The pappalysins and their substrates in early pregnancy

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Abstract

Abnormal levels of pregnancy-associated plasma proteins -A and -A2 (PAPP-A and PAPP-A2) have been associated with adverse pregnancy outcomes, including preeclampsia and intrauterine-growth-restriction, two leading causes of maternal and perinatal mortality. The goal of this thesis was to assess the roles of PAPP-A, PAPP-A2, and their substrates, insulin-like growth factor-binding proteins -4 and -5 (IGFBP-4 and IGFBP-5), in early pregnancy. In contrast to previous findings with PAPP-A, first trimester maternal serum levels of PAPP-A2 were elevated in pregnancies that subsequently developed preeclampsia. In a model of early placental development, IGFBP-4 and IGFBP-5 inhibited the migration-stimulating effects of insulin-like growth factors -I and -II, although to different extents. Investigation of the location of IGFBP-4 and IGFBP-5 in the placenta revealed sites of protein localization that suggest previously unknown functions. Taken together, these findings suggest different roles of PAPP-A and PAPP-A2 in the first trimester placenta.

Keywords: pappalysins; insulin-like growth factor-binding proteins; preeclampsia; intrauterine growth restriction; small-for-gestational-age; trophoblast migration

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List of Acronyms

ADAM	A disintegrin and metalloprotease
CT	Cytotrophoblast
ECM	Extracellular matrix
EVT	Extravillous trophoblast
HELLP	Haemolytic anemia, elevated liver enzymes and low platelet count
IGF	Insulin-like growth factor
IGF1R	Type 1 IGF receptor
IGF2R	Type 2 IGF receptor
IGFBP	Insulin-like growth factor binding protein
IUGR	Intrauterine growth restriction
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MoM	Multiples of the median
PAPP-A	Pregnancy-associated plasma protein A
PAPP-A2	Pregnancy-associated plasma protein A2
PE	Preeclampsia
proMBP	Proform of eosinophil major basic protein
SGA	Small for gestational age
ST	Syncytiotrophoblast
TGF-β	Transforming growth factor β
Wnt	Wingless (pathway)

1. Introduction

1.1. The Placenta

The placenta is a multi-functional organ of finite lifespan that is crucial to healthy fetal development. In addition to the transfer of nutrients, gases and wastes, the placenta also serves as an immune barrier (1) as well as a source of hormones required to sustain pregnancy (2). The placenta is composed of tissue from two distinct genetic origins: the maternal placenta (decidua) which develops from the uterine tissue, and the fetal placenta (chorion), which develops from the blastocyst (3). Between the two layers is the intervillous space, a sinus within the placenta which fills with maternal blood supplied by the uterine arteries. The maternal blood bathes tree-like structures called chorionic villi which originate from the chorion and serve as the interface where gas and nutrient exchange takes place between the fetal and maternal circulatory systems. Some villi span the distance of the intervillous space, serving as a place of attachment to the uterine wall and so are termed “anchoring” villi, whereas others float freely in the maternal blood and are termed “floating” villi (3,4).

Several different cell types make up the chorionic villi. These include the fetal endothelial cells lining the fetal blood vessels, the villous mesenchyme, the villous cytotrophoblast (CT) and the multinucleated syncytiotrophoblast (ST), which makes up the outermost cellular layer of the chorionic villi and is in direct contact with the maternal blood (2,4,5). The ST is maintained by differentiation and fusion of the highly proliferative CT, which makes up the layer immediately beneath the ST (4).

Another trophoblast cell type, the extravillous trophoblast (EVT), arises from the proliferation and differentiation of stem cells within the CT layer (4). EVT cells are key players in processes crucial to normal placental development: EVT migration and invasion, and spiral artery remodeling (Figure 1.1). Upon migrating from the anchoring

villous columns, EVT cells penetrate the maternal decidua and first third of the myometrium where they invade and restructure the maternal uterine arteries (4,6). By invading and replacing the uterine vessel endothelial cells, they transform the blood vessels into wide diameter spiral arteries that can accommodate the blood flow required to sustain pregnancy (4,6,7). This invasion occurs between 6 and 18 weeks of gestation, with spiral artery remodeling being more or less complete before 20 weeks of gestation (8).

1.2. Abnormal placental development and pregnancy complications

Adverse pregnancy outcomes such as preeclampsia (PE) and intrauterine growth restriction (IUGR) are associated with placental insufficiency resulting from impairment of processes critical to placental development including but not limited to EVT migration/invasion and spiral artery remodeling (8-11). Together, IUGR and PE affect approximately 5-7% of all pregnancies, and represent leading causes of perinatal and maternal mortality, respectively (12,13). Symptoms of PE typically appear in the late second or third trimester, and include acute hypertension and proteinuria in the mother, with risk of organ failure and seizure (8,14). Presently, there exists no cure for PE, with the symptoms of PE only being relieved upon delivery. As a result of delivery being the only treatment for severe PE, PE is responsible for 18% of premature births, representing a significant risk to the life of the baby as premature infants suffer twice the risk of neonatal death as infants from uncomplicated pregnancies (15). IUGR is often, although not completely concurrent with PE, and results from alterations of blood flow that are detrimental to fetal growth and development, producing a small-for-gestational-age infant (12). Apart from an increased risk of perinatal mortality as mentioned above, IUGR is also associated with sepsis, asphyxia and several adult-onset diseases including type 2 diabetes, stroke, hypertension and coronary heart disease (12,16).

1.3. The pappalysins, the IGFBPs, and the IGFs

Levels of pregnancy-associated plasma proteins A and A2 (PAPP-A and PAPP-A2, respectively), have been consistently associated with pregnancy complications. Abnormally low first trimester levels of PAPP-A in the maternal circulation have been associated with both PE (17-21) and IUGR (17,18,22-24). PAPP-A2 shares 45% amino acid identity with PAPP-A (25-27), and although less well studied than PAPP-A, has been found to be upregulated in term placentae from pregnancies afflicted by PE and HELLP (Hemolytic anemia, Elevated Liver enzymes, and Low Platelet count) (28-35) and is elevated in the circulation of women with PE in late gestation (30). The vast majority of PAPP-A circulates as a covalent, heterotetrameric 2:2 complex with the proform of eosinophil major basic protein (proMBP), which inactivates PAPP-A (36,37). In contrast, it has been suggested that PAPP-A2 is secreted as a non-covalently associated dimer (38,39), with no known inhibitor.

PAPP-A is a protease of insulin-like growth factor binding proteins -4 and -5 (IGFBP-4 and IGFBP-5, respectively) (40-43), while PAPP-A2 cleaves only IGFBP-5 (25-27). In total, six IGFBPs (IGFBP-1 through -6) have been identified, and function to modulate the bioavailability of insulin-like growth factors (IGF-I and IGF-II) by various routes. These routes may include 1) binding and sequestering the IGFs such that they cannot exert their effects, 2) potentiating the effects of the IGFs, or 3) concentrating the IGFs in particular regions (4,40,44-50). The insulin-like growth factors (IGFs) themselves play important roles in the development of the placenta and spiral artery remodeling by stimulating processes including CT proliferation and EVT migration in the first trimester (51). Previous work has suggested that IGF-I and IGF-II mediate most of their effects by binding to the type 1 IGF receptor (IGF1R) (52-60). In binding to IGF1R, IGF-I and IGF-II have been shown to exert effects on CT proliferation and ST formation via activation of the MAPK (mitogen-activated protein kinase) pathway, and effects on survival via activation of the phosphoinositide 3-kinase pathway (60). EVT invasion has been shown to be stimulated by IGF-I activation of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin pathways via IGF1R (61,62), and IGF-II activation of the MAPK pathway and involvement with inhibitory G proteins via the type 2 IGF receptor (IGF2R) (63). As gestation progresses, levels of IGF-I in the maternal circulation steadily increase, potentially due to increased growth of

maternal tissues responsible for its secretion (such as muscle and adipose tissue) (51). In contrast, circulating levels of IGF-II quickly stabilize after their initial increase, likely in association with pre-partum maturation of the placental tissue responsible for its secretion (51). Although the roles of IGF-I and IGF-II in late gestation are less well-characterized in humans, animal studies have suggested that they impact fetal growth indirectly via influences on maternal metabolism, nutrient partitioning, and/or placental growth and function (including endocrine function and growth of the surface area for gas and nutrient exchange) (51).

As the proteases of the IGFBP-4 and -5, PAPP-A and PAPP-A2 are expected to increase the amount of unbound IGF-I and IGF-II, thus leading to increased placental growth and function. From this it follows that altered expression of either PAPP-A or PAPP-A2 could contribute to placental dysfunction, either by altered bioavailability of the IGFs due to sequestration by IGFBP-4 and/or -5, or via altered IGF-independent effects of the IGFBPs. The most well-studied of the IGFBPs is IGFBP-1, the most abundant IGFBP in the placenta (64). IGFBP-1 has been found to have IGF-independent effects stimulating EVT invasion (40,65-67), and is present at abnormal levels in the maternal circulation in women with PE (68-74). Meanwhile, IGFBP-4 and IGFBP-5 have been less well-studied, although IGFBP-4 was recently found to be elevated in the maternal circulation of pregnancies complicated by IUGR (75), and IGFBP-5 has been shown to abrogate the migration-stimulating effects of IGF-II in an *in vitro* model of EVT (76). Currently it is unknown if PAPP-A2 may exert effects via IGFBP-independent routes, however one recent study has noted IGFBP-independent effects of PAPP-A in zebrafish development (77).

Notably, PAPP-A and PAPP-A2 are not the only IGFBP proteases that have been implicated in adverse gestational outcomes. ADAM12 (a disintegrin and metalloprotease 12) also cleaves IGFBP-5 as well as IGFBP-3, and is highly expressed by the placenta. Similar to PAPP-A, abnormally low levels of ADAM12 in the first trimester maternal circulation have been associated with gestational hypertension (78), PE (79,80) and IUGR (22,81,82). Previous work has suggested that ADAM12 plays a role in EVT migration (83). Matrix metalloproteinases (MMPs) also cleave IGFBPs, in addition to various other substrates (74). MMP-9 and MMP-2 have both been shown to

be elevated in the maternal circulation of pregnancies that subsequently develop PE (84-86), with MMP-2 also being elevated in the second trimester amniotic fluid (87), although these findings have not been consistently reproduced (88-90). MMP-2 and MMP-9 are thought to contribute to placental development via degradation of the extracellular matrix (ECM) thus enabling ET invasion (91,92), but their importance in the IGF pathway via IGFBP proteolysis is unclear.

Many other growth factors, binding proteins, ECM components and adhesion molecules play important roles in EVT migration and invasion that are distinct from the IGF pathway. Apart from the IGFs and IGFBP-1 as mentioned above, endothelin 1 (ET-1) has been shown to stimulate EVT migration and invasion via binding to receptors expressed on both CT and EVT cells and signaling through G_q-coupled receptors (4). Signaling via the wingless (Wnt) pathway has also been shown to be stimulatory of EVT invasion and migration (93). In contrast, transforming growth factor β (TGF-β), decorin (a proteoglycan in the ECM), and melanoma cell adhesion molecule have all been found to inhibit EVT migration and invasion (4), again via routes that are independent of the IGF pathway.

1.4. Thesis work and objectives

The objective of this thesis has been to look at patterns and mechanisms through which PAPP-A and PAPP-A2 might be acting to help ascertain whether the observed associations with PE and IUGR are causal or a compensatory reaction in response to abnormal placental development. In chapter two, I describe an experiment in which I measured first trimester maternal serum PAPP-A2 levels using immunofluorescent Western blotting. Serum samples were collected as part of first trimester screening and included samples from 17 pregnancies resulting in small for gestational age (SGA) infants, 6 pregnancies which developed PE, 1 pregnancy which developed PE and resulted in a SGA infant, and 37 gestational age matched controls. The objective was to determine whether first trimester levels of PAPP-A2 in the maternal circulation differed between uncomplicated pregnancies and those that subsequently developed PE and/or resulted in a SGA infant. Understanding the timing of the upregulation of PAPP-A2 in

pregnancies afflicted by PE is important to understanding the mechanisms underlying associations between PAPP-A2 and PE.

In chapter 3, I describe experiments in which I measured the effects of IGFBP-4 and IGFBP-5 in an *in vitro* model of EVT migration using HTR-8/SVneo cells. The objective of this work was to test whether IGFBP-4 and IGFBP-5 function to simply inhibit the migration-stimulating effects of IGF-I and IGF-II, or if they also exert IGF-independent effects and/or potentiate the effects of the IGFs. It was also of interest to see whether IGFBP-4 and IGFBP-5 exert equal effects modulating EVT migration, as this is informative as to the relative effects of each of the pappalysins. Also described in chapter 3 is immunohistochemistry that was performed in order to determine whether IGFBP-4 and IGFBP-5 are present in first-trimester villi so as to shed light on ambiguities surrounding the current understanding of the location and timing of the expression of IGFBP-4 and -5 in the placenta. Information regarding the effects of the IGFBPs on EVT migration as well as their expression patterns within the placenta is necessary to determine whether the pappalysins might play a causal role in pregnancy complications.

In chapter 4, I will summarize the results and conclusions reached in chapters 2 and 3, and provide an overall synthesis of the findings of this thesis. Chapter 4 will also include suggestions for future directions of PAPP-A and PAPP-A2 research that will serve as follow-up for the work performed as part of this thesis.

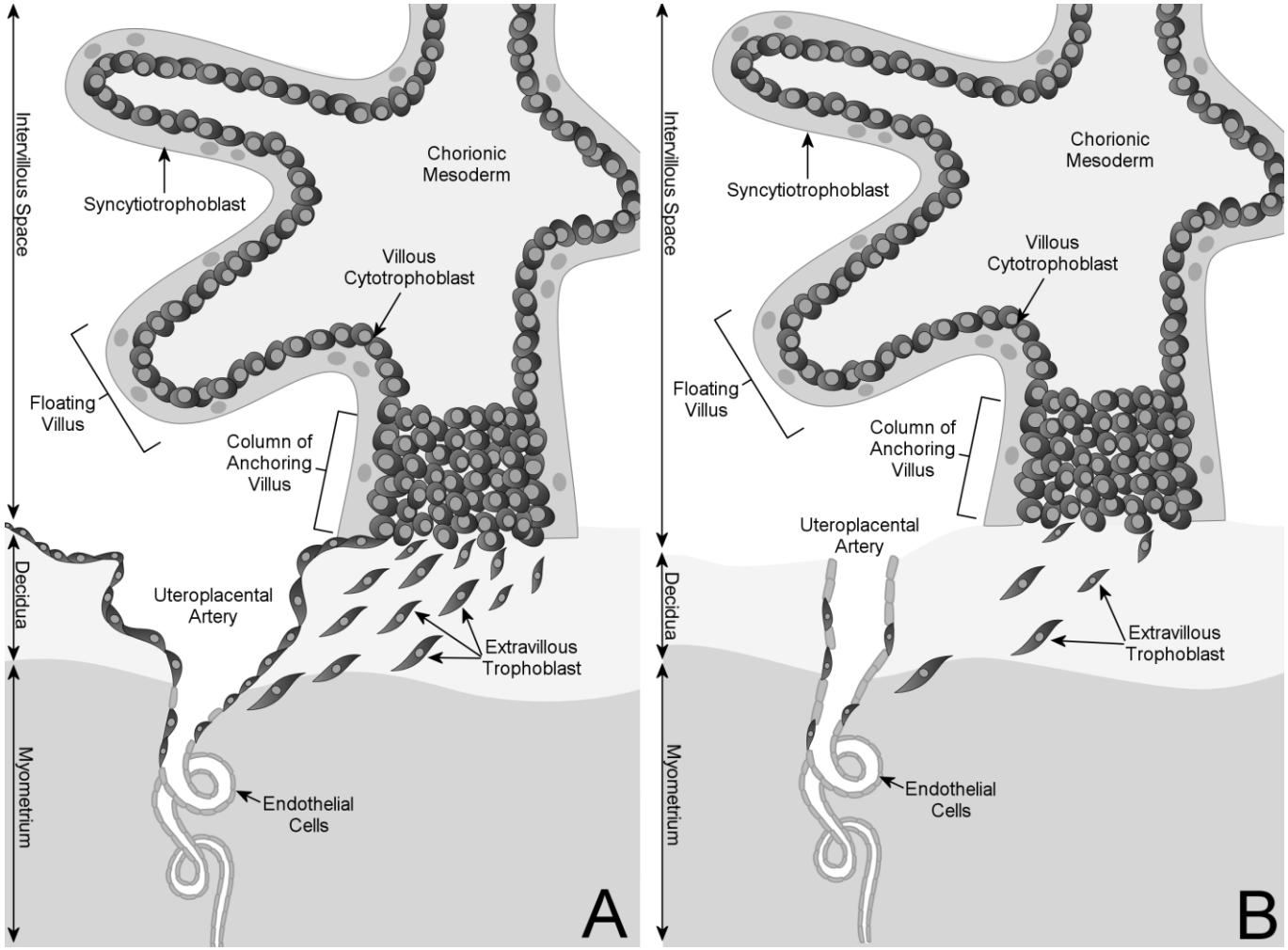

Figure 1.1. Extravillous trophoblast (EVT) invasion and spiral artery remodeling
In normal placentation, EVT cells migrate from the anchoring villi into the decidua and first third of the myometrium where they invade and restructure the maternal epithelium of the uteroplacental arteries, transforming these vessels into wide diameter spiral arteries (A). The placental insufficiency that contributes to PE and IUGR is associated with impaired EVT migration and insufficient spiral artery remodeling (B).

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2. First trimester levels of pregnancy-associated plasma protein A2 (PAPP-A2) in the maternal circulation are elevated in pregnancies that subsequently develop preeclampsia

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2.1. Abstract

Recent studies have consistently found pregnancy-associated plasma protein-A2 (PAPP-A2) to be upregulated in preeclamptic placentae at term. We tested whether first trimester circulating PAPP-A2 levels differed between complicated and uncomplicated pregnancies. We measured maternal PAPP-A2 levels at 10-14 weeks gestational age in 17 pregnancies resulting in small for gestational age (SGA) infants, 6 that developed preeclampsia (PE), 1 that developed PE and resulted in a SGA-infant, and 37 gestational age matched controls. The concentration of the PAPP-A2 isoform corresponding to the full-length protein was significantly higher in pregnancies that

developed PE (35 ng/mL) compared with those that did not (23 ng/mL; $P < 0.044$). In contrast, we found no difference in PAPP-A2 levels between pregnancies that did or did not result in a SGA-infant. The upregulation of PAPP-A2 that has previously been observed in PE at term appears to begin early in pregnancy, well before symptoms develop.

Keywords: PAPPA2, preeclampsia, small-for-gestational-age

2.2. Introduction

Abnormal placental development is associated with a wide range of adverse gestational outcomes, including preeclampsia (PE) and intrauterine growth restriction (IUGR) (1-3). PE, a gestational syndrome characterized by acute hypertension and proteinuria in the mother, affects 5-7% of all pregnancies and remains a leading cause of maternal mortality for which there is presently no cure other than delivery of the fetus and the placenta (2,4). Meanwhile, IUGR resulting from placental insufficiency carries consequences such as increased risk of perinatal mortality, sepsis and asphyxia and an increased risk of several adult-onset diseases such as type-II diabetes, stroke, hypertension and coronary heart disease (5). PE and IUGR are often, though not completely, concurrent.

The placental insufficiency that is associated with PE and IUGR has been characterized by defective remodeling of the uterine spiral arteries, coinciding with impaired invasion of extravillous trophoblast (EVT) into the maternal decidua (3). Insulin-like growth factors I and II (IGF-I and –II, respectively), stimulate the EVT migration and invasion that is key to spiral artery remodeling (6,7). The bioavailability of IGFs-I and –II is modulated by insulin-like growth factor-binding proteins (IGFBPs) (6,8-10). Pregnancy-associated plasma protein-A (PAPP-A) is a protease of IGFBP-4, IGFBP-5 and possibly IGFBP-2, and thus is expected to play a role in increasing the bioavailability of the IGFs and potentially affect processes such as EVT migration and invasion (6,11-13). PAPP-A circulates in the maternal blood at high levels during pregnancy, and is already in clinical use as a first trimester biomarker for Down syndrome, as abnormally low levels of PAPP-A early in gestation have been associated with elevated risk of chromosomal abnormalities (14). Abnormally low levels of PAPP-A are also associated

with an increased risk of PE (15-19) and IUGR (15-18,20), but the predictive value of PAPP-A alone for detecting these conditions is poor, with only 8–23% of PE cases and 11-24% of IUGR cases having PAPPA serum levels below the 5th percentile (21).

Pregnancy-associated plasma protein-A2 (PAPP-A2) shares 45% amino acid identity with PAPP-A, and cleaves IGFBP-5 and possibly IGFBP-3, thus potentially modulating the activity of the IGFs (22-24). While low first trimester levels of PAPP-A are associated with an increased risk of PE, recent studies have shown PAPP-A2 to be upregulated in term placentae from pregnancies afflicted by PE and HELLP syndrome (Hemolytic anemia, Elevated Liver enzymes, and Low Platelet count) (25-32), with PAPP-A2 being one of the top 5 most consistently upregulated genes in a meta-analysis of microarray studies of preeclamptic placentae (32). Additionally, third trimester circulating levels of PAPP-A2 have also been found to be elevated in women with PE (27).

The objective of the present study was to determine whether PAPP-A2 levels in the maternal circulation are elevated in the first trimester of pregnancies that subsequently develop PE and/or result in a small for gestational age (SGA) infant, compared with uncomplicated pregnancies. Understanding whether the elevation of PAPP-A2 in complicated pregnancies occurs early, near the time when placental dysfunction is developing, or later in pregnancy, in response to fully-developed placental pathology, will shed light on the mechanisms underlying the associations between PAPP-A2 upregulation and preeclampsia.

2.3. Methods

2.3.1. Samples and control matching

First trimester serum samples were collected as part of unrelated first trimester screening and stored at the Pacific Centre for Reproductive Medicine (PCRM), in Burnaby, BC, Canada. For women who delivered at BC Women's Hospital, pregnancy outcomes associated with each sample were obtained from the Provincial Health Services Authority. The use of these samples for a retrospective study was approved by

both the University of British Columbia Children's and Women's Research Ethics Board and the Simon Fraser University Research Ethics Board.

Samples included 24 case samples, comprising 17 pregnancies resulting in SGA infants, 6 pregnancies which developed PE, and 1 pregnancy which developed PE and resulted in a SGA-infant. SGA was defined as birth weight within the lowest 5th percentile, and PE was defined as gestational hypertension with significant proteinuria (33). Only pregnancies resulting in singleton births with no other known pathologies (including congenital abnormalities) were included in this study. For control matching we obtained 37 serum samples from women who experienced normal pregnancies (normotensive women who gave birth to babies with birth weights above the 10th percentile). The earliest gestational age at birth was 34 weeks. We matched case samples to controls according to gestational age at the time of first trimester sampling, as well as length of time in storage, with some case samples being matched to two controls and others to only one, giving priority in matching to gestational age at the time of sampling. First trimester PAPP-A2 levels were too low to be detected using an ELISA adapted from Nishizawa *et al.* (2008) (27) (data not shown) and therefore were quantified using immunofluorescent Western blot.

2.3.2. Immunofluorescent Western blot

Patient serum was thawed and diluted 1/8 in PBS with 0.1% BSA before being aliquoted and refrozen (for a total of one additional freeze-thaw cycle prior to assay). PAPP-A2 protein levels in serum were quantified using a previously described Western blotting method (34,35), using wet transfer from gel to nitrocellulose membrane instead of semi-dry transfer. Twenty microliters of diluted serum samples were mixed with 5 µL of 6X SDS loading buffer and heated for 10 minutes at 100 °C. Samples were run for 1 hour and 50 minutes through a 4% stacking and 8% separating polyacrylamide gel to achieve separation of proteins in the 100 – 250 kDa region. Wet transfer to nitrocellulose membrane (Bio-Rad, Hercules, CA) was performed for 75 minutes at 100 V (constant) on the Criterion Wet Blotter System (Bio-Rad, Hercules, CA) in CAPS buffer with 10% MeOH (Sigma Aldrich, St. Louis, MO). Initial optimization studies established that in a range of transfer times from 30 minutes up to 2 hours, 75 minutes of wet

transfer to nitrocellulose was optimal to ensure complete transfer of proteins without blow-through. Nitrocellulose was then blocked using Odyssey® Blocking Buffer For Quantitative Westerns (Li-Cor Biosciences, Lincoln, Nebraska) for 1 hour before overnight incubation with 1:500 polyclonal goat anti-human PAPP-A2 antibody (R&D Systems, AF1668) in the same blocking buffer with 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO). Membranes were washed 5 times with sterile PBS containing 0.1% Tween-20 (PBST) at room temperature before being incubated in a solution containing 1:10000 fluorescently-labelled IRDye 800 secondary antibody (Li-Cor Biosciences) diluted in blocking buffer, 0.1% Tween-20 and 0.1% SDS for 45 minutes in the dark. The membranes were washed again for 5 minutes each in PBST at room temperature, rinsed with filter-sterilized PBS, and scanned with an Odyssey Infrared imaging system.

2.3.3. Quantification

PAPP-A2 patient serum concentrations were derived from the optical density values of PAPP-A2-immuno-specific bands and a standard curve of recombinant PAPP-A2 protein (R&D Systems, 1668-ZN) included on the same blot. Optical density values for each of the PAPP-A2-immuno-specific bands were calculated with Odyssey Infrared Imaging System Application Software (Version 2.1.12) using rectangles of the same size and shape for all bands, selected by an observer who was blind to the outcome of the samples. PAPP-A2 immuno-specificity was determined via comparison of identical membranes developed either with polyclonal goat anti-human PAPP-A2 antibody or polyclonal goat anti-human immunoglobulin-G (IgG) as the primary antibody (Figure 2.1). PAPP-A2-specific bands are present at ~290 kDa, ~250 kDa and ~130 kDa (close to the expected size of a splice variant of PAPP-A2), consistent with previous findings (23,27,35).

Each sample was measured in triplicate, with each replicate run on a separate gel. Each gel contained its own standard curve of recombinant PAPP-A2 protein (5 wells, 20 - 1.25 ng/mL) and 3 to 4 case/control groups, i.e., case samples were always run adjacent to their control(s) (Figure 2.2). Each case/control group replicate was run on three separate gels, and was run adjacent to different case/control groups in each

replicate. Group position on the gel always varied between replicates to control for potential gel edge effects.

Pregnancy associated plasma protein-A (PAPP-A) levels in maternal serum were quantified using the DELFIA(R) Xpress PAPP-A immunoassay kit (PerkinElmer; Waltham, MA) and were converted to multiples of the median (MoM) using LifeCycle(TM) Elipse (PerkinElmer; Waltham, MA).

2.3.4. Statistical analyses

The optical densities of the 290, 250 and 130 kDa PAPP-A2 immunospecific bands were converted to serum concentration in ng/mL for each replicate using the standard curve included on the same gel/blot. For each band, all three replicate values for each sample were analysed using a general linear model including the effects of sample identity, blot, and lane on the gel. In these analyses, the sample identity term was highly significant for the 290, 250 and 130 kDa PAPP-A2 bands ($P < 0.0001$ for each), indicating that the variation between samples was significantly greater than the variation among triplicate values. These analyses yielded one least-squares mean for each sample, and these least-squares means were used for further analyses. De-identified information regarding gestational age at time of sampling, ethnicity, maternal age, weight and parity was used to test whether these factors influence first trimester PAPP-A2 levels among women who went on to have uncomplicated pregnancies. To determine whether first trimester PAPP-A2 levels differ between uncomplicated controls, preeclamptic pregnancies and/or pregnancies resulting in SGA, each of the three PAPP-A2 bands reactive bands at 290, 250 and 130 kDa was analysed by a general linear model including the effects of PE (yes/no) and SGA (yes/no).

2.4. Results

2.4.1. Biological factors affecting PAPP-A2 in uncomplicated pregnancy

There were no significant differences in maternal age, weight, ethnicity, parity or sampling variables (gestational age on draw date of sample or total freeze time of sample) between the women who developed PE and/or gave birth to SGA-infants and controls (Table 2.1). Among our controls were 5 East Asian women, 1 Asian woman and 31 Caucasian women (Table 2.1). Sixteen were nulliparous, 20 were parous and 1 woman was of uncertain parity (Table 2.1).

Among controls, there were no correlations between the concentrations of any of the PAPP-A2 isoforms or PAPP-A MoM and gestational age at the time of first trimester sampling or maternal age or weight at the time of first trimester sampling, whether or not preterm births (prior to 37 weeks, but at least 34 weeks, of which there were two) were included ($P > 0.05$ in all cases). Similarly, there was no difference in PAPP-A2 or PAPP-A concentrations between women who had not previously given birth and those who had, whether or not preterm births were included ($P > 0.05$ in all cases). Excluding pregnancies which resulted in preterm births, birth weight was positively correlated with levels of the 290 kDa PAPP-A2 isoform ($r = 0.379$, $P < 0.02$) and the PAPP-A MoM ($r = 0.343$, $P < 0.03$), but not with levels of the 250 kDa or 130 kDa isoforms of PAPP-A2 ($r= 0.078$, $P > 0.6$ and $r = -0.176$ and $P > 0.25$, respectively). Additionally, significant correlations were found between PAPP-A MoM and the 290 kDa PAPP-A2 isoform ($r = 0.375$, $P < 0.02$), as well as between PAPP-A MoM and the 250 kDa PAPP-A2 isoform ($r = 0.399$, $P < 0.01$), but not between PAPP-A MoM and the 130 kDa PAPP-A2 isoform ($r = -0.126$, $P > 0.4$). There was no significant correlation between the 290 kDa and 250 kDa PAPP-A2 isoforms ($r = 0.254$, $P > 0.1$), the 290 kDa and the 130 kDa PAPP-A2 isoforms ($r = 0.227$, $P > 0.15$) or the 250 kDa and 130 kDa PAPP-A2 isoforms ($r = 0.208$, $P > 0.15$).

2.4.2. Relationship between PAPP-A2 development of PE and/or SGA

In a general linear model including the effects of PE (yes/no) and SGA (yes/no), first trimester 250 kDa PAPP-A2 serum concentration was significantly higher in women who subsequently developed PE than in those who did not ($F = 4.25$, $P < 0.044$) but was not different between women who did or did not give birth to SGA-infants ($F = 0.04$, $P > 0.844$). Very similar results were obtained if preterm births, i.e., prior to 37 weeks, but at least 34 weeks were excluded (excluded samples included one preterm PE sample plus its two gestational-age matched controls, of which one was preterm and one was non-preterm, as well as one other preterm control plus its matched non-preterm SGA case sample; data not shown). This difference was also significant when analysed by nonparametric tests (Wilcoxon $Z = 2.43$, $P = 0.015$; Kruskal-Wallis $\chi 2 = 5.97$, $P = 0.015$). Levels of the 250 kDa isoform of PAPP-A2 were approximately 51% higher in women who developed PE compared to controls, with a mean serum concentration of 35 ng/mL in PE pregnancies compared to 23 ng/mL in controls (Table 2.1, Figure 2.3). The concentrations of 290 kDa and 130 kDa PAPP-A2 did not differ between pregnancies that did or did not subsequently develop PE, or that did or did not result in a SGA infant ($P > 0.05$ in all cases). First trimester PAPP-A MoM was significantly lower in women who subsequently gave birth to SGA babies than in those who did not ($F = 6.64$, $P < 0.01$), but was not different between women who did or did not develop PE ($F = 0.01$, $P > 0.9$). The difference between SGA pregnancies and controls was significant if preterm births were excluded and/or nonparametric tests were used (data not shown). PAPP-A MoM was approximately 37% lower in women with SGA-infants (0.76) compared to controls (1.20) (Table 2.1, Figure 2.4).

2.5. Discussion

This study measured the IGFBP protease PAPP-A2 in serum samples collected in the first trimester of pregnancy from women who subsequently gave birth to SGA-infants and/or developed PE. Concentrations of the 250 kDa isoform of PAPP-A2 were significantly higher in pregnancies that subsequently developed PE, but not in those that resulted in SGA-infants.

2.5.1. PAPP-A2 immunospecific bands

Of the three bands immunospecific for PAPP-A2, levels of the 250 kDa protein were significantly elevated in samples from pregnancies that subsequently developed PE (but not SGA). This band is the size expected for the longer isoform, and has been previously found to be present at higher levels in the serum of pregnant women compared with non-pregnant women using the same antibody (35). In contrast, neither the 130 kDa nor 290 kDa isoforms were associated with the development of PE or SGA. We speculate that the 290 kDa band represents a glycosylated or otherwise modified variant of the same protein, as glycosylation has been shown to add between 40-50 kDa to the molecular weight of PAPP-A2 in recombinant protein (http://www.sinobiological.com/PDF/10528-H08H.pdf). However, because the 290 kDa band did not differ between complicated and uncomplicated pregnancies, we did not investigate this further. The 130 kDa band represents a known splice variant of PAPP-A2 (23,35). Previous work has found PAPP-A2 proteolytic activity against IGFBP-5 in pregnancy plasma, but only among proteins greater than 150 kDa, suggesting that the 130 kDa splice variant of PAPP-A2 does not contribute to this proteolytic activity (36).

2.5.2. PAPP-A2 and preeclampsia

PAPP-A2 is consistently upregulated in placentae at term in pregnancies afflicted by PE and HELLP (25-32), and also circulates at higher levels in late gestation in women afflicted with PE (27). The late-gestation upregulation of PAPP-A2 has been suggested to be a mechanism to compensate for the abnormal placentation by increasing IGFBP proteolysis to raise local IGF levels to promote feto-placental growth (37). Placental hypoxia, known to occur in PE, has been hypothesized as a mechanism by which PAPP-A2 might be upregulated in placental pathologies, since hypoxia upregulates PAPP-A2 mRNA and protein expression in BeWo cells and late-gestation placental explants (31,38), but this has not been examined in first trimester tissue. The present study suggests that upregulation, whatever the cause, occurs early in complicated pregnancies, and therefore may mitigate developmental abnormalities that might otherwise have more severe effects.

While a growing number of studies have found associations between PE and PAPP-A2 at term, we know of only one study to examine circulating PAPP-A2 concentrations early in gestation, described in a meeting abstract (39). Our observation of elevated levels of circulating PAPP-A2 in the first trimester of pregnancy in women who subsequently develop PE is consistent with this study (39), but in contrast with the frequent findings of lower first trimester PAPP-A circulating levels in pregnancies that develop PE (15-17,19,40) and other complications (14-17,20). Although we found PAPP-A2 levels were elevated in pregnancies that subsequently developed PE, PAPP-A concentrations were significantly lower in pregnancies that resulted in a SGA infant, consistent with previous work. This finding suggests that while PAPP-A and PAPP-A2 are both IGFBP proteases, they have different functions and/or regulation and therefore play different roles in adverse gestational outcomes. As a result, the potential of first trimester PAPP-A and PAPP-A2 levels to jointly predict PE deserves further attention.

2.5.3. PAPP-A2 and SGA

Levels of PAPP-A2 were not found to be significantly different in pregnancies resulting in SGA-infants compared with controls. However, previous work has shown PAPP-A2 to be elevated in maternal circulation and upregulated in term placentae from pregnancies afflicted with IUGR (41). A potential explanation for this discrepancy is that, in our study, SGA was defined as simply having birth weights below the lowest 5th percentile, whereas Whitehead *et al*. (2013) examined pregnancies complicated by IUGR that required delivery before 34 weeks with antenatal evidence of uteroplacental insufficiency (asymmetrical growth + abnormal umbilical artery Doppler velocimetry, ± oligohydramnios or abnormal fetal vessel velocimetry) (41). Accordingly, it is possible that elevation of PAPP-A2 may not be specific to PE, and may be elevated in cases of preterm IUGR as well.

Our sample sizes are small, limiting our ability to adjust for potential confounding effects. However, the observation that the elevation in PAPP-A2 levels was significant despite this small sample size, even though the well-established association between PAPP-A levels and PE was not significant, suggests that the dysregulation of PAPP-A2 may be substantial and consistent. This is the first report of elevated first trimester

PAPP-A2 in PE, which warrants further investigation with expanded sample sizes as well as the development of a more sensitive PAPP-A2 ELISA or perhaps other proteomic techniques to improve and streamline quantification.

2.6. Conclusion

We observed elevated first trimester levels of PAPP-A2 in the maternal circulation in pregnancies that developed PE, but not in those that developed SGA. The findings presented here suggest that the elevation in PAPP-A2 expression in PE that has consistently been observed at term actually begins early in pregnancy and so is not simply a response to fully-developed placental pathology. This result underlines the need for further research into the functions of PAPP-A2 early in pregnancy.

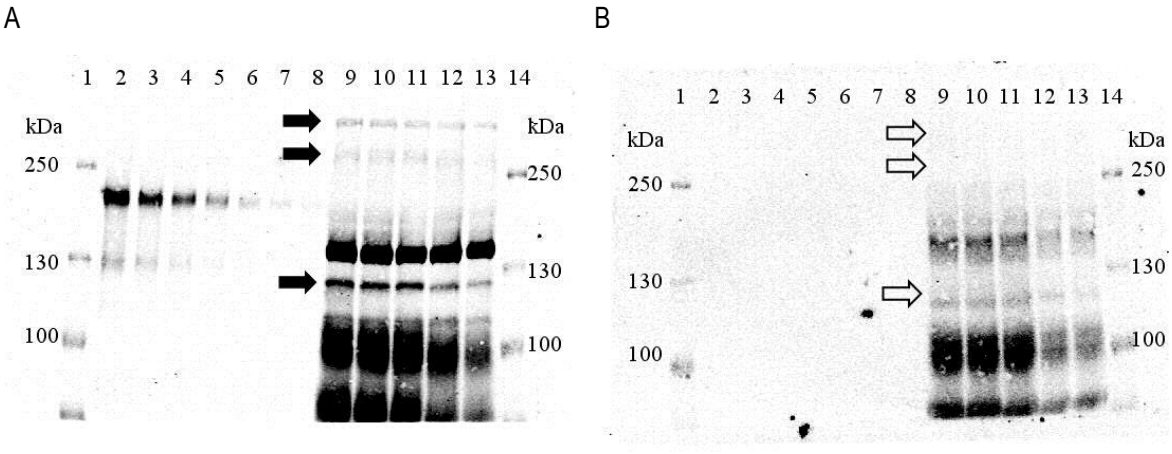

Figure 2.1. Determination of PAPP-A2 immunospecific bands
Western blots of first trimester serum with (A) polyclonal goat anti-human PAPP-A2 or (B) polyclonal goat anti-human IgG. Lanes 2-8: PAPP-A2 recombinant protein standard curve from 40 ng/mL to 0.625 ng/mL; Lanes 9-11: Serum sample diluted 1/8 in sterile PBS+0.1%BSA in triplicate; Lanes 12-13: Serum sample diluted 1/16 in sterile PBS+0.1%BSA; Lanes 1 and 14: Ladder. Recombinant human PAPP-A2 protein is not full-length as it does not include the prepro region (residues 1-233) and the C-terminal region (residues 1397-1791), and so has an expected size of 168 kDa in SDS-PAGE reducing conditions. Solid arrows (A) highlight the presence of PAPP-A2 immunospecific bands at 290, 250 kDa and 130 kDa (splice variant) with polyclonal goat anti-human PAPP-A2 as the primary antibody. Hollow arrows in (B) show the absence of these same bands in the negative control.

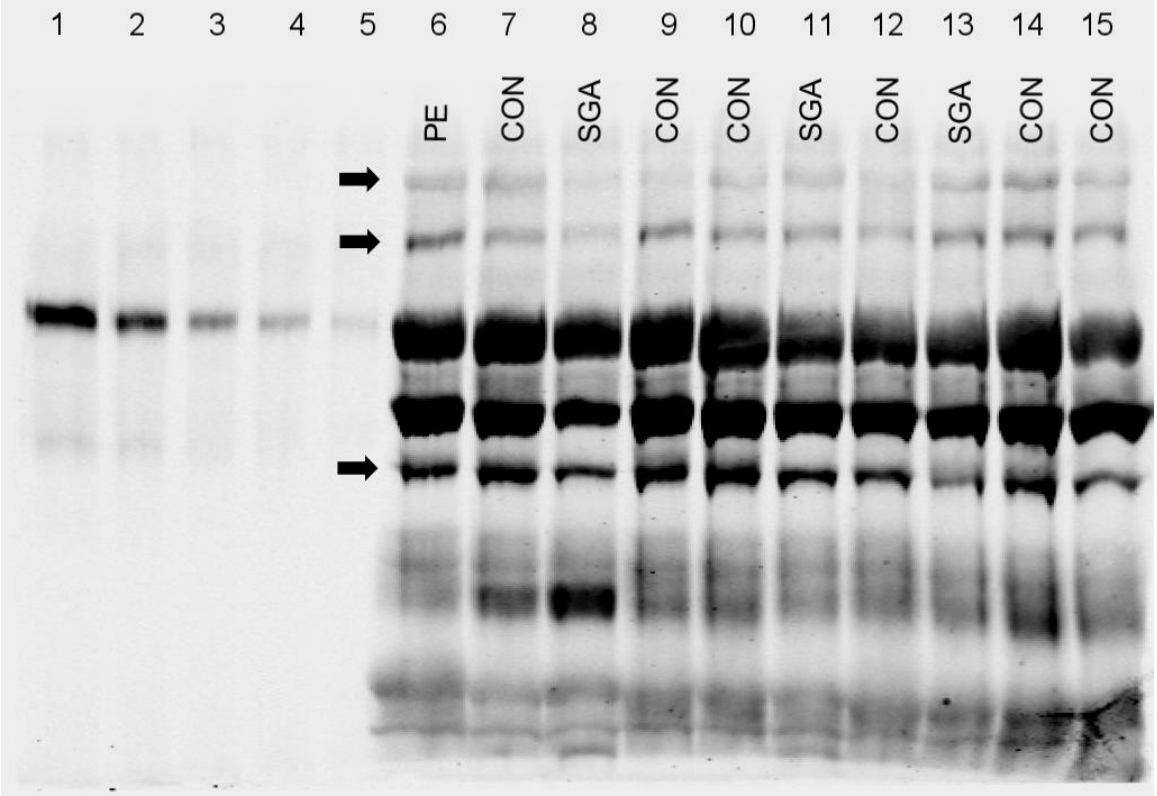

Figure 2.2. Representative Western blot
A representative Western blot of the 21 that were run to quantify each case/control group in triplicate. Solid arrows show PAPP-A2 immuno-specific bands that were quantified in this analysis at 290, 250 and 130 kDa (top to bottom). Lanes 1-5: PAPP-A2 protein standard (20 - 1.25 ng/mL); Lanes 6-15: 4 groups of cases and matched controls.

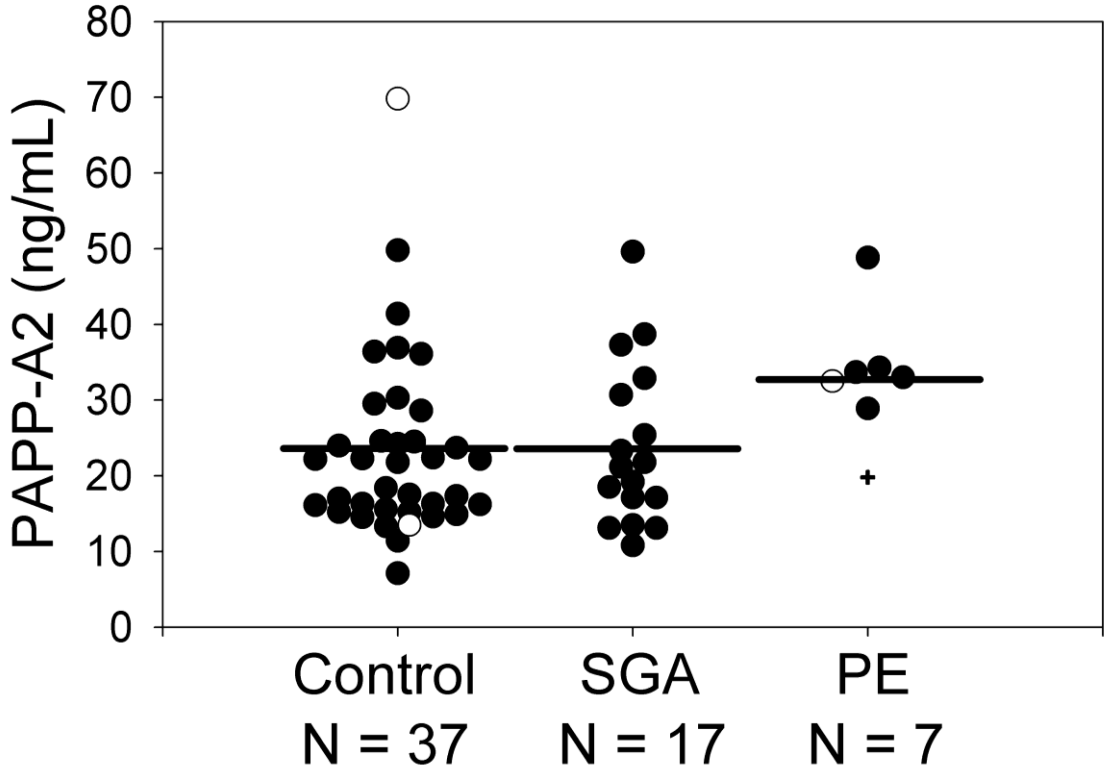

Figure 2.3. First trimester maternal serum levels of 250 kDa PAPP-A2
First trimester maternal serum levels of 250 kDa PAPP-A2 (measured via immunofluorescent Western blot) were significantly elevated in PE compared with control, but not in PE/SGA or SGA, regardless of whether or not preterm samples (prior to 37 weeks, but at least 34 weeks, indicated by open symbols) were included. One sample (plus symbol) was from a pregnancy that subsequently developed PE and resulted in a SGA infant. Horizontal lines represent the least squares means from a general linear model including the effects of PE and SGA.

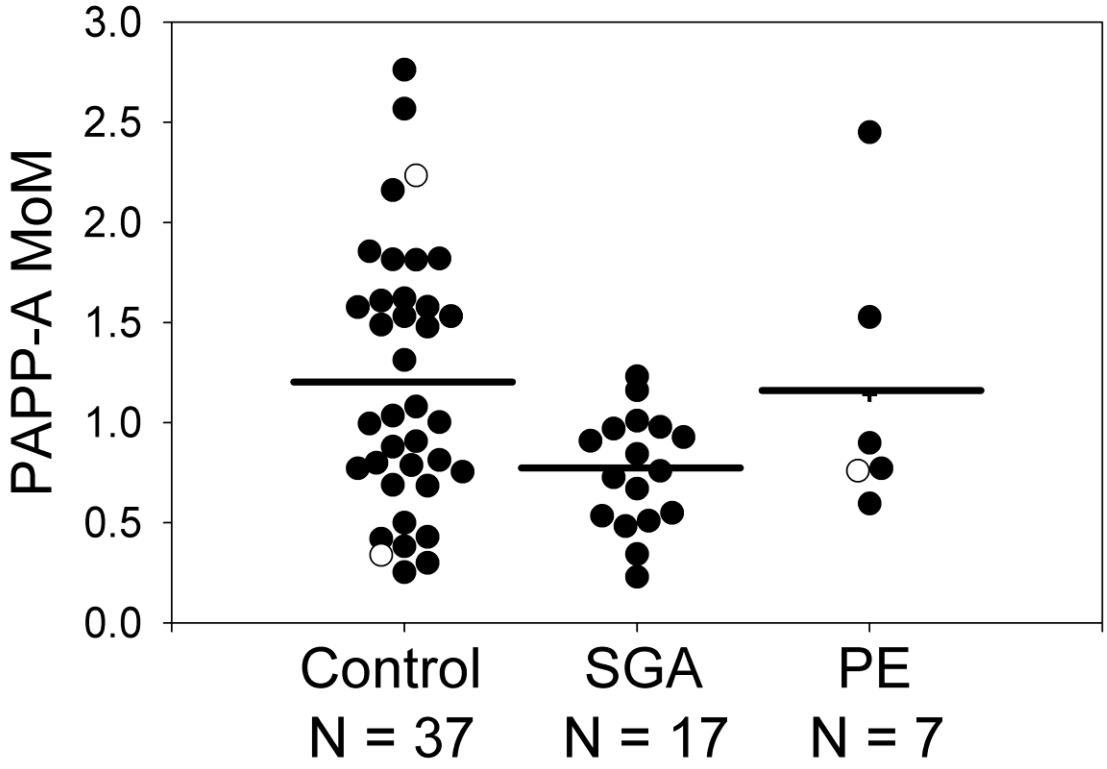

Figure 2.4. First trimester maternal serum levels of PAPP-A
First trimester levels of PAPP-A (measured via the DELFIA(R) Xpress PAPP-A immunoassay kit) were significantly lower in pregnancies resulting in a SGA-infant compared with controls, but not in PE/SGA or PE, regardless of whether or not preterm samples (prior to 37 weeks, but at least 34 weeks, indicated by open symbols) were included. One sample (plus symbol) was from a pregnancy that subsequently developed PE and resulted in a SGA infant. Horizontal lines represent the least squares means from a general linear model including the effects of PE and SGA.

Table 2.1. Maternal and Sample variables examined in first trimester serum (means ± standard error of the mean)

	Control (n=37 including 2 preterm)	PE (n=6 including 1 preterm)	PE/SGA (n=1)	SGA (n=17)
Maternal age at draw date (years)	34.3 ± 0.6	36.7 ± 1.3	36.0	34.9 ± 0.6
Maternal weight (kg)	64.4 ± 2.0	62.9 ± 2.7	61.0	59.9 ± 2.9
Gestational age on draw date (weeks)	11.8 ± 0.1	12.4 ± 0.4	11.7	11.6 ± 0.2
Freeze Time (months)	42.4 ± 1.2	42.0 ± 4.3	46.0	41.2 ± 2.6
% Parous	54%	33%	100%	24%
Ethnicity Caucasian/Asian*/East Asian	31 / 1 / 5	3 / 1 / 2	1 / 0 / 0	9 / 0 / 8
Birth weight (g)				
Including preterm(s)	3468 ± 81.7	3138 ± 321.0	**	**
Excluding preterm(s)	3551 ± 60.8	3426 ± 174.5	2180	2633 ± 31.1
Gestational age at delivery (weeks)				
Including preterm(s)	39.1 ± 0.3	37.5 ± 0.8	**	**
Excluding preterm(s)	39.4 ± 0.2	38.2 ± 0.6	37.0	40.0 ± 0.2
Mean PAPP-A2 (ng/mL)				
Including preterm(s)	23.3 ± 2.0	35.2 ± 2.8	**	**
Excluding preterm(s)	22.2 ± 1.6	35.7 ± 3.4	19.8	23.7 ± 2.6
MoM PAPP-A	1.20 ± 0.1	1.17 ± 0.3	1.14	0.76 ± 0.1

*(e.g., India, Pakistan, Bangladesh)
**(the PE/SGA and SGA sample sets contained no preterms)

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3. Effects of IGFBP-4 and -5 on trophoblast migration and localization within first trimester placental villi

3.1. Introduction

Adverse gestational outcomes such as preeclampsia (PE) and intrauterine growth restriction (IUGR) are associated with placental insufficiency that has been attributed to deficiencies in processes critical to placental development. These processes include but are not limited to cytotrophoblast (CT) proliferation as well as extravillous trophoblast (EVT) migration. Proliferation and fusion of cells in the CT layer of the placental villi function to maintain the outer syncytiotrophoblast (ST) layer which serves as the interface between the fetal placenta and the maternal blood, as well as a site of placental endocrine secretion (1). Migration of EVT cells from the placental villi into the maternal decidua is a necessary step for the remodeling of uterine spiral arteries, whereby EVT invade and replace the vessel endothelium, thus increasing vessel diameter to ensure adequate blood flow required for oxygen and nutrient delivery to the placenta (2,3). In both human villous explants and primary trophoblast cultures, insulin-like growth factors I and II (IGF-I and IGF-II, respectively), have been found to stimulate trophoblast proliferation and EVT migration, as well as several other processes including hormone secretion, and glucose and amino acid uptake (4,5).

In general, the bioavailability of IGF-I and -II is modulated by six insulin-like growth factor-binding proteins (IGFBPs) (4,6-8), with the bioavailability of the IGFs thus being increased with proteolysis of IGFBPs (7) . In addition to reducing the availability of the IGFs, IGFBPs have also been found to increase the half-life of IGFs, concentrate them in particular regions and/or potentiate their effects (9). Furthermore, IGFBP-1(10,11), IGFBP-2 (12-14), IGFBP-3(15-17) and IGFBP-5 (18-21) have all been shown to exert IGF-independent effects on cellular processes including apoptosis, cellular

proliferation, cell adhesion and cellular mobility within cell models of various tissues including bone, breast, prostate, muscle, kidney, placenta and gliomas (4,22-24). Specifically, in the context of the placenta, IGFBP-1 was found to stimulate migration in an IGF-independent manner in HTR-8/SVneo cells, an immortalized EVT cell line and commonly used model of EVT migration and invasion (11). In BeWo cells, an immortalized choriocarcinoma cell line and commonly used model of villous CT, IGFBP-3 was found to inhibit proliferation in an IGF-independent manner (17). The proteolytic fragments of cleaved IGFBP-3 and -5 have also been shown to have effects modulating the mitogenic abilities of IGFs in mouse embryonic fibroblasts and osteoblast cells, respectively (22,25,26).

The pappalysins pregnancy-associated plasma proteins-A and -A2 (PAPP-A and PAPP-A2, respectively) are two IGFBP proteases that have been consistently associated with a wide range of pregnancy complications. PAPP-A is a protease of IGFBP-4 and IGFBP-5, and thus is expected to play a role in increasing the bioavailability of the IGFs which could potentially affect processes such as EVT migration and invasion (4,27-29). PAPP-A circulates in the maternal blood at high levels during pregnancy, and abnormally low levels are associated with an increased risk of PE (30-34) and IUGR (30-32,35). Pregnancy-associated plasma protein-A2 (PAPP-A2) shares 45% amino acid identity with PAPP-A, and cleaves IGFBP-5 and possibly IGFBP-3 (36-38). PAPP-A2 levels are higher in the placenta and maternal circulation of preeclamptic pregnancies compared with controls at term (39-46), and are also elevated in the first-trimester maternal serum of pregnancies that subsequently develop preeclampsia (47).

A mechanistic link between pappalysin levels and complications resulting from placental dysfunction has yet to be elucidated. If altered pappalysin expression plays a causal role in placental pathologies, it is likely through the proteolysis of their IGFBP substrates. Of the IGFBPs, IGFBP-1 is the most abundant within the maternal decidua (48), and is also the most well-studied. While IGFBP-1 has been found to have both IGF-dependent and independent effects modulating human trophoblast invasion *in vitro* (4,11,49,50), and has also been found to be present at abnormal levels in the serum of women afflicted with preeclampsia (51-57), the roles of the pappalysin substrates

IGFBP-4 and -5 in placental development have received little attention (4). Recently it was found that elevated circulating levels of IGFBP-4 in early pregnancy are strongly associated with the subsequent development of fetal growth restriction, a consequence of abnormal placental development (58). Prior to this, increasing doses of IGFBP-5 were shown to inhibit the migration-stimulating effects of IGF-II in EVT cells *in vitro* (59).

Currently, it is unclear through which mechanisms the pappalysins may play a role in abnormal placental development, whether via effects on IGF availability or through effects on other actions of the IGFBPs. I therefore employed a well-established model of first trimester EVT, HTR-8/SVneo cells, to elucidate the effects of exogenous IGFBP-4 and IGFBP-5 on the migration-stimulating effects of IGF-I and IGF-II. I also attempted to complement conclusions reached via migration assays with invasion assays on HTR-8/SVneo cells.

Furthermore, the location and timing of expression of the substrates of PAPP-A and PAPP-A2 is relevant to the mechanisms by which the pappalysins might influence placental development. Previously it had been thought that expression of IGFBP-4 and IGFBP-5 in the first trimester was isolated to the maternal decidua, with expression in the chorionic mesoderm not being observed until later in gestation (48). Should expression of the pappalysin substrates be isolated to the maternal decidua, then PAPP-A and PAPP-A2 could potentially play a role influencing trophoblast migration and invasion. However, recently IGFBP-4 immunoreactivity was observed in villi at 10-13 weeks, suggesting potential roles for IGFBPs, and therefore for their respective proteases (58), within the villi early in gestation. Immunohistochemical staining of placental sections was therefore performed, in order to determine more precisely the timing and location of the presence of IGFBP-4, IGFBP-5 and PAPP-A2 within the placental villi. I also attempted to investigate potential effects of IGFBP-4 and IGFBP-5 on a process occurring within the villi, CT proliferation, using BeWo choriocarcinoma cells as a model.

3.2. Methods

3.2.1. Cell lines and cell culture

HTR-8/SVneo cells, an immortalized cell line that is a well-established model of first trimester human trophoblasts, were obtained from Dr. Caroline Dunk with the permission of Dr. Charles Graham (Queen's University, Kingston, ON, Canada) (60). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 U/mL of streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cell culture media and reagents were purchased from Life Technologies (Burlington, ON, Canada).

BeWo cells, an immortalized human choriocarcinoma cell line and well established model of villous CT (61) were obtained from Dr. Andrée Gruslin of the University of Ottawa. Cells were cultured in Ham's F-12K medium (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

3.2.2. HTR-8/SVneo cell wounding assay

HTR-8/SVneo cells were seeded at a density of 40,000 cells per well in 24-well polystyrene tissue culture plates and allowed to grow to confluence before serum starving for at least 4 hours. Wounding was performed with a 20 µL pipette tip across the horizontal midsection of each well, and photographs were taken at two points along each wound. The XY coordinates of each point were saved using Simple PCI coordinate mapping software and photographed again 24 hours after treatment (described below). The percentage wound closure was calculated as (1 – (area of wound at 24 hours/area of wound at time 0))*100%, as quantified using ImageJ software (Figure 3.1). Recombinant human IGF-I, IGF-II, IGFBP-4 and IGFBP-5 were all purchased from R&D Systems (Minneapolis, Minnesota). Initial experiments assessed effects of dose ranges of 2-25 nM for IGF-I and –II and established a dose of 2 nM as a minimum dose at which a significant increase in migration of HTR-8/SVneo cells was observed. An effective dose range of IGFBP-4 and IGFBP-5 was expected to be 1-25 nM according to literature

and preliminary experiments (59). Migration experiments examining the stimulatory effects of the IGFs were performed by treating HTR-8/SVneo cells for 24 hours with either 2 nM IGF-I or IGF-II. Migration experiments examining the inhibitory effects of the IGFBPs were performed by co-treating HTR-8/SVneo cells with either 20 nM IGFBP-4 or IGFBP-5, and one of each of the two IGFs at 2 nM. Experiments examining IGF-I potentiating effects of low doses of IGFBP-5 were performed across a range of concentrations from equimolar amounts of IGF-I and IGFBP-5, to an 8 fold excess of IGF-I against IGFBP-5 based on previous work with IGFBP-1 (62,63). Preliminary experiments examining potential IGF-independent effects of HTR-8/SVneo cells treated with 20 nM IGFBP-4 or 20 nM IGFBP-5 for 24 hours were also performed.

3.2.3. HTR-8/SVneo invasion assay

To assess the effects of treatments on invasive behaviour, the ability of HTR-8/SVneo cells to penetrate Matrigel-coated (BD Biosciences, Bedford, MA) 8 µm pore size polycarbonate membrane Transwell inserts was measured (Costar, Corning Inc., Corning, NY) (64). Optimization experiments included coating inserts with either 50 or 100 µL of high concentration, growth-factor–reduced Matrigel (BD Biosciences), diluted to either 0.6, 1 or 3 mg/mL. Various polymerization protocols were attempted, including overnight incubation at 37°C in a humidified atmosphere, or “drying” overnight at room temperature under sterile conditions, with next-day reconstitution at 37°C for 1 hour with serum-free RPMI 1640.

3.2.4. BeWo proliferation assay

BeWo cell proliferation was analyzed with a 5-bromo-2’-deoxy-uridine (BrdU) labelling and detection kit according to the manufacturer’s instructions (Roche Diagnostics; Mannheim, Germany). BeWo cells were grown over a range of cell densities in 96-well tissue culture plates for 24 h in 10% serum supplemented medium before being serum starved for 24 hours. Cells were then treated with either vehicle or IGF-I or IGF-II at various concentrations (2 nM, 10 nM or 25 nM) for 24 or 48 hrs. After labelling with BrdU for 4 hrs, the cells were fixed and BrdU incorporation into DNA was measured by an ELISA technique. BeWo cell proliferation was also measured in a

manual cell counting experiment using Hoechst staining. Briefly, cells were seeded at a density of 10,000 cells per well in a 24-well polystyrene tissue culture plate, and allowed to grow 24 hours before being serum starved for 24 hours. Cells were treated with either vehicle, 2 nM IGF-I or 10 nM IGF-I for 24 hours, and then stained with Hoechst fluorescent stain for 20 minutes before washing and imaging. Manual cell counting was performed using Image J software.

3.2.5. Immunohistochemistry

Human placental tissue was collected from elective terminations of normal pregnancies between 5 and 20 weeks of gestation. Upon collection, samples were fixed overnight in 4% paraformaldehyde, paraffin-embedded, sectioned (4-5 μm) and mounted. Immunohistochemical staining was performed using the HRP-AEC goat kit (R&D Systems) according to the manufacturer's instructions. Briefly, sections were deparaffinized in xylene and rehydrated with a graded series of alcohol. Antigen retrieval was performed by heating sections over a hot plate in 10 mM citrate buffer. Sections were then rinsed in PBS + 0.1% Tween twice for two minutes before incubation with Peroxidase Blocking Reagent. Adjacent sections were incubated with primary antibodies against one of CD31, PAPPA2, IGFBP-4 and IGFBP-5, followed by incubation with biotinylated secondary antibodies and then streptavidin-Horse Radish Peroxidase (HRP) incubation. Sections were visualized with DAB and counterstained with hematoxylin stain. Polyclonal anti-human PAPP-A, PAPP-A2, IGFBP-4 and IGFBP-5 antibodies were obtained from R&D Systems. Negative controls were prepared using non-specific goat IgG (also from R&D Systems) in place of primary antibodies.

3.2.6. Statistical analyses

Effects of treatments were analyzed using a general linear model including the effects of treatment and date of experiment. Treatment replicates were distributed across different experimental dates, with not all treatments performed on the same day. All experiments contained the appropriate controls (controls, and IGF-I and/or –II alone) to allow for comparison across dates (with sample sizes comparable to each of the treatments within the experiment).

3.3. Results

3.3.1. IGF-I, IGF-II and HTR-8/SVneo migration

Preliminary experiments with 2 nM, 10 nM or 25 nM of either IGF-I or IGF-II showed 2 nM to be the minimum tested dose at which a significant stimulatory effect on HTR-8/SVneo cells was observed (for preliminary IGF-I experiments, $F= 28.65$, $P<.0001$; for preliminary IGF-II experiments, $F= 12.86$, $P<.0001$) (Table 3.1). Treating HTR-8/SVneo cells with either IGF-I or IGF-II at 2 nM significantly increased migration compared with controls, with IGF-I increasing migration significantly more than IGF-II ($F= 38.11$, $P<.0001$) (Table 3.2). Over 24 hours, treatment with IGF-I or IGF-II at 2 nM produced 63% and 53% wound closure, respectively, compared with 38% wound closure in controls (Table 3.2).

3.3.2. IGFBP-4, IGFBP-5 inhibit effects of IGF-I and IGF-II on HTR-8/SVneo migration

While IGFBP-4 and IGFBP-5 both significantly reduced the effects of 2 nM IGF-I, 20 nM IGFBP-4 almost completely blocked the effects of 2 nM IGF-I, while 20 nM IGFBP-5 significantly reduced the effects of 2 nM IGF-I, but not to control levels ($F= 38.11$, $P<.0001$) (Table 3.2). HTR-8/SVneo cells showed 41% wound closure following treatment with 20 nM IGFBP-4 and 2 nM IGF-I compared with 38% in controls, while HTR-8/SVneo cells treated with 20nM IGFBP-5 and 2 nM IGF-I showed 56% wound closure. Either 20 nM IGFBP-4 or 20 nM IGFBP-5 completely blocked the effects of 2 nM IGF-II ($F= 38.11$, $P<.0001$) (Table 3.2). Treatment with 20 nM IGFBP-4 and 2 nM IGF-II showed 36% wound closure, and treatment with 20 nM IGFBP-5 and 2 nM IGF-II showed 42% wound closure; neither treatment was significantly different from controls. There was no significant effect of IGFBP-4 or IGFBP-5 alone at 20 nM in preliminary experiments, suggesting an absence of IGF-independent effects (data not shown). Moreover, there was no significant potentiation effect of low doses of IGFBP-5 with 2 nM IGF-I (Table 3.3).

3.3.3. Strong immunoreactivity for IGFBP-4, IGFBP-5 and PAPP-A2 in the syncytiotrophoblast

The presence of IGFBP-4, IGFBP-5 and PAPP-A2 was examined in serial sections of placental villi at 5, 6, 7 and 13 weeks of gestation. As a negative control, non-specific goat IgG was used in the place of the primary antibodies. In addition, a primary antibody against CD31 was used as a negative control for immunoreactivity in the ST, since staining was primarily in the endothelium of fetal vessels. Across all gestational ages, IGFBP-4 and IGFBP-5 showed localization within the ST, with stronger immunoreactivity observed for IGFBP-4 than IGFBP-5 (Figure 3.2). IGFBP-4 and IGFBP-5 also appeared in the chorionic mesoderm. PAPP-A2 in contrast, showed little to no immunoreactivity in the chorionic mesoderm at 5 and 6 weeks, but did show strong immunoreactivity in the ST (Figure 3.2).

3.3.4. HTR-8/SVneo invasion assay

Findings of HTR-8/SVneo invasion assay were inconsistent and inconclusive, regardless of treatment or polymerization protocol employed. Trial assays and results are summarized in Appendix A.

3.3.5. BeWo proliferation assay

No significant effects of IGF on proliferation were observed when measuring proliferation either by BrdU incorporation or manual cell counting in ImageJ, regardless of cellular density, treatment or treatment time employed. Cellular proliferation is expressed as percent compared to the control (Appendix B).

3.4. Discussion

The primary objective of this study was to elucidate the effects of pappalysin substrates IGFBP-4 and IGFBP-5 on IGF-I and IGF-II in a model of EVT migration to shed light on potential roles of the pappalysins in placental development. A related objective was to clarify ambiguities regarding the current understanding of the location

and timing of the presence of IGFBP-4 and IGFBP-5, shown in immunohistochemical staining of placental sections in the first trimester.

3.4.1. IGF-I and IGF-II stimulate HTR-8/SVneo migration

Both IGF-I and IGF-II at 2 nM significantly stimulated migration of HTR-8/SVneo cells in a cell-wounding assay, with IGF-I being more stimulatory than IGF-II. These migration-stimulating effects are consistent with previous findings in various models of first trimester human EVT, including HTR-8/SVneo cells, primary cultures of EVT cells, and placental explants (49,50,65-69). The disparate effects of IGF-I and –II in my results are potentially explained by the presence of both type 1 and type 2 IGF receptors (IGF1R and IGF2R) in HTR-8/SVneo cells (59,69). Previous work has shown that the majority of the effects of IGF-I and –II are mediated through IGF1R, which has a higher binding affinity for IGF-I than it does for IGF-II, depending on the cell type studied and the conditions of the binding assay (70-77). Higher binding affinity of IGF1R for IGF-I is consistent with IGF-I having greater stimulatory effects on migration than IGF-II. Conversely, IGF2R has been described as an IGF-II antagonist, binding to IGF-II with higher affinity than to IGF-I, inactivating IGF-II by targeting it for lysosomal degradation (70,78-80). However, at least one study of HTR-8/SVneo cells has shown IGF-II to stimulate migration through the IGF2R receptor (69).

IGF-I and -II have different primary physiological sources in the placenta. IGF-II is strongly expressed by EVT and ST in the human placenta (4,5,48,77,81), whereas the predominate source of IGF-I in the placenta is the maternal circulation, as it is only weakly expressed by the placenta (5,48). Low levels of IGF-I mRNA relative to IGF-II mRNA have been detected in the CT, mesodermal core and endothelium of human placental villi (in first, second and third trimesters) (77,82), but not in EVT (65,77). In contrast, IGF-I protein has been detected less consistently. In one study, IGF-I protein was undetectable in human placental lysates (83), and in another IGF-I protein was detected in first trimester villous CT, but to a much lesser extent than IGF-II (77).

3.4.2. No IGF-I potentiating effects of IGFBP-5, or IGF-independent effects of IGFBP-4 or IGFBP-5 observed in HTR-8/SVneo cells

In the wound-healing migration assay, there was no significant potentiation of the IGF-I migration-stimulating effect by low doses of IGFBP-5, and no significant IGF-independent effects of either 20 nM IGFBP-4 or IGFBP-5. There are several potential explanations for the lack of effect in both sets of experiments. In the case of IGF-potentiation, it could be that interaction between IGFBP-5 and extracellular matrix is required for IGF-potentiation (84), or that other factors that may require optimization, including incubation time of the treatments or molar ratio of IGF-I to IGFBP-5. The lack of a significant effect of either IGFBP-4 or IGFBP-5 alone at 20 nM in HTR-8/SVneo cells may be attributed to either the absence of as-of-yet-uncharacterized cell surface IGFBP-4 and -5 receptors (85), a required interaction with an extracellular matrix (84), or that proteolysis of IGFBP-5 is required for IGF-independent effects (22,26).

3.4.3. Differential effects of IGFBP-4, IGFBP-5 imply different roles of PAPP-A and PAPP-A2

IGFBP-4 and -5 show different levels of inhibition of the migration-stimulating effects of IGF-I and –II. Specifically, IGFBP-4 was able to block the migration-stimulating effects of both IGF-I and IGF-II such that migration of treated HTR-8/SVneo cells was not significantly different from controls. In contrast, IGFBP-5 was able to significantly inhibit the migration-stimulating effects of IGF-I, but not to control levels. IGFBP-5 was however able to completely inhibit the effects of IGF-II. While previous work has shown IGFBP-5 blocks IGF-II stimulation of migration in a cell line model of extravillous trophoblasts (59), this is to my knowledge the first data regarding the effects of exogenous IGFBP-4 on trophoblast phenotypes. IGFBP-4 has however been found to influence migration and invasion in cancer studies, demonstrating contrasting effects depending on the cell type examined. Overexpression of IGFBP-4 has been shown to promote invasion and migration in primary renal cancer cells (86). In contrast, studies in other tissue types have shown IGFBP-4 to be a cancer inhibitor (87-89), with cleavage of IGFBP-4 by PAPP-A enhancing migration of malignant pleural mesothelioma cells via release of IGF-I (90). While IGFBP-5 has been shown to stimulate apoptosis and

influence differentiation in normal breast epithelial cells, the effects of IGFBP-5 on cellular proliferation and invasion in various breast cancer studies appears to be cell line dependent (91). In situ studies and cellular models of prostate and other cancers suggest IGFBP-5 may have growth-stimulatory effects on tumour cells (91).

Given that IGF-I and IGF-II have different physiological sources in pregnancy, that IGF-I has greater effects on EVT migration than IGF-II, that IGFBP-4 and IGFBP-5 have disparate effects on each IGF, and that PAPP-A and PAPP-A2 have shown contrasting patterns of regulation in relation to adverse pregnancy outcomes (with PAPP-A being down-regulated (30-35) and PAPP-A2 being upregulated in association with pregnancy complications (39-47)), it may be that PAPP-A and PAPP-A2 play very different roles in normal placental development and disease (Figure 3.3). PAPP-A is a protease of both IGFBP-4 and IGFBP-5, whereas PAPP-A2 only proteolyzes IGFBP-5. Therefore, PAPP-A2 is expected to have less stimulatory effect on EVT migration compared to PAPP-A, as IGFBP-5 shows less inhibition of IGF-I, which has been shown to stimulate more migration than IGF-II. Additionally, PAPP-A2 is expected to have a relatively stronger effect modulating the availability of locally produced IGF-II versus IGF-I from the maternal circulation. In mice, deletion of an IGF-II promoter transcript specifically expressed in the labyrinthine trophoblast of the placenta led to reduced placental growth followed by fetal growth restriction (92). While the same promoter transcript may not necessarily play the same role in humans, these findings support the role of placenta-specific IGF-II in modulating nutrient demand and transport to the placenta, and thus placental and fetal growth (70,93).

3.4.4. Co-localization of IGFBP-4, IGFBP-5 and PAPP-A2 in placental villi

PAPP-A and PAPP-A2 are expressed by the ST as well as the invasive EVT (41,58,94). It was previously thought that the only location of expression of IGFBP-4 and IGFBP-5 in the first trimester of pregnancy was in the maternal decidua (48), with immunoreactivity of IGFBP-5 in the ST and in the chorionic mesoderm of the villi having only been observed in the second and third trimester (41,48). More recently, IGFBP-4 immunoreactivity has been observed in the chorionic mesoderm of placental villi at 10-

13 weeks (58). My findings suggest that IGFBP-4 and IGFBP-5 may be present in the ST of placental villi as early as 5 weeks of gestational age. PAPP-A and PAPP-A2 may therefore influence steps of early placental development other than the invasion of the decidua and remodeling of the spiral arteries by EVT. While PAPP-A and PAPP-A2 may affect placental development via interaction with IGFBP-4 and IGFBP-5 through paracrine dialogue between the villi and the decidua, we suggest that interaction between the pappalysins and IGFBPs within the placental villi may also be taking place, and earlier in gestation than previously supposed. Co-localization of IGFBP-4, IGFBP-5 and PAPP-A2 in the ST suggests potential roles of these proteins in CT proliferation, or fusion to ST.

BeWo choriocarcinoma cells, a model of villous CT cells, were thus intended for examination of the effects of IGFBP-4 and -5 on CT proliferation. Unfortunately, preliminary experiments do not lend confidence to either the methods or assays employed, as a significant effect of either IGF-I or IGF-II was not detected (Appendix B). This is inconsistent with previous findings, which have shown that BeWo cells dosed with 10 nM IGF-I and IGF-II show significant increases in mitosis and proliferation (95,96). However, these studies did not employ BrdU incorporation or Hoechst nuclei staining as their quantification methods. An alternate direct counting method or fluorescence-activated pulse cytophotometry (FACS) may be useful to employ in follow-up from this work (97).

3.4.5. Limitations of cell culture

As with any study using transformed cell lines as a model of physiological processes, there exist inherent limitations to the work described in this study. HTR-8/SVneo cells are an established and often used model of first trimester EVT. They are distinct from other commonly used trophoblast cell lines in that they were derived directly from first trimester trophoblast (then rendered immortal via transfection with simian virus pSV40neo), as opposed to having been derived from a choriocarcinoma or trophoblastic tumor. HTR-8/SVneo cells share expression of many of the cellular markers of parental primary EVT cells including human leukocyte antigen (HLA) framework, which is instrumental to the interaction between the EVT and uterine natural killer cells that is

required for spiral artery remodeling (24). However, a recent study using GeneChip analyses to compare gene expression profiles between primary EVT and several commonly used trophoblast cell models revealed that HTR-8/SVneo cells were not more similar to parental EVT compared to other cell lines, including several derived from choriocarcinomas (98). Given this information, it would be beneficial to confirm the work described here with work performed in placental explants.

The same limitations that apply to work performed with HTR-8/SVneo cells also apply to the work performed using BeWo cells. As a tetraploid, transformed cell line derived from choriocarcinoma, they are an imperfect model for villous CT *in situ*. Follow-up work investigating the effects of the pappalysins and their substrates on trophoblast proliferation would be best confirmed with work in more than one immortalized cell line, or placental explants.

3.5. Conclusion

The findings of this study showed that IGFBP-4 and IGFBP-5 have disparate effects on each IGF in an *in vitro* model of EVT migration and that IGFBP-4 and IGFBP-5 may be co-localized in the placental villi alongside PAPP-A and PAPP-A2. Taking into account previous findings that IGF-I and IGF-II have different physiological sources in pregnancy and that PAPP-A and PAPP-A2 have shown contrasting patterns of regulation in adverse pregnancy outcomes, I propose that the stimulatory effects of PAPP-A2 on EVT migration may be less than those of PAPP-A, and that PAPP-A2 may function principally to modulate IGF-II produced locally in the placental via its effects on IGFBP-5. Further work could include migration/invasion studies in human placental explants, as well as improved CT proliferation assay methods.

Table 3.1 Preliminary dose experiments examining effects of IGF-I and IGF-II on trophoblast migration measured using wound healing assay in HTR-8/SVneo cells.

Treatment	n	% Wound Closure (Mean)*	Standard Error	Tukey's Test
Control	6	45%	4%	B
2 nM IGF-I	6	82%	4%	A
10 nM IGF-I	6	84%	4%	A
25 nM IGF-I	6	86%	4%	A
Treatment	n	% Wound Closure (LS Mean)**	Standard Error	Tukey's Test
Control	14	34%	3%	B
2 nM IGF-II	14	49%	3%	A
10 nM IGF-II	14	55%	3%	A
25 nM IGF-II	6	58%	4%	A

* Treatments all conducted within same day; therefore average effect on wound closure calculated as mean ($F=28.65$, $P<.0001$).

** Effects measured in two experimental trials with replication of treatments in each trial; effect on wound closure therefore calculated using a general linear model including the effects of treatment and date of experiment, and displayed as the least square mean ($F=12.86$, $P<.0001$).

Table 3.2 Effects of IGF-I, IGF-II, IGFBP-4 and IGFBP-5 on trophoblast migration measured using wound healing assay in HTR-8/SVneo cells (F=38.11, P<.0001).

Treatment	n	% Wound Closure (LS Mean)	Standard Error	Tukey's Test
Control	56	38%	1%	C
2 nM IGF-I	58	63%	1%	A
2 nM IGF-I + 20 nM IGFBP-4	30	41%	2%	C
2 nM IGF-I + 20 nM IGFBP-5	24	56%	2%	B
2 nM IGF-II	62	53%	1%	B
2 nM IGF-II + 20 nM IGFBP-4	26	36%	2%	C
2 nM IGF-II + 20 nM IGFBP-5	26	42%	2%	C

Table 3.3 Preliminary experiments examining IGF-I potentiating effects of low doses of IGFBP-5 on trophoblast migration measured using wound healing assay in HTR-8/SVneo cells (F=4.38, P<0.0048).

Treatment	n	% Wound Closure (LS Mean)	Standard Error	Tukey's Test
Control	6	35%	6%	B
2 nM IGF-I	20	60%	3%	A
2 nM IGF-I + 0.25 nM IGFBP-5	14	55%	4%	AB
2 nM IGF-I + 0.5 nM IGFBP-5	20	57%	3%	A
2 nM IGF-I + 2 nM IGFBP-5	11	55%	4%	A

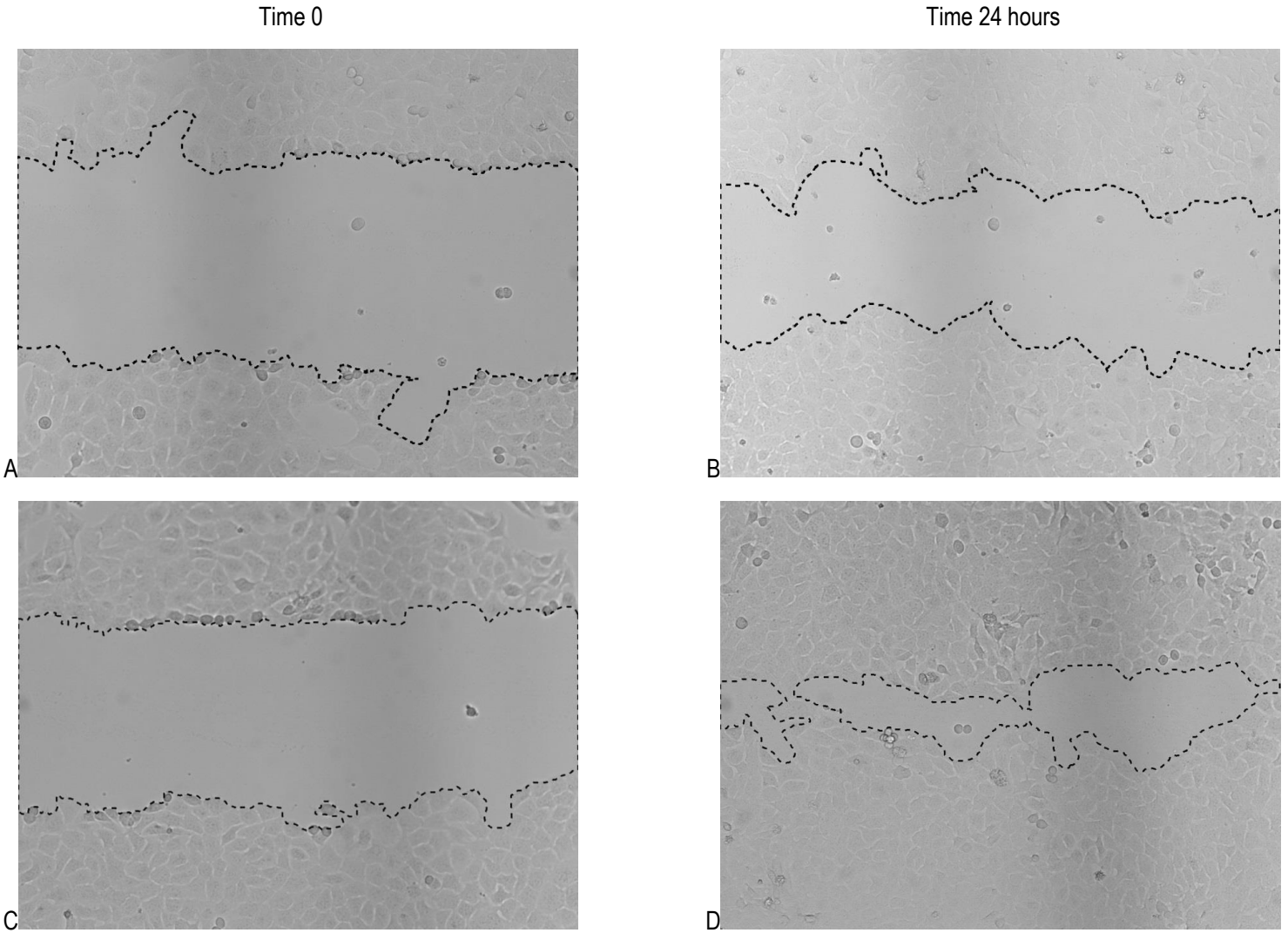

Figure 3.1. HTR-8/SVneo cells at time 0 (A & C) and 24 hours later following treatment with either vehicle (B) or 2 nM IGF-I (D).

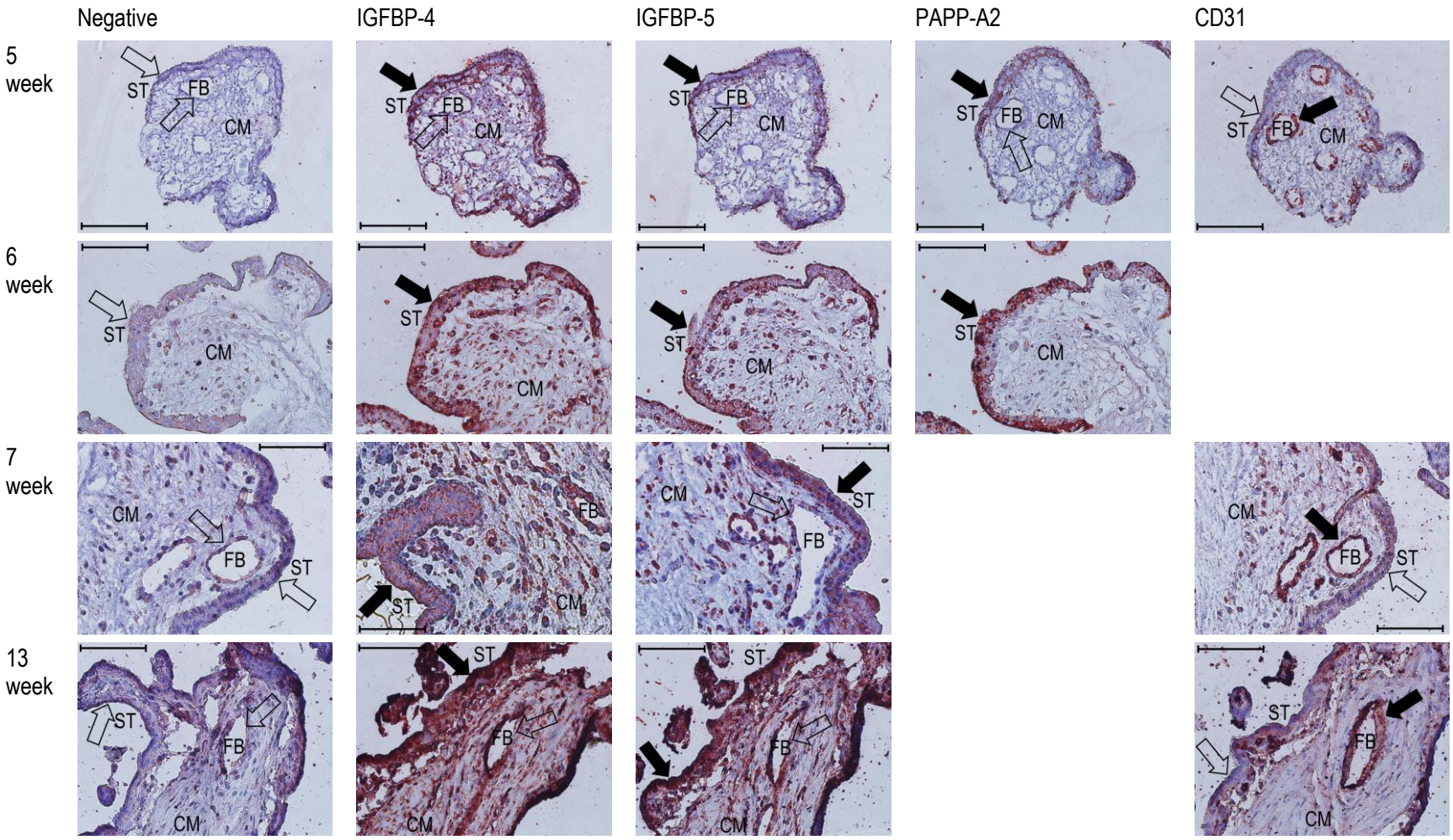

Figure 3.2. **Immunoreactivity (red colour) against IGFBP-4, -5, and PAPPA2 is seen in the syncytiotrophoblast of first trimester placental villi. Serial cross sections of placental villi at 5, 6, 7 and 13 weeks of gestation were stained for IGFBP-4, IGFBP-5, PAPP-A2 and CD31. Negative control was non-specific goat IgG used in the place of the primary antibodies. FB = fetal blood vessel, CM = chorionic mesoderm, ST = syncytiotrophoblast. Opaque arrows indicate immunoreactivity, hollow arrows indicate absence of immunoreactivity. Scale bars in each image denote 100 µm.**

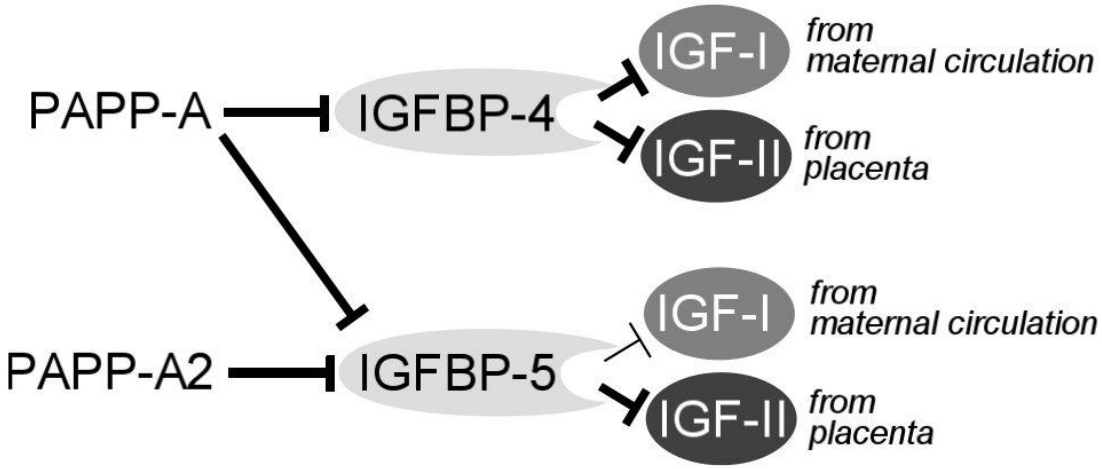

Figure 3.3. **PAPP-A and PAPP-A2 have potentially different functions due to the different actions of their substrates on IGF-I and -II in placental development, and the different physiological sources of each IGF in pregnancy.**

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4. Conclusion

4.1. Summary of results

Preeclampsia (PE) and intrauterine-growth restriction (IUGR) affect 5-7% of all pregnancies and represent two leading causes of maternal and perinatal mortality, respectively (1,2). PE and IUGR are often, although not completely, concurrent and are both associated with placental insufficiency that can result from impaired extravillous trophoblast (EVT) invasion and inadequate spiral artery remodeling (3-6). Abnormally low first trimester levels of pregnancy-associated plasma protein A (PAPP-A) in the maternal circulation have been associated with PE (7-11) and IUGR (7,8,12-14). In contrast, abnormally high levels of pregnancy-associated plasma protein A2 (PAPP-A2) have been found to be elevated in term placentae and the third trimester maternal circulation from pregnancies afflicted by PE and HELLP (Hemolytic anemia, Elevated Liver enzymes, and Low Platelet count) (15-22). PAPP-A and PAPP-A2 are proteases of insulin-like growth factor binding proteins -4 and -5 (IGFBP-4 and IGFBP-5) (23-26), and IGFBP-5 respectively (27-29), and are thus players in the insulin-like growth factor (IGF) pathway, which is critical to cellular processes involved in feto-placental growth including EVT invasion and spiral artery remodeling (30). The goal of this thesis was to look at patterns and mechanisms through which PAPP-A and PAPP-A2 might be acting to help ascertain whether observed associations are causal or a compensatory response to abnormal placental development.

Chapter 2 of this thesis describes work which assayed whether first trimester levels of PAPP-A2 in the maternal circulation differed between uncomplicated pregnancies and those that subsequently developed PE and/or resulted in a small-for-gestational-age (SGA) infant. With the exception of one other study (31), most work to date has been focused on associations between the upregulation of PAPP-A2 and PE in the placenta and maternal circulation at term (15-22). Understanding whether the

elevation of PAPP-A2 in complicated pregnancies occurs early, near the time when placental dysfunction is developing, or later in pregnancy, in response to fully-developed placental pathology, is important to uncovering the mechanisms underlying associations between PAPP-A2 upregulation and PE. The findings of this study showed that first-trimester PAPP-A2 serum concentrations were significantly higher in women who subsequently developed PE than in those who did not, whether or not preterm births were included in analysis. As mentioned above, this is in contrast with previous findings of abnormally low first trimester circulating levels of PAPP-A in pregnancies that go on to develop PE (7-11). Although PAPP-A2 was not significantly different between women who did or did not give birth to SGA infants, first trimester PAPP-A MoM were significantly lower in women who subsequently gave birth to SGA babies than in those who did not, which is consistent with previous findings (7,8,12-14). These findings suggest that although PAPP-A and PAPP-A2 are both IGFBP proteases, they may have different functions and/or regulation and therefore play different roles in adverse gestational outcomes.

Interestingly, the PAPP-A2 immunospecific bands quantified in chapter 2 (at 290, 250 and 130 kDa) do not correspond with the predicted molecular weight of full length PAPP-A2 or known splice variants based on amino acid sequence. The molecular weight of mature PAPP-A2 protein and of the PAPP-A2 splice variant are predicted to be approximately 172 kDa and 82 kDa, respectively (28,29). Given that PAPP-A2 contains multiple sites for glycosylation, it is possible that native full-length PAPP-A2 circulates in a modified form *in situ*. Previous work has shown PAPP-A2 immunoreactive bands at 250 kDa in both human and murine placental lysates, murine embryos, and human pregnancy serum (17,32), and has included the use of an antibody different from the one employed in this study(17). Although one recent study showed PAPP-A2 immunoreactivity in human pregnant serum at 220 kDa and not 250 kDa (33), I suggest this may be due to a difference in the timing of serum sampling, as different isoforms / modified forms of PAPP-A2 may be present at term compared to in the first trimester.

Chapter 3 describes experiments in which the effects of IGFBP-4 and IGFBP-5 were measured in an *in vitro* model of EVT invasion. These experiments investigated the ability of IGFBP-4 and IGFBP-5 to not only inhibit the migration-stimulating effects of

IGF-I and IGF-II, but also to what extent they may have IGF-independent effects, as well as IGFBP-5's ability to potentiate the effects of IGF-I. IGFBP-4 was able to inhibit the migration-stimulating effects of both IGF-I and IGF-II, such that HTR-8/SVneo cells showed wound closure that was not significantly different from controls. IGFBP-5 was also able to significantly inhibit the migration-stimulating effects of each of the IGFs. However, while cells co-treated with both IGF-II and IGFBP-5 showed wound closure that was not significantly different from controls, cells co-treated with both IGF-I and IGFBP-5 showed significantly reduced wound closure, but not to control levels. In preliminary experiments, neither IGFBP-4 or IGFBP-5 demonstrated significant IGF-independent effects on the migration of HTR-8/SVneo cells, and low doses of IGFBP-5 did not demonstrate an ability to significantly potentiate the effects of IGF-I.

IGF-I and IGF-II have different primary sources in pregnancy; IGF-I is predominantly supplied to the fetus and placenta via the maternal circulation (30,34), whereas IGF-II is highly expressed by the EVT and syncytiotrophoblast (ST) (23,30,34-36). When considering that IGF-I was significantly more stimulatory of HTR-8/SVneo cell migration than IGF-II, that IGFBP-4 and IGFBP-5 inhibited each IGF to different extents, and that PAPP-A has been found to be abnormally low in first trimester maternal serum of pregnancies that go on to develop PE (7-11), whereas PAPP-A2 is shown in Chapter 2 to be abnormally high, it follows that PAPP-A and PAPP-A2 play different roles in normal placental development and disease. As such, the findings of Chapter 3 support the hypothesis derived from the results of Chapter 2.

Chapter 3 also describes immunohistochemistry performed on placental explants from the first trimester of gestation, and showed co-localization of IGFBP-4, IGFBP-5 and PAPP-A2 in the ST of placental villi. This is in contrast with the previously held thought that the maternal decidua was the only location of expression of IGFBP-4 and IGFBP-5 in the first trimester (34). From this followed the hypothesis that PAPP-A and PAPP-A2 may be involved in processes other than EVT migration and invasion, such as cytotrophoblast (CT) proliferation or fusion to ST. However, experiments examining the effects of IGF-I and IGF-II on proliferation in BeWo cells, an *in vitro* model of villous CT, did not yield significant results, which is inconsistent with previous work (37,38). The co-localization of IGFBP-4, IGFBP-5 and PAPP-A2 to the ST indicates that the possibility of

autocrine dialogues between the pappalysins and their substrates deserves further attention. The pappalysins may potentially play important roles in CT proliferation and maintenance of the ST that have yet to be described.

4.2. Synthesis of conclusions

Previous work has contributed to the current understanding of the consequences of abnormal PAPP-A2 expression and factors affecting PAPP-A2 regulation. In a mouse line that was genetically homogenous except for a quantitative trait locus (QTL) containing the PAPPA2 gene, PAPPA2 mRNA and protein levels differed by a magnitude of 2.5 (39). However, in spite of this difference in placental PAPP-A2 expression, neither placental mass nor embryonic growth were significantly different between groups, suggesting that alternations in placental PAPP-A2 expression are not causal of pregnancy complications. Notably, placental hypoxia, a consequence of the placental insufficiency that is associated with PE, has been found to increase PAPPA2 mRNA expression in BeWo cells, as well as primary placental explants (31,40). Taken together, these findings suggested that the observed upregulation of PAPP-A2 is more likely a compensatory response to abnormal placental development, rather than a cause.

Overall, given that the migration assays described in chapter 3 indicate that the effects of IGFBP-5 are restricted to inhibition of the IGFs, this suggests that PAPP-A2 functions to stimulate EVT invasion via liberation of IGF-I and IGF-II. Also, in finding that IGFBP-5 is more inhibitory of IGF-II than IGF-I, it can be hypothesized that PAPP-A2 plays a role primarily facilitating the effects of placental IGF-II, which has been shown to be important to nutrient absorption and feto-placental growth as well as EVT migration and invasion (30,41). These findings paired with my finding of high first trimester maternal serum levels of PAPP-A2 in pregnancies that subsequently develop PE support the hypothesis that upregulation of PAPP-A2 represents a compensation attempt for placental dysfunction by increasing the amount of unbound IGF-II and IGF-I in the placenta, and further suggest that this compensation may be taking place as early as in the first trimester.

4.3. Future directions

While the findings of this thesis have elucidated further upon the timing of PAPP-A2 upregulation in PE, as well as potential mechanisms by which PAPP-A and PAPP-A2 might be acting to influence EVT migration, further study is required in order to fully understand the roles of the pappalysins in adverse pregnancy outcomes. Although the elevation of maternal serum PAPP-A2 levels in pregnancies that subsequently developed PE was found to be significant despite a small sample size, expanding the study with a larger sample size and more sensitive proteomic techniques could prove informative. For instance, previous work has shown that ethnicity has a significant impact on biochemical markers such as PAPP-A in prenatal testing for trisomy 21 (42), and that Afro-Caribbean women are at a higher risk of PE and IUGR than Caucasian women (43,44). A systemic review from 2005 showed that additional maternal variables such as increasing maternal age, nulliparity, previous PE, a family history of PE, multiple pregnancies (twins or triplets), raised body mass index and pre-existing medical conditions such as diabetes and hypertension, are all risk factors of PE (45). In chapter 2, I found no significant correlation between maternal variables and levels of PAPP-A2 in uncomplicated pregnancies, but this could potentially be confounded by an underrepresentation of different ethnic groups and certain variables in the patient group (for instance, no women of Afro-Caribbean descent were captured in the study). Pairing first trimester screening and assessment of maternal risk factors can be used to establish suitable surveillance to detect PE in individual women. Given that the predictive value of PAPP-A alone for detecting PE and IUGR is poor (46), the potential for first trimester PAPP-A and PAPP-A2 levels to jointly predict PE thus deserves further attention, as does investigation of the impact of etiologic factors in PAPP-A2 upregulation and the prediction of PE and IUGR.

Following up work performed in HTR-8/SVneo cells with experiments on first trimester placental explants would provide an important correlate to the work described in this thesis, as it would be valuable to examine migration and invasion in a system in which PAPP-A, PAPP-A2, IGFBP-4 and IGFBP-5 are all expressed. HTR-8/SVneo cells are a useful model for EVT invasion in that they mimic many of the phenotypic and functional behaviours of primary EVT cultures (47), but they do not strongly express

PAPP-A or PAPP-A2. However, placental explants represent a closer model to the *in vivo* conditions of placental development (48) , and the presence of the ST would mean expression of both pappalysins and their substrates, would potentially be occurring (as suggested in chapter 3 of this thesis). Furthermore, placental explants may express as of yet uncharacterized receptors of IGFBP-4 and -5 that are not expressed by immortalized cell lines such as HTR-8/SVneo cells (49), and would be a promising model in which to investigate potential IGF-independent effects of both of the pappalysin substrates.

The prospect that PAPP-A, PAPP-A2 and their substrates could be affecting processes within the placental villi such as CT proliferation and maintenance of the ST also merits further attention. It has been suggested that dysregulation of trophoblast turnover and syncytialization may be a cause of PE, either by reduction of the number of invasive EVT due to excessive CT fusion, or by release of excess cellular debris from the ST into the maternal circulation (50). Although measuring the effect of IGF-I and IGF-II on BeWo proliferation via incorporation of 5-bromo-2'-deoxy-uridine (BrdU) or manual nuclei counting via Hoechst staining did not yield significant results, this could be due to problems with the assay methods employed. Previous work dosing BeWo with 10 nM IGF-I or IGF-II has shown a significant increases in mitosis and proliferation, as measured by counting methods other than BrdU incorporation (37,38). Notably, at least one other study also failed to show a significant effect of IGF-I on BeWo cell proliferation despite using a cell counting method other than BrdU incorporation, however they employed comparably lower doses (0.1 nM and 1 nM) (51). In follow-up work in BeWo cells, an alternate direct counting method or fluorescence-activated cell-sorting (FACS) could prove more successful in assessing the effects of the pappalysins and their substrates on CT proliferation (52).

4.4. References

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Appendix A. HTR-8/SVneo invasion assays

Abbreviations used in this table: Exp. = Experiment, Mat. = Matrigel, Tx = Treatments

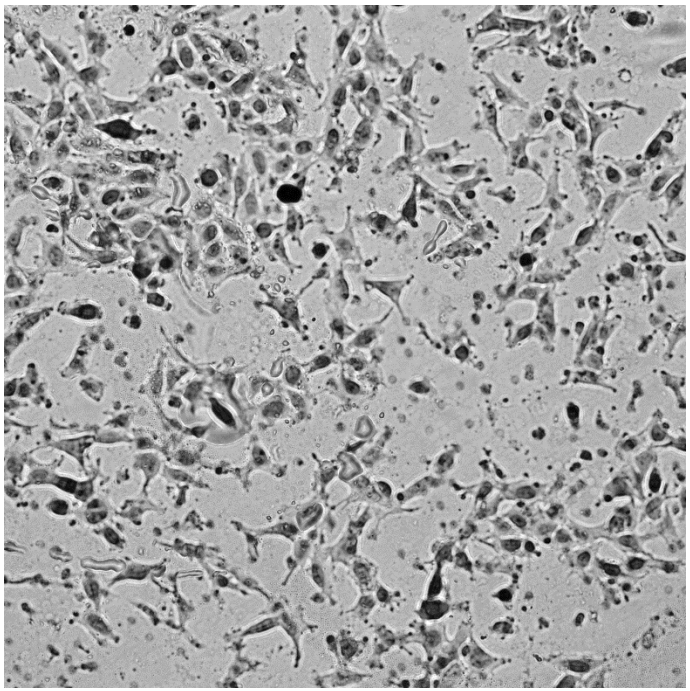
Exp. Date	[Mat.]	Mat. µL	Mat. Diluent:	# of inserts:	Polymer-ization:	Rehydration:	Serum Starve (hrs):	Cells Seeded In:	Cell Density (cells / well):	Tx:	Invasion Time (hrs):	Results	Comments
July 15, 2013	None	N/A	SFM	2	N/A	N/A	0	1% Serum Media	20,000	None	24	Lots of invasion	
Aug 1, 2013	1:30 dilution	100	SFM	2	overnight, biosafety cabinet (lid ajar)	100 µL 1% serum media, 1 hour in biosafety cabinet	24	1% Serum Media	20,000	None	24	No invasion	Perhaps matrigel did not dry properly
Aug 9, 2013	1:30 dilution	100	SFM	3 (1 uncoated)	overnight, biosafety cabinet (lid ajar)	100 µL 1% serum media, 1 hour in biosafety cabinet	24	1% Serum Media	20,000	None	24 48	• 24, Uncoated: Invasion • 24, Coated: No invasion • 48, Coated: Some invasion	
Aug 16, 2013	1:30 dilution	100	SFM	4 (1 uncoated)	overnight, biosafety cabinet (lid ajar)	100 µL 1% serum media, 1 hour in biosafety cabinet	24	0% Serum Media*	20,000	None	24 48 72	• 24h, Uncoated: Invasion • 24h, Coated: No invasion • 48h, Coated: V. little invasion • 72h, Coated: V. little invasion	Try treating cells with IGF (to generate a significant effect)

Exp. Date	[Mat.]	Mat. µL	Mat. Diluent:	# of inserts:	Polymer-ization:	Rehydration:	Serum Starve (hrs):	Cells Seeded In:	Cell Density (cells / well):	Tx:	Invasion Time (hrs):	Results	Comments
Aug 26, 2013	1:30 dilution	100	SFM	4(1 uncoated)	overnight, biosafety cabinet (lid ajar)	100 µL 1% serum media, 1 hour in biosafety cabinet	24	0% Serum Media	20,000	10 nM IGF2 25 nM IGF2	72	• Control, Uncoated: Invasion • Control, Coated: No invasion except edge • 10 nM IGF2, Coated: No invasion except edge • 25 nM IGF2, Coated: No invasion except edge	Revert back to seeding with 1% serum media.
Sept 18, 2013	1:30 dilution	50 100	SFM	8	overnight, 37°C cell culture incubator	N/A	24	1% Serum Media	20,000	10 nM IGF1	72	• 100µL, Control: Some/lots • 100µL, 10 nM IGF1: little invasion; patchy • 50µL, Control: little invasion, patchy • 50µL, 10 nM IGF1: little invasion, patchy	Matrigel didn't polymerize properly overnight; suspect matrigel concentration may be too dilute. Going forward: do an experiment testing optimal matrigel concentrations
Sept 29, 2013	0.64 mg/mL 1.0 mg/mL 3.0 mg/mL	50 100	SFM	12	overnight, 37°C cell culture incubator	N/A	24	1% Serum Media	20,000	None	48	• Matrigel at 0.64 mg/mL does not gel properly • 50 µL of 1 mg/mL matrigel showed the best invasion (see Figure 1 below) • Poor/no invasion was seen from inserts coated with 3mg/mL matrigel (at either volume) or 1 mg/mL matrigel at 100 µL	Go forward coating inserts with 50 µL of matrigel at 1.0 mg/mL
Oct 31, 2013	1.0 mg/mL	50	SFM	12	overnight, 37°C cell culture incubator	N/A	24	1% Serum Media 0% Serum Media	20,000	10 nM IGF1 25 nM IGF1	48	• Control 1% Serum: No/Little invasion • 10 nM IGF1 1% Serum: No/ Little invasion • 25 nM IGF1 1% Serum: No/ Little invasion • Control 0% Serum: No/ Little invasion	Seeding in 0% Serum doesn't seem different from 1% seum. No apparent difference in treatments. Invasion does not match that observed on 2013-09-29.

Exp. Date	[Mat.]	Mat. µL	Mat. Diluent:	# of inserts:	Polymer-ization:	Rehydration:	Serum Starve (hrs):	Cells Seeded In:	Cell Density (cells / well):	Tx:	Invasion Time (hrs):	Results	Comments
Nov 9, 2013	1.0 mg/mL	50	SFM	12	overnight, 37°C cell culture incubator	N/A	24	0% Serum Media	20,000 DUNK 20,000 CFRI	10 nM IGF1	48	Overall very little invasion.	Have not been able to replicated invasion seen on 2013-09-29. Going forward: revise way that matrigel is polymerized.
Nov 14, 2013	1.0 mg/mL	50	SFM	Petri Dish	overnight, 37°C cell culture incubator, overnight, biosafety cabinet (lid ajar)	50 µL 1% serum media, few hours in cell culture incubator	N/A	N/A	N/A	N/A	N/A	Found that dehydrating then reconstituting matrigel is necessary in order to create a true gel.	Will dry matrigel overnight in biosafety cabinet in future trials.
Nov 17, 2013	1.0 mg/mL	50 100	SFM	12	overnight, biosafety cabinet (lid ajar)	100 µL 0% or 1% serum media, few hours in cell culture incubator	24	1% Serum Media 0% Serum Media	20,000	None	48	No consistent invasion	

*Recommendation made at committee meeting Aug 15, 2013

A



B

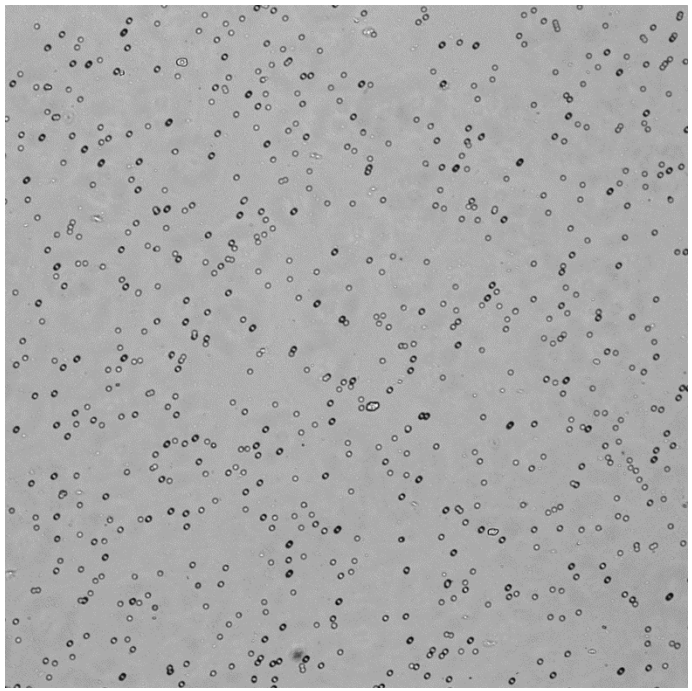


FIGURE A.1: Representative image of invasion seen on September 29, 2013 when coating inserts with 50 µL of matrigel at 1.0 mg/mL (A), and invasion observed with all other matrigel constitutions (B). Subsequent trials were unable to reproduce invasion shown in (A). Invasion in most trials was extremely sparse or nil as shown in (B).

Appendix B. Cytotrophoblast proliferation experiments

Experiments prior to September 25, 2013 did not include shaking in the plate reader protocol, contributing to variability between reads. Experiments considered representative should thus include only those including and onward from September 30, 2013

Experiment Date	Cell Line	Cell Density (Cells/Well)	Grow Time Before Serum Starving (Hrs)	Serum Starved Before Treatment (Hrs)	Time Treated (Hrs)	Treatment	Results	Comments
July 29, 2013	BeWo	20,000	72	24	24	Control 2 nM IGF-I 10 nM IGF-I 25 nM IGF-I 2 nM IGF-II 10 nM IGF-II 25 nM IGF-II	No significant difference	*Shaking not included in plate reader protocol.*
Aug 9, 2013	BeWo	20,000 10,000	72	24	24	Control 10 nM IGF-I 10 nM IGF-II	No significant difference	*Shaking not included in plate reader protocol.* Suggested at committee meeting that cells are overconfluent by time of BrdU labeling and detection. Going forward: reduce growth time and try lower density.

Experiment Date	Cell Line	Cell Density (Cells/Well)	Grow Time Before Serum Starving (Hrs)	Serum Starved Before Treatment (Hrs)	Time Treated (Hrs)	Treatment	Results	Comments
Aug 19, 2013	BeWo	20,000 10,000 5,000	24	24	24	Control 10 nM IGF-I 10 nM IGF-II	No significant difference	*Shaking not included in plate reader protocol.*
Aug 25, 2013	BeWo	20,000 10,000	24	24	24	Control 2 nM IGF-I 10 nM IGF-I 25 nM IGF-I 2 nM IGF-II 10 nM IGF-II 25 nM IGF-II	No significant difference	*Shaking not included in plate reader protocol.* Observed large amount of variability between treatments in this trial as well as previous trials. Going forward: try experiment examining effects across a range of fixation times to see if improves variability.
Sept 25, 2013	BeWo	20,000	24	24	24	Control 2 nM IGF-I 10 nM IGF-I 25 nM IGF-I 2 nM IGF-II 10 nM IGF-II 25 nM IGF-II fixed at 10, 20, 30, 40, 50, 60 minutes	No significant difference	*Shaking not included in plate reader protocol.* Found that time of fixation step had no strong effect. Found in this trial that tapping the plate before reading greatly reduced variability between treatments. Going forward: add shaking to plate reader protocol for all future experiments.

Experiment Date	Cell Line	Cell Density (Cells/Well)	Grow Time Before Serum Starving (Hrs)	Serum Starved Before Treatment (Hrs)	Time Treated (Hrs)	Treatment	Results	Comments
Sept 30, 2013	BeWo	20,000 10,000	24	0; treated and serum starved on same day	24	Control 2 nM IGF-I 10 nM IGF-I 25 nM IGF-I 2 nM IGF-II 10 nM IGF-II 25 nM IGF-II	No significant difference	Going forward: do not treat and serum starve on same day (inconsistent with literature and previous trials).
Oct 4, 2013	BeWo	20,000 10,000	24	24	24 48	Control 2 nM IGF-I 10 nM IGF-I 25 nM IGF-I 2 nM IGF-II 10 nM IGF-II 25 nM IGF-II	No significant difference	See Table B.1 below Although not significant, in most cases, 25nM IGF1 and 25nM IGF2 seemed to have a detrimental effect on proliferation in BeWo compared to controls. 2nM IGF1 and 2nM IGF2 seem to have a positive effect on proliferation in BeWo compared to controls when seeded at 10,000 cells/well, but not when seeded at 20,000 cells/well.
Oct 31, 2013	BeWo	10,000 5,000	24	24	24	Control 2 nM IGF-I 10 nM IGF-I	No significant difference	Attempted to follow up on trends observed on October 4, 2014 by including a lower cell density and increasing sample size for each treatment (n=16 for each treatment within each density). Going forward: try a manual cell counting method.

Experiment Date	Cell Line	Cell Density (Cells/Well)	Grow Time Before Serum Starving (Hrs)	Serum Starved Before Treatment (Hrs)	Time Treated (Hrs)	Treatment	Results	Comments
Nov 5, 2013	BeWo	10,000	24	24	24	Control 2 nM IGF-I 10 nM IGF-I	No significant difference	Quantified by manual counting using Hoescht nuclear staining Seeded 24 well plate on Nov 5, 2013 Serum starved BeWo on Nov 6, 2013 Treated Cells on Nov 7, 2013 (Control, 2nM IGF-I, 10nM IG1) On Nov 8, 2013 aspirated media from each well added 1000 µL of SFM added 1 µL of Hoesct stain to each well and incubated for 20 minutes in 37C incubator aspirated media and washed 2x in 1000 µL PBS before imaging each well was imaged twice, at two equidistant points along the midsection of the well (as measured using Simple PCI coordinate mapping software) Found heterogeneous growth of BeWo across plate Found heterogeneous Hoescht staining (bright and dim nuclei) See results in Table B.2 below
Nov 15, 2013	HTR-8/Svneo	10,000 5,000 2,500	24	24	Cells Dead			Cells died prior to being treated; may have needed an extra day to grow and establish themselves before being serum starved.

Experiment Date	Cell Line	Cell Density (Cells/Well)	Grow Time Before Serum Starving (Hrs)	Serum Starved Before Treatment (Hrs)	Time Treated (Hrs)	Treatment	Results	Comments
Nov 18, 2013	HTR-8/Svneo	10,000 5,000 2,500	48	24	24	Control 10 nM IGF-I	No significant difference	For 5000 and 2500 cells/well densities no cells survived

Table B.1: Results of October 4, 2013 experiment; effects of IGF-I and IGF-II on BeWo proliferation as measured using a BrdU labelling and detection kit (n=6 for all treatments)

Treatment Time (hrs)	Treatment	Cell Density (cells per well)					
		20,000			10,000		
		Mean (% of Control)	Standard Error (%)	Tukey's Test	Mean (% of Control)	Standard Error (%)	Tukey's Test
24	Control	100	5	A	100	11	A
	2 nM IGF-I	95	3	A	109	12	A
	10 nM IGF-I	98	4	A	98	10	A
	25 nM IGF-I	89	5	A	96	7	A
	2 nM IGF-II	93	4	A	99	10	A
	10 nM IGF-II	91	4	A	91	7	A
	25 nM IGF-II	89	3	A	100	9	A
48	Control	100	9	A	100	6	AB
	2 nM IGF-I	91	16	A	105	8	A
	10 nM IGF-I	90	9	A	99	4	AB
	25 nM IGF-I	78	4	A	77	10	AB
	2 nM IGF-II	88	6	A	94	4	AB
	10 nM IGF-II	96	11	A	82	6	AB
	25 nM IGF-II	79	2	A	74	6	B

24 hr treatment, 20,000 cells/well: F=1.13, P>0.3663
24 hr treatment, 10,000 cells/well: F=0.33, P>0.9139
48 hr treatment, 20,000 cells/well: F=0.79, P>0.5854
48 hr treatment, 10,000 cells/well: F=3.44, P<0.0089

TABLE B.2: Results of November 5, 2013 experiment; effects of IGF-I on BeWo proliferation as measured by manual counting of Hoescht stained nuclei (F Ratio = 1.3784, P > 0.2749)

Treatment	n	Mean cell count per visual field	Standard Error	Tukey's Test
Control	7	524	32	A
2 nM IGF-I	8	575	30	A
10 nM IGF-I	8	594	30	A