EXPRESSION LEVELS AND LOCALIZATION OF PREGNANCY-ASSOCIATED PLASMA PROTEIN-A2 (PAPP-A2) IN MOUSE PLACENTA AND EMBRYO

by

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ABSTRACT

Pregnancy-associated plasma protein-A2 (PAPP-A2) is a metalloproteinase that specifically cleaves insulin-like growth factor binding protein-5 (IGFBP-5), a major modulator of insulin-like growth factor (IGF) bioavailability. Among several murine tissues surveyed, PAPP-A2 is most highly expressed in the placenta, and is also present in pregnancy serum and embryo. In the placenta, PAPP-A2 is localized in the junctional zone, where its degradation of IGFBP-5 may lead to the release of IGFs and so promote the development of the fetal portion of the placenta into the uterine wall. These expression patterns suggest that PAPP-A2 may play important roles during murine pregnancy. Cloning of the gene to further investigate its biochemical actions and allelic variation was also attempted. This work offers insight into the potential importance of a poorly-understood protein which may play crucial roles during pregnancy and fetal development in both mice and humans.

Keywords:
Pregnancy-associated plasma protein-A2
IGFBP-5 proteolysis
Mouse placenta
Pregnancy
Trophoblast invasion
Junctional zone
Gene cloning

To My Family

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CHAPTER 1: INTRODUCTION

1.1. Introduction

Insulin-like growth factors (IGFs) are polypeptides that are essential for normal growth and development (Le Roith *et al.* 2001). IGFs I and II, cell-surface IGF-I and IGF-II receptors, IGF binding proteins (IGFBPs) and an ever-growing list of proteolytic enzymes that cleave IGFBPs make up the IGF-axis (Beattie *et al.* 2006) (Figure 1.1). As implicated by their names, certain regions of the IGFs have similar sequences to insulin (Blundell *et al.* 1978). Similarly, the IGF-I receptor also shows a high degree of structural and functional similarity with the insulin receptor (Sara and Hall. 1990). On the other hand, the IGF-II receptor is not homologous to either the IGF-I or insulin receptor, yet it binds to IGF-II with high affinity, and is involved in the sequestering of IGF-II for intracellular degradation (Brown *et al.* 2008).

IGFs is mainly associated with cell growth, survival, and differentiation (Siddle *et al.* 2001), and the specific actions of IGFs appear to be concentration-dependent (Ewton *et al.* 1994). Binding of IGFs to the IGF-I receptor, a protein with an extracellular IGF-binding site and a transmembrane domain bearing tyrosine kinase activity, triggers the activation of the tyrosine kinase, which subsequently phosphorylates various cytoplastmic substrates such as the insulin receptor substrate (IRS)-1/4, and the Shc (Src homology collagen) proteins (Laviola *et al.* 2007). Upon interaction with the IGF-I receptor, the phosphorylated substrates recruit and activate more kinases, thereby

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prompting a cascade of actions leading to downstream effects such as glucose transport, glycogen and protein synthesis, mitogenesis, anti-apoptosis, and regulation of gene transcription (Shepherd et al. 1998). Because of its important roles and the variety of its target cell types, such as osteoblasts (Wang et al. 1995), skin cells (Hodak et al. 1996), myoblasts (Kaliman et al. 1998), pancreatic β-cells (Kulkarni et al. 1999), overexpression of IGF-I is associated with changes in phenotype in mice, such as substantial bone and muscle growth (Mathews et al. 1988), whereas a targeted mutation in the IGF-I receptor in transgenic mice result in severe growth retardation, thin epidermis, and early mortality presumably due to under-developed respiratory muscles (Liu et al. 1993). Like IGF-I, IGF-II is also important for growth, particularly during prenatal development and placental formation in rodents (Constancia et al. 2002; Forbes and Westwood. 2008).

Because of their crucial functions, the bioavailability of IGFs is tightly modulated by a group of proteins known as the IGFBPs. The IGFBPs bind to IGFs with high affinity and therefore regulate their access to the IGF receptors (Butt et al. 1999). IGFs have both endocrine and paracrine effects (Mohan et al. 1996), and IGFBPs are also observed to influence both the local (Roberts et al. 2008; Giudice et al. 2002; Hamilton et al. 1998) as well as endocrine actions of IGFs (Miyakoshi et al. 2001b; Sugimoto et al. 1997). To date six IGFBPs have been discovered and characterized (Clemmons. 1998; Rajaram et al. 1997), and the bioavailability of these IGFBPs is further modulated by different types of proteases (Beattie et al. 2006). Like all other IGFBPs, IGFBP-5 binds to IGFs, restricting access to IGF receptors and hence downstream effects (Beattie et al. 2006). It should be noted that IGFBP-5 has much greater affinity for IGFs than do IGF receptors;

IGFBP-5 thus plays an important role in regulating IGF bioavailability (Beattie et al. 2006). The interaction between IGFs and IGFBP-5 is also interesting; IGF-I has been shown to stimulate the expression of IGFBP-5 in the mouse brain (Ye and D'Ercole. 1998), and IGFBP-5 is less vulnerable to proteolysis when bound to IGF (Camachohubner et al. 1992). Moreover, some evidence suggests that IGFBP-5 has mitogenic effects independent of the presence of IGF in some systems (Schneider et al. 2002) especially in bone cell formation (Andress and Birnbaum. 1992; Mohan et al. 1995; Richman et al. 1999). As a result, IGFBP-5 has been studied extensively, and it appears that it is subjected to proteolysis by various proteolytic enzymes (Busby et al. 2000; Hou et al. 2005; Mohan et al. 2002). Recently, a novel proteinase, pregnancy-associated plasma protein-A2 (PAPP-A2) was identified (Overgaard et al. 2001) that cleaves IGFBP-5 in humans (Overgaard et al. 2001), and is a candidate gene for a quantitative trait locus (QTL) affecting skeletal growth (Christians et al. 2006; Christians and Senger. 2007) and levels of circulating IGFBP-5 in mice (Christians et al. 2006). This QTL is located at mouse chromosome 1 and spans an approximately 0.93-MB region containing 4 genes, of which one is PAPP-A2 (Christians et al. 2006). In a mouse line that only segregates (differs) at this QTL region, but is otherwise genetically homogenous, significantly different tail lengths and circulating IGFBP-5 levels are found among the genotypes, implying that PAPP-A2 may be responsible for the effects of the QTL (Christians et al. 2006). When comparing the sequence variation of the two different genotypes, 15 synonymous single nucleotide polymorphisms (SNPs) and 14 non-synonymous SNPs were found to exist in the PAPP-A2 coding region (Christians et al. 2006). These non-synonymous SNPs may cause differential proteolytic activity of

PAPP-A2, leading to phenotypic variation (i.e. skeletal growth and IGFBP-5 physiology) (Christians *et al.* 2006).

PAPP-A2 codes for a proteolytic enzyme that specifically cleaves insulin-like growth factor binding protein-5 (IGFBP-5) *in vitro* (Overgaard *et al.* 2001). Although the biochemical properties of PAPP-A2 are well-characterized (Overgaard *et al.* 2001), very little is known about its expression profile and tissue-specific functions. In contrast, a structurally similar protease, pregnancy-associated plasma protein A (PAPP-A), has been extensively studied in various cell types (Bunn *et al.* 2004; Conover *et al.* 2004a; Lawrence *et al.* 1999). PAPP-A is known to be the major protease of IGFBP-4 in human pregnancy serum (Byun *et al.* 2001), and it also cleaves IGFBP-5 (Laursen *et al.* 2001). In the human placenta, PAPP-A is a critical player in the proteolysis of IGFBP-4, elevating local IGF bioavailability (Giudice *et al.* 2002). PAPP-A is detected during early gestation (Hossenlopp *et al.* 1990) and its circulating levels in pregnant women have been used for diagnosing fetal disorders such as Down syndrome (Berry *et al.* 1997; Casals *et al.* 1999) and Cornelia de Lange syndrome (Aitken *et al.* 1999). Moreover, PAPP-A levels are positively correlated with birth weight (Peterson and Simhan. 2008), and reduced PAPP-A levels are associated with higher risk of preterm delivery (Dugoff *et al.* 2004), pregnancy loss (Krantz *et al.* 2006) and pre-eclampsia (Smith *et al.* 2002; Spencer *et al.* 2008). PAPP-A is expressed in trophoblasts, which are invasive cells that facilitate embryo implantation in the uterus. As a result, IGF-mediated trophoblast invasion into the maternal tissue is regulated by PAPP-A (Jones and Clemmons. 1995; Sun *et al.*

2002). Interestingly, while PAPP-A is expressed at high levels in human placenta, PAPP-A is not particularly abundant in mouse placenta (Qin *et al.* 2002).

Even though PAPP-A is not highly expressed in murine placenta during pregnancy, it has been found to be important in embryonic development in mice (Conover *et al.* 2004b). Disruption of the PAPP-A gene led to delayed skeletal growth, retarded development as well as impaired IGF-mediated growth (Conover *et al.* 2004b). As PAPP-A2 transcripts have been shown to be expressed in mouse embryos (Christians *et al.* 2006), it is possible that it also plays important roles during embryonic growth by regulating IGFBP-5 bioavailability. Because IGFBP-5 is responsible for various developmental processes such as bone formation (Miyakoshi *et al.* 2001a), muscle development (Wang *et al.* 1995), and mammary gland morphogenesis (Ning *et al.* 2007), it will be beneficial to identify specific embryonic tissues that express PAPP-A2 in order to study the function of PAPP-A2 and its interaction with IGFBP-5.

To gain a better understanding of PAPP-A2's role *in vivo*, my primary task was to identify where the gene is expressed. I was particularly interested in examining PAPP-A2 expression in two types of tissues, embryo and placenta, because of the important roles played by PAPP-A during embryonic development and it is possible that PAPP-A2 has similar functions in these tissues. In my project, I examined PAPP-A2 expression at the mRNA level in whole mouse embryos by *in situ* hybridization and at the protein level in placenta sections by immunohistochemistry, and compared PAPP-A and PAPP-A2

transcript levels by quantitative PCR. Also, I determined whether the protein is secreted into circulation during pregnancy.

I also attempted to express PAPP-A2 in vitro. Recombinant human PAPP-A2 has been shown to possess proteolytic activity specifically against human IGFBP-5 (Overgaard *et al.* 2001), and although mouse and human PAPP-A2 proteins demonstrate 80% identity at the amino acid level (Ensembl. 2008), it is not known whether mouse PAPP-A2 shows the same substrate specificity, or whether it also cleaves other IGFBPs. Moreover, as there are 14 non-synonymous SNPs between QTL alleles in the coding region of PAPP-A2 (Christians *et al.* 2006), it would be interesting to investigate whether the allelic variation leads to different proteolytic activity of the translated protein.

1.2. Figures and Figure Legends
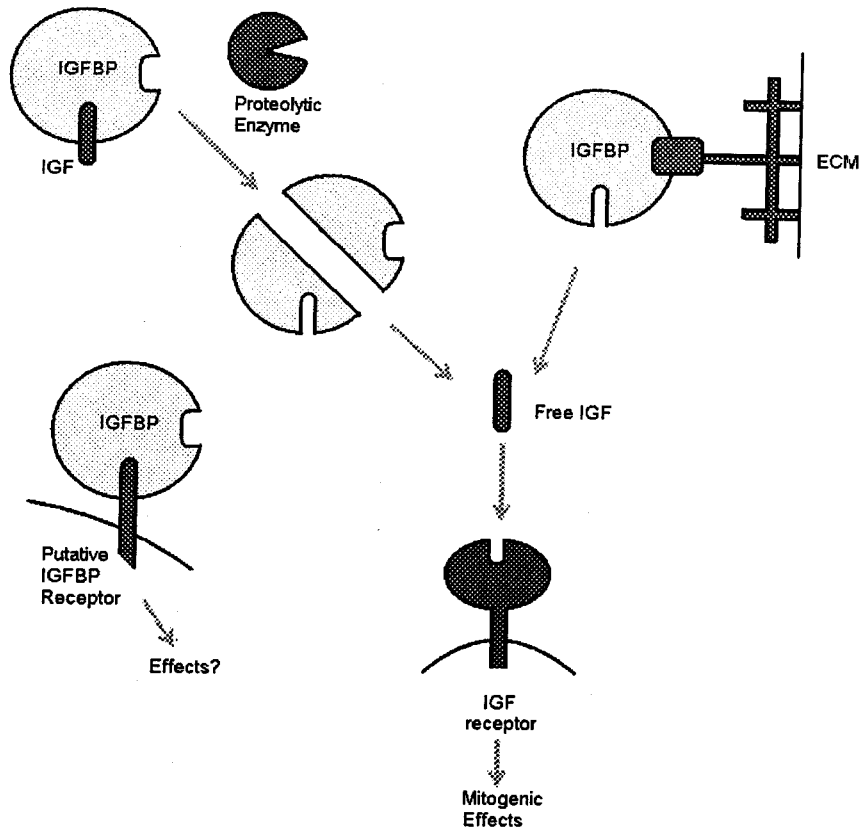
Figure 1.1: Components of the insulin-like growth factor (IGF) system. Intact insulin-like growth factor binding proteins (IGFBPs) bind to IGFs and regulate their bioavailability. When the IGFBPs are cleaved by proteases, IGFs are released and free to bind to receptors. Some IGFBPs also have been shown to have their own cell-surface receptors or bind to extracellular matrix (ECM). IGFBP-5 bound to ECM has substantially reduced affinity for IGFs, allowing the IGFs to interact with their receptors more freely (Clemmons. 1998) (Figure adapted from Marshman *et al*. 2002).

CHAPTER 2: LOCALIZATION AND EXPRESSION LEVELS OF PREGNANCY-ASSOCIATED PLASMA PROTEIN-A2 (PAPP-A2) IN MOUSE PLACENTA AND EMBRYO

2.1. Introduction

The placenta acts as the interface between the fetus and the mother during gestation (Cross *et al.* 1994). Abnormal placental development leads to serious consequences for both fetal and maternal health, such as intrauterine growth restriction (IUGR) (Jackson *et al.* 1995; Salafia *et al.* 1995), and hypertensive disorders such as pre-eclampsia (Redman and Sargent. 2005). During the formation of the placenta in primates and rodents, the epithelium of the uterus is eroded by embryonic trophoblasts, which leads to direct contact between maternal blood and fetal trophoblastic villi, facilitating efficient diffusion between maternal and fetal blood (Rossant and Cross. 2001). The invasive action of trophoblasts is aided by proteases that regulate the bioavailability of growth factors (Salamonsen. 1999; Lala and Hamilton. 1996). One of the critical proteases involved in normal placental development is pregnancy-associated plasma protein-A (PAPP-A) (Sun *et al.* 2002), which is produced by human trophoblasts (Tornehave *et al.* 1984) where it is the major proteolytic enzyme cleaving insulin-like growth factor binding protein-4 (IGFBP-4) (Boldt and Conover. 2007). Degradation of IGFBP-4 by PAPP-A leads to the release of insulin-like growth factor II (IGF-II) (Giudice *et al.* 1998; Giudice *et al.* 2002) which promotes the development of the placenta through its positive influence on trophoblast invasion as well as growth and permeability of this highly specialized organ (Constancia *et al.* 2002). Moreover, PAPP-

A is secreted into the maternal circulation (Folkersen *et al.* 1981), and can be a useful marker for certain diseases and pregnancy complications. For example, unusually low levels of PAPP-A are indicative of fetal genetic disorders such as Down's syndrome (Brambati *et al.* 1993) and Cornelia de Lange syndrome (Aitken *et al.* 1999). Furthermore, low PAPP-A levels in maternal blood are associated with higher risk of low birth weight (Smith *et al.* 2002), preterm delivery (Dugoff *et al.* 2004), miscarriage (Kwik and Morris. 2003) and pre-ecampsia (Spencer *et al.* 2008), which are characterized by impaired trophoblast invasion.

Although PAPP-A is highly expressed in human placenta (Tornehave *et al.* 1984), its expression in the placenta is not higher than that in a number of other adult tissues in the mouse (Qin *et al.* 2002; Soe *et al.* 2002). Nevertheless, PAPP-A plays an important role in fetal development as PAPP-A deficient mice show delayed and impaired growth (Conover *et al.* 2004b).

Recently, another protease similar to PAPP-A was identified (Farr *et al.* 2000; Overgaard *et al.* 2001; Page *et al.* 2001), and designated PAPP-A2 (earlier names included PAPP-E and Plac3). Like PAPP-A, PAPP-A2 is abundantly expressed in the human placenta (Farr *et al.* 2000; Page *et al.* 2001), and also cleaves an IGFBP (Overgaard *et al.* 2001). Unlike PAPP-A, which proteolyses both IGFBP-4 and IGFBP-5 (Boldt *et al.* 2004), PAPP-A2 cleaves IGFBP-5 and may also show a lower proteolytic activity against IGFBP-3 (Overgaard *et al.* 2001). PAPP-A2 has also been identified as a candidate gene for a quantitative trait locus (QTL) affecting skeletal growth and levels of

circulating IGFBP-5 in mice (Christians *et al.* 2006; Christians and Senger. 2007). Christians *et al.* semi-quantitatively surveyed PAPP-A2 expression in various murine tissues, and found its expression to be strongest in embryo, stomach, and skin, but this study did not examine the placenta (Christians *et al.* 2006). Therefore, the goal of the current study is to examine the expression and localization of PAPP-A2 in placenta and embryo.

2.2. Materials and Methods

2.2.1. Animals and sample collection

Mice (Charles River Laboratories, QC, Canada) were housed in the Animal Care Facility at SFU and all procedures were in accordance with the guidelines of the Canadian Council on Animal Care. Six CD1 female mice were time-mated with CD1 studs, and the day the seminal fluid plug was found was designated as day 0.5 post-coitus (dpc). On 12.5 dpc, females were sacrificed by CO_2 inhalation. Embryos, placentae, stomach, kidneys, and liver were quickly dissected out in DEPC-treated 10X PBS (Nagy *et al.* 2003), and these samples were immediately either frozen at -20 °C for protein work, fixed in 4 % paraformaldehyde overnight for *in situ* hybridization (placentae and embryos only), or placed in RNAlater® (Ambion, Foster City, CA) overnight at 4 °C and then stored at -20 °C prior to quantitative PCR. Non-pregnancy serum samples were collected from live mice from the saphenous vein, and pregnancy serum samples were collected from sacrificed mice by cardiac puncture. Blood samples were centrifuged, and serum was collected and stored at -80 °C until further use.

2.2.2. RNA Isolation and Quantitative PCR

Quantitative PCR was performed to compare expression levels of PAPP-A2 and PAPP-A transcripts (n=6). Total RNA was extracted from various tissues, including the placenta, liver, kidney, and stomach, using QIAshredder homogenizers (Qiagen, ON, Canada) and RNeasy spin columns (Qiagen) following the manufacturer's instructions. Each sample was standardized to contain 50 ng/μL of total RNA. To assess the expression levels of PAPP-A and PAPP-A2 in tissue samples relative to the expression of each gene in the placenta, a reference sample was prepared by combining aliquots of placental samples, and this reference sample was included in every assay. Placental samples were used as a reference because preliminary work showed that they produced a consistently strong signal. The inclusion of a reference sample in every assay enabled me to account for variation between assays. The relative expression levels of each gene of interest were measured in the tissue samples and reference sample by quantitative PCR with the MJ Mini Thermal Cycler (Bio-Rad, Hercules, CA) using primers and probes (Integrated DNA Technologies, Coralville, IA) described in Table 2.1. The expression of a "housekeeping gene", β-actin, was also measured. All primers were designed to span introns to avoid detection of genomic DNA. iScript One-Step RT-PCR Kit for Probes (Bio-Rad) was used to reverse-transcribe and amplify the RNA template for 40 cycles, and the cycle at which the signal rose above a fixed threshold (Ct) was determined. The quantitative PCR amplification was performed using 25 μL reaction volumes containing reaction mix (Bio-Rad), Protector Rnase Inhibitor (Roche Applied Sciences, QC, Canada), 0.5 U of iScript RTase, 8.5 μL of RNA template (i.e., 425ng), 0.25 mM of each primer, and 175 nM of probe. The quantitative PCR program consisted of an initial

reverse transcription of 30 minutes at 50 °C, an initial PCR activation step of 15 minutes at 95 °C followed by 40 cycles of 1 minute at 94 °C, 1 minute at 55 °C, and 1 minute at 72 °C.

Samples were measured in triplicate and the Ct values for PAPPA-2 and PAPP-A were normalized to those of β-actin using the method of Pfaffl (Pfaffl. 2001), which involves calculating the efficiency of the PCR for each gene using serial-dilutions of samples. Using this method, expression in a sample is calculated relative to the reference sample measured in the same assay, e.g., a value of 1.5 indicates a sample has 50 % more of a particular transcript than the reference sample, correcting for β-actin.

In order to compare transcript levels between PAPP-A2 and PAPP-A, we estimated transcript copy number using a standard curve constructed for each gene. cDNA samples corresponding to the regions amplified during quantitative PCR were generated with a OneStep RT-PCR Kit (Qiagen). The DNA concentration of each cDNA sample was measured using a NanoDrop spectrometer, and copy number was calculated using cDNA concentration and amplicon length (http://www.uri.edu/research/gsc/resources/cndna.html). cDNA samples were then diluted serially and used as template for quantitative PCR to construct the standard curves.

2.2.3. Western Blotting

Tissue samples were homogenized in T-PER™ Tissue Protein Extraction Reagent (PIERCE, Rockford, IL) and incubated on ice for 15 minutes to allow cell lysis. The

homogenates were then centrifuged at 13000 rpm and supernatant was collected and stored at -80 °C until further use. Complete Protease Inhibitor Cocktail (Roche Applied Sciences) was added to all samples (including serum samples) to prevent protein degradation.

Samples containing 30 μg of total protein were mixed with 5X SDS loading buffer and boiled for 10 minutes. Samples and pre-stained molecular weight markers (Precision Plus Protein Prestained Standards, Bio-Rad, Canada) were loaded onto a 4 % stacking gel and run through a 8 % polyacrylamide gel under reducing conditions. The gels were then equilibrated in transfer buffer and proteins were transferred onto pure cast nitrocellulose membranes (Bio-Rad) using a semi-dry transfer machine (Bio-Rad). After transfer, the membranes were rinsed with filtered PBS and blocked in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, Nebraska) for 1 hour at room temperature. The membranes were then incubated overnight at 4 °C with 1:1000 polyclonal anti-human-PAPP-A2 antibody (R&D Systems, Minneapolis, MN) diluted in Odyssey Blocking Buffer containing 0.1 % Tween-20 (Sigma, ON, Canada). According to the manufacturer, this antibody shows less than 1 % cross-reactivity with PAPP-A. Membranes were washed 4 times for 5 minutes each at room temperature in filtered PBS containing 0.1 % Tween-20 on a shaker to remove excess unbound primary antibody. The membranes were subsequently incubated with 1:10000 fluorescently-labeled secondary antibody diluted in Odyssey Blocking Buffer containing 0.1% Tween-20 and 0.01 % SDS for 45 minutes at room temperature on a shaker. Incubation took place in a dark box to avoid degradation of the fluorescent dye. The membranes were then rinsed 4 times for 5 minutes each at room temperature in filtered PBS containing 0.1 % Tween-20 with gentle

shaking. After washing, membranes were rinsed with filtered PBS to remove residual Tween-20. Lastly, membranes were scanned with an Odyssey infrared imaging system (Li-Cor Biosciences).

2.2.4. Immunohistochemistry

Cross sections of mouse placenta at different stages of pregnancy were obtained from Zyagen (San Diego, CA). Slides were deparaffinized in xylene washes and rehydrated with graded series of ethanol. Antigen retrieval was performed by heating the slides in a microwave for 15 minutes in citrate buffer (pH 6.0). Sections were then incubated in PBS with 3 % H_2O_2 for 10 minutes to inactivate endogenous peroxidase. Blocking steps were performed using serum blocking reagent G, avidin blocking reagent, and biotin blocking reagent (R&D Systems), followed by incubation with polyclonal anti-human PAPPA-A2 or polyclonal anti-mouse IGFBP-5 antibody (1:50, R&D Systems) in PBS overnight at 4 °C. After 3 washes in PBS, samples were incubated with biotinylated secondary antibody (R&D Systems) for 30 minutes at 37 °C, followed by streptavidin conjugated with HRP, and visualized with DAB substrate (R&D Systems). The slides were then counterstained with hematoxylin. To evaluate the extent of non-specific immunostaining, primary antibodies were substituted with goat anti-rabbit IgG (1:50) (Sigma) as negative controls.

2.2.5. Probe synthesis for in situ hybridization

Mouse placental mRNA was reverse-transcribed into cDNA using the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, ON, Canada), and

600 bp of the PAPP-A2 gene was amplified using the following primers: 5'-CAGAGGGAGGACAGAGCAA-3' and 5'-GTAAAGGTGACAGAATCTCAGG-3'. The 600 bp cDNA was inserted into TA TOPO cloning vector (Invitrogen, ON, Canada) and used as template for *in vitro* transcription. A 660 bp fragment of the IGFBP-5 gene was also amplified (forward primer: 5'-ACGAGAAAGCTCTGTCCATGTGTC-3', reverse primer: 5'-GCTTCATTCCGTACTTGTCCACAC-3') and cloned into TA TOPO cloning vector. Similarly, a 434bp fragment of the IGF-II gene was produced using the following primers: 5'-TTCTCATCTCTTTGGCCTTCGCCT-3' and ACGATGACGTTTGGCCTCTCTGAA. Antisense and sense probes were synthesized by either SP6 or T7 RNA polymerase depending on insert orientation. DIG-labeled RNA was subsequently purified by Quick Spin Columns (Roche).

2.2.6. Whole-mount in situ hybridization

The brain cavity and heart of mouse embryos were punctured to facilitate exchange of solutions and avoid probe trapping. Embryos were rehydrated by passage through 75 %, 50 %, 25 % methanol and twice through PBS containing 0.1 % Tween-20 (PBST). To permeabilize tissues, embryos were treated with 10 μg/ml proteinase K in PBST for 35 minutes at room temperature, and rinsed briefly in PBST containing 2 mg/ml of glycine. Postfixation was in 4 % PFA with 0.1 % glutaraldehyde for 20 minutes. After one rinse and one wash in PBST, embryos were equilibrated in 1:1 PBST/hybridization mix at room temperature. Hybridization mix was composed of 50 % formamide, pH 5 1.3X SSC, (Invitrogen), 5mM EDTA, 50 μg/ml yeast RNA core particle (Sigma), 0.2 % Tween-20, 0.5 % CHAPS (Sigma), 100 μg/ml heparin (Sigma)

and RNAse-free water, adjusted to pH 8 (Correia and Conlon. 2001). Embryos were prehybridized at 65 °C for at least 1 hour and hybridized for 36 hours at 65 °C with 1 μg/mL of either antisense or sense (for negative controls) DIG-labeled RNA probes. After hybridization, embryos were rinsed twice, followed by two 30-minute washes with prewarmed hybridization buffer at 65 °C. The solution was then replaced by 0.1 M maleic acid containing 0.1 % Tween-20 (MABT) at room temperature. The embryos were incubated in MABT containing 2 % Boehringer Blocking Reagent (BBR) (Roche) for one hour at room temperature with gentle shaking, and the solution was replaced with MABT containing 2 % BBR and 20 % heat-treated goat serum (Sigma) for at least one hour. The embryos were subsequently incubated with MABT + 2 % BBR + 20 % sheep serum +1/5000 dilution of sheep anti-digoxigenin Fab fragment covalently coupled to alkaline phosphatase (Roche) on rotation overnight at 4 °C. The next day embryos were rinsed three times with MABT, and washed at least five times for 2-4 hours each and incubated with fresh MABT overnight. To visualize probes bound to the embryo, a colour substrate that reacts with alkaline phosphatase, NBT/BCIP, was added to the detection buffer consisting of 100mM NaCl, 100 mM Tris (pH 9.5) and 0.1 % Tween-20. The embryos were incubated in the solution until colour had developed. Subsequently, the embryos were rinsed once and washed at least twice with PBST. Embryos were fixed again in 4 % PFA/0.1 % glutaraldehyde for two hours at room temperature. Embryos were then stored in PBST+0.1% azide at 4 °C (Nagy *et al.* 2003).

2.3. Results

2.3.1. Expression of PAPP-A2 measured by quantitative PCR

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Previously, PAPP-A2 was found to show highest transcript levels in mouse stomach, skin and embryo, less in the kidney, and was undetectable in liver (Christians *et al.* 2006). However, this previous study did not examine PAPP-A2 transcript levels in the placenta. In the current study, the mRNA levels of PAPP-A2 and PAPP-A in different tissues (placenta, kidney, liver and stomach) were compared to a reference sample composed of a mixture of placental samples, and normalized to β-actin transcript level. PAPP-A transcript levels were not particularly high in mouse placenta compared to other tissues (Fig. 2.1A), but PAPP-A2 expression in the placenta was much higher than in other tissues (Fig. 2.1B). To account for variation between assays, the expression of each gene was reported in relation to a reference sample that consisted of a pool of placental RNA samples. Therefore, the relative abundance of PAPP-A and PAPP-A2 in the placenta were both close to 1 and the expression levels shown in Fig. 2.1 cannot be used to compare expression between genes.

For placental samples, the Ct values for PAPP-A ranged from 24 to 28, whereas the Ct values were in the 16-19 range for PAPP-A2. Although this suggests that the mRNA levels of PAPP-A2 are higher than those of PAPP-A, it is possible that this difference is caused by differences between genes in the efficiency of the quantitative PCR. To estimate the copy number of each gene transcript in placenta samples, Ct values were converted into copy number using standard curves constructed by plotting the log-transformed value of initial cDNA copy number (calculated from cDNA concentration) against its Ct value. The r^2 values were 0.9951 for PAPP-A and 0.9933 for PAPP-A2,

showing a strong correlation between Ct values and copy number. The copy number of

PAPP-A2 in the placenta was estimated to be nearly 1000-fold higher than that of PAPP-

A (PAPP-A: 141 ± 149 copies; PAPP-A2: $1.3\times10^5\pm0.7\times10^5$ copies).

2.3.2. Western Blotting

Using polyclonal anti-human PAPP-A2 antibody, I examined PAPP-A2

expression in mouse tissues. Western blot analysis (Fig. 2.2) showed that PAPP-A2

protein was strongly expressed in the murine placenta, to a lesser extent in the embryo

and that it was also detectable in pregnancy serum. The estimated size of PAPP-A2 was

approximately 250 kDa in mouse (Fig. 2.2). PAPP-A2 protein was not detectable in

mouse non-pregnancy serum, liver, kidney, and stomach (Fig. 2.2).

2.3.3. Localization of PAPP-A2 in the placenta

The spatial and temporal expression pattern of PAPP-A2 protein in the mouse

placenta from mid-placentation to late pregnancy was determined by

immunohistochemistry. Fig.2.3A. shows a schematic illustration of the mouse placenta,

which is composed of three main layers: a layer of decidual cells of maternal origin, the

labyrinth of fetal origin and the intermediate junctional zone. I examined placenta cross-

sections at different gestational stages, 10.5 to 16.5 dpc. Throughout pregnancy, PAPP-

A2-positive signals were detected primarily in the junctional zone (Fig. 2.3B). At 11.5

dpc, IGFBP-5 was expressed in the decidual part of the placenta, adjacent to PAPP-A2 expression (Fig. 2.3B).

2.3.4. Whole mount in situ hybridization

Previously, a cDNA expression panel of various mouse tissues revealed relatively high PAPP-A2 expression level in the embryo, stomach, and skin (Christians *et al.* 2006). In order to determine specific sites of PAPP-A2 expression in the embryo, whole mount *in situ* hybridization was performed. At embryonic day 12.5, PAPP-A2 transcripts were found to be present in the nasal region, forebrain, dorsal side and the sides of the tail (Figs. 2.4A,B). To compare the expression of PAPP-A2 with that of its substrate, *in situ* hybridization was also carried out with IGFBP-5 RNA probes. In agreement with previous findings (Allan *et al.* 2001), IGFBP-5 transcripts were expressed throughout the body except in the brain region, developing whisker barrels and feet (data not shown). IGFBP-5 expression in the tail occurred laterally and along the midline (Fig. 2.4C). There was some co-expression of PAPP-A2 and IGFBP-5 such as in the tail and nasal region. Moreover, IGF-II was found to be expressed in the forelimbs, hindlimbs and tail (Fig. 2.4D).

2.4. Discussion

In the present work, I showed that PAPP-A2, but not PAPP-A, is highly expressed in the mouse placenta. Although PAPP-A is substantially elevated in human placenta (Sun *et al.* 2002), PAPP-A expression in the murine placenta is not particularly high

compared to other tissues (Qin *et al.* 2002; Soe *et al.* 2002; this study). In contrast, PAPP-A2 is expressed at much higher levels in the murine placenta than in other tissues analyzed, such as the kidney, stomach, and liver. Previously, PAPPA2 expression was found to be higher in stomach than in a variety of other adult tissues, including brain, kidney, heart, lung, testis, pancreas and prostate gland (Christians *et al.* 2006). In humans, PAPP-A2 has also been found to be expressed much more strongly in placenta than in other adult tissues (Farr *et al.* 2000; Page *et al.* 2001).

I quantitatively compared the transcript abundance of PAPP-A and PAPP-A2 in mouse placenta and found that PAPP-A2 mRNA levels were nearly 1000-fold higher than those of PAPP-A. Given the fact that PAPP-A plays important roles during pregnancy, the high PAPP-A2 expression in the placenta suggests that it may also have crucial functions in placental physiology and fetal development.

To identify specific sites of PAPP-A2 expression, immunohistochemistry was performed in murine placenta. PAPP-A2 was primarily expressed in the junctional zone, located between the maternal decidua and the fetal labyrinth zone. The junctional zone is mainly composed of two cell types: the spongiotrophoblasts and glycogen trophoblast cells (Coan *et al.* 2005). Glycogen trophobast cells have been proposed to be a specialized subtype of spongiotrophoblast, and potentially involved in the invasion of the decidua by fetal tissue (Adamson *et al.* 2002). The substrate of PAPP-A2, IGFBP-5, is expressed in the metrial gland stroma and the maternal vessel endothelium at the mRNA level in the murine placenta (Carter *et al.* 2006). Given that PAPP-A2 contributes to the

proteolysis of IGFBP-5 (Overgaard *et al.* 2001), its expression in the junctional zone likely leads to the breakdown of IGFBP-5 in the neighbouring decidua, freeing IGF-II molecules to promote growth of fetal tissue into the maternal compartment (Gratton *et al.* 2002). In the human placenta, PAPP-A2 has been found to be expressed in the syncytiotrophoblast layer of placental villi (Nishizawa *et al.* 2008; Winn *et al.* 2009), again consistent with a role in promoting invasiveness into the maternal decidua.

I show for the first time that PAPP-A2 is detectable in the circulation during pregnancy in mice. This observation, together with its potential role in placental invasion, raises the possibility that PAPP-A2 may be a useful biomarker of placental dysfunction, as is the case with PAPP-A. Abnormal levels of human maternal serum PAPP-A in the first trimester have been associated with pre-eclampsia and several other pregnancy-induced complications (Smith *et al.* 2002; Spencer *et al.* 2008). Although the physiological roles of PAPP-A2 *in vivo* remain unclear, the results of several recent studies suggest that PAPP-A2 may be useful for diagnosing problematic pregnancies. Buimer *et al.* (2008) measured mRNA expression levels in placental tissue obtained from mothers with pre-eclampsia, HELLP (Hemolysis, Elevated Liver Enzymes and Low Platelets) syndrome and controls. High expression of PAPP-A2 and several other genes distinguished the expression profiles of HELLP patients from those with severe pre-eclampsia and controls. Similarly, Winn *et al.* (2009) and Nishizawa *et al.* (2008) have found that PAPP-A2 expression levels are altered at both the mRNA and protein levels in pregnancies complicated by pre-eclampsia. Sitras *et al.* further confirmed that PAPP-A2 is upregulated by nearly 3 fold in pre-eclamptic patients (Sitras *et al.* 2009). As elevated

levels of PAPP-A2 are associated with disease, and the localization patterns of PAPP-A2 are similar in both mouse and human placentae, the mouse may be a suitable animal model for studying the physiological roles of PAPP-A2. Also, it is interesting to note that in spite of their similar biochemical functions, abnormally low levels of PAPP-A are indicative of pregnancy complications (Dugoff et al. 2004; Kwik and Morris. 2003; Smith et al. 2002; Spencer et al. 2008) while elevated expression of PAPP-A2 is associated with pre-eclampsia, and this difference suggests that these two IGFBPases may play different roles during pregnancy.

PAPP-A2 is also expressed in mouse embryo (Christians et al. 2006). Using whole mount in situ hybridization, I found that PAPP-A2 gene is expressed in the tail and dorsal side of the embryo, forebrain, and the nasal region. While PAPP-A is present throughout the embryonic tail (Conover et al. 2004b), PAPP-A2 transcripts are laterally located in the tail and the dorsal side of the embryo. The substrate of PAPP-A2, IGFBP-5, has been found to be expressed in various embryonic tissue types, including the developing tail (Allan et al. 2001; Green et al. 1994; Han et al. 1996), as confirmed in this study. The co-localization of PAPP-A2 and IGFBP-5 in certain embryonic tissues suggests that PAPP-A2 may play a role in modulating IGFBP-5 bioavailability in the mouse fetus, and hence affecting prenatal growth. The localization of PAPP-A2 expression in the tail is also consistent with the hypothesis that PAPP-A2 is the gene responsible for a quantitative trait locus affecting tail length (Christians et al. 2006).

This study demonstrates that PAPP-A2 is expressed in both the placenta and embryo in the mouse, suggesting that it plays important roles in early development. The mouse, therefore, may be a useful model for studying the role of PAPP-A2. A better understanding of the role of PAPP-A2 in placental physiology will help to evaluate its potential as a biomarker of placental dysfunction and complications such as preeclampsia and intrauterine growth restriction which have lasting health consequences for the mother and child.

2.5. Figures and Figure Legends
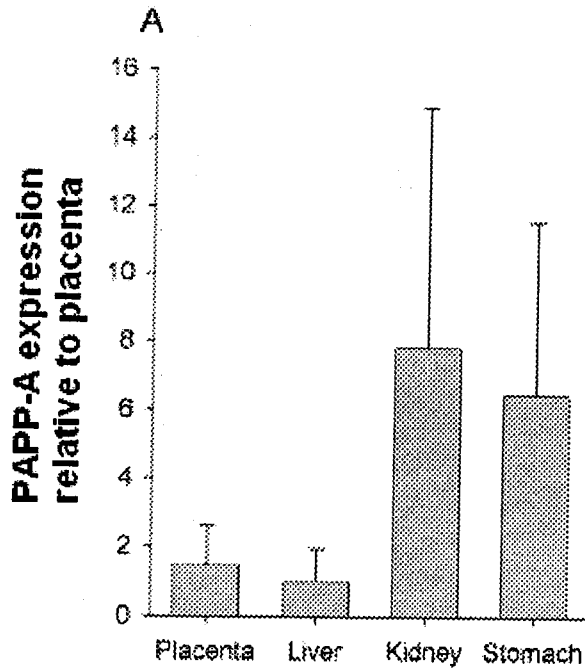
Figure 2.1A. mRNA expression levels of PAPP-A in mouse placenta (n=6), liver (n=3), kidney (n=3) and stomach (n=3). Values are expressed relative to a reference sample composed of a mixture of placental samples, and normalized to β-actin, where a value of 1 is the average expression of PAPP-A in the reference sample. Each column represents the mean ± SD.
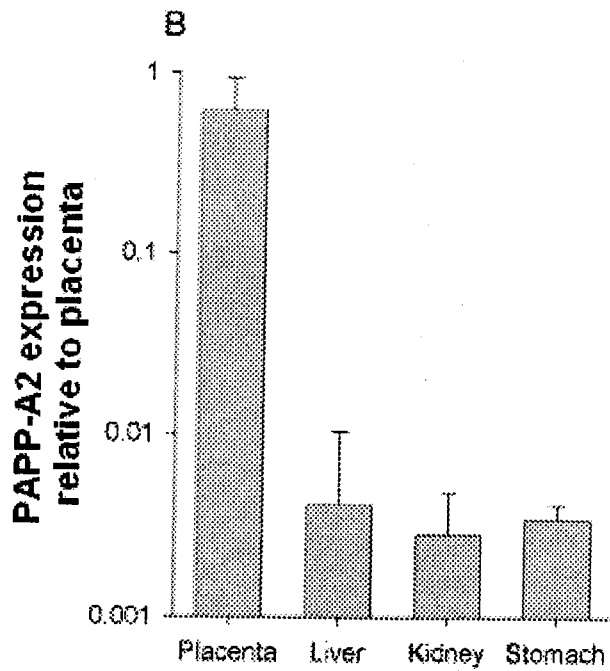
Figure 2.1B. mRNA expression levels of PAPP-A2 in mouse placenta (n=6), liver (n=3), kidney (n=3) and stomach (n=3). Values are expressed relative to a reference sample composed of a mixture of placental samples, and normalized to β-actin, where a value of 1 is the average expression of PAPP-A2 in the reference sample. Each column represents the mean ± SD.
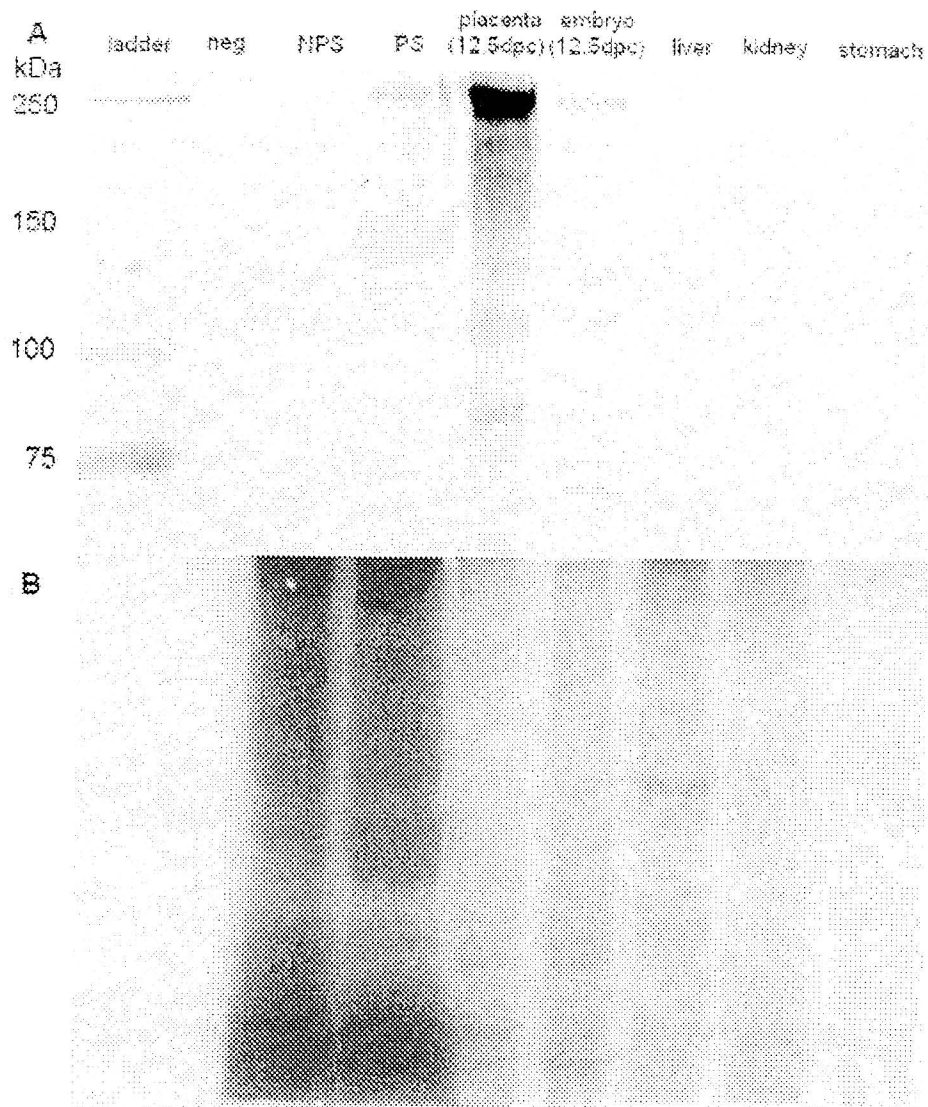
Figure 2.2A. Western blot of PAPP-A2 in various murine tissues. The blot shown is representative of blots obtained from 6 different mice. neg: negative control (water), NPS: non-pregnancy serum; PS: pregnancy serum; dpc: day post coitum. B. EZblue-stained SDS-PAGE gel loaded with the same protein samples as used for immunodetection.
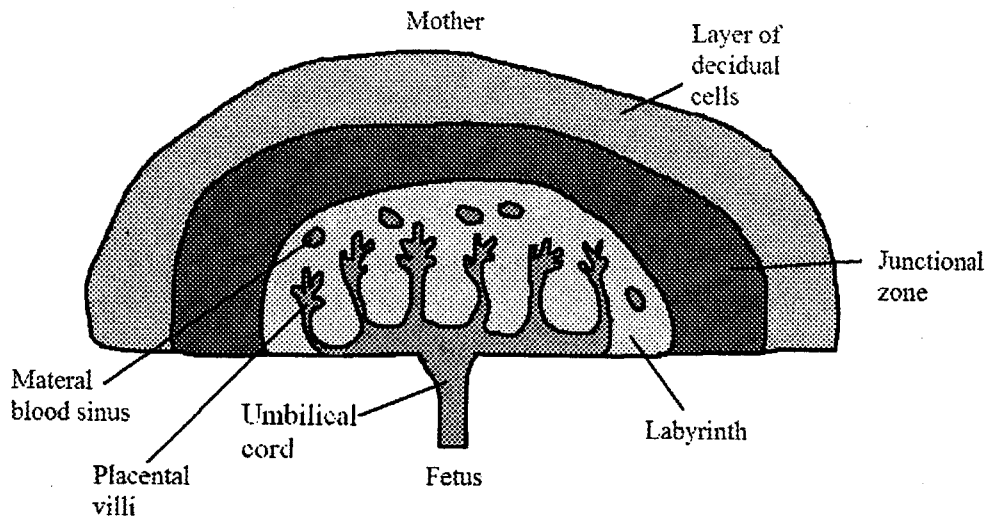
Figure 2.3A. Cross-section of mouse placenta (Adapted from Tycko *et al*, 2002). The placenta is composed of three layers: the decidua, junctional zone, and labyrinth.

PAPP-A2   10.5 dpc   PAPP-A2   11.5 dpc   PAPP-A2   10.5 dpc   20X   PAPP-A2   11.5 dpc   20X

PAPP-A2   12.5 dpc   PAPP-A2   13.5 dpc   PAPP-A2   14.5 dpc   PAPP-A2   15.5 dpc

PAPP-A2   16.5 dpc   PAPP-A2 (neg)   11.5 dpc   IGFBP-5   11.5 dpc   IGFBP-5 (neg)   11.5 dpc
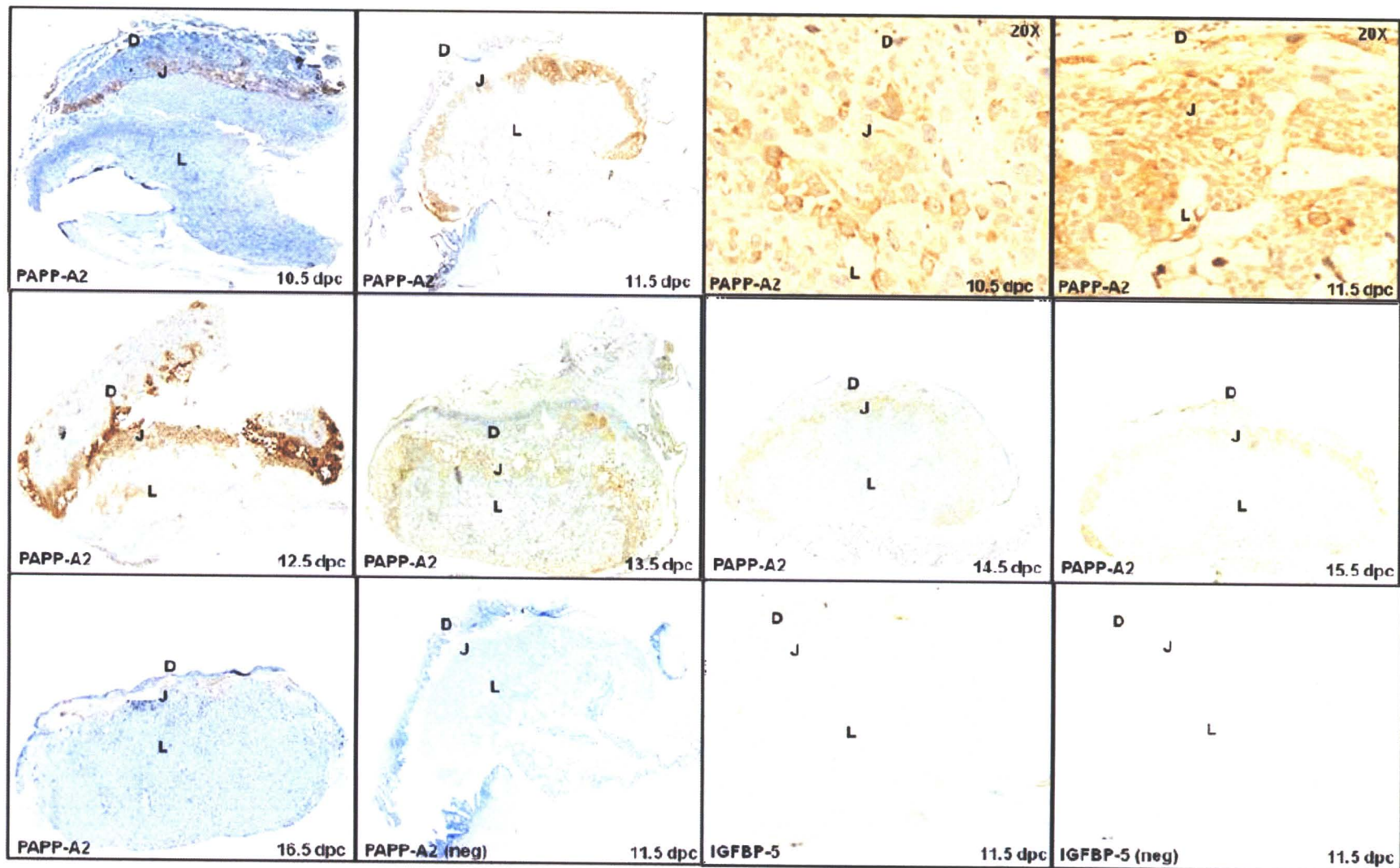
Figure 2.3B. Expression of PAPP-A2 was strongest in the junctional zone in the mouse placenta. IGFBP-5 protein was expressed in the decidual part in the mouse placenta at 11.5 dpc. No staining was observed when non-immune goat IgG was used for immunohistochemical detection in mouse placenta (neg). **D**: decidua, **J**: junctional zone, **L**: labyrinth, dpc: day post coitum. Note that the section showing IGFBP-5 expression was not counterstained with hematoxylin due to low signal level. A negative control is also included in this figure (IGFBP-5-neg) for comparison.
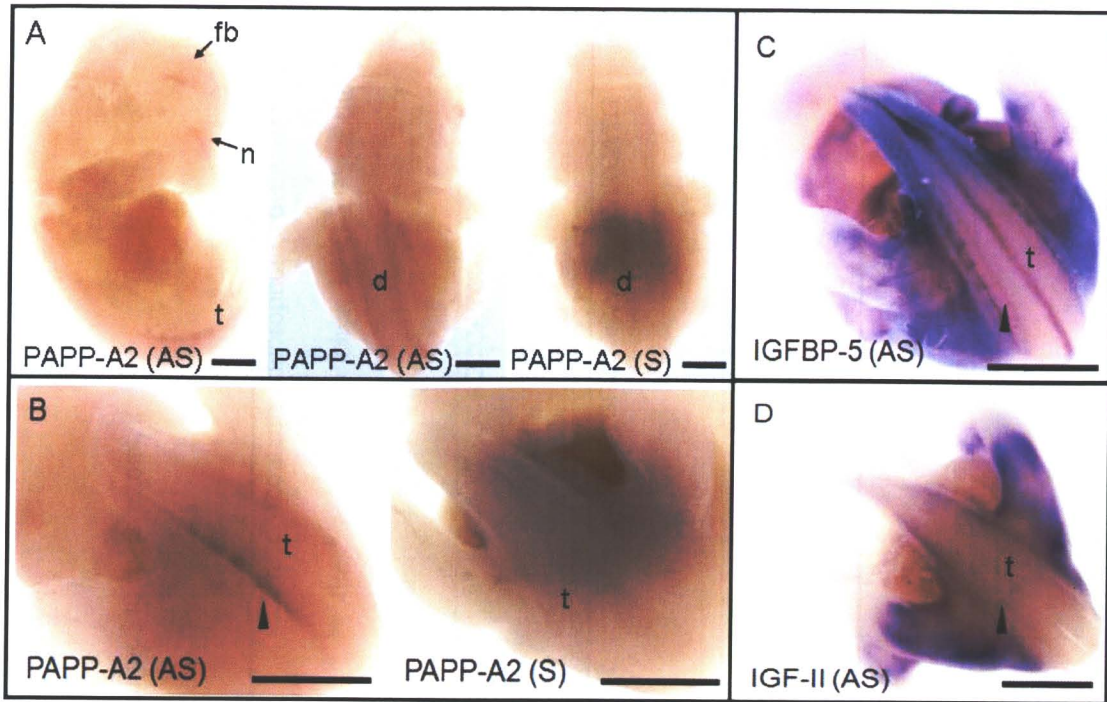
Figure 2.4. PAPP-A2, IGFBP-5, and IGF-II expression in mouse embryos at e12.5. Low levels of PAPP-A2 transcripts were present in the nasal region (n), forebrain (fb), dorsal side (d), and tail (t) (A,B). The expression of PAPP-A2 in the tail laterally co-localizes with that of IGFBP-5, which in turn also co-localizes with IGF-II expression (C,D) (indicated by arrowheads). AS: Antisense probes, S: Sense probes. Scale bar represents 1 mm.

2.6. Tables

Table 2.1. Quantitative PCR primer and probe sequences. All primers were designed to span introns.

Gene	Forward Primer	Reverse Primer	Probe (contains fluorophore, 6-FAM™, on the 5'end and quencher, BHQ-1™, at the 3' end)
PAPP-A	5'-CACAATGGACTCTGTGAT GCT-3'	5'-TCTCCCTTCTAGGCAAAG GT-3'	5'-TGGTTCCCACCCATCGATGG-3'
PAPP-A2	5'-GGGACAAGGAAGCTCTCA GT-3'	5'-CAGGGATCATCACAGGAT TC-3'	5'-CATGCTTGGCCACACCAACATCATG ATCCA-3'
β-actin	5'-CCTAAGGCCAACCGTGAA AAG-3'	5'-CTACGTACATGGCTGGGG TGTT-3'	5'-ACCCAGATCATGTTTGAGAC-3'

CHAPTER 3: CLONING OF PAPP-A2

3.1.   Introduction

Insulin-like growth factor-I (IGF-I) and -II are polypeptides produced in various cell types and secreted into the bloodstream (Clemmons. 2005). They exert their effects in either endocrine or paracrine fashion, and are involved in the regulation of cell division, differentiation and anabolic reactions in different cell types (Beattie *et al.* 2006; Han *et al.* 1987). The bioavailability of IGFs is modulated by IGF binding proteins (IGFBPs) that have higher affinity for the IGFs than the IGF-I receptor (Clemmons. 1998). As a key component affecting the interaction between the IGFs and type I IGF receptor (De Meyts *et al.* 1994), the action of IGFBPs is also highly regulated. Among the six IGFBPs characterized to date (IGFBP-1 – 6), IGFBP-5 has been found to play important roles in many cellular processes, such as embryonic development (Allan *et al.* 2001) and skeletal development (Mohan *et al.* 1995). Moreover, IGFBP-5 possesses stimulatory effects independent of the presence of IGF in certain cellular systems (Miyakoshi *et al.* 2001a). Because of its important roles, the mechanisms regulating IGFBP-5 bioavailability has been extensively studied, and a number of proteolytic enzymes that cleave IGFBP-5 have been identified, such as complement C1s (Busby *et al.* 2000), ADAM-9 (Mohan *et al.* 2002), ADAM-12 (Loechel *et al.* 2000), plasmin (Campbell and Andress. 1997), kallikrein (Iwadate *et al.* 2003), PAPP-A (Laursen *et al.* 2001) and PAPP-A2 (Overgaard *et al.* 2001). Among the characterized proteases, pregnancy-associated plasma protein-A2 (PAPP-A2) is a newly identified metalloproteinase that shows proteolytic activity against insulin-like growth

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factor binding protein-5 (IGFBP-5) and possibly -3 (IGFBP-3) in humans (Overgaard *et al.* 2001).

In humans, PAPP-A2 is highly expressed in the placenta, mammary gland, and kidney (Page *et al.* 2001). In mice, PAPP-A2 is also highly expressed in the placenta and embryo, and is detectable in pregnancy serum (Chapter 2). In the murine placenta, PAPP-A2 is specifically expressed in the junctional zone where trophoblast cells responsible for the invasion of fetal tissue into the maternal decidua reside (Adamson *et al.* 2002). The expression of PAPP-A2 in the junctional zone may contribute to the proteolysis of IGFBP-5 in the neighbouring decidua, releasing IGF-II molecules to stimulate growth of fetal tissue into the maternal compartment. This suggests that PAPP-A2 may play an important role in the development of the murine placenta through regulation of IGFBP-5 bioavailability (Chapter 2).

In a previous study, PAPP-A2 was identified as a candidate gene for a quantitative trait locus (QTL) affecting skeletal size and levels of circulating IGFBP-5 in mice (Christians *et al.* 2006). QTLs are regions of chromosome that contribute to naturally-occurring phenotypic variation in continuous traits. The aforementioned QTL region contains only 4 genes, one of which is PAPP-A2. In a mouse line that varies only at this region, but is otherwise genetically homogenous, tail lengths and circulating IGFBP-5 levels differ significantly among the genotypes, strongly suggesting that PAPP-A2 may be the causal gene underlying effects of this QTL (Christians *et al.* 2006). There are 14 non-synonymous single nucleotide polymorphisms (SNPs) between QTL alleles in the coding region of the PAPP-A2 gene (Christians *et al.* 2006). In order to determine whether these SNPs cause differential proteolytic activity of PAPP-A2, I attempted to clone and express the proteins coded by different alleles *in vitro* and investigate

their level of proteolytic activity against IGFBP-5. This functional assay would provide additional evidence regarding whether or not PAPP-A2 is the causal gene underlying the effects of QTL.

In addition, expression of the mouse gene would be useful for comparing the biochemical actions of human and mouse PAPP-A2, particularly with regards to their substrates. Circulating IGFBP-3 levels are also affected by the QTL described above (Christians *et al.* 2006), which is an intriguing observation as human PAPP-A2 has shown limited proteolytic activity against IGFBP-3 (Overgaard *et al.* 2001). Therefore, it would be useful to examine whether mouse PAPP-A2 also cleaves IGFBP-3, or other IGFBPs.

3.2. Materials and Methods

3.2.1. RNA extraction and cDNA synthesis

Total RNA was extracted from mouse placentae using QIAshredder homogenizers (Qiagen, ON, Canada) and RNeasy spin columns (Qiagen) following the manufacturer's instructions. The quality of RNA was tested by agarose gel electrophoresis. cDNA synthesis was performed with RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, ON, Canada). Approximately 4 μg total RNA and 100μM oligo(dT)$_{18}$ primer were mixed together and denatured at 65°C for 5 minutes. Subsequently, 5X reaction buffer, RiboLock™ RNase inhibitor, 10mM dNTP mix were added to the reaction and incubated at 37°C for 5 minutes. Lastly, 200U RevertAid™ H Minus M-MuLV Reverse Transcriptase was added and the reaction was incubated at 42°C for 60 minutes and terminated by heating at 70°C for 10 minutes. The

quality of the cDNA was then confirmed by three primer pairs that amplify the beginning, middle and end regions the PAPP-A2 cDNA (i.e. PAPP-A2F + Cr02R, Cr21F + Cr21R, and Cr12F + PAPP-A2R) (Primer sequences reported in Christians *et al.*, 2006). A schematic diagram of primer locations is shown in Fig. 3.1.

3.2.2. Long Range PCR

The predicted size of mouse PAPP-A2 cDNA based on the annotated sequence is approximately 5.4 kb (Ensembl. 2008). To improve PCR efficiency and fidelity, a Long Range PCR mix (Fermentas) containing both *Taq* DNA polymerase and a proofreading DNA polymerase was used for cDNA amplification. Primers used were PAPP-A2F: 5'-GAAGCTGACTTTGGGTTCTAAG-3', and PAPP-A2R: 5'-CTGGTTTTCTTCTGCCTTGG-3', and the region spanned by these primers contains the full-length PAPP-A2 cDNA including the start and stop codons. One µL of cDNA was used as template for long range PCR amplification in a 24 µL reaction mixture containing 1X PCR buffer with 15 mM $MgCl_2$, 1 µM of each primer, 0.2 mM dNTP, 4% dimethyl sulfoxide, and 0.5U of long PCR enzyme mix. The PCR procedure consisted of an initial denaturation of 3 minutes at 94°C followed by 10 cycles of 20 seconds at 95°C, 30 seconds at 50°C and 4 minutes at 68°C. The next 30 cycles were done under the same conditions with a 10 second time increase per cycle during the elongation step at 68°C. The final elongation step was at 68°C for 10 minutes before the termination of the reaction. PCR product of correct size was extracted and purified from 1% agarose gel with QIAquick Gel Extraction kit (Qiagen).

3.2.3. Transformation and Transformant Analysis (TOPO TA Cloning, Invitrogen)

3.2.3.1. Ligation and transformation

To enhance cloning efficiency, a post-amplification addition of 3' A-overhangs was done by incubating PCR product with 0.5 unit of *Taq* polymerase, buffer and 2mM dATP for 10 minutes at 72°C. The PCR product was incubated with pCR®II-TOPO vector and salt solution (1.2 M NaCl, 0.06 M MgCl$_2$) (TOPO TA Cloning Kit, Invitrogen, ON, Canada) overnight at room temperature. Chemically-competent *E. coli* cells were thawed on ice, incubated with 2 µL of the ligation product and heat-shocked for 30 seconds at 42°C. Subsequently S.O.C. medium (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, and 20 mM glucose) was added to the transformed cells and the cells were shaken horizontally (200 rpm) at 37 °C for one hour. These chemically-transformed *E. coli* cells were subjected to blue/white selection (40 mg/mL X-gal) and ampicillin (50 µg/mL) screening. White colonies were picked and inoculated in 5 mL LB medium containing 50 µg/mL ampicillin overnight at 37 °C. Plasmid DNA was prepared with GeneJet Plasmid Miniprep kit (Fermentas).

3.2.3.2. Transformant Analysis

The presence of insert was confirmed by either restriction digest or PCR. HindIII, which cut once in the vector and once in the insert, was used to digest the plasmids, and two bands, 3400 bp and 6000 bp, were expected to be generated. Alternatively, circular plasmids were linearized by XbaI, which only cut once in the vector, to determine the total size of the plasmid.

To directly analyze positive transformants using PCR, selected white colonies were resuspended individually in 20μL of water. The cells were then incubated at 94°C for 10 minutes to lyse the cells and inactivate nucleases. To amplify a ~1000 bp fragment of the PAPP-A2 cDNA, PAPP-A2F and PAPP-A2R primers were used in combination with one of the Cr primers which hybridizes within the PAPP-A2 cDNA (sequences provided in Christians et al. 2006) (Fig. 3.1). Polymerase chain reaction (PCR) amplification was performed using 20 μL reaction volumes containing reaction buffer (Fermentas), 0.5U of Taq DNA polymerase (Fermentas), 0.2 mM dNTP, 1 μM of each primer, 2.25 mM $MgCl_2$, 0.2 mM cresol red, and 12% sucrose. PCR consisted of an initial denaturation of 4 minutes at 95 °C followed by thirty cycles of 30 seconds at 95°C, 30 seconds at 50°C and 30 seconds at 72 °C, then a final extension of 7 minutes at 72 °C.

3.2.4. Transformation and Transformant Analysis (CloneJET™ PCR Cloning Kit, Fermentas)

3.2.4.1. Blunt-end ligation and transformation

After using the TOPO TA cloning kit for 3 months without success, the CloneJET™ PCR Cloning kit, designed for cloning large inserts up to 10 kb, was used as a second method of cloning. Gel-purified PCR product was blunted by a DNA blunting enzyme at 70 °C for 5 minutes prior to incubation with pJet1.2/blunt cloning vector and T4 DNA ligase. Ligation time was increased from 5 minutes (as suggested by the supplier) to overnight at room temperature to allow more time for the insert to be taken up by the vector. Bacterial transformation and plasmid isolation were carried out as described above.

3.2.4.2. Transformant Analysis

The presence of insert was determined by either PCR or restriction digest. pJET1.2 forward primer (5'-CGACTCACTATAGGGAGAGCGGC-3'), or pJET1.2 reverse primer (5'-AAGAACATCGATTTTCCATGGCAG-3'), which hybridizes with the vector, was used in combination with one of the Cr primers (Christians et al. 2006). XbaI, which cuts the vector once, was used to analyze the total size of the plasmid.

3.2.5. Transformation and Transformant Analysis (Gateway, Invitrogen)

3.2.5.1. BP Clonase Reaction

Three months after switching to the CloneJET™ PCR Cloning kit, positive transformants carrying the PAPP-A2 fragment had still not been identified. Therefore, a third method of cloning, the Gateway cloning system was employed. This system uses an enzyme (BP clonase) that recombines DNA segments containing specific sequences (attB sites) into vectors that contain the recombination sites (attP sites). The attB sites have to be added to the PCR product using primers containing the attachment sequences. Attachment sites attF and attR were introduced at the 5'- and 3- ends of PAPP-A2 cDNA by PCR with primers PAPP-A2attF 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GAC TTT GGG TTC TAA GGA ACA-3' and PAPP-A2attR 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAC TGG TTT TCT TCT GCC TTG-3' (bold letters indicate att sites added to the PCR product and the remaining sequence indicates PAPP-A2 specific sequence). PCR product (designated PAPP-

A2att to distinguish from the version of PAPP-A2 cDNA used for TOPO TA cloning and CloneJET cloning) was gel-purified with QiaQuick Gel Extraction Kit (Qiagen), and incubated with pDONR221 vector and BP Clonase II enzyme mix at room temperature overnight (Invitrogen). The ligation product was introduced into *E. coli* cells via electroporation. The transformed cells were then plated out on LB agar plates containing 50μg/mL kanamycin, and grown at 30°C overnight. A lower temperature was used to maintain insert stability. Colonies were then picked and inoculated at 30°C for plasmid isolation.

3.2.5.2. Transformant Analysis

The presence of insert was determined by digestion with BamHI, which cut once in the insert. In addition, two plasmid samples were chosen to be sequenced (NAPS, University of British Columbia, BC, Canada) to confirm the accuracy of the PCR and recombination reactions. The efficiency of the BP clonase reaction was further verified by carrying out the reaction with pEXP7-tet control DNA (1.4kb; Invitrogen) and plating transformed colonies on LB agar plates containing 20μg/mL tetracycline. The control DNA sequence should confer tetracycline resistance. An additional positive control experiment was performed with a 2kb DNA fragment generously provided by Adam Foster (Biological Sciences, SFU). The presence of insert was confirmed by restriction enzyme using EcoRV which cuts once in the insert and once in the vector.

3.3. Results

3.3.1. Gel purification

PCR product was gel-purified to eliminate non-specific bands and primer-dimers that might be preferentially cloned into a vector during a ligation reaction (Fig. 3.2). Gel-extraction was performed collectively five times on independent PCR products for the preparation of purified DNA for TOPO TA Cloning and CloneJET™ PCR Cloning methods. Gel-extraction was carried out twice to purify PAPP-A2att PCR product for BP clonase reaction. However, the yield of purified DNA was extremely low (2-10 ng/μL), leading to inappropriate insert:vector ratio and potentially causing unsatisfactory ligation efficiency.

3.3.2. TOPO TA cloning

In total, three PCR trials produced fragments of the correct size. These fragments were gel-purified individually, and one of the purified DNA was modified with 3' A-overhangs. Subsequently, three individual transformation reactions were carried out with these PCR products. In general, transformation of *E. coli* cells with the TOPO TA ligation product resulted in fewer than 20 white colonies on each plate, implying that the PCR product was not effectively taken up by the PCR-II TOPO vector. When PCR was used to analyze the presence of the insert in isolated plasmids or white colonies with various primer pairs binding to the central region of the cDNA, no product was generated, suggesting that the full-length cDNA was not cloned into the vector. Moreover, when the plasmids were digested using different enzymes, the size of linearized bands indicated that the vector did not contain any insert of the correct size.

3.3.3. CloneJET™ PCR Cloning Kit

Overall, two PCR trials resulted in products of the correct size, and three transformations were done with the CloneJET™ PCR Cloning kit. The presence and integrity of insert in positive transformants were analyzed using PCR. As blunt-end ligation reactions allowed the gene to be inserted in 2 possible orientations, it was important to determine the direction of insertion. Therefore, in addition to amplifying the central segment of the cDNA, two primer pairs that each had one priming site within the pJet1.2/blunt cloning vector (pJET1.2F or pJET1.2R) and another within PAPP-A2 were also used for PCR. The primers would then amplify the DNA fragment only if the insert was positioned in a certain direction. Some transformants were also linearized with XbaI to confirm the size of the vector. Although a large number of transformants were analyzed either by PCR or restriction digest, only two samples (Clones A6 and B7) were found to contain a substantial portion of the cDNA. When A6 was used as a template with primer pairs Cr26, Cr28, Cr11, and Cr13, bands of expected sizes were amplified. However, when Cr21 and Cr24 primer pairs were used, no band was generated. Collectively, Cr26, Cr28, Cr11, and Cr13 primers spanned a region approximately 2.7 kb in size near the 3' end of the transcript. Therefore, it appeared that half of the transcript had been truncated during the cloning process. When B7 was used as a template for PCR, all the primer pairs used (Cr21-29, 11-14) resulted in bands of the correct size. However, when the plasmid DNA was digested with HindIII or BsmI, fragments of the expected size were not produced. HindIII was supposed to cut once in the insert at 3345 bp and once in the vector at 624 bp, producing two bands approximately of 6 kb and 2.2 kb; however, only one band of 8 kb was present when B7 was digested with HindIII. Another restriction enzyme, BsmI, theoretically would cut three times in the insert at 2981 bp, 4994 bp,

and 5375 bp, and once in the vector at 722 bp, producing four bands of approximately 5.6 kb, 2 kb, 381 bp, and 332 bp. However, when BsmI was used to digest B7, only 3 bands were produced, and their sizes were approximately 5 kb, 1.5 kb and 400 bp. The result of restriction digest implied that the entire cDNA had not been inserted into the vector, and part of the sequence that contained the restriction sites might have been deleted or mutated during the cloning steps. As PAPP-A2 was manipulated very frequently in our laboratory, bands produced with Cr primers during PCR could be due to possible contamination present in the environment, subsequently leading to the discrepancy between the PCR and restriction digest. Therefore, no full-length PAPP-A2 cDNA was successfully cloned in using the blunt-end cloning kit.

3.3.4. BP Clonase Reaction

PAPP-A2att fragments were gel purified from 2 separate PCR reactions and six transformations were done with the BP clonase reaction. For all BP clonase reaction trials, both PAPP-A2att and pEXP7-tet (control DNA) were used as templates in parallel, allowing the efficiency of ligation and transformation of each DNA fragment to be compared. Transformation with the control DNA always resulted in more colonies on the transformation plates than with the PAPP-A2att DNA. This difference was likely due to the fact that the insert to vector ratio was much higher for the control DNA than for PAPP-A2, and the control DNA is also only 25% of the size of PAPP-A2att. To confirm whether the intact pEXP7-tet control DNA was successfully cloned, transformed *E. coli* cells were plated on LB agar plates containing tetracycline, and the presence of colonies showed that the control DNA was cloned and the tetracycline-resistant gene was expressed. *E. coli* cells transformed with PAPP-A2att ligation

product were used as a negative control and would not grow in the presence of tetracycline. Also, the 2kb DNA fragment provided by Adam Foster was successfully cloned and when analyzed by restriction digest, fragments of the correct size were produced.

The restriction enzyme BamHI, which cut the PAPP-A2att cDNA once, was used to linearize the plasmid, yet no DNA of the correct size was ever found. However, the linearized vector appeared to be slightly larger than just the empty vector alone, therefore it seemed that some form of contamination might be present, and small segments of PCR product were being cloned. Indeed, when the generic M13F and M13R primers were used to amplify the region flanked by M13 sites on plasmid DNA isolated from a clone, a 700 bp band was produced. This finding demonstrated that a small fragment was actually recombined into the pDONR vector during the BP clonase reaction, because the region flanked by the M13 sites would be approximately 2500 bp in the original pDONR vector (if no recombination had occurred). Subsequently, the 700 bp band was sequenced, and it was found that the fragment contained the beginning and end parts of the PAPP-A2att cDNA including the att sites and primer sequences, while the central region (approximately 5000 bp) of the cDNA was missing (Fig. 3.3).

To determine how frequently this excision event occurred, PAPP-A2attF and PAPP-A2attR primers, which were expected to produce a 513 bp band based on the sequencing result, were used to screen 25 more isolated plasmids by PCR, and 21 samples gave bands of approximately 500 bp or less in size (Fig. 3.4). The screening was carried out again with PAPP-A2 primers (without the att sites) to confirm that the PCR was amplifying the PAPP-A2 sequence specifically, and the product was expected to be 450 bp. The same 21 out of 25

plasmids generated bands of 450 bp or less in size, agreeing with the previous screening result (Fig. 3.5). Collectively these results show that a specific region in the PAPP-A2 cDNA was frequently being removed, hence the remaining segment was approximately of the same size in the majority of the plasmids screened. This finding was not likely to be the result of selection against *E. coli* cells carrying the insert, because if that was the case, random portions of the vector would be excised, and the PAPP-A2 primers would have amplified bands of varying sizes in different clones.

To determine whether the removal of part of the PAPPA2 cDNA occurred during the ligation reaction or after transformation, M13F and PAPP-A2*att*R or M13R and PAPP-A2*att*F primer sets were used to amplify a BP clonase ligation product (i.e., prior to transformation) and sequenced plasmid, and bands of expected sizes, approximately 600 bp for both primer pairs, were generated by both the BP clonase reaction and the plasmid, verifying that the removal had occurred during the recombination reaction steps (Fig. 3.6).

4. Discussion

In humans, PAPP-A2 shows proteolytic activity against IGFBP-5 and possibly -3 (Overgaard *et al.* 2001). However, the biochemical action of PAPP-A2 in mice remains unclear. Furthermore, allelic variation in the PAPP-A2 gene has been suggested to be the underlying cause for the effects of a QTL affecting body size and the level of circulating IGFBP-5 in mice (Christians *et al.* 2006). To determine whether PAPP-A2 has similar biochemical actions in both humans and mice, and whether PAPP-A2 proteins coded by different alleles have different

enzymatic activity against its substrate(s) such as IGFBP-5, I attempted to clone and express the mouse PAPP-A2 *in vitro*.

RNA extracted from the mouse placenta, a major site of PAPP-A2 expression (Chapter 2), was used for cDNA synthesis. The 5.4 kb, full-length PAPP-A2 cDNA was then amplified using PCR. However, attempts to introduce the PCR insert into the pCR-II TOPO (Invitrogen) vector failed, possibly because the efficiency of TOPO reaction starts to drop when the insert is larger than 3 kb (Invitrogen technical support, personal communication). The next attempt with the blunt-end pJET1.2 vector (Fermentas) also did not yield any positive transformant containing the full-length cDNA. Two colonies carrying substantial portions of the PAPP-A2 cDNA were identified (A6 and B7), but in A6 the middle of the cDNA had been deleted, and this excision event was also observed in the next section with the BP clonase reaction. Subsequently, I switched to the Gateway Cloning Technology (Invitrogen), which is capable of cloning an insert up to 6 kb (Fisher *et al.* 2006), and allows an insert to be cloned into the entry vector via site-specific recombination. Another advantage of using the Gateway system is that the insert in the entry clone can be directly shuttled into a compatible expression vector, again by recombination. This technology could potentially save the time and effort compared with conventional cloning methods, where the insert would have to be transferred into another expression vector by restriction digest followed by ligation. Although no colonies containing the full-length PAPP-A2 have been identified, many screened transformants appeared to contain the beginning and end parts of the cDNA based on sequencing and PCR. As the deletion appeared to occur in a specific region of the insert, it was unlikely to be the result of selection against *E. coli* cells carrying the full length insert, because if that was the case, random segments of the insert would have been

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missing. Instead, it was found that the recombination products created during the BP clonase reaction and the plasmids harvested from transformed colonies yielded PCR products of the same size, implying that the deletion occurred during the BP clonase reaction. However, it is unclear why the deletion occurred. It is possible that secondary structure of the cDNA causes the excision event during the cloning reaction. Also, one of the components in the BP Enzyme mix, integrase, is known to have splicing activity against single-stranded DNA (Chow *et al.* 1992), even though the insert generated by PCR should have been double-stranded. Following PCR, the product was gel-purified to eliminate smaller DNA fragments that might be cloned in preferentially during the BP clonase reaction. Although the gel extraction step itself would not have adverse effects on the integrity of the DNA, additives such as dimethyl sulfoxide (DMSO) in the PCR reaction could destabilize the double-stranded DNA (dsDNA) (Masoud *et al.* 1992). Moreover, as gel-extraction removes residual salts from the PCR reaction, dsDNA becomes less stable due to lower ionic concentrations (Tan and Chen. 2006). Therefore, it may be useful to extract fresh PCR product and use it as template for BP clonase reaction in a timely manner.

Once the mouse PAPP-A2 gene has been cloned, expressed and purified, a number of functional analyses can be performed to further investigate the biochemical activity of the protein *in vitro*. For example, it will be possible to determine whether mouse PAPP-A2 cleaves IGFBP-5 specifically, or whether it also cleaves other IGFBPs. This is of particular interest since human PAPP-A2 has been observed to have some proteolytic activity against IGFBP-3 (Overgaard *et al.* 2001), and the allelic variation in the QTL region mentioned above also has a significant effect on the levels of circulating IGFBP-3 in mice (Christians *et al.* 2006). Moreover, to further understand how PAPP-A2 interacts with other players in the IGF-axis, it will be

important to determine whether its action is IGF-mediated, as IGF is not required for IGFBP-5 cleavage by human PAPP-A2 (Overgaard *et al.* 2001). The proteolytic activity of PAPP-A2 against IGFBP-5 availability can be measured using Western blots of IGFBP-5 detecting the levels of intact and cleaved IGFBP-5. As IGFBP-5 has been observed to have IGF-independent stimulatory effects in certain cell types (Andress and Birnbaum. 1992), the proteolytic activity of PAPP-A2 against IGFBP-5 will likely decrease the rate of cell differentiation or metabolism. This hypothesis can be tested by transfecting an appropriate cell type with PAPP-A2, and observing whether the overexpression of PAPP-A2 would lead to reduced IGFBP-5 mediated cell growth *in vitro*. In addition, once a reliable cloning protocol has been established, it will be possible to clone PAPP-A2 coded by different alleles with relative ease. There are examples where allelic variation can affect protein abundance and enzyme activity (Chen *et al.* 2004; LeVan *et al.* 2001), as well as transcriptional level (Pugliese *et al.* 1997). By cloning PAPP-A2 alleles containing non-synonymous SNPs in the coding region, PAPP-A2 proteins with different amino acid sequences will be produced and possibly have different activities against their substrate(s). Therefore, the cloning of PAPP-A2 will allow us to further investigate whether PAPP-A2 is the causal gene underlying the effects of the mouse QTL that affects the levels of circulating IGFBP-5 and -3 (Christians *et al.* 2006).

In summary, the cloning and expression of the mouse PAPP-A2 gene would be very useful in addressing questions such as its biochemical actions compared to that of its human homologue, as well as how allelic variation may contribute to variation in enzyme activity. As attempts made so far have not been successful, more effort needs to be invested to solving the

problems associated with the cloning conditions, but it should be feasible to express the

recombinant protein and study its functions in the future.

Figures and Figure Legends
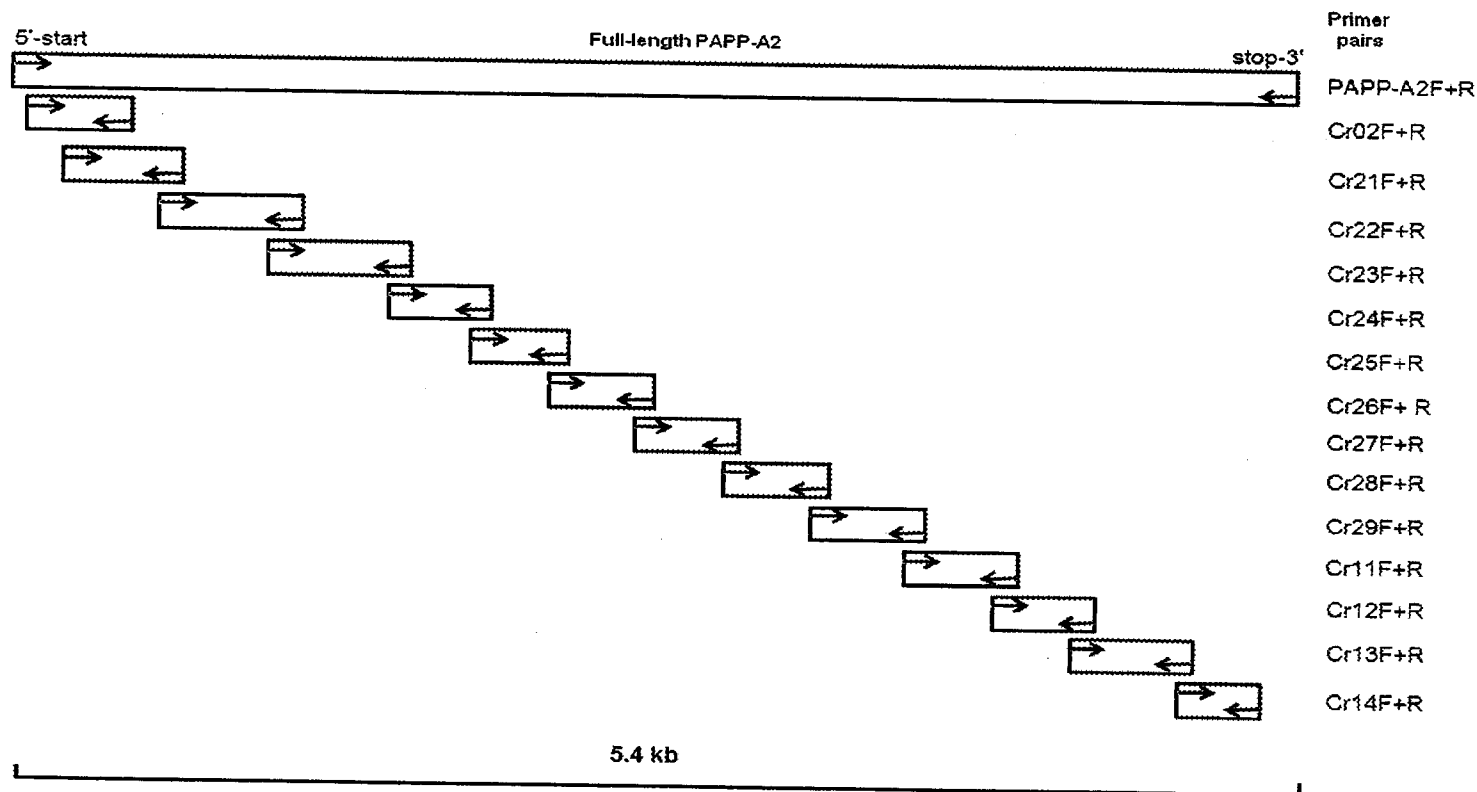
Figure 3.1 Locations of primers used in this study; PAPP-A2F and PAPP-A2R primers span the full-length PAPP-A2 cDNA and Cr primers span different regions within the cDNA. All Cr primers except Cr02L and Cr02R span an intron. Forward (F) primers are at the left end of each box and reverse (R) primers are at the right end of each box. Cr primer sequences are provided in the supplemental data file of Christians *et al.* (2006).
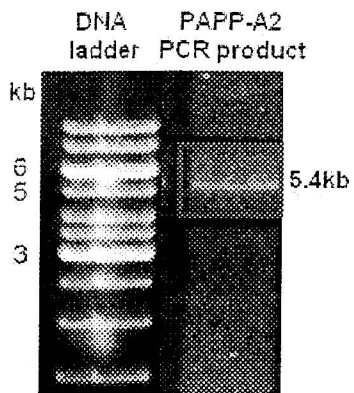
Figure 3.2. PCR product of approximately 5.4 kb was extracted from agarose gel containing EtBr.

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NNTCAGGAAACNAGCTATGANCANNNNNTGTAAAACGNCGGCCAGTNTTAAGCTCGGG
CCCCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATG
AGCAATGCTTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTTGACTTTGGGTTC
TAAGGAACAAGCTGAGGGAGAATGCCCACCACCCACCTCAGAGCTGAATTCTGTGGAG
TACAAGTGTGGACAGGGATATGGGAACGGTGCAGTGTGCTCCCCATCATGTGTAATCC
CCCCTAGTGACCCTGTGATACTACCAGAGAATGTGACTACTGACACTTTGGAGCACTG
GATGGAACCTGTCAAAGTCCAGAGTATCGTGTGTACCGGCAGGCGCCAGTGGCACCCA
GATCCCTCTCTGGTGCACTGCATCCAGTCATGTGAGCCTTTCCAAGCAGATGGTTGGT
GTGACACTATTAATAGCCGGGCCTACTGCAACTATGACGGGGGAGACTGCTGCTCTTC
CACACTCTCTTCCAAGAAGGTCATTCCATTTGCTGCTGATTGTGACTTGGATGAATGC
ACCTGTCGAGACCCCAAGGCAGAAGAAAACCAGTAAACCCAGCTTTCTTGTACAAAGT
TGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGT
CAAAATAAAATCATTATTTGCCATCCAGCTGATATCCCCTATAGTGAGTCGTATTACA
TGGTCATANNGTTTNCCTGANNN
```

Figure 3.3. Sequence of the sequenced M13 PCR product obtained from isolated plasmid, containing binding sites for M13, PAPP-A2*att*, and PAPP-A2 primers (forward and reverse), and parts of the PAPP-A2 gene. Red: M13F and M13R; blue: PAPP-A2*att*F and PAPP-A2*att*R; blue underlined: PAPP-A2F and PAPP-A2R; black bold: 5' end of PAPP-A2; brown: 3' end of PAPP-A2.
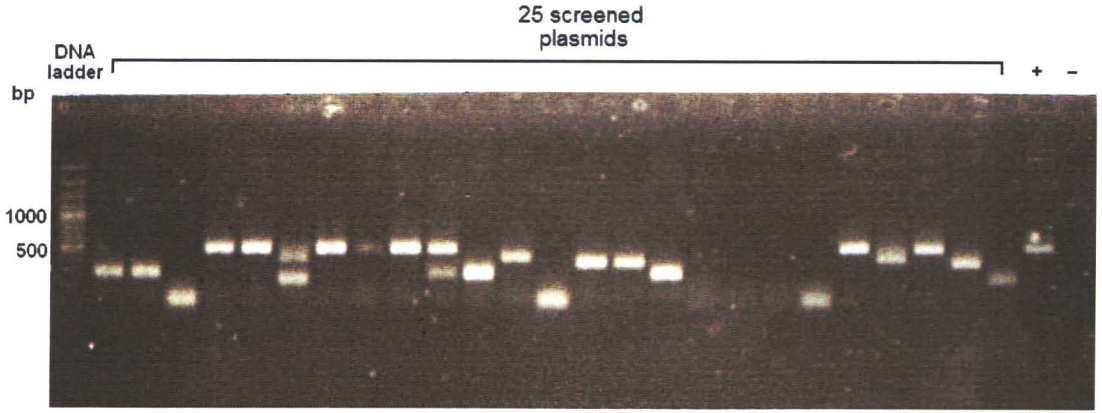
Figure 3.4. PCR with PAPP-A2*att*F and PAPP-A2*att*R primers on plasmids from 25 different clones. Most screened plastmids and the sequenced plasmid produced bands of the same size, 500bp. +: sequenced plasmid; -: pDONR221 vector containing control DNA sequence (thus no binding sites for PAPP-A2*att* primers).
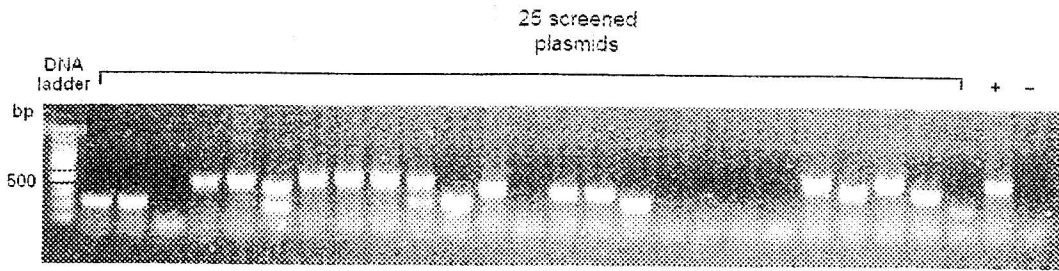
Figure 3.5. PCR with PAPP-A2F and PAPP-A2R primers on plasmids from 25 different clones. Most screened plasmids and the sequenced plasmid produced bands of the same size, 450bp. +: sequenced plasmid; -: pDONR221 vector containing control DNA sequence (thus no binding sites for PAPP-A2 primers).
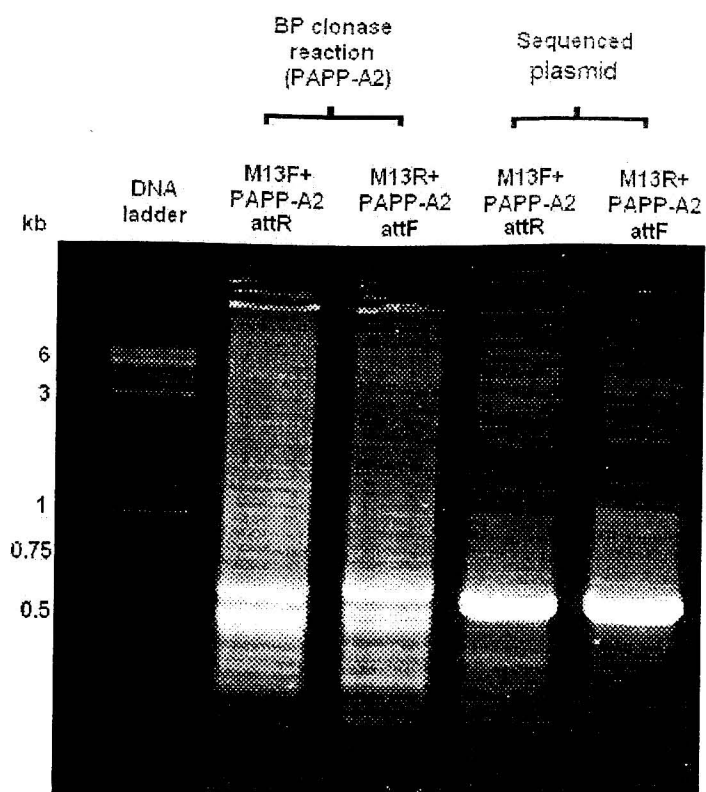
Figure 3.6. Long range PCR with M13F+PAPP-A2*att*R and M13R+PAPP-A2*att*F primers on 2 different samples. Bands of similar sizes were produced in both the BP clonase reaction with PAPP-A2 and the sequenced plasmid.

CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

The primary goal of my study was to characterize the expression pattern of a metalloproteinase, PAPP-A2, in various mouse tissues. Specifically, it was my particular interest to examine where PAPP-A2 is localized in the mouse placenta and embryo. In addition, I attempted to clone and express mouse PAPP-A2 to determine its biochemical actions *in vitro* and whether allelic variation would lead to differential enzyme activity. Although the human PAPP-A2 gene was first identified nearly a decade ago (Overgaard *et al.* 2001), no study has investigated its *in vivo* roles and only one study to-date has attempted to characterize its expression profile in animal models such as the mouse (Christians *et al.* 2006). As a result, PAPP-A2 remains poorly understood and its physiological importance is yet to be revealed. Human PAPP-A2 has been found to cleave IGFBP-5 (Overgaard *et al.* 2001), which is a crucial player in many processes such as embryonic development and skeletal formation (Allan *et al.* 2001; Miyakoshi *et al.* 2001a), as well as regulating the bioavailability of IGFs (Clemmons. 1998). Moreover, the amino acid sequence of the PAPP-A2 protein shows 62% homology to another well-characterized metalloproteinase, PAPP-A (Farr *et al.* 2000), which is a major IGFBP-4 protease (Lawrence *et al.* 1999). In humans, PAPP-A is highly expressed during pregnancy and is synthesized by trophoblasts in the placenta (Tornehave *et al.* 1984) where it degrades IGFBP-4 and modulates the levels of IGF-II available for promoting the development of the placenta (Giudice *et al.* 1998; Giudice *et al.* 2002). PAPP-A has also been found to be medically important as abnormal levels of circulating PAPP-A are associated with fetal chromosomal disorders such as Down's syndrome (Brambati *et al.* 1993), as well as higher risk

of low birth weight (Smith *et al.* 2002), preterm delivery (Dugoff *et al.* 2004), miscarriage (Kwik and Morris. 2003) and pre-eclampsia (Spencer *et al.* 2008). Like PAPP-A, PAPP-A2 is also highly expressed during human pregnancy (Farr *et al.* 2000; Page *et al.* 2001); however, no previous study has investigated whether PAPP-A2 is expressed in the murine placenta. Because the mouse serves as a good model for studying placentation in terms of the cell types involved in trophoblast invasion (Adamson *et al.* 2002; Carter 2007) and genes regulating placental development (Carter 2007), I was interested in examining whether the expression of PAPPA2 was similar in mouse and human placenta. Moreover, while its expression profile in the murine placenta remains unclear, there is some evidence that PAPP-A2 is physiologically important in mice. PAPP-A2 has been proposed to be the causal gene underlying the effects of a QTL that affects tail length and circulating levels of IGFBP-5 and -3 in mice. The expression of mouse PAPP-A2 *in vitro* would allow us to better study its biochemical actions and whether proteins coded by different PAPP-A2 alleles have different proteolytic activity.

In this study, it was found that the expression of PAPP-A2 is much higher in the mouse placenta than in other tissues analyzed, such as stomach, liver and kidney (Chapter 2), which have been found to express PAPP-A2 at relatively high levels in a previous study (Christians *et al.* 2006). Moreover, the number of PAPP-A2 transcripts in the mouse placenta was calculated to be nearly 1000-fold higher than that of PAPP-A. Given the important functions that PAPP-A plays during human pregnancy, it is conceivable that PAPP-A2 also plays crucial roles in mouse placentation and fetal development. To better characterize the expression profile of PAPP-A2 in the mouse placenta, I examined PAPP-A2 protein expression pattern in early to mid gestation, and found that PAPP-A2 is specifically expressed in the junctional zone, located between the maternal decidua and fetal labyrinth. The junctional zone contains cell types responsible for the

invasion of the fetal component into the maternal tissue during placentation (Adamson *et al.* 2002). As IGFBP-5 mRNA is present in the neighbouring maternal decidua ((Carter *et al.* 2006), Chapter 2), the expression of PAPP-A2 in the junctional zone likely contributes to IGFBP-5 proteolysis. The degradation of IGFBP-5 would increase the bioavailability of IGF-II, which is known to have crucial roles in regulating the growth of placenta (Constancia *et al.* 2002; Hamilton *et al.* 1998). In addition, PAPP-A2 is detectable in the maternal circulation during mid-gestation in mice (Chapter 2). This observation, together with its potential functions in placental development, suggests that PAPP-A2 may be useful as a biomarker for monitoring problematic placental formation and function. Indeed, several recent studies have reported elevated PAPP-A2 mRNA and protein levels in human patients suffering from pre-eclampsia (Nishizawa *et al.* 2008; Sitras *et al.* 2009; Winn *et al.* 2009) and HELLP syndrome (Buimer *et al.* 2008). However, these studies examined human placenta and serum samples obtained at delivery or at the time of the diagnosis of pre-eclampsia, and it would be more useful to investigate the relationship between PAPP-A2 levels early in maternal circulation and occurrence of pre-eclampsia for preventive screening. Moreover, it is certainly interesting that abnormally low levels of PAPP-A are associated with placental or fetal disorders, whereas elevated PAPP-A2 levels are associated with pre-eclampsia. This contrast between the two proteins suggests that even though they are structurally and functionally similar, they may play different roles during pregnancy.

In agreement with a previous report (Christians *et al.* 2006), this study also found that PAPP-A2 is expressed in the mouse embryo. PAPP-A2 transcripts are expressed in the tail and dorsal side of the embryo, forebrain, and nasal region. Interestingly, the substrate of PAPP-A2, IGFBP-5, is also expressed in the developing tail (Allan *et al.* 2001). The co-localization PAPP-

A2 and IGFBP-5 in the embryonic tail suggests that PAPP-A2 may play a role in modulating IGFBP-5 bioavailability in the mouse fetus, and is consistent with the hypothesis that PAPP-A2 is the causal gene responsible for a QTL affecting tail length (Christians *et al.* 2006). To further determine the roles of PAPP-A2 during embryonic development, a knockout mouse needs to be developed.

A knockout mouse that is deficient in PAPP-A2 can be useful for addressing several questions: is PAPP-A2 necessary for proper fetal development? How many functional PAPP-A2 alleles are required for the development of the wild-type phenotype? If PAPP-A2 deficient embryos are viable, will they show the same growth rate/pattern as the wild-type embryos? Does the deletion of PAPP-A2 have lasting effects into adulthood and affect fitness such as longevity and fertility? Also, how does the absence of PAPP-A2 affect the levels of circulating IGFBP-5 and -3? Moreover, if a conditional knock-out mouse can be developed in which PAPP-A2 is deleted specifically in the placenta, the effects of PAPP-A2 on placental development can be observed, and the effects of potential placental dysfunction due to PAPP-A2 ablation on embryonic growth *in utero* can also be investigated. The development of a knockout mouse would, therefore, be very useful for investigating the *in vivo* actions of PAPP-A2. Furthermore, it will be interesting to examine PAPP-A2 expression and placental morphology in mouse strains, such as BPH/5 (US Patent 7045674) that spontaneously show pre-eclampsia-like symptoms such as gestational hypertenstion, proteinuria, and endothelial dysfunction (Davisson *et al.* 2002; Dokras *et al.* 2006). This study potentially would offer more insight into the relationship between PAPP-A2 and the pathophysiology of pre-eclampsia.

Outside pregnancy, PAPP-A2 may also play physiological roles in other tissues as it has been detected in skin, stomach, kidney, brain, heart, lung, testis, pancreas, and prostate gland in mice (Christians *et al.* 2006), and kidney, fetal brain, pancreas, mammary gland, and testis in humans (Page *et al.* 2001). Indeed, PAPP-A2's functional counterpart, PAPP-A, has been shown to be involved in the healing of human skin (Chen *et al.* 2003). Moreover, PAPP-A has been implicated in the development of carotid atherosclerotic plaques (Sangiorgi *et al.* 2006). As a result, PAPP-A is being examined as a potential marker of coronary syndromes (Bayes-Genis *et al.* 2001; Consuegra-Sanchez *et al.* 2009). In addition, variation in the PAPP-A gene appears to be associated with risk of heart attack (Park *et al.* 2007). Furthermore, PAPP-A has been found to have regulatory functions on bone formation *in vitro* (Conover *et al.* 2004a). Because of the functional and structural similarities between PAPP-A and PAPP-A2, it is possible that PAPP-A2 is involved in some, if not all, of these diverse physiological systems, and thus it will be reasonable to investigate whether PAPP-A2 plays similar roles as PAPP-A in these areas.

In conclusion, my work has offered insight into the potential importance of PAPP-A2 which may play crucial roles during pregnancy and fetal development in mice. This finding further presents the possibility that the mouse can be used as an animal model for studying PAPP-A2 *in vivo* which will be important for understanding its physiological roles in humans. As PAPP-A2 is likely involved in the pathophysiology of pre-eclampsia (Nishizawa *et al.* 2008; Sitras *et al.* 2009; Winn *et al.* 2009) and potentially plays important roles in embryonic development and other processes, extending the scope of the research presented in this thesis will be necessary to better characterize this protein. A better understanding of PAPP-A2 will hopefully shed light on the underlying mechanism of certain pathological conditions.

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