal connective tissue (Grosser, 1927, 1909). Recently, as a result of detailed histological evidence accumulated by F.B.P. Wooding and others, it is now understood that the uterine epithelium can be altered, but remains intact for the most part. Instead of an erosion of uterine epithelium, specialized trophoblasts (binucleate trophoblast cells) can fuse with uterine epithelial cells to form a feto-maternal syncytial cell layer (Wooding, 1982b, 1984, 1982a). Hence, the term 'syndesmochorial placenta' has been revised to the more accurate 'synepitheliochorial placentation', wherein 'syn' stands for the feto-maternal syncytium and 'epitheliochorial' signifies the persistence of a maternal epithelium at the fetal-maternal interface (Wooding, 1992).

In ruminant ungulates, the morula enters the uterus about 4-5 dpc (Rowson and Moor, 1966). The blastocyst sheds the zona pelucida around 8-9 dpc (Rowson and Moor, 1966), and becomes positioned in the center of the uterine horn ipsilateral to the ovulated ovary (Lee et al., 1977). Transuterine migration of blastocysts is rarely reported in cows, but is seen in 8% of the ewes with single ovulations (Scanlon, 1972). The positioning is predominantly due to myometrial contractions of the uterus. Once positioned, the embryo does not implant immediately but instead starting around day 10 (sheep) to day 12 (cattle) pc begins to elongate from a spherical to a tubular form and eventually transforms into a filamentous structure (Betteridge et al., 1980; Spencer et al., 2004; Wintenberger-Torres and Flechon, 1974). The elongating embryo by 13 dpc in sheep and d15 in cattle

establishes firm apposition with the maternal epithelium by developing small chorionic villus projections or papillae that penetrate into the openings of the uterine glands (Guillomot and Guay, 1982; Wooding and Staples, 1981; Wooding et al., 1982). Interestingly, goats lack these papillae. The conceptus then expands progressively and fills the entire uterus by d16 pc in sheep and d19-20 pc in cattle (King, 1993b; Wales and Cuneo, 1989; Wintenberger-Torres and Flechon, 1974). The pre-attachment phase is much longer than in the human and mouse, allowing for greater development prior to implantation. Not surprisingly then, ungulate embryos have a much greater reliance on uterine glandular secretions than do those species that implant immediately. Furthermore, once implantation does occur, it is not a blastocyst that implants but a much more differentiated conceptus.

Attachment and implantation begins near the embryonic disk, and spreads to the ends of the conceptus (King et al., 1982). The events of implantation are similar between sheep and cattle, being delayed by 2-3 days in cattle relative to sheep. In sheep, the process is initiated with a transient loss of microvilli on the tropectodermal cells between 13 and 15 dpc; however, the microvilli remain intact on the luminal epithelium (Guillomot et al., 1982; Guillomot et al., 1993; Guillomot et al., 1981). This loss of microvilli in the chorion (trophectoderm) permits a close apposition of trophoblast with the luminal epithelium. By 16 dpc, the maternal microvilli begin to penetrate the folds of the chorionic cells. The microvilli on the trophoblast soon reappear and interdigiation begins by d18 and is completed by d22. In cattle it is completed by d27 and in goat between days 25 and 28 (King et al., 1982).

In the regions of the maternal uterine caruncles (focally dense connective tissue formations covered by columnar epithelium), the initial attachments are reinforced and the surface area increased due to the penetration of chorionic villi (cotyledons) into crypts within the caruncular endometrium. Lengthening and branching of the villi, along with coincident development of a blood supply in the expanding villi, results in the production of mature 'placentomes' (Figure 8) (King, 1993a). Since the cotyledons are the most prominent feature of the mature ruminant placenta, it is often referred to as a 'cotyledonary placenta'. However, an exception to this generalization is in the Tragulidae family which includes species such as the mouse deer. The mouse deer has a diffuse placenta, with no caruncles or placentomes (Kimura et al., 2004) To date, this is the only animal classified in the *Ruminantia* suborder that does not possess a cotyledonary placenta. However, these animals do possess binucleate trophoblast cells (described below) dispersed within the trophoblastic villi (Kimura et al., 2004). These observations suggest that the placenta of mouse deer represents an intermediate stage between the epitheliochorial placenta of swine and the derived cotyledonary synepitheliochorial placenta of most other ruminant ungulates.

The number of caruncles that are present within the uterus determines the maximum possible number of placentomes that can be established. This number is species specific and is fixed at birth. It ranges anywhere from 3-8 in deer to 20-150 in sheep, goats and cattle (Mossman, 1987; Wooding and Flint, 1994). The decrease in the number of placentomes within deer is presumably offset by a corresponding increase in the size of the individual placentomes. This also holds true for twin pregnancies in sheep and goat. The twins, though in theory, have only about half the total number of available

caruncles compared to singletons (though singletons do not use all the available caruncles), the individual size and total mass of placentomes is larger than that observed in singleton pregnancies. In fact, there is a positive correlation between placentomal mass and the size of the fetus (Bell, 1984, 1991; Wooding and Flint, 1994). Across species, the placentomes also differ in gross morphology, being convex in cattle, concave in sheep and goats, and flat in antelopes (Figure 8).

The mature placentome develops an extensive blood supply and is the main site of exchange for easily diffusible nutrients. The caruncular regions are separated by glandular endometrium that is responsible for the secretion of large macromolecules into the uterine lumen. By the fourth week of pregnancy, absorptive areas form within the chorionic membrane over the uterine glands, providing further indication that the glandular regions are involved in the transport of larger, less soluble nutrients (Wilmsatt, 1950). There are also reports of hemophagous zones within the placentomes of some species. They are presumably formed by the leakage of blood from the blood vessels at the base of the chorionic villi, subsequently undergoing hemolysis and forming colored deposits. These zones are thought to be an important source of iron for the growing fetus. However, these observations are somewhat inconsistent and there are no such zones in the placenta of the cow (Byorkman, 1969; Myagkaya and Vreeling-Sindelarova, 1976).

Binucleate cells of ruminants:

A unique feature of the ruminant placenta is the population of fetal chorionic binucleate cells (BNCs). The BNCs first become apparent around d16 pc in sheep (Boshier, 1969), d18 pc in goat (Wango et al., 1990b) and d17 pc in cattle (Greenstein et al., 1958). These timepoints correspond roughly to the period of trophoblast attachment to the uterine epithelium. The BNCs eventually constitute 15 to 20 % of the chorionic epithelium within the mature placenta (Figure 9) (Wooding, 1992). Their numbers remain fairly constant throughout gestation, until one or two days prior to parturition, when they suddenly dwindle in sheep and goat (Wooding and Flint, 1994; Wooding et al., 1986). There are no known stem cells or reserves for the genesis of binucleate cells and they are believed to originate from mononuclear trophoblast cells (MNC) (Wooding and Flint, 1994; Wooding et al., 1997). In addition, there are no developing cues or signals that underpin the progenitors of BNCs; apparently any MNC can give rise to a BNC. At first, the MNC undergoes a horizontal division to give rise to two MNCs. One of the MNCs is included within the tight junctions of trophectoderm and the other is free (Boshier and Holloway, 1977; Lawn et al., 1969). The latter undergoes a nuclear division without subsequent cytokinesis to give rise to a cell with two nuclei (Wooding et al., 1997). This BNC is a terminally differentiated cell that has lost its ability to divide, but it does undergo further development and matures. At maturity, it possesses two nuclei and an extensive network of rough endoplasmic reticulum and large golgi bodies. The golgi bodies produce a substantial number of cytoplasmic granules that make up to 50% of the total cell volume. Although, endoreduplication has been opined as the basis for BNC production, there is no conclusive evidence to rule out the possibility that a fusion event

between two MNCs could occur as a means for generating BNCs. Nevertheless, the BNC once matured, sets its migration towards the uterine epithelium. It forms a cytoplasmic vesicle as it squeezes its contents through the microvillar junctions of the trophoblast and luminal epithelia. It fuses with an apposing uterine epithelial cell forming a hybrid feto-maternal trinucleate cell (TNC) (Figure 9) (Wooding et al., 1997). During the fusion event, the cytoplasm and nuclei of the two cells mix thoroughly and the secretory granules of the BNC that were originally in the basolateral region make their way towards the maternal side of the nascent TNC (Wooding, 1987). Soon after, the granular contents are expelled toward the maternal stroma (Figure 9 and 10). Following fusion, the microvillar junctions reappear in the TNC; it reforms part of the apical tight junctions with the adjacent trophoblast cells, thus retaining a protective seal between the two interfacing epithelia (Wooding et al., 1997).

The newly formed trinucleate cells can undergo expansion as a result of continued binucleate cell migration and fusion to form syncytial plaques (Wooding, 1984). The extent of syncytium formation is dependent on the species. In the placentomes of sheep, the syncytium is quite extensive. These syncytial plaques contain up to 20-24 nuclei, they are bordered by tight junctions and they persist throughout pregnancy (Morgan and Wooding, 1983). The syncytium is presumably maintained by the continued migration of binucleate cells, since nuclear divisions are not observed within the syncytium (Wooding et al., 1981; Wooding et al., 1993). In cattle, no extensive syncytium is present beyond about d40 of gestation (King et al., 1979; King et al., 1982; Wathes and Wooding, 1980b). After d 40, the migration and fusion of binucleate cells is limited to the generation of short lived trinucleated cells that are soon replaced by epithelial cells (Fig

12b) (Wooding and Wathes, 1980). In the intercotyledonary regions of sheep and goat, syncytial plaques are found initially, but they are soon replaced by proliferating cells of the glandular epithelium (King and Atkinson, 1987; King et al., 1981; Wooding and Flint, 1994). Thereafter, occasional migrations of BNCs result in the formation of TNCs, but not a syncytium. An endogenous Jaagsiekte Sheep Retrovirus (enJSRV) envelope (env) protein mRNA and its endogenous receptor hyaluronidase 2 (HYAL2) message has been found to be localized exclusively to BNCs and syncytial plaques within the placentomes of sheep throughout gestation (Dunlap et al., 2005). This is a notable observation since, a similar retroviral envelope protein, 'syncytin', produced by a human endogenous retrovirus-W has been found in the syncytial trophoblast of human. Syncytin is a fusogenic membrane protein that is involved in the fusion of cytotrophoblast cells to form syncytia (Mi et al., 2000). With the identification of an additional retroviral envelope protein in sheep, the argument about a critical role of retroviruses in the evolution of placenta and viviparity in mammals gains further momentum (Harris, 1998; Muir et al., 2004; Stoye and Coffin, 2000; Villarreal, 1997).

Binucleate cells are endocrine cells that produce steroid (progesterone), prostaglandin (PGI_2, PGE_2), and protein hormones (placental lactogens), as well as protein products with no known function (pregnancy-associated glycoproteins) (Fig 12) (Duello et al., 1986; Green et al., 2000; Reimers et al., 1985; Wooding et al., 2005; Xie et al., 1991b). These proteins, which are packaged in the BNC secretory granules, are released toward the maternal tissues subsequent to BNC fusion. They can then diffuse into the maternal capillary network to be distributed throughout the maternal circulation (Green et al., 2005; Wooding, 1992). Therefore, binucleate cells have two principle

functions during pregnancy: to form the fetomaternal syncytium required for successful implantation and placentomal growth and to produce and deliver steroid and protein hormones.

The fusion of binucleate cells with uterine epithelial cells is the extent of invasive implantation in ruminant ungulates. However, it almost certainly allows the conceptus to establish a more intimate physiological 'dialog' with the mother than is observed in species such as the pig, where no erosion of the uterine epithelium occurs.

Role of peptidases in establishment and maintenance of pregnancy

During pregnancy, an array of peptidases is induced in the trophoblast, maternal endometrium and at the maternal-fetal interface. These peptidases interact with a wide repertoire of substrates. The resulting interactions between these enzymes and their natural substrates form the basis for a multitude of functions, which culminate in successful implantation and pregnancy. In the section below, the documented functions of peptidases in pregnancy are briefly reviewed. Some well described roles are:

A. Hatching of embryo from zona pellucida (ZP):

Following fertilization, the subsequent development of the embryo until blastocyst formation takes place within the glycoproteinaceous casing called the zona pellucida (ZP). Therefore, the crucial first step in the establishment of a successful pregnancy is the liberation of the expanding blastocyst (EB) from the ZP. Hatching in mammals is characterised by the nicking of the ZP by the EB, followed by extrusion

involving repeated contraction-expansion cycles. Two serine peptidases, implantation serine peptidases- 1 and 2 (ISP -1 and -2), have been implicated in hatching in mammalian embryos (Colleen M. O'Sullivan, 2002). While ISP1 is produced by the embryo, ISP2 is produced by the uterine glands (Colleen M. O'Sullivan, 2002). Additionally, a membrane associated serine peptidases known as strypsin/hepsin, which is expressed as early as the two-cell stage and later throughout the early blastocyst stage of development, has also been implicated in blastocyst hatching (Vu et al., 1997). Besides, the two well characterized serine peptidases, prtn3 (peptidase 3) and prss23 (peptidase, serine, 23) were recently reported to have a role in murine blastocyst development and hatching (Chen and Tzeng, 2007). Apart from the serine peptidases, members belonging to other classes such as metallopeptidases of the astacin family (invertebrates and vertebrates) (Hishida et al., 1996; Yasumasu et al., 1992) and cysteine peptidases (hamster) have also been found to play an important role in the hatching process (Mishra and Seshagiri, 2000). Therefore, it is possible that additional peptidases expressed by the developing embryo may have a redundant role in this critical process as well.

B. Regulating attachment of embryo to luminal epithelium (LE) of endometrium:

Once the expanded blastocyst is liberated from the ZP, it begins attachment to the receptive uterus. Different peptidases facilitating this process and their likely mechanisms are documented below.

1) Remodelling of surface glycocalyx:

The uterine luminal epithelium (LE) like many other epithelial surfaces contains a protective layer of apical glycocalyx which allows diffusion of small molecules but prevents cell-cell and cell-ECM interactions (Brayman et al., 2004; Thathiah and Carson, 2002). The blastocyst because of its relatively large size cannot access the LE for the sake of apposition and successive attachment. The non-adhesive properties of the glycocalyx are partly imparted due to cell surface expression of the mucins, MUC-1 and -4 (Aplin JD, 1995; McNeer et al., 1998). In mice and rats, Muc1 is downregulated precisely at the time of implantation. Another surface mucin, Muc4, is also downregulated in rats (Bowen et al., 1996; Johnson et al., 2001b; McNeer et al., 1998). This period therefore corresponds to the initiation of apposition between LE and trophoblast (TR) and thereby onset of implantation. In sheep for example, MUC-1 at the apical surface of the LE is reduced dramatically by D9 and is nearly undetectable by D17 of pregnancy when intimate contact between LE and TR begins (Johnson et al., 2001a). Although steroidal regulation has been prompted as a cause for this downregulation, experimental studies with hatched embryos on monolayer culture of endometrial epithelial cell layer have shown other molecular players may be involved as well. These experiments revealed a potential role for a metallopeptidase ADAM17/TACE in the disappearance of MUC-1 in an area adjacent to embryo (Thathiah et al., 2003).

2) **Modulating integrin mediated trophoblast adhesion:**

Integrins (IGs) are hetero-dimeric transmembrane glycoprotein receptors that mediate cellular differentiation, motility and adhesion (Albelda and Buck, 1990). The IGs

are found on the LE of endometrium and TR. Of all the known IGs, IG αvβ3 has been suggested to be most crucial in the attachment of embryo in species such as human, mouse, rabbit and farm animals (Burghardt et al., 2002); although, several other candidates, αV β3, α4β1 and αV β1 IGs are also expressed during the implantation window. The IGs are known to recognize and bind to the RGD motif found in diverse candidates such as osteopontin, fibronectin, vitronectin, tenascin, thrombospondin and possibly laminin (Kimber and Spanswick, 2000).

It has been shown that, IGs are prone to proteolytic processing both intra- and extracellularly. For example, urokinase-type plasminogen activator (uPA), a serine peptidase, was reported to process IGs extracellularly (αVI) (Demetriou et al., 2004). Interestingly, this peptidase is expressed in the TR within the implantation window (see below) and is known to cleave the ECM component, fibronectin, which is the known ligand for IGs. Additionally, Calpains, a family of intracellular cysteine endopeptidases, were shown to endoproteolytically process the cytoplasm domain of ' β' subunits (Du et al., 1995). Therefore, the peptidases such as uPA and calpains, which are capable of proteolytically processing IGs, can influence the dynamics of adhesion between the trophoblast and receptive uterus.

In species with invasive placenta, an alternative mechanism involving IGs and peptidases can be envisioned that is analogous to the mechanism proposed for cancer progression, wound healing and angiogenesis. According to this proposed mechanism, IGs signal the induction of metallopeptidases such as MMPs, and upon synthesis conjugate with them. On the other hand, the IGs also bind components of ECM, thereby bringing the peptidase and its substrate in close proximity, facilitating cleavage in defined

locations (Brakebusch et al., 2002). In other words, the IGs may utilize peptidases such as MMPs as a 'spear-head' in clearing the way for progression into uterine tissue.

Another interesting scenario, involves a ligand for IG, osteopontin (SPP-1). SPP-1 is a secretory product of endometrial glands and is associated with the apical surface of LE, glandular epithelium and conceptus trophoblast (Apparao et al., 2001). In sheep, SPP-1 is a component of the histotroph that increases in uterine flushings from pregnant ewes between D11 and 17. Between days 40 and 80 of gestation, SPP-1 mRNA expression increases remarkably and isolated protein concentrations increase 30 fold. Osteopontin binds to the IG heterodimers displayed by trophoblast and LE and helps induce adhesion for implantation and placentation (Johnson et al., 2001a; Johnson et al., 2003). Upon treatment with peptidases, the native 70kDa SPP-1 is converted to 45KDa and 25KDa fragments. Interestingly, the 45kDa form of SPP-1 was detected continuously and abundantly along the apical surface of LE, on conceptus trophectoderm, and along the uterine placental interface of both interplacentomal and placentomal regions through d120 of pregnancy in sheep. The 45kDa SPP-1 is more stimulatory to cell attachment and migration than the native 70kDa form. Therefore, paradoxically a cleavage event associating with the substrate of IG actually aids adhesion.

These observations therefore provide multiple scenarios for likely modulation of the adhesion of TR to uterus via IGs, which is a very critical event for establishment of pregnancy.

C. Regulating the bioavailability of various growth factors:

Numerous factors like insulin, leukemia inhibiting factor (LIF), leptin, heparin-binding epidermal growth factor (HB-EGF), granulocyte-macrophage colony stimulating factor (GM-CSF), insulin like growth factor 1 (IGF-1), etc., have been shown to increase either the rate of embryo development to blastocyst stage, blastocyst cell number in culture, blastocyst survival, etc. Studies in genetically modified mice have also demonstrated a clear role for these factors (Diaz-Cueto and Gerton, 2001; Hardy and Spanos, 2002; Robertson et al., 2001; Sjoblom et al., 1999). Therefore, regulating the relative levels of these factors within the uterine milieu can influence the outcome of pregnancy.

The role of peptidases in regulating bioavailability of growth factors has been well documented in the Insulin-like growth factor (IGF) system. The IGFs are mitogenic proteins that regulate cell proliferation, differentiation, and are important for fetal and placental growth and differentiation during pregnancy (Han and Carter, 2000). Levels of IGF are regulated by binding proteins known as insulin- like growth factor binding proteins (IGFBP), which are numbered 1-6 in the order of their discovery. Among these binding proteins, IGFBP-1 is the major binding partner and IGFBP-4 is the second most abundant binding protein for IGFs. IGF-1 is important in syncytiotrophoblast steroidogenesis and, glucose and amino acid uptake in the villous portion of the human placenta (Kniss et al., 1994; Nestler, 1990). It is expressed abundantly in decidual cells at the site of implantation. The reduction in fertility associated with IGF-1 null mutant mice is consistent with a role for this protein in implantation and decidualization (Liu et al.,

1993). IGF-II on the other hand is also critical for pregnancy, since, placental specific deletion of IGF-II resulted in decreased placental size and, thereby, reduced fetal size (Consancia et al., 2002). Pregnancy associated plasma protein A, a product of the placenta and decidua is a known IGFBP-4 peptidase (Sun et al., 2002). It's presumed functions at the maternal fetal interface is to proteolyze IGFBP-4 and thus increase IGF bioavailability locally in the placenta (Sun et al., 2002). Both matrix metalloproteinase -3 (MMP -3) (decidual cells) and MMP-9 (trophoblast) also proteolyse IGFBP-1, another major secretory protein of deciduas (Coppock et al., 2004). The kallikreins found in humans, as well as flushes of early pregnant and cycling pigs also were identified as the IGFBP-peptidases (Geisert et al., 2001; Réhault et al., 2001).

Besides the IGF system, there has been mounting evidence to show the role of diverse growth factors in regulating other aspects of pregnancy and, therefore, serving as attractive candidates for proteolytic action by various peptidases. Such peptidases could modulate their levels or activity by binding or degrading binding proteins or the growth factors themselves. Peptidases can also modulate growth factor activity by proteolytic processing of the native proteins into an active form. A brief description of these phenomena is discussed below.

A member of epithelial growth factor (EGF) family, Heparin-binding epidermal growth factor (HB-EGF) was induced in the LE of the endometrium immediately adjacent to the blastocyst, solely at the site of implantation in mice (Das et al., 1994). Transforming growth factor-alpha (TGF –α) another member of the EGF family is found to be expressed in the mouse uterus throughout the peri-implantation period (Munson et

al., 1996) and in goats, detected in the luminal epithelium between days (D) 22-30 post coitum (Flores et al., 1998). Both HB-EGF and TGF- α are synthesized and translocated as inactive transmembrane precursors to the cell surface. On the cell surface, they are subjected to proteolytic cleavage that causes them to be released as soluble and diffusible growth factors (Massague and Pandiela, 1993). Integral membrane metallopeptidases with a disintegrin domain (ADAMs) are also located on the cell membrane and are capable of cleaving various membrane-bound proteins including ligands, receptors and ligand-receptor complexes (Black and White, 1998; Blobel, 2000). Among the well studied ADAMs that are known to proteolytically process EGF-like ligand precursors are Tumor necrosis factor –α converting enzymes (TACE)/ ADAM-17, Kuzbanian /ADAM-10 and ADAM-9 which are capable of acting as sheddases releasing membrane bound EGF ligands such as HB-EGF (Izumi, 1998; Yan et al., 2002). Some of the members of the ADAMs family were reported in trophoblast and uterus, therefore suggesting a likely role for these peptidases in their bio-availability (Kim et al., 2005).

Therefore it is evident from these discussions that peptidases regulate the effective levels of numerous growth factors by either targeting their binding partners or the growth factors themselves.

D. **Promoting trophoblast invasion into uterine endometrium:**

In species with invasive placentation such as humans and mouse, the blastocyst invades the LE and underlying stroma of the endometrium. This invasion process is mediated by peptidases that are positioned on the cell surface of the invasive trophoblast cell population. These peptidases include members of the MMPs, and serine peptidases

plasminogen activators (PA) and plasmin. Various members of MMPs- collagenases, gelatinases and stromelysins are produced by the subset of trophoblasts (Figure 11) which are capable of degrading collagen IV and other components of ECM (Matrisian, 1992; Pistola et al., 1989).

Upa secreted by trophoblasts may directly contribute to the breakdown of stromal ECM or may activate plasminogen to convert into plasmin (Queenan et al., 1987), which is a potent peptidase. Besides its proteolytic activity on components of ECM , it can activate procollagenase, progelatinaseB and pro-MT1-MMP (Lala and Hamilton, 1996).Therefore, it is clear that the peptidases mutually aid each other in the peptidase cascade involved in trophoblast invasion.

Besides those species with invasive placentation, ungulates (with non-invasive placenta forms) such as cattle, sheep, pig, express a preponderance of peptidases as well; one such example is the vast family of aspartic peptidases known as Pregnancy-associated glycoproteins or PAGs. They have been reported as early as the blastocyst stage. A detailed discussion of these peptidases is discussed in later sections. In addition, to PAGs, the trophoblast layer of pig and cow also express PA and kallikreins (Dyk and Menino, 1991; Fazleabas et al., 1983; Mullins et al., 1980). Therefore, in principle, the trophoblast of these species is equipped to invade the uterus. However, such an invasion does not occur; a plausible explanation for this is discussed below.

Role of inhibitors in modulating peptidase activity during pregnancy

The establishment of successful pregnancy in species with invasive placentation hinges on delicate balance between the peptidases that facilitate invasion and the inhibitory substances that regulate them. The trophoblast populations are inherently invasive. Studies with trophoblasts of species with non-invasive placenta such as pig, have shown apparent invasiveness (Samuel, 1971; Samuel and Perry, 1972). Therefore, it is imperative that the uterus in itself or in conjunction with the physiological state induced by pregnancy must be regulating the invasiveness of trophoblast cell populations. The uterus can check the invasion of trophoblast by the production of inhibitory proteins to peptidases, as well as through signaling molecules that inhibit their expression. For example, transforming growth factor - beta (TGF-β) produced by the uterus induces the expression of tissue inhibitor of metallopeptidases (TIMP) in decidual cells and, in a paracrine manner (Graham and Lala, 1992), limits the invasiveness of trophoblast by inducing their differentiation into non-proliferative syncytiotrophoblasts (Graham and Lala, 1992).

The deciduas of rodents and primates produce a non-specific peptidase inhibitor known as α_2-macroglobulin (α_2M). α_2M is a large multimeric protein (720 kDa)(Gu et al., 1995; Sayegh et al., 1995) that inhibits peptidases by entrapping them (Borth, 1992). The peptidase - bound α_2M interacts with a cell surface receptor resulting in internalization and degradation of the bound peptidase. In addition, it has been proposed that α_2M associates with TGF- β (O'Conner-McCourt and Wakefield, 1987) prolonging

its half-life (Borth and Luger, 1989). Consequently, α_2M exhibits two-pronged inhibitory effects on the peptidases.

While the physiological importance of inhibitors in species with invasive placenta can be envisioned, a similar role in epitheliochorial and non-epitheliochorial placentas such as swine and cow warrants detailed explanation. As discussed above, since it has been shown that the trophoblasts in these species are inherently capable of invasion, there ought to be some regulatory mechanisms in place to limit that invasive character. The evidence for such regulation has been gathered from uterine flushings of pregnant sows. The flushes from the pregnant uteri of pigs showed high levels of PA inhibitor (Mullins et al., 1980) known as urokinase type plasminogen inhibitor (UPTI). The UPTI is a low molecular weight protein that belongs to the Kunitz family of serine peptidase inhibitors. This inhibitor showed high level of inhibition towards plasmin, trypsin, and, to a lesser extent, chymotrypsin (Fazleabas et al., 1982). The UPTI is expressed at high levels in the progesterone -primed uterus as well as by the conceptus itself (Duffy et al., 1997; Stallings-Mann et al., 1994). Recently, additional members of Kunitz domain containing family of peptidase inhibitors have been cloned from the trophoblast of cattle and sheep which are known as trophoblast Kunitz domain proteins (TKDPs). The members of these families possess a conserved kunitz domain, the principal inhibitory motif in the inhibitors, and multiple amino terminal domains (Chakrabarty et al., 2006a; Chakrabarty et al., 2006b; MacLean et al., 2003; MacLean et al., 2004).

Besides, the Kunitz domain serine peptidases, the uterus of the ruminants and pigs, also produce inhibitors which belong to the serpin (serine protease inhibitors) class of peptidases. The members of this class include the uterine milk proteins of ruminants

(Mathialagan and Hansen, 1996) and the uteroferrin-associated basic protein of pigs (Malathy et al., 1990). Interestingly both classes of peptidase inhibitors constitute a major fraction of the uterotroph. Therefore, the abundant expression of inhibitors from both tissue types (uterus and trophoblast) likely contributes to establishment of the noninvasive epitheliochorial placenta of pig.

Aspartic peptidases

Introduction and Background:

Research into aspartic peptidases began in the 19th century with studies involving digestive secretions of man and nepenthes (pitcher plant). Today we have a thorough understanding of the role of aspartic peptidases not only as digestive enzymes but also as critical players in other cellular processes. The pepsin family first attracted the attention of biochemists as a group of peptidases that were active in the pH range of 1-5 (Queenan et al., 1987). The aspartic peptidases really came of age when Tang and coworkers defined the sequence of porcine pepsin (Lala and Hamilton, 1996). Because of this important early work, most comparative discussions of mammalian aspartic peptidases are described relative to porcine pepsin A. Consequently, the amino acid numbering provided in the descriptions below is based on amino acid positions relative to the porcine pepsin A sequence, unless otherwise indicated.

Structure of mammalian aspartic peptidases:

The secondary structure of most aspartic peptidases consists largely of beta-strands, with four short alpha helices (Northrop et al., 1948). The mammalian proteins consist of two distinct lobes that are roughly symmetrical (Figure 12) (Tang et al., 1973). The lobes form a binding cleft of about 40 angstroms in length and can accommodate a peptide substrate of seven to eight amino acids. Two ψ- shaped loops extend from the lobes. The catalytic aspartic acid residues Asp32 and Asp215 are located on the ends of the loops. Strongly conserved residues flank the catalytic aspartic acids: hydrophobic-F/I/L-D-T-G-S in the N-terminal domain and hydrophobic-D-T/S-G-S/T in the C-terminal domain (Cooper et al., 1990). The carboxyl groups of these two aspartic acids interact with one another and with a solvent water molecule by a network of hydrogen bonds. The hydrogen bonding involving conserved threonine or serine residues are important for maintaining the structure of the catalytic center (Tang and Wong, 1987). Another critically-important structure is a hairpin loop known as the "flap", which is constituted by residues 65-85 and extends over the cleft of the catalytic center. The flap is purportedly involved in contributing to substrate specificity of the enzyme molecule (Davies, 1990).

Aspartic peptidases are produced as zymogens/pro-enzymes. Functional activation of the zymogens involves proteolytic processing of the amino-terminal propeptide. The propeptides range in length from 35-50 amino acids and are highly basic with a preponderance of His, Ala and Leu residues. Most notable differences in the sequences of the prosegments are seen at the C-terminus region connecting the propeptide to the active enzyme (Navia et al., 1989; Tang). At neutral pH, electrostatic

interactions, particularly the ion pairs between the highly conserved propeptide residue Lys-36p ('p' stands for propepdide) and the two catalytic aspartates, Asp-32 and Asp-215, maintain the zymogen in its inactive form by stabilizing the position of the prosegment in the substrate binding cleft. This interaction, along with the hydrogen bonds between Tyr-37p and Asp-215 and between Tyr-9 and Asp-32, render the catalytic aspartates unavailable for catalysis at neutral pH. At pH values below 5.0, acidic residues in the active enzyme portion become protonated, disrupting the electrostatic interactions between the prosegment and the active enzyme. The resulting change in the conformation displaces the propeptide and exposes the substrate-binding cleft. At low pH (pH 1-3) a series of intramolecular cleavages remove the propeptide. However, at slightly higher pH values, another peptidase is necessary to remove the propeptide (Bohak, 1973; Hartsuck et al., 1992; James and Sielecki, 1986; McPhie, 1976).

Mechanism of catalysis:

In accordance with recent data, it has been shown that the mechanism of action does not involve a covalent intermediate (Figure 13) between the substrate and the enzyme. The accepted mechanism of action for APs is a model of general acid-base catalysis (Davies, 1990; Dunn, 2002; Szecsi, 1992). According to this model, a water molecule is coordinately bound via a series of hydrogen bonds by the two highly-conserved aspartic acid residues (Asp32 and Asp 215). One of the two aspartates, Asp215 acts as a general base extracting a proton from the water molecule, thereby activating it. The activated water molecule now acts as a nucleophile in the attack on the carbonyl carbon of the substrate scissile bond. The other aspartate, Asp32 behaves as a general

acid donating a proton to the oxygen in the carbonyl moiety of the scissile bond. This results in a transient unstable tetrahedral intermediate. In the resulting tetrahedral intermediate, Asp215 is hydrogen bonded to the attacking oxygen atom (originally part of the water molecule), while the hydrogen remaining on that oxygen is hydrogen bonded to the oxygen on Asp32. Transfer of a proton from Asp215 to the nitrogen of the scissile bond occurs by inversion around the nitrogen atom. The C-N bond then breaks, forming the two products (Bohak, 1973).

Pregnancy-Associated Glycoproteins

Pregnancy associated glycoproteins (PAGs) represent a complex and diverse family of trophoblast secreted proteins. The first member of the family to be purified was called 'Pregnancy Specific Protein B (PSPB)'. A range of molecular weights (47-90kDa) and isoelectric points (3.7-4.4) has been reported for purified PSPB. A radioimmunoassay (RIA) with an anti-PSPB antiserum was developed and used to demonstrate that PSPB could enter the maternal circulation. The detection of PSPB has formed the basis of a successful test for pregnancy, which is accurate as early as 30-35 days following insemination in cattle (Butler et al., 1982). Immunologically similar antigens were eventually discovered in pregnant sheep (Ruder et al., 1988), mule deer, white tail deer (Wood et al., 1986) and musk oxen (Rowell et al., 1989). In 1991, another antigen from bovine fetal cotyledons was purified and was named Pregnancy-Associated Glycoprotein (PAG) (Zoli et al., 1991). Later, it was reported that both PSPB and PAG were the same, or, very similar proteins (Lynch et al., 1992).

Bovine and ovine PAGs were first cloned from placental cDNA libraries by using an antiserum directed against bovine PAG. These two proteins shared 86% identity in nucleotide sequence with one another. In recent years, many more PAG molecules have been cloned (Figure 14), including 22 cDNAs from cattle, nine from sheep, 12 from goats, ten from white-tailed deer and two from pigs (Brandt et al., 2007a; Green et al., 2000; Szafranska et al., 1995; Vawter et al., 2004b).

The most thoroughly-studied members of the PAG family are those in cattle. In situ hybridization of mRNA has revealed that most of the bovine PAGs are confined to trophoblast BNCs, while others are expressed in all trophoblasts (Green et al., 2000). Coincidentally, the BNC-specific PAGs have been shown to be a more recently evolved subgroup – an observation that is consistent with their BNC-restricted expression. The synepitheliochorial placenta, with its unusual BNC trophoblasts, is believed to have arisen from the epitheliochorial placenta 50-55 MYA concurrent with the evolution of the *Ruminantia* suborder (Hughes et al., 2000). In contrast, the ancient members of the PAG gene family are predicted to have arisen about 86 MYA - a time-frame that is consistent with the period at which the *Artiodactyla* order is believed to have arisen (Hughes et al., 2000).

Recent studies have expanded our current understanding of PAG distribution. Immunolocalization with antibodies raised against the BNC and ancient PAGs have shown that the ancient PAGs are expressed in all trophoblast cells and are particularly abundant at the trophoblast-endometrial interface (Figure 15a-c) (Wooding et al., 2005). Likewise, the modern PAGs, apart from being localized to BNCs, are also localized to the extracellular matrix (ECM) of the superficial maternal stroma (Fig 15a-d).

Presumably, they are becoming bound to the ECM of the stroma upon release from the BNCs. It is clear that many of the released BNC-PAGs are also able to enter the maternal circulation where their presence can be detected soon after BNCs begin to fuse with uterine LE. It is however worth mentioning here that the localization of the ancient PAGs have not been studied extensively, with the exception of bovine PAG-2. Much of the ancient PAG localization work has been performed with an antibody raised against a mixture of ancient PAGs. Therefore, it still remains unclear as to where most individual ancient PAGs are localized.

The PAGs are members of the aspartic peptidase gene family and they share around 50% amino acid sequence identity with human pepsin A. Similar to nearly all aspartic peptidases, most PAGs can bind to pepstatin A, a specific hexapeptide inhibitor of aspartic peptidases (Green et al., 2005; Landon et al., 1999; Wooding et al., 2005). However, despite the similarity in structure to aspartic peptidases, many PAGs have accumulated mutations in key residues comprising the catalytic center (Table 1) (Green et al., 2000; Guruprasad et al., 1996). Examples of such mutations can be observed in bovine PAG-9, caprine PAG-9 and ovine PAG-1. In boPAG-9, Asp 215 in the c-terminal lobe has been replaced by a histidine. In caprine PAG-9, both catalytic aspartates were replaced; Asp 32 was replaced by an aspargine and Asp 215 by a glycine residue. Similarly, in ovine PAG-1, Asp 215 has been replaced by a Glycine residue (Table 1) (Green et al., 2000). While, replacement of critical aspartates in caprine PAG-9 and ovPAG-1 would likely result in inactivation of their proteolytic activity, the replacement of Asp215 by His in boPAG-9, might result in proteolytic activity similar to Histo-aspartic proteases (HAP) (Banerjee et al., 2002) found in plasmodium food vacuole. The

His residue might constitute a catalytic dyad with Asp32 and may act as a general acid or base, depending on the pKa of His within the microenvironment of the active site (the pKa range of free histidine is between 6 and 7) (Banerjee et al., 2002). In addition to inactivating mutations within the catalytic aspartates, mutations within conserved neighboring residues flanking the catalytic aspartates are also known to exist in several other PAGs. Interestingly, all those bovine, ovine and caprine PAGs that have accumulated mutations within the catalytic center belong to the BNC-group. In contrast, the ancient PAGs are largely intact and possess the hallmarks of typical aspartic peptidases (Green et al., 2000). However, to date, no proteolytic activity has been demonstrated for these proteins.

As was described in the previous section, the PAG family in ruminants is quite large and complex. Comparatively, the PAG family in swine is relatively simple with with only two distict PAG transcripts (porcine PAG-1 and 2) (Figure 14)(Szafranska et al., 1995). Recently additional members of this family have been discovered; however, they differ by 1% or less in amino acid sequence from the known PAGs and therefore likely represent variants. The two porcine PAG genes poPAG -1 and -2 share 48 - 57% identity in terms of amino acid sequence with ruminant PAGs. These proteins have multiple putative N-linked glycosylation sites and are glycoproteins with a size ranging from ~50 to ~70 kDa (Szafranska et al., 1995). As was the case for many of the ruminant PAGs, porcine PAG-1 possesses key mutations within the catalytic center that would likely render the protein incapable of proteolysis. In contrast, porcine PAG-2 has all of the hallmarks of a typical aspartic peptidase (Szafranska et al., 1995). The expression of the PAG proteins was detected as early as day 15 of pregnancy and was detectable until

day 90 of pregnancy (Szafranska et al., 1995)(pregnancy in the pig is ~114 days). As for the localization of the protein, immunohistochemical studies performed on porcine PAG-2 revealed that, copious amounts of this protein were found lining the maternal-fetal interface (Szafranska et al., 2005).

PAG -like molecules or pepF

Other molecules related to PAGs have been identified in species outside the *Artiodactyla* order, namely in the horse (*Perissodactyla*), cat (*Carnivora*), rabbit (*Lagomorpha*), mouse and rat (*Rodentia*) (Chen et al., 2001; Green et al., 1998a; Kageyama et al., 1990). The first member of the PAG –like proteins to be described was that from the rabbit (Kageyama et al., 1990). It was found to be transiently expressed in the neonatal gastric mucosa and was given the name 'pepsinogen F' (pepF). The orthologous proteins in mice and horses were found to be localized to the placenta as well as to the neonatal stomach, but not the adult stomach. Indeed, the expression of pepF in both of these organs distinguishes these genes from the PAG family. Some additional characteristics that distinguish pepF from PAGs are that pepF possesses little, if any, glycosylation. In addition, recombinant equine and mouse pepF do exhibit general proteolytic activity (Chen et al., 2001; Green et al., 1998a).

As described earlier, the PAGs are expressed exclusively in trophoblasts. The pepFs, on the other hand, have shown some unexpected species-specific differences in their placental expression. For example, in the horse, pepF was found in the trophoblast of the chorioallantoic membranes (Figure 16b) (Green et al., 1998a). However, in the mouse, the mRNA for pepF was localized to the parietal and visceral yolk sac endoderm

(Fig- 8.1) (Chen et al., 2001). The expression was stronger in visceral than compared to parietal yolk sac (Figure 16a). In rodents, the visceral yolk sac in particular is involved in active digestion and absorption of nutrients from macromolecule precursors (Chen et al., 2001). The presence of this enzyme in two organs involved in selective protein degradation suggests that pepF has specialized digestive functions (Chen et al., 2001).

Pepsinogen F has been identified in species representing four distinct orders (*Perrissodactyla*, *Carnivora*, *Lagomorpha*, and *Rodentia*) that diverged at least 90-100 million years ago (MYA)(Novacek, 1992). The oldest PAGs originated approximately 85 MYA (Hughes et al., 2000), corresponding to the time when the lineage leading to the modern day artiodactyls diverged from other hoofed mammals (Novacek, 1992). The PAGs appear to have arisen from a pepF gene by a duplication event around that time (Hughes et al., 2000), whereas, the pepF group has retained considerable structural conservation and appears not to have undergone further rounds of duplication. In the *Artiodactyla*, the PAGs duplicated extensively and seem to be undergoing rapid functional diversification, including loss of proteolytic activity in some family members.

In summary, the pepF proteins more closely resemble PAG family members than other aspartic peptidases, including pepA and cathepsin D. Nonetheless, the general proteolytic activity of the pepF group suggests that they are functionally distinct from the other ruminant PAGs and, unlike the diverse multigene PAG family of the ruminant ungulates, only a single pepF gene is present in species outside the *Artiodactyla*.

Summary and aims of dissertation

It is apparent from the discussions detailed above, that the PAGs are an intriguing group of proteins. They are restricted in their expression to a single phylogenetic order (*Artiodactyla*) (Hughes et al., 2003). In those species in which they are expressed, they comprise one of the major transcript populations in the placenta (Green et al., 2000; Green et al., 1998b; Szafranska et al., 1995; Xie et al., 1997b; Xie et al., 1991b). However, the family members differ quite remarkably from one another in regard to sequence identity, and the different groupings in the family are known to exhibit differential spatial and temporal expression patterns between family members (Brandt et al., 2007a; Garbayo et al., 2000; Green et al., 2000; Hughes et al., 2000). Furthermore, the PAGs are continuing to diverge as is evidenced by the fact that they are accumulating amino acid changing mutations at a rate that is higher than would be predicted by chance (Hughes et al., 2000; Hughes et al., 2003). Upon secretion from placental trophoblasts, the PAGs either accumulate to high concentrations at the placenta-uterine interface or they build up in the maternal uterine stroma until they eventually enter the maternal circulation (Green et al., 2005; Wooding et al., 2005). The detection of circulating PAGs in maternal blood has been used as the basis for an effective pregnancy diagnostic tool in cattle and related species (Green et al., 2005; Sasser et al., 1986).

Yet, despite the considerable amount of research devoted to the PAGs, exceedingly little is known about these proteins in terms of their genetic organization, transcriptional regulation, and their function in reproductive biology. Therefore, a major goal of the projects described in this dissertation was to bridge some of these gaps

regarding PAG biology by performing an annotation of the bovine PAG gene family and by examining the putative peptidase activity of PAGs in two model species (cattle and pig).

During the process of analyzing the PAG-gene family, emphasis was placed on analyzing the promoter regions to identify putative elements responsible for transcriptional regulation of the PAGs. For those projects focused on defining the proteolytic activity of PAGs in cattle, boPAG-2 was chosen as a candidate for investigation, since it is the most abundant of all known bovine PAG genes. Parallel experiments were performed upon boPAG-12, since this is the closest paralog to boPAG-2, thus providing a useful opportunity for a comparative analysis. Due to key differences between bovine and swine pregnancies, there was interest in studying cross-species similarities/differences between PAG family members. Therefore, similar experiments were also performed on poPAG-2 since it was the only porcine PAG predicted to possess proteolytic activity. Since poPAG-2 is known to be transcribed in the early embryo, some experiments were performed to determine if it was possible to detect acid peptidase release from embryos.

The specific goals of this dissertation included:

A. Annotation of the PAG-gene family in the bovine genome
B. Exploring the putative proteolytic activity of boPAGs -2 and -12
C. Investigating the putative proteolytic activity of poPAG-2, and finally,
D. Determining if it is possible to detect proteolytic activity in the secretome of embryos.

The experimentation involving each of these specific objectives was expanded and discussed as chapters below. They were documented in a standard manuscript format, starting with an abstract, introduction, materials and methods, results and discussion. A detailed bibliography of these research projects is included at the end of the dissertation.

Chapter –I figures

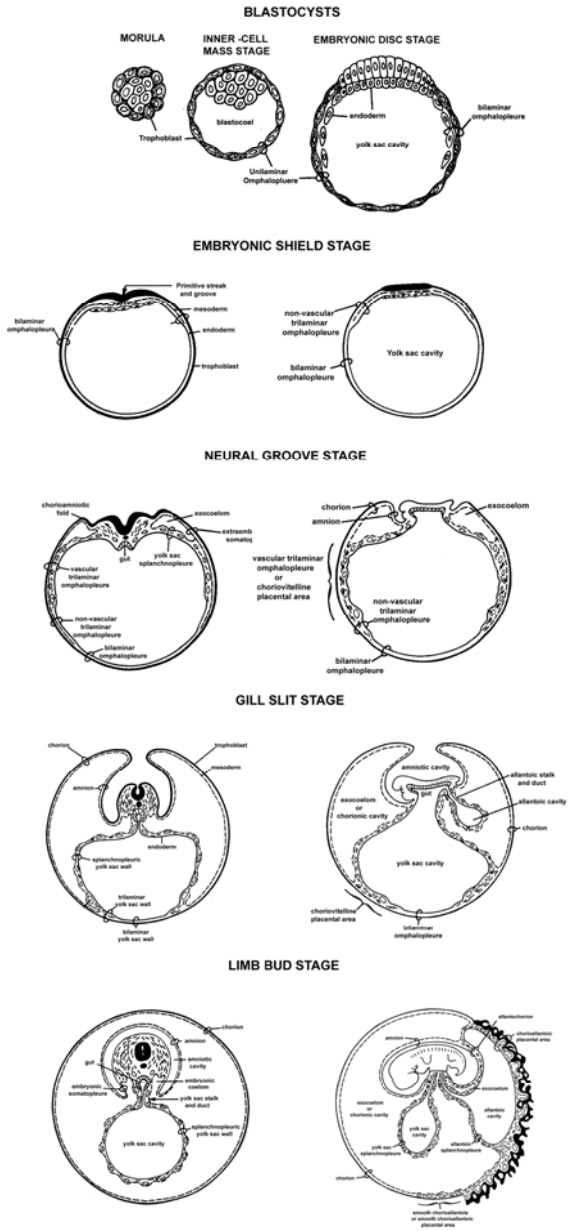


Figure -1. Stylized diagrams of transverse (left side) and sagittal (right side) sections of eutherian embryos, depicting, fetal membranes in developmental transition. These diagrams illustrate many of the tissues and structures described throughout chapter -I. [Modified from (Mossman, 1987)]

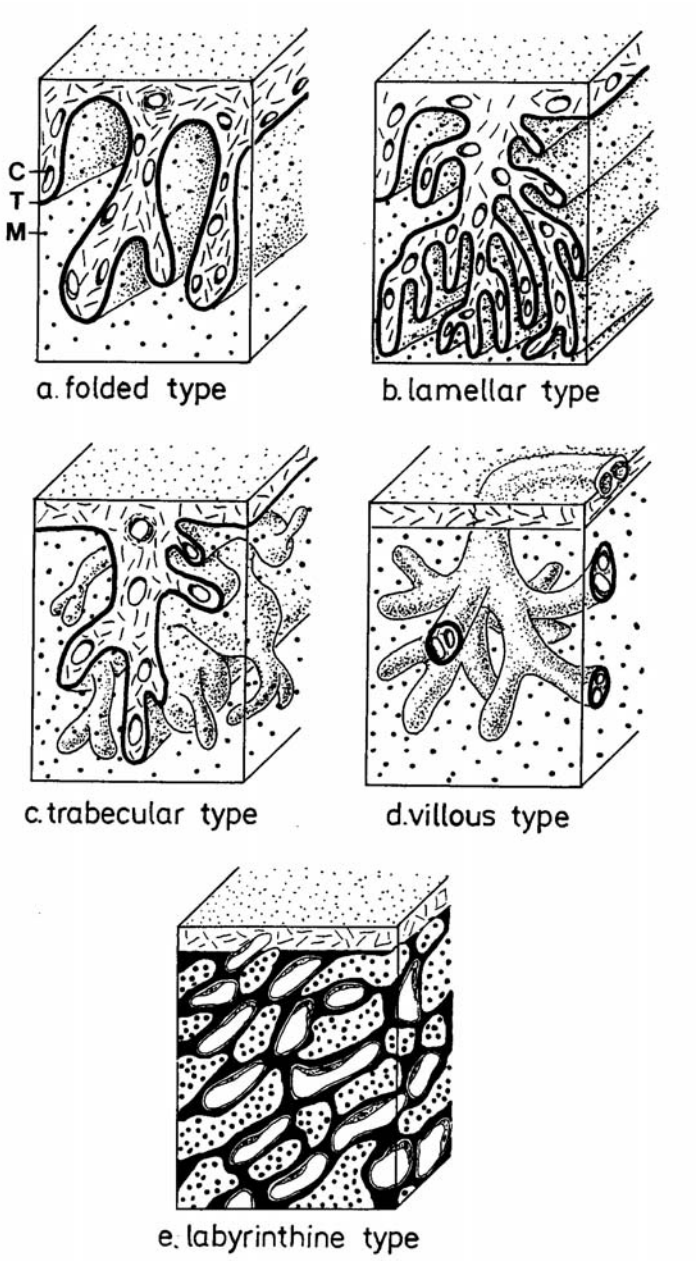


Figure -2. Illustrations of the different types of interdigitation of fetal chorion and maternal tissues. M: maternal tissues; T: trophoblast (black); C: fetal capillaries and connective tissue. [From (Kaufmann and Burton, 1994)]

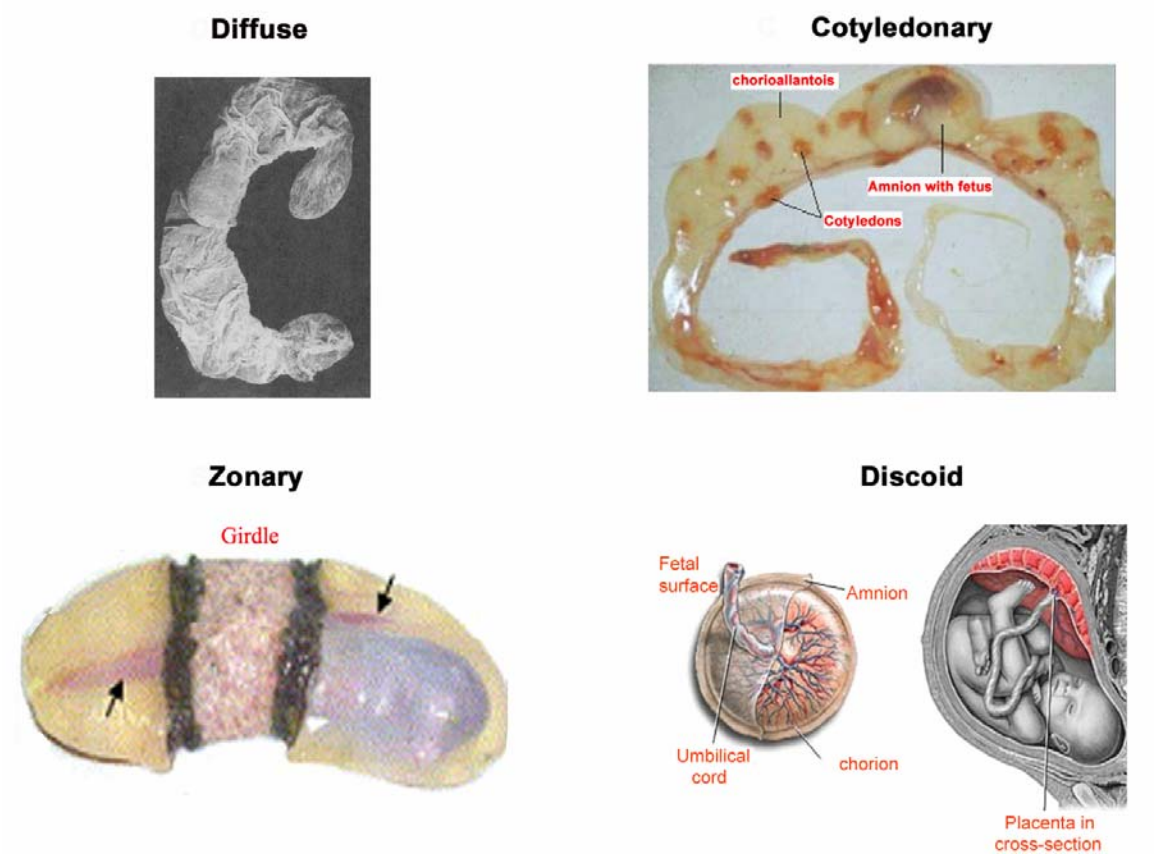
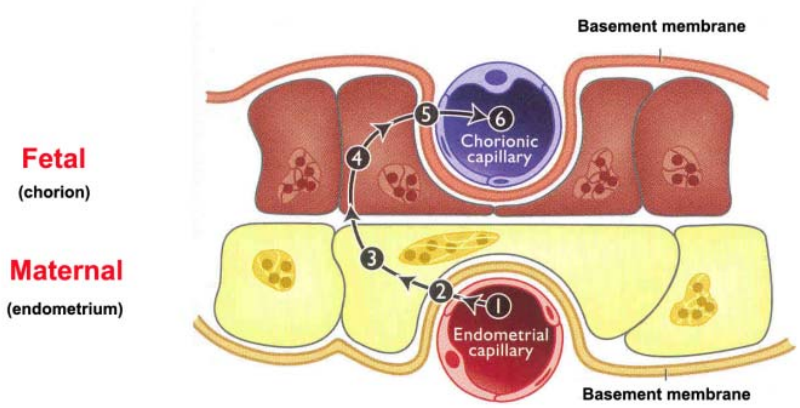
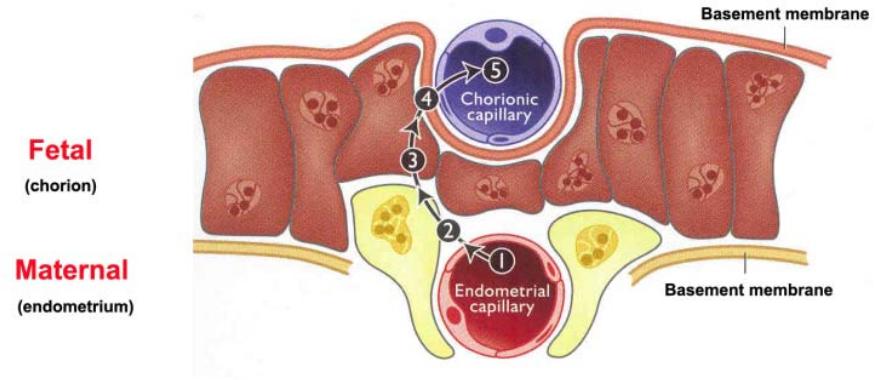


Figure -3. Illustrations of four main placenta classifications based on the gross shape of the vascularized fetal membranes in contact with maternal tissues. The four categories are diffuse (pig placenta), cotyledonary (bovine placenta), villous (dog placenta) and discoid (human placenta) [A montage from various sources].

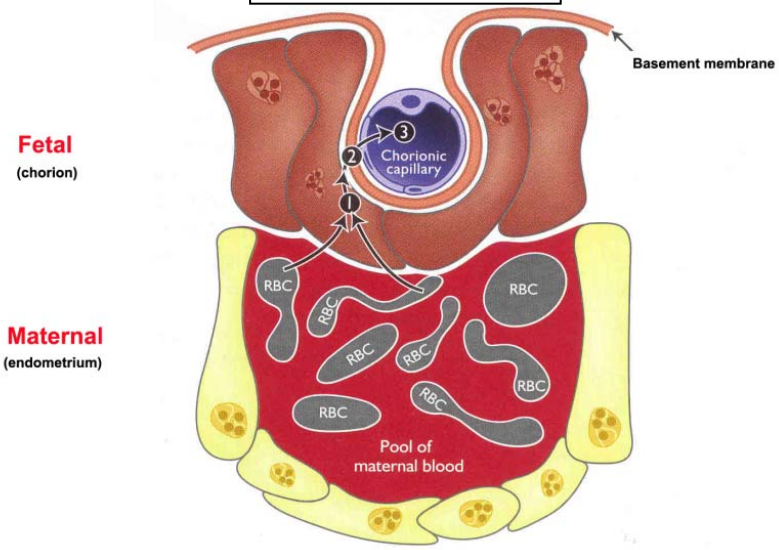
Epitheliochorial



Endotheliochorial



Hemochorial



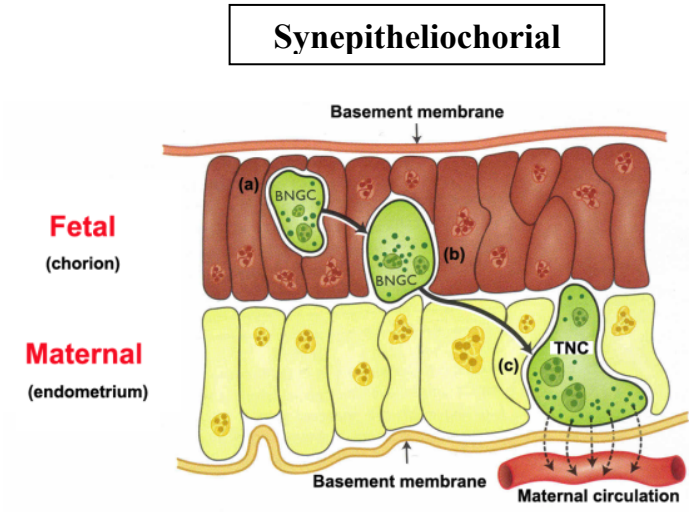


Figure -4. Pictorial illustrations of the placental types based on the number of tissue layers separating the fetal and maternal vasculatures, according to the revised Grosser's placental classification system. The number of tissue layers constituting the inter-hemal membrane (separating the fetal and maternal circulations) is shown numbered in the picture. The three main categories are epitheliochorial (six tissue types separating fetal and maternal blood), endotheliochorial (four tissues) and hemochorial (three tissues). The fourth placental type – the Synepitheliochorial placenta is a derivation from the epitheliochorial form seen in the ruminant ungulates. The number of cell layers constituting inter –hemal membrane are similar to Epitheliochorial placenta. This placenta form however, has a specialized trophoblast population capable of fusing with uterine epithelial cells to form a syncytial cell type (TNC, or trinucleated cell) or a syncytial cell layer, depending on the species. The migration route and the subsequent formation of a trinucleated cell is traced by alphabets in the figure [Modified from (Senger, 2003)] .

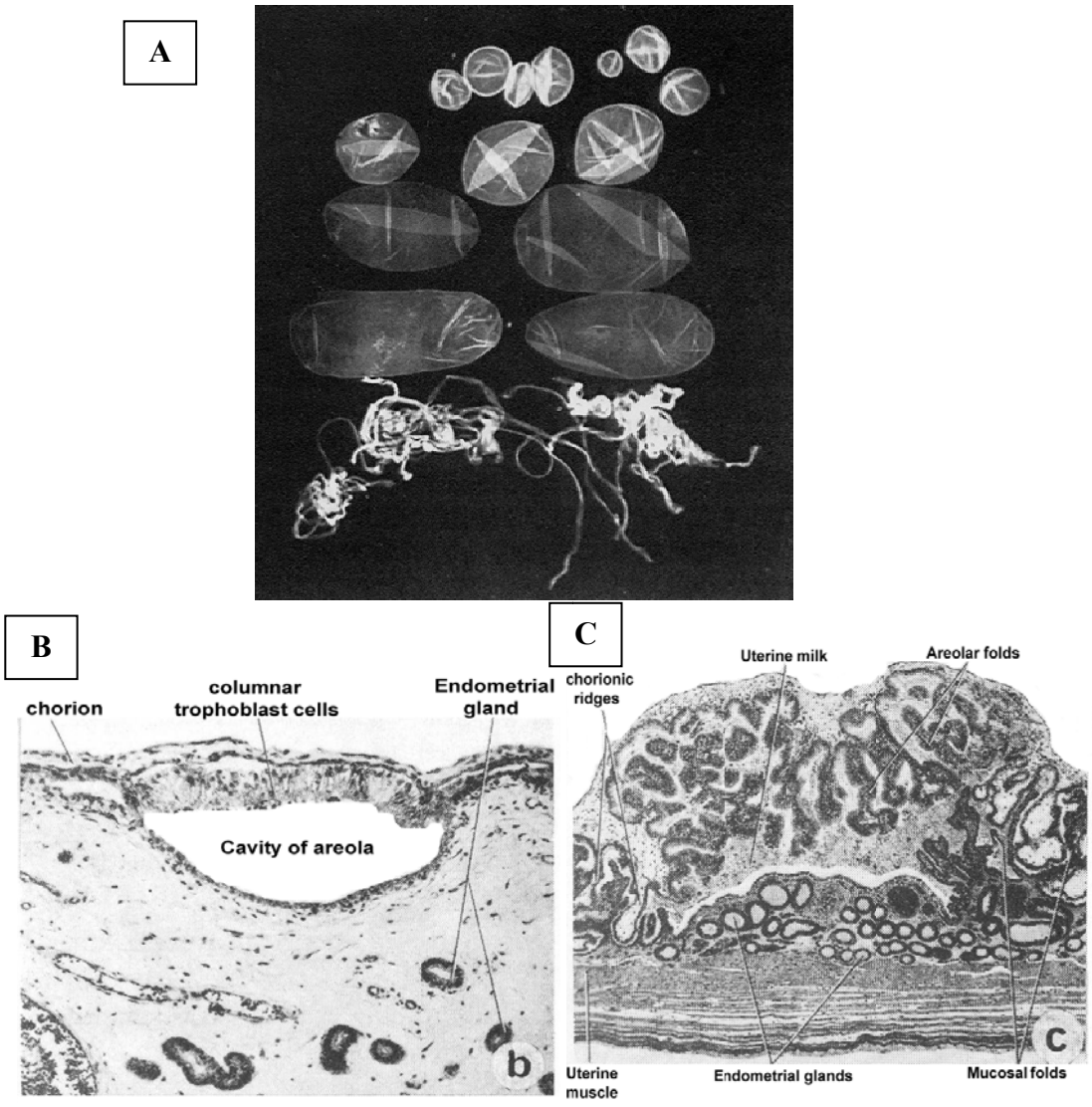


Figure -5. Early conceptus development in swine and the establishment of specialized trophoblasts for the uptake of uterine gland secretions. 5a) Picture illustrating the transition of porcine conceptuses from small spherical to long filamentous structures (from (Roberts and Bazer, 1988)). Figures - 5b and c: The development of specialized absorptive trophoblasts (areolae) over the openings of uterine glands. 5b) was taken from a 23 dpc conceptus and (5c) was taken from a mature placenta at 111 dpc. [From (Wooding and Flint, 1994)].

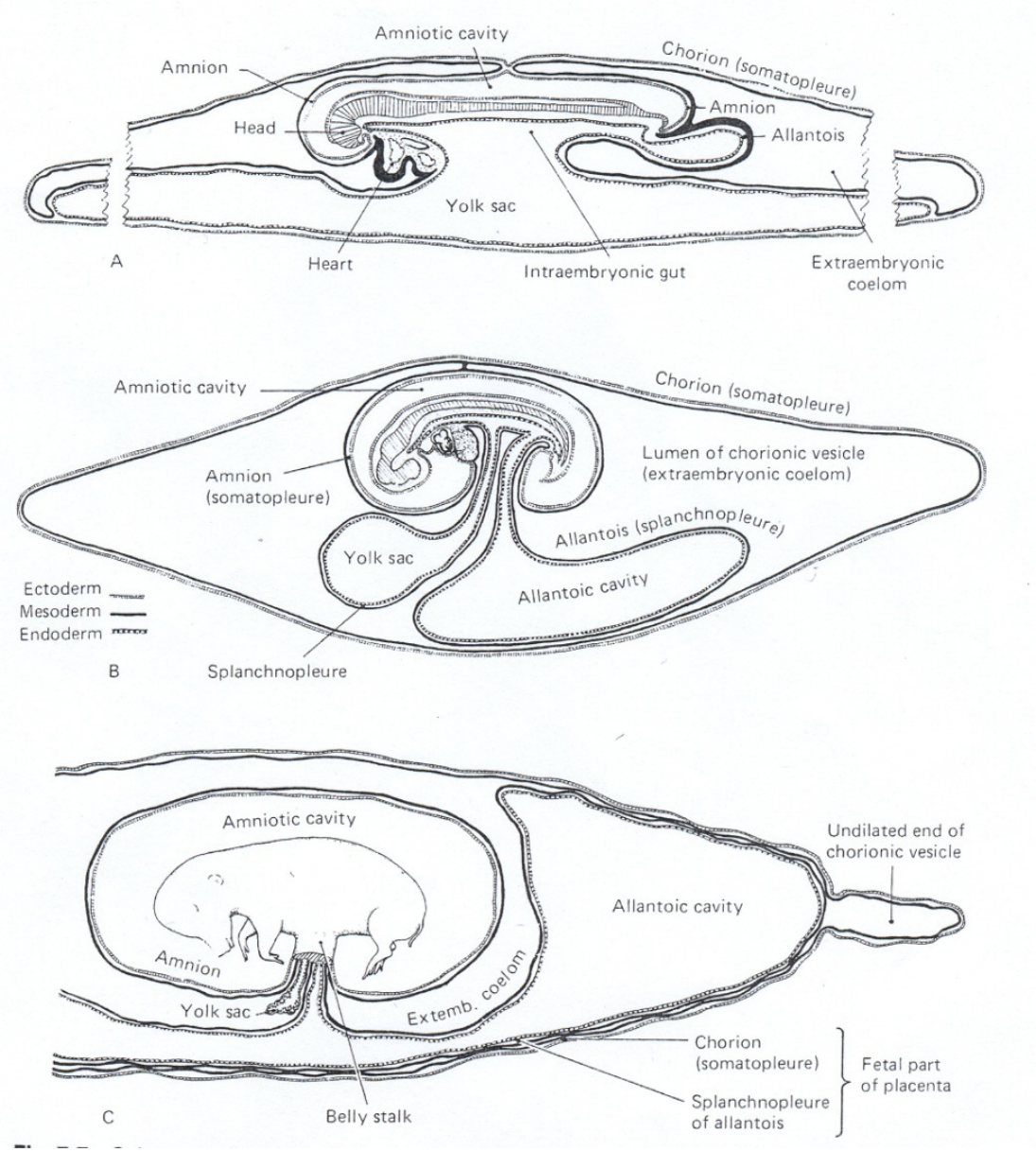


Figure -6. Diagrams showing porcine extraembryonic membranes (proportions are not to scale). The various developmental events can be clearly seen, such as the formation of somatopleure (chorion) and splanchnopleure (allantois) and the fusion of the two membranes to generate the allanto-chorion, the definitive placenta. A: Representative of embryos with 15-20 somites. B: Representative of embryos 4-6 mm in length. C: Representative of embryos approximately 30 mm in crown-rump length [From (Carlson, 1981)]

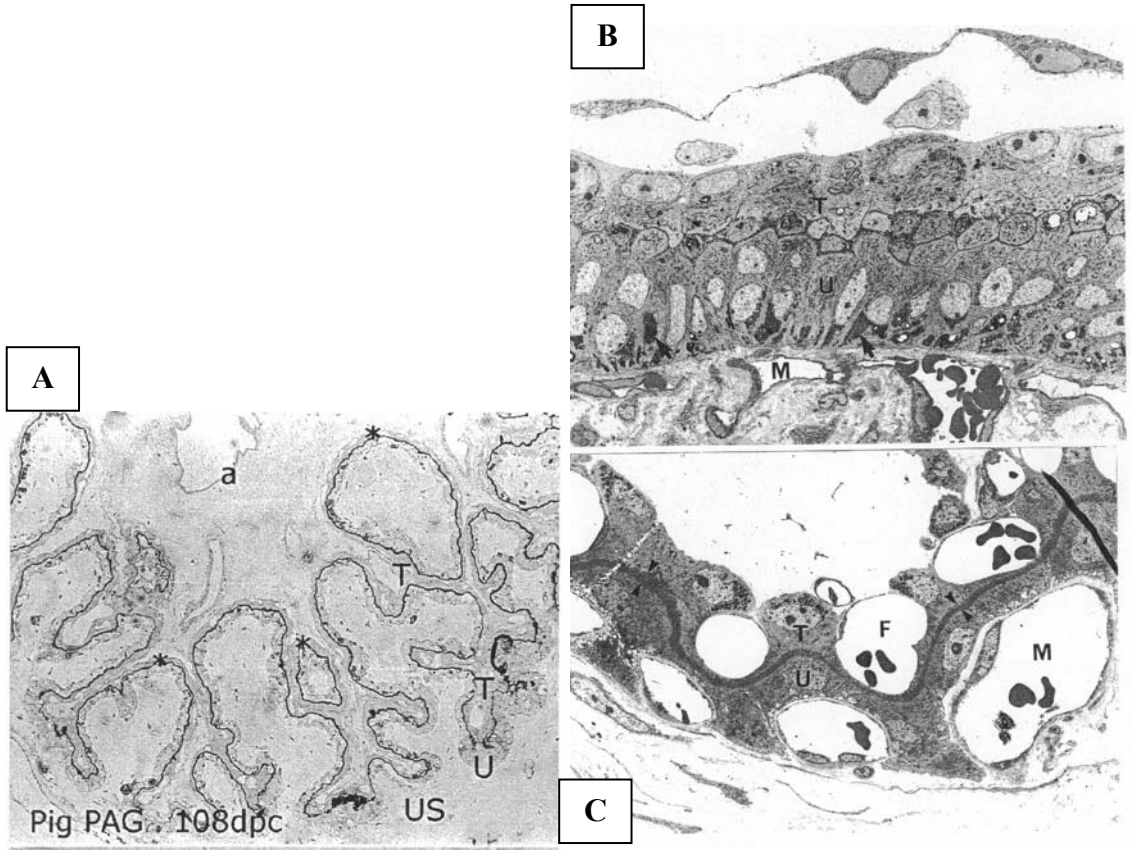


Figure -7. Histological sections of the fetal-maternal interface in domestic pigs. Panel A: Immunocytochemistry of a 108 dpc pig placenta with an antibody to the trophoblast product, porcine pregnancy-associated glycoprotein 2. The intense staining highlights the microvillar junction between cellular trophoblasts (T) and the intact uterine epithelial cells (U) From (Wooding et al., 2005). Right panels: Electron micrographs of the fetal-maternal interface at 16 dpc (panel B) and 109 dpc (panel C). Of particular interest is the narrow diffusion distance between the fetal (F) and maternal (M) capillaries in the mature placenta from (Wooding and Flint, 1994).

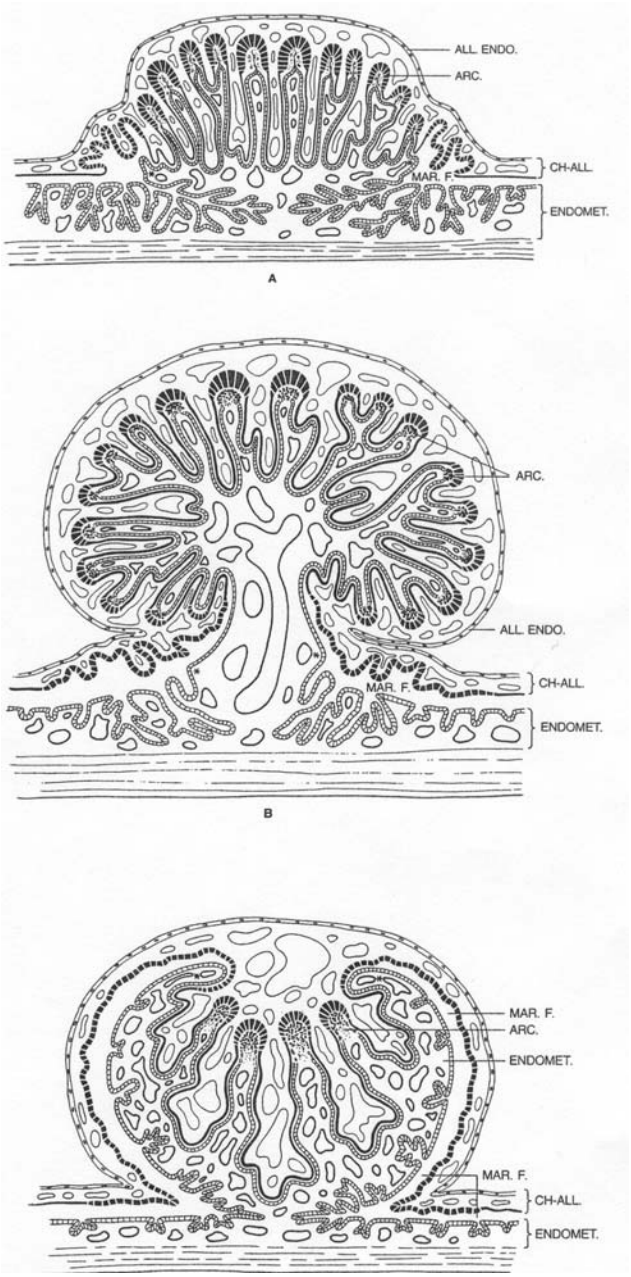


Figure -8. Gross shape of ruminant placentomes. Top: A 'flat' placentome found in some deer species. The cotyledons are straight and relatively unbranched. Middle: A 'convex' placentome typically found in Bovinae, giraffes and some deer. Bottom: A 'concave' placentome characteristic of Caprinae (sheep and goats). ALL. ENDO.: endoderm of the allantois; ARC: arcade; CH-ALL: chorioallantois; ENDOMET: endometrium; MAR. F.: marginal folds of the allantochrorion; *: approximate border between the glandular endometrium and the aglandular caruncular endometrium. From (Mossman, 1987)

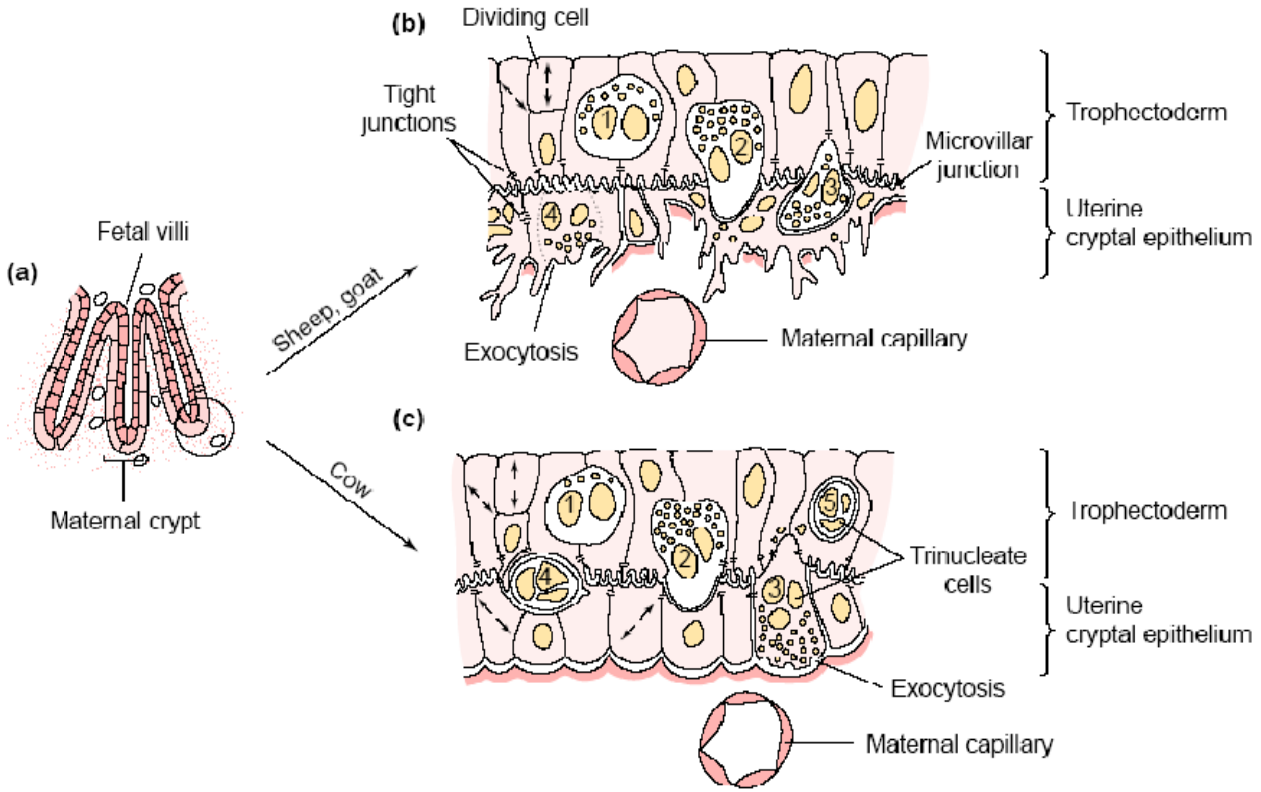


Figure -9. Diagram illustrating the characteristics of trophoblast binucleate cells in the ruminant placentome. A: Cotyledonary villi penetrating crypts of the uterine caruncles. B: In some ruminant species, extensive migration and fusion of trophoblast binucleate cells with uterine epithelia produces a fetal-maternal syncytial cell layer. C: In cattle, binucleate cell fusion with uterine epithelial cells produces short-lived trinucleate cells that are resorbed upon release of secretory granule products into the maternal stroma. [From (Green et al., 1998b)]

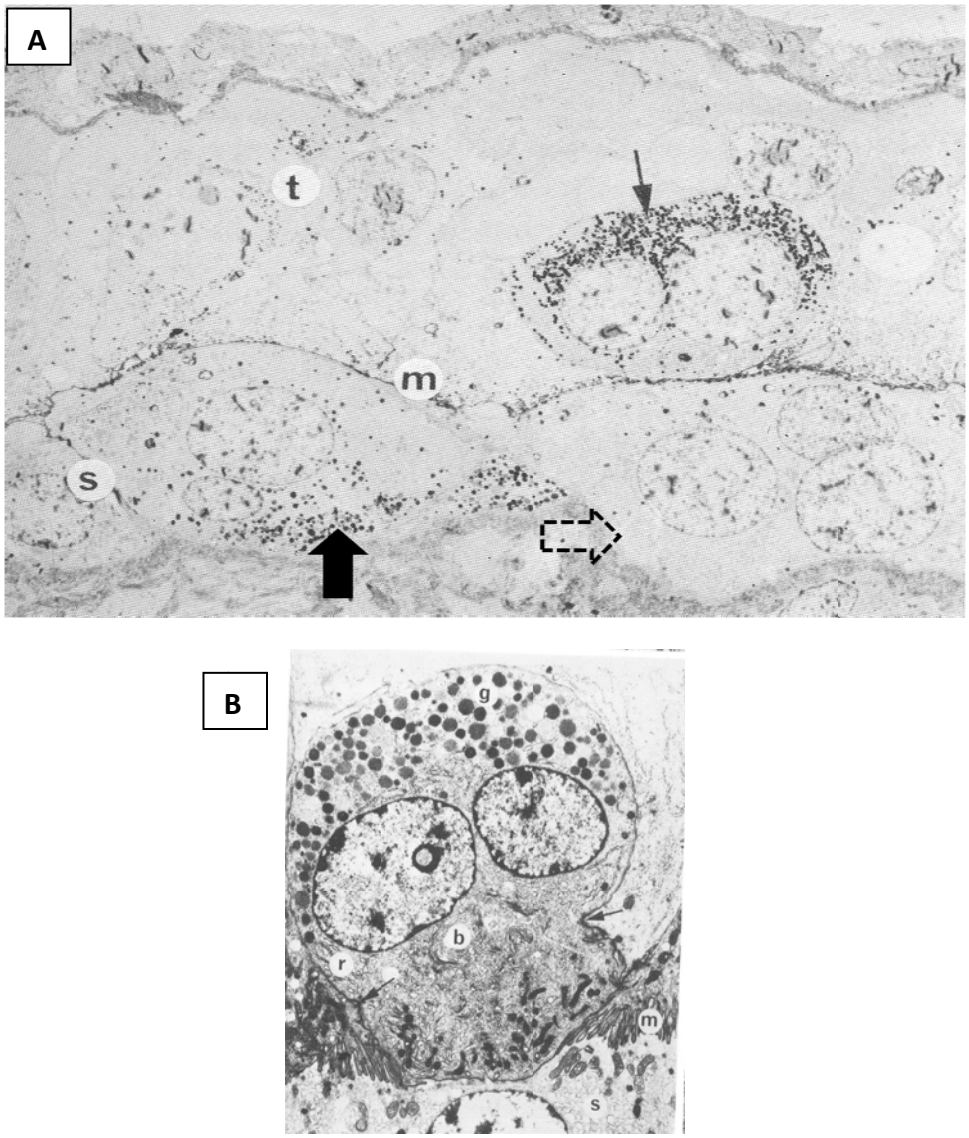


Figure -10. Electron micrograph of the bovine placenta-uterine interface. The picture displays the three stages of binucleate cell (BNC) migration. A thin arrow in the figure depicts a BNC initiating migration from the trophoblast layer (t). A dense arrow depicts a BNC that crossed the maternal (m) - trophoblast interface and fused to an endometrial epithelial cell, and a serrated arrow with no fill shows a trinucleated cell formed as a result of fusion of BNC with endometrial cell. Note the secretory granules have already been released from this cell. Panel b) shows the ultrastructure of a BNC. The BNC is seen with two nuclei and densely scattered granules (g) in the process of migration towards maternal epithelium. [From (Wooding and Flint, 1994)]

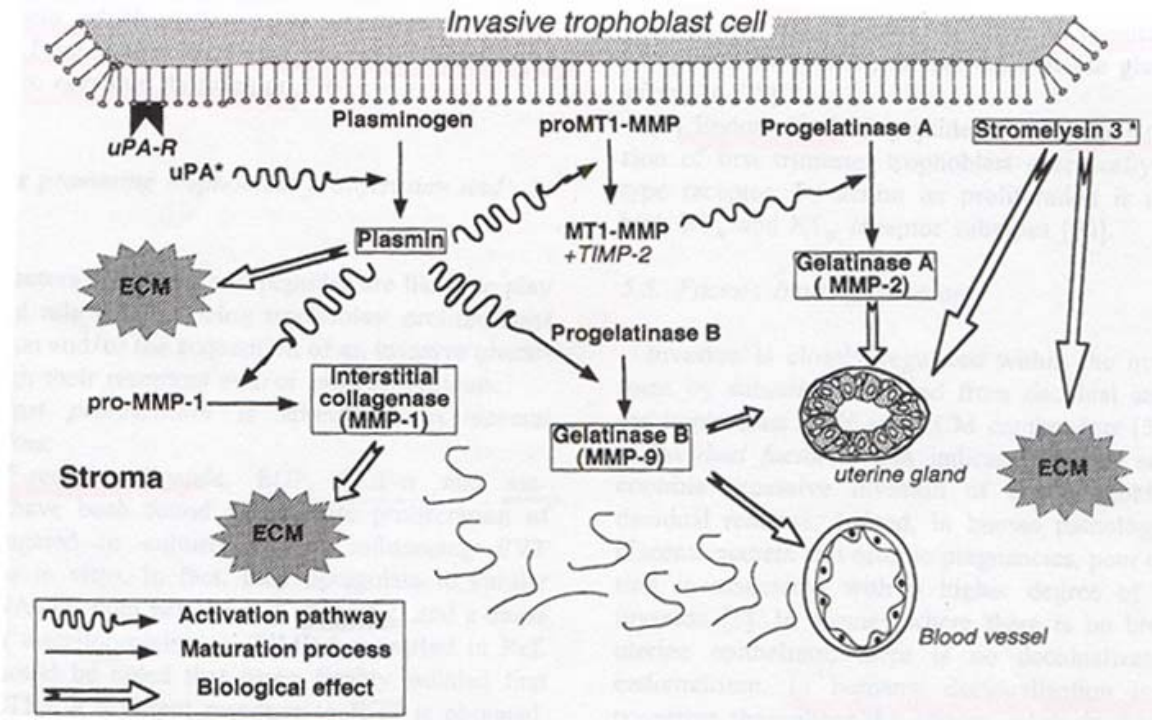


Figure -11. Schematic showing synergistic activity of various peptidases in degradation of the ECM of uterus. The invasive trophoblast cells produce a wide variety of enzymes such as plasminogen (pro-form of plasmin), pro-MT1 MMP (matrix type-I MMP), progelatinase and stromelysin. It also expresses receptors for uPA (urokinase- type plasminogen activator), which binds to uPA and localizes its activity regionally. The uPA, proteolytically activates plasminogen into an active form plasmin. Plasmin is a very robust enzyme, which in turn activates a host of other proforms which includes pro-MMPs, apart from the ones described above. The net result is a large increase in the amount of matrix -degrading peptidases which aids in the invasion of embryo into uterus. [From (Lala and Graham, 1990)].

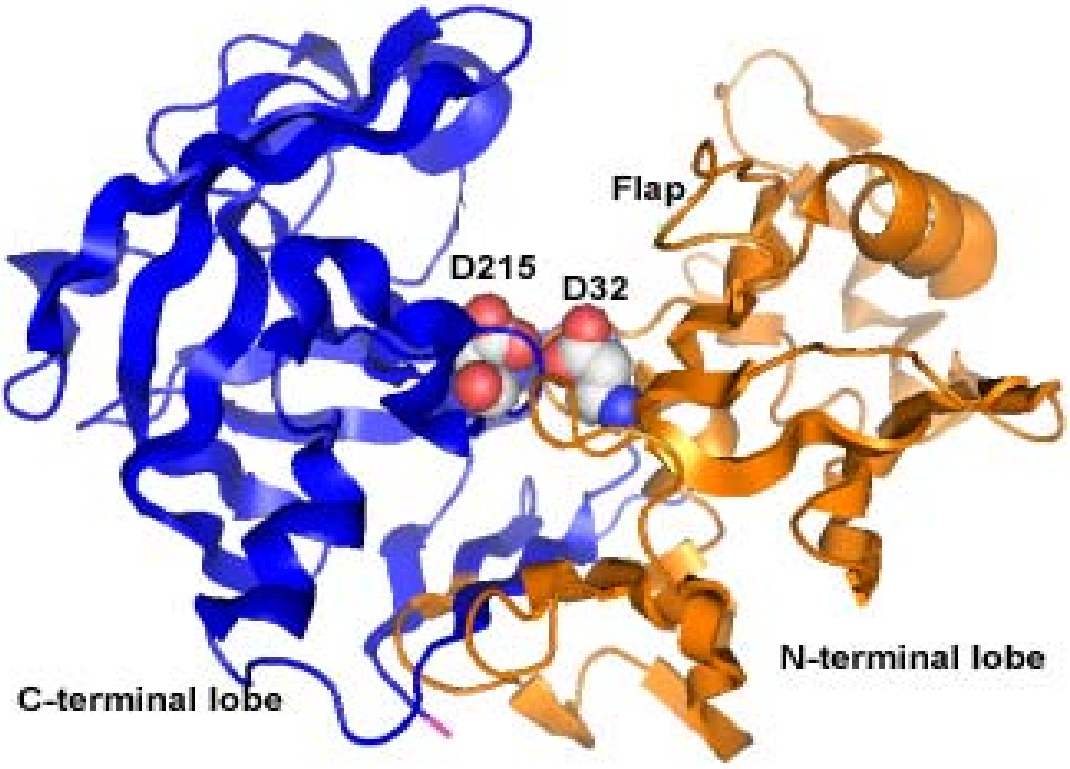


Figure -12. A ribbon diagram of porcine pepsin. Notice the two distinct N and C terminal lobes which were shaded differently for the sake of convenience. Also, note the two critical aspartic acid residues (Asp 32 and 215) in the bottom of the substrate binding cleft formed by the two lobes. A conserved structural overhand on the substrate binding site known as the 'Flap' was also shown in this view.

(source: www. clas.ufl.edu/jur/200404/images/jeung_1.jpg).

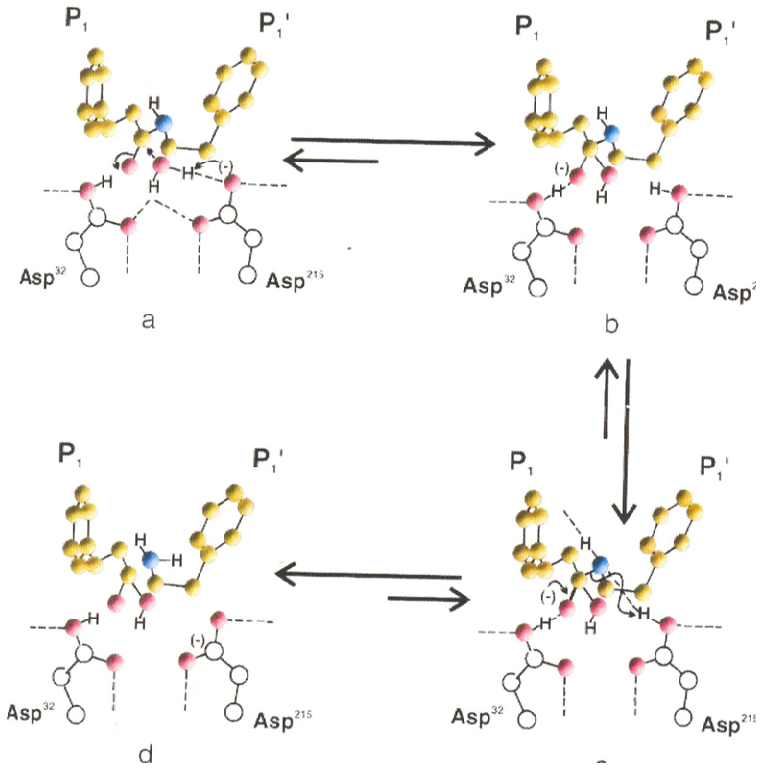


Figure -13. The proposed catalytic mechanism of aspartic peptidases. a) Note the coordination of a water molecule by the two catalytic aspartates, Asp-32 and Asp-215. One of the two aspartates, Asp215 acts as a general base extracting a proton from the water molecule, thereby activating it. b) The activated water molecule now acts as a nucleophile in the attack on the carbonyl carbon of the substrate scissile bond. The other aspartate, Asp32 behaves as a general acid donating a proton to the oxygen in the carbonyl moiety of the scissile bond. c) This results in a transient unstable tetrahedral intermediate. c and d) In the resulting tetrahedral intermediate, Asp215 is hydrogen bonded to the attacking oxygen atom (originally part of the water molecule), while the hydrogen remaining on that oxygen is hydrogen bonded to an oxygen on Asp32. Transfer of hydrogen from Asp215 to the nitrogen of the scissile bond occurs by inversion around the nitrogen atom. The C-N bond then breaks, forming the two products (Tang, J. Chapter-III: Pepsin A. Handbook of proteolytic enzymes, 2nd edition).

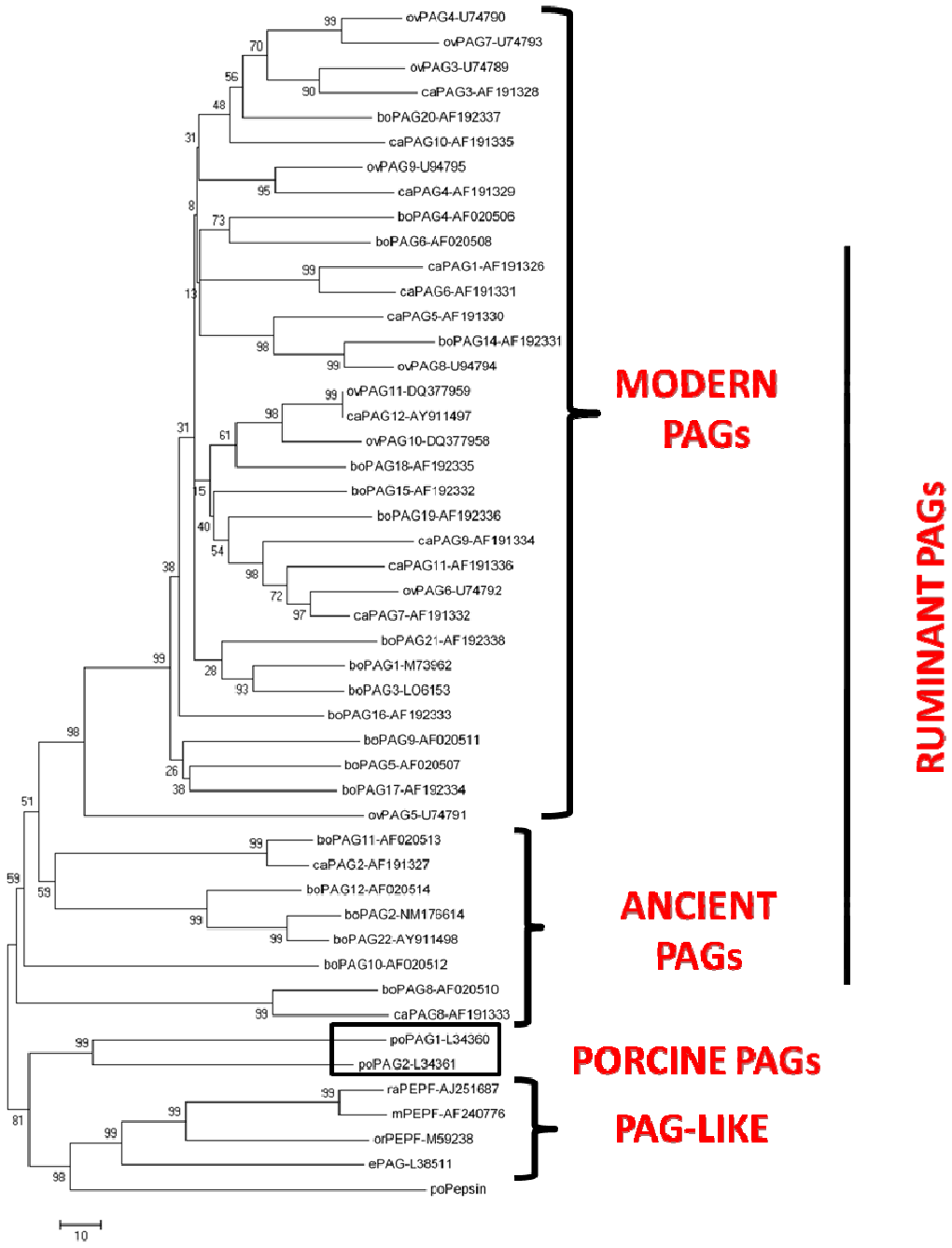


Figure -14. A comprehensive Neighbor-Joining phylogenic tree inferred from amino-acid sequences depicting the evolutionary relationship between representative ruminant PAGs, porcine PAGs and Pepsinogen F (PAG like proteins) from several species. Notice the clear separation of the ruminant PAG-gene family (bovine, ovine and caprine) into a relatively large grouping of tightly clustered 'modern-PAGs' and a small grouping of

loosely clustered ‘ancient-PAGs’. Also notice that the PAGs are clearly isolated from the ‘PAG-like’ molecules grouping, which consists of equine PAG and pepF molecules from mouse, rat and rabbit. A distinctly small grouping of porcine PAGs could also be seen sandwiched between the PAG-like grouping and ruminant- PAGs. The porcine pepsin is used to root the tree. Note that the ruminant-PAGs and porcine PAGs are somewhat more closely related to PAG-like proteins as apposed to the conventional aspartic peptidase, pepsin.

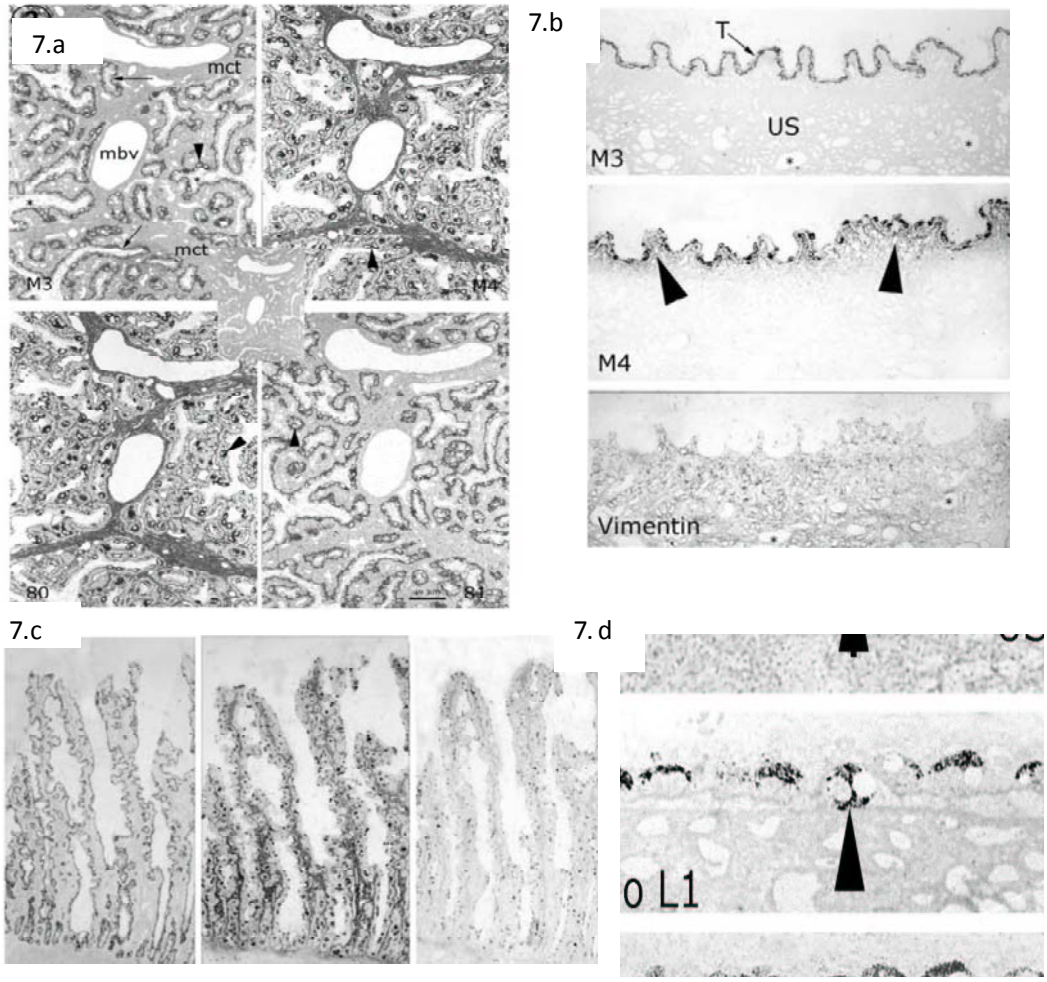


Figure -15. Light microscope immunocytochemistry of bovine placentome sections. M3, M4: polyclonal antibodies recognizing **ancient** and **modern** PAGs respectively. #80: anti-ovine **PAG-1** (a modern PAG) polyclonal antibody.#81: anti-**boPAG-2** (an ancient PAG) antibody.

15a) Light microscope immunocytochemistry of adjacent sections of a 150 Days post estrus (dpe) bovine placentome. M3 and 81 antibodies show equivalent localization restricted to the microvillar junction (arrows) and BNC granules (arrowheads); M4 and

80 antibodies labeled the BNC more strongly (arrowheads) and the maternal connective tissue (MCT). Fetal connective tissue (asterisks) was not labeled by any of these antibodies. The inset in the centre is a control section without antibody.

15b) Light microscope immunocytochemistry of adjacent sections of a 37 dpe placentome. The villi (e.g. at arrowheads on the M4 section) have just started to form, covered with trophoblast (T). The microvillar junction (mvj), trophoblast apices and BNC granules labeled with M3; M4 predominantly localized to the BNCs and did not label the mvj, although it did label the maternal connective tissue within the villi (M4, arrowheads) but did not label the deeper uterine stromal (US) connective tissue. Vimentin antibody labeled all the uterine connective tissue, including that in the newly formed villi. Profiles of the same blood vessels are indicated (asterisks) in the M3 and Vim panels.

15c) Light microscope immunocytochemistry of adjacent sections of a 55 dpe placentome. The M3 antibody labeled only the microvillar junction (mvj) clearly and the BNC weakly; M4 labeled BNCs and maternal villus connective tissue strongly and minimally labeled the mvj. The maternal endometrial connective tissue (US) below the villi is unlabeled. The monoclonal L1 labeled only the BNC granules.

15d) Light microscope immunocytochemistry of adjacent sections of an implanting 30 dpe conceptus, without counterstain. All antibodies labeled every BNC (arrowheads) in the trophoblast (T) at this early stage (M3, 80, 81 not shown) the uterine stroma (US) showed no significant labeling [From(Wooding et al., 2005)].

Amino acid sequences adjacent to the catalytic Aspartates (D) of aspartic peptidases

	N –terminus						C -terminus						
Pepsin (porcine)	**F**	**D**	**T**	**G**	**S**	**S**	**V**	**D**	**T**	**G**	**T**	**S**	
PepF (mouse)	**L**	**D**	**T**	**G**	**S**	**S**	**M**	**D**	**T**	**G**	**T**	**S**	**PAG –like Molecule**
PAG-1 (ovine)	**F**	**D**	**T**	**G**	**S**	**A**	**V**	**G**	**T**	**G**	**T**	**S**	**Modern PAGs**
PAG -9 (bovine)	**F**	**D**	**T**	**G**	**S**	**S**	**V**	**H**	**T**	**G**	**T**	**S**	
PAG -9 (caprine)	**F**	**N**	**T**	**G**	**S**	**S**	**V**	**G**	**T**	**G**	**A**	**G**	
PAG -1 (bovine)	**F**	**D**	**T**	**A**	**S**	**S**	**V**	**D**	**T**	**G**	**T**	**S**	
PAG -15 (bovine)	**F**	**D**	**T**	**G**	**S**	**S**	**V**	**D**	**T**	**G**	**V**	**S**	
PAG -2 (bovine)	**F**	**D**	**T**	**G**	**S**	**S**	**V**	**D**	**T**	**G**	**T**	**S**	**Ancient PAGs**
PAG -2 (ovine)	**F**	**D**	**T**	**G**	**S**	**S**	**V**	**D**	**T**	**G**	**S**	**S**	
PAG -2 (porcine)	**F**	**D**	**T**	**G**	**S**	**S**	**V**	**D**	**T**	**G**	**T**	**S**	
PAG -2 (caprine)	**F**	**D**	**T**	**G**	**S**	**S**	**V**	**D**	**T**	**G**	**T**	**S**	

Table -1. Sequences of amino acids around the catalytic aspartic acid (D) of both bovine and ovine PAGs are aligned for comparison. Pig Pepsin is used as an example of a catalytically active aspartic peptidase, displaying conserved catalytic residues. Notice mutations in either the N-terminal or C-terminal domains in modern PAGs. The similar regions in ancient PAGs do not display any deviations.

Figure 16a

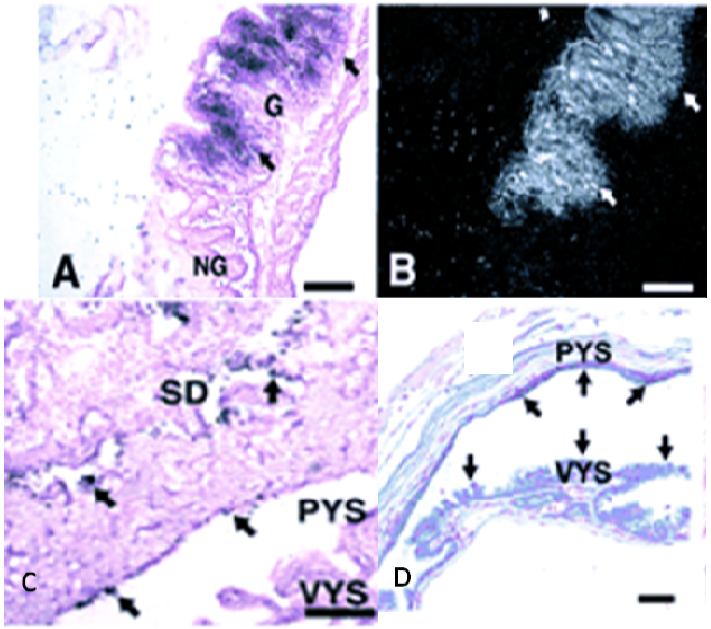


Figure 16b

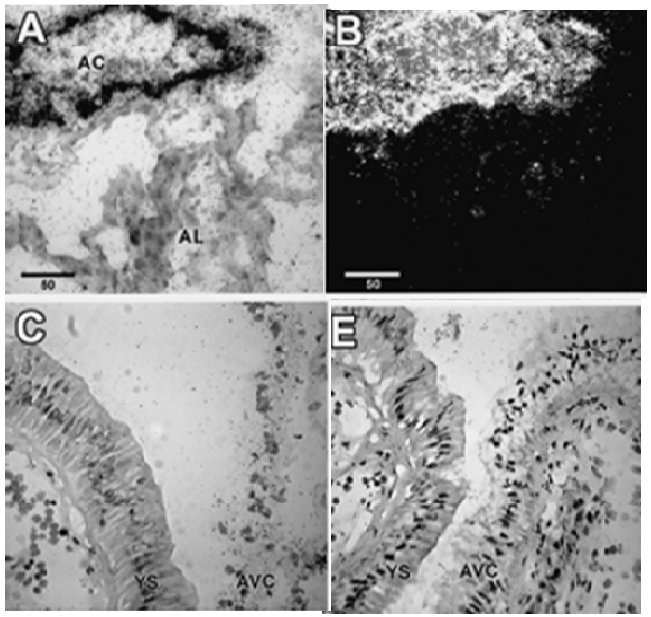


Figure -16. 16a) In situ hybridization for mpepF in the neonatal stomach and placenta. Photomicrograph of sections hybridized with a radioactive mpepF antisense probe; a, bright field; B, dark field. Notice the signal in gastric chief cells (arrows). C) Placenta sections exhibiting a signal in the parietal yolk sac and sinus of Duval. D) In yolk sac the signal was predominantly localized in the parietal yolk sac (PYS) (arrows), yet, detectable signal was also present in the visceral yolk sac layer (arrows) (VYS). (Chen et al, 2001)

16b) In situ localization of mRNA for ePAG in extraembryonic membranes obtained from pregnant mares at Day 25 of pregnancy. Shown are brightfield (A, C, and D) and darkfield (B) illuminations. Notice the ePAG mRNA localization to the AC and AVC. AC, Allantochorion; AL, allantois; YS, bilaminar yolk sac; AVC, avascular chorion. (Green et al, 1999).

Chapter –II

Characterization of the bovine Pregnancy-associated glycoprotein gene family-analysis of the gene sequences, regulatory regions within the promoter and expression of genes.

Abstract

The Pregnancy-associated glycoproteins (PAGs) belong to a large gene family of putative aspartic peptidases expressed exclusively by trophoblasts in species belonging to the Artiodactyla order. In cattle, the PAG gene family is rather large and consists of at least 22 distinct transcribed genes, in addition to the numerous variants and pseudogenes reported. The vast PAG family in cattle can be classified into two distinct groups, the 'ancient' and 'modern' PAGs, based on their phylogenetic relationships. Apart from differences in the gene sequence between (and among) the two groups, there are clear distinctions in their spatio-temporal distribution, as well as in their relative level of expression. With this underlying complexity in mind, we designed three broad goals to study this gene family. One of the objectives was *in silico* analysis of the current bovine genome assembly to further characterize the PAG gene family. The second objective was to scrutinize the proximal promoter sequences of the PAG genes to evaluate the evolution pressures operating on them in addition to identifying crucial regulatory regions. The final objective was to study the relative expression and perform preliminary characterization on some putative regulatory elements of a candidate PAG gene (boPAG-2; 'bo'-for bovine). From our analysis of the current bovine genome build, we found 18 distinct PAG genes and 14 pseudogenes. The analysis of the proximal promoter regions of all the boPAG genes revealed that the first 500 base pairs (bp) upstream of the translational start point (TSP) were highly conserved in all the boPAG genes and,

therefore, would likely harbor the crucial regulatory elements of the genes. Within this critical region, we found multiple regions of conservation shared by all boPAGs, however, a preponderance of these conserved regions were unique to modern boPAG grouping, but not to the ancient boPAGs. Some of these conserved regions harbored recognition sites for putative transcriptional factors, implicating a potential role for transcriptional regulation, unique to this group. We also gathered evidence by means of Real-time PCR and EST frequency data to show that boPAG-2 is the most abundant transcript of all the boPAGs in the bovine genome. Finally, we performed electrophoretic mobility shift experiments to provide experimental evidence for the role of the Ets and c-Rel group of transcription factor (TF) in the regulation of boPAG-2 gene. These experiments mark the crucial first step in discerning the complex transcriptional regulation operating within the boPAG gene family. Future efforts will be directed at collaborating with the bovine genome consortium for further annotation of the boPAG gene family, as well as systematically analyzing promoter sequences for delineating transcriptional regulatory networks.

Introduction

Duplication of a region of chromosome containing a gene, also known as 'gene duplication' results in two copies of the parent gene. Following the origin of a new gene-pair, both the members may undergo a period of accelerated mutagenesis (Prince and Pickett, 2002); more often, however, stringent selection is placed on one of the gene pairs, allowing it to maintain the gene structure, while the redundant copy relieved from the selection pressure accrues random mutations. In most cases, as a consequence of random mutagenesis, one of the paired genes will acquire inactivating mutations and will

undergo degeneration to a pseudogene and, eventually to disappear from the genome due to chromosomal remodelling and/or locus deletion. This process is known as 'non-functionalization'(Haldane, 1933). While the genomes of all studied species have evidence for such pseudogenes, there are a few examples wherein, alterations within coding or regulatory sequences exposes the formerly redundant gene to new functions, a process known as 'neo-functionalization'(Prince and Pickett, 2002). While neo-functionalization is a rare outcome, a distinct and more likely alternative is 'sub-functionalization' (Force et al., 1999; Lynch and Force, 2000). According to this model, complementary partial 'loss-of-function' mutations in both genes results in the sharing of a full complement of functions that had been performed by the single ancestral gene (Prince and Pickett, 2002). It has been emphasized that such changes leading to sub-functionalization might happen primarily at the level of regulatory regions of the promoter affecting gene regulation, rather than amino acid sequences influencing protein function (Yuh et al., 2001). Complementary changes in regulatory elements might result in two discrete expression patterns that together would recapitulate the more complex expression pattern of the single ancestral gene. Therefore the end result of neo- and sub-functionalization is the birth of novel gene pairs and potentially expanded gene families (Prince and Pickett, 2002; Yuh et al., 2001).

All sequenced genomes to date revealed, among other things, a predominance of gene families, whose products are expressed in reproductive organs, including the placenta (Bailey et al., 2002; Green, 2004; Lynch and Conery, 2000; Sol-Church et al., 2002). Within the placenta, the chorionic trophoblast constitutes the interface between the maternal uterus and the fetus and performs a range of transport and endocrinological

functions that provide support to the growing fetus. At times, the physiological demands of the fetus are not necessarily compatible with the interests of the mother to provide for current and future offspring. Consequently, the groundwork is laid for genetic conflict between these individuals. As part of the interface between the fetus and the mother, gene products that are functional in trophoblast are targets of selective pressures arising from this so called "genetic arms race". Such selective pressures are thought to drive the birth of novel gene families (Green, 2004; Haig, 1996). Indeed, examples of such gene families do exist in the placenta of cattle and related species. One such example is the family of purported aspartic peptidases, pregnancy-associated glycoproteins or PAGs.

The PAGs represent one of the major trophoblast secretory products of species in the *Artiodactyla* order (swine, cattle, deer, camels, etc) (Brandt et al., 2007a; Garbayo et al., 2000; Green et al., 2000; Szafranska et al., 1995; Vawter et al., 2004b; Xie et al., 1997a; Xie et al., 1997b). The PAGs are related to the aspartic peptidases, a diverse grouping that includes gastric enzymes such as pepsin and chymosin, lysosomal enzymes such as cathepsin D and the enzyme renin that is critical in maintaining salt homeostasis and blood pressure (Davies, 1990). Mammalian aspartic peptidases are strikingly conserved in regard to their gene structure (most mammalian aspartic peptidase genes consists of nine exons and eight introns) (Azuma et al., 1992; Davies, 1990; Hayano et al., 1988; Ishihara et al., 1989; Ord et al., 1990; Sogawa et al., 1983). The aspartic peptidases (APs) also have a conserved bi-lobed structure (Davies, 1990; Hartsuck et al., 1992; Szecsi, 1992; Tang and Wong, 1987) with the two lobes of the enzyme being roughly symmetrical and enclosing a substrate binding cleft between them. Positioned within this cleft is a catalytic center that contains two critical aspartic acid residues (one

from each lobe) flanked by conserved amino acids (Davies, 1990; Green et al., 1998b). These aspartates are involved in the catalytic mechanism.

In species within the *Ruminantia* suborder, the PAGs constitute a large and diverse family (Brandt et al., 2007a; Garbayo et al., 2000; Green et al., 2000; Vawter et al., 2004a; Xie et al., 1997a; Xie et al., 1997b). In cattle for example, 22 distinct PAG cDNAs have been reported, in addition to numerous variants and pseudogenes. However, the exact number of PAG genes remains a mystery. The distinct PAG cDNAs that have been cloned in cattle, fall into two main groupings: the evolutionarily more 'ancient PAGs' whose members are transcribed in all cotyledonary trophoblasts, and a second group that arose more recently, the 'modern PAGs', which are transcribed exclusively by specialized trophoblasts called binucleate cells (BNC) (Garbayo et al., 2000; Green et al., 2000; Hughes et al., 2000). Interestingly, many of the PAGs in the modern-grouping have amino acid substitutions at normally conserved positions within the catalytic center that are believed to preclude these PAGs from functioning as enzymes (Green et al., 1998b; Guruprasad et al., 1996; Hughes et al., 2003; Xie et al., 1997b; Xie et al., 1991a). The ancient PAGs on the contrary, were not confined in their expression to the invasive BNCs, but were present throughout the trophoblast. In addition, the ancient PAGs possess all the hallmarks of typical aspartic peptidases and are predicted to be active enzymes (Green et al., 1998b). It is also worth noting that the PAGs are not expressed coordinately throughout pregnancy. Some are expressed early while others are not detectable until later in the pregnancy (Green et al., 2000; Patel et al., 2004). Another, crucial differentiating characteristic is that, the relative abundance or expression of individual PAGs in either group seems to differ from one another. For example, boPAG-

2 was found to be a relatively abundant PAG, compared to closest relatives in the ancientPAG grouping (J. Green; personal communication). While differences in the coding sequences between the two PAG groupings have received much attention in the past, one interesting aspect of their biology, that has received relatively less attention so far, is their transcriptional regulation.

The sequencing and ensuing assembly of the bovine genome has provided two unique opportunities. One of them was to 'peer' into the bovine genome to identify and evaluate all the potential PAG and PAG-like genes. The other advantage was to gain access to full length sequences of the PAG genes, especially their promoters, thus facilitating analyses and insight into PAG transcriptional regulation. The goal of this current work was to take advantage of both these opportunities to perform *in silico* analysis to annotate the PAG genes within the bovine genome, as well as their promoter regions. The analysis of the promoter was extended somewhat to gain additional understanding into the transcriptional regulation of the PAG genes. Particular attention was focused on the regulatory elements of boPAG-2, which appears to be the most abundant PAG transcript, and to characterize its relative expression compared to its closest paralog, boPAG-12.

Material and Methods

A. Annotation of PAG-genes within bovine genome (Build 3.1):

To annotate the PAG genes in the bovine genome, guidelines set forth for annotation by the bovine genome consortium were followed (http://genomes.tamu.edu/bovine//annotation_information.html). Previously, an extensive

official gene set for the bovine genes was developed by using a powerful gene prediction algorithm known as GLEAN (Elsik et al., 2007), which was provided to the annotation community. The first step in the annotation process was to identify the best matching protein homolog or GLEAN model for a candidate boPAG. The nucleotide sequence of the known RefSeq mRNA or cloned boPAG cDNA (if Refseq was not available) was used as a query sequence for searching the GLEAN model on the bovine genome consortium web browser (http://genomes.tamu.edu/bovine//blast/blast.html). The closest matching protein homolog-GLEAN model, was used to locate and retrieve the original gene model from the bovine genome database, by using the Apollo Genome Annotation and Curation Tool (v. 1.6.5). The sequence obtained from the gene model was visually verified for the presence of a putative translation start site, intact exon-intron boundaries and defined 5′ and 3′ UTR's. More importantly, the coding sequence (CDS) was scrutinized thoroughly for any mismatches with known RefSeq or cDNA clones by performing megaBLAST on the NCBI database (for highly related nucleotide sequences) and a BLASTN search on the bovine genome browser (GBROWSE). Any lingering incompatibility was further evaluated by performing megaBLAST or cross-species megaBLAST against the bovine EST database in Genbank. The sequence which had the best EST support was accepted and, based on these results, the GLEAN-models were accepted, rejected and/or refined. Refined models were submitted to the genome annotation curators for independent review and acceptance.

In addition to the known boPAG-genes, there were other putative PAG-like genes predicted by the GLEAN software and a GLEAN#ID was assigned to them. We performed comprehensive BLASTN search in the EST database as well as the

discontiguous megaBLAST in the others nucleotide collection (nr/nt) database to verify if these predicted genes were actively transcribed. The accession numbers for the original boPAG cDNA sequences and their corresponding RefSeq mRNA in Genbank that were used to retrieve the query sequences were listed in Table -2.

B. Phylogenetic relationships of bovine PAG-genes:

The amino acid sequences of the boPAG genes, that were used to evaluate the phylogenetic relationships of the PAG gene family were shown highlighted (Regular bold, font) in Table -2. As could be verified from Table -2, the amino acid sequences used in the analysis were predominantly obtained from verified GLEAN models. For those boPAG genes that have a known mRNA sequence, but were not represented in the current bovine genome build (or for whom a GLEAN sequence could not be retrieved), the amino acid sequences were inferred from their mRNA sequence. The resulting amino acid sequences from all the boPAG genes were aligned by using CLUSTALW software. All positions within the alignment that contained gaps or missing data were ignored during pairwise comparisons. An initial tree was generated by using the Neighbor-Joining method (Saitou and Nei, 1987), following which Minimum Evolution (Rzhetsky and Nei, 1992) and bootstrapping (from 1000 replicates) were performed, and the inferred consensus tree was displayed. The tree was drawn to scale, and the percentage representations obtained from the bootstrap analysis were shown next to the appropriate branchpoint (Felsenstein, 1985). Phylogenetic analyses were conducted by using the MEGA4 program (Tamura et al., 2007).

C. Analysis of repeat elements within bovine PAG-genes:

RepeatMasker software, version 3.1.9 (http://www.repeatmasker.org) was used to scan for inserted transposable elements (TE) in the entire gene sequence, as well as 3000bp upstream of the translational start point (TSP-ATG) and 3000bp downstream of the translational termination codon (TAA, TGA and TAG) of each representative boPAG gene. The parameters used for the analysis were described elsewhere (Almeida et al., 2007). Briefly, 'cross match' was used as the search engine, cow (*Bos taurus*) was identified as the DNA source, simple repeats and low complex repeats were requested not to be masked and the matrix was set to sub-loci optimization pre-runs.

D. Analysis of proximal promoters:

I) Analysis of selective pressures operating on the proximal promoter sequence of boPAG-genes

The selective pressures operating on the open reading frames (ORFs) of various boPAG genes have been analyzed systematically in prior publications (Hughes et al., 2000; Hughes et al., 2003). The availability of full length gene sequences has made it possible to extend similar types of analyses to the PAG promoter regions. Two different lengths of promoter sequence were chosen for comparison [1000 bp as well as 500 bp proximal to the 'ATG' translational start point (TSP)] between several ancient (boPAGs-2, 8, 10, 11 and 12) and modern PAGs (boPAGs-1, 3, 4, 5, 6, 7, 15, 18, 19, 20 and 21). The nucleotide sequences were aligned by using CLUSTALW in the MEGA4 software suite. All the deletions and gaps arising from the alignment were eliminated by using the pairwise deletion option. The aligned boPAG sequences were subjected to pairwise

comparisons in MEGA4 by using the Maximum composite likelihood method with 1000 bootstrap replicates to calculate the p-distance (number of differences/total length of sequence analyzed).

In order to understand the type of evolutionary pressures operating on the promoter regions, we plotted the inferred p-distances obtained from the promoter analysis against the proportion of synonymous changes per synonymous site (dS) estimated for the corresponding boPAG- ORFs. The underlying assumption for this approach was that, dS within the ORFs would approximately reflect the rate of nucleotide change in the locus in the absence of selection. In other words, if the p-distance of the promoter equals dS of the corresponding exons of the gene (p-distance/dS = 1), then the boPAG-promoter is accumulating substitutions in this region at a rate that corresponds to that expected based on normal mutation rates. A value >1 would indicate that nucleotide changes are occurring faster than would be predicted and a value <1 would suggest stringent purifying selection, with fewer substitutions being tolerated and hence retained.

II) Multiple sequence alignment of the proximal promoter regions of selected bovine PAG genes for identification of incorporated Transposable elements (TE) and conserved regulatory regions:

a) Analysis of TE with the proximal promoter region:

To explain for the apparent differences within the non-coding proximal promoter sequences of the boPAG genes obtained from the evolutionary analysis of the promoter sequences described above, 1000bp upstream of the translational start codon (ATG) were

analyzed for transposable elements. RepeatMasker software, version 3.1.9 (http://www.repeatmasker.org) was utilized for analyzing 1000bp upstream of the translation start point (TSP) of the boPAG genes using the parameters detailed above. A multiple sequence alignment was performed utilizing CLUSTALW software, on the analyzed sequences and the insertions of TE identified by the repeat masker program within the promoter elements were visually detected and mapped to the boPAG promoter sequences.

b. Analysis of the conserved regulatory regions in the proximal promoter for transcription factor binding (TF):

DiAlign TF, a component of comprehensive promoter analysis software - Genomatix-GEMS launcher (http://www.genomatix.de/products/GEMSLauncher/) was used to align, and search for conserved transcription factor (TF) binding sites within the proximal promoter regions of select PAGs. Approximately, 1000bp upstream of the TSP (proximal promoter) of eight boPAG genes [4 ancient (boPAGs-2, -8, -11, -12), and 4 modern PAGs (boPAGs -3, -5, -15 and -18)], which were recognized by the GEMS database were used in the analysis. The following parameters were selected for performing the analysis: Matrix library 7.0 was used as the default library to match the TF binding sites and 'all' the matrix groups, from 'embryo' tissue type were selected as a reference. The software was used to align the input sequence, and map regions conserved in more than 50% of the input sequences (4 out of 8), which closely matched the known TF- binding sites.

E. Estimation of relative frequency of various PAG ESTs found within bovine genome:

In order to estimate how the observed differences within the boPAG promoters might reflect true differences in expression, we searched the bovine EST database, to estimate relative levels of transcription based on EST frequency. The boPAG cDNAs were used as the query sequence. All the ESTs that came up as positive hits were analyzed for their percent identity and all those matching ESTs that exceeded 98% nucleotide identity were considered to be a positive match with a particular PAG.

F. Quantitative Real-time PCR:

It was noted from analysis of proximal promoters and EST frequencies, that there were some distinct differences in the EST frequencies and in the regulatory regions of boPAG-2 and boPAG-12. These two PAG genes share 88% similarity in the nucleotide sequence within the promoter (500bp). They differ in only a few conserved regions that are likely recognized by TFs, for example a MYBS TF binding site at -333bp, (marked in red font color, the deviant residues are boxed). Another major difference was a gap in the promoters (missing in boPAG-2) at position -251-258 relative to the translational start point, which matched a TF, AP4R binding site in boPAG-2 (Figure -21). In order to determine if these minor differences in the purported promoter elements influence the relative expression of two closely related boPAGs, we performed quantitative Real-time PCR (Q-PCR) to measure boPAG-2 and boPAG-12 transcripts in placental RNA harvested from different stages of pregnancy.

To perform Q-PCR, RNA was extracted from placentomes at various stages of pregnancy (days 60, 75, 90, 140, 170, 220 and 280), by using STAT-60 RNA extraction

reagent. The extracted RNA preparations were treated with amplification grade DNAse I (Invitrogen, CA, USA) at room temperature according to the manufacturer's recommendations. The DNA-free RNA samples were quantitated and analyzed for quality (260/280). Two micrograms of high quality RNA from each sample were reverse transcribed by using an oligo-dT primer and SSIII-reverse transcriptase (First strand cDNA synthesis kit-Invitrogen, CA, USA) at 50˚C for 1hr.

Oligonucleotides for Q-PCR were designed to span exons of both boPAGs to prevent unwarranted amplification of any trace carry-over contamination from the genomic DNA. Oligonucleotides were also designed for four putative control genes in cattle, YWHAG (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide), HPRT (hypoxanthine phosphoribosyl transferase), ALDH (aldehyde- dehydrogenase) and beta-actin. Power SYBR© Green PCR master mix (Applied Biosystems, CA, USA) reagent and the Applied Biosystems 7300 Real-Time PCR system were employed for the Q-PCR. Initially, the amplification efficiency as well as the dynamic range for each primer set was determined according to the methods provided by the manufacturer. Following the analysis, YWHAG was determined to be the best control gene for this assay, since it displayed similar amplification efficiency compared to the boPAG targets and was represented uniformly across all the stages examined (data not shown). Following the preliminary evaluation, the optimum primer sets were selected (Table -3). The Q-PCR for each candidate gene was set up with a duplicate of biological replicates and duplicate technical replicates. The cycling conditions used were: pre-heating for : 50°C for 2 min (1cycle); followed by a pre-run to activate the polymerase at 95°C for 10 min (1 cycle) followed by 40 cycles of 95°C for

15 sec, 65°C for 30 sec and 75°C for 1 min, with the data being acquired in the 75°C window. The data was analyzed by the ABI-PRISM 7000 sequence detection system software and the results from the analysis were graphed.

G. Characterization of boPAG-2 gene promoter:

Since boPAG-2 transcripts are abundant compared to all the other known PAGs, additional experiments were performed on its promoter region.

I. Primer extension analysis for determination of the transcription start point of boPAG-2:

A bovine PAG-2 specific primer of 37 nucleotides (GTGATGTTACCCACGTAGGCAGTATCCAGATAGTTCC), complementary to positions 182-218 of the boPAG-2 cDNA, was constructed and end labeled with 20 μCi of [^{32}P- γ] ATP by using T4 polynucleotide kinase. Following purification on a G-50 sephadex column (Amersham biosciences, NJ), 200,000 cpm of the labeled oligonucleotide was annealed to bovine placental RNA from Days 80, 90 and 120 of pregnancy. Bovine ovary and fetal RNA were used as negative controls. Annealing of the labeled oligonucleotide to the RNA and reverse transcription (with AMV reverse transcriptase, 42° C for 2 hrs) were performed by following standard protocols (Sambrook et al., 1989). The amplified fragment was subjected to electrophoresis in formamide buffer on a 7% acrylamide denaturing gel with a dideoxy sequencing ladder generated from pBluescript SK+ vector (Stratagene, CA) as a molecular sizing ladder (Sambrook et al., 1989).

II. Electrophoretic mobility shift assays (EMSA):

Oligonucleotides (Integrated DNA Technologies, IA) were designed to encompass sequences in the regulatory region that were predicted to be involved in transcriptional regulation of the boPAG-2 gene. These regions included a putative Ets site (-229 to -232) and unique repeated regions in the promoter (-286 to -293 and -303 to-316). The oligonucleotides were annealed and end-labeled with [^{32} P-γ] ATP by using T4 poly nucleotide kinase. Binding reactions were performed by using radiolabeled probe (10,000-20,000 cpm/25fmol) with 20 μg of JAr choriocarcinoma cell nuclear extracts in the presence of 1 μg of nonspecific competitor (poly dI:dC; Sigma, MO). The nuclear extracts for EMSA were prepared as described by Dignam et al. The composition of the buffer used was 20% (v/v) glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dTT, 250 mM NaCl, 50 mM Tris-HCl of pH 8.0 containing 2% (v/v) CHAPS detergent and 10mg/mL BSA (Sigma, MO). For competition assays, a 50-250 molar excess of unradiolabeled competitor DNA (cold probe) was used. The Ets- 2 antibody competition assays were performed by mixing 2 μg of Ets antibody (Santa Cruz Biotechnologies, CA) with 20 μg of nuclear extracts. The mixture was incubated on ice for ½ hr followed by addition of the radiolabeled probe and incubation at room temperature for ½ hr.

Results

A. The PAG gene family in cattle:

The results from annotation of the boPAG-gene family were compiled and displayed in Table -2. The table contains, accession numbers of the mRNA sequence representing cDNA or Ref Seq in addition to the GLEAN model best matching their

sequence and corresponding locations on the chromosome. Out of the 37 potential boPAG genes (known and predicted), there were **18** full length, functional PAG genes that were represented and properly annotated in the bovine genome. **Four**, boPAG genes, which were previously characterized as distinct boPAG cDNAs were not represented in the current build. There were no matching GLEAN models for their cDNAs. There were **3** boPAG-like genes, which were predicted by the *in silico* gene prediction analysis, as having the conserved 9-exon structure of PAGs (GLEAN-IDs: 24769, 10319, 10323). Out of the three predicted genes, the boPAG-19 like gene (GLEAN_10323) had 100% identity with the boPAG-19 gene, both in the open reading frame and the proximal promoter regions. Therefore, we identify this as a recently duplicated copy of boPAG-19 gene within the bovine genome. The other two predicted genes were not shown to be actively transcribed. Apart from the known and full length boPAG-like genes, there were an additional 12 predicted genes, which seem to be either fragments or legitimate boPAG genes with few missing exons (Table -2). These boPAG-like genes that are missing exons are likely pseudogenes; since, no ESTs were found matching these sequences, even after thorough searching (data not shown). Therefore, in total, there were 18 unique, full length functional PAG genes, represented in the current bovine genome build (version 3.1). All the annotated boPAG-genes that were assigned a chromosome location were found to be clustered on chromosome 29. Many were placed on undefined chromosomal fragments ('ChrUn.').

B. Evolutionary relationships of PAG genes in cattle: The phylogenetic relationships of various annotated PAGs in cattle were based on their predicted amino-acid sequences (Figure -17). The boPAGs were grouped into two distinct sub-classes, one of the two groupings, the 'modern PAGs' comprised the bulk of the PAGs represented in the bovine genome build. They were found to be relatively tightly grouped with short branch lengths, implicating the apparent 'modern-ness' of this cluster (Hughes et al., 2000; Hughes et al., 2003). The others comprised a much smaller grouping (the ancient PAGs) and had relatively longer branch lengths and were loosely clustered.

C. Analysis of the Repetitive and transposable elements within the boPAG genes:

The incorporation of TE within genes results in modifications of the gene structure and provides information about the evolutionary history of genes. In order to evaluate the implications of such transposition events on boPAG gene structure, a preliminary evaluation was performed on the sequence of each PAG gene (including 3000bp 5′ and 3′ to the gene itself). The Repeat Masker software revealed that TEs were distributed only within the intronic and non-coding regions of the PAG genes. Consequently, the TEs are not directly influencing the reading frame of boPAGs.

The observations noted in Figure-18a, are derived from analysis of the boPAG genes for insertions from putative TEs. From our preliminary analysis, we noted that among the boPAG genes, the ancient group in general and boPAG-8 and 10 in particular, had more insertions from TEs, when compared to the modern boPAGs (Figure -18b). The ancient boPAGs on an average had 30.25 repeats incorporated into their sequence when compared to 23.3 for modern boPAGs. Of all the boPAGs that were analyzed boPAG-8

had more elements incorporated into its gene with a total of 37. This was closely followed by boPAG-2 and -10 with 31 repeats (Figure -18b). In the modern boPAGs, boPAG-5 had the largest number (29) of TE insertions, followed by boPAG-7 and -4 with 25 and 24 elements incorporated, respectively (Figure -18b).

Regardless of the total number of repeats incorporated, the total amount of sequence contributed by the introduced TEs did not seem to differ much between the two groups. For example, in the ancient boPAGs the average size of sequence contributed by the elements was around 36.6% when compared to 35.2% in the modern boPAGs. Again, of all the boPAGs, the percentage of total gene length contributed by the TEs was highest in boPAG-8 (44.5%) followed by boPAG-10 (43.6%) (Figure -2c). Interestingly, the average contribution of TEs to the size of the boPAG genes was much less than the average of 45% in bovine genes in general – although the ancient PAG members, boPAG-8 and boPAG-10 did approach this proportion (Figure -2C)(Almeida et al., 2007). While these constitute interesting observations, the significance of such results can only be derived from more systematic analysis of the sequences of the inserted TEs, themselves.

D. Analysis of the promoters of bovine PAG genes:

I. Selective Pressure operating on bovine PAG promoter sequences:

It was noted that the regulatory region of boPAGs did not possess extended lengths of conservation with other genes whose expression is restricted to trophoblast (data not shown). This analysis sought to evaluate how the proximal promoters of boPAGs were conserved across different family members. The evolution of these regions

was studied by performing pairwise comparisons on the aligned promoter nucleotide sequences of either 500bp (Figure 19b) or 1000bp (Figure -19a) sequence upstream of the TSP of boPAGs.

In order to verify the significance of results obtained from pairwise comparisons of promoter regions and understand the selective pressures operating on the promoters, the observed p-distance of the promoters were plotted against dS obtained for the nucleotide sequences of corresponding ORF. There were two principal assumptions, within the analysis; these were 1) that dS of the exons of each analyzed gene pair was under neutral selection and would reflect the normal mutation rate for this chromosomal location, and 2) if the calculated p-distance within the promoter is equal to the dS of the exons, then the promoter is mutating at a rate that is expected for this location. If the observed ratio is above 1, it was considered positive selection for nucleotide substitutions and if below one, it was purifying selection.

The analysis was performed with two variable lengths of promoter sequence. When we mapped the p-distance vs the ORF for the proximal 1000bp, we noticed that almost all of the boPAGs were undergoing neutral to purifying selection (Figure 19 -a and b), with the exception of boPAG-10 and -6, which had ratios of more than one (Figure -19a). These promoters seem to have accumulated more mutations than was predicted by molecular clock. The analysis, when confined to the first 500 bp, it generated similar results except that both boPAG-6 and -10 showed a ratio close to neutrality (Figure 19b). Overall, the boPAG promoters are being conserved, particularly in the first 500 bp upstream of the translational start site (Figure 19b).

II. Multiple sequence alignment of the boPAG promoters for TE and conserved transcription factor (TF) binding sites:

a. Analysis of TE with the proximal promoter region:

To account for observed differences within the proximal promoter elements, the 1000 bp upstream of the translational start site was characterized for the presence of repeat element insertions. The sequences of the promoters were aligned and the position and types of TE insertions were identified and mapped (Figure -20). We found that among all the boPAG promoters analyzed there were no TE insertions within the proximal 600 bp region with the exception of boPAG-10 which had a SINE (MIRb) insertion at -317 bp (TSP being +1). An interesting observation was that the type of TEs incorporated into individual boPAG promoters deviated between modern and ancient boPAGs. In boPAG-10 for instance, there was a long SINE-element insertion from -524to -1066 bp. The corresponding region was occupied by DNA element Charlie-8 in all modern boPAGs and an additional LINE element (L2) in boPAGs-4, -5, - 7, and -15 (Figure -20). In the ancient boPAGs there was an ~200 bp DNA MER-1element upstream of -750 bp that was conserved in all the ancient boPAGs, with the exception of boPAG-10. This analysis, therefore, reveals that the two groups of the boPAG promoters deviated in regard to the types of TEs that invaded their sequences upstream of -500bp, which probably was the reason for the large deviations in the observed p-distances between them. Similarly, because of a lengthy SINE insertion in the boPAG-10 promoter in the corresponding region, which was not found in the rest of the boPAG promoters, the boPAG-10 promoter diverged considerably from the remainder of the boPAG promoters.

b. **Conservation of regulatory regions within the boPAG-promoters:**

From our previous observations, we realized that the boPAGs exhibited differences in spatial and temporal expression patterns (Garbayo et al., 2000; Green et al., 2000; Xie et al., 1997b). The availability of the full-length promoter sequences provided an opportunity to identify any obvious differences in the conserved regulatory elements, that are unique to either sub-classes of boPAGs-modern or ancient, that could potentially explain the observed differences in temporal and spatial expression pattern.

For this analysis, the first 1000bp upstream of the TSP of various boPAGs was examined by using the DiAlign TF program of Genomatix-GEMS launcher. Among the aligned boPAG-promoter sequences, there were regions of conservation that were shared by all the boPAGs; however, there were also isolated regions, that were unique to either ancient or modern boPAGs, and some of these regions matched known transcription factor binding sites. Examples of such regions within the first 400 bp were boxed and listed in the Figure -21. It became very apparent that many of these conserved putative TF binding sites were highly prevalent in modern boPAGs. For example, there were predicted binding sites for these TFs: MADS at position -120, RPOA at -131, a FKHD at -135, E2FF at -161, EREF at -168, DTCF at -187, HOMF at -216, DTCF at -256 and CSKN at -325 bp (TSP is +1). In addition, an atypical Ets site was conserved in all boPAGs and was found at position -239 bp. Besides these sites, there were two tandem repeats (TTCTCCA) 11 bp apart at positions -286 and -304 bp, respectively. Of these two repeats, the distal repeat was predicted to be recognized by a c-Rel class of transcription factors (DDVL-drosophila dorsal – ventral factor). These repeats were conserved in most

of the boPAGs and were referred to as 'bovine repeats' (BR), as was described previously (Szafranska et al., 2001).

E. The relative distribution of boPAG-ESTs in the bovine EST database:

In order to verify if apparent differences observed in the promoter sequence might be associated with the relative levels of transcription of various genes, we searched the EST databases for the relative distribution of various boPAG transcripts. We noticed that of all the boPAGs that were investigated, boPAG-2 had the highest occurrence, with 92 ESTs represented. The next most abundant member was boPAG-11 with 46 ESTs (Figure -22). Of the modern boPAGs that were probed, boPAG-1 had the highest number of EST matches of 28 followed by boPAG-17 with 25 matches (Figure -22).

F. Real-Time PCR of bovine PAGs -2 and-12:

We observed from previous experiments in our lab and from the analysis above that boPAG-2 was an extremely abundant transcript. Therefore, we set out to study the relative expression of boPAG-2, in comparison to its closest relative boPAG-12. Real time quantitative PCR of bovine PAGs -2 and -12 were performed relative to an endogenous control, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (YWHAG) in placentomal samples at different stages of pregnancy starting from d 60 till term. The relative fold changes of both boPAG-2 and-12 from the assay were graphed and shown in Figure -23. The results revealed that bovine boPAG-2 is a highly abundant transcript relative to boPAG-12 under similar experimental conditions, with transcript abundance level ranging from 186-1745 times

greater than YWHAG, depending on the stage of pregnancy. In contrast, boPAG-12 varied 0.16 to 1.37 times the YWHAG transcript. Assuming, that the efficiencies of primers were identical between both the boPAG-2 and -12 primer sets, the transcript abundance of boPAG-2 relative to boPAG-12 was at the least an order of 100 more.

G. Systematic Analysis of the boPAG-2 promoter:

Since boPAG-2 was the most abundant transcript observed in the bovine genome, we set out to study its promoter in some detail. We performed primer extension analysis to define the putative transcription start site and experimentally verified the validity of some of the conserved elements within its promoter.

I. Primer extension analysis for determination of the transcription start site (tss) of boPAG-2:

Primer extension analysis was performed by using a ^{32}P labeled antisense oligonucleotide specific to the boPAG-2 gene (Figure -24). Based on the size of the extended products, the *tsp* was found to be 25 bp downstream of a putative TATA box and 48 bp upstream of the ATG start codon.

II. Electrophoretic mobility shift assays:

Ets-2 is a key transcription factor involved in the regulation of numerous placenta specific genes such as IFN-tau (Ezashi et al., 1998) and the hCG beta subunit (Ghosh et al., 2003). Electrophoretic mobility shift assays (EMSA) were performed as a first step to determine if the putative Ets site might also be involved in the regulation of PAG genes

and we chose boPAG-2 as the candidate. Competition and super shift assays (Figure -25 a, and -25b) were performed with ^{32}P-labeled oligonucleotides representing the putative Ets site at -239. Nuclear extracts obtained from JAr human choriocarcinoma cells indicated the presence of a protein(s) capable of specific association with the oligonucleotide probe. The complex could be competed away by excess unlabeled probe and could be decreased by the addition of an anti-Ets antibody. Likewise, to determine if proteins are present in human JAr cells capable of binding to the unique tandem repeats (BR-1 and -2), similar EMSA were performed. A specific complex was identified that could be competed away with an excess of non radiolabeled specific competitor (Figure -25C and D).

Discussion

Trophoblasts comprise the outermost cells of the placenta and are, therefore, the cells most directly involved in maternal interactions. Trophoblasts express and secrete numerous peptide hormones and other proteins, most of which have yet to be fully studied. The PAGs clearly fall into this category. To date, dozens of PAG cDNAs have been cloned from species within the *Artiodactyla* order (Brandt et al., 2007a; Garbayo et al., 2000; Green et al., 2000; Szafranska et al., 1995; Vawter et al., 2004b; Xie et al., 1997b). Adding to the numerical complexity associated with this gene family is the pattern of their distribution within the placenta of ruminants. Some members are expressed throughout all trophoblasts, while the majority are confined to the invasive trophoblast BNC population (Green et al., 2000). In cattle, these two expression patterns (BNC-specific v. expression in all trophoblasts) happen to correspond to the phylogenetic

relationships among the bovine PAGs (Green et al., 2000; Hughes et al., 2000; Hughes et al., 2003). Those members expressed in all trophoblasts are relatively ancient, having arisen more than 80 MYA (Hughes et al., 2000). In contrast, those PAGs whose expression is restricted to BNCs are relatively recently evolved genes that are predicted to have arisen 50-55 MYA (Hughes et al., 2000). This time frame corresponds approximately to the period in which the ruminant ungulates, with their unique synepitheliochorial type of placentation, are believed to have diverged from the swine lineage (Kumar and Hedges, 1998). The BNCs are the hallmark of the synepitheliochorial placenta. These large cells, which comprise ~20% of the total trophoblast population, can fuse with uterine epithelial cells to form either a syncytium or short-lived trinucleated cells – depending on the species (Wathes and Wooding, 1980a; Wooding, 1992; Wooding, 1983; Wooding et al., 1997; Wooding et al., 1994). This fusion event is the extent of invasiveness in ruminant ungulates and is quite unique among eutherians (King, 1993a; King et al., 1982; Leiser and Kaufmann, 1994). This type of placentation probably developed from the completely noninvasive epitheliochorial placenta observed in nonruminants, such as camels and pigs (Amoroso, 1952a; Skidmore et al., 1996). Similarly, the origin of the *Artiodactyla* order itself has been estimated at about 83 MYA (Kumar and Hedges, 1998), a value that is very close to the estimate of when the PAG genes as a whole first began to duplicate. It is tempting to speculate that the burst of duplications that gave rise to the PAG gene family initially were associated with the formation of the *Artiodactyla* order to fulfill a role distinctly required of the epitheliochorial placenta. Likewise, the formation of the BNC-PAG group may have been

linked to the emergence of the sub-specialized synepitheliochorial placental type of the *Ruminantia*.

With reference to cattle, the PAG gene family was found to be very extensive. A total of 22 distinct PAG cDNAs have been deposited into GENBANK, in addition to numerous variants (and pseudogenes), which underscores the complexity of this gene family within the bovine genome. Therefore, it was difficult to arrive at an exact number of PAG genes within the bovine genome. Needless to say, the annotation of such an extensive gene family is prone to errors in the assembly because of the repetitive nature of duplicated genes which are often arranged in tandem. Therefore, one of the several objectives of this chapter was to annotate the PAG genes within the most recent publicly available bovine genome build. When we annotated the PAG genes within the current build, it was notable that, out of the 22 cDNAs that have been described, 18 distinct, full length functional PAGs were represented in the assembly. In addition to the 18 boPAG genes, there was a boPAG-19-like gene, which was found to be an additional copy of the existing gene. There were also, 2 full length boPAG-like genes that seemed to be intact, but appear to be trancriptionally silent due to a lack of ESTs corresponding to these predicted PAG genes. In addition to these genes, there were 12 obvious pseudogenes. We still consider this to be a conservative estimate of the number of boPAG genes, since some of the known boPAG cDNAs were not represented in the build and we could not rule out the possibility of additional PAG-like genes that may have been unrecognized and not included in the assembly.

One of the other main objectives of this paper was to study the promoter regions of the boPAG genes to gain insight into the evolutionary pressures operating on them and

to probe for likely evidence that might explain some of the known differences in expression patterns. Toward this end, we analyzed the proximal promoter regions comprising of 500 or 1000 bp regions upstream of the TSP. When we analyzed the promoter regions, we noticed that sequence conservation was limited to a short region ~500bp upstream of the TSP, implying that critical regulatory elements responsible for trophoblast expression may be positioned within this region. Distal to this region from about -500 to -1000 bp we noticed numerous insertional events by divergent TEs that likely contribute to differences within the promoter regions. We noticed that boPAG-10 accumulated a novel SINE element within this region and, therefore, is quite distinct from the rest of the boPAGs. The functional significance of these inserted TEs is not known, but a potential role for these elements in influencing the expression of boPAGs could not be ruled out.

We next focused our attention to the critical regulatory regions (~500 bp) of boPAG genes which may likely explain some of the observed differences in the expression of PAGs, both in terms of spatial regulation as well as the relative abundance of each message. We observed that among the boPAG genes there were regions that were conserved in both the ancient and modern PAGs and are therefore likely critical for trophoblast-specific expression. However, there were a discordant number of conserved regions that were unique to either modern or ancient boPAG groupings, the most predominant being confined to modern PAGs. We discovered that some of these conserved regions corresponded to consensus sequences for TF binding, suggesting the unique possibility that the divergence of such elements were responsible for the observed differences in temporal and spatial distribution pattern in the boPAG groups.

We then probed the available EST database to check for differences in relative expression of boPAGs as a function of the differences within these promoter elements. We noticed that boPAG-2 was the most predominant transcript represented. A more systematic analysis by means of Q-PCR revealed an extremely high level of transcript abundance for boPAG-2 relative to boPAG-12. The comparisons were performed at several stages of pregnancy and both results were normalized to the same reference control (YWHAG) gene. Even by the most conservative estimates, boPAG-2 mRNA was at least 100 times more abundant than boPAG-12 at all the different stages of pregnancy examined [beginning at day 60 to term (day 280)].

Since boPAG-2 was the most abundant PAG transcript, we chose to further analyze the boPAG-2 gene. Analysis of proximal 500 bp of the promoter identified likely recognition sites for GATA, ETS as well as the c-rel group of TFs. Of these elements we noted that ETS-2 and purportedly the c-Rel sites were bound by proteins present within the Jar (human) choriocarcinoma cell line. The transcription factor Ets-2 has been implicated in the transcription of trophoblast-specific genes (Ezashi et al., 1998; Ezashi et al., 2001; Ghosh et al., 2003; Yamamoto et al., 1998). Therefore, the possible involvement of Ets-2 in the regulation of the boPAG-2 gene was determined. We therefore envision a possibility for systematically gaining additional understanding of the role of Ets and other transcription factors in driving trophoblast-specific transcription of the PAG genes for future experimentation.

Chapter –II figures

Table -2. Summary of results from the analysis of PAG genes in bovine genome.

Gene name	Transcript ID	REF SEQ	GLEAN	EST -FREQ	Location	Comment
PAG-1	M73962	NM_174411	**10324**	28	ChrUn.13	full-length
PAG-2	L06151	NM_176614	**19448**	92	Chr29.48	full-length
PAG-2 like	XM_001252523		**10441**	0		partial;pseudogene
PAG-3	XM_615231		**24765**	3	Chr29.54	full-length
PAG-4	AF020506	NM_176615	**10334**	5	ChrUn.13	full-length
PAG-4 like	XM_600174		**24769**	0		predicted;full length
PAG-5		NM_176616	**18735**	2	ChrUn.278	full-length
PAG-5 like	XM_001252636		13899	0	ChrUn.1071	pseduogene
PAG-5 like	XM_001252636		**10439**	0	ChrUn.833	partial;pseudogene
PAG-5 like	XM_598365		10317	0		pseduogene
PAG-6	AF020508	NM_176617	**24763**	10	Chr29.54	full-length
PAG-7	**BC133469**		NR	19		full-length
PAG-7 like		**NM_176618**	NR	0		Splice variant
PAG-7- like		NM_001109978	13897	0	ChrUn.1071	fragment;pseudogen
PAG-8	AF020510	NM_176619	**24771**	39	Chr29.54	full-length
PAG-9	AF020511	NM_176620	**18917**	19	ChrUn.1099	full-length
PAG-10	AF020512	NM_176621	**19477**	25	Chr29.48	full-length
PAG-10-like	XM_864803		19475	0	Chr29.48	fragment;pseudogen
PAG-10-like	XM_864803		19476	0	Chr29.48	fragment;pseudogen
PAG-11	AF_020513	**NM_176623**	24761;24762	46	Chr29.54	full-length
PAG-12	AF_020514	NM_176622	**19478**	9	Chr29.48	full-length
PAG-12-like			10442	0	ChrUn.833	partial;pseudogene
PAG-13	AF_192330		NR	0		No ref seq
PAG-14	**AF_192331**		NR	0		No ref seq
PAG-15	AF_192332	NM_176624	**10338**	1	ChrUn.13	full-length
PAG-16	AF_192333	NM_176625	**10332**	9	ChrUn.13	full-length
PAG-16- like	XM_596391		10319	0	ChrUn.13	predicted;full length
PAG-17	**AF_192334**	NM_176627	17225*	25		* error
PAG-17-like	XM_001252975		**10321**	0	ChrUn.13	partial;pseudogene
PAG-18	AF_192335	NM_176626	**18733**	2	ChrUn.278	full-length
PAG-19	AF_192336	NM_176628	**10322**	2	ChrUn.13	full-length
PAG-19-like	XM_001253033		**10323**	0	ChrUn.13	predicted;full length
PAG-19-like			10327	0	ChrUn.13	pseudogene
PAG-19-like			10328	0	ChrUn.13	pseudogene
PAG-20	AF_192337	NM_176629	**10330**	5	ChrUn.13	full-length
PAG-21	AF_192338	NM_176630	**10329**	4	ChrUn.13	full-length
PAG-22	AY911498		NR	0		variant

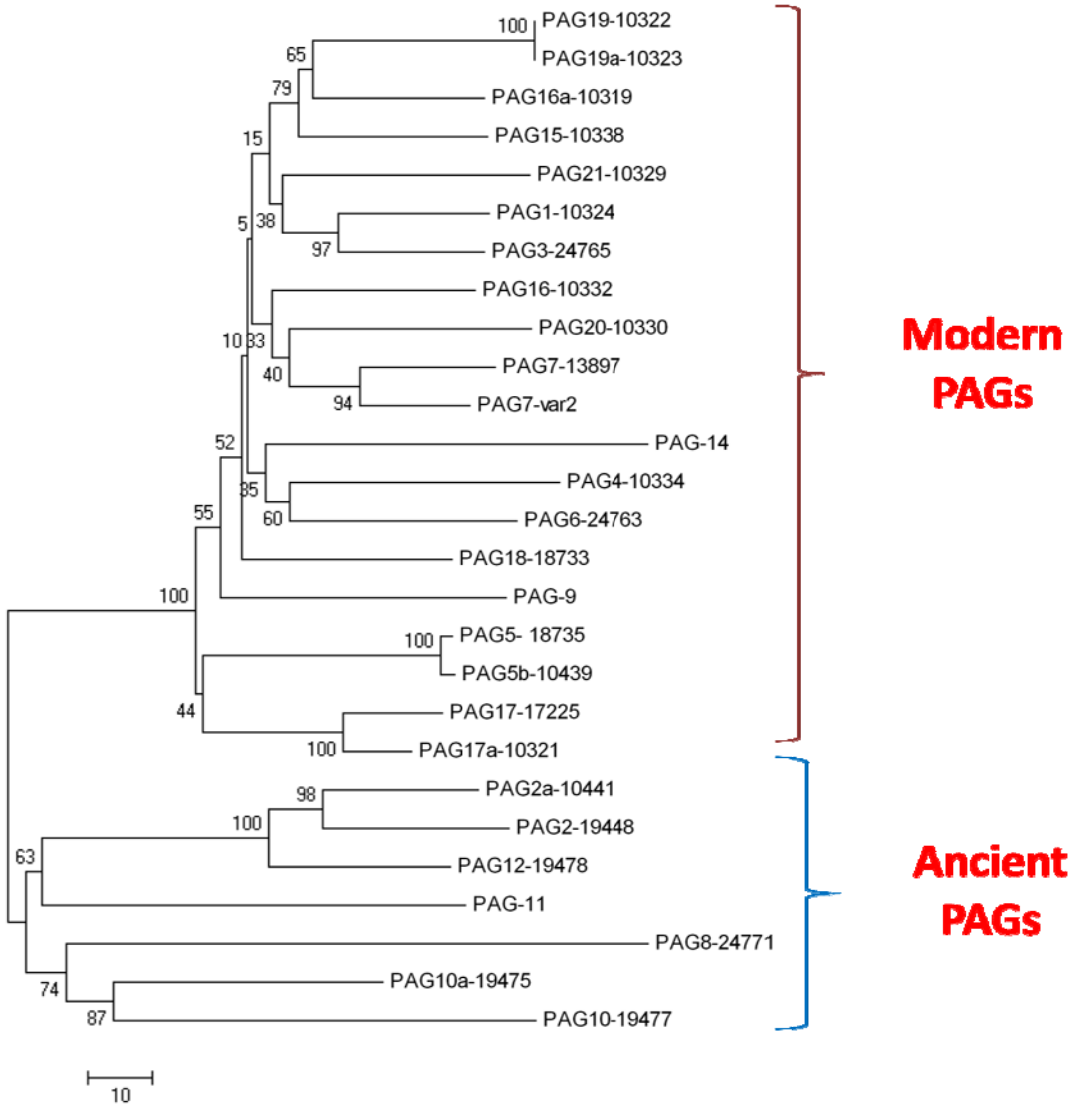


Figure -17. Evolutionary relationships among the bovine PAG-genes represented in the bovine genome build 3.1. The tree was created from the inferred amino acid sequences by using the Minimal evolution method in the MEGA 4.0 program. Bootstrap confidence values for each branch point are indicated. Notice the clear separation of the PAG gene family into two groups, the modern and the ancient PAGs. Also notice the relatively shorter branch lengths in the modern PAG clusters compared to the ancient PAGs.

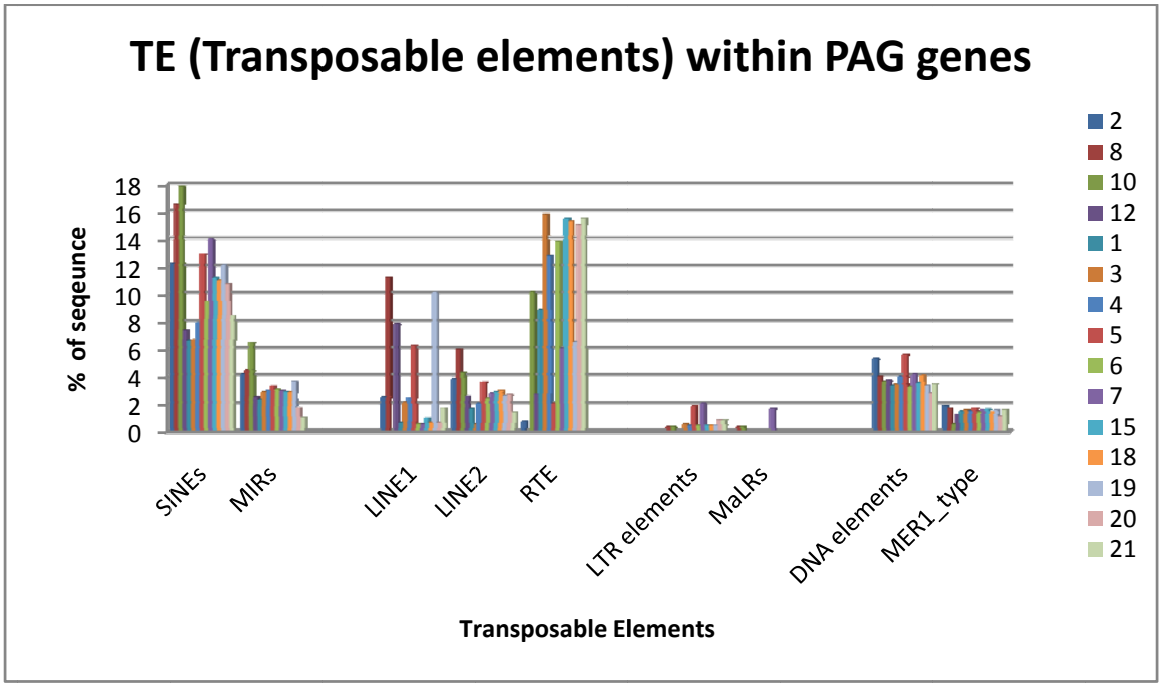
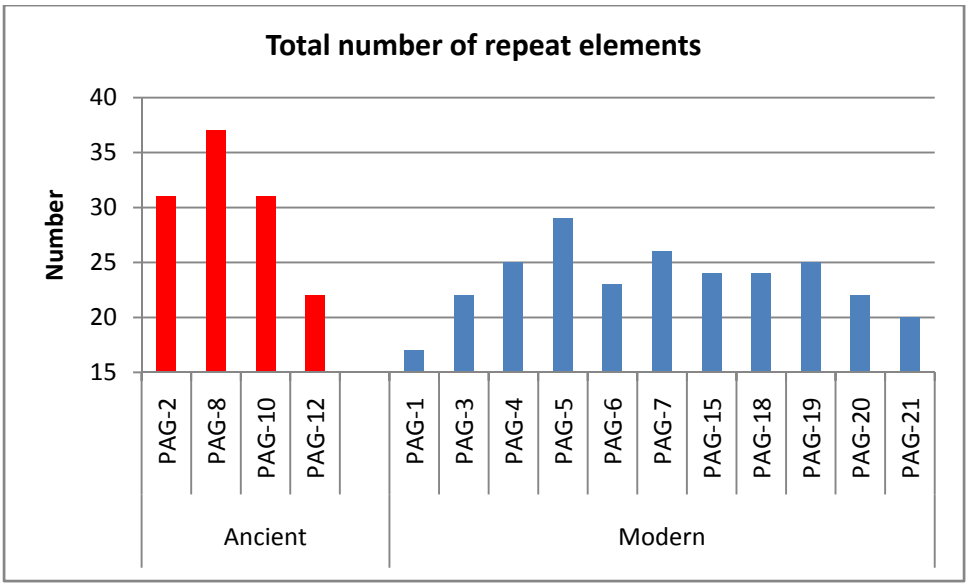


Figure -18a. Relative distribution of non-LTR (SINE, LINE), LTR and DNA – transposable elements (TEs) within bovine PAG genes. Each colored bar represents the relative distribution of the corresponding element in each candidate boPAG gene. The TE elements were shown on the –X axis. The relative % of the sequence contributed by each element is shown on Y–axis. The expansion of the acronyms used in the figure: LTR: long terminal repeat; SINE: short interspersed element; LINE: long interspersed element; MIR: mammalian wild- interspersed repeat (sub-class of SINE); RTE: retrotransposable elements; MaLR: mammalian apparent long terminal repeat; MER: medium reiterated element.



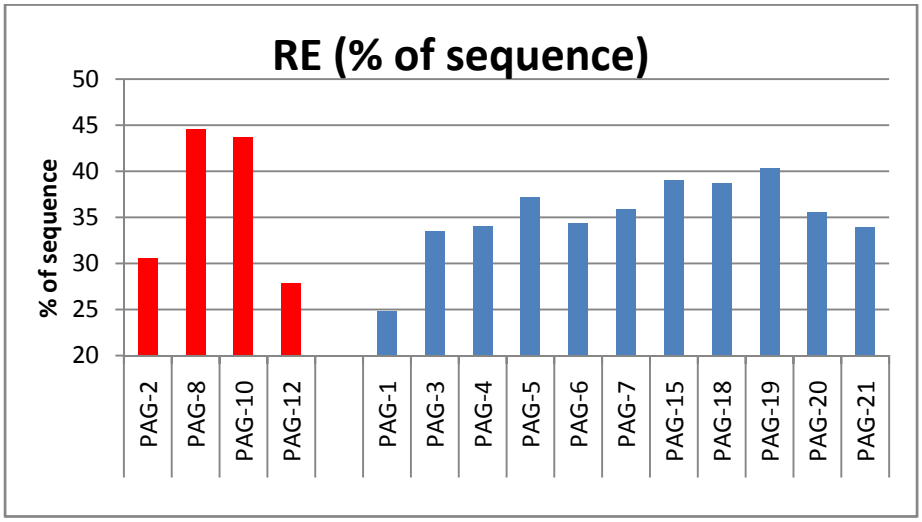
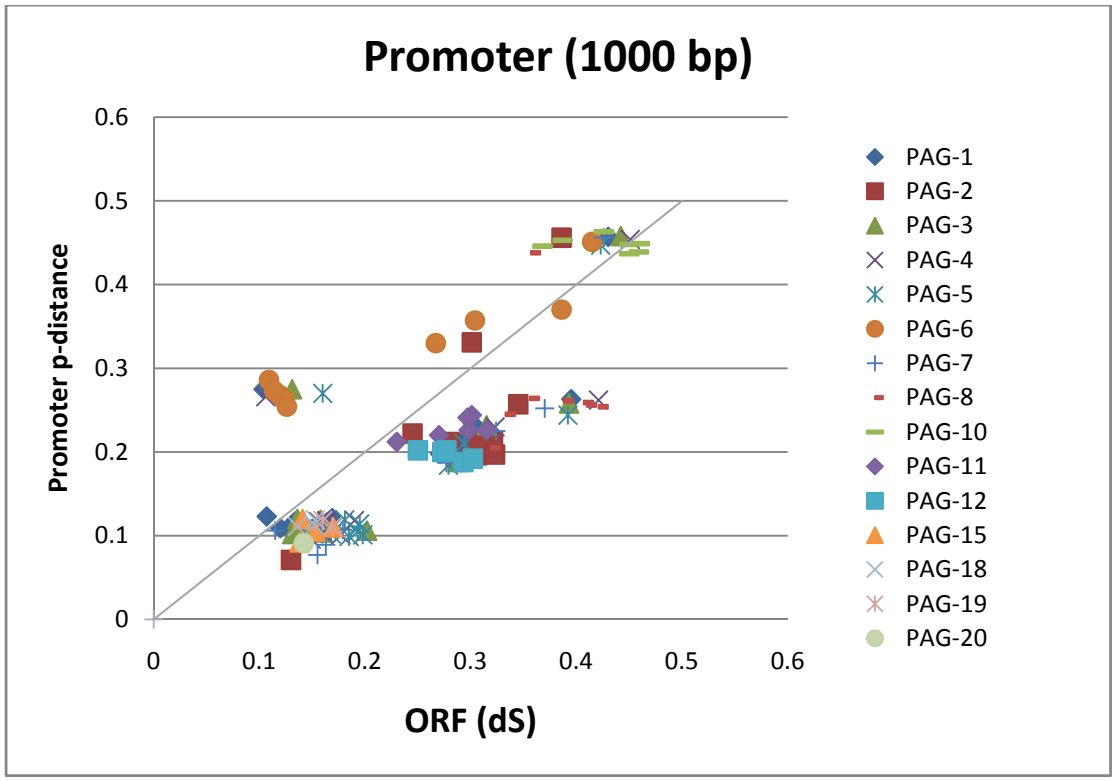


Figure -18b and C) show the cumulative total of the number of TEs, as well as the % contribution to the sequence of individual boPAGs. Fig-18b: represents the cumulative total of the all the different kinds of the elements reperesented in individual PAG genes. Figure -18c: shows the % make-up of the PAG genes by the TEs.


Promoter (1000 bp)
Promoter p-distance
ORF (dS)
0
0.1
0.2
0.3
0.4
0.5
0.6
PAG-1
PAG-2
PAG-3
PAG-4
PAG-5
PAG-6
PAG-7
PAG-8
PAG-10
PAG-11
PAG-12
PAG-15
PAG-18
PAG-19
PAG-20

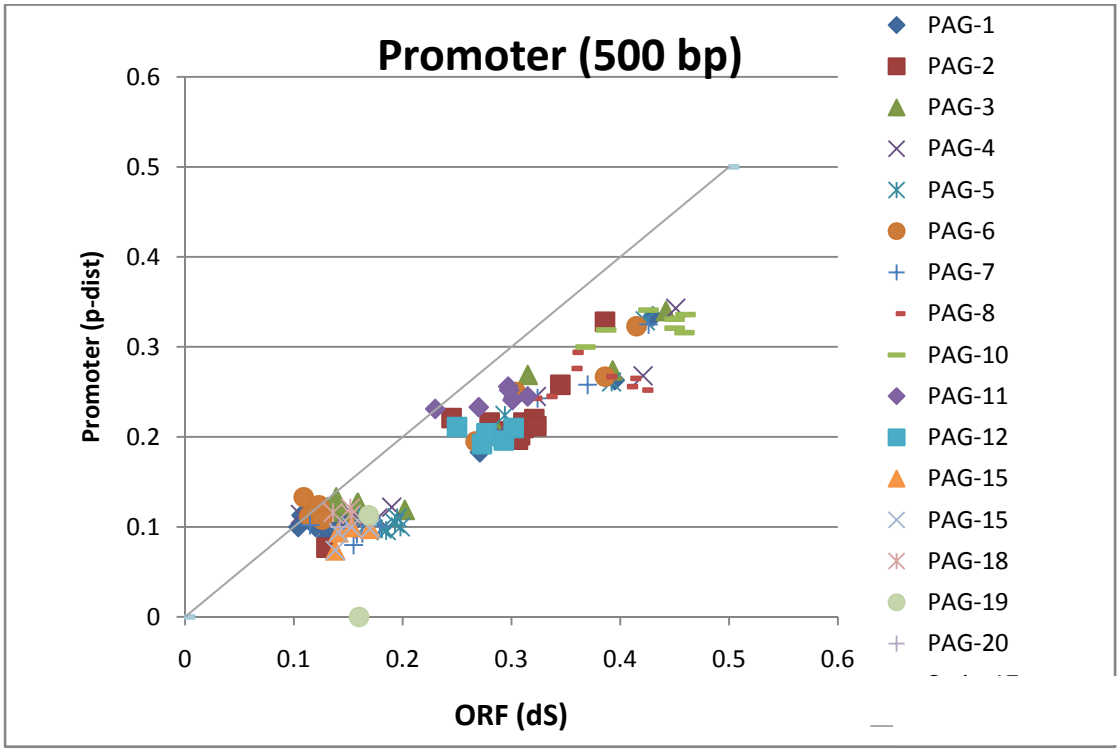
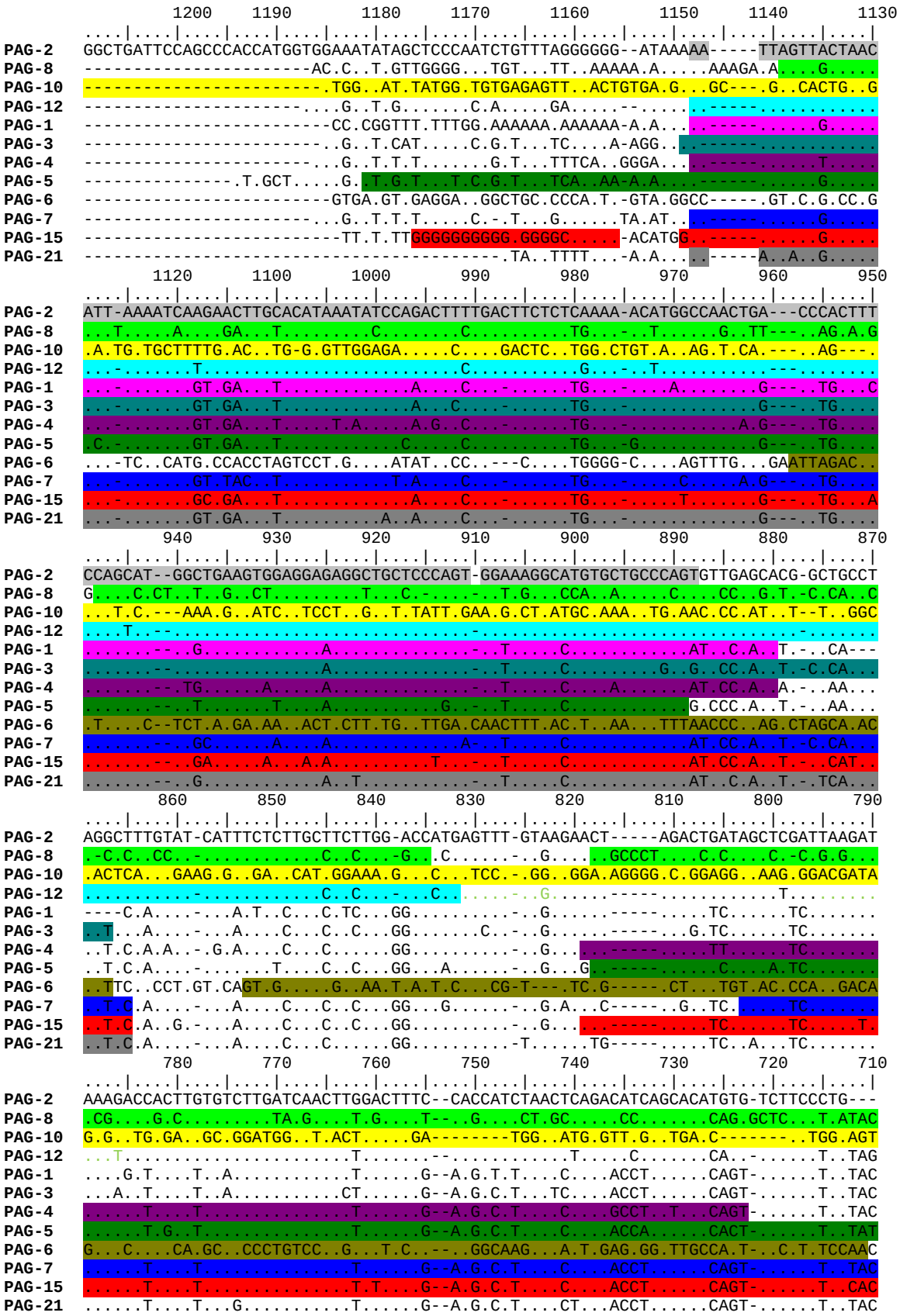
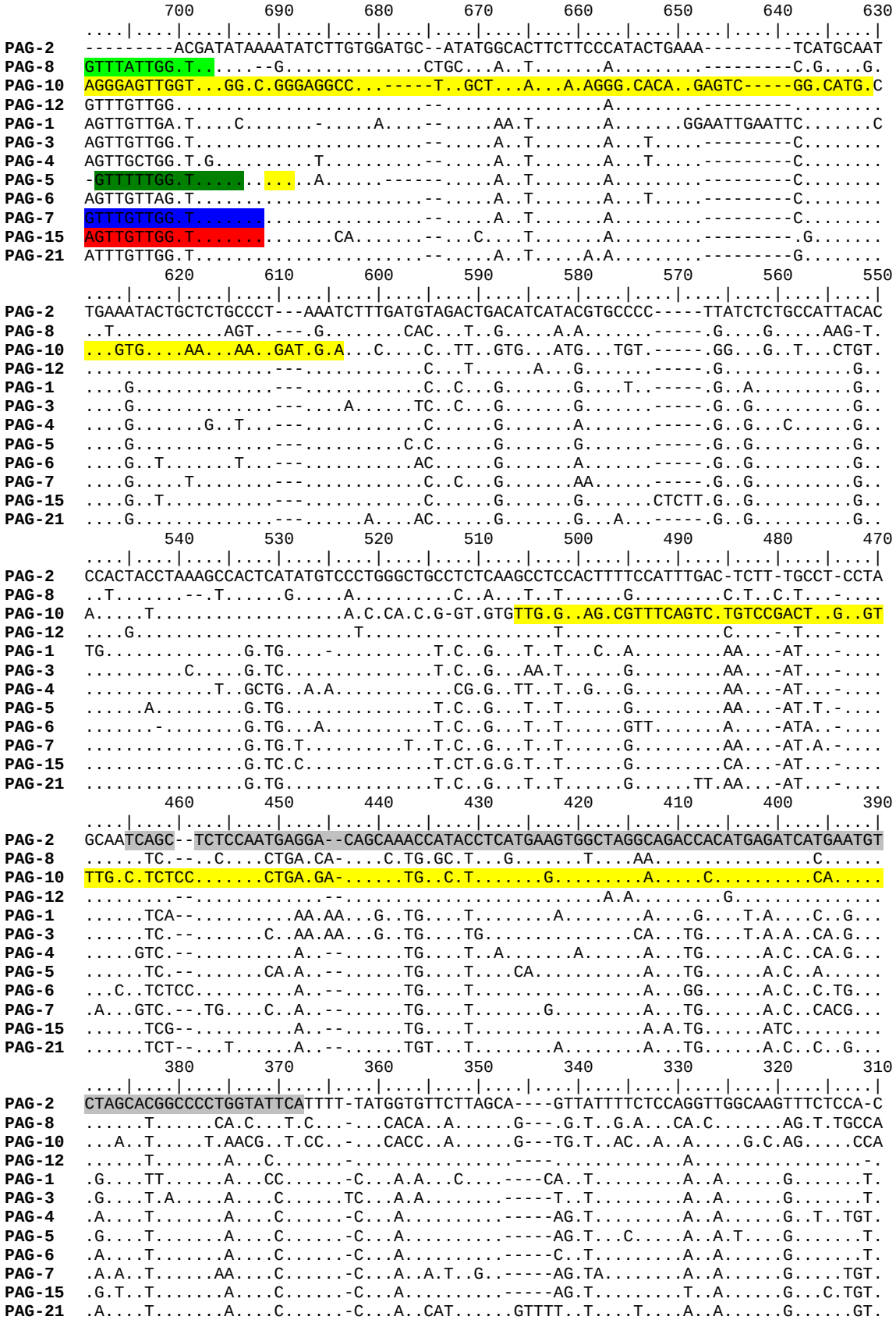

Promoter (500 bp)
Promoter (p-dist)
ORF (dS)
0
0.1
0.2
0.3
0.4
0.5
0.6
PAG-1
PAG-2
PAG-3
PAG-4
PAG-5
PAG-6
PAG-7
PAG-8
PAG-10
PAG-11
PAG-12
PAG-15
PAG-15
PAG-18
PAG-19
PAG-20

Figure -19. The ratio of p-distance of the promoter regions versus predicted nucleotide mutation rate [calculated as dS (proportion of synonymous substitutions per synonymous site in the exons] in pairwise comparisons for each PAG gene represented in the genome build.

a) Comparisons with the proximal 1000bp of the promoter region
b) Comparisons with the proximal 500bp of the promoter region.

The p-distance of the promoters was shown on the Y-axis and the dS of their protein coding regions were displayed on the X-axis. The unique marks of a particular color and shape in the figure represent the pairwise comparisons of candidate boPAG against each of the other PAGs included in the analysis. The listing of PAG genes and their indicators are shown in the legend.


1200 1190 1180 1170 1160 1150 1140 1130
PAG-2 GGCTGATTCCAGCCCACCATGGTGGAAATATAGCTCCCAATCTGTTTAGGGGGG--ATAAAAA-----TTAGTTACTAAC
PAG-8 ----------AC.C..T.GTTGGGG...TGT...TT..AAAAA.A.....AAAGA.A....G.....
PAG-10 ----------.TGG..AT.TATGG.TGTGAGAGTT..ACTGTGA.G...GC---.G..CACTG..G
PAG-12 ----------....G..T.G.......C.A.....GA.....-.....-----.....
PAG-1 ----------CC.CGGTTT.TTTGG.AAAAAA.AAAAAA-A.A.....-----.....G.....
PAG-3 ----------..G..T.CAT.....C.G.T...TC....A-AGG..-----.....
PAG-4 ----------...G..T.T.T.......G.T...TTTCA..GGGA...-----.....T.....
PAG-5 ----------.T.GCT.....G..T.G.T...T.C.G.T...TCA..AA-A.A.....-----.....G.....
PAG-6 ----------GTGA.GT.GAGGA..GGCTGC.CCCA.T.-GTA.GGCC-----.GT.C.G.CC.G
PAG-7 ----------...G..T.T.T.....C.-.T...G.....TA.AT..-----.....G.....
PAG-15 ----------TT.T.TTGGGGGGGGG.GGGGC.....-ACATGG..-----.....G.....
PAG-21 ----------.TA..TTTT...-A.A.....-----A..A..G.....
1120 1100 1000 990 980 970 960 950
PAG-2 ATT-AAAATCAAGAACTTGCACATAAATATCCAGACTTTTGACTTCTCTCAAAA-ACATGGCCAACTGA---CCCACTTT
PAG-8 ...T.....A....GA...T.....C.....C.....TG...-..T.....G..TT----.AG.A.G
PAG-10 .A.TG.TGCTTTTG.AC..TG-G.GTTGGAGA.....C....GACTC..TGG.CTGT.A..AG.T.CA.-----.AG---.
PAG-12 ...-.....T.....C.....G...-..T.....--.....
PAG-1 ...-.....GT.GA...T.....A....C...-.....TG...-....A.....G----.TG...C
PAG-3 ...-.....GT.GA...T.....A...C....-.....TG...-.....G----.TG....
PAG-4 ...-.....GT.GA...T.....T.A.....A.G..C...-.....TG...-.....A.G----.TG....
PAG-5 .C.-.....GT.GA...T.....C.....C.....TG...-G.....G----.TG....
PAG-6 ...-TC..CATG.CCACCTAGTCCT.G....ATAT..CC..---C....TGGGG-C....AGTTTG...GAATTAGAC..
PAG-7 ...-.....GT.TAC..T.....T.A....C...-.....TG...-.....C.....A.G----.TG....
PAG-15 ...-.....GC.GA...T.....A....C...-.....TG...-.....T.....G----.TG...A
PAG-21 ...-.....GT.GA...T.....A..A....C...-.....TG...-.....G----.TG....
940 930 920 910 900 890 880 870
PAG-2 CCAGCAT--GGCTGAAGTGGAGGAGAGGCTGCTCCCAGT-GGAAAGGCATGTGCTGCCCAGTGTTGAGCACG-GCTGCCT
PAG-8 G....C.CT..T..G..CT.....T...C.-....-..T.G...CCA..A.....C....CC..G.T.-C.CA..C
PAG-10 ...T.C.---AAA.G..ATC..TCCT..G..T.TATT.GAA.G.CT.ATGC.AAA..TG.AAC.CC.AT..T--T..GGC
PAG-12 ....T..-.....-.....
PAG-1 .....---..G.....A.....-..T.....C.....AT..C.A..T.-..CA---
PAG-3 .....--.....A.....-..T.....C.....G..G..CC.A..T.-C.CA...
PAG-4 .....---.TG.....A....A.....-..T.....C....A.....AT.CC.A..A.-..AA...
PAG-5 .....-...T.....A.....G..-..T.....C.....G.CCC.A..T.-..AA...
PAG-6 .T....C--TCT.A.GA.AA..ACT.CTT.TG..TTGA.CAACTTT.AC.T..AA...TTTAACCC..AG.CTAGCA.AC
PAG-7 .....---.GC.....A....A.....A-..T.....C.....AT.CC.A..T.-C.CA...
PAG-15 .....--..GA.....A...A.A.....T...-..T.....C.....AT.CC.A..T.-..CAT..
PAG-21 .....---..G.....A..T.....-..T.....C.....AT..C.A..T.-.TCA...
860 850 840 830 820 810 800 790
PAG-2 AGGCTTTGTAT-CATTTCTCTTGCTTCTTGG-ACCATGAGTTT-GTAAGAACT-----AGACTGATAGCTCGATTAAGAT
PAG-8 .-C.C..CC..-.....C..C...-G..C.....-..G....-.GCCCT....C.C....C.-C.G.G...
PAG-10 .ACTCA...GAAG.G..GA..CAT.GGAAA.G...C...TCC.-.GG..GGA.AGGGG.C.GGAGG..AAG.GGACGATA
PAG-12 .....-.....C..C...-...C..-...G.....-----.....T.....
PAG-1 ----C.A....-...A.T..C...C.TC...GG.....-..G.....----.....TC.....TC.....
PAG-3 ..T...A....-...A....C...C..C...GG.....C..-..G.....---...G.TC.....TC.....
PAG-4 ..T.C.A.A..-.G.A....C...C.....GG.....-..G...-.....TT.....TC.....
PAG-5 ..T.C.A.....-.....T....C..C...GG...A.....-..G...G.-----.....C....A.TC.....
PAG-6 ..TTC..CCT.GT.CAGT.G.....G..AA.T.A.T.C...CG-T---.TC.G-----.CT...TGT.AC.CCA..GACA
PAG-7 ..T.C.A....-...A....C...C..C...GG...G.....-..G.A...C------..G..TC.....TC.....
PAG-15 ..T.C.A..G.-...A....C...C..C...GG.....-..G...-.....TC.....TC.....T.
PAG-21 ..T.C.A....-...A....C...C.....GG.....-T.....TG----.....TC..A...TC.....
780 770 760 750 740 730 720 710
PAG-2 AAAGACCACTTGTGTCTTGATCAACTTGGACTTTC--CACCATCTAACTCAGACATCAGCACATGTG-TCTTCCCTG---
PAG-8 .CG....G.C.....TA.G....T.G....T-..G....CT.GC.....CC.....CAG.GCTC...T.ATAC
PAG-10 G.G..TG.GA..GC.GGATGG..T.ACT.....GA--------TGG..ATG.GTT.G..TGA.C--------..TGG.AGT
PAG-12 ...T.....T.....--.....T.....C.....CA..-.....T..TAG
PAG-1 ....G.T....T..A.....T.....G--A.G.T.T....C....ACCT.....CAGT-.....T..TAC
PAG-3 ...A..T....T..A.....CT.....G--A.G.C.T...TC....ACCT.....CAGT-.....T..TAC
PAG-4 .....T....T.....T.....G--A.G.C.T....C....GCCT..T...CAGT-.....T..TAC
PAG-5 .....T.G..T.....T.....G--A.G.C.T....C....ACCA.....CACT-.....T..TAT
PAG-6 G...C....CA.GC..CCCTGTCC..G...T.C..-...GGCAAG...A.T.GAG.GG.TTGCCA.T-...C.T.TCCAAC
PAG-7 .....T....T.....T.....G--A.G.C.T....C....ACCT.....CAGT-.....T..TAC
PAG-15 .....T....T.....T.T....G--A.G.C.T....C....ACCT.....CAGT-.....T..CAC
PAG-21 .....T....T...G.....T.....G--A.G.C.T....CT...ACCT.....CAGT-.....T..TAC


700 690 680 670 660 650 640 630
PAG-2 ---------ACGATATAAAATATCTTGTGGATGC--ATATGGCACTTCTTCCCATACTGAAA---------TCATGCAAT
PAG-8 GTTTATTGG.T..----G.............CTGC...A..T.......A.........---------C.G....G.
PAG-10 AGGGAGTTGGT...GG.C.GGGAGGCC..-----T..GCT...A...A.AGGG.CACA..GAGTC-----GG.CATG.C
PAG-12 GTTTGTTGG.....-.....A.........---------.....
PAG-1 AGTTGTTGA.T....C.......-.....A....--.....AA.T.......A.......GGAATTGAATTC.......C
PAG-3 AGTTGTTGG.T.....-.....A..T.......A...T.....---------C.....
PAG-4 AGTTGCTGG.T.G.....T.....--.....A..T.......A...T.....---------C.....
PAG-5 -GTTTTTGG.T.....A.....-.....A..T.......A.........---------C.....
PAG-6 AGTTGTTAG.T.....-.....A..T.......A...T.....---------C.....
PAG-7 GTTTGTTGG.T.....-.....A..T.......A.........---------C.....
PAG-15 AGTTGTTGG.T.....CA.....---.C....T.......A.........---------.G.....
PAG-21 ATTTGTTGG.T.....-.....A..T.....A.A.........---------G.....
620 610 600 590 580 570 560 550
PAG-2 TGAAATACTGCTCTGCCCT---AAATCTTTGATGTAGACTGACATCATACGTGCCCC-----TTATCTCTGCCATTACAC
PAG-8 ..T.....AGT..----G.....CAC...T..G.....A.A.....-----.G....G.....AAG-T.
PAG-10 ...GTG....AA...AA..GAT.G.A...C....C..TT..GTG...ATG...TGT.-----.GG...G..T...CTGT.
PAG-12 .....---.....C...T.....A...G.....-----.G.....G..
PAG-1 ....G.....---.....C..C...G.....G....T.-----.G..A.....G..
PAG-3 ....G.....---....A.....TC..C...G.....G.....-----.G..G.....G..
PAG-4 ....G.....G..T.---.....C.....G.....A.....-----.G..G...C.....G..
PAG-5 ....G.....---.....C.C.....G.....G.....-----.G..G.....G..
PAG-6 ....G..T.....T..---.....AC.....G.....A.....-----.G..G.....G..
PAG-7 ....G.....T.....---.....C..C...G.....AA.....-----.G..G.....G..
PAG-15 ....G..T.....---.....C.....G.....G.....CTCTT.G..G.....G..
PAG-21 ....G.....---.....A....AC.....G.....G...A..-----.G..G.....G..
540 530 520 510 500 490 480 470
PAG-2 CCACTACCTAAAGCCACTCATATGTCCCTGGGCTGCCTCTCAAGCCTCCACTTTTCCATTTGAC-TCTT-TGCCT-CCTA
PAG-8 ..T.....--.T.....G.....A.....C..A...T..T.....G.....C.T..C.T...-....
PAG-10 A.....T.....A.C.CA.C.G-GT.GTGTTG.G..AG.CGTTTCAGTC.TGTCCGACT..G..GT
PAG-12 ....G.....T.....T.....-.....C....-.T...-....
PAG-1 TG.....G.TG....-.....T.C..G...T..T...C..A.....AA...-AT...-....
PAG-3 .....C.....G.TC.....T.C..G...AA.T.....G.....AA...-AT...-....
PAG-4 .....T..GCTG..A.A.....CG.G..TT..T..G...G.....AA...-AT...-....
PAG-5 .....A.....G.TG.....T.C..G...T..T.....G.....AA...-AT.T.-....
PAG-6 .....-.....G.TG...A.....T.C..G...T..T.....GTT.....A....-ATA..-....
PAG-7 .....G.TG.T.....T..T.C..G...T..T.....G.....AA...-AT.A.-....
PAG-15 .....G.TC.C.....T.CT.G.G.T..T.....G.....CA...-AT...-....
PAG-21 .....G.TG.....T.C..G...T..T.....G.....TT.AA...-AT...-....
460 450 440 430 420 410 400 390
PAG-2 GCAATCAGC--TCTCCAATGAGGA--CAGCAAACCATACCTCATGAAGTGGCTAGGCAGACCACATGAGATCATGAATGT
PAG-8 .....TC.---.C....CTGA.CA-....C.TG.GC.T...G.....T....AA.....C.....
PAG-10 TTG.C.TCTCC.....CTGA.GA-.....TG..C.T.....G.....A.....C.....CA.....
PAG-12 .....--.....-.....A.A.....G.....
PAG-1 .....TCA--.....AA.AA...G..TG....T.....A.....A....G....T.A....C..G...
PAG-3 .....TC.--.....C..AA.AA...G..TG....TG.....CA...TG....T.A.A..CA.G...
PAG-4 .....GTC.--.....A..--.....TG....T..A.....A.....A...TG.....A.C..CA.G...
PAG-5 .....TC.--.....CA.A..--.....TG....T....CA.....A...TG.....A.C..A.....
PAG-6 ...C..TCTCC.....A..--.....TG....T.....A...GG.....A.C..C.TG...
PAG-7 .A...GTC.--.TG....C..A..--.....TG....T.....G.....A...TG.....A.C..CACG...
PAG-15 .....TCG--.....A..--.....TG....T.....A.A.TG.....ATC.....
PAG-21 .....TCT--...T.....A..--.....TGT...T.....A.....A...TG.....A.C..C..G...
380 370 360 350 340 330 320 310
PAG-2 CTAGCACGGCCCCTGGTATTCATTTT-TATGGTGTTCTTAGCA----GTTATTTTCTCCAGGTTGGCAAGTTTCTCCA-C
PAG-8 .....T.....CA.C...T.C...-...CACA..A.....G---.G.T..G.A...CA.C.....AG.T.TGCCA
PAG-10 ...A..T.....T.AACG..T.CC..-...CACC..A.....G---TG.T..AC..A..A.....G.C.AG.....CCA
PAG-12 .....T.....A...C.....-.....----.....A.....-.
PAG-1 .G....TT.....A...CC.....-C...A.A...C.....-CA..T.....A..A.....G.....T.
PAG-3 .G....T.A.....A....C.....TC...A.A.....-----T..T.....A..A.....G.....T.
PAG-4 .A....T.....A....C.....-C...A.....-----AG.T.....A..A.....G..T..TGT.
PAG-5 .G....T.....A....C.....-C...A.....-----AG.T...C.....A..A.T....G.....T.
PAG-6 .A....T.....A....C.....-C...A.....-----C..T.....A..A.....G.....T.
PAG-7 .A.A..T.....AA....C.....-C...A..A.T..G.-----AG.TA.....A..A.....G.....TGT.
PAG-15 .G.T..T.....A....C.....-C...A.....-----AG.T.....T..A.....G...C.TGT.
PAG-21 .A....T.....A....C.....-C...A..CAT.....GTTTT..T....T....A..A.....G.....GT.

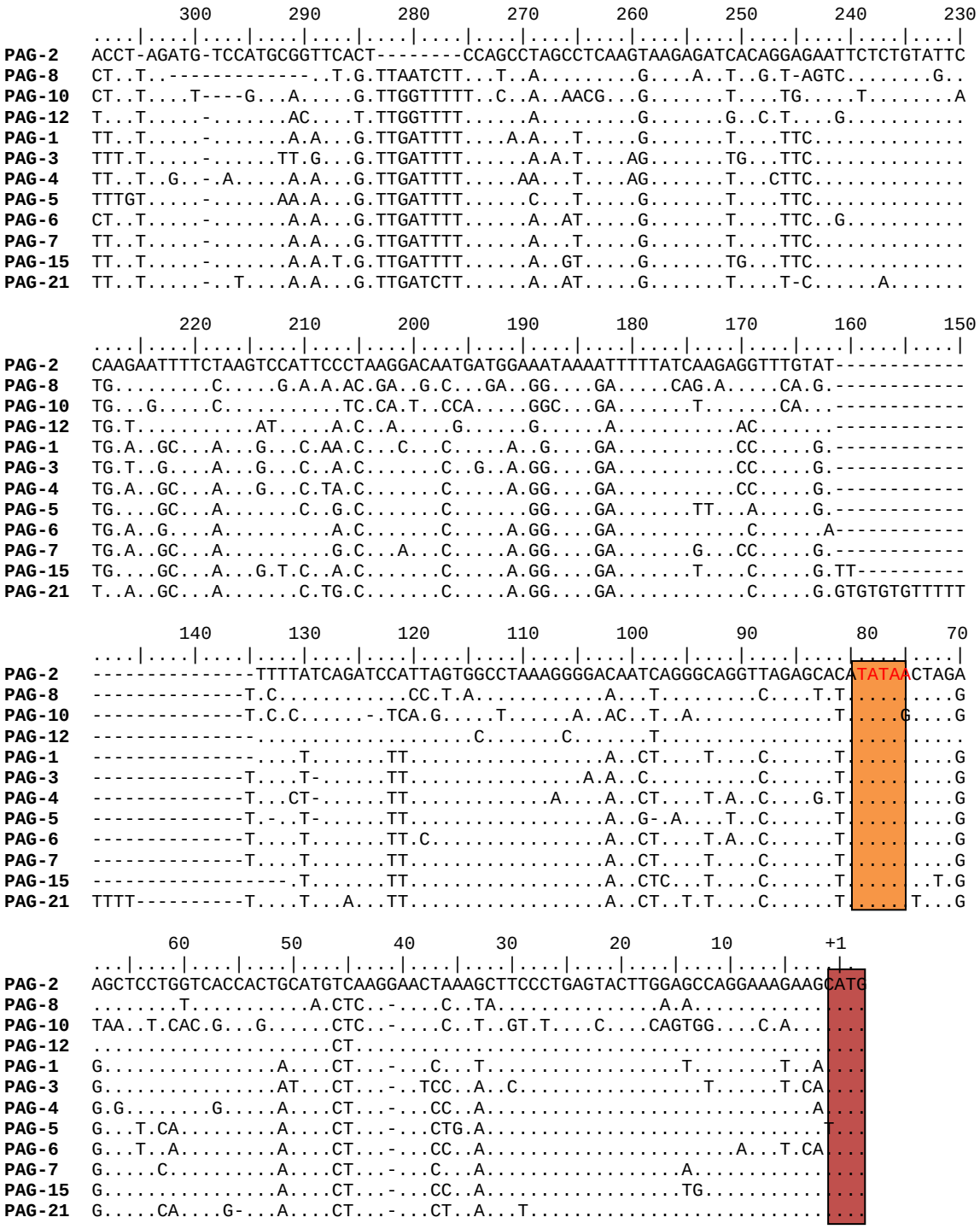
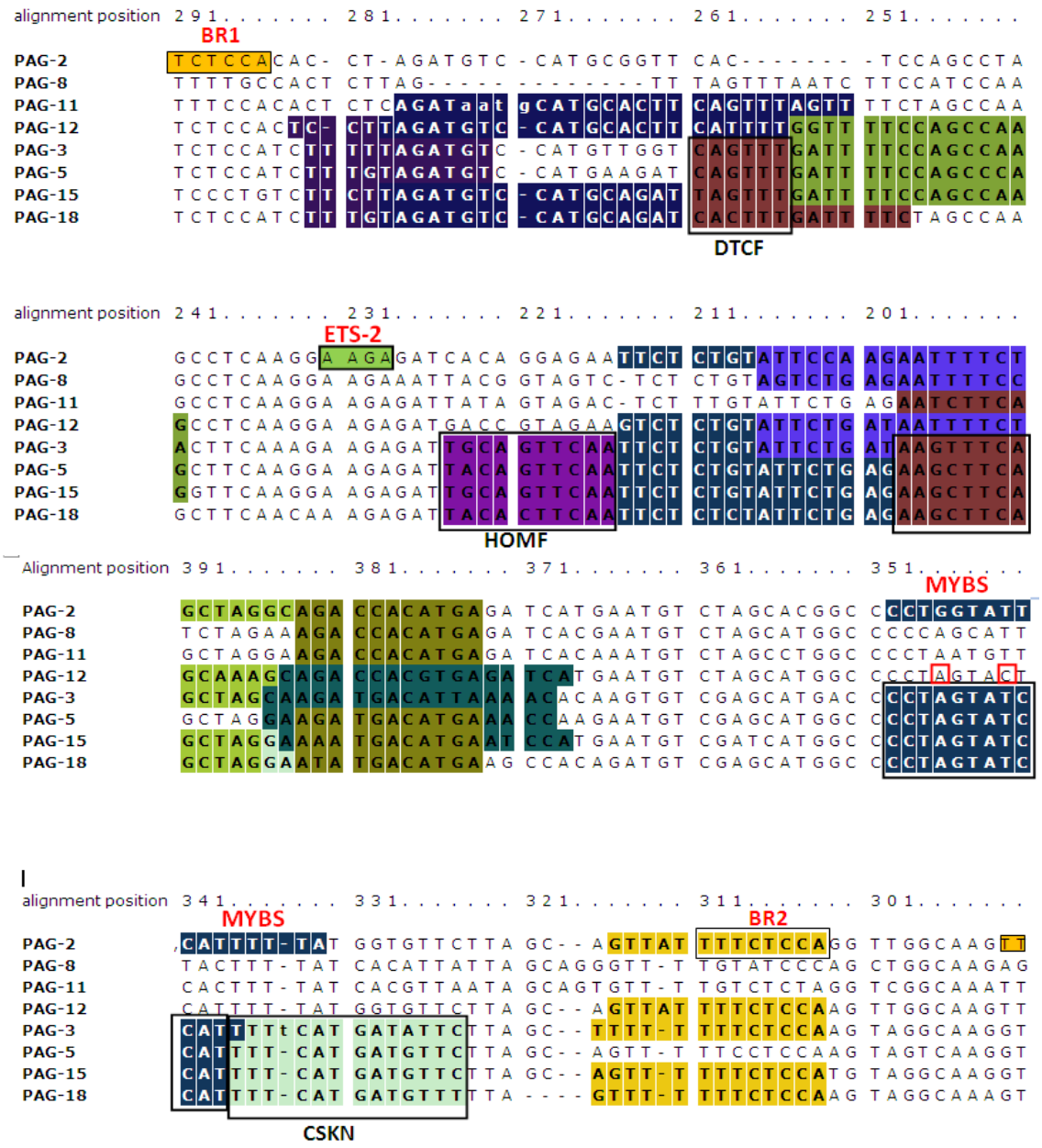


Figure -20. Multiple sequence alignment of the proximal 1000 bp of the promoters of boPAG-genes. Regions of the promoter sequence that are occupied by the TEs are highlighted. Each colored highlight identifies the region of the sequence that corresponds

to a TE insertion in a particular gene. Note that within the proximal 500 bp there are no insertional events within all the PAG genes. In addition to the TEs, TATA box and translational start codon were also highlighted within the genes.



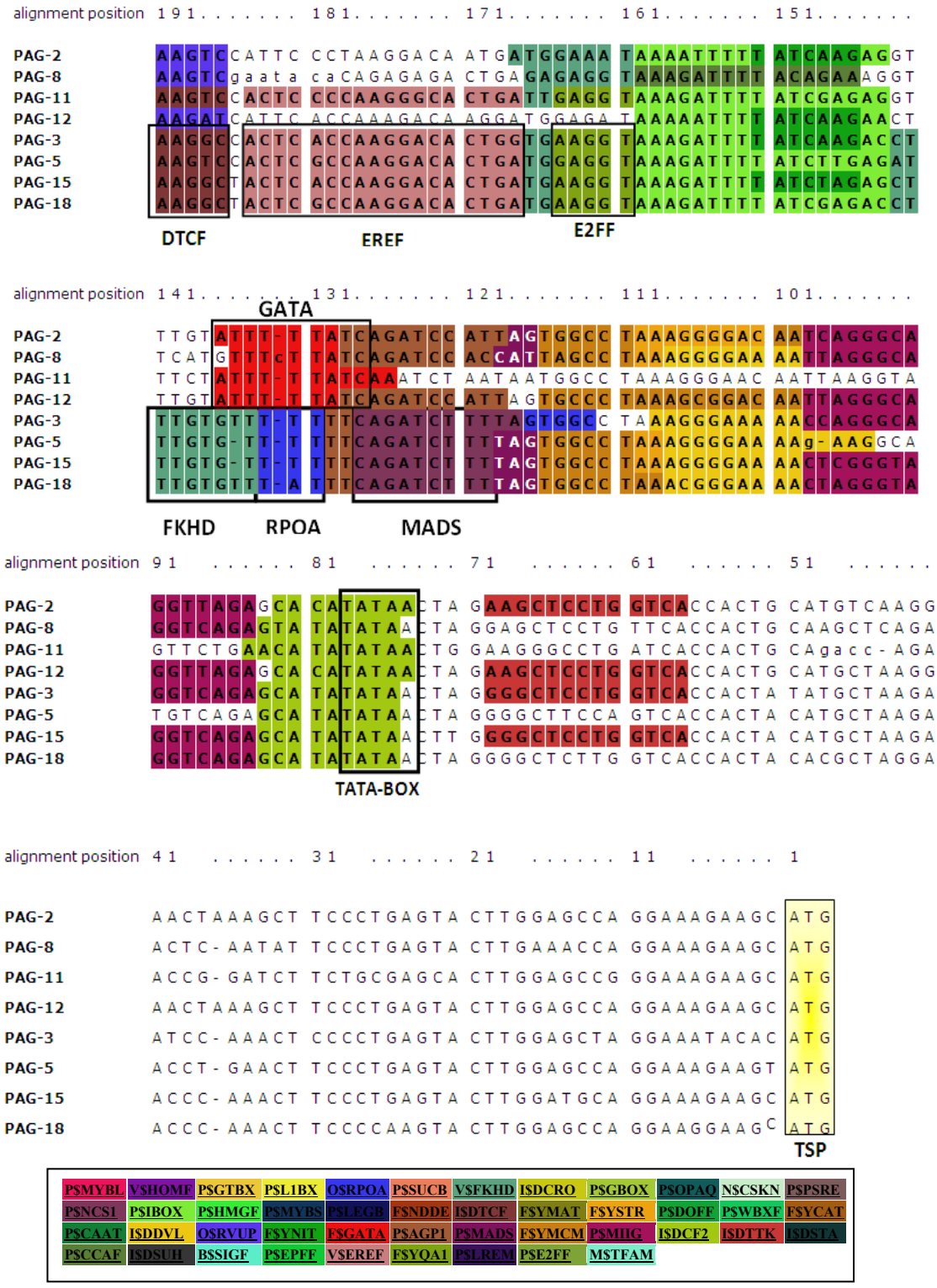

alignment position 191 181 171 161 151
PAG-2
PAG-8
PAG-11
PAG-12
PAG-3
PAG-5
PAG-15
PAG-18
DTCF
EREF
E2FF
alignment position 141 131 121 111 101
GATA
FKHD
RPOA
MADS
alignment position 91 81 71 61 51
TATA-BOX
alignment position 41 31 21 11 1
TSP
PSMYBL VSHOMF PSGTBX PSL1BX OSRPOA PSSUCB VSFKHD ISDCRO PSGBOX PSOPAQ NSCSKN PSPSRE
PSNCS1 PSIBOX PSHMGF PSMYBS PSLEGB FSNDDE ISDTCF FSYMAT FSYSTR PSDOFF PSWBXF FSYCAT
PSCAAT ISDDVL OSRVUP FSYNIT FSGATA PSAGP1 PSMADS FSYMCM PSMIIG ISDCF2 ISDTTK ISDSTA
PSCCAF ISDSUH BSSIGF PSEPFF VSEREF FSYQA1 PSLREM PSE2FF MSTFAM

Figure -21. Multiples sequence alignment of proximal 391bp of the promoter region showing conserved pockets bearing recognition sequences for transcription factors (TFs). Regions conserved in at least 50% of the input sequence (4/8) that had putative TF binding sites were shown as boxed regions in the figure. Each color shade corresponds to the unique site for a matching color shaded TF shown in the bottom of the alignment. The regions that are of importance for discussion were boxed in the figure.

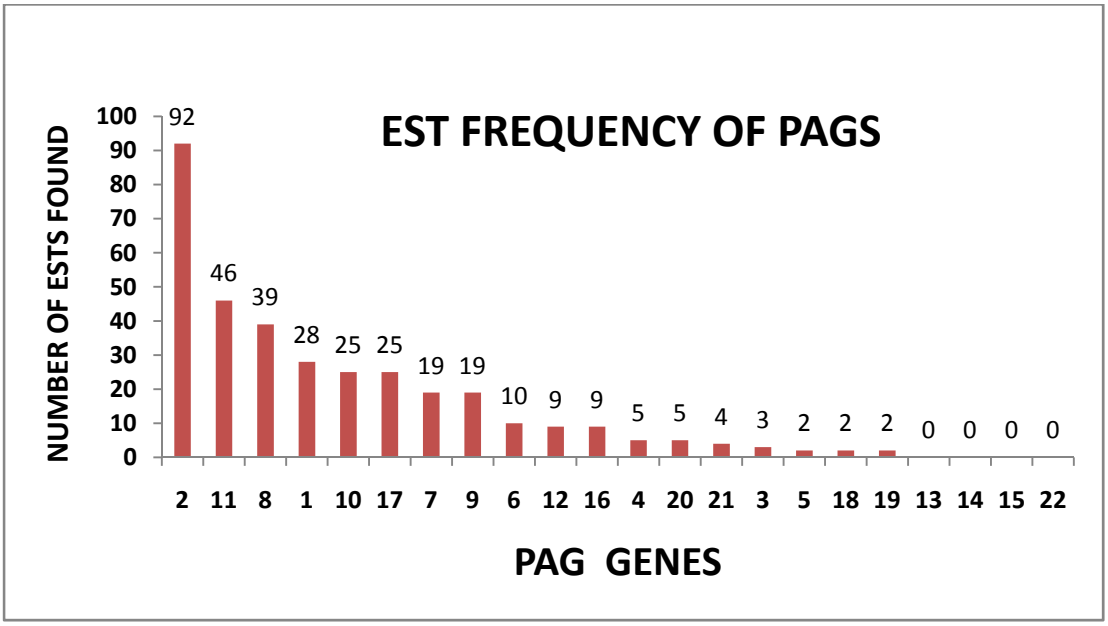


Figure -22. The relative distribution of ESTs of candidate boPAGs represented in the bovine EST database. The total numbers of ESTs that matched the coding sequences with more than 98% nucleotide identity were considered a match and were shown in the figure. Note the relative abundance of the ESTs corresponding to boPAG-2 in comparison to the other members of the PAG family.

Table -3. Oligonucleotide sequences used for relative quantification of boPAGs -2 and -12 as well as the control gene, YWHAG.

GENE	PRIMER	SEQUENCE
PAG-2	FOR	GTAGGCTCGCCTATCACCATCTTC
	REV	CCTCTGGCTTGTTTGTGTTCAAGTAG
PAG-12	FOR	ACACACCAGCCTATTAGCATCTCC
	REV	CGGCTGGCATGTGTTCAAGTAG
YHWAG	FOR	AGCACATGCAGCCCACTC
	REV	TCGTCGAAGGCGGTCTTG

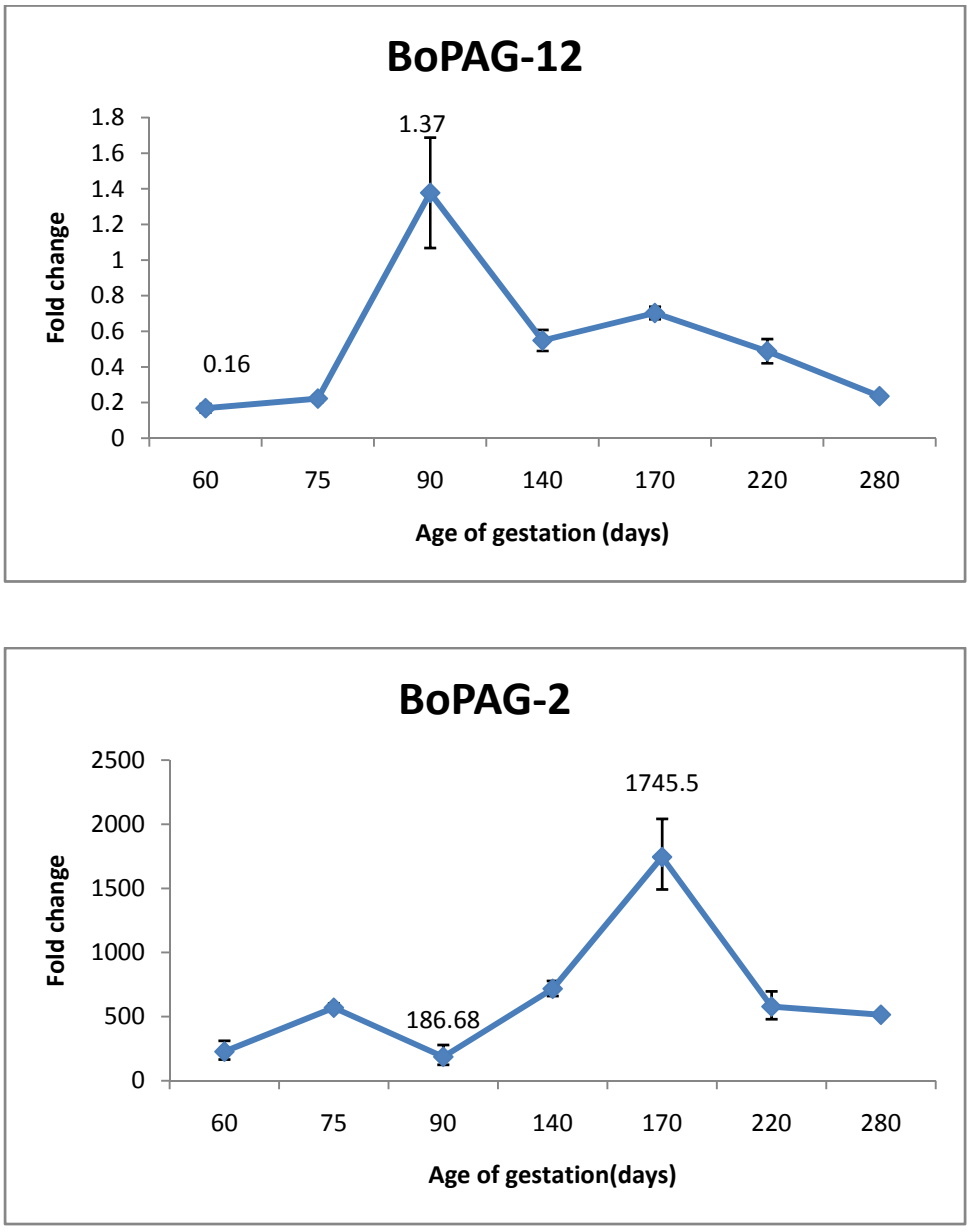


Figure -23. Quantitative real time PCR results for boPAG -2 and -12. The relative fold changes compared to an external control gene (YWHAG) were shown. The different stages of pregnancy investigated were shown on the X -axis and the fold change on the Y –axis. Note the difference in scale between each panel showing the apparent disparity in message between both genes.

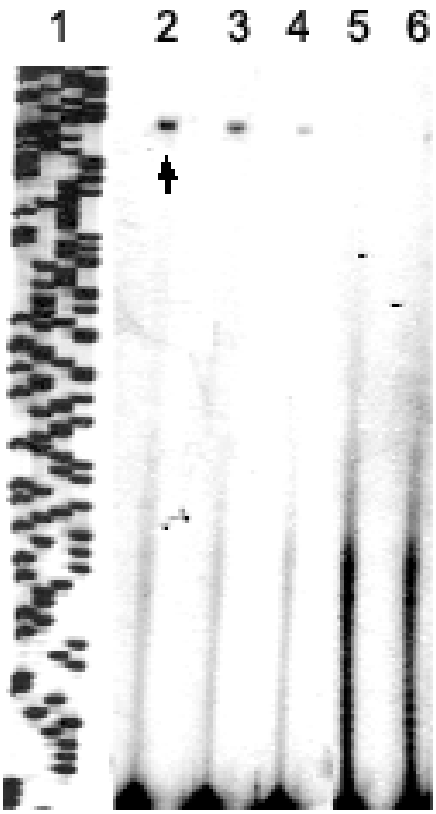


Figure -24. Primer extension analysis of boPAG-2 transcripts for determination of the transcription start site. Lanes 2, 3 and 4 denote the extension products with bovine placental RNA at d 80, 90 and 120, respectively. Lane 5 and 6 are negative controls comprised of bovine ovary and fetus RNA. Lane 1 is a dideoxy sequencing reaction performed up pBluescript SK+ plasmid for use as a single-base sizing ladder to calculate the exact size of the extended product. The arrow indicates the extended product. Neitherof the control RNA samples showed any product formation.

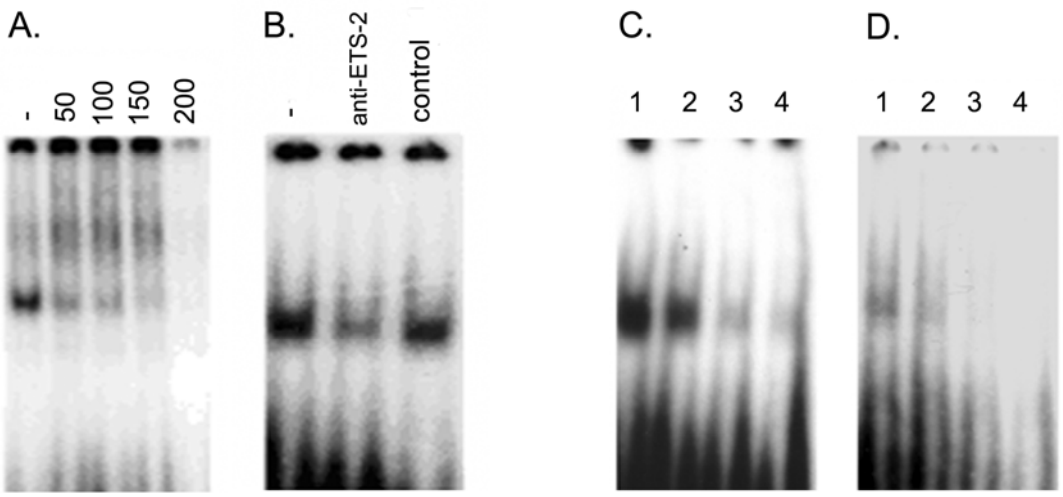


Figure -25. Electrophoretic mobility shift assays demonstrating that the putative Ets site and the repeated elements in the bovine PAG-2 promoter are capable of binding proteins in trophoblasts nuclear extracts. A. Competition of ETS-2 binding activity (20µg protein) with cold ETS-2 probe. Nuclear extracts were incubated with 1µL of 50 pmol probe, in the absence or presence of the indicated molar excess of cold probe (indicated along the top). B. The ETS-2 complex composition was examined by depleting ETS-2 with an antibody specific to ETS-2. Preincubation of the ETS antibody with the nuclear extracts followed by binding reaction with the probe resulted in specific dissociation of the complex. Control: normal rabbit serum. C and D. Competition assays indicating specificity of association of, as yet unknown, transcription factors capable of binding to the unique bovine tandem repeats, BR1(C) and BR2 (D). Lane 1: labeled probe and nuclear extract; Lane 2: same as lane 1 except for addition of a 50-fold molar excess of unlabeled double-stranded oligonucleotide; Lane 3: 250-fold molar excess of unlabeled probe; Lane 4: 500-fold molar excess.

Chapter –III

Closely related members of the bovine Pregnancy-Associated Glycoprotein family (bovine PAGs- 2 and 12) are peptidases that possess distinct proteolytic activity and specificity.

Abstract

Pregnancy-associated glycoproteins or 'PAGs' represent a complex and diverse group of trophoblast expressed proteins that are structurally related to aspartic peptidases. They are expressed exclusively by the placentas of species in the Artiodactyla order. In ruminants, especially in cattle, they constitute a rather large gene family with dozens of expressed genes. Based on their sequence identity and evolutionary relationships, the members of this gene family can be segregated into two distinct classes - the 'ancient' and the 'modern' PAGs. Many modern PAGs are characterized by accumulation of mutations in highly conserved residues that make-up the catalytic center and, therefore, are purportedly inactive as proteases. The ancient PAGs, on the other hand, have retained all the hallmarks of proteolytically active aspartic peptidases although such activity has yet to be determined. The goal of this present study was to investigate 1. if ancient bovine PAGs (boPAGs) have proteolytic activity, and 2. if there is any evidence for differences in activity and substrate specificity between closely related members of the ancient grouping, boPAGs-2 and -12. Recombinant boPAGs-2 and -12 were expressed by using a baculovirus insect cell expression system and engineered to contain a 'FLAG'-peptide sequence at the amino-terminal end of the protein. The proteins were affinity purified

over anti-flag resin and the resultant proteins were analyzed for their proteolytic activity against a synthetic commercial fluorescent cathepsin D/E substrate. It was noticed that both boPAG-2 and 12 have proteolytic activity against this substrate with an acidic pH optimum characteristic of most mammalian aspartic peptidases. The boPAG-2 had maximal activity at around pH 4; boPAG-12 at pH 3.5. An estimation of kinetic parameters revealed that the catalytic efficiency of boPAG-2 against this substrate at 37°C was almost 3 fold higher than that of boPAG-12. In addition, the specific activity of each candidate PAG toward synthetic fluorescent substrate libraries revealed that there seems to be a clear distinction in substrate preference between these related members of the gene family. Taken together, these results reveal that the ancient boPAGs are proteolytically active and that there seems to be clear differences in activity and specificity between closely related members, suggesting a complex role for these peptidases at the trophoblast-uterine interface. The physiological role of these proteolytic enzymes in the context of maternal-fetal interactions remains to be elucidated.

Introduction

Aspartic peptidases (AP) are a class of proteolytic enzymes that typically require acidic conditions for optimal activity (Davies, 1990). Not unexpectedly, most mammalian AP tend to be localized predominantly in the gastric mucosa (e.g. pepsin A, pepsin C, chymosin), lysosomes (e.g. cathepsin D) and other intracellular compartments (e.g. cathepsin E) within the cell, where acidic environments are found (Davies, 1990; Dunn, 2002). One notable exception to this tendency is renin, which is operational under

physiological pH conditions. It is produced by the juxtaglomerular cells of the kidney and its major physiological role is to cleave angiotensinogen to angiotensin I, and thereby indirectly regulate salt homeostasis and blood pressure (Cody, 1994).

The pepsin-like APs are bi-lobed proteins with both the N-terminal and C-terminal lobes being roughly symmetrical to one another (Tang and Wong, 1987). At the base of the two lobes is a binding cleft, which can accommodate a peptide substrate of seven to eight amino acids. Two conserved catalytic aspartic acid residues Asp32 and Asp215 (porcine pepsin numbering) are present at the catalytic site (Dunn, 2002). These aspartic acids assist in the cleavage of the substrate bound within the cleft by activating a water molecule positioned between them to act as a nucleophile on the carbonyl carbon of the scissile peptide bond (Davies, 1990; Dunn, 2002; Szecsi, 1992). The current model suggests that Asp215 acts as a general base to remove a proton from the water molecule while Asp32 donates a proton to the carbonyl oxygen of the scissile bond. In the resulting tetrahedral intermediate, Asp215 is hydrogen bonded to the attacking oxygen atom (originally part of the water molecule), while the hydrogen remaining on that oxygen is hydrogen bonded to an oxygen on Asp32. Transfer of a hydrogen from Asp215 to the nitrogen of the scissile bond occurs by inversion around the nitrogen atom. The C-N bond then breaks, forming the two products.

Pregnancy- associated glycoproteins (PAGs) are a recently discovered family of proteins that are structurally related to the APs. As far as is known, they are expressed exclusively by trophoblasts - the outer cell type of the placenta that is in direct contact with maternal tissues. This gene family is found exclusively in species within the *Artiodactyla* order (swine, cattle, sheep, deer, etc.) (Garbayo et al., 2000; Green et al.,

2000; Green et al., 1998b). As the name suggests the PAGs are glycoproteins and they range in molecular weight between 45,000 and 90,000 (Green et al., 1998b). The mature unglycosylated molecular mass of most PAGs is ~37,000 (Green et al., 1998b). This range in molecular weights is due predominantly to the number of available glycosylation sites and the extent of glycosylation (Klisch et al., 2006; Klisch and Leiser, 2003). Besides glycosylation, these proteins undergo additional modifications such as phosphorylation (Xie et al., 1996).

In ruminant ungulates, the PAG family of proteins is rather large and complex. Dozens of distinct cDNAs, and numerous variants, have been cloned from cattle, sheep, goat, deer and pig placentae (Brandt et al., 2007b; Garbayo et al., 2000; Garbayo et al., 1998; Green et al., 2000; Szafranska et al., 1995; Xie et al., 1997a). In species outside *Artiodactyla* order, proteins that are distantly related to the PAGs have been identified in the rabbits (*Lagomorpha*) (Kageyama et al., 1990), rodents (rats and mice-*Rodentia*) (Chen et al., 2001; Kageyama et al., 2000), cats and dogs (*Carnivora*) (Gan et al., 1997) and zebras and horses (*Perissodactyla*) (Green et al., 1998a). These proteins are known as 'pepsinogen F' and they resemble PAG family members somewhat more closely than other aspartic peptidase such as pepsin A and cathepsin D. *Pepsinogen F* exists as a single copy and does not appear to accrue significant glycosylation (Chen et al., 2001; Green et al., 1999). In addition, pepsinogen F is expressed in the neonatal stomach and extraembryonic membranes, such as the yolk sac (mouse pepsinogen F), instead of being restricted to trophoblasts like the PAGs (Chen et al., 2001; Kageyama et al., 1990).

The PAG gene family in ruminants is comprised of two evolutionarily distinct groups based on their shared sequence identity and phylogenetic relationships (Green et

al., 2000; Hughes et al., 2000; Hughes et al., 2003). One grouping, known as the 'ancient PAGs' is transcribed in all trophoblast cell types (Green et al., 2000; Wooding et al., 2005). The other class of PAGs, the 'modern PAGs', is transcribed exclusively in specialized, moderately invasive trophoblasts known as 'binucleate' cells (BNC) (Green et al., 2000; Hughes et al., 2003; Wooding et al., 2005). The different spatial localization of PAG transcripts is also reflected in the localization of these proteins at the placenta-uterine interface. The ancient PAGs are found in vesicles within mononucleate and binucleate trophoblasts; upon secretion, they accumulate at the microvillar junction of the maternal-fetal interface (Wooding et al., 2005). The modern PAGs are localized to BNCs, but also become associated with the maternal caruncular stroma adjacent to chorionic villi (Wooding et al., 2005). Coincident with differences in spatial expression, there seems to be variations in temporal expression pattern as well. For example, some PAGs are expressed relative early in gestation, other PAGs appear later in pregnancy (Green et al., 2000; Patel et al., 2004). Notably, there are obvious differences in their levels of expression. For example, boPAG-2 is the most abundant transcript among all the PAGs identified to date (comprising as much as 25% of the total PAG message - J.G. unpublished results). A closely related family member, boPAG-12, is substantially less abundant in the placenta (Figure -23, chapter -II).

Finally, for those PAGs that are predicted to act as peptidases, there are differences in residues that predicted to contribute to activity and substrate specificity, attesting further to the underlying complexity that exists within the family (Guruprasad et al., 1996; Xie et al., 1997b; Xie et al., 1991b). Since, PAGs are closely related to pepsin, molecular models, based on porcine pepsin (and bovine chymosin) crystal structures,

revealed that most of the modern PAGs appeared to have accumulated mutations within the catalytic center and are likely inactive as proteolytic enzymes (Green et al., 2000; Guruprasad et al., 1996; Xie et al., 1997b). On the other hand, the ancient PAGs have retained most of the characteristics of typical APs and are likely capable of proteolytic activity. However, no such activity has been attributed to the PAGs so far. In this report, we sought to confirm or reject the suggestion that some PAGs are capable of proteolytic activity. For these experiments, two closely related ancient PAG members, boPAG-2 and boPAG-12, were chosen as the focus of the analysis.

Materials and methods

Evolutionary relationships of PAGs:

The evolutionary history of PAGs was inferred from amino acid sequence by the Minimum Evolution method (Rzhetsky and Nei, 1992). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the analyzed PAGs (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. All positions containing gaps and missing data were eliminated only from pairwise comparisons (Pairwise deletion option). There were a total of 388 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Cloning and expression of recombinant bovine PAGs-2 and 12 (*r*boPAG-2 and 12):

The BD Baculogold™ Baculovirus insect cell expression system (BD biosciences Pharmingen, San Diego, CA) was used to express the recombinant proteins. BoPAG-2 was cloned into the pvl-92 transfer vector by using the following oligonucleotides, sense: 5' GAC TGA ***GCGGCCGC***ATG**GATTACAAGGACGAT GACGATAAG**ATAGTCATTTTGCCTCTA 3' and antisense: 5' GTCAGTC AGAGTCAGAGTCATGACTAGAG***TCTAGA***TGACTATTACACTGCCGGAGCCAG 3'. BoPAG-12 was cloned into the pacmp-3 transfer vector with the following oligonucleotides, sense: 5' GAC***TCTAGA***ATG**GATTACAAGGACGATGACGATAAG** ATAGTCATTTTGCCTCTA 3' and antisense: 5'GATCTATGATCTCAGTACT ***GCGGCCGC***TCACTATTACACCTGTGCCAGGCCAAT 3'. The recombinant proteins were expressed as fusion proteins with flag-tag in the N-terminus of the protein. A sequence encoding for a flag tag (DYKDDDDK) shown as regular bold in the sense oligonucleotide was engineered into the sequence and, thereby, incorporated into the N-terminus of the proforms of both PAG-2 and -12. Sequence encoding for restriction enzymes (bold italicized) Not-1 and Bgl-2 (New England Biolabs, MA USA) were also engineered into the sense and antisense oligonucleotides to permit directional cloning into the corresponding transfer plasmids. Once the integrity and frame of the sequences in the transfer vectors was verified by sequencing, the vectors were transfected into Sf-9 cells along with BD baculogold linearized Baculovirus DNA by using the BD baculogold transfection kit according to the manufacturer's recommendations. Following

transfection, the viruses were extracted, amplified and were used to infect Sf-9 cells to generate recombinant proteins as described elsewhere (1999; O'Reilly et al., 1992; Summers and Smith, 1987). Infected cells were harvested, chilled on ice, centrifuged at 600 g for 5 min at 4ºC followed by two wash cycles under similar conditions with cold 1x PBS (2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl and 8.10 mM Na_2HPO_4, pH 7.2). The final cell pellet was stored at -80ºC until use.

Purification of recombinant boPAG-2 and 12:

For purification of the recombinant protein, the corresponding frozen pellets were lysed on ice with I-Per insect cell protein extraction reagent (Pierce, IL, USA). A standard cocktail of protease inhibitors, which included 0.4mM Pefabloc SC-AEBSF (Roche Applied Science), 5 µg/mL aprotinin, 10 µM E-64, 1 mM EDTA (Sigma, MO, USA) along with 1mM DTT, was supplemented to the lysis buffer just before use. Following mixing and incubation with lysis buffer for at least 15 min on ice, the lysate was cleared by centrifugation at 15,093 g for 30 min and dialyzed overnight in a 30 K MWCO dialysis tubing in a buffer containing 20 mM Tris-HCl, 250 mM NaCl pH 7.4 at 4ºC. All the downstream purification procedures were performed in a refrigerated room at 4-6ºC. The dialysed lysate was fractionated on a Sephadex-200 size exclusion column (1.5cmx106cm) equilibrated in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. All fractions that were determined to have FLAG peptide present (by dot-blot with an anti-FLAG M2 antibody) were pooled and subsequently affinity purified by using anti-FLAG M2 agarose (Sigma, MO, USA). For affinity chromatography, the matrix was equilibrated with TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), following which, the FLAG-

containing protein samples were loaded twice onto the column by gravitational flow at approximately 0.2 mL/min. The column was then subjected to subsequent washes with 20 column volumes of wash buffer (20 mM Tris-HCl, 150 mM NaCl, pH7.4), 20column volumes of high salt buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.4) and finally 20 column volumes of high-salt buffer supplemented with 0.1% Tween. The matrix re-equilibrated with 10 column volumes of wash buffer, to remove residual detergent, and 10 column volumes of pre-elution buffer (10 mM phosphate buffer, pH 7.2). The column was then eluted with 5 column volumes of 50 mM phosphate buffer, 2 M $MgCl_2$, pH 7.2. The protein sample was desalted by dialysis in 20 mM Tris-HCl, 250 mM Salt, pH8.0 and concentrated by using an Amicon-ultra-15 with ultra cell-30 membrane (Millipore, MA, USA). The concentrated protein samples were supplemented with the inhibitor cocktail and cold (4ºC) glycerol (to a final concentration of 10%, v/v). In most cases, the protein samples were immediately used in the assays. For long term storage, the protein sample was stored in 50% glycerol at -80ºC.

Western blot analysis:

For screening the proteins by western blots, the expressed recombinant proteins were separated on a SDS-PAGE gel and electrophoretically transferred on to Immobilon PVDF-membrane (Millipore, MA, USA). The membranes were washed once with excess of 1x TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween, pH 7.5) and blocked in blocking buffer which consisted of 3% bovine serum albumin and 3% non fat dry milk (Sigma, MO, USA) in 1XTBST. The blots were subsequently incubated with either 1:1000 dilution of monoclonal anti-FLAG antibody (Sigma, MO, USA) or 1:2000 polyclonal anti-boPAG-2 or 12 anti -serum in blocking buffer. The blots were then washed and incubated with 1:2000 dilution of anti-mouse (for anti-flag) or anti-rabbit IgG (for boPAG-2 and 12 antisera) conjugated to alkaline phosphatase (Promega, WI, USA) for 45 min. The blots were finally washed and stained with a mixture of NBT and BCIP according to manufacturer's instructions.

Determining optimal pH for activity studies:

To estimate the optimal pH for each PAG, the recombinant PAGs were incubated in various buffers with ionic strength adjusted to 100 mM with NaCl. All the pH activity experiments were conducted at 35ºC. A synthetic fluorescent FRET cathepsin D substrate MOCAC-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2 (Peptides International, KY, USA) was used to investigate the activity of bovine PAG-2 & -12. The substrate was dissolved in 330 µL of 10% DMSO solution to a final concentration of 200 µM, aliquoted into 10 µL samples and stored in the dark at -80ºC until use. Equal amounts of protein sample (approximately 500 ng in 20 µL) were incubated with 20 µM

of substrate. For determination of optimal pH the following buffers were used: 0.1M glycine-HCl buffer for pH 2.5, 3.0 and 3.5, 0.1M sodium citrate-citrate buffer for 4 and 4.5. 0.1 M sodium acetate-acetic acid for 5 and 5.5, 0.1 M Bis-tris-HCl buffer for 6 and 6.5, 0.1 M Hepes-sodium hydroxide for pH 7 and 0.1 M Tris-HCl for 7 and 7.5 buffers. Each reaction solution also contained NaCl at a final concentration of 100 mM. The final volume of the reaction was maintained at 100 µL. The reaction was performed at 35ºC for 20 min and was terminated by addition of 900 µL of 5% TCA as described previously (Yasuda et al., 1999a). The resultant mixture was further diluted to 2 mL with 5% TCA and the resultant fluorescence in the mixture was read in a PC1^{TM} Photon counting spectofluorimeter at 328 nm (excitation) and 393 nm (emission) wavelengths as described previously (Yasuda et al., 1999a). All experiments were set up in duplicate. The results from duplicate reads and from two successive experiments were used to compile the data.

Estimation of pH profile of activated and proform of recombinant boPAG-2:

A well known feature of APs is the ability to auto-activate themselves (by removal of the propeptide) by either an intra- or intermolecular mechanism (Barret, et al., 2004; Hazel, et al., 1992; Whittlin, et al., 1999; Koelsh, et al., 1994). After systematic characterization of activation procedures for boPAG-2 (data not shown), an optimal activation scheme was identified which encompassed addition of one-third volume of activation buffer (0.25 M Glycine-HCl, pH 3.5) to protein sample (calibrated and stored in 20 mM Tris-HCl, pH 8.0, 250 mM NaCl buffer), mixing and incubation for 1 min at RT. To estimate the profile of this activated protein and compare it to the activity of

zymogens, approximately 620 ng of total protein either proform or activated was incubated with 20 μM of substrate in buffers of gradient pH as described in the earlier experiment. The samples were placed in a 96-well costar black microtitre plate (Corning, USA) and subsequently incubated at 37˚C for 10 min in Synergy-HT Fluorescent plate reader (Biotek, USA) and the endpoint reads were obtained. The experiment was conducted in triplicate to estimate the experimental noise.

Determining the pH stability of recombinant PAG preparations:

To understand the stability and subsequent activity of both zymogen as well as active recombinant boPAG-2, in different pH conditions, approximately 620 ng of total protein was incubated in a range of buffers described above in a total volume of 90 μL and incubated in water bath for 2 hrs. Following incubation for 2 hrs, the protein samples were transferred to a Costar black microtitre plate and the experiment was initiated by addition of 10 μL of 20 μM substrate which was dissolved in sterile water and pre-equilibrated to 37˚C in the plate reader. The concentration of buffer in the final reaction was maintained at 100 mM and the NaClconcentration at 100 mM. Following the addition of substrate, the kinetic reads were obtained for the first 10 minutes of the reaction and the initial rates were displayed as relative fluorescent units (rfu)/min. The experiments were conducted in triplicate for statistical strength.

Enzyme assays of boPAG-2 &12:

The assays for the determination of kinetic parameters for boPAG-2 & -12 were estimated from progress curves as described previously (Palmier and Van Doren, 2007a).

Concentration of total protein in the purified samples was estimated by BCA protein assay (Pierce Thermo Scientific, Rockford, IL). Accurate estimates of specific activity of PAG preparations were obtained by active site titrations against the tight-binding inhibitor of aspartic peptidases, pepstatin A, as described elsewhere (Copeland, 2000; Knight, 1995) and the K_i for PAGs was estimated by fitting the data to the following equation by a procedure detailed in the reference (Neumann et al., 2004):

$v = (vo/2Eo)\{1 - [(Eo + Io + Ki) - (Eo + Io + Ki)^2 - (4EoIo)^{0.5}\}$. Progress curves were used for accurate measurement of k_{cat}/K_m using the following equation:

$y = (F_{max}*(1-\exp(-(k_{cat}/K_m)*E_t*x)))+B$, where y is the relative fluorescence intensity, F_{max} is the maximum change in fluorescence intensity during the reaction, E_t is the enzyme active site concentration in the assay, x is time in seconds, and B is a Y axis offset correction (Balcon and Fitch, 1970; Duggleby and Morrison, 1977, 1978; Orsi and Tipton, 1979). All kinetic experiments were performed in 0.1M sodium citrate, 0.1 M NaCl (pH 4.0) buffer at 37˚C with a Fluorescent FRET - Cathepsin D substrate in 3x3mm cuvettes to reduce the path length and Inner filter effect (Lakowicz, 1999; Matayoshi et al., 1990; Y.Liu et al., 1999). The data were obtained by monitoring product development in a PC1 ^{TM} Photon counting spectofluorimeter (ISS Inc., IL, USA).

Nonlinear fitting of data:

Origin Pro 7.5 (Microcal) was used for nonlinear and global regression of kinetic data according to previously published methods (Palmier and Van Doren, 2007a). Optimization of fitted parameters used iterations of the simplex algorithm followed by Levenberg-Marquardt minimization as described elsewhere (Palmier and Van Doren,

2007b). Because global fits of enzyme kinetics are very sensitive to the concentration of active sites, we carefully titrated the active sites before experiment for accuracy.

Determining differences in primary peptide specificity between boPAG-2 and boPAG-12 and comparison with porcine gastric pepsin and bovine spleen cathepsin D:

To determine differences in substrate preferences, if any, between boPAG-2 and -12 a synthetic 25-x FRET substrate library (Peptides International, KY, USA) was employed. The library consisted of 5 different amino acids at position Y (Pro, Tyr, Lys, Ile and Asp) and Z (Phe, Ala, Val, Glu and Arg) for each residue at position X [D-A2pr(Nma)-Gly-$(Z_{aa})_5$-$(Y_{aa})_5$-X_{aa}-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg. There are 19 different primary substrate libraries with each natural amino acid at position X with the exception of cysteine. Therefore, the libraries represented of 475 different peptides for experimentation. The library has utility in delineating optimal substrate sequence for a given peptidase based on initial rates against the 19 different primary libraries by the peptidase followed by more refined analysis. This experiment sought to determine if there were any obvious differences in substrate preference between the two PAGs and to compare them to two well defined aspartic peptidases, cathepsin D and pepsin. Pre-extracted native preparations of cathepsin D (bovine spleen) and pepsin (porcine stomach) were obtained (Sigma, MO, USA) and used in the assay. All the preparations were active site titrated, and equal amounts (5 nM) of active enzyme and 20 μM substrate were used in the assay. The assay was performed in duplicate under conditions described for the kinetic analysis [0.1 M sodium citrate, 0.1 M NaCl (pH 4.0) buffer at 37˚C]

above. The kinetic readings were obtained against the primary substrate libraries for first 10 minutes of the reaction using the fluorescent plate reader and the initial velocities were calculated and displayed.

Results

Evolutionary relationships of bovine PAG gene family:

The analysis of evolutionary relationships between different PAG members based on their differences in amino-acid substitutions at synonymous sites; revealed branching and separation of PAGs into two distinct classes (Figure -26). The larger class of PAGs represents recently evolved members of the PAG gene family and is referred to as the 'modern' PAGs. The smaller class which contains a roster of 5 different PAG genes (boPAGs-2, 8, 10, 11 and 12) are referred to as ancient PAGs. Note that of the 5 PAG members, boPAGs-2 and 12 are evolutionarily closely related and are clustered together.

Expression and purification of recombinant boPAG-2 & -12:

Bovine PAGs-2 and -12 were expressed as fusion proteins with an N-terminal FLAG-tag in a Baculovirus insect cell expression system. The FLAG peptide at the N-terminus allowed for affinity purification of fusion proteins by using an anti-FLAG M2 antibody matrix. The purified sample from affinity purification when desalted on a sizing column resulted in a preparation highly homogeneous for soluble PAG fraction (Figure -27). The expressed full length proteins following purification were of the expected molecular weight. BoPag-12, however, did exhibit a higher molecular weight fraction accompanying the regular pro-form, probably because it was cloned into pACMP2 transfer vector, that facilitates cytoplasmic glycosylation (Baculovirus expression

manual, Pharmingen)(Pharmingen, 1999). Both the expressed PAGs were identified by both the anti-FLAG antibody and a polyclonal (anti-boPAG-2) antibody by western blot (Figure 27).

Estimating optimum pH profile for activity studies:

The optimal pH for maximal activity for boPAG-2 was found to be around pH 4.0 (Figure -28). When the raw values (relative fluorescent units) were transformed into relative percentage values with maximal activity defined as 100%, we found that while maximal activity was found to be at pH 4.0, high activity was also observed between pH 3.0 and 4.5 (Figure -28). Bovine PAG-12 had optimal activity at pH 3.5, albeit with a relatively broad pH activity range from pH 2.5 to 4.5 (Figure -28). It was notable that, while boPAG-12 had high activity at pH 2.5, boPAG-2 had relatively little activity at this pH. The optimal activity of boPAG-2 and 12 under acidic conditions is consistent with pH preferences of most other aspartic peptidases (Davies, 1990; Dunn, 2002; Kay and Dunn, 1992; Szecsi, 1992).

pH profile of activated recombinant boPAG-2:

One hallmark of many APs is their ability to remove their propeptides when placed in an acidic environment (Barrett et al., 2004; Hazel et al., 1992; Koelsch et al., 1994; Richter et al., 1998; Wittlin et al., 1999). When boPAG-2 was incubated in activation buffer (0.25 M Glycine - HCl, pH 3.5) for 1 min at RT, the ability of boPAG-2 to exhibit activity at higher pH became evident. When activated protein was compared in the assay alongside the pro-form of boPAG-2 (which was not subjected to a pre-

activation step), there was significant agreement in activity between both of them within the pH optimum determined above (pH 3 - 4.5) (Figure -29). However, the activity within the pH range 5 - 8 deviated substantially; there was increased activity in the activated preparation, as opposed to the zymogen form, within this pH range. Greatest activity was recorded at pH 6.0. These experiments were replicated multiple times and the observations were quite consistent.

pH stability of recombinant boPAG-2:

To assess the stability of the zymogen and activated forms of boPAG-2 under different pH conditions, the proteins were incubated for 2 hrs at 37˚C in the buffers indicated above before addition of substrate and recording of peptidase activity. Both forms of PAGs exhibited activity profile similar to the one described above within the optimum pH range (pH 3.0 - 4.5) (Figure -30). This signifies that both forms of PAGs are very stable in the acidic pH between 3.0 and 4.5 which was found to be optimum for maximal activity. It may be noted that following 2 hrs of incubation in buffers ranging from pH 4.5 to 6.0, most of the zymogen seems to have been converted to activated form resulting in much greater activity and subsequently a much broader curve when compared to the previous experiment (Figure – 29), where in the zymogen was exposed to these buffers only for the duration of assay. This resulted in apparent narrowing of curves between the zymogen and activated sample within this pH range. Besides, in the activated protein sample, the high level of activity was sustained between pH 5.0 and 6.0 in contrast to the zymogen. Once again, the high activity of the activated protein sample

between pH 5.0 to 6.0 was consistent with our earlier observation when the protein was exposed to short assay periods.

Comparison of kinetic parameters of recombinant boPAG-2 and -12:

The Michaelis-Menton kinetic parameters k_{cat}, K_m and k_{cat}/K_m were determined from progress curves (Figure -31) and the K_i for pepstatin A was determined and listed in Table -4. The kinetic parameters revealed obvious and significant differences in terms of affinity and catalytic efficiency toward the cathepsin D substrate between boPAG-2 and 12. BoPAG-2 has high affinity (indirect measure-K_m), turnover (k_{cat}) and subsequently a high catalytic efficiency (k_{cat}/K_m) against the substrate when compared to boPAG-12 (Figure -31, Table -4). Under identical reaction conditions, the catalytic efficiency of boPAG-2 (272168 ± 277 $M^{-1}s^{-1}$) was found to be approximately 3.9 fold greater than boPAG-12 (68638 ± 415 $M^{-1}s^{-1}$). The k_{cat} for boPAG-2 ($0.96 \pm 0.08 s^{-1}$) was approximately 3.3 fold higher than boPAG -12 (0.29 ± 0.03 s^{-1}) and the Km (3.53 ± 0.03 μM) was lower compared to boPAG -12 (4.2 ± 0.3 μM). The K_i for pepstatin A was lower in boPAG-2 (0.56 nM) compared to boPAG-12 (7.5 nM) (Table -4).

Substrate preferences of boPAG-2 and boPAG-12 in comparison to porcine gastric pepsin and bovine spleen cathepsin D:

Inorder to identify the primary peptide specificity for boPAG-2 and -12, both boPAG -2 and -12 were investigated for their activity against FRET -25X peptide libraries alongside canonical APs porcine pepsin (stomach) and bovine cathepsin D (spleen). While porcine pepsin and cathepsin D displayed good activity against the

primary substrate libraries, boPAG-2 and 12 failed to produce reasonable velocities (Figure -32). These observations were repeatable even when the experiments were conducted at relatively high concentrations of enzymes (100 nm of boPAG-2). This observation distinguishes them from cathepsin D, which is an AP found within most cell types, even trophoblast. However, of the two candidate PAGs, PAG-12 produced more activity than PAG-2 against the range of substrate libraries, suggesting another difference between these closely related members.

Discussion

Pregnancy-associated glycoproteins are structurally related to APs and are abundant within the placenta of ruminants and other species of the *Artiodactyla* order. Although PAGs have been studied extensively, their role within the ungulate placenta remains largely obscure. Despite being structurally related to APs, many of the modern PAGs have accumulated mutations within key residues in and around the catalytic site that likely inactive them as peptidases. The ancient PAGs, in contrast, have all the definitive characteristics of typical APs and were predicted to have proteolytic activity. Therefore, the goal of this project was to address two important questions: (1) do ancient PAGs have proteolytic activity and, if so, (2) how do closely related family members compare to one another in regard to kinetic parameters? To address these fundamental questions, boPAGs-2 and -12 were chosen as candidates for these experiments. BoPAG-2 was an obvious choice to begin with, since it is the most abundantly transcribed member of the bovine PAG group (it represents roughly 25% of the total PAG message in the

bovine placenta) (J.Green; unpublished data). It is also among the first PAGs to have been identified and characterized in some detail (Wooding et al., 2005; Xie et al., 1994). BoPAG-12, on the other hand, is closely related to boPAG-2; it shares 89% identity at nucleotide level and 83% identity in amino acid composition (Green et al., 2000; Hughes et al., 2000).

A baculovirus insect cell expression system was chosen for expression of PAGs as soluble recombinant full-length PAGs since it is amenable for expression of recombinant proteins, especially acid peptidases, since the cytoplasm in these cells is not extremely reducing and is maintained close to a pH of 7.0 – these conditions were predicted to help prevent the activation, and subsequent loss, of acid peptidases by autocatalysis. The inclusion of a synthetic coding sequence for Flag-tag allowed for preparing relatively homogenous preparations of purified proteins by using a standard anti-flag affinity purification scheme (Medina et al., 1995).

The purified protein preparations were tested for optimal activity against a synthetic FRET substrate MOCAC-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2. This sequence was derived from a consensus substrate sequence for APs [Lys-Pro-Ile-Gln-Phe*Nph-Arg-Leu (Nph: nitro-phenylalanine)] described elsewhere (Beyer et al., 2005). The fluorescent substrate was initially designed and tested for utility against other well characterized APs, Cathepsin D andE (Yasuda et al., 1999a). Both boPAG-2 and -12 exhibited activity towards this substrate. The optimal activity was observed to be around pH 4 and 3.5 for PAG-2 and -12, respectively. Such acidic pH optima are typical for most APs. Of the two candidate PAGs tested, boPAG-12 had substantially greater activity at pH 2.5 compared to boPAG-2. When comparing their kinetic parameters, it

was apparent that boPAG-2 and -12 have substantial differences in activity, despite that they are closely related. It was determined that boPAG-2 had a kinetic efficiency in cleaving the substrate at least 3.9 times greater than boPAG-12 under the same experimental conditions. In addition, the affinity of boPAG-2 for pepstatin A was also more than an order of magnitude greater than boPAG-12.

The experiments performed with the synthetic 25-x FRET substrate libraries suggested that boPAG-2 and -12 may have stringent requirements in regard to the length of the substrate required for optimal activity. Both porcine pepsin and bovine cathepsin D exhibited activity toward these libraries in a manner consistent with what is already known regarding their substrate specificities (Abad-Zapatero et al., 1990; Barrett et al., 2004; Scarborough and Dunn, 1994). However, both boPAGs-2 and 12, when used at the same molar concentration, failed to produce any product liberation from these libraries. This lack of activity is reminiscent of studies performed with Furin, which is fastidious when it comes to substrate sequences required for optimal activity; in these experiments, furin failed to show activity against these substrate libraries (Reported by PeptidesInt-product brochure). The lack of activity against these libraries could be arise from the fact that the library failed to provide peptide sequences of the appropriate length or combination for optimal activity with both PAG-2 and 12. However, it is worth mentioning that PAG-12 did seem to have produced at least some activity when compared to PAG-2, which suggests that PAG-12 might be more flexible in regard to its substrate requirements. Evidence for stringent substrate specificity for boPAG-2 was suggested from some unpublished preliminary experiments with general peptidase assays

(milk clotting assays, fluorescent casein assays); no activity was observed in these experiments (data not shown).

The fact that these ancient bovine PAG members (2 and 12) have peptidase activity, the fact that measurable differences in activity exists, and the indication that they are not capable of acting as general peptidases, suggests that they likely have somewhat refined roles in proteolytically cleaving substrates - either within the trophoblast itself or at the fetal-maternal interface. The PAGs are secreted glycoproteins, hence they are exposed to acidic environments, with an acidic pH as low as 5.2 within the secretory pathway (Paroutis et al., 2004b). The PAGs might have a major role within the secretory pathway, where they might be activated by an unidentified peptidase or by auto-activation (Davies, 1990; Dunn, 2002). These activated PAGs might play a role in proteolytic processing and maturation of candidate proteins within the secretory vesicles before they exit the cell. Alternatively, they might be diverted towards the endo-lysosomal pathway where they function by digestion of endocytosed agonist bound receptors or proteins taken up from uterine secretions (uterotroph). Since both the identified PAGs seem to be highly substrate specific, it may be safe to hypothesize that, the ancient PAGs may not function as general degradative enzymes like cathepsin D and E, which are lysosomal and endosomal peptidases, respectively (Godbold et al., 1998; Ishidoh and Kominami, 2002). Another, interesting possibility is that boPAG-2/-12, which are constitutively secreted and accumulate at the maternal-fetal interface, may play roles as sheddases to activate latent growth factors or as peptidases involved in inactivating growth factor binding proteins, etc., (Munger et al., 1998; Rifkin et al., 1999)

within the pericellular microenvironment. Some data exist to indicate that this environment may be slightly acidic (pH 6.5) (Punturieri et al., 2000). In either situation, such proteolytic conversion of trophic factors may have an important role in promoting trophoblast/placentomal growth and differentiation in the ruminant placenta (Grundker and Kirchner, 1996; Ko et al., 1991; Mathialagan and Roberts, 1994; Rifkin et al., 1999; Spencer and Bazer, 2004; Tanaka et al., 1998; Yelich et al., 1997). Future efforts will be directed toward exploring these varied possibilities.

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Chapter –III figures

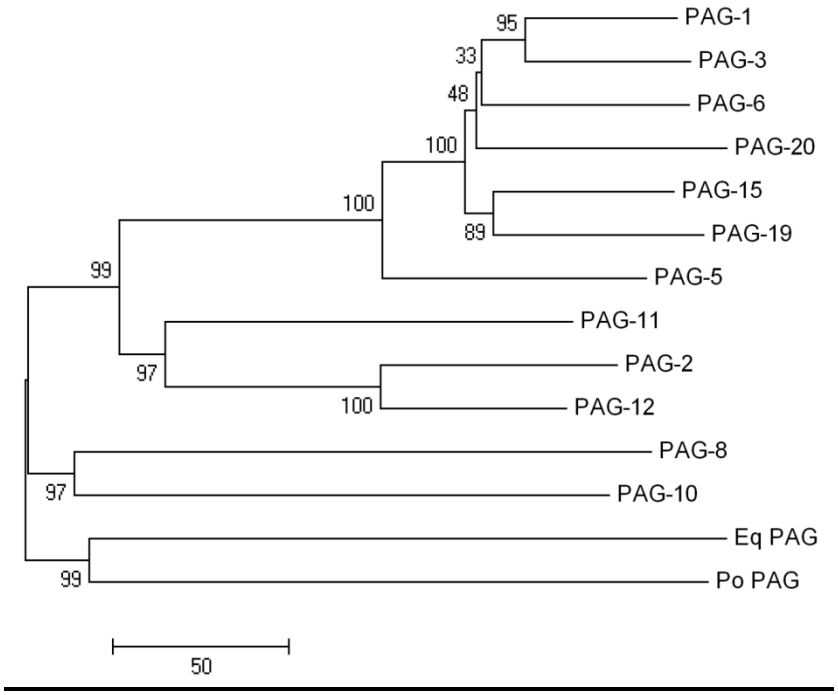


Figure -26. Evolutionary relationships of PAGs and PAG-like proteins:

The phylogenetic relationships of boPAGs were computed based on amino acid sequence by the Minimal evolution method. Notice the clear branching and separation of modern and ancient PAGs. Also notice the clustering of both boPAGs-2 and 12 within the ancient PAG grouping.

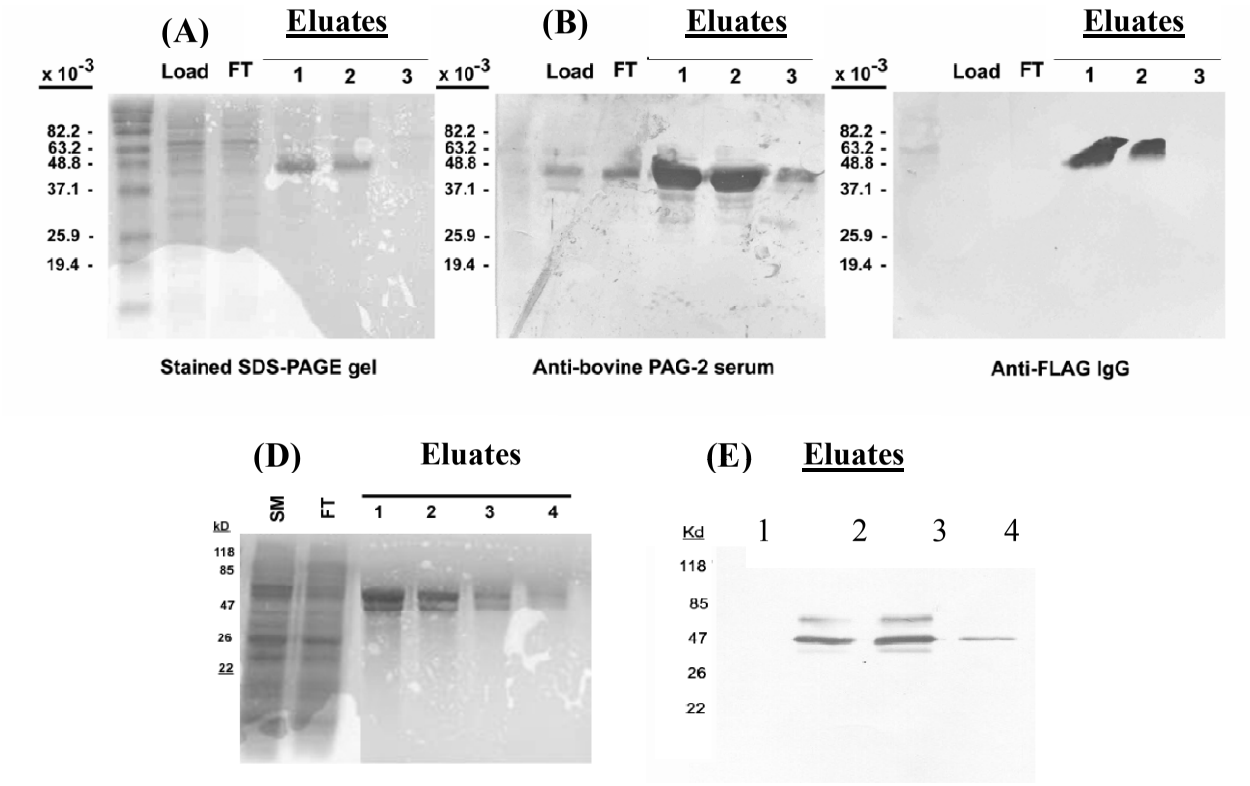


Figure -27. SDS-PAGE gel stained with coomassie blue and Western-blots of expressed recombinant bovine PAGs-2 and 12. Figure -27a) shows the SDS-gel stained by coomassie blue. Lane-1: represents the total proteins from the lysed boPAG-2 pellet. Lane-2: Flow-through of lysate from the anti-flag column. Lanes 3-5 represents respective elution fractions of recombinant boPAG-2 from the column in numerical order. Figure 27b and c) shows the Western blot images of the transferred proteins from identically loaded gels immuno-blotted with anti-PAG-2 polyclonal and anti-flag monoclonal antibodies, respectively. Figure -27d) SDS-PAGE gel of expressed recombinant boPAG-12 stained with coomassie blue stain. Lane-1 represents total proteins from lysate. Lane-2: flow-through from the column. Lane-3-5: Elution fractions in numerical order. Fig-2e) Western blot image obtained by immuno-blotting with anti-flag monoclonal antibody.

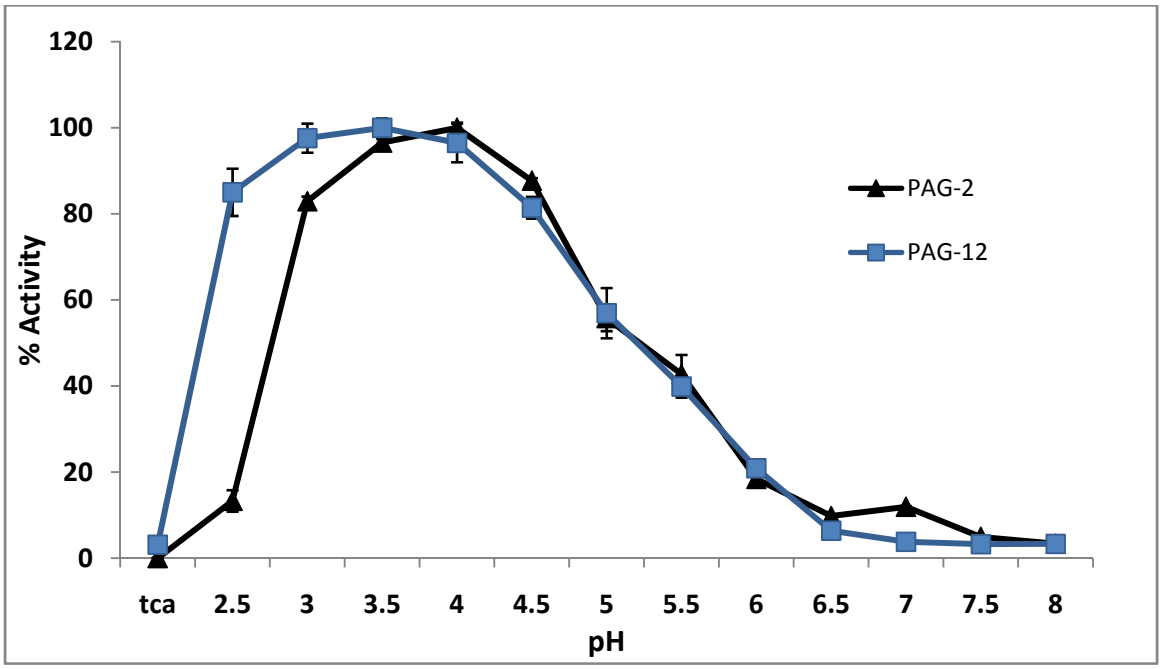


Figure -28. Graph showing the relative activity profiles of boPAG-2 and -12. The raw-fluorescent values obtained from spectro-fluorimeter readings were transformed with the highest activity being 100%. The activity profiles for both bovine PAGs-2 (▲) and -12 (■) were overlapped for comparison.

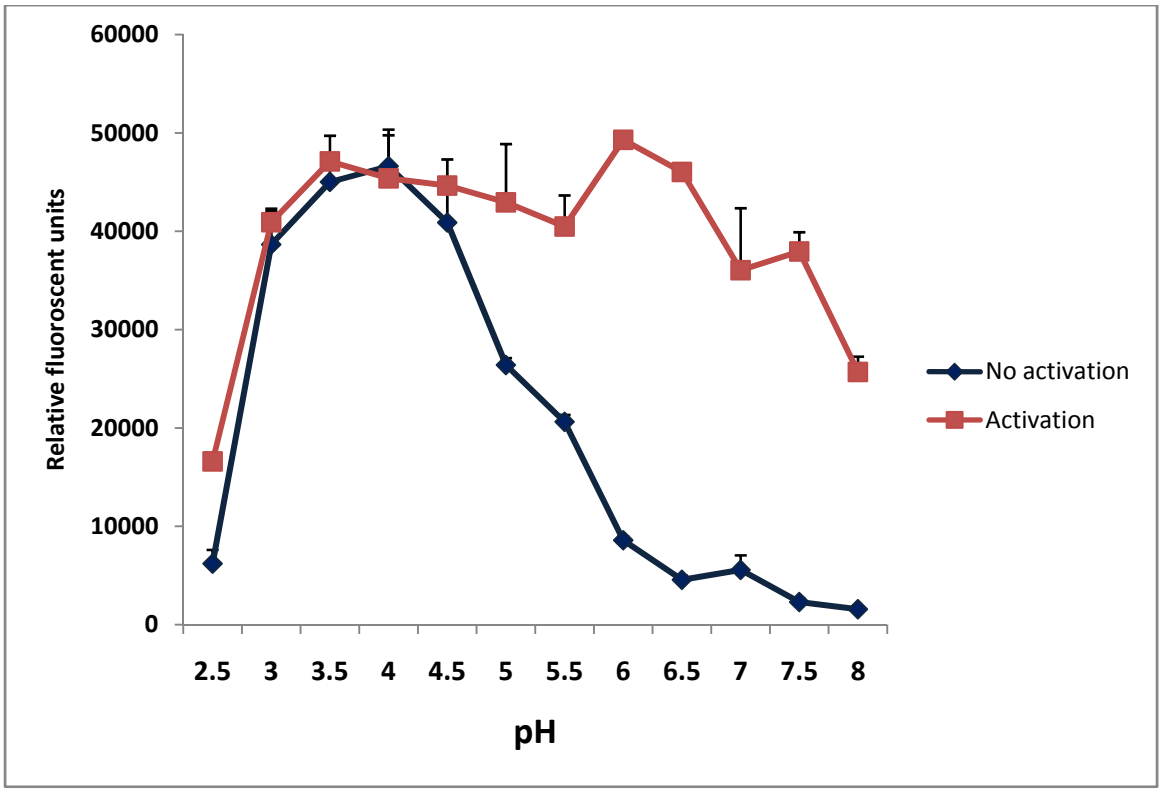


Figure -29. pH profiles of recombinant boPAG-2 before and following activation. The graph represents the relative activity of boPAG-2 material before (◆) and following (■) activation. The net activity of each candidate PAG at each respective pH is displayed as relative fluorescent units (Rfu). The assays represent end-point reads.

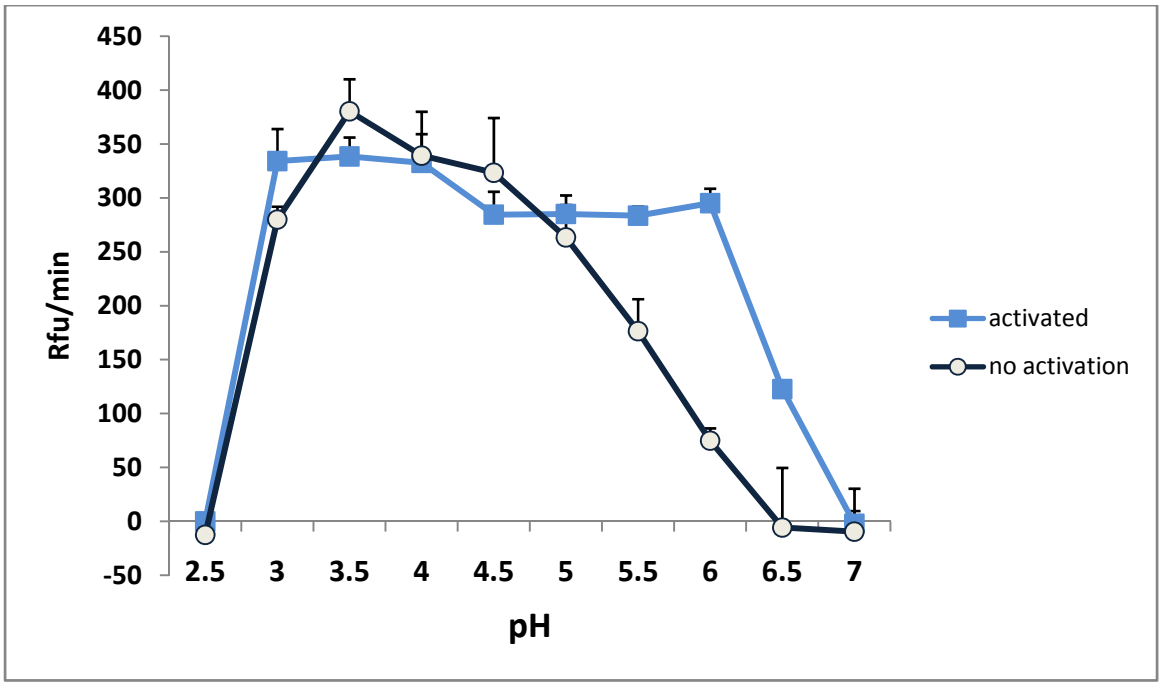


Figure -30. pH stability assay. The graph represents the stability of recombinant boPAG-2 before and following activation in the buffers indicated in the picture. The zymogen (◯) and activated protein (■ pH 3.5 for 1 min at RT) were incubated in the pH conditions indicated in the graph for 2 hrs at 37˚C. Following the incubation period, the substrate was added and assayed at the same temperature, in respective pH conditions. The initial rates of cleavage of substrate (Rfu/min) were shown in the picture.

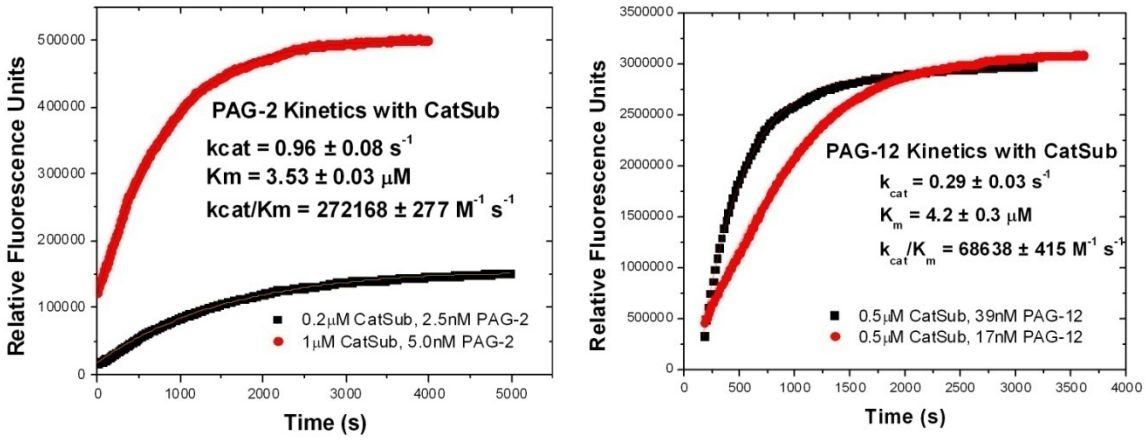


Figure -31. Two progression curves are shown for obtaining k_{cat} and K_m by global fitting. The enzyme and substrate concentrations for Upper (red) and lower (black) curves for bovine PAG-2 are 0.5 µM substrate and 39 nM enzyme and 0.5 µM substrate and 17 nM enzyme respectively. For the two different curves of boPAG-12 the combination of substrate and enzyme black and red are 0.5 µM substrate and 39 nM enzyme and 0.5 µM substrate and 17 nM enzyme, respectively.

Table -4: Respective kinetic parameters for boPAG-2 and 12 obtained from global fitting of progress curves.

PAG	K_m (µM)	k_{cat} (s^{-1})	k_{cat} / K_m ($M^{-1}s^{-1}$)	Pepstatin K_i (nM)
BoPAG-2	3.53±0.03	0.96±0.08	272,168±277	0.56
BoPAG-12	4.2±0.3	0.29±0.03	68,638±415	7.5

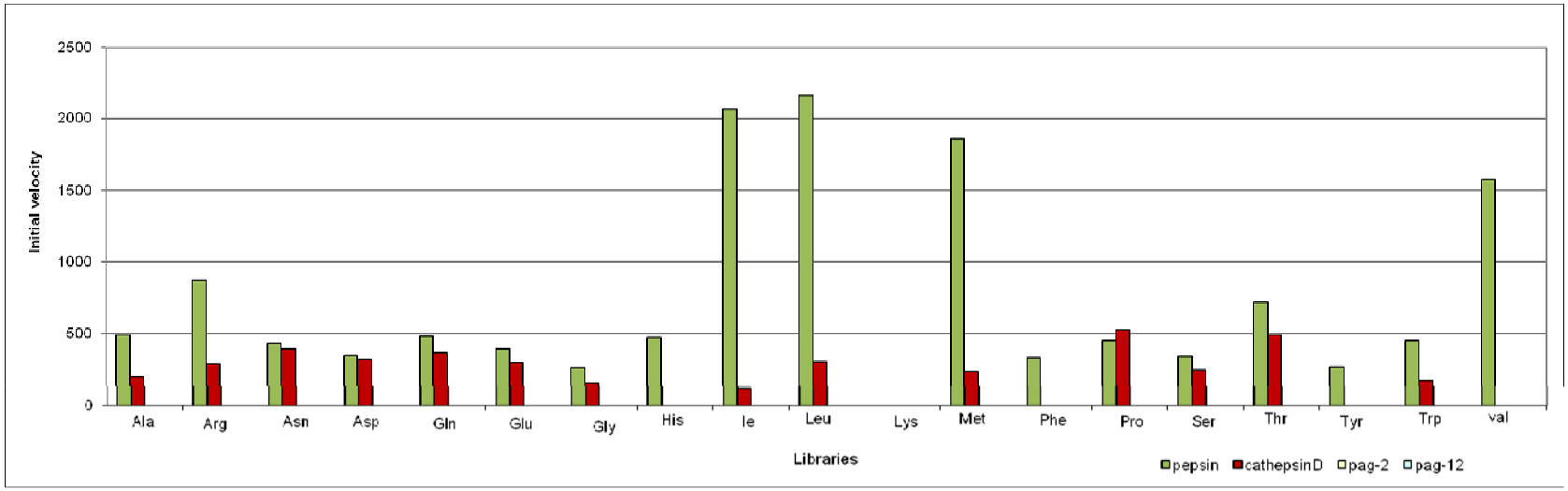


Figure -32. Initial velocities obtained from the proteolysis of several fluorescent peptide substrate libraries (designated by a three letter amino acid code). The libraries are named after the amino acid occupying position-X in the library. The velocities obtained from the four different peptidases, porcine gastric pepsin, bovine spleen cathepsin D, boPAG-2 and -12 against each representative substrate library are showed in the figure. Note that significant velocities were obtained only with pepsin and cathepsin D.

Chapter -IV

Identification of Porcine Pregnancy-Associated Glycoprotein-2 (poPAG- 2) as a proteolytically active aspartic peptidase.

Abstract

The pregnancy-associated glycoproteins (PAGs) represent a large gene family of aspartic peptidases expressed exclusively in trophoblasts of even toed ungulates such as pigs, ruminants, etc. In ruminants, multiple members of the PAG gene family have been described. However, in pigs, only two clearly distinct PAG transcripts have been identified, although several variants have been deposited in Genbank. One of the transcripts, porcine PAG-1 (poPAG-1) was predicted to be proteolytically inactive due to inactivating mutations within the catalytic center. The second transcript, poPAG-2, possessed a conserved catalytic center and has been predicted, but not experimentally shown, to have proteolytic activity. Since poPAG-2 possesses all the characteristics of a proteolytically active aspartic peptidase, the thrust of this work was to test such a possibility. The two specific goals of this present study were to investigate if poPAG-2 has any detectable proteolytic activity and to determine its kinetic parameters. PoPAG-2 was expressed as a recombinant fusion protein with an amino-terminal 'FLAG -tag' in a Baculovirus- insect cell expression system. The expressed proteins were affinity purified by using an anti-flag antibody conjugated resin. The purified preparations were then analyzed for proteolytic activity against a fluorescent substrate. It was noticed that, poPAG-2 was able to cleave the substrate with an optimal proteolytic activity around pH 3.5. Against this substrate, it had a k_{cat}/K_M of 1.2 $\mu M^{-1}s^{-1}$ and was inhibited by the

aspartic peptidase inhibitor, pepstatin A, with a K_i of 12.5 nM. Since the proteolytic activity of PAGs in the pig has now been established, the search for putative substrates to gain insight into the physiological role of PAGs will likely be the focus of future investigations.

Introduction

Aspartic peptidases (AP) are a class of proteolytic enzymes that have a wide distribution. They are found in retroviruses, fungi, plants and vertebrates (Szecsi, 1992). Examples of mammalian aspartic peptidases include pepsin, cathepsins D and E, napsin and renin (Szecsi, 1992). The principal defining characteristic of the vast majority of APs is their near universal requirement for an acidic pH for maximal activity (one exception to this rule is renin). Another common characteristic is that most APs are inhibited by a statine containing peptide, pepstatin A (Davies, 1990; Kay and Dunn, 1992).

The eukaryotic aspartic peptidases are comprised of a two-domain or bi-lobed structure, probably resulting from a duplication event involving a single domain from an ancestral retroviral AP (Dunn, 2002; Tang and Wong, 1987). The APs as their name suggests utilize two aspartic acid residues, Asp32 and Asp215 (porcine pepsin numbering), in the catalytic center that participate in hydrolysis of peptide substrates (Davies, 1990; James and Sielecki, 1986). The generally accepted mechanism of action for APs is a model of general acid-base catalysis (Davies, 1990; Dunn, 2002; Szecsi, 1992). According to this model, a water molecule is coordinated between the two highly-conserved aspartic acid residues (Asp32 and Asp 215) via a series of hydrogen bonds. One of the two aspartates, Asp215 acts as a general base extracting a proton from the water molecule, thereby activating it. The activated water molecule now acts as a

nucleophile in the attack on the carbonyl carbon of the substrate scissile bond. The other aspartate, Asp32 , behaves as a general acid donating a proton to the oxygen in the carbonyl moiety of the scissile bond. This results in a transient unstable tetrahedral intermediate. Rearrangement of this intermediate leads to protonation of the scissile amide and eventual hydrolysis of the substrate.

A unique multi-member family of proteins that are structurally related to aspartic peptidases, known as pregnancy- associated glycoproteins (PAGs), has been described in the placental trophoblast cells of even-toed ungulates such as swine, cattle, sheep, deer, etc. (Garbayo et al., 2000; Green et al., 2000; Green et al., 1998b). In ruminant ungulates (cattle, sheep) it has been found that the PAG gene family is extensive and is comprised of dozens of expressed genes (Brandt et al., 2007; Garbayo et al., 2000; Garbayo et al., 1998; Green et al., 2000; Szafranska et al., 1995; Xie et al., 1997a). In contrast, swine possess only two distinct transcripts (Szafranska and Panasiewicz, 2002; Szafranska et al., 1995). In species beyond the *Artiodactyla* order, proteins resembling PAGs, known as 'pepsinogen F (pepF)' have been identified in rabbits (Kageyama et al., 1990), rodents (Chen et al., 2001; Kageyama et al., 2000), cats and dogs (Gan et al., 1997) and equids (Green et al., 1998a). But unlike PAGs, the pepF locus did not undergo expansion and, therefore, only a single gene copy has been found in all these species. An additional differentiating characteristic of the pepF gene is that, unlike PAGs, it is not restricted in expression to trophoblasts. It is found expressed in the neonatal stomach mucosa and in other extraembryonic membranes, such as the yolk sac (mouse pepF) (Chen et al., 2001; Kageyama et al., 1990).

The two transcripts encoding for porcine (po) PAGs, poPAG-1 and -2 were initially cloned from a d13-17 pig conceptus cDNA library (Szafranska et al., 1995). The transcripts share 79% identity in nucleotide and 64% in amino acid sequences. Recently, additional poPAG members have been reported which have been classified as belonging to either the poPAG-2 subfamily (poPAGs-4, -6, -8 and -10) or poPAG-1 subfamily (poPAG-1, -3 and 5) (Panasiewicz et al., 2004; Szafranska and Panasiewicz, 2002). However, these transcripts seem to more appropriately be considered as variants of poPAG-1 and -2, since they differ by only 1% or less in amino acid identity.

Of the two predominant PAGs in swine, poPAG-2 is the most abundant transcript in the conceptus and the poPAG-2 transcript could be identified as early as the 4-cell stage of embryo development (Do et al., 2001; Szafranska et al., 1995). Mirroring the high abundance of message, copious amounts of poPAG-2 protein have been identified in explant cultures of pig trophoblast *in vitro* (Dore et al., 1996; Szafranska et al., 2003; Szafranska et al., 1995). In addition, major secretory products of implanting pig embryos known as porcine basic proteins (pBP) were identified as poPAG-2 (Baumbach et al., 1988; Dore et al., 1996; Godkin et al., 1982; Godkin et al., 1985). Upon establishment of a mature placenta, profuse amounts of the poPAG-2 protein line the maternal fetal interface *in vivo* (Majewska et al., 2006).

Besides apparent differences in the relative abundance of message and protein between the two porcine PAGs, there exists a key difference in respect to their putative proteolytic activity. Porcine PAG-1 seems to be proteolytically inactive due to mutations within the catalytic center. On the other hand, poPAG-2 has no such mutations within the catalytic center and, therefore, was predicted to be capable of acting as a peptidase

(Guruprasad et al., 1996; Szafranska et al., 2001; Szafranska et al., 1995). However, no such activity has been demonstrated for this protein to date. In this report, we sought to determine if a PAG from the pig, specifically poPAG-2, is an active AP and to investigate its kinetic parameters if peptidase activity is observed.

Materials and methods:

Evolutionary relationships of poPAGs:

The evolutionary relationship of two poPAGs-1 and -2, in relationship to other ruminant PAGs, PAG-like molecules (pepF) and other mammalian APs was inferred from amino acid sequences by using the Minimum Evolution method (Rzhetsky and Nei, 1992). The amino acid sequences were aligned by CLUSTALW and all positions containing gaps and missing data were eliminated from each pairwise comparison (Pairwise deletion option). Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree, and bootstrap analysis with 1000 replicates (Felsenstein, 1985) was performed to build the consensus tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 447 positions in the final dataset. Phylogenetic analyses were conducted by using the MEGA4 program (Tamura et al., 2007).

Cloning and expression of recombinant porcine PAG-2:

Recombinant protein for poPAG-2 was expressed by using the BD Baculogold^{TM} Baculovirus insect cell expression system (BD biosciences Pharmingen, San Diego, CA). PoPAG-2 was cloned into the pvl-92 transfer vector by using the following oligonucleotides, sense: 5' GAC AGT ***GCGGCCGC***ATG**GATTACAAGGACGAT GACGATAAG**TTAGTCATGATCCCTCTC 3' and antisense: 5' GTCAGTC AGAGTCAGAGTCATGACTAGAG***TCTAGA***TGACTATTATGTGGCCTGAGCCAG 3'. A sequence encoding for a flag tag (DYKDDDDK) was incorporated into the forward primer (sequence in regular bold) allowing for the expression of the FLAG-epitope at the N-terminus of the recombinant poPAG-2 protein. Sequence recognition sites for restriction enzymes (bold italicized) Not-1 and Bgl-2, (New England Biolabs, MA USA) were also incorporated into the sense and antisense oligonucleotides to permit directional cloning into the transfer plasmid. The integrity of the cloned sequence was verified by sequencing. The cloned transfer vector was then co-transfected with linearized Baculovirus DNA (BD baculogold) into Sf-9 insect cells, by using the BD baculogold transfection kit according to the manufacturer's recommendations. Following transfection, the recombinant viruses were extracted from the culture media and were amplified in two successive rounds of infection of fresh Sf-9 cells. Once the viruses reached an appropriate titre (~2X10^{7} plaque forming units/mL), the recombinant viruses were used to infect fresh insect cells to generate recombinant proteins according to the methodologies described elsewhere (1999; O'Reilly et al., 1992; Summers and Smith, 1987). Following three days of culture, the infected cells were harvested, chilled on ice and centrifuged at 600 g for 5 min at 4ºC. This was followed by two wash cycles under

similar conditions with cold 1x PBS (2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl and 8.10 mM Na_2HPO_4, pH 7.2). The final cell pellet was stored at -80ºC until use.

Affinity purification of recombinant poPAG-2 on an anti-flag antibody column:

For purification of recombinant poPAG-2, the frozen insect cell pellets expressing poPAG-2 were lysed on ice with I-Per insect cell protein extraction reagent (Pierce, IL, USA). A standard cocktail of protease inhibitors, which included recommended concentrations of various inhibitors 0.4 mM Pefabloc SC-AEBSF (Roche Applied Science, USA), 5 µg/mL Aprotinin, 10 µM E-64, 1 mM EDTA (Sigma, MO, USA) along with 1 mM DTT, was added to the lysis buffer just before use. The pellets were thoroughly mixed with the lysis buffer by pipeting or vortexing and were left to incubate on ice for 15 min. The lysate was then clarified by centrifugation at 15,093 x g for 30 min at 4˚C, and dialyzed overnight against a buffer containing 20 mM Tris-HCl, 250 mM NaCl pH 7.4 in 30 K MWCO dialysis tubing at 4ºC. All the downstream purification procedures were performed in a refrigerated room at 4-6ºC. Following dialysis, the lysate was fractionated on a sephadex-200 size exclusion column (1.5cmx106cm), using 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl buffer. The fractions obtained from sizing column were analyzed by dot-blot with an anti-FLAG M2 antibody and all fractions that were determined to have FLAG peptide were pooled and subsequently affinity purified by using anti-FLAG M2 agarose resin (Sigma, MO, USA). For affinity chromatography, the matrix was equilibrated with TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), following which, the pooled FLAG-contained protein sample obtained from size-fractionation was loaded twice onto the column by gravitational flow at approximately

0.2 mL/min. The column was then subjected to subsequent washes with 20 column volumes of wash buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4), 20 column volumes of high salt buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.4) and finally 20 column volumes of high-salt buffer supplemented with 0.1% Tween. The matrix re-equilibrated with 10 column volumes of wash buffer, to remove residual detergent, and 10 column volumes of pre-elution buffer (10 mM phosphate buffer, pH 7.2). The column was eluted with 5 column volumes of 50 mM phosphate buffer, 2 M $MgCl_2$, pH 7.2 buffer. The eluted protein sample was desalted by dialysis in 20 mM Tris-HCl, 250 mM NaCl, pH 8.0 and concentrated on Amicon-ultra-15 with ultra cell-30 membrane (Millipore, MA, USA). The concentrated protein samples were supplemented with the inhibitor cocktail and for short term storage, cold (4ºC) sterile glycerol (autoclaved) was supplemented to a final concentration of 10%, v/v. For long term storage, the protein sample was mixed with sterile glycerol to a final concentration of 50% and stored at -80ºC, until use.

Western blot analysis:

For Western blot analysis of the recombinant proteins, the protein samples were resolved on a 12% SDS-PAGE gel and electrophoretically transferred on to an Immobilon PVDF-membrane (Millipore, MA, and USA). Following transfer, the membranes were washed once with an excess of 1x TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween, pH 7.5) and blocked in a blocking buffer consisting of 3% bovine serum albumin and 3% non fat dry milk (Sigma, MO, USA) in 1XTBST. The blots were subsequently incubated with either 1:1000 dilution of monoclonal anti-FLAG antibody (Sigma, MO, USA) or 1:2000 polyclonal anti-poPAG-2 anti-serum in blocking buffer.

The blots were then washed and incubated with a 1:2000 dilution of anti-mouse (for anti-flag) or anti-rabbit IgG (for poPAG-2 antisera) conjugated to an alkaline phosphatase for 45 min (Promega, WI, USA). The blots were finally washed and stained with a mixture of NBT and BCIP according to manufacturer's instructions.

Determining optimal pH for activity studies:

To estimate the optimal pH for poPAG-2 activity, the recombinant PAG was incubated in various buffers with ionic strength adjusted to 100 mM with NaCl. All the pH activity experiments were conducted at 40ºC. A synthetic fluorescent FRET substrate MOCAC-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2 (Yasuda et al., 1999) (FRET-cathepsin D/E substrate, Peptides International, KY, USA), which was previously utilized to test for activity of bovine PAGs (Manuscript in preparation), was used to measure the activity of poPAG-2. The various buffers used for determination of an optimal pH for poPAG-2 activity were: 0.1 M glycine-HCl buffer for pH 2.5, 3.0 and 3.5, 0.1 M sodium citrate-citrate buffer for 4 and 4.5. 0.1M sodium acetate-acetic acid for 5 and 5.5, 0.1 M Bis-tris-HCl buffer for 6 and 6.5, 0.1 M Hepes-sodium hydroxide for pH 7 and 0.1 M Tris-HCl for 7 and 7.5 buffers. All the buffers were filter sterilized before use. A reaction mixture was prepared by mixing 10 µL of 200 µM substrate with each buffer. The final volume of the reaction mixture was adjusted to 80 µL with sterile double distilled water (autoclaved and filter sterilized to remove residual particles if any). The reactions were set up in individual wells of a costar black 96-well plate and allowed to pre-equilibrate to 40˚C by incubating in a Synergy-HT plate reader. The reactions were then initiated by simultaneous transfer of 20 µL of fresh protein sample (approximately

750 ng) with the help of a multi-well dispensing pipette. The reactions were thoroughly mixed and kinetic readings were obtained for 10 min by using a Synergy-HT fluorescent plate reader (Bio-tek, USA) with 320/20 excitation and 380/20 emission filters. The initial readings were used to estimate the initial velocities which were displayed as relative fluorescent units (rfu)/min. All the reactions were performed in triplicate and the experiment was replicated multiple times. The initial velocities were then transformed to % activity with the highest rate being 100%.

Determining the pH stability of recombinant poPAG-2 preparation:

To understand the stability of recombinant poPAG-2 in different pH conditions, 520 ng of total protein was incubated in a range of different buffers described above in a total volume of 90 µL and incubated in a water bath for 2 h at 40˚C. Following the incubation, the peptidase assay was initiated by addition of 10 µL of 200µM substrate. The concentration of buffer and salt concentration in the final reaction was maintained at 100 mM. Following the addition of substrate, the reactions were carried out for an additional 10 minutes in the water bath. The reactions were terminated by an addition of 100 µL of 10% TCA. The resultant volume was split into 2 wells of a 96-well plate described above and the end-point readings were obtained. The resultant reads were transformed to a percent activity with the highest value being 100%. The reactions were performed in duplicate to calculate experimental error in the reads.

Enzyme assays for poPAG-2:

The concentration of total protein in the purified poPAG-2 preparation was estimated by BCA protein assay (Pierce Thermo Scientific, Rockford, IL). The amount of active enzyme within the protein preparation was obtained by titrating the active sites by using pepstatin A as described elsewhere (Copeland, 2000; Knight, 1995). Briefly, eight different concentrations of pepstatin A were incubated with 100 nM of total protein at room temperature for 30 min. A 'reaction mixture' consisting of 20 μM substrate in 100 mM sodium citrate-citric acid buffer, pH 4 with100 mM NaCl was prepared and dispensed into multiple wells in a costar black 96-well microtitre plate. The reactions were then incubated at 37˚C for 5 min (pre -heated) in a Synergy-HT fluorescent plate reader (Bio-Tek, USA) prior to the addition of the enzyme-pepstatin mixture. The kinetic readings for the first 10 min of the reaction were obtained in the plate reader by using 320/20 and 380/20 absorption and emission filters. The initial velocities from the reads were determined and the data was fitted by nonlinear regression analysis to the following equation to obtain the k_i of pepstatin A for poPAG2 (Neumann et al., 2004):

$$v = (vo/\,2Eo)\ \{1- [(Eo + Io + Ki)-(Eo+Io+ Ki)^2 -(4EoIo)]^{0.5}\}$$

For the determination of kinetic parameters and to facilitate comparisons with other PAG family members (manuscript in preparation), the reactions were performed in conditions similar to those employed for determination of kinetic parameters for boPAGs (100 mM sodium citrate-citric acid buffer, pH 4.0, 100 mM NaCl, at 37˚C). The assays were performed with two different concentrations of active enzyme (7.5 nM and 10.0 nM) and six different concentrations of the cathepsin D/E - Fret substrate (2.5, 5, 7.5, 10,

20 and 40 μM). The substrate was thoroughly mixed with the reaction mixture (described above) and the reactions were set up in duplicate for each data point. The reaction mixtures were placed in a 96 well costar black round bottom microtitre plate and preheated to 37˚C for 10 min, prior to the addition of enzyme (7.5 nM and 10 nM). The resulting fluorescence emitted was measured by the Synergy HT plate reader by using 320/20 and 380/20 emission and excitation filters. The initial velocities were calculated from the first 10 min of the kinetic reads. The K_M and V_{max} values were evaluated from the intercepts and slopes of double-reciprocal plots of rate (μM/min^{-1}) *versus* substrate concentration (μM), by using curve fitting software (GOSA-fit). The k_{cat} value was calculated from the equation $V_{max} = k_{cat}$ [E]; where [E] corresponds to the amount of active site titrated enzyme (Yasuda et al., 1999).

Results

Evolutionary relationships of porcine PAG gene family:

The evolutionary relationships of poPAGs to ruminant PAGs, PAG-like proteins or pepF and the APs, cathepsin D and pepsinogen A revealed an expected branching pattern, where the two poPAGs were clustered together as a small group separated from ruminant PAGs. The analysis also revealed that they are more closely related to PAGs than to the PAG-like proteins (equine PAG and mouse PepF) and other APs such as cathepsin D and pepsinogen A. In addition, even though the two poPAGs grouped together, there were relatively long branch-lengths in the poPAG-1 and -2 pairing, demonstrating that they are rather divergent from one another (Figure -33). Based on this

analysis, it is clear that even though the poPAGs are related to the ruminant PAGs, they constitute a rather unique grouping within the family that warrants further attention.

Expression and purification of recombinant poPAG-2:

The recombinant form of poPAGs-2 was expressed in a Baculovirus insect cell expression system. The recombinant proteins were expressed with a FLAG peptide at the N-terminus that allowed for affinity purification of fusion proteins by using an anti-FLAG- M2 antibody matrix. The bound proteins were eluted from the affinity column by using a high salt buffer that yielded a full length PAG protein. The expressed full length proteins following purification were of the expected molecular weight. The expressed PAG was identified by western blot by using both an anti-FLAG monoclonal antibody and a polyclonal anti-serum for poPAG-2 (Figure -34).

Estimating optimum pH profile for activity studies:

The optimal pH for maximal activity for poPAG-2 was found to be around pH 3.5 (Figure -35) based on the initial rates obtained from the experiment. Although it had maximal activity at pH 3.5, approximately 76% of the activity was retained at pH 3.0 and 65% at pH 4.0. It had very low activity at pH 2.5 (19%) and the activity was nearly undetectable at pH 6.0 (3%). The optimal pH of 3.5 for poPAG-2 was consistent with pH preferences of most other aspartic peptidases (Davies, 1990; Dunn, 2002; Kay and Dunn, 1992; Szecsi, 1992).

pH stability of recombinant poPAG-2:

To investigate the stability of poPAG-2 under various pH conditions, the proteins were incubated for extended periods of time (2 h) at 40˚C in various buffers before addition of substrate to record remaining peptidase activity, if any. In these experiments, the optimal pH for poPAG-2 shifted from 3.5 to 4.0, suggesting that poPAG-2 may be stable at pH 4.0, when compared to pH 3.5 following extended periods of incubation (Figure -36). It was also noted that the activity of poPAG-2 improved substantially in the buffers with pH ranging from 4.5 to 6.0 relative to the activity seen at pH 4.0, suggesting a possibility that much of the zymogen may have become activated within this timeframe.

Kinetic parameters of recombinant poPAG-2:

The Michaelis-Menten kinetic parameters k_{cat}, K_M and k_{cat}/K_M and the K_i for pepstatin A were determined and listed in Table -5. The kinetic parameters revealed that poPAG-2 is a relatively robust enzyme with a k_{cat}/K_M of $1.2 \times 10^{-6} \pm 485$ $M^{-1}s^{-1}$. It displayed a turnover rate of 9.49 ± 0.01 s^{-1} and had a K_M of 7.66 ± 0.03 μM (Figure -37 and Table -5). The K_i for pepstatin A was calculated to be 12.5 nM (Table -5).

Discussion

The pregnancy-associated glycoproteins (PAGs) in the placenta of the domestic pig make up a simple group, with only two clearly distinct PAG genes, poPAG-1 and -2 compared to the complex and heterogeneous grouping found in cattle and other ruminants. In those species, dozens of PAG genes have been identified and characterized in the placenta (Green et al., 2000; Green et al., 1998b; Wooding et al., 2005). Based on phylogenetic studies, the PAGs belong to the AP family of genes (Green et al., 1998b;

Hughes et al., 2003; Xie et al., 1997b; Xie et al., 1991). Interestingly, several members of the family have been shown to have unusual residues in and around the catalytic site that are predicted to preclude enzymatic activity in these PAGs (Green et al., 2000; Guruprasad et al., 1996; Xie et al., 1997b). Other PAGs have all the hallmarks of typical active APs. However, peptidase activity has yet to be shown for these potentially active peptidases. Therefore, the goal of this work was focused on determining if poPAG-2 was capable of acting as a peptidase and, if so, to perform an analysis of its kinetic characteristics. Since poPAG-2, is the only candidate that possesses characteristics of an active peptidase, we chose to express poPAG-2 as a recombinant protein and investigate its activity and kinetic parameters.

A baculovirus insect cell expression system was used to express a recombinant form of poPAG-2. This system was particularly selected because; it has proven its ability to express soluble, functional PAGs. The PAGs were expressed as a fusion protein with an N-terminal 'FLAG-tag'. The presence of the FLAG-peptide allowed for monitoring the expression of proteins as well as purification of poPAG-2 to homogeneity with antibodies specific to the epitope. The purified proteins of poPAG-2 were found to be proteolytically active against a synthetic fluorescent substrate, MOCAC-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2. This peptide was originally designed as a substrate for cathepsins D&E (Yasuda et al., 1999). Perhaps, the activity against this substrate could be explained by the fact that the backbone sequence for this substrate was derived from a general substrate consensus sequence for APs [Lys-Pro-Ile-Gln-Phe*Nph-Arg-Leu (Nph: nitro-phenylalanine)] described elsewhere (Beyer et al., 2005). We noticed that, against this substrate, poPAG-2 had maximal activity at around pH 3.5. This

pH optima was not surprising given that most APs have an acidic pH optima. However, following incubation in various buffers for 2 h prior to analysis, we noticed that poPAG-2 activity was more prominent at pH 4.0. When the kinetic parameters for poPAG-2 in pH 4.0 buffer at 37˚C were calculated against this substrate, it was apparent that poPAG-2 is a relatively robust enzyme with a k_{cat} of 9.49 s^{-1} and k_{cat}/K_M of approximately 1,238,903.394 $M^{-1}s^{-1}$. In addition, the estimated K_i for pepstatin was found to be 12.5 nM. These kinetic parameters, when compared to cathepsin D, a canonical aspartic peptidase, against the similar substrate, revealed that cathepsin D had approximately an order of magnitude higher activity than poPAG-2 with a k_{cat}/K_M of 15,600,000 $M^{-1}s^{-1}$ and a turn-over rate of 57.8 s^{-1} and K_M of 3.3 μM (Note: 50 mM sodium citrate buffer, pH 4.0; at 40˚C were the reaction conditions for those studies (Yasuda et al., 1999).

The placenta in swine is a simple epitheliochorial type, where the trophectoderm is simply apposed and attached to the uterus with no invasion into maternal uterine tissues (King, 1993). However, in cattle, there are focal points of invasion and fusion of an invasive cell type of trophoblast called ‘binucleate cells’ with maternal endometrium (Wooding, 1992; Wooding, 1982b, 1982a). Since, the binucleate cells are the source of many PAGs, the invasion into the maternal uterus results in the eventual accumulation of PAGs in the maternal circulation (Green et al., 2005; Lee et al., 1986b). The PAGs in cattle are, therefore, positioned to perform a potentially systemic role. In swine, since there are no invasive cell types in the placenta and no reports of invasion into the maternal uterus, it is unlikely that the poPAGs enter maternal blood. However, porcine PAGs, especially poPAG-2, has been found to accumulate at the maternal-fetal interface (i.e. at the microvillar junction between trophoblasts and the uterine epithelia) and this

location is likely to be where porcine PAGs function (Majewska et al., 2006; Wooding et al., 2005).

A multifaceted role for poPAG-2 proteolytic activity could be envisioned either within the secretory pathway of trophoblasts or at the maternal-fetal interface. During the transit via the secretory pathway, poPAG-2 is likely exposed to a pH as low as 5.2 (Paroutis et al., 2004). This pH might allow for maturation of the poPAG-2 zymogen by an intra- or intermolecular mechanism. Indeed, many APs are capable of removing their own propeptide (or that of a neighboring AP), under acidic conditions, thereby exposing the peptide-binding cleft for interaction with substrates (Beyer and Dunn, 1996; Davies, 1990; Dunn, 2002; Koelsch et al., 1994). Alternatively, poPAG-2 may become activated by an as yet unknown pro-protein convertase in a manner similar to other proteins (Seidah and Prat, 2002; Taylor et al., 2003). The activated PAG-2 could subsequently cleave propeptides from other poPAGs or may become involved in maturation of other candidate precursor proteins.

Once activated, poPAG-2 has been proposed to be involved in degradation of the microvillar surface ('thinning of microvilli') on the endometrium, allowing greater surface area for apposition and attachment by the placenta (Szafranska and Panasiewicz, 2002). Alternatively, poPAG-2 might increase the bioavailability of trophic factors by cleaving their binding partners/inhibitors, thereby promoting such diverse mechanisms as angiogenesis, growth of the placenta, etc. A discrete plausible alternative is that, poPAG-2 may be functioning as a degradative enzyme. The poPAG-2 may become rerouted during secretion to endosomes or it may be actively endocytosed back into the trophoblast along with the uterotroph, which is the principal source of nutrition for the

growing fetus (Bazer et al., 1984). The PAGs in the endo-lysosomal pathway could then function in proteolytically digesting the nutrients. Finally, since there are only a few closely related proteolytically active PAGs (the poPAG-2 subfamily) in the pig, poPAG-2 might be an ideal candidate to help to dissect PAG peptidase function during ungulate pregnancy.

Chapter –IV figures

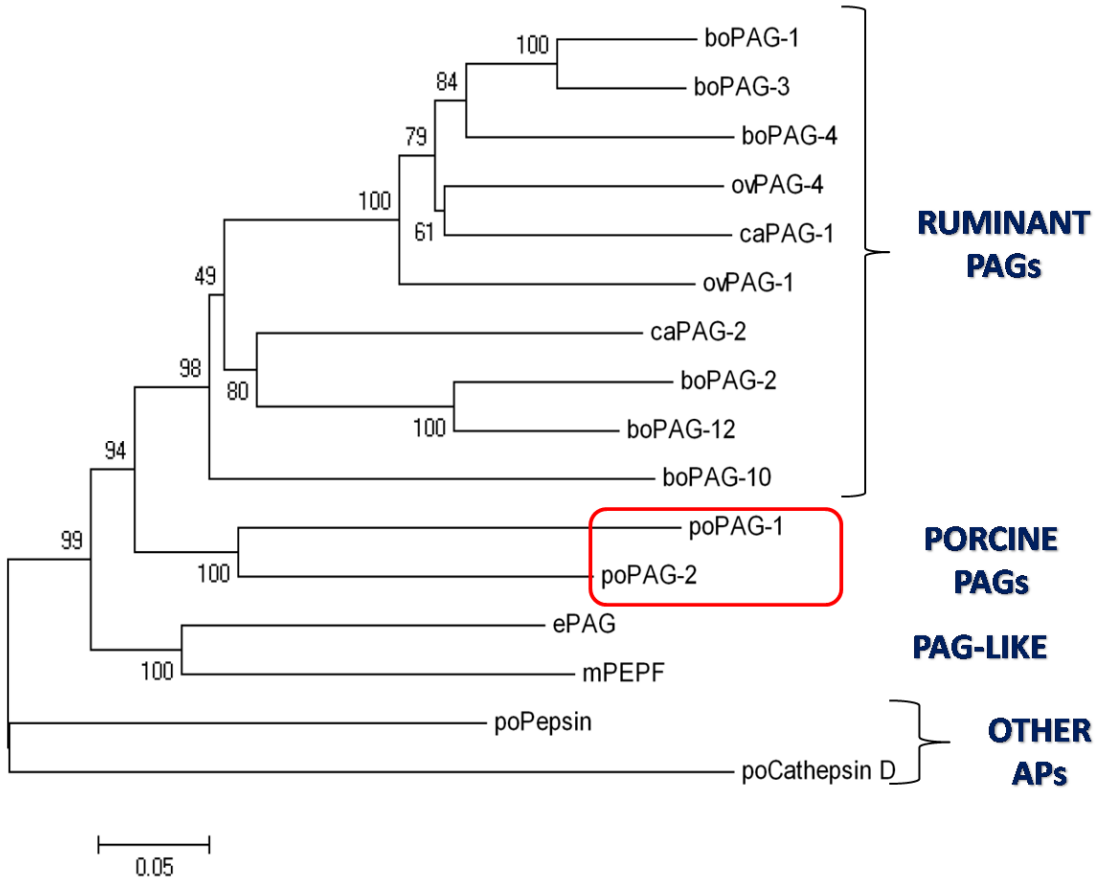


Figure -33. Evolutionary relationships of poPAGs, ruminant PAGs, PAG-like proteins and other APs:

The phylogenetic relationships of poPAGs with other PAGs, PAG-like molecules and aspartic peptidases (APs) were computed based on amino acid sequence by Minimal evolution method and were displayed in this figure. Notice the clear branching and separation of ruminant PAGs (bo: bovine; ca: caprine; ov: ovine) and PAG-like proteins (ePAG: equine PAG and mpepF: mouse pepsinogenF). Also notice that the two poPAGs are clustered together, and they are found branching from PAGs rather than PAG-like proteins. A APs, porcine pepsin (poPepsin) and cathepsinD (poCathepsinD), can be seen branching out as outliers.

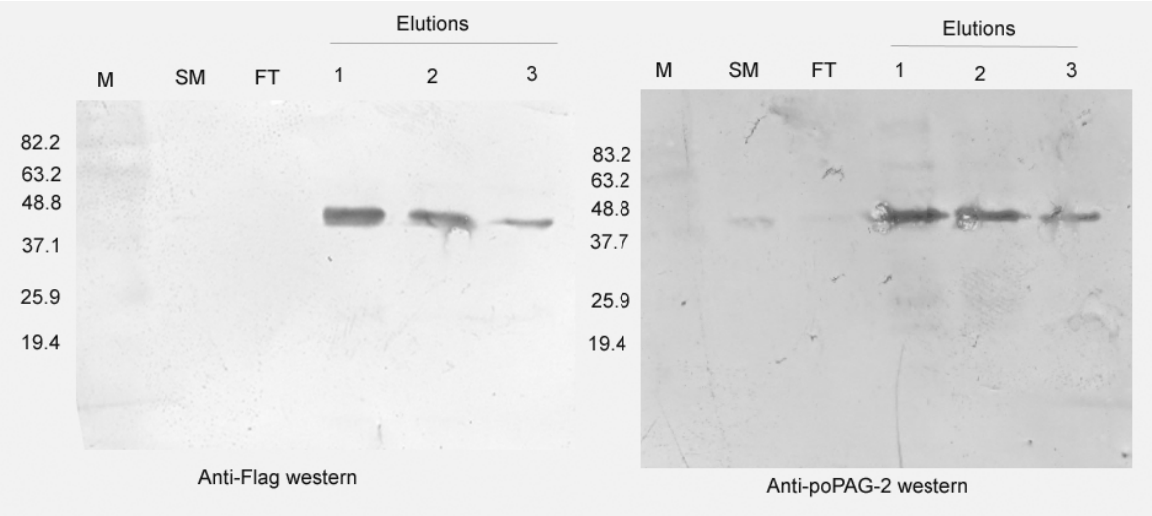


Figure -34. Western blot images of the transferred proteins from identical SDS-PAGE gels immuno-blotted with anti-flag monoclonal (figure 34a) and anti-poPAG-2 polyclonal antibodies (figure 34b), respectively. Lane-1: represents the total proteins from the lysed poPAG-2 pellet. Lane-2: Flow-through of lysate from the anti-flag column. Lanes 3-5 represents respective elution fractions of recombinant poPAG-2 from the column in numerical order.

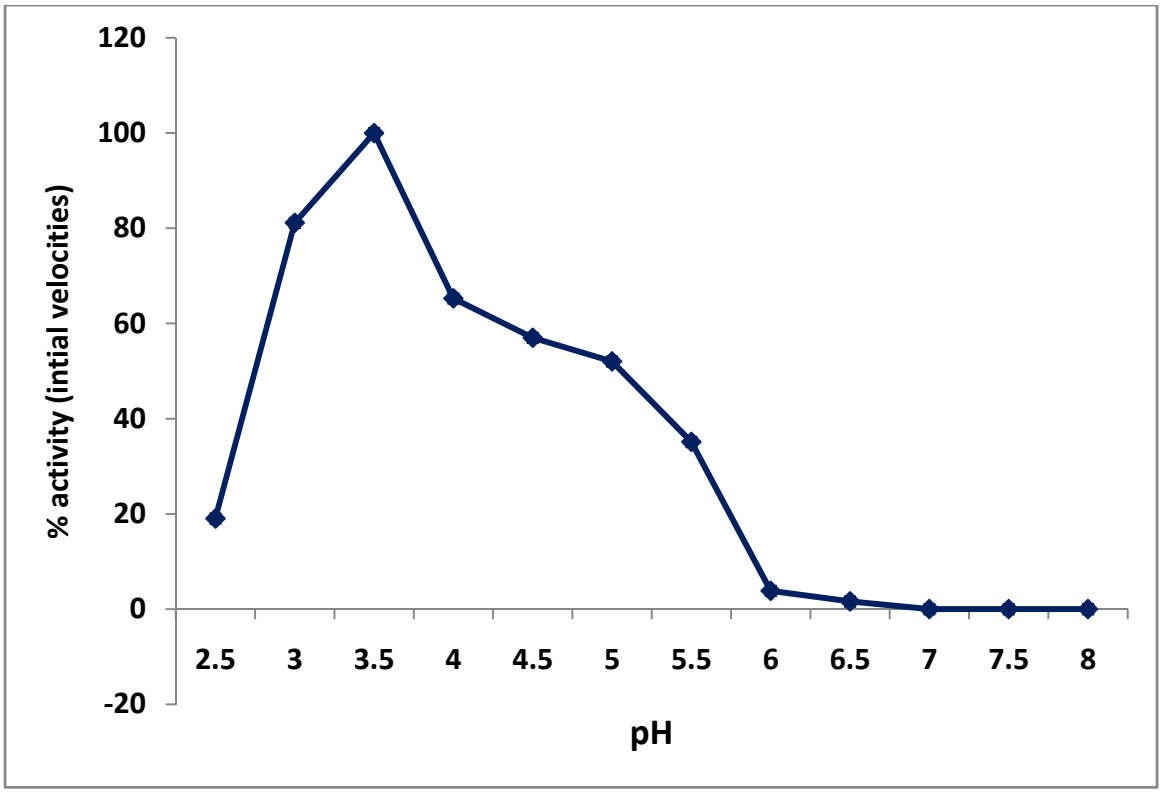


Figure -35. A graph showing the pH activity profile for recombinant poPAG-2. The initial rates obtained from cleavage of the fluorescent substrate were transformed. The highest activity observed was set at 100% and remaining activities were adjusted relative to it.

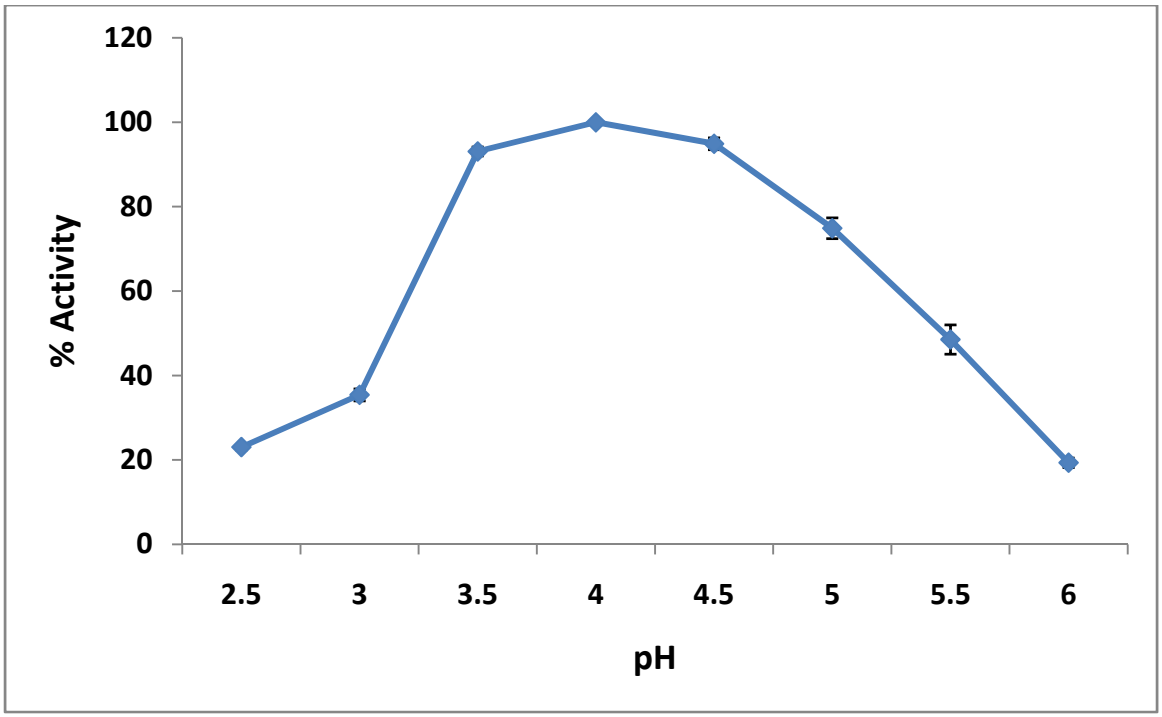


Figure -36. pH stability for recombinant poPAG-2: The graph represents the stability of recombinant poPAG-2 in pH conditions and buffers (indicated in the picture). The protein was incubated in the different buffers, as indicated in the graph, for 2 hrs at 40˚C. The substrate was then added to the incubated mixture and assayed at the same temperature. The values obtained were transformed into % activity.

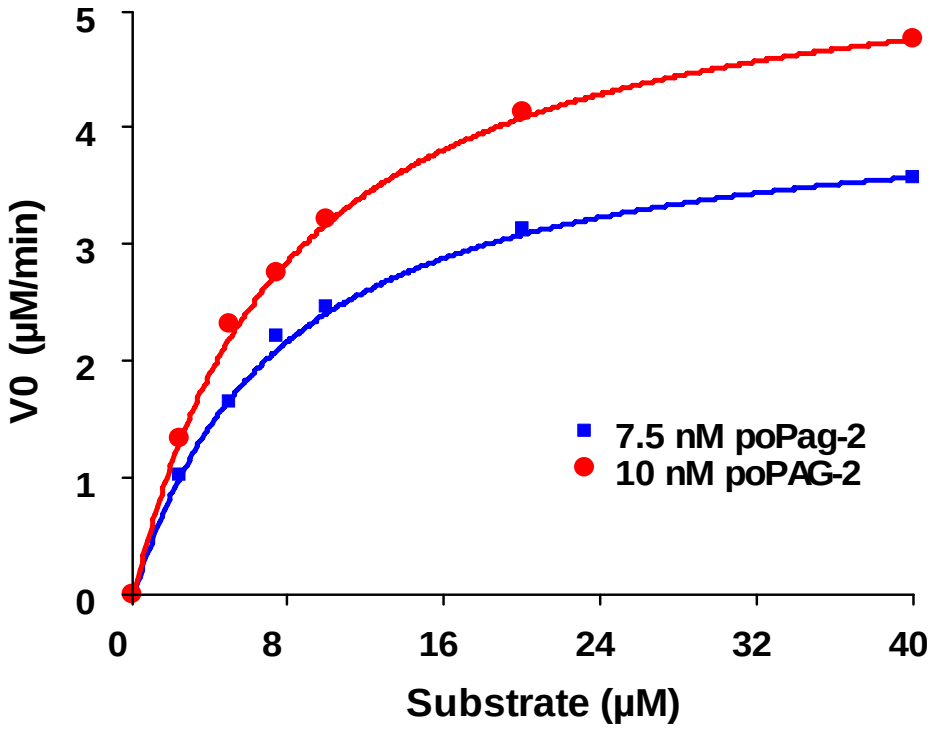


Figure -37. Estimation of kinetic parameters for poPAG-2 (k_{cat}, K_m, and k_{cat}/K_m) by global fitting. The concentrations of substrate (µM) used in the experiments are shown on the X-axis, the relative rates obtained (µM/min) are displayed on the Y-axis. Two different concentrations of the enzyme 7.5 nM and 10 nM, were used in the assay. The resulting activity achieved against these concentrations of the enzyme were shown in the figure.

Table -5: Respective kinetic parameters for poPAG-2 obtained from global fitting of initial rates and substrate concentrations.

PAG	Km (µM)	k_{cat} (s^{-1})	k_{cat}/Km ($M^{-1}s^{-1}$)	Pepstatin K_i (nM)
popag-2	7.66±0.032	9.49 ±0.016	1,238,903.394 ± 485	12.5

Chapter -V

Acid peptidase activity as a marker for predicting developmental competence of *in vitro* produced porcine embryos

Abstract

The ability to efficiently and reproducibly create high quality embryos, competent in producing normal viable offspring *in vitro*, facilitates diverse technological advancements in animal agriculture, assisted reproduction, etc. Unfortunately, the current *in vitro* culture methods are largely ineffective in creating good quality embryos consistently. While much of the impetus was laid on improving the conditions and techniques for efficient culture *in vitro*, a better system for objectively assessing the quality of the produced embryos needs similar attention. The current methods for evaluation of the embryos are predominantly based on morphological characteristics which are prone to potential bias of the individual scorer. As an alternative, metabolic and genetic markers have been explored for quality assessment, but, they are cost prohibitive or require longer periods of time for evaluation of the embryos, respectively. Therefore, an ideal marker has long been sought that is endowed with the following traits: (a) the marker can act as a predictor of embryo quality (b) it is released into the culture media to permit ready access and measurement of the marker, (c) it is easy to identify, and most importantly (d) it is economical to measure. We hypothesized that secreted proteins - especially acid peptidases that are known or suspected to be produced and secreted by the developing embryo - can fulfill the above criteria. In this report, we

provide evidence that the medium conditioned by the culture of porcine embryos either individually or in pools for variable lengths of time possess acid peptidase activity (APA) that can be readily detectable via the use of sensitive fluorescent substrates. We also observed that such activity correlated with stage and quality of embryos (assessed morphologically) *in vitro* and, therefore, could serve as a marker by itself or in combination with other evaluation methods. Future efforts will be directed at identifying the source of the APA in embryos as well as verifying if the observed APA can be correlated with subsequent development *in vivo*.

Introduction

It is generally not economical or feasible to routinely use embryos of *in vivo* origin for experimentation. Therefore, the advent of culture methods that permit the production and growth of embryos *in vitro*, facilitated widespread usage of these methods (Gardner and Lane, 1998; Schoolcraft et al., 1999). For example, many assisted reproductive technologies including ICSI, embryo culture out to the blastocyst stage, embryo transfer, as well as fertilization with normal or X- or Y-sorted sperm, etc (Greve and Callesen, 2004; Prather et al., 2003), make use of *in vitro* matured oocytes and cultured embryos. However, *in vitro* produced embryos are known to be developmentally delayed and/or less competent when compared to their *in vivo* counterparts (Armant, 2005; Machaty et al., 1998a; Machaty et al., 1998b; Wheeler et al., 2004a; Wheeler et al., 2004b). There are several factors that contribute to the poorer quality of *in vitro* cultured embryos, a few of these include, poorer gamete quality (Balaban et al., 2001; Sirard et al., 2006; Urman et al., 2002), ineffective reprogramming following fertilization, culture

medium composition (Vanroose et al., 2001), culture techniques (Behr and Wang, 2004; O'Neill, 1991), etc. Consequently, there is significant variation in the number as well as the quality of embryos derived - not only between different laboratories, but between different replicates with in a particular laboratory. Much of the experimentation involving embryos, such as transfer of embryos into surrogates, are most effective when developmentally-compentent/high-quality embryos are used. While the generation of high quality embryos *in vitro* presents one important challenge, the ability to objectively assess the quality of the resulting embryos presents another.

An additional option for grading or evaluation of embryos will result in significant improvement in the utility as well as the reliability of the *in vitro* produced embryos. Several strategies are currently in place to predict embryo quality. One of the most prevalent techniques is the grading of blastocyst stage embryos based on morphological parameters according to the criteria proposed by Dokras et al.(Dokras et al., 1993) or by Gardner et al.(Gardner et al., 2000). A similar morphology-based grading system was extended to embryos on day 4 or 5 of their development by Alikani et al. (Alikani et al., 2000). Although, there have been many studies that correlate embryo morphology with embryo quality, evaluation based on these methods is still prone to potential bias (Hoelker et al., 2006; Zaninovic et al., 2001). Other methods for evaluation, involve genetic screening for detecting defects associated with aneuploidy. Such screens are effective and useful. However, they require a biopsy of the embryo, which can result in loss of viability, and a longer duration in culture for follow-up evaluation (Derhaag et al., 2003; Magli et al., 2000).

Clearly, an additional method for evaluation of embryo quality would have utility, particularly if the method utilizes a non-subjective marker, preferably a biochemical or metabolic one that can be readily assayed without undo manipulation of the embryo itself (e.g. something released into the culture medium). The detection of such marker can be used either alone or in combination with the current morphological evaluation systems. The search for such an elusive marker(s) has already begun. Lopes et al., developed an 'Embryo respirometer' for determining the respiration rates of the embryos which has been found to have positive correlation with the quality of embryo (Lopes et al., 2007). Lane and Gardner studied glucose consumption and subsequent lactic acid production in the medium as a predictor of embryo quality (David et al., 2001). Jurisicova et al., reported that SP1 (pregnancy specific β-1 glycoprotein) secreted into the conditioned media had a significant correlation with embryo cell number (Jurisicova et al., 1999). However, follow up studies on SP1 showed conflicting results (Saith et al., 1996). Alternatively, besides SP1 protein, there are several other proteins that are actively secreted into the surrounding environment; one such class of proteins is the proteolytic enzymes (peptidases). Peptidases play crucial roles during embryo development. For example, the extracellular matrix degrading peptidases such as urokinase plasminogen activator (uPA) and matrix metalloproteinase -9 (MMP-9) promote blastocyst invasion (Aflalo et al., 2005; Eliahu et al., 2004; Salamonsen, 1999), an implanation specific serine proteinase (ISP1)(Colleen M. O'Sullivan, 2002; O'Sullivan et al., 2001) and Hepsin, a membrane associated serine protease are involved in hatching of the embryo (Vu et al., 1997).

Another peptidase suspected to be secreted from trophoblasts of pig embryos is porcine pregnancy-associated glycoprotein-2 (poPAG2). The message for poPAG2 was shown to be transcribed in porcine embryos as early as the 4-cell stage (Do et al., 2001). It is an aspartic peptidase (AP) and a recombinant form of the protein was shown to be capable of cleaving a synthetic fluorescent Cathepsin D/E- FRET substrate with an activity optima of pH 4.0 (described in chapter-III). It is also known to be secreted from trophoblasts of the placenta (Szafranska et al., 2001; Szafranska and Panasiewicz, 2002). We hypothesized that this trophoblast marker would accumulate in media during embryo culture and, therefore, could be detected based on the presence of acid peptidase activity (APA) in embryo conditioned media.

The principle objective of the current study was to assay for acid proteolytic activity in media conditioned by the culture of embryos and, if such activity is present, to determine if any correlation exists between the amount of APA released and the developmental competence of embryos based on their morphological parameters.

Materials and Methods

Oocytes and IVF:

Gilt-derived ovarieses were acquired from the Premium Standard Farms slaughter facility in Milan, MO. The oocytes were aspirated from 3-5mm follicles and matured in TCM 199 (Gibco BRL) maturation medium which was supplemented with 0.1% PVA (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 75 µg/mL penicillin G, and 50 µg/mL streptomycin (Sigma, MO, USA). To the above medium, freshly prepared components such as 0.57 mM cysteine, 0.5 µg/mL luteinizing hormone, 0.5 µg/mL

follicle stimulating hormone, 10 ng/mL epidermal growth factor, were added just before use. Fertilization was performed according to the method described elsewhere (Lai and Prather, 2003). Briefly, following maturation of the oocytes in the maturation medium, the oocytes were stripped of their cumulus cells by using hyaluronidase according to previously published methods (Lai and Prather, 2003)-. The cumulus-free oocytes were washed three times in IVF medium (a modified Tris-buffered medium (mTBM) containing 2 mg/mL BSA and 2 mM caffeine). Following which, approximately 30-35 oocytes were transferred into 50 μL droplets of IVF medium covered with mineral oil that had been equilibrated for 24 hr at 38.5°C in 5% CO_2 in air and incubated until sperm were added for fertilization. The semen for IVF was obtained from a proven boar known to produce low polyspermy rates. For IVF, one 0.1 mL frozen semen pellet was thawed at 39°C in 10 mL of sperm washing medium [Dulbecco's phosphate-buffered saline (dPBS; Gibco) supplemented with 1 mg/mL BSA (pH 7.3)]. After washing 2 times by centrifugation (1900 × g, 4 min), the pelleted spermatozoa were resuspended with fertilization medium (Hao et al., 2007; Hao et al., 2006) to a concentration of 2×10^6 cells/mL. Fifty μL of the sperm sample were added to the fertilization droplets containing the oocytes, giving a final sperm concentration of 1×10^6 cells/mL. Oocytes were co-incubated with sperm for 6 h.

Embryo Culture:

Six-hours post-insemination, the oocytes from fertilization droplets were washed 3 times and pools of 50 zygotes were cultured in 500 μL of PZM3 embryo culture medium in 4-well Nunc dishes and incubated in a humidified atmosphere at 38.5°C, with

5% CO_2 in air. The base formulation of PZM3 embryo culture medium used in the experiment was: 0.1% polyvinyl alcohol, 108 mM NaCl (Sigma S5886), 10 mM KCl (Sigma P4504), 0.35 mM KH_2PO_4 (Sigma P5655), 0.40 mM $MgSO_4*7H_2O$ (Sigma M1880), 25.07 mM $NaHCO_3$ (Sigma S8875), 0.20 mM Na-Pyruvate (Sigma 4562), 2.0 mM Ca-$(lactate)_2*5H_2O$ (Fisher C110-500), 1 mM L-Glutamine (Sigma G7029), 5.0 mM Hypotaurine (Sigma H1384), 20 mL/L BME amino acids solution (Sigma B6766), 10 mL/L MEM (non-essential amino acids solution) (Sigma M7145), and 0.05 mg/mL Gentamicin (GibcoBRL 15710-064). Following 28 hrs in culture, embryos that progressed to cleavage were selected and approximately 20 embryos were placed in a droplet of 25 µl PZM3 medium in a Corning (60x15) suspension culture dish. The embryos were incubated in a humidified atmosphere with 5% O_2, 5%CO_2 and 90% N_2 at 39°C and cultured in pools for different periods of time based on the experimental design described below. The conditioned medium was harvested and analyzed for acid peptidase activity.

Single embryo cultures:

In addition to culturing the embryos in pools, individual embryos were also subjected to culture in small droplets of PZM-3 medium. Briefly, multiple droplets of PZM-3 medium were deposited in a total volume of 1.5 µL onto a Corning suspension culture dish and overlaid with paraffin-oil. The culture dishes were then placed in a humidified incubator and equilibrated in an atmosphere of 5% O_2, 5%CO_2 and 90% N_2 at 39°C. Dependent on the experimental design, individual embryos at various developmental time points (day 3, 4, 5, 6) were isolated from cultured embryo pools,

placed into 1.5µL PZM-3 medium droplets and cultured for defined time. Note that, although long-term culture of single embryos was found to be detrimental for development (Vajta et al., 2000), we have not identified such effects, particularly when the single embryos were cultured in small volumes (Appendix -II).

Measurement of acid peptidase activity (APA):

Proteolytic activity in the embryo conditioned media was estimated at either pH 3.5 or 4 depending on the experimental design discussed below. The standard reaction mixture consisted of 0.05M citric acid-sodium citrate buffer (pH 3.5 or 4.0), 100mM NaCl and 5µM of substrate. We utilized a commercial synthetic fluorescent FRET-substrate, MOCAC-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2 (Peptides International, KY, USA) to measure the proteolytic activity. This fluorescent decapeptide was derived from a consensus polypeptide sequence for aspartic peptidases, which are the predominant class of proteolytic enzymes capable of operating under these pH conditions. Since, the substrate was initially designed and tested for optimal cleavage by Cathepsin D and E it was referred to as FRET-cathepsin D/E substrate (Yasuda et al., 1999b). Following addition of the components, the total volume of the reaction was adjusted to 100 µL by addition of sterile filtered double distilled water and the resulting reaction mixtures were incubated in a water bath at either 37˚C or 40˚C depending on the experiment. Following incubation (specific incubation times differed depending on the experimental paradigm), the reactions were terminated by addition of 100 µL of 10% TCA. The resultant 200 µL mixture was split equally into two wells (for technical duplicate reads) in a 96 well Costar black plate (Corning, USA) and fluorescence of

liberated product was read by using a Synergy-HT fluorescent plate reader (Biotek, USA) at 320/20 and 380/20 excitation and emission filter settings.

Experimental design:

Experiment-1: Assessing APA in conditioned medium obtained from day7 embryos cultured in pools:

To determine if there was any APA in conditioned media of embryos in culture, a pool of 20 cleavage-stage embryos were placed in a total volume of 25 µL of PZM-3 medium and incubated for 7 days. Of the 20 embryos, the number of embryos that developed to the blastocyst stage were recorded and removed. The remaining 'embryo-conditioned' medium (25 µL) was collected and tested for APA. Equal amounts of fresh PZM-3 embryo culture medium, which was not used for embryo culture, was also tested in the assay to establish base-line/background fluorescence for the assay. The reactions were performed at 37˚C for 3.5 hrs and the resulting readings were tabulated.

Experiment-2: Inhibition of APA in the embryo conditioned medium by pepstatin-A, but not by other class of inhibitors:

In light of the reaction conditions employed, the APA was assumed to be arising from an aspartic peptidase. To test this prediction, the medium obtained from a pool of embryos and fresh PZM-3 medium was incubated with inhibitors against different classes of proteolytic enzymes. The inhibitors were 0.4 mM Pefabloc SC-AEBSF (serine peptidases)(Roche Applied Science), 5 µg/mLAprotinin (serine peptidases), 10 µM E-64 (Cysteine peptidase), 1 mM EDTA (Metallo peptidases) (Sigma, MO, USA). The

medium pools were then split into two groups each, with one of the two groups being inhibited with 1 mM pepstatin A (Sigma, MO, USA) and the other group with equal amounts of the carrier DMSO (Sigma, MO, USA). The resultant mixture was incubated at room temperature for 30 min, following which, the different groups were tested for acid peptidase activity in pH 4 buffer at 37˚C for 3.5 h. The resultant net fluorescent values obtained from reading in a fluorescent plate reader were tabulated.

Experiment-3: Assessment of APA in d7 blastocyst-conditioned medium after 24hrs in culture obtained from one, two or three-embryo cultures:

In this experiment we sought to identify whether conditioned medium from 24 hr embryo cultures contained APA. The media samples were obtained from the culture of one, two or three embryos per droplet. The goal of this experiment was to determine if embryo culture for only one day would generate enough APA to be detectable. Pools of embryos were cultured in PZM3 medium until d6 and the development of the embryos to blastocyst was assessed. Those embryos that cavitated, and showed signs of blastocyst formation were collected and washed three times in fresh PZM3 medium. Blastocysts were then isolated and placed individually or in groups of two to three in 1.5, 3 or 4.5 µL droplets of culture medium, respectively, and cultured for an additional day. Individual droplets of 1.5, 3 or 4.5 µL were left empty and co-cultured in the same dish. These served as controls. On d7, the blastocysts were removed from the culture medium and assessed for quality. The corresponding conditioned media in addition to the unconditioned control media were tested for acid peptidase activity by incubating in pH 4 buffer at 37˚C for 3.5 hrs.

Experiment-4: Estimation of total APA in conditioned medium of solitary d7 blastocysts in culture for 1day and comparison to multiple unconditioned medium droplets.

There was clearly a range in APA arising from individual embryos in the experiments shown in Figure 3. As an extension to experiment 3, eight blastocysts were obtained from pools on d6, washed three times in fresh culture medium and cultured individually in 1.5μL droplets for 24 h in standard conditions. In order to determine if the variations observed between different blastocyst media samples were truly reflective of genuine biochemical differences in secreted APA or if they fall within the normal variation associated with the experiment, 11 control droplets were set up along with the embryo conditioned droplets and also assayed for APA as described above on d7. The assessment of activity from multiple control droplets served two important purposes, first, it allowed for estimation of the general variation associated with the experiment. This variation was reflected as standard deviation (STD) from the control mean, which was used to estimate the coefficient of variation (mean/STD) associated with the assay. Second, it facilitated the determination of a true 'threshold' to define whether or not a particular sample was truly higher than the background activity with confidence. The calculation of such a threshold value is discussed below.

Experiment-5: Estimating the amount of APA in d6 conditioned medium of solitary embryos in culture for 2days:

This experiment was performed to verify if enough product would accumulate in two days of single-embryo culture in order to determine if APA could be assessed on d6 instead of d7 – a time that is more amenable to embryo transfer. In these experiments, multiple embryos were cultured in pools until four days post-fertilization. Individual embryos were then removed and cultured for an additional two days along with "empty" medium droplets similar to the experiment described above. On d6, the embryos were removed and assessed for quality (described below), and the conditioned medium was tested for APA. From our experiments (results not shown) for determining optimal reaction conditions, we identified that performing the reactions at pH 3.5 and 40˚C for 2 h was most optimal. Therefore, these slightly different reaction conditions were adopted for this experiment as well as subsequent ones.

Experiment-6: Determination of the amount of APA in conditioned medium of single d4 embryos on two successive days of culture (d5 and d6):

To investigate whether the APA could be observed earlier in development and to determine if this activity correlates to the stage and quality of embryos, single embryo cultures were set up on d4 and the conditioned medium, following 24 h in culture, was assessed for APA on two successive days. To do this, embryos were cultured in pools until day 4. Forty-four individual embryos were isolated, 'rolled' (washed) in fresh culture medium to minimize carryover of residual APA, and placed in a fresh individual droplet of 1.5 µL culture medium and cultured for one day. On d5 the embryos were

removed, assessed for their stage and quality according to the modified standard IETS guidelines:

Stage: **-3**: 4-7cells; **-2**: 8cells; **-1**: 16cells; **0**:morula; **1**: early blastocyst; **2**: mid-blastocyst; **3**: blastocyst; **4**:expanded blastocyst.

Quality score; **1**: perfect; **2**: very good (<50%cells dead); **3**:good (> 50% cells dead); **4**: dead.

After scoring the embryos were then placed in a fresh droplet for an additional day of culture. The conditioned medium obtained on d5 was named 'd4-5 medium'. After the second 24 h culture period the scoring and media harvest was repeated. The conditioned medium obtained on d6 was referred to as 'd 5-6 medium'. Ten control droplets of medium (with no embryos) were co-cultured in the same dish and the control droplets collected on each of the respective days were assayed alongside the conditioned media samples. The raw fluorescent values obtained from individual embryos from two different experiments were transformed as to fold change relative to the mean of the control media samples. The experiment was performed twice.

Experiment-7: Determination of the amount of APA in conditioned medium of individual d3 embryos on two different days of culture (d5 and d6):

This experiment was a variation of experiment 6. The experimental design is very similar with the exception that, instead of initializing the individual embryo culture on day 4, the experiment was initiated a day earlier, on day 3. The cleavage-stage embryos were cultured for 48 hrs and the resulting conditioned medium was referred to as 'd3-5 medium'. Subsequently, the embryos were cultured for an additional 24 hrs to obtain

'd5-6 medium'. Again, the embryos were assessed for quality and stage of development on both days 5 and 6. The experiment was replicated with 55 embryos in the first experiment and 33 embryos in the second experiment. The assays were performed and analyzed as described above.

Statistical analysis:

Unpaired t-tests were performed to determine if there were significant differences in mean APA between embryo-conditioned media and control droplet groups. The mean APA of the control media, plus three standard deviations, was used to establish a threshold background, above which one could confidently state that APA in the conditioned media samples were above background. In other words, any data point within the embryo group that had an activity above the threshold was considered significantly greater than background with ≥99% confidence. To assess if there was any significant correlation between embryo stage and quality on the observed peptidase activity, PROC GLM in SAS was used to estimate the type III sum of squares of the means and to determine the p-value.

Results

APA in conditioned media from d7 embryos:

There was a significant amount of APA in conditioned media from mixed-embryo culture after incubation for 7 days. The amount of product liberated which was reflected as net fluorescence emitted from reaction was at least 3-fold greater compared to the control unconditioned media samples. The experiment was repeated and results from both

experiments were represented in Figure-38. Note that, of the different pools tested, the amount of activity appeared to be a reflection of the number of embryos that developed to the blastocyst stage.

APA activity in d7 embryo conditioned medium was predominantly due to one or more aspartic peptidases:

The APA observed in the embryo conditioned medium was was not observed, when AP inhibitor, pepstatin A, but not inhibitors directed towards other peptidase classes was added to the medium. The inclusion of pepstatin A reduced the amount of product liberated by almost 90% compared to another aliquot of the sample, which had instead been treated with only the vehicle used for dissolving pepstatin A, DMSO (Figure -39). The total activity was diminished and the net fluorescence emitted was similar to that found in the reagent blank control. One notable observation was that there was activity observed in the control culture medium. This control activity was also diminished by pepstatin, suggesting that the residual activity in the control media could be due to a contaminating AP, probably emanating from BSA or some of the other components used to prepare the medium.

APA in single blastocyst or small groups of blastocyst cultures:

Based on the results shown in Figures 38 and 39, it was clear that APA in conditioned media from pools of d7 blastocyst cultures was readily detectable. An experiment was then performed to determine if APA could be detected in: 1, single embryo cultures, and 2, if 24 h was sufficient time to detect such activity. The experiment

was performed in a similar manner as those shown in Figures 38 and 39, except that embryos were removed on day 6 after fertilization, washed, placed in individual 1.5 uL droplets and cultured for another day. A few sets of two- and three-embryo pools were also incubated in a similar manner.

Several of the single embryo and embryo pools exhibited APA that was substantially greater than their corresponding control media samples (Figure -40, experiment-1). The amount of activity from the pools of two to three embryos was generally higher than the single blastocyst cultures, although such activity was not found to be additive. It is also worth noting that there was quite a bit of heterogeneity in this experiment. Although, several of the single blastocyst cultures displayed relatively high activity, there were others that failed to generate signal much greater than controls (Figure-40, experiments-1&2). These differences in activity between blastocysts, which are at similar developmental stages and morphology, suggested that the APA may serve as a biochemical marker to distinguish classes of morphologically similar embryos – possibly even as a way to predict developmental competence.

APA can be observed in d7 conditioned media after embryo culture for 24 hours:

To verify the results identified in the experiment above (where a few single blastocysts cultured in 1.5µL droplets for as little as 24 h could generate enough measurable APA), another eight blastocysts were isolated on d6 and cultured in individual 1.5µL droplets for 24 h along with 11 control medium droplets. On d7 the media were harvested and assayed for APA. The results from this experiment demonstrated that the mean APA obtained from the blastocyst conditioned medium

samples deviated significantly from the mean of the control medium ($p<0.001$) (Figure-41b). The activity observed in each individual blastocyst was significantly higher (denoted by *) when compared to the threshold value that was calculated by adding the average fluorescent readings obtained from control media and three standard deviations (Figure -41a). In addition, there were significant differences between individual samples within the embryo-conditioned media group with some (i.e. embryos #7 and 8) exhibiting almost twice the activity of others (i.e. embryo #1) (Figure -41a).

Experiment-5: APA in embryo conditioned medium of day6 embryos in culture for 2 days:

Pools of embryos were cultured until day 4, at which time single embryos were cultured individually for an additional 48 hours and the media was assayed for APA (Figure -42)**.** APA was clearly detectable and significantly higher than the threshold in some of the samples (Figure-42: denoted by an *), but was absent or very low in others. This biochemical difference in the samples was not clearly associated with embryo quality scores; i.e. several embryos that were morphologically indistinguishable from one another, exhibited markedly different secretion levels of acid peptidase released into the medium. This observation was similar to those from the previous experiments where there were differences in secreted APA among a cohort of embryos that were at similar developmental stages, suggesting that this difference in APA may reflect a biochemical difference between embryos that is not readily apparent by standard embryo scoring systems.

Experiment-6: APA in day 5 and day 6 conditioned media from single embryo cultures after 24 hrs of culture, beginning on day 4:

The conditioned medium obtained from single embryo cultures on day 5 and 6 were analyzed for APA. We noticed from these data sets that a considerable number of embryos displayed an APA that was significantly above the threshold (Figure -43a: denoted by a bold highlight). However, the average APA activity was much higher on day 6 (2.26 fold greater than the control mean) than on day 5 (1.62 fold greater than the control mean). These data suggest that the source of APA is released to a greater extent between days 5-6 compared to period between days 4-5. However, apart from comparisons between average APA, the coefficient of variation (mean/standard deviation), which is a reflection of the amount of variation within a population, deviated substantially between conditioned media and the control samples on d6 (70% vs. 16.6%) compared to d5 (38% vs. 28.3%). Again, that extent of variation in the samples on day 6 suggested that embryo-to-embryo differences were a reflection of real differences in APA release and not merely due to normal experimental 'noise' (Figure -43b).

Experiment-7: APA in conditioned media of single embryo cultures of d3 embryos on day 5 and 6:

Another experiment was performed in a similar format for estimation of APA on d5 and 6. However, the culture of individual embryos was initiated on d3 instead of d4. The rationale behind this experiment was that the somewhat weak signal observed in d5 media in experiment 6 might be enhanced if more time were allotted to allow for the accumulation of additional APA in the media. Therefore, data were obtained from

analysis of d3-5 media and d6 media, and the results were analyzed and formatted similar to experiment 6 (Figure- 44a &b). A similar trend as was observed in the previous experiment. The average APA activity was higher on d6 (1.8 times greater than the control mean) when compared to d5 (1.24 times greater than control) and the CV deviating significantly from control samples on d6 (58% vs. 21%) compared to d5 (27.8% vs. 20.1%)(Figure-44b).

An ANOVA analysis on d6 embryo APA from experiments 6 and 7 revealed that there was a trend towards positive correlation (p-value: 0.113) between the APA and the morphological stage and quality of the individual embryos.

Discussion

The current embryo grading systems in use are mostly based on morphological and genetic evaluations that do have limitations. Therefore, the continued search for markers to predict embryo quality has been the focus of intense research (David et al., 2001; Jurisicova et al., 1999; Lopes et al., 2007). Of all the different types of potential markers explored, a biochemical marker would be particularly useful, especially if it is released from an embryo and tools were available to permit detection of such a marker. In this chapter, we propose that peptidases that are actively secreted by embryos are candidates that might satisfy these criteria.

Porcine embryos are known to express a class of APs, known as Pregnancy-associated glycoproteins (PAGs), in trophectoderm even as early as the 4-cell stage. The PAGs represent a large gene family and are expressed in a host of other species such as cattle, sheep, goats, etc., (ruminants) (Garbayo et al., 2000; Green et al., 2000; Xie et al.,

1991b). Proteins similar to this class are expressed in rodents (Chen et al., 2001) and equids (Green et al., 1998a; Green et al., 1999). The PAGs are actively secreted by placental trophoblasts and some are known (Chapters - 3 and 4) or predicted to be proteolytically active. Those that have been shown to possess such activity do so under acidic pH conditions. With these observations in mind, we proposed that acid peptidase activity would likely accumulate in embryo-conditioned media, resulting from the secretion of aspartic peptidases such as PAGs. Such activity could be monitored and could serve as either a positive or negative indicator or embryo quality.

To measure APA present in culture media, a commercial FRET substrate was used to measure the amount of peptidase present. The substrate was initially designed for cleavage by aspartic peptidases cathepsin D/E (Yasuda et al., 1999b), however, we discovered from other experiments in our laboratory, that the substrate has utility in proteolytic experiments involving a range of other APs, including the PAGs. The experiments described in this chapter were performed in acidic pH buffers of 3.5 or 4.0 – such conditions specifically favor activity from APs.

Initially we investigated whether there was any detectable APA within conditioned medium obtained from the culture of pooled embryos for one week. We noticed that there was significant activity in the conditioned medium when compared to fresh culture medium which was not been exposed to embryos. Unexpectedly, the control media also exhibited some residual activity compared to the 'experimental blank' sample, which included only reaction reagents and substrate. We assumed that this activity was due to a trace contaminant within the medium. Because of this, control media samples (often several) were always employed for comparison to the embryo-conditioned media.

In the next experiment, we verified whether the APA found in the medium was due to an AP. In this experiment, aliquots of conditioned medium from d7 blastocyst culture were either treated with pepstatin A, a specific inhibitor of APs, or DMSO, the solute used to dissolve the pepstatin. We noticed that the APA within the conditioned medium could be inhibited by pepstatin A but not by DMSO verifying that the source of APA in conditioned medium was due to at least one AP. It was also notable that the residual APA in the control media samples was inhibited by pepstatin A suggesting that an AP is the likely source of that residual activity.

Subsequent experiments demonstrated that the APA was detectable in media conditioned by small numbers of embryos or even from a single embryo after culture for as little as 24 hrs. Interestingly, the amount of APA detectable in the media was not always additive, meaning that measured APA often did not correspond to the numbers of embryos present in the droplet. Such results suggested that there were embryo to embryo differences in the amount of APA released.

Many of the initial experiments focused on embryos cultured for up to seven days. In situations where embryo-transfer is to be employed, the earlier embryos can be graded/ assessed, the better. Experiments were then performed to detect measurable APA at earlier timepoints. Again, activity was detectable; particularly on day 6 embryos (activity on day 5 was generally much lower). These experiments also lent further support to the observation that embryos at the same developmental stage and with similar quality grades, displayed marked differences in the amount of APA in the media. One obvious concern that needed to be addressed, however, was whether or not the observed embryo-to-embryo variations were really a reflection of biochemical differences between

embryos or were such differences merely normal experimental variation in the assay system. The use of multiple control droplets helped define the extent of intra-experiment variation (coefficient of variation or 'CV'). By also determining the CV for the APA in conditioned media samples, it was clear that the embryo-to-embryo differences were substantially greater than those observed in the control media droplets, particularly in the day 6 culture samples. In other words, not only was the mean APA in the conditioned samples significantly greater than that observed in the control media, but the extent of embryo-to-embryo variation was greater than what would have been predicted if the samples had merely exhibited normal experimental variation. Because there are clear differences between embryos, it seems possible that APA in the media might be used as a positive or negative indicator of embryo quality or developmental potential to distinguish between embryos that are morphologically identical.

To further characterize the APA, the amount of APA in the media was compared to the embryo quality and stage scores. This analysis suggested that there was only a trend (P=0.113) towards significance between the morphological evaluation and APA.

With these observations in hand, several next-step experiments are warranted. For example, it is important to identify a likely candidate(s) contributing to the APA in the conditioned medium. The secreted AP, poPAG-2, is a product of trophoblasts and would be a likely candidate since the activity observed in the conditioned media mirrors that of the profile observed with recombinant poPAG-2 (Chapter 4). However, the APA may be coming from another peptidase capable of activity under similar conditions. One obvious candidate is cathepsin D. Other candidates such as legumain-an asparginyl endopeptidase was also proposed as a likely candidate (MU-porcine EST project). Future

experiments are focused on testing any association between relative APA and developmental competence *in vivo* should also be a focus of future efforts as well as determining if this activity is secreted by embryos from other species besides swine.

Chapter –V figures

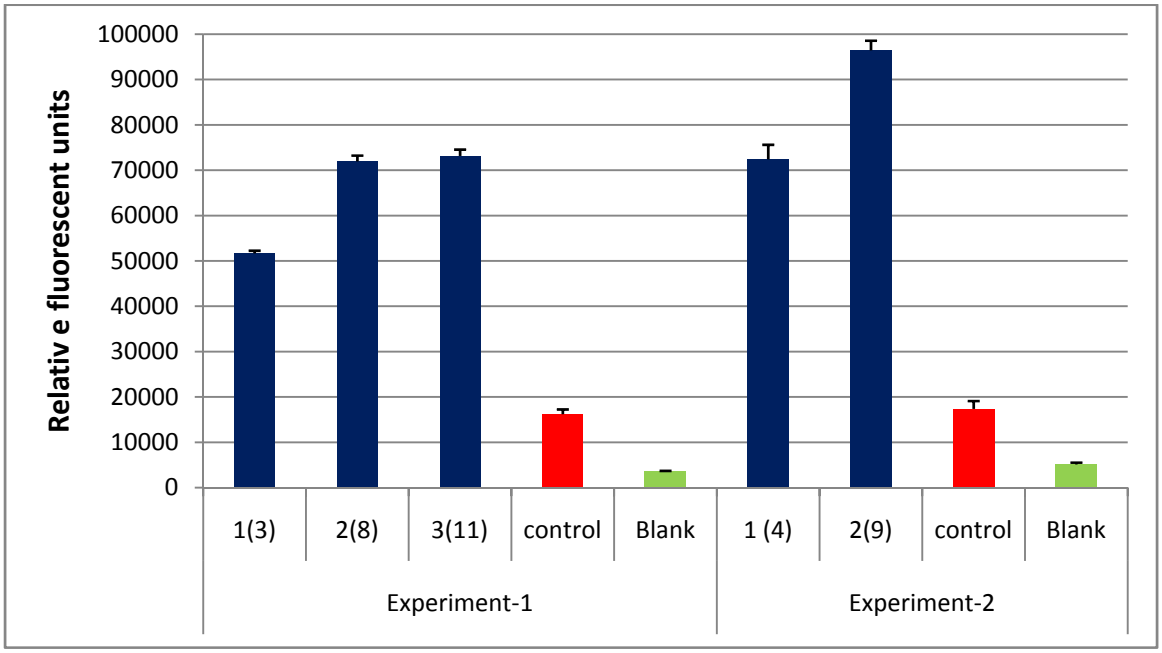


Figure -38. Results from two experiments investigating acid peptidase activity (APA) in 25µL of d7 pooled embryo-conditioned media compared to equal amounts of fresh PZM-3 culture medium (control) and sterile double distilled water (blank). Note the high amount of fluorescence emitted (shown as relative fluorescent units or RFU) in the embryo-conditioned media compared to the control and blank samples. The numbers in parentheses indicate the number of blastocysts remaining in the droplet on d7 after fertilization (20 cleavage-stage embryos were initially placed in each droplet). The error bars represent the variation between technical replicates of each sample.

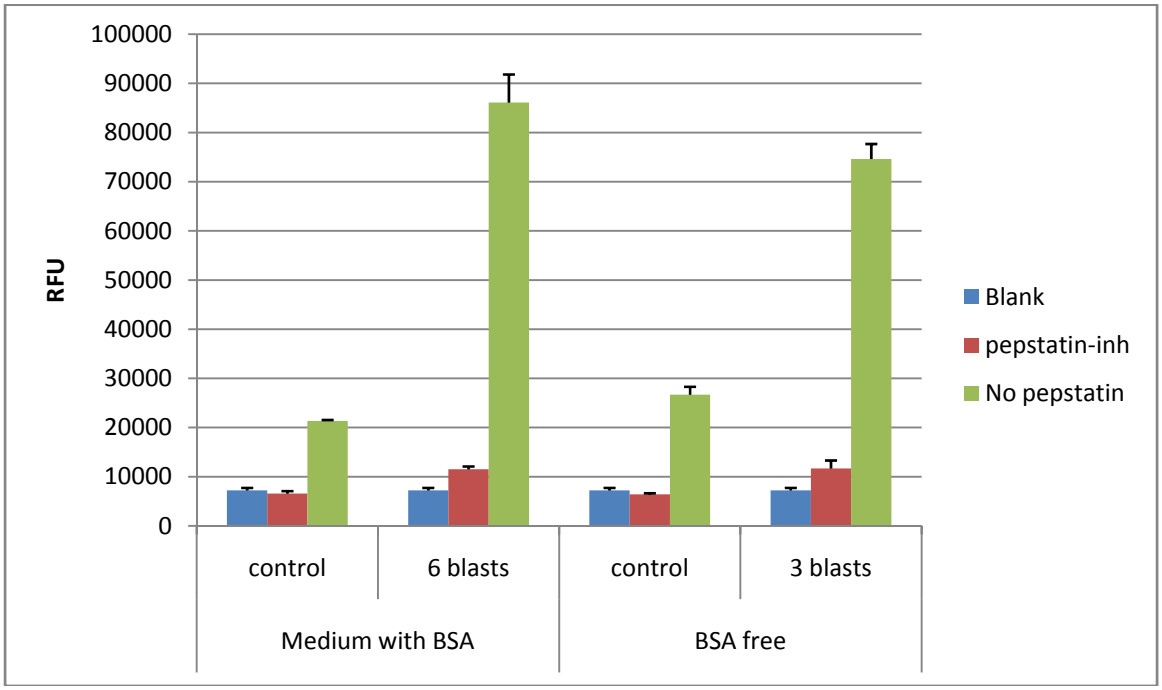


Figure -39. Inhibition of APA in culture media by pepstatin A - a specific inhibitor of aspartic peptidases (AP). Notice the decline in APA of two different conditioned media [PZM-3(with BSA) and PZM-4(PZM-3 without BSA)] treated with pepstatin A. Notice that the residual APA observed in the control media samples could also be inhibited with pepstatin A. The number of blastocysts remaining in each d7 pool is indicated at the bottom of each dataset.

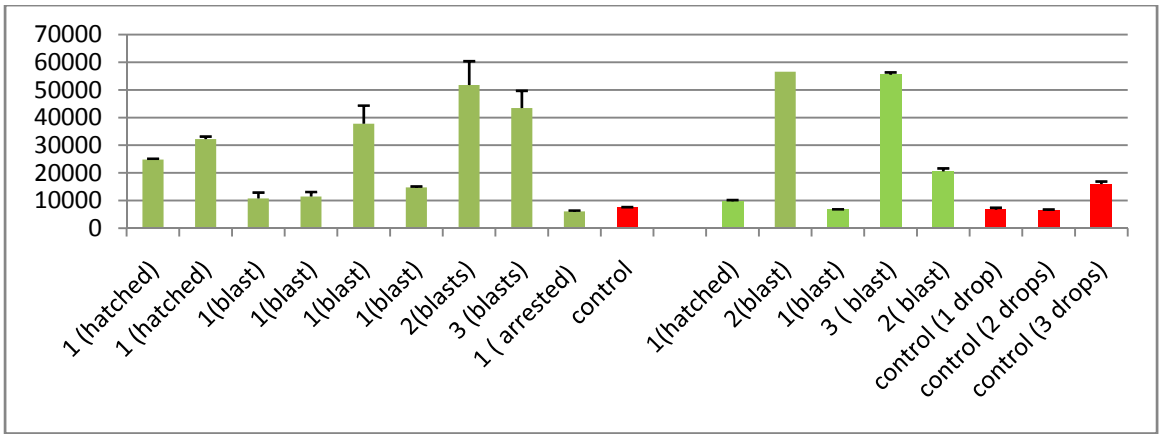


Figure -40. APA in conditioned medium obtained from solitary or groups (2 or 3) of d7 blastocysts after 24 hrs in culture. Results obtained from two different experiments were displayed. APA from single-embryo cultures or groups of embryos, along with their stage of development (in parentheses), are shown. APA in control medium cultures corresponding to 1.5, 3 or 4.5 μL media droplets (1, 2 or 3 drops) are also shown.

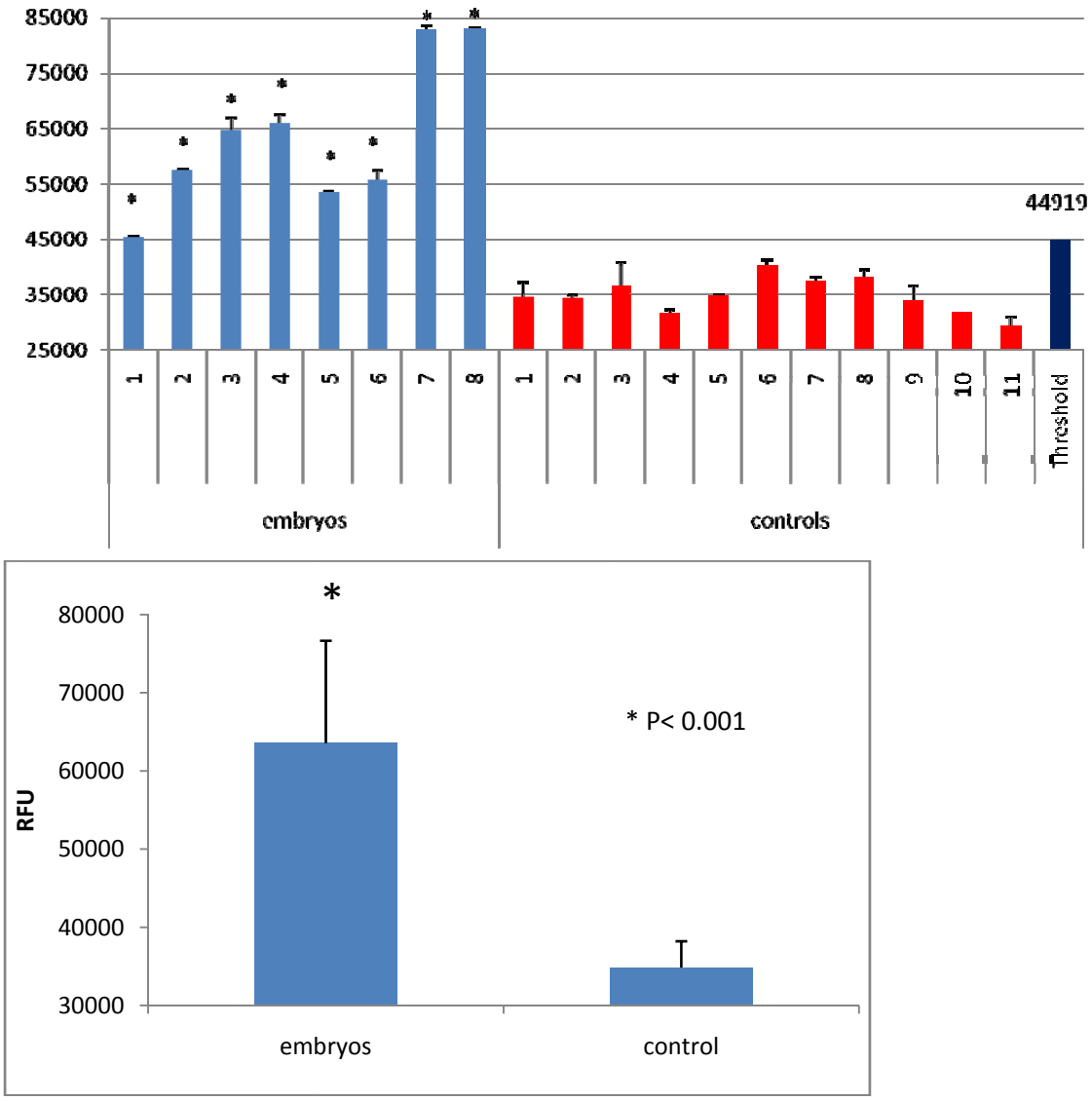


Figure -41a. The APA obtained from single d7 embryo conditioned media (cultured for 24hrs) as well as multiple droplets of unconditioned control medium. A threshold bar representing the calculated threshold (mean plus 3 Standard deviations of the control

medium) was also shown in the picture. The embryos with activity significantly higher than the control activity were identified by an asterisk (*) symbol.

41b) The mean APA from embryo-conditioned media compared to the control average. A t-test was used to determine if the two means differed at a p-value of <0.001.

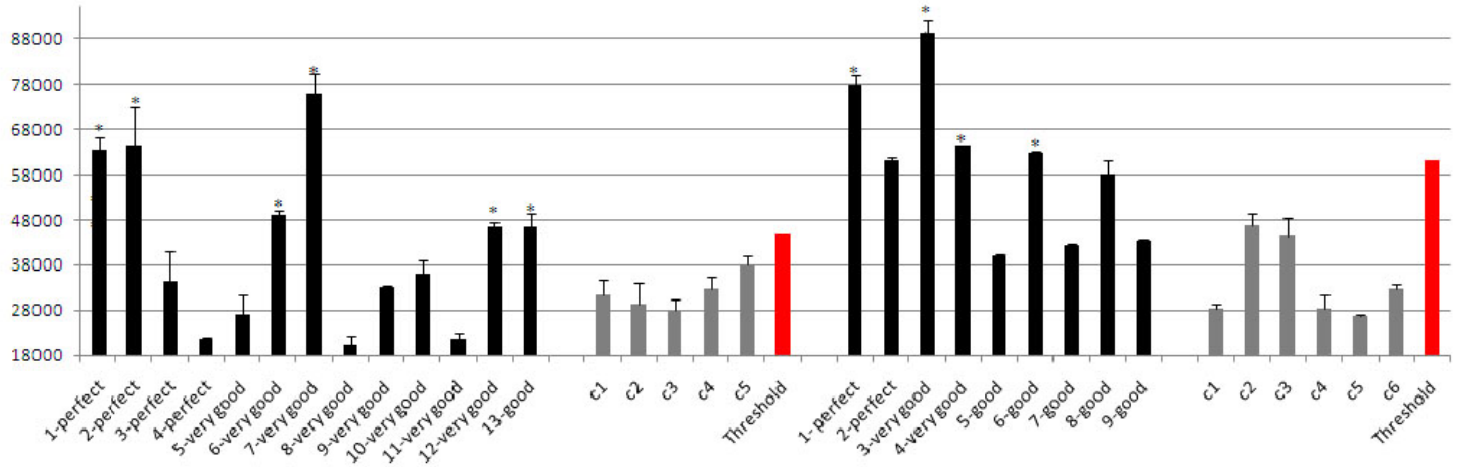
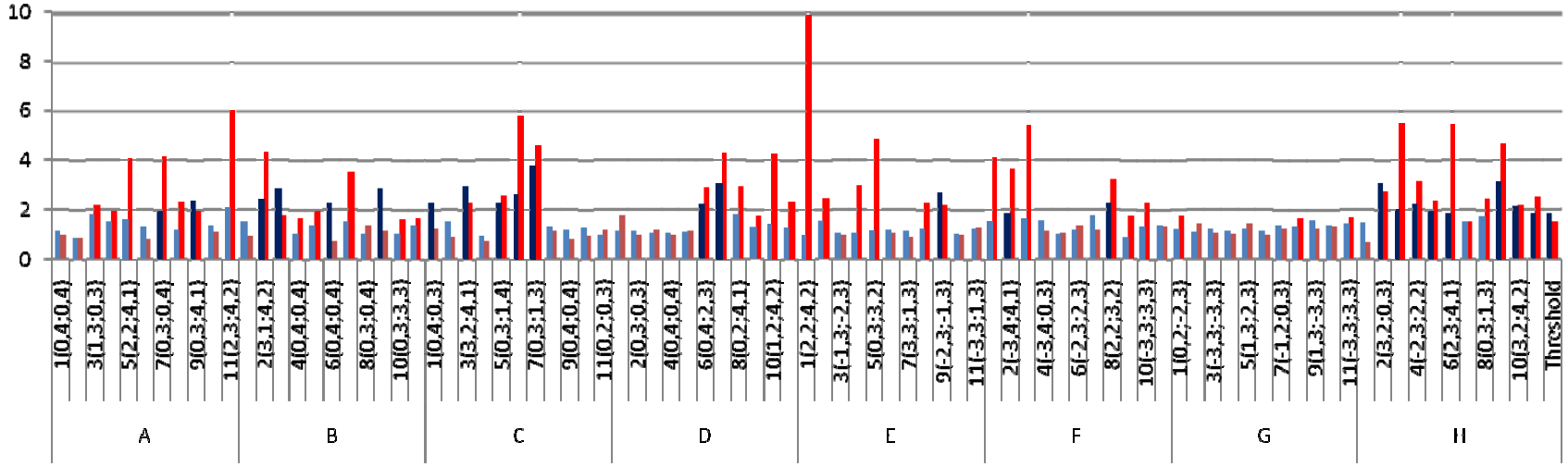


Figure -42. Estimation of APA in d6 conditioned medium obtained from single embryos after culture for two days. Two replicate experiments are shown with their associated control media samples (c 1- 5/6). The quality score assigned to each individual embryo is shown in parenthesis. All samples that had activity significantly above background are represented by an asterisk. Note that in this particular experiment, the control media samples had relatively high amounts of APA. Even so, there were embryo-conditioned samples that were significantly greater than the controls.



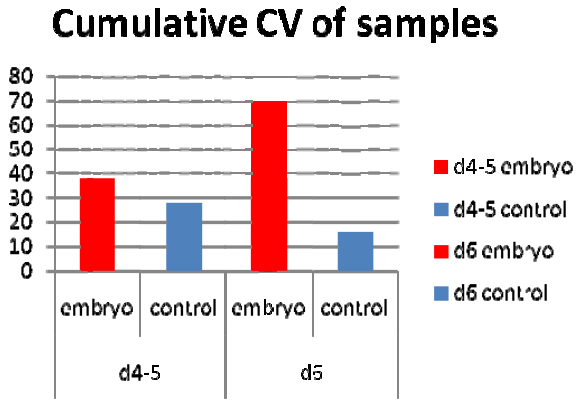


Figure -43. APA in d4-5 and d6 conditioned medium of single embryo cultures. Pools of cleavage-stage embryos were cultured to day 4. Individual embryos were then placed in separate droplets and cultured for an additional 24 h. Media were harvested and fresh medium was added to each embryo and cultured for an additional 24 h. The d5 and d6 media samples were assayed for activity. Embryo developmental stage and quality scores were recorded for both collection times. The raw fluorescent values obtained from media-derived APA were transformed into a fold change relative to the mean APA for the control samples from the same experiment. The transformed values of individual embryos on both days 5 and 6 were displayed side-by-side. The threshold values (control media APA, plus 3 standard deviations) determined for samples on both d5 and d6 were included for reference. All the embryos that had activity significantly above the threshold value were highlighted. The stage and quality score assigned to each embryo on d5 and d6 are shown in parenthesis and separated by a semicolon. Day 5 media APA is shown in blue; Day 6 media APA is shown in red.

43b) The coefficient of variation (CV) calculated for the groups of embryos as well as the control samples on both days 5 and 6 are displayed. Notice the high CV values in the d6 samples compared to their corresponding controls and compared to the day 5 samples.

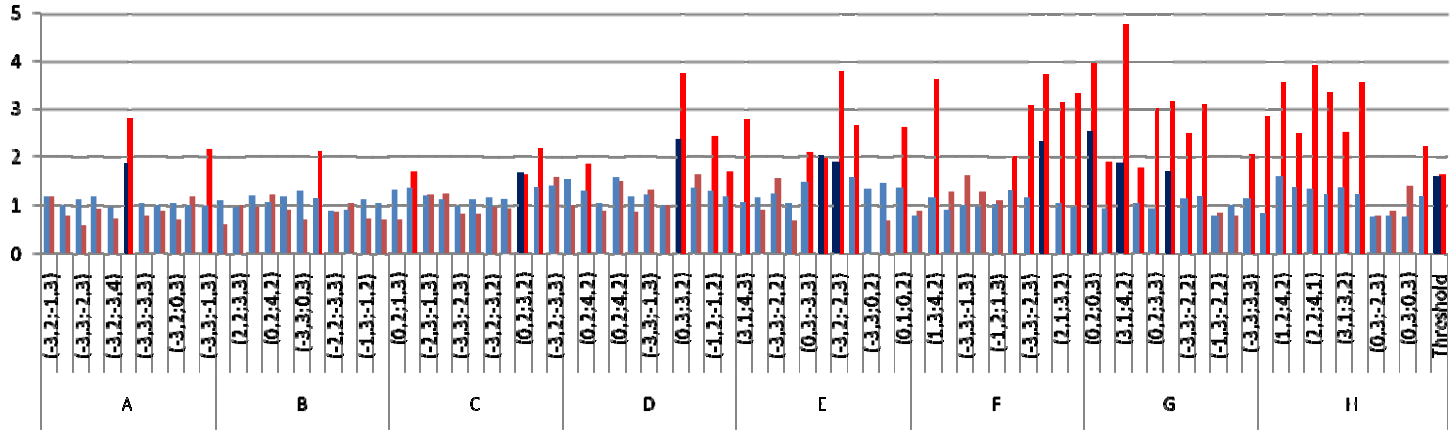


Figure -44. APA in d3-5 and d6 conditioned medium of single embryo cultures. Pools of cleavage-stage embryos were cultured to day 3. Individual embryos were then placed in separate droplets and cultured for an additional 48h until day 5. Media were harvested and fresh medium was added to each embryo and cultured for an additional 24h to day 6. The d5 and d6 media samples were assayed for activity. Embryo developmental stage and quality scores were recorded for both collection times. The transformed values of APA obtained from d3- 5 and d6 media of individual embryos on respective days were displayed next to each other. The embryos showing significant activity above that for the control media droplets are highlighted. The stage and quality score assigned to each blastocyst on days 5 and 6 are shown in parenthesis as for Figure 6. Day 5 media APA is shown in blue; Day 6 media APA is shown in red.

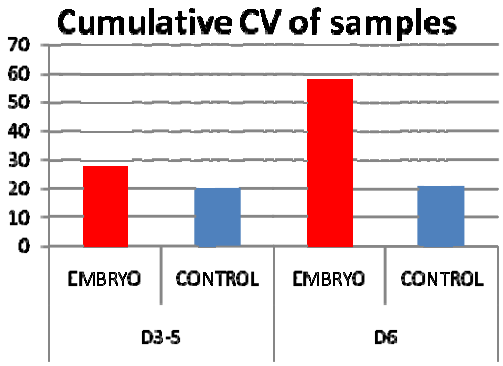


Figure -44b. The coefficient of variation calculated for the groups of embryos as well as the control samples on both days 5 and 6 are displayed. Notice the high CV associated with samples on d6 compared to the d3-5 samples.

APPENDIX

PROTEOLYTIC ASSAYS AS A MEANS FOR DETECTING PREGNANCY-ASSOCIATED GLYCOPROTEINS IN SOLUTION

[POTENTIAL APPLICATIONS FOR DETERMINATION OF PREGNANCY IN CATTLE]

Abstract

A biochemical test for pregnancy diagnosis in cattle is currently available that makes use of RIA or ELISA formats for the detection of pregnancy-associated glycoproteins (PAGs) present in the blood of pregnant cows and heifers. PAGs are related to a group of proteins known as aspartic peptidases, which are enzymes capable of cleaving protein substrates at relatively acidic conditions. In this report we sought to establish an alternative means for measuring PAGs, in simple solutions or in complex biological fluids (including serum/plasma), by taking advantage of the ability of some PAGs to cleave peptide substrates. Measurement of this activity could serve as a way to detect the presence of PAGs in conjunction with an antibody-binding type of approach. These experiments use antibodies specific to certain PAGs to 'trap' or immobilize the PAGs from serum or other biological fluid onto a solid support such as an ELISA plate. The presence of PAGs, sequestered by the anti-PAG antibodies, were then identified by adding a fluorescent peptide substrate and monitored for product liberated as a result of the enzymatic activity. The results provided here demonstrate that proteolytic activity is present in purified native PAGs as well as recombinant bovine PAGs-6, -8, -9 and -10, in

addition to bovine PAGs -2 and -12 that have already been characterized. In addition, these results demonstrate the ability to specifically immobilize PAGs from a complex protein mixture such as cell lysates, as well as serum, by using PAG-specific antibodies. The PAGs immobilized by the antibodies displayed activity towards the fluorescent substrate. These experiments serve as a 'proof of principle' demonstrating that measurement of PAG peptidase activity could potentially be used to detect PAGs in biological fluids from cattle and other ungulates. Ongoing and future work will be focused on optimizing PAG activity conditions and in identifying PAG-specific substrate peptides.

Introduction

Pregnancy diagnosis is a major part of reproductive management in the cattle industry. In general, artificial insemination is successful less than 50% of the time and the producer must either rely on overt signs of return to estrus (that are easily missed) or delay re-synchronizing until pregnancy failure is confirmed by ultrasound, palpation or by assay of circulating pregnancy-specific antigens. Such delays are extremely costly and constitute a major economic loss to the industry.

An accurate pregnancy test for cattle, which can be performed early in pregnancy, continues to be an active area of research. Several methods and test for pregnancy are currently available, including a milk progesterone assay (Markusfeld et al., 1990; Oltenacu et al., 1990), estrone sulfate analysis (Hatzidakis et al., 1993; Holdsworth et al., 1982; Warnick et al., 1995), rectal palpation (Day et al., 1995), ultrasound (Beal et al., 1992; Cameron and Malmo, 1993), and blood tests for pregnancy-specific antigens (Green et al., 2005; Sasser et al., 1986).

Pregnancy-associated glycoproteins (PAGs) are a family of proteins structurally related to aspartic peptidases such as pepsins, and are expressed in the outer epithelial cell layer (trophoblasts) of the placenta of even-toed ungulates (Green et al., 2000; Hughes et al., 2000; Xie et al., 1997b). There are no published reports showing that ruminant PAGs are capable of acting as peptidases, although each appears to possess a cleft capable of binding peptides (Guruprasad et al., 1996). It is estimated that cattle, sheep, and most probably all ruminant *Artiodactyla* possess dozens of PAG genes. Other artiodactyls, such as swine, also have PAG genes. Polypeptides with somewhat related sequences are also known to exist in species outside the *Artiodactyla*, including horses, cats and rodents (Chen et al., 2001; Green et al., 1999).

Many members of the ruminant PAG family are able to enter the maternal circulation during pregnancy. Due to the placenta-specific origin of these proteins, their presence in maternal blood can serve as a means of determining pregnancy status of bred cattle. Indeed, immunological-based assays have been used as the basis for making such determinations (*e.g.* United States Patent No. 6,869,770) (Green and Roberts, 2006; Green et al., 2005).

Phylogenetically, the ruminant PAGs are a highly diverse family that fall in two distinct groupings: the more recently evolved 'modern' PAGs and the 'ancient' PAGs. Even in initial protein purification studies (Butler et al., 1982; Xie et al., 1994; Xie et al., 1996; Xie et al., 1991b; Zoli et al., 1991), it was clear that the boPAGs were heterogeneous in molecular weight and charge, and as more isoforms were purified, it was evident that they differed in their amino terminal sequences (Atkinson et al., 1993;

Xie et al., 1997b). Despite their relationship to aspartic peptidases, many PAGs – particularly those in the modern grouping - are incapable of proteolytically activity due to site-specific mutations within the catalytic site (Green et al., 1998b). Other PAGs, however, have all the hallmarks of typical aspartic peptidases and are functional peptidases (Chapters -III and IV).

A biochemical test for pregnancy, such as the PAG assay, should possess three fundamental attributes: specificity, accuracy and early detection. Although the current immune-based PAG assay has shown considerable utility as a way to identify pregnant animals, it may fall somewhat short in regard to the first of these criteria because the antibodies currently employed in the assay are known to bind to multiple PAGs (Green et al., 2005). The measure of PAG proteolytic activity – particularly the use of PAG-specific peptide substrates - could potentially improve overall specificity of the current PAG assay platform. In addition, the use of peptidase substrates could provide a different approach to pregnancy diagnosis and may help streamline the current PAG assay by decreasing the number of steps required to measure these proteins in biological samples.

Thus, despite the availability of several assays to detect pregnancy, there remains some need to provide additional assay platforms for accurate and early detection of pregnancy, especially in agriculturally important animals such as cattle. Thus in this appendix, we tried to establish 3 broad objectives: 1) to show that native preparations and additional recombinant forms of PAGs are proteolytically active. 2) to use monoclonal and polyclonal antibodies to specifically trap PAGs and finally, 3) to provide evidence that the trapped-PAGs are still proteolytically active and can liberate products that can be assayed.

Materials and Methods

Purification of native bovine PAGs

Native PAGs were partially purified initially from bovine placenta according to the method of Green *et al.* (2005) and Wooding *et al.* (2005). The native bovine PAGs were further purified via affinity chromatography and ion-exchange chromatography. Subsequent to the initial isolation, the PAG preparation was applied to a Pepstatin A-agarose affinity column at pH 7.0, and eluted in the presence of high salt (1 M NaCl) and Triton® X-100 (1%) at pH 10.0, this preparation was termed "7/10". Modifications of these binding and elution conditions resulted in different native PAG preparations such as 5/7, 5/8, 7/8 and 7.5/8 where the pH for binding to pepstatin column is indicated preceding to the pH used for elution.

Following affinity chromatography the enriched PAG preparations were further purified by DEAE-anion exchange chromatography, to remove excess Triton X-100 and other contaminating proteins (Green et al., 2005; Wooding et al., 2005). PAGs bound to anion exchange column were eluted by subjecting the column to a gradient of salt concentration. The fractions representing purified PAGs eluted from the anion exchange column are displayed in the figures and tables in parenthesis after the original pepstatin A purified preparation for e.g., 7/10(60) or 7/10 (100).

Proteolytic activity of purified PAGs

Purified native PAGs were incubated with a cocktail of peptidase inhibitors to inhibit different classes of peptidases [1 mM EDTA (Sigma) for metallopeptidases; 10 μM E64 (Sigma) for cysteine peptidases and 4 mM AEBSF (Roche) for serine peptidases]. A preliminary experiment to identify an optimal pH for proteolytic activity of the purified native PAG preparation 7/10(60), the experiment was conducted similar to the procedure described in detail in Chapter III. Briefly, each protein sample was incubated in buffers ranging from pH 3.5-6.0 with increment of 0.5 pH units. A commercial fluorescent Cathepsin D/E substrate MOCac-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 (*e.g.* Peptides International KY, USA) was used at a final concentration of 20 μM. The buffers and NaCl were both at a final concentration of 100 mM. The final volume of the reaction was maintained at 100 μL. To confirm that the activity within the acidic pH range arose from an aspartic peptidase, the sample was inhibited with pepstatin A prior to performing the assay. The reactions were performed at 35ºC for 20 min and were terminated by addition of 900 μL of 5%TCA as described previously (Yasuda et al., 1999a). The resultant mixture was further diluted to 2 mL with 5% TCA and the resultant fluorescence in the mixture was read in a PC1^{TM} Photon counting spectofluorimeter at 328 nm (excitation) and 393 nm (emission) wavelengths as described previously (Yasuda et al., 1999a). All experiments were set up in duplicate. The results from duplicate reads were used to compile the data.

Generation of recombinant bovine PAGs:

Production of recombinant bovine PAGs with an N-terminal flag-tag was discussed in detail in Chapters II and III and will not be discussed here. Sf-9 insect cells

infected with recombinant baculovirus expressing ancient boPAGs -2, 8, 10, 12 and modern boPAGs -6, 9, 17, 19 and 20 as well as porcine PAG-2 were used in the assays. In addition, recombinant equine-TSH beta as well as wild-type virus (AcNPV) infected insect cells were incorporated as negative controls in the assays.

Enrichment of PAGs from solution with antibodies prior to peptidase assay - "Immuno-trap peptidase assay":

Experiment-1: Several antibodies were tested for their ability to selectively bind and immobilize native PAGs from solution in a standard 96-well ELISA plate (Corning, USA). The antibodies employed were (a) polyclonal antibodies (POLY) raised against a mixture of native PAGs (Green *et al*., 2005; Wooding *et al*., 2005); (b) an equimolar mixture of three specific monoclonal antibodies (MONO) directed toward selected PAGs (Green *et al*., 2005); and (c) a rabbit polyclonal anti-PAG-10 (PAG-10) antibody raised against recombinant bovine PAG-10 that had been produced and purified following standard protocols in the laboratory. In each case, 1 µg of the antibodies were coated in the wells of a standard ELISA plate (coating overnight followed by storage at 4°C). Remaining non-specific binding sites were blocked by incubation with bovine albumin for 1-2 hours. Purified PAGs (a mixture of pepstatin-purified PAGs that represented 7/8, 7/10, 5/7 and 5/8 preparations) were then applied to the plate as either 5 or 50 ng of total protein in 100 µL (Table -6 and Table -7). The blank (Blank) consisted of 50 ng of bovine serum albumin and the background control (Rabbit-Ig) was rabbit immunoglobulins loaded in the same amount (1 µg per well) as the other trapping immunoglobulins. After an incubation period, the plates were washed twice with wash buffer (saline with 1% tween) and 100 µL of assay buffer (100 mM sodium citrate-citric

acid, pH 3.5; 100 mM salt) with 5 μM Cathepsin D/E substrate was added to the wells and incubated at 37°C for 8hrs in an incubator. The reaction was terminated by addition of 100 μL of 10% TCA and the amount of product liberated was measured with a fluorescent plate reader (Synergy- HT, BioTek, USA) using 320/20 nm excitation and 380/20 nm emission filters. All the treatments were run in duplicate wells and the average of the raw fluorescent units were displayed. A schematic of the immune-trap peptidase assay is illustrated in Figure -48.

Experiment-2:

A modification of the immuno-trap peptidase assay of native PAG preparations was performed with the PAG mixture enriched to a greater extent than used for the experiment-1. Polyclonal antibody ("#20"; the same anti-PAG antibody that was used in experiment-1) and a mixture of monoclonal antibodies were used to trap PAGs from solution. Native PAGs used in the assay were 5/7 (60), 5/8 (60) and "Mixed PAG" which represents pooled native PAGs from various preparations that was also used in the experiment-1. Bovine serum albumin (BSA) was loaded at the same concentration as native PAGs and served as an experimental control. To determine if the antibodies are capable to trap PAGs from complex biological solutions, the native PAGs in the assay were mixed with non-pregnant heifer serum and the resultant mixture was incubated with the antibodies. Following incubation O/N with protein samples at 4ºC, and three wash cycles, the proteolytic activity of the trapped PAGs was tested by addition of 5 μM substrate in assay buffer and incubation for 2 hrs at 37ºC. For all subsequent experiments,

the incubation time was reduced to 2 hrs, and the resultant fluorescent emission was estimated as described above.

Enrichment of PAGs from serum of pregnant cows with antibodies prior to peptidase assay:

Immuno-trap peptidase assay was performed with sera from two pregnant animals at different stages of gestation (Table -8). A mixture of monoclonal antibodies were used to trap PAGs from the serum samples collected from known stages of pregnancy (Days 0, 21, 30, 40, 150 and 200) and the assay was performed as described above.

Enrichment of recombinant PAGs in insect cell lysates with PAG-specific and anti-flag antibodies prior to peptidase assay:

As a variant to the above experiments which utilized native PAGs in the assay, we tried to determine if the recombinant bovine PAGs expressed as fusion proteins with a synthetic N-terminal FLAG-tag could be utilized in a similar format.

Experiment-1: A commercially available anti-FLAG monoclonal antibody (M2; Sigma, MO, USA) was used to selectively immobilize recombinant proteins bovine PAG 2, boPAG 12 and poPAG-2 by binding to the FLAG peptide present on the amino-terminus of the recombinant proteins (described in Chapters II and III). The solution added to the wells was a complex protein mixture consisting of a crude insect cell lysate containing expressed recombinant PAG proteins, and control wells which consisted of protein control or antibody control. The protein control insect cell lysate consisted of insect cells

(sf9 cells) infected with empty Baculovirus and the antibody control (Ab-control) constituted of a trapping antibody with no added lysate. As an additional control empty wells were set up with only buffer and substrate with no trapping antibody and protein source. These three different controls served to help define the background fluorescence in the experiment. These experiments also included a polyclonal antibody raised against recombinant porcine PAG-2 (Table -9).

Experiment-2: Several additional recombinant bovine PAGs-6, 8, 9, 10, 17, 19 and 20 that were expressed as fusion proteins with a FLAG epitope tag were immobilized in the wells of an ELISA plate by using anti-FLAG in an immuno-trap peptidase assay format (as described above) and were analyzed for their proteolytic activity against the Cathepsin D/E FRET substrate at pH increments ranging from 3-7. Recombinant equine-TSH-beta was expressed and extracted in the same manner as the PAGs in the experiment. As equine-TSH-beta does not possess proteolytic activity, this protein was used as a negative control in the assay to establish the baseline for background fluorescence (Table -10).

Experiment-3: To test whether the individual monoclonals (A6, L4, J2) as well as a mixture of them ("Mixed") have any difference in affinity towards individual recombinant PAGs, five different PAGs (6, 8, 9, 10 and 21) were tested with these antibodies in the immune-trap peptidase assay format. Recombinant equine-TSH beta which should not bind to any of these antibodies was used as a control in the experiment. As an experimental positive control, anti-flag antibody was used to trap poPAG-2 with equine-TSH beta as the loading control (Table -11).

Results

Purification of native bovine PAGs:

Purification of native PAG preparations was performed by chromatography with modified binding and/or elution pH. Thus, preparation "7/8" was obtained by binding to a pepstatin A affinity column at pH 7.0, as above, followed by elution at pH 8.0 in the presence of increasing osmolarity (up to 1 M NaCl), including detergent (1% Triton X-100). Different PAGs bind with differing affinities to the aspartic peptidase inhibitor, pepstatin A, and it is possible to isolate different members of the family by systematically altering binding and elution conditions (*e.g.* Green *et al*., 2005; Wooding *et al*., 2005). Further purification of the PAG-containing fractions was performed by DEAE anion exchange chromatography. The majority of PAGs bound to the anion exchange column eluted as two major peaks at elution fractions 60 and 100, which corresponded to NaCl concentrations of 75 and 150 mM, respectively.

Proteolytic activity of purified PAGs

A pH profile of peptidase activity of the purified 7/10 (60) PAG preparation that employed a fluorescent Cathepsin D/E substrate MOCac-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 is shown in Figure -45. Units on the left axis denote fluorescence intensity liberated by cleavage of substrate as represented by relative fluorescence units (Rfu). Significant peptidase activity was seen with a pH optimum of 3.5-4.5, consistent with our earlier observations from recombinant boPAGs (Results - Chapter III). This activity could be inhibited by the aspartic peptidase inhibitor pepstatin A.

A similar experiment was performed with two anion exchange elution fractions (60 and 100) of two different bovine PAG preparations 7/8 and 7.5/8, albeit with a narrow pH range (pH 3.5, 4 and 5.5) (Figures -46 and 47). The results from 7/8 (60) differed from the other experiments in that the highest activity was recorded around pH 3.5 instead of 4. In both these experiments we saw highest activity with elution fraction 60, when compared to fraction 100.

Enrichment of native PAGs from solution followed by peptidase assay -Immuno-trap peptidase assay:

Experiment-1: Figure- 48 shows schematic illustrating the immune-trap peptidase assay method. Table -6 shows the relative fluorescence (counts) of product emanated from a representative "immuno-trap" peptidase assay with purified native bovine PAG standards. In each case, the anti-PAG antibodies were able to selectively immobilize proteolytically active PAGs from the solution as was indicated by the increase in fluorescence following proteolytic cleavage of the substrate by trapped PAGs which was significantly above the blank wells consisting of BSA (protein control) and rabbit IgG wells (antibody control).

Experiment-2: Relative fluorescence was also assayed by an immuno-trap peptidase assay of native PAG preparations enriched to a greater extent than the PAG mixture used for the experiments in Table -6. Table -7 details the ability of polyclonal ("#20"; the same anti-PAG antibody that was used earlier in Table -8) and monoclonal antibodies to trap PAGs from solution (readings in italics are different from the control). An asterisk (*) in Table 9 denotes that heat-inactivated BSA was used in those assays as blocking

protein and as a loading control to determine the extent, if any, that BSA might be contributing to the fluorescent signal. Row 4 of Table -7 represents results in which representative native PAGs were mixed with non-pregnant heifer serum (NPHS). The numbers in italics represent readings that are significantly different from the controls **($p<0.05$)**. In each experiment, the monoclonal and polyclonal antibodies were able to trap proteolytically active PAGs from the Mixed PAG and the 5/8 (60) preparations in solution or serum. The 5/7 (60) preparation was somewhat more variable; only one of the monoclonal trap experiments exhibited activity above the BSA control.

Immuno-trapping and enrichment of native PAGs from sera of pregnant cows with PAG-specific antibodies prior to peptidase assay:

Results from trapping PAGs from sera of two different cows collected from known stages of pregnancy (Days 0, 21, 30, 40, 150 and 200) using monoclonal antibodies, and detecting the immobilized PAGs via their proteolytic activity was displayed in Table -8. The higher values in samples from D30 onwards are reflective of increasing concentrations of circulating PAG in serum. Numbers in italics are significantly different from the Day 0 control.

Immuno-trap peptidase assay of recombinant PAGs in insect cell lysates:

Experiment-1: Table -9 displays the results obtained from trapping recombinant PAGs from insect cell lysates by using an anti-Flag antibody. The anti-flag antibody was able to successfully immobilize recombinant bovine PAGs-2, 12 and poPAG-2 via the N-

terminal Flag-tag, which as the numbers in italics indicated, have greater fluorescence when compared to the control wells. An antibody which is specific to poPAG-2 was also used in the experiment; it was able to specifically trap recombinant poPAG-2, but not boPAG 2 and boPAG 12, from its corresponding lysate.

Experiment-2: Table -10 show results of peptidase activity (fluorescence) from immuno-trap assays of several recombinant PAGs. Numbers in italics represent readings significantly different from the TSH-beta control protein. The assay shows that recombinant bovine PAGs -6, 8, 9 and 10 have proteolytic activity with acidic pH optima. Whilst, bovine PAGs-17, 19 and 20 did not display proteolytic activity significantly above the background fluorescence estimated from inclusion of TSH-beta.

Experiment-3: Table -11 shows results from an assay demonstrating how candidate PAGs can be differentially recognized by anti-PAG monoclonal antibodies. Numbers in italics represent readings significantly different from controls. PAG-10 was not captured by any of the candidate monoclonals. The monoclonals either have no affinity for PAG-21 or it is proteolytically inactive under the tested conditions. TSH-beta was used as a negative control to establish the background level of fluorescence. For comparison, an anti-flag antibody (FLAG) control and poPAG-2 lysate were used as positive controls for the experiment.

Discussion

In the dairy industry, years of selection for such traits as high milk production have had a significantly negative impact on reproductive traits such as fertility, and increasing loss post-conception(Lucy, 2001). These reproductive management concerns necessitate a tool(s) for diagnosis of pregnancy in cattle that is accurate, not technically cumbersome and provides a cheaper alternative to the current pregnancy detection methods which are lacking in one of these faculties. Since the estimates that employ molecular markers and utilize sensitive techniques such as ELISA and RIA are more reliable and sensitive, there was a need to develop a tool along similar lines that would obviate the need for trained veterinarians or technicians to perform rectal palpations or ultrasound. In addition, since many of the pregnancies in dairy herds are lost in the first 60-90 days of pregnancy, there was a need to do multiple checks of pregnancy after the initial pregnancy determination. This necessitated a diagnostic tool that used such markers which are a) products of the fetus b) circulating within the maternal circulation or excreted and c) have a relatively short circulating half life.

Several pregnancy diagnostic assays and kits are currently in the market or in the planning stages for commercialization. One such diagnostic assay, which was developed and reported earlier by Green et al, (Green et al., 2005) utilizes circulating PAGs as a means of pregnancy diagnosis. Since PAGs are products of the placenta of the fetus and since some of these PAGs enter maternal circulation and attain significant concentrations, they serve as ideal candidates for pregnancy diagnosis. They also serve to provide an estimate of the health of the surviving fetus. The current assay utilizes polyclonal and monoclonal antibodies raised against a mixture of PAGs purified from placental extracts

and are utilized in a standard ELISA format to trap PAGs in maternal serum and for detection by using chromogenic substrates. This diagnostic procedure has proven to be more accurate than previously reported PAG assays. However, it has some innate faults such as a lack of confidence in the specific PAGs identified in the assay.

PAGS ARE PROTEOLYTICALLY ACTIVE:

Since some of the PAGs are proteolytically active, a combination of PAG-specific antibody, which traps a specific PAG within the well, and the addition of a substrate (particularly one specific to a PAG) should enhance the overall sensitivity of the assay. Since, the procedure requires trapping or binding of PAG to the antibody followed by proteolysis of the substrate by the bound PAG, we refer to this as "immune-trap peptidase assay". As a proof of principal we performed several experiments that provided some preliminary evidence that such an assay may have merit. For example, 1) the available polyclonal and monoclonal antibodies were able to trap purified preparations of native PAGs from solution. We were also able to demonstrate that the soluble native PAGs in complex solutions such as non-pregnant heifer serum could be trapped by the antibodies. Such trapped native PAGs when exposed to a pH of 3.5 which is more optimal for its activity showed significant release of product which can be estimated by reading in a fluorescent plate reader. Coincidentally, the low pH of the assay probably also provides for the elution of bound PAGs from the antibody, thereby preventing the antibody from interfering with PAG activity. The liberated product provess much higher activity than solution in control wells.

2) We further verified that circulating PAGs in maternal circulation of pregnant animals could also be successfully trapped by utilizing a mixture of monoclonal antibodies specific to native PAGs. Such trapped PAGs displayed higher activity than controls. There was also a trend towards a pregnancy-dependent increase in PAG concentration which was manifested by an increase in signal with advancement of pregnancy.

3) We further extended the utility of this procedure to show that various recombinant bovine PAGs in complex mixture such as insect cell lysates could be effectively trapped in a similar procedure to native PAGs and that this procedure could be utilized to estimate if the recombinant PAG is proteolytically active and if so its optimal activity range. Since the recombinant PAGs are expressed with a synthetic sequence encoding for a 'Flag' epitope, an antibody specific to the Flag-tag could be utilized as a trapping antibody. This obviated the necessity to characterize the affinity of each individual PAG antibody towards the candidate recombinant PAG. We realized that the PAGs whose activity has already been established such as boPAG-2, 12 and poPAG-2 were successfully immobilized which was evident from the high fluorescence from the wells. Of the PAGs whose activity was not previously been established, this method proved effective in determining their proteolytic activity as well as insight into their preferred reaction conditions. Of the bovine PAGs investigated (PAGs- 6, 8, 9, 10, 17, 19 and 20), we found that PAGs-6, 8, 9 and 10 exhibited proteolytically activity with reactivity at low pH ranges.

4) Finally, we could establish that it is possible to trap specific PAGs out of the complex mixture by using monoclonal and polyclonal antibodies. We could show that a polyclonal antibody raised against recombinant poPAG-2 was able to bind to recombinant poPAG-2 but not boPAG-2 and 12. Similarly, monoclonal antibodies either individually, or in combination, could bind to PAG-6, 8 and 9 but not 10 and 21.

Therefore we could demonstrate in principal that circulating PAGs in complex mixtures such as maternal serum could be trapped and assayed by monitoring their proteolytic activity. This unique combination of specific antibodies towards candidate PAGs and a PAG-specific substrate should provide an improved means for pregnancy diagnosis.

Future experiments focused on identifying PAG-specific substrates may lead to an assay that no longer requires the use of PAG antibodies and could, theoretically, form the basis for a stand-alone PAG assay.

Appendix-figures

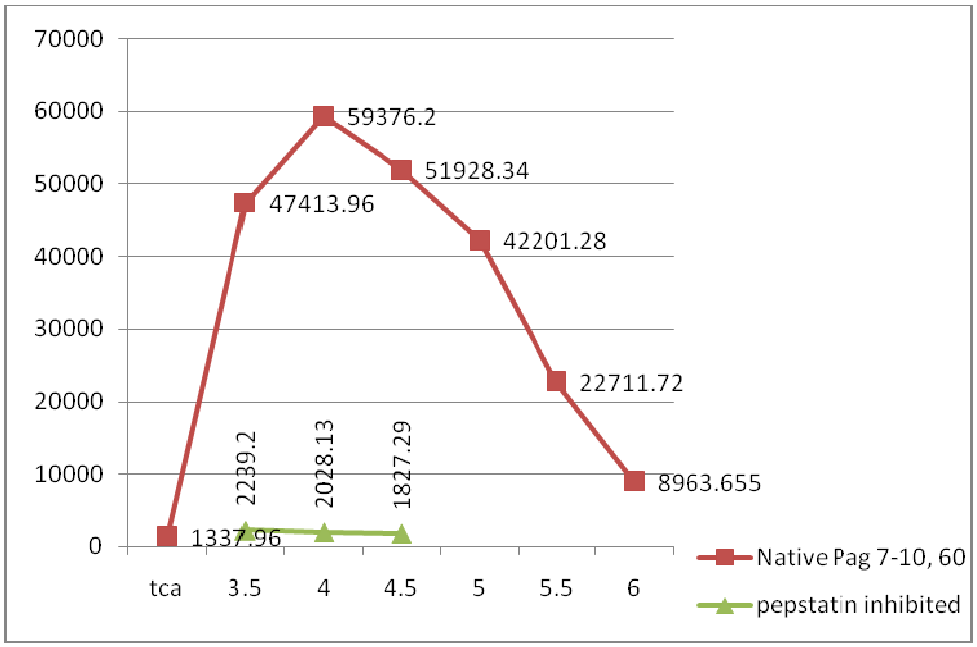


Figure -45. pH profile of the activity of a native bovine PAG (preparation 7-10*) against a fluorescent peptidase substrate.

Native bovine PAG was bound to PepstatinA-agarose affinity column at pH 7.0 and eluted at pH 10.0 in the presence of the detergent, Triton X-100. The PAG was purified further over a DEAE-anion exchange column to remove excess Triton-100 and other contaminating proteins. The units on the left axis reflect relative fluorescence intensity as a result of cleavage of the substrate. Note the high peptidase activity of the PAG preparation at pH 3.5 through 4.5, which could be inhibited by the aspartic peptidase inhibitor, pepstatin A.

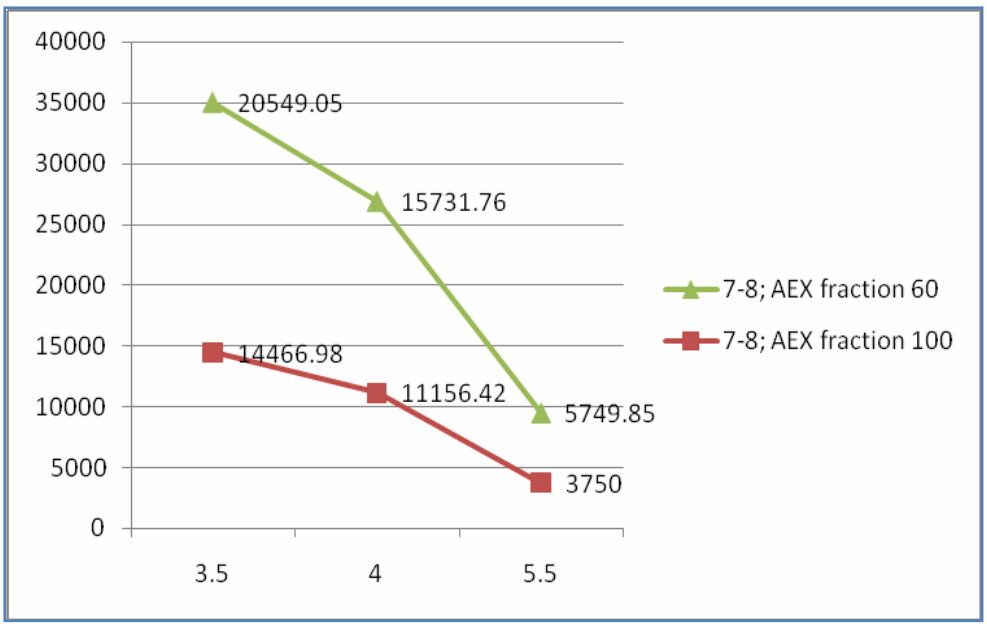


Figure - 46. pH profile of the activity of native bovine PAG (preparation 7-8) against a fluorescent peptidase substrate. Native bovine PAG was bound to PepstatinA-agarose affinity column at pH 7.0 and eluted at pH 8.0 in the presence of the detergent, Triton X-100. Further purification was performed over a DEAE-anion exchange (AEX) column and two elution fractions (60 and 100; corresponding to 75 and 150 mM NaCl, respectively) were assayed

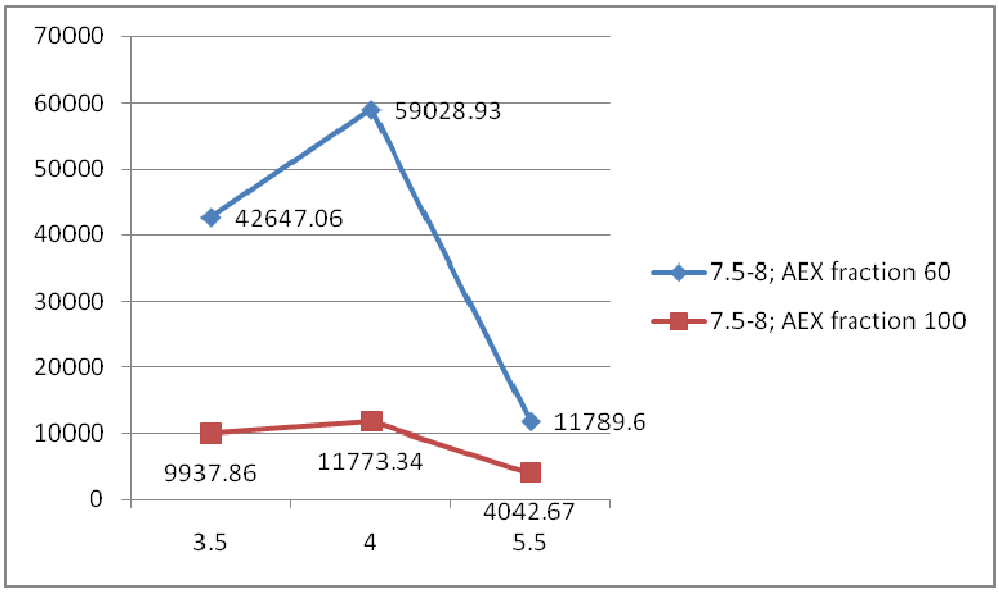


Figure - 47. pH profile of the activity of native bovine PAG preparation 7.5-8 against a fluorescent peptidase substrate. Native bovine PAG bound to PepstatinA-agarose affinity column at pH 7.5 and eluted at pH 8.0 in the presence of the detergent, Triton X-100. Further rounds of purification over a DEAE-anion exchange (AEX) column were performed exactly as for the experimental data represented in Figure 2. Again, fractions 60 (75 mM NaCl) and 100 (150 mM NaCl) were assayed.

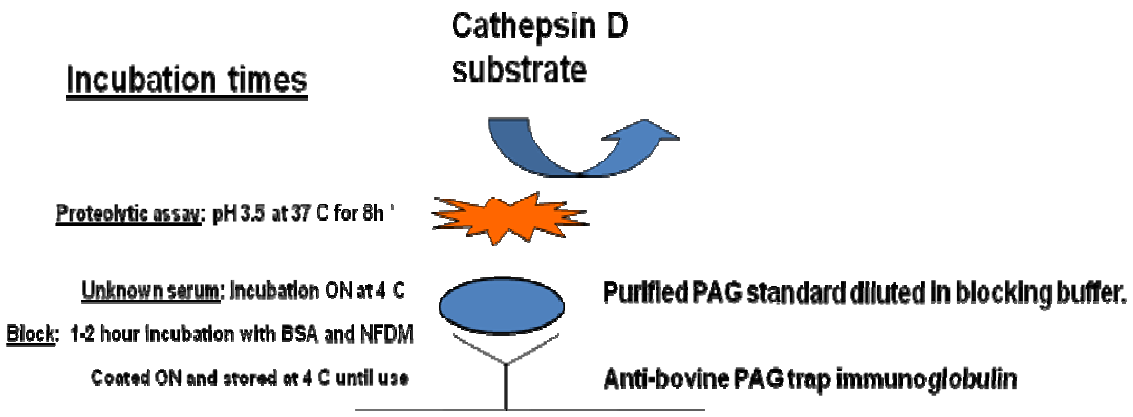


Figure - 48. A schematic illustrating the immune-trap peptidase assay that makes use of antibodies to selectively immobilize PAGs onto an ELISA dish, followed by detection of the bound PAGs by measuring their peptidase activity against a peptide substrate.

TRAP Ab	50ng-std	5ng-std	50ng-Blank
POLY	575724	573585	37731
MONO	103779	63068	30892
PAG-10	75774	46290	15173

Rabbit-Ig
6037

Table -6. Results from Immuno-trap peptidase assay with native PAG-standards.

The antibodies employed to selectively trap PAGs from solution in this experiment were 1. polyclonal antibodies (POLY) raised against a mixture of native PAGs (Green et al., 2005; Wooding et al., 2005); 2. three specific monoclonal antibodies (MONO) directed toward selected PAGs (Green et al., 2005); 3. a polyclonal anti-PAG-10 (PAG-10) antibody raised against recombinant bovine PAG-10. The blank (Blank) consisted of 50ng of bovine serum albumin and the background control (Rabbit-Ig) was rabbit immunoglobulins loaded in the same amounts as the other trapping immunoglobulins.

Table -7. Fluorescent product release from immuno-trap peptidase assay,

Row #	Antibody	Protein Source	BSA	5/7 (60)	5/8 (60)	MIXED PAG
1	**Polyclonal (#20)**	Native PAGs	10292	10797	***19688***	***20492.5***
2	**Monoclonal Trap**	Native PAGs	3444	***13761***	5000	***11024***
3	**Monoclonal Trap**	Native PAGs	3911*	***6947***	***8650***	***9738***
4	**Monoclonal Trap**	Native PAGs in NHPS	7584.5	7265	***16953.5***	***12324.5***

Rows 1-3: Polyclonal (#20) and monoclonal antibodies were utilized to trap native PAG preparations from solution. PAG preparation 5/7(60), 5/8 (60) and Mixed PAG (pooled native PAGs) from various preparations. (*) in the table denotes that Heat-inactivated BSA was used in this assay as a loading control. **Last row:** represents an assay in which representative native PAGs were added to the non-pregnant heifer serum (NPHS). Note that monoclonal antibodies were able to specifically pull PAGs with the exception of the 5/7(60) sample from non pregnant sera and that the resultant activity was significantly higher when compared to heat inactivated BSA control wells.

Table -8. Selectively trapping PAGs from pregnant cow sera as detected by proteolysis of a fluorescently tagged peptidase substrate.

	Pregnant cow	Day 0	Day 21	Day 30	Day 40	Day 150	Day 200
Monoclonal Trap	A	7214.5	4754	5584.5	***8410***	***7653.5***	***9892.5***
	B	3808.5	3420	***8424.5***	4094	***8979***	***6370***

The table shows results from trapping PAGs (by using monoclonal antibodies) from sera of two different cows at defined stages of pregnancy and detecting them via proteolytic activity. Note the higher values in samples from D30 onwards reflective of high concentrations of circulating PAG in serum.

Table - 9. Immuno-trap assay of recombinant PAGs in insect cell lysates.

	boPAG-2	boPAG-12	poPAG-2	Control lysate	Ab-control	Empty wells
Anti-FLAG	*27048*	*42298*	*96768*	4116.5	3812	4008
Anti-FLAG	*26547.5*	*22998.5*	*92254.5*	7758.5	6068.5	5299.5
Anti-poPAG-2	1783	3013	*10936*	5717.5	4443	4469

Anti-FLAG monoclonal antibody was used to selectively immobilize recombinant proteins, bovine PAGs 2and 12 and poPAG-2 by binding to the FLAG peptide present on the amino-terminus of the recombinant proteins. The resultant activity from these were significantly higher than the control insect cell lysate, which represented insect cells (sf9 cells) infected with empty Baculovirus. Controls consisted of trapping antibody, but no lysate added (Ab-control) as well as empty wells used to estimate the background fluorescence. Also note for comparison that a polyclonal antibody raised against porcine PAG-2 was able to specifically trap recombinant poPAG-2, but not boPAGs 2 and 12 from its corresponding lysate and that the activity was significantly higher than the control wells.

Table -10. Relative fluorescent product release from recombinant bovine PAGs obtained from immuno-trap peptidase assay with anti-FLAG monoclonal antibody.

Recombinant PAG	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0
6	***83970.5***	***77910.5***	***66336.5***	***31300.5***	24296
8	***80567.5***	***63634***	***56086.5***	***31924***	26426.5
9	***72112.5***	***55013***	***48137.5***	27758	24401.5
10	***52075***	***42734.5***	***38844***	26525	23581.5
17	34647	28612	28519.5	23267	23673
19	40253.5	32507.5	32086.5	24268.5	23271.5
20	38630.5	31285	31407.5	24524.5	23780
tsh (control)	38349	33908	34996.5	28222.5	26590.5

Notice that of the different recombinant PAGs tested, PAGs-6, 8, 9 and 10 displayed proteolytic activity within acidic range (pH 3 to 5).

Table -11. Relative fluorescent product release from recombinant bovine PAGs obtained by using an immuno-trap peptidase assay with PAG-specific monoclonal antibodies.

Monoclonal	boPAG -6	boPAG-8	boPAG-9	boPAG-10	boPAG-21	TSH-beta
Mixed	*64554*	*85319.5*	*89343*	48468.5	53946	53027
A6	*67510*	*81389.5*	*91777.5*	52183.5	55432.5	52778
L4	*66321.5*	*80958*	*91452.5*	54533	57730	53980.5
J2	*62766.5*	*86421.5*	*97480.5*	57481	-	-
	poPAG-2	tsh-beta				
FLAG	*97357*	47019.5				

The results from the table indicate that bovine PAGs 6, 8 and 9 were identified by each of the individual monoclonals (A6, L4, J2) as well as a mixture of them (Mixed). PAG-10 was not trapped by any of the candidate monoclonals. The monoclonals either have no affinity for PAG-21 or it is proteolytically inactive. TSH-beta was used as a negative control to establish the baseline for background. An anti-flag antibody (FLAG) and poPAG-2 lysate was used as a positive control for the experiment.

Appendix –II

Comparison of cell numbers of embryos cultured in isolation or in groups

Individual cultures	Group Cultures
19	18
21	24
17	38
30	6
27	16
17	19
15	22
22	17
8	14
21	20
7	14
	27
	30
	29
	19
	13
	20
$\bar{x}$ 18.5	$\bar{x}$ 20.35

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VITA

Bhanu Prakash V.L. Telugu was born to Krishna Veni and Suri Babu Telugu on August 24, 1977, in Srikakulam, Andhra Pradesh, India. After obtaining secondary education from prestigious A.P. Residential Junior College in Nagarjuna Sagar, A.P. India, he attended College of Veterinary Science, Tirupati, A.P. India, where he graduated with honors in November, 2001, and received Bachelors of Veterinary Science and Animal Husbandry degree. Following, undergraduation in veterinary school, he began pursuing doctoral degree under the able guidance of Dr. Jonathan A. Green in the Division of Animal Science at University of Missouri in Columbia. During his graduate career, he was supported by Miller graduate fellowship and graduate research assistantships. On May 9, 2008, he completed the requirements of graduate school and became eligible for graduation in summer semester of the year 2008. He joined Dr. R. Michael Roberts's laboratory in the capacity of a postdoctoral fellow.