# THE ROLE OF THE HUMAN AMNION IN AMNIOTIC FLUID VOLUME REGULATION

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| | |
|---|---|
| AEC | amniotic epithelial cell |
| AFI | amniotic fluid index |
| cDNA | complementary DNA |
| CT | cycle threshold |
| DMEM | Dulbecco's Modified Eagle Medium |
| EIA | enzyme immunoassay |
| FBS | fetal bovine serum |
| NAD | Nicotinamide adenine dinucleotide |
| NAMPT | Nicotinamide phosphoribosyltransferase |
| NS | nonstretch |
| PAEC | placental amniotic epithelial cell |
| PBEF | pre-B-cell colony-enhancing factor |
| PBS | phosphate buffer saline |
| P13K | phosphatidylinositol-3-kinases |
| PLAC | placental region of amnion |
| PLCγ | phospholipase C gamma |
| PPROM | preterm premature rupture of the membranes |
| PROM | premature rupture of the membranes |
| qRT-PCR | reverse transcriptase real-time polymerase chain reaction |
| RAEC | reflected amniotic epithelial cell |
| REF | reflected region of amnion |
| RIPA | radioimmunoprecipitation assay |
| RNA | ribonucleic acid |
| S | stretch |
| Sflt-1 | splice variant of VEGFR-1 |
| Src | proto-oncogene tyrosine-protein kinase src |
| VEGF | vascular endothelial growth factor |
| VEGRR2 | vascular endothelial growth factor receptor 2 |

# CHAPTER 1. BACKGROUND

## 1.1 PRETERM BIRTH

The leading cause of infant mortality is preterm birth, contributing globally to 35% of neonatal death (Center for Disease Control and Prevention 2015). This accounts for more than 1 million children a year losing their lives due to preterm birth related complications. Although the percentage of preterm birth in the United States decreased from 12.8% in 2006 to 11.4% in 2013, preterm birth rates in Hawaii and southern states remain the highest in the nation, reaching over 14.6% (National Center for Health Statistics 2015). Preterm birth is defined by the World Health Organization as the birth of a live baby before the completion of 37 of a normal 40 weeks gestation. These last three weeks of gestation are critical because although much of development is complete, during these weeks the vital organs such as the brain, lungs and liver are still maturing within the fetus. Therefore, with insufficient time to finish development in the womb, a preterm baby may incur serious complications such as respiratory distress syndrome, patent ductus arteriosus, intraventricular hemorrhage of the brain and hypothermia, among a slew of other short-term and long-term complications (Callaghan *et al*. 2006; Park *et al*. 2001). In extreme cases of prematurity, when a baby is born prior to 32 weeks of gestation, the baby has been able to complete even less of its developmental program and can therefore suffer from more severe disorders such as necrotizing enterocollitis, neurological defects, infections, weighing less than 3.3 pounds and be at an increased risk of mortality (Werner *et al*. 2011).

The efforts to reduce preterm birth rates between 2006 to 2013 focused on predicting and preventing preterm birth. While this is not a new approach to solve prematurity issues, as methods of predicting preterm birth have been explored for decades, the methods of prediction and prevention have been met with both advancements and setbacks. Progress was made with

the use of the cervical/vaginal fetal fibronectin test as a predictor of labor. In this test fetal fibronectin equal to or more than 50 ng/mL at 24 weeks gestation proved to be a positive predictor of imminent labor, providing a reliable result in more than 50% of the tested subjects, although the sensitivity of the test decreased with later preterm births (Goldenberg *et al*. 1996). A more reliable predictor of preterm birth is the history of prior preterm birth, as women with a previous preterm birth carry a 2.5-fold increase in the risk of preterm birth in their current pregnancy (Cooper *et al*. 1996; Mercer *et al*. 1999). As effective as this method of prediction is, by definition it is not useful for a woman carrying her first child. Therefore risk factors for a first-time mother, include having a mother or sister who gave birth preterm or maternal family history of diabetes or preeclampsia (Dekker *et al*. 2012). Unfortunately, predicting preterm birth will not prevent complications associated with preterm birth but merely offers extended time to prepare for preterm birth. Thus advancements have been sought to provide preventative measures to help deter preterm birth. During pregnancy the cervix, the lower part of the uterus that connects to the vagina, gradually softens, decreases in length and dilates as the body prepares for labor. With the early onset of cervical softening, shortening and dilation, premature birth becomes a great risk as a decreasing cervical length is indicative of proximity to labor. To prevent preterm birth in such cases, the administration of daily vaginal progesterone has been shown to reduce preterm birth by 45% (Werner *et al*. 2011). Progesterone administration has also been shown to increase gestational duration and thus improve fetal development due to the increased times *in utero*, but the long-term effects of the treatment on the health of the baby have yet to be determined although the method has been shown to be ineffective in twin pregnancies (Keirse 1990).

A couple of other treatments have been tested and used on mothers experiencing the onset of preterm birth complications. Magnesium sulphate had been used to inhibit uterine activity in women with preterm labor, but proved to be linked to increased infant mortality (Crowther *et al*. 2002). Another more traditional treatment for preventing preterm birth is maternal bed-rest. Even though bed-rest treatment had been assumed to be safe, research shows that it is ineffective in preventing preterm birth as well as increasing infant birth weight. Bed-rest leads to dangerous side effects such as maternal muscle atrophy, bone loss, weight loss, depression and decreased fetal birth weight and gestational age at birth (Maloni *et al*. 2011).

The administration of preventative measures to address preterm birth has been met with immense challenge because pregnant women are the least favorable subjects for clinical trials. Modern contemporary pharmaceutical practice follows the theory that a drug should only be used if the benefits outweigh the risks, the catch being that the risks may be wholly unknown. Because of the potential risks that medications may pose to the fetus, it is difficult for pregnant women to receive even the most thoroughly tested medications. Unfortunately the difficulties of predicting and preventing preterm birth are exacerbated by the understandable challenges inherent in administering clinical drug trials using pregnant women. Some argue that the benefits of including pregnant women in drug trials exceed their risks, but this is a subject of contention.

## 1.2 THE FINANCIAL BURDEN OF PRETERM BIRTH

Economically, preterm birth places a great financial burden on society, costing the United States over $26 billion annually (Hodek *et al*. 2011). This exorbitant fee includes the cost of the care of preterm infants as 50% of infants born before 28 weeks gestation require admission to neonatal intensive care within 24 hours of birth. Children who overcome the immediate health

complications associated with preterm birth are also at a higher risk for long-term health and developmental issues such as attention deficit disorder, motor impairments, metabolic disease, diabetes, cardiovascular disease, chronic lung disease and respiratory disorders, all of which present high costs for care. Although the advances in perinatal and neonatal care have profoundly improved survival rates of preterm babies, these babies accrue expenses about ten times more than a term baby without including the potential long-term adult health costs of preterm birth. Of the $26 billion a year spent on the care of preterm babies, $18.8 billion contributed to total medical care of the baby and mother, $611 million on early intervention services, $1.1 billion on special education services and $5.7 billion has been estimated as lost household and labor market productivity due to the disabilities causes by premature birth (Hodek *et al*. 2011; Institute of Medicine 2007). This exorbitant financial burden weighs on various facets of society from healthcare to education and demonstrate the importance of this issue.

### 1.3 CAUSES OF PRETERM BIRTH

When a baby is born prematurely it can be because (1) the uterus has become prematurely active leading to spontaneous labor, (2) the fetal membranes have weakened leading to preterm premature rupture of the membranes (PPROM) or (3) the cervix has softened and opened leading to cervical insufficiency. These three main etiologies of preterm birth can occur independently but also often happen in various combinations.

About 10 percent of pregnancies exhibit premature rupturing of the membrane (PROM), which is defined as tearing of the fetal membranes causing amniotic fluid leakage before the commencement of contractions. When PROM occurs prior to 37 weeks of gestation, it is termed preterm premature rupture of the membranes (PPROM), which occurs in about 1 percent of pregnancies (Millar *et al*. 2000).

The two mechanisms underlying PPROM that are most understood include infection and over distension (or overstretching) of the fetal membranes (Goldenberg *et al*. 2008). One of the main reasons why PPROM is a significant problem during pregnancy is that it presents an increased risk for infection of the fetus and mother. This happens because the uterine compartment is open to the potential ascension of bacteria from the vagina causing bacterial vaginosis. The standard treatment procedure if the membranes rupture before labor is to administer corticosteroids and antibiotics to the mother to accelerate fetal lung development and to prevent infection in the remaining amniotic fluid and uterus and in an attempt to stop any infection from becoming systemic within the mother and fetus. The administration of antibiotics does not have an overall benefit to neonatal outcomes and actually increases the risk for neonatal mortality.

Over distension of the fetal membranes by multi-fetal pregnancy or by polyhydramnios, a pregnancy disorder in which excessive amniotic fluid can lead to distension of the fetal membranes, can lead to preterm birth, but the mechanism by which this happens is still uncertain. In the third trimester of pregnancy the fetal membranes terminate proliferation, and consequently the intact membranes experience about 70% distension at term (Oyen *et al*. 2005). *In vitro* studies have shown that with stretching of the fetal membranes several cytokines and enzymes are upregulated which promote collagen breakdown and eventual membrane rupture. Pre-B-cell colony-enhancing factor (PBEF) is the most robust of these stretch-responsive cytokines, which is constitutively expressed in all cellular layers of the fetal membrane. During the third trimester of normal pregnancy, the fetal membranes become distended and begin to remodel. An increase in PBEF expression is associated with initiating the events leading to parturition such as the weakening of the mechanical integrity of the fetal membranes, leading to

rupture and birth (Institute of Medicine 2007). If this normal progression of events is accelerated, it can lead to PPROM and preterm birth.

## 1.4 AMNIOTIC FLUID DYSREGULATION

A critical factor contributing to the health of a growing fetus is the need to balance the volume of amniotic fluid that surrounds it. Mis-regulation in the volume of this fluid can cause serious disorders leading to congenital abnormalities, preterm birth and even fetal demise. Over distension of the uterus and amniotic sac can be associated with the pregnancy disorder polyhydramnios, in which the amniotic sac contains excessive fluid (Spong *et al*. 2011). In pregnancy, sufficient volume of amniotic fluid is imperative for correct organ development but also to buffer against potential fetal trauma while it remains in utero (Brace 1997). In a healthy pregnancy, the volume of amniotic fluid rises as gestation progresses to a maximum of one liter at 37 weeks, but in cases when there is too much fluid produced it can rise to more than two liters and polyhydramnios is diagnosed. The amniotic fluid index (AFI) is a method of measuring the amount of amniotic fluid in the uterine cavity as seen by ultrasonography. The median AFI at week 20 to 35 in a normal pregnancy is 14 cm, while in polyhydramnios it can rise to 20-24 cm. Oligohydramnios is also a disorder of amniotic fluid dysregulation that presents with insufficient amniotic fluid at an AFI less than 5 cm. Oligohydramnios usually occurs during the third trimester of pregnancy and can also lead to birth defects, miscarriage, Potter Syndrome, premature birth and stillbirth, complications caused by a compressed space for development. In olygohydramnios these problems arise as a result of space limitations within the amniotic sac that cause fetal compression and orthopedic abnormalities such as clubbed feet and a potential compressed umbilical cord leading to oxygen deprivation for the baby. Potter Syndrome refers to the physical appearance of a neonate as a direct result of compression *in*

*utero* from oligohydramnios. All of the causes for oligohydramnios are not known, but some causes include preexisting diabetes, renal agenesis and PPROM. The only current treatment option for oligohydramnios is amnioinfusion where the provider introduces saline solution to the uterus through the cervix. However, if after amnioinfusion the AFI remains below 5 cm, labor may be induced to prevent infection of the fetus (Venturini *et al*. 2005).

Polyhydramnios on the other hand develops in the late second or early third trimester of pregnancy, and can be caused by fetal anomalies impairing the fetus to swallow amniotic fluid properly. Polyhydramnios can lead to placental abruption when the placenta peels away from the inner wall of the uterus before delivery, umbilical cord prolapse when the umbilical cord falls into the vagina ahead of the baby, heavy bleeding for the mother after delivery due to lack of uterine muscle tone, premature birth, and stillbirth. Current treatments for polyhydramnios include amniocentesis to remove excess fluid and a medicine called indomethacin to decrease levels of urine produced by the fetus, which contributes to amniotic fluid volume.

The mechanisms underlying the cause of polyhydramnios and oligohydramnios are important to define because, although oligohydramnios only occurs in 4% of pregnancies and polyhydramnios in 1%, the current treatment methods of amniocentesis, amnioinfusion and induced birth may actually cause additional health consequences for the mother and fetus. Thus, increasing the understanding of the mechanisms by which amniotic fluid volume is normally regulated during pregnancy and also dysregulated in polyhydramnios and oligohydramnios is crucial to establish novel therapies for these disorders. By further understanding these mechanisms, methods for early detection and therapeutic treatments may be developed and implemented.

## 1.5 AMNIOTIC FLUID VOLUME REGULATION

A shift in the volume of amniotic fluid reflects an imbalance between the production of the fluid by the fetus and a removal of fluid via the umbilical cord. Although the mechanism with which amniotic fluid is regulated is still being explored, it has been shown in the ovine model that the fetus contributes to the bulk of amniotic fluid flux by secreting lung fluids and releasing urine and removes the fluid via swallowing and intramembranous absorption (Figure 1) (Brace 1997).

Intramembranous absorption can be described in this instance as the transfer of amniotic water and solutes from the amniotic cavity through the amnion into the underlying vasculature of the fetal surface of the placenta (Brace 1995; Brace *et al*. 2014). Since gas and nutrient exchange occur through the placenta, the tissues overlying the placenta have been suggested to have the potential to be involved in intramembranous absorption of amniotic fluid. The amnion is an avascular layer of fetal tissue of which the innermost layer of epithelial cells faces the amniotic fluid and developing fetus. The chorion is a vascular tissue, also derived from fetal cells, that lies beneath the amnion.

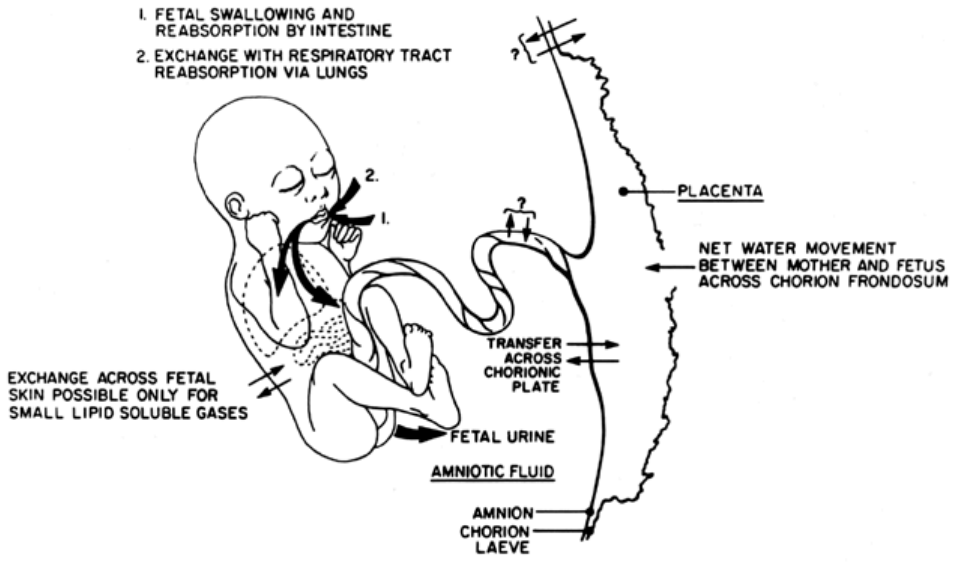



**Figure 1.1. Human Amniotic Fluid Dynamics.** Amniotic fluid is secreted from various sites: through fetal skin, through the amnion and chorion, from lung secretions and via urination. Amniotic fluid is eliminated by three methods: fetal swallowing and reabsorption by the intestine (1), exchange with the respiratory tract via reabsorption by the lung (2) and through the amnion and chorion over the placenta. (Figure adapted from: Gynecology and Obstetrics, Vol 3. New York, Harper & Row, 1989.)

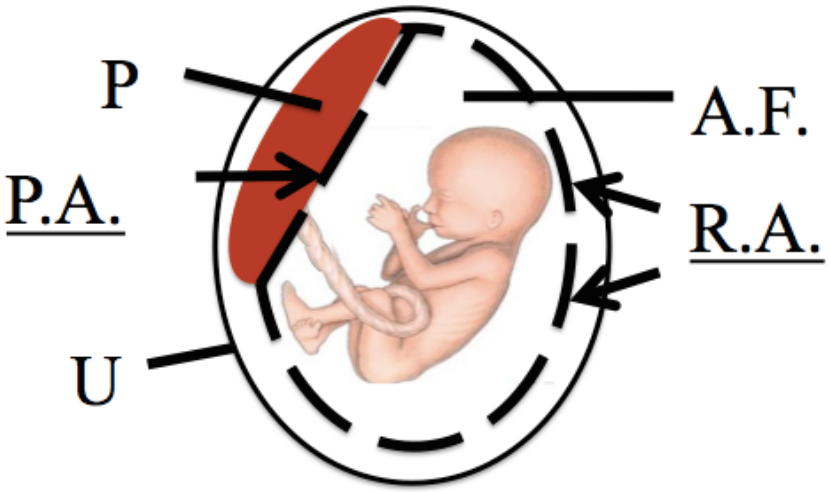



**Figure 1.2. The developing fetus within the amniotic cavity.** P.A. = placental amnion, R.A. = reflected amnion, A.F. = amniotic fluid, U= uterine wall, P = placenta

Substantial headway towards understanding fluid regulation across the amnion was made in ovine models, as this work has elegantly illustrated the disparity in volume between fetal swallowing and fetal urine and lung secretion output (Brace 1995). This work led researchers to explore the potential transfer of fluids across the placental amnion (Brace *et al*. 2004) (Figure 1.2). For ethical reasons, mechanisms key to pregnancy in humans cannot easily be studied using human subjects because *in vivo* studies would be too invasive, thus until recently the ovine model was utilized to make progress in understanding of fluid regulation. While the ovine model is by no means a perfect model for human placental function, the sheep has very similar placental structure to the human without the same ethical conflicts that are associated with studying human models (Barry and Anthony 2008). The human placenta is discoid shaped with the maternal side divided into lobules or cotyledons comprised of fetal vessels, chorionic villi and intervillous space. Similarly the sheep placenta also has cotelydons of structural similarity to that of the human placenta. Both can be divided into a stem and intermediate and terminal

villi. The fetal vessels within the villi in both the human and the sheep are comprised of stem veins and arteries, intermediate venules and arterioles, and terminal capillaries. These structural similarities in fetal placental vasculature when coupled with the capability to provide repetitive fetal and maternal blood samples allows researchers to use the sheep as a suitable model of placental fluid regulation, nutrient exchange and vascular development. For experimental purposes, the ovine model provides an added benefit of allowing repetitive sampling by surgically placed catheters that can be maintained in the maternal and fetal vasculature.

Thus in the past decade the ovine model has been used to show that reabsorption of amniotic fluid and solutes into the fetal surface of the placenta play an important role in governing amniotic fluid volume homeostasis. More specifically they have demonstrated that intramembranous absorption rates are regulated by cytokines in the amniotic fluid (Anderson *et al*. 2013).

## 1.6 STRETCH AS A POTENTIAL REGULATOR OF AMNIOTIC FLUID VOLUME

The amnion is a naturally distendable membrane that becomes increasingly stretched during the third trimester of pregnancy to accommodate fetal growth (Kendal-Wright *et al*. 2010). Although the amnion is a continuous membrane, multiple regions define it both morphologically and functionally. The region overlying the placental disc is termed the placental amnion and the region extending across the remainder of the uterus is the reflected amnion (Figure 1.2). These regions are both lined by amniotic epithelial cells which have been shown to have distinctive regional cell morphology and gene expression patterns, leading researchers to speculate whether their biological roles, including in fluid regulation, may also differ (Kim *et al*. 2011). The amniotic epithelial cells from the reflected region have been

manipulated *in-vitro* to demonstrate their response to stretch while both amniotic epithelial cells from the reflected and placental regions have been explored for their potential roles in amniotic fluid volume regulation.

## 1.7 STUDY PURPOSE

The purpose of this study was to elucidate the role of the human placental amnion in amniotic fluid volume regulation, by specifically looking at (1) stretch as a potential physiological stimulus to regulate amniotic fluid volume and (2) to understand the regulation of potential down stream pathways that may be activated by stretch to regulate fluid absorption across the amnion.

By further characterizing the mechanisms of amniotic fluid volume regulation via intramembranous absorption, we hoped to further the understanding of the mechanisms underlying pregnancy disorders such as polyhydramnios, where stretch of the amniotic sac may not be eliciting efficient intramembranous absorption.

# CHAPTER 2. SPECIFIC AIM 1

## 2.1 INTRODUCTION

Pre-B Cell Colony Enhancing Factor (PBEF) is a 52-kDa protein found in organisms ranging from bacteria to humans. It is an evolutionarily conserved protein comprised of 491 amino acids in the human, chimp, cattle, pig, rat and mouse, with the canine amino acid sequence being 96% identical to that of the human. Cross species conservation of the PBEF protein demonstrates the maintenance of the gene through evolution despite the divergence of species. This evolutionary conservation of PBEF signifies its important role in basic cellular function; indeed complete knockdown of this protein leads to cell death (Sonoli *et al*. 2011).

In the body, PBEF is endogenously expressed in all cells, taking on various roles ranging from promoting angiogenesis to inducing cellular expression of inflammatory cytokines. PBEF is identified by two other names that are associated with its various functions, Nampt (Nicotinamide phosphoribosyltransferase) and Visfatin. PBEF is recognized for its three different roles, acting intracellularly as Nampt, out of the cell as PBEF, and in visceral fat as the adipokine Visfatin. First identified in the prokaryote *Haemophilus ducreyi* for its critical role in the biosynthesis of NAD+ (Nicotinamide adenine dinucleotide), the role of Nampt in mammalian NAD biosynthesis was confirmed when its increased dosage was shown to cause an increase in cellular NAD level (Martin *et al*. 2001; Rongvaux *et al*. 2002; Revollo *et al*. 2004). In this metabolic role it regulates intracellular NAD levels by acting as the rate-limiting enzyme in the salvage pathway of NAD biosynthesis.

In its secreted form PBEF has been shown to have a role in the immune system, as it was first defined as a cytokine acting on early B-lineage precursor cells (Samal *et al*. 1994), enhancing the maturation of B-cell precursors. PBEF also induces cellular expression of

inflammatory cytokines such as TNF-α, IL-1β, IL-8 and IL-6. Patients with inflammatory diseases have elevated levels of PBEF in their circulation, since PBEF is produced by both the cells of the immune system and tissues experiencing inflammation (Luk *et al*. 2008).

Lastly, Visfatin is an adipokine that is highly expressed in and secreted by visceral fat. In the past decade it has become clear that adipose tissue has an important endocrine/immune function as it secretes adipokines. Adipokines affect fat and glucose metabolism and are key players in the inflammatory response. With the development of obesity, and its associated tissue inflammation and hypoxia, Visfatin serum expression level is increased since Visfatin is an adipokine involved in a network of cellular signaling and communication (Adeghate 2008).

While some of the mechanisms for the roles of PBEF in cellular metabolism, immunity and in visceral fat have been studied, another function of PBEF as a key protein in pregnancy has been unveiled. PBEF is a stress responsive cytokine that is constitutively expressed in all layers of the fetal membranes although its highest expression is in the cells of the amnion compared to the chorion or placenta. In addition, this expression *in vitro* is known to increase with stretch of fetal membranes and primary amniotic epithelial cells (AEC's) isolated from the amnion (Kendal-Wright *et al*. 2010; Kendal-Wright 2007).

PBEF was detected in the amniotic fluid during pregnancy, with its concentration increasing with maturing gestational age as well as with infection (Mazaki-Tovi *et al*. 2008). Exogenous PBEF induces the production of Vascular Endothelial Growth Factor (VEGF), by both the placental and reflected amnion, with indications that the reflected amnion may produce more VEGF than the placental. Along with this, PBEF-induced increase in VEGF, another interesting component of this interaction was revealed by the up-regulation of the VEGF Receptor 2 (VEGFR2) by the placental amnion. Thus, when the VEGF ligand is up regulated by

PBEF, it can bind to VEGFR2, which levels are also increased ,on the placental amnion. By enhancing VEGFR2 expression in the placental amnion via PBEF and VEGF treatments, it has been shown that the placental amnion is primed for a VEGF-mediated increase in permeability. This series of data, from the presence of exogenous PBEF in the amniotic fluid, to increased VEGF production, to increased VEGFR2 production, to increased permeability of the amnion have established a potential mechanism for the intramembranous absorption of amniotic fluid across the amnion. Whether the fluid absorption is via paracellular or transcellular mechanisms is yet to be determined, as is the mechanism for stimulating this pathway leading to fluid absorption (Astern *et al*. 2013).

One such possibility to stimulate this pathway is the stretching of the human amnion as the fluid volume increases. Stretching of human amniotic epithelial cells from the reflected amnion *in vitro* has been shown to increase the expression of PBEF, but this is not yet known in the placental amnion since to our knowledge it has not been stretched *in vitro*. It is imperative to understand the behavior of amniotic epithelial cells from both the reflected and placental regions to fully explain their relative roles in the maternal regulation of amniotic fluid. This may be of particular significance as it is the placental amnion, not the reflected amnion that has been shown to be responsive to proposed induction pathways that elicit changes in fluid permeability. It is assumed that the difference in response between these two regions of the amnion to experimental treatments can be attributed to their unique anatomy and physiology *in vivo*. The structures underlying the placental amnion differ greatly from that of the reflected amnion in that they are composed of an underlying highly vascularized placenta through which fluid absorption is highly plausible.

The amnion is a continuous membrane that lines the amniotic sac and is comprised of a single layer of cuboidal epithelial cells, a basement membrane and a collagen rich layer (Figure 2.1). The reflected amnion rests on a fairly thick chorion comprised of a basement membrane, a thin collagen layer, reticular fibers and a stromal layer. Under the chorion is a layer of intermediate trophoblast cells that rests on a decidual layer. At the site of the placental disc, a single layer of cuboidal amniotic epithelium faces the fetus and this rests upon a thinner collagenous chorion consisting of large blood vessels. Beneath a fibrinoid layer is the intervillous space where the maternal blood circulates around the fetal villi. This is the location of exchange of oxygen and nutrients from the mother to the fetus. Therefore, what sets the placental amnion apart from the rest of this structure is that it rests upon a highly vascularized placenta while the reflected amnion does not.

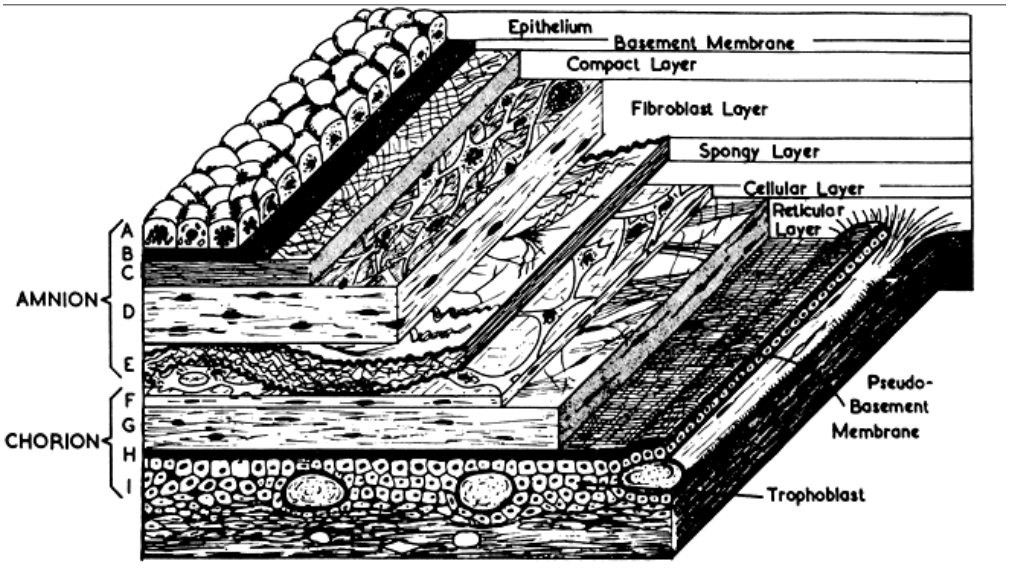



**Figure 2.1. Layers of the Amnion and Chorion.** (Adapted from: Bourne GL. The microscopic anatomy of the human amnion and chorion. Am J Obstet Gynecol 79:1070, 1960.)

Given that the mechanism to stimulate intramembranous absorption of amniotic fluid is unknown and that PBEF is stretch-responsive in the reflected amnion and this has yet to be investigated in the placental amnion where intramembranous absorption takes place *in vivo*, Aim 1 of this thesis was to determine the effect of stretch on PBEF production by amniotic epithelial cells from the placental versus the reflected regions of the human fetal membranes. The amniotic epithelial cells from these tissues have been shown to have differing characteristics and the stark difference in the underlying structures in these tissues begs the question of their relative roles in fluid permeability and this has been achieved by addressing three sub-aims.

## 2.1 AIM 1

### AIM 1.1: To test the hypothesis that static stretch increases *PBEF* gene expression in AEC's.

Rationale: Since the *PBEF* gene must be transcribed prior to potential translation of the protein, a gene expression assay provides evidence for the earliest appearance of *PBEF* irrespective of whether the protein is translated or not and irrespective of whether that protein will function in its secreted or intracellular form. Thus, *PBEF* trasncript level was measured in the placental and amniotic epithelial cells as a consequence of stretch in order to characterize the difference in stretch responsive *PBEF* between differing regions of the amnion. With increased stretch, *PBEF* gene expression was expected to increase since PBEF is a distension responsive protein.

### AIM 1.2: To test the hypothesis that intracellular PBEF protein expression increases with stretch.

Rationale: With higher gene expression it was expected that greater protein expression will be detected. However, it is possible that although the gene is expressed, it is not translated into

protein. Therefore, it was crucial to characterize protein expression in response to stretch and to look at this along with the gene expression data in order to decipher the precise effect of stretch. Although it is known that PBEF is distension responsive in cells from the reflected amnion, it was possible that the protein is released from intracellular stores in reflected amniotic epithelial cells (RAEC) or that gene changes may be detected without the following translation into protein.

**AIM 1.3: To test the hypothesis that expression of secreted PBEF protein increases with stretch.**

Rationale: In Aim 1.3 the secreted form was measured. Secreted PBEF acts as a cell-signaling cytokine, which has been shown to increase in response to stretch (Revollo *et al*. 2007). Thus, it was expected that with increased stretch, there will be an increase in secreted PBEF. By analyzing the response of stretch to PBEF secretion in reflected versus placental amniotic epithelial cells, the role of these differing regions of the amnion in PBEF secretion was characterized.

## 2.2 MATERIALS AND METHODS

### Tissue collection

During the summer of 2013, placental and reflected amnion membranes were collected from 6 repeat Caesarean section live births at Kapiolani Women and Children's Hospital (Honolulu, HI, USA). The collection of this tissue was approved by the Institutional Review Board as an exempt protocol. After the random collection of a small rectangle of amniochorionic membrane from the placental and reflected regions that was processed for histology, the amnion was peeled away from the chorion. Cell isolation followed the MacDonald-Casey method (Casey and

MacDonald 1996). Both regions of the amnion were washed with PBS (phosphate buffer saline), spread onto a cutting board and scraped of excess blood, then incubated with trypsin (Gibco BRL, Life Technologies, Inc., Grand Island, NY) as follows. The tissue was minced and placed in a spinner flask with 100 mL DMEM/trypsin (1g trypsin in 500 mL DMEM, Dulbecco's Modified Eagle Medium). The flask with the tissue was kept at 37°C in a water bath, then mixed at 1.62 g for 30 minutes. Then the media was discarded and the tissue minced again then incubated for the second time with stirring at 1.62 g in DMEM/trypsin (100mL) for 30 minutes. The contents of the flask were then poured over gauze to separate the amniotic epithelial cells (AEC's) from the pieces of tissue. Once the AEC's were pelleted by centrifugation, they were suspended in media (F12:DMEM; 1:1; v:v) (Invitrogen, Carlsbad, CA) with FBS (fetal bovine serum) (10%, v:v), penicillin (200 U/mL), streptomycin (200 µg/mL), fungizone (0.5 µg/mL), kanamycin (200 g/mL) and gentamycin (200 Ig/mL). The tissue that remained was minced again and incubated in DMEM/trypsin (100 mL) a total of four times for 30 minutes with mixing at 1.62 g. For the reflected amniotic epithelial cells, the $2^{nd}$ and $3^{rd}$ trypsinizations were collected. For the placental amniotic epithelial cells, the $2^{nd}$, $3^{rd}$, and $4^{th}$ trypsinizations were collected. Then the AEC's were maintained in a primary monolayer culture in a humidified atmosphere of air and $CO_2$ (5%). Media was changed every 2-3 days and the cells were used once reaching confluency in 6-10 days.

**Primary cell culture:** Cells were seeded at $5 \times 10^5$ cells per well on a Collagen IV coated silicone lined stretch plate (Flexcell Inc, PA, USA) in 10% FBS DMEM/F12. Then the cells were incubated in an incubator until 70-80% confluence.

**Stretch experiments:** Once sub-confluency (75-80%) was reached (average of 6-10 days) 3mL of 10% FBS and DMEM/F12 was added to serum starve the cells overnight. The following day

the stretch plates were placed into Flexcell apparatus (Flexcell Inc, PA, USA) and the FX-4000 stretch program that stretches the cells by 20% was initiated for durations of T0, 1, 2, 4, 10, and 24 hours (Casey and Macdonald 1996). As a control, parallel samples of nonstretched cells on stretch plates were incubated alongside those undergoing stretch. The cells were collected as soon as the program ended and were divided into two groups, one for RNA isolation and one for the collection of cellular protein. The conditioned media from these cells was also collected.

**1. Media sample collection:** Media was removed from all samples, centrifuged for 5 minutes at 865 g, the supernatant removed and then aliquotted into 15mL tubes for storage at -80ºC.

**2. Samples for RNA isolation:** After media removal 500µL of trypsin was added to each well. 500µL of complete media was added when all cells had detached, as determined by the absence of cells on the base of the plate. The cells were centrifuged for 5 minutes at 96 g, supernatant removed, then the cells were washed with 1mL ice cold PBS, centrifuged for 5 minutes at 96 g, supernatant removed, then the cells were flash frozen in liquid nitrogen and stored at $-80^0$C.

**3. Samples for cellular protein analysis:** After media removal, the cells were washed with 500µL of ice-cold 1X PBS. This was removed and then 200µL of RIPA (radioimmunoprecipitation assay) buffer (50mM Tris, pH 7.4, 1% NP-40, 0.2% Sodium deoxycholate, 150mM NaCl, 1mM EGTA, 0.4M EGTA, 1mM PMSF, 1µg/mL apiotimin, 1µg/mL pepstatin, 1mM Na3VO4, 1mM NaF) was added to each well. Using a cell scraper the cells were removed from the stretch plate wells and placed into an eppendorf tube to be stored at $-80^0$C until use.

**RNA isolation and qRT-PCR:** qRT-PCR was performed to quantify PBEF gene expression in the amniotic epithelial cells from the placental and reflected regions that had been stretched.

RNA was isolated using a silica-membrane spin column (RNeasy Mini Kit, Qiagen, Germany). The RNA underwent a 20µL reverse transcription polymerase chain reaction to synthesize single-stranded cDNA from total RNA. The reverse transcriptase reaction consisted of the Buffer (dNTPs) and Enzyme (MulV Reverse Transcriptase and RNase inhibitor) and 0.07µg RNA/9µL (High-Capacity RNA-to-cDNA Kit, Life Technologies, CA, USA). The reagents and RNA were aliquotted on ice, centrifuged for 5 minutes at 96 g and inserted into a thermocycler, which heated the reaction to 37°C for 60 minutes then to 95°C for 5 minutes. The cDNA was then be amplified through a real-time polymerase chain reaction to detect the PBEF and 18s genes. In the reaction, 18µL of cDNA was used for the PBEF gene and 1.5µL of cDNA for the 18s gene. To the cDNA for either gene, TaqMan Gene Expression Master Mix (Life Technologies, CA, USA), which consisted of DNA polymerase and dNTPs, was added. To this 4µL of primer for Nampt (PBEF) or 4µL of primer for 18s (TaqMan Gene Expression Assay, Life Technologies, CA, USA) were added. The DNA Polymerase from the Master Mix amplified the target cDNA sequence using the sequence-specific primer. The reaction volume of 20µL was aliquoted into a 96-well plate in triplicate was then placed into the Real-Time PCR instrument (ABI Biosystems, MA, USA). The thermocycler held at 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds to denature the cDNA, then 60°C for 1min to anneal and extend. Using the standard curve a relative amounts of PBEF and 18s were found. 18s gene expression was used as a housekeeping gene control to normalize the PBEF gene expression by dividing the mean relative concentration obtained during qRT-PCR by the average of the housekeeping gene.

**Western blot of cell lysate and conditioned media:** PBEF protein was detected using the cell lysate samples from the stretch experiments. For all western blots, 10µg of protein (as measured by Pierce BCA Assay Kit, Life Technologies, CA, USA) was denatured in LaemmLi buffer at 95°C for 10 minutes then loaded into the gel. The denatured proteins were separated based on size in a 10% SDS polyacrylamide gel by gel electrophoresis at room temperature for 20 minutes at 75V then 40 minutes at 150V. The proteins were then transferred via an electric current for 90 minutes at 100V onto a polyvinylidene difluoride (PVDF) membrane, membranes blocked with 5% non-fat dry milk for 1 hour and probed with anti-PBEF monoclonal antibody (1:1000 dilution with 5% non-fat dry milk, Phoenix Pharmaceuticals, CA, USA). A secondary antibody (1:5000, Anti-Rabbit Horseradish Peroxidase, GE Healthcare, UK) was used to complex with the primary antibody and the complex was detected by the oxidation of luminol (Enhanced ChemiLuminescence, Amersham ECL, GE Healthcare, UK) to emit light. Then the membrane was exposed to film via a developer and the intensity of light emission translated into a dark band. Once the bands were associated with the protein size on the reference ladder, densitometry via ImageJ was used to measure the intensity of stain and to quantitate amount of protein present in comparison to the loading control and positive control. As a loading control, the membranes were reprobed with beta-actin (40 kDa) at 1:300,000 primary antibody dilution (Abcam, MA, USA). The film was scanned into the computer, opened on Image J, and the density was measured after setting the image at 16-bits gray. Maintaining the same rectangle size from band to band within one membrane, the density was divided by the area, the inverse divided by the inverse of the density of the beta-actin loading control for the sample and the product was finally divided by the density of the positive control then by the time zero density for the experiment.

**Enzyme immunoassay:** EIA was performed using a PBEF C-Terminal (Human) EIA Kit (Phoenix Pharmaceuticals, CA, USA). 50 µL of sample was added to each well and a standard curve produced by using the positive control provided. Twenty-five µL of a working solution of primary antibody was added and the sample incubated for 2 hours while shaking at room temperature. The wells were washed with distilled water and 100µL Streptavidin Horseradish peroxidase (SA-HRP) was added to each well then incubated for 1 hour while shaking at room temperature. One hundred µL of tetramethylbenzidine (TMB) substrate solution was added and incubated upon shaking for another hour. Finally, 100µL HCL was added to each well to stop the reaction. Absorbance was read at a wavelength of 450nm on a microplate reader (VersaMax Plate Reader, Molecular Devices, Sunnyvale, CA). The final PBEF protein concentration was determined using the standard curve equation derived from the standard curve on each EIA plate.

**Statistical analysis:** Statistical analyses were conducted in collaboration with the Biostatistics Core at University of Hawaii John A. Burns School of Medicine. Statistical analyses on the data were completed using the Mixed Model Repeated Intercepts method. An overall and across-time analysis was conducted on individual patient gene expression data using mixed-model-based estimates. The following relationships were looked at in the analysis: baseline placental nonstretch versus reflected nonstretch, placental nonstretch versus placental stretch and reflected nonstretch versus reflected stretch. A power analysis using a t-test was also conducted to achieve 80% power on each of the downstream assays.

## 2.3 RESULTS

The results of Sub-Aims 1.1, 1.2 and 1.3 were generated to answer two main questions. (1) Is there a difference in the baseline expression of PBEF in placental amniotic epithelial cells (PAEC) versus reflected amniotic epithelial cells (RAEC) without stretch? (2) Is there a difference in the response of the PAEC versus RAEC to stretch? The results of Sub-Aims 1.1, 1.2 and 1.3 will be discussed in depth, focusing on these two main questions.

### Aim 1.1 PBEF gene expression determined by qRT-PCR

PBEF gene expression in the stretch experiment cells was assessed using qRT-PCR. A minimum of 0.07μg in 9μL was needed for PCR analysis. This was determined by performing an RNA titration on amounts of amniotic epithelial cell (AEC) RNA to determine the minimum amount of RNA to obtain a detectable and robust CT value, or cycle threshold value indicating the number of cycles of PCR required for the fluorescent signal to cross the threshold or background level which is inversely proportional to the amount of target nucleic acid in the sample. The amount of RNA chosen for subsequent experiments was 0.07μg based on a satisfactory CT value and a value that would maximize the number of samples that could be used for qRT-PCR (Figure 2). A CT of 28 using 0.07μg of RNA was chosen because it fell below a relatively unreliably high CT of 30 and was a compromise between the CT values of 29 and 27 (Figure 2). Since the qRT-PCR reaction on the PBEF gene required 0.07μg of RNA in a 9μL volume, samples that contained less than this amount of RNA were excluded from the study. It is important to note that of the total 132 samples, 33 samples were omitted from the qRT-PCR due to insufficient quantity of available RNA. In addition, samples that contained large amounts

of RNA had to be diluted to obtain the 0.07µg of RNA in 9µL. Of these samples that were diluted, 9 samples were omitted because the volume of RNA was too high (>9µL) after dilution.

The pooled qRT-PCR data from all of the patients tested was analyzed by normalizing to the housekeeping gene 18s rRNA gene (Figure 3). The 18s exhibited high plate-to-plate and sample-to-sample variation of relative gene expression (Table 1) even though the same initial amount of RNA (0.07µg) was used in the pipetted volume of 1.5µL. Due to this variation, 18s as a housekeeping gene was eliminated as an endogenous control. Thus the gene expression data presented in this Chapter, except for Figure 3, is not normalized to 18s.

The data were quantified according to a standard curve of AEC cDNA. AEC cDNA were used to create the standard curve because they are known to endogenously express the PBEF, the gene of interest. The standard curve was prepared with 10-fold dilutions of AEC cDNA, with the highest standard at 0.5µg RNA and the lowest 0.00005µg RNA, designed to capture all potential CT values of the samples. PBEF relative expression values are expressed in relation to this standard curve (Figure 4).

After reviewing the pooled patient qRT-PCR data a couple of initial observations can be made. It can be seen that the nonstretched cell baseline PBEF relative expression levels for both the placental and reflected regions are similar (Figure 4). This observation helps to answer the first line of questioning we had for our data, which was to compare the baseline regional expression levels. Subsequently individual patient data in the reflected and placental regions were reviewed. Beginning with the reflected region, it was seen that patient-to-patient variation, as defined by the variation in time zero untreated controls, was small (standard deviation = 0.280) (Table 2). With regards to the effect of stretch on PBEF gene expression, a modest stretch effect can be seen in 4 patients. Patient 1 exhibits an increase in PBEF gene expression

with stretch at the 4 and 10-hour time points (Figure 5). Patient 2 also shows a stretch effect at 10-hours. A stretch effect at the 2-hour time point was seen in both Patients 3 and 6 (Figure 5). Proceeding to the placental region, the data demonstrated that patient-to-patient variation was larger than that of the reflected region of relative PBEF gene expression (Table 2). Stretch-induced PBEF expression can be seen in Patient 2 at the 4-hour time point (Figure 6).

After evaluating both the pooled and individual patient qRT-PCR data, a statistical analysis was performed on the pooled data. A mixed model random intercepts analysis method was utilized to account for repeated measurements on multiple patients. The first observation from this analysis was that the baseline PBEF gene expression in the placental nonstretched samples was higher compared to that of the reflected nonstretched samples but that this difference did not reach significance ($p=0.252$) (Table 3). At all time points except for 2-hours, the PBEF gene expression in the placental nonstretched region was higher than that of the reflected nonstretched region (Figure 7). When looking at the subtraction of mean reflected nonstretch subtracted from mean placental nonstretch PBEF gene expression to compare baseline expression, a positive value indicates that placental nonstretch is larger. This difference between the placental nonstretch and reflected nonstretch PBEF gene expression values is most prominent at the 1-hour time point and evident at the 4, 10 and 24-hour time points (Figure 8, Table 4). The second observation from the statistical analysis was that the PBEF gene expression in the reflected region appeared slightly more affected by stretch than the placental region, $p= 0.445$ and 0.820, respectively (Table 3).

Lastly, a power analysis was conducted on the qRT-PCR data to determine the appropriate sample size for future studies that would obtain 80% power (Table 5). With 80% power, there is an 80% chance of obtaining a p-value of less than 5% in a statistical test. The

time point of 4-hours on which the power analysis was conducted was based on the peak response time in previous studies on AEC PBEF gene expression. Power analyses were done on the 3 comparisons of interest: placental nonstretch versus reflected nonstretch, placental nonstretch versus placental stretch and reflected nonstretch versus reflected stretch. It was found that the required sample size for the first two comparisons is larger than 100, and for the final comparison is 32.

**Aim 1.2 Western blot data on intracellular PBEF protein expression**

AEC from the six patients that underwent stretch were analyzed by western blotting to measure intracellular PBEF protein expression in response to stretch in the placental and reflected regions of the amnion (Figure 9A). The pooled patient data highlights the detection of higher baseline intracellular PBEF protein levels in the placental AEC as opposed to the reflected AEC (Figure 9B).

Individual patient data were reviewed (Figure 10) to examine trends within individuals. Every patient demonstrated higher baseline of intracellular PBEF protein expression in the placental region versus the reflected region and a stretch-induced PBEF expression effect was noted at specific time points. In the reflected amnion, 5 patients exhibited an increase in intracellular PBEF protein expression with stretch at 2 hours, 4 patients at 4 hours, and 4 patients at 10 hours. In the placental region, 4 patients exhibited an increase in intracellular PBEF protein expression with stretch at 1 hour and 4 patients at 2 hours (Figure 10).

When statistical analysis was conducted on the western blot densitometry data, a couple of critical findings were made. A mixed model random intercepts analysis method was utilized again to account for repeated measurements on multiple patients. Firstly, the placental nonstretch samples were shown to have a statistically significantly higher baseline intracellular

PBEF protein expression level than the reflected nonstretch samples ($p < 0.005$) (Table 6). Secondly, as seen in Figure 11B and 11C, as time progressed, an increase, although statistically insignificant, in the difference between stretch and nonstretch in both regions was observed. A greater patient-to-patient variation, resulting in a standard variation of 0.403 between patient unstretched controls, is evident in placental samples (Table 2), versus a standard deviation of 0.155 in the reflected.

Lastly, the power analysis for the intracellular PBEF protein western blot indicated that a sample size of 41 samples necessary for the placental nonstretch versus reflected nonstretch comparison, 69 samples necessary for the placental nonstretch versus placental stretch comparison and over 100 samples needed for the reflected nonstretch versus reflected stretch comparison (Table 5).

**Aim 1.3 Western blot and EIA data for secreted PBEF protein expression**

**Western blotting of secreted PBEF protein**

AEC from six patients that underwent stretch were analyzed by western blotting to measure PBEF protein secreted into the media in response to stretch in the placental and reflected regions of the amnion. The pooled data of all of the patient samples highlights the presence of higher amounts of secreted PBEF protein in the placental amnion stretched samples compared to the reflected amnion stretched samples (Figure 12). When individual patient data are reviewed the same trend can be seen in Patients 1, 2, 5 and 6 (Figure 13). In the reflected region, stretch-induced release of PBEF protein was most prominently observed at the 2 and 24-hour time points for 5 patients (Figure 13). In the placental region, stretch-induced release of

PBEF protein was most noticeable at 4-hours for all patients then at 10-hours for all patients but one (Figure 13).

Statistical analysis was conducted using a mixed model random intercepts analysis method to account for repeated measurements on multiple patients. When looking at the difference between means between placental nonstretched and reflected nonstretched PBEF secretion, a positive value indicates that the placental nonstretch value is higher than the reflected nonstretch value, this was noted at the 1, 2, 4 and 10-hour time points (Figure 14). An important outcome was also noted that with stretch of the placental AEC, a statistically significant increase in PBEF secretion was found ($p < 0.05$) (Table 7) when compared to that of the reflected AEC. A greater patient-to-patient variation, as calculated by standard variation between data points, is evident in placental samples (Table 2).

Lastly, the power analysis for the secreted PBEF protein western blot indicated that a sample size of over 100 samples necessary for the placental nonstretch versus reflected nonstretch comparison and placental nonstretch versus placental stretch comparison, and 47 samples necessary for the reflected nonstretch versus reflected stretch comparison (Table 5).

**EIA on secreted PBEF protein**

PBEF protein secreted from the stretch experiment cells was also assessed using an EIA assay. In the pooled patient data it can be seen that secreted PBEF was higher in the reflected region than the placental region (Figure 15). The pooled patient data shows greater patient-to-patient variation in secreted PBEF levels in the reflected region, with a standard deviation between nonstretched samples at 11.030 in the placental samples versus 6.802 in the reflected (Table 2). Individual data showed peak response times inconsistent between patients. Statistical

analysis performed on the EIA data was done using the mixed model analysis. Due to the outlying data observed in Patient 5 (Table 8), statistical analyses were performed with and without the inclusion of Patient 5.

The reflected stretched samples exhibited greater amounts of secreted PBEF protein than the reflected nonstretched samples with a mean difference of -0.25 (Table 9). When looking at the difference between the means of reflected nonstretch and reflected stretch, an increase in PBEF secretion can be seen with stretch at the 1, 2, 4 and 10-hour time points (Figure 16A, Table 10). The placental nonstretched samples had higher secreted PBEF protein than the placental stretched samples with a mean difference of 0.29 (Table 9). When looking at the difference between the means of placental nonstretch and placental stretch, a higher secreted protein level of PBEF secretion in the nonstretched samples can be seen at the 1, 4, 10 and 24-hour time points (Figure 16B, Table 10).

Lastly, the power analysis for the secreted PBEF EIA indicated that a sample size of 21 samples is necessary for the placental nonstretch versus reflected nonstretch comparison, 22 samples for the placental nonstretch versus placental stretch comparison, and 38 samples necessary for the reflected nonstretch versus reflected stretch comparison (Table 5).

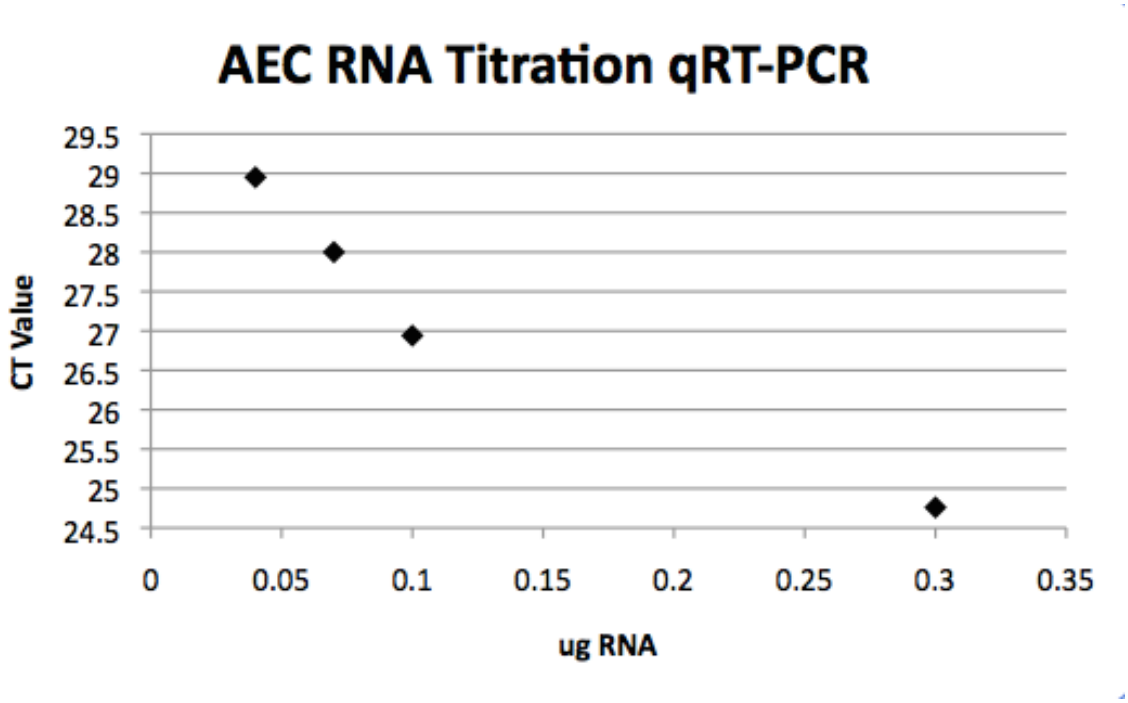



**Figure 2.2.** AEC (amniotic epithelial cell) RNA titration for qRT-PCR reaction on PBEF Gene. A qRT-PCR titration on AEC RNA at quantities of 0.04µg, 0.07µg, 0.1µg and 0.3µg was conducted to titrate the minimum amount of RNA to obtain amplified product.

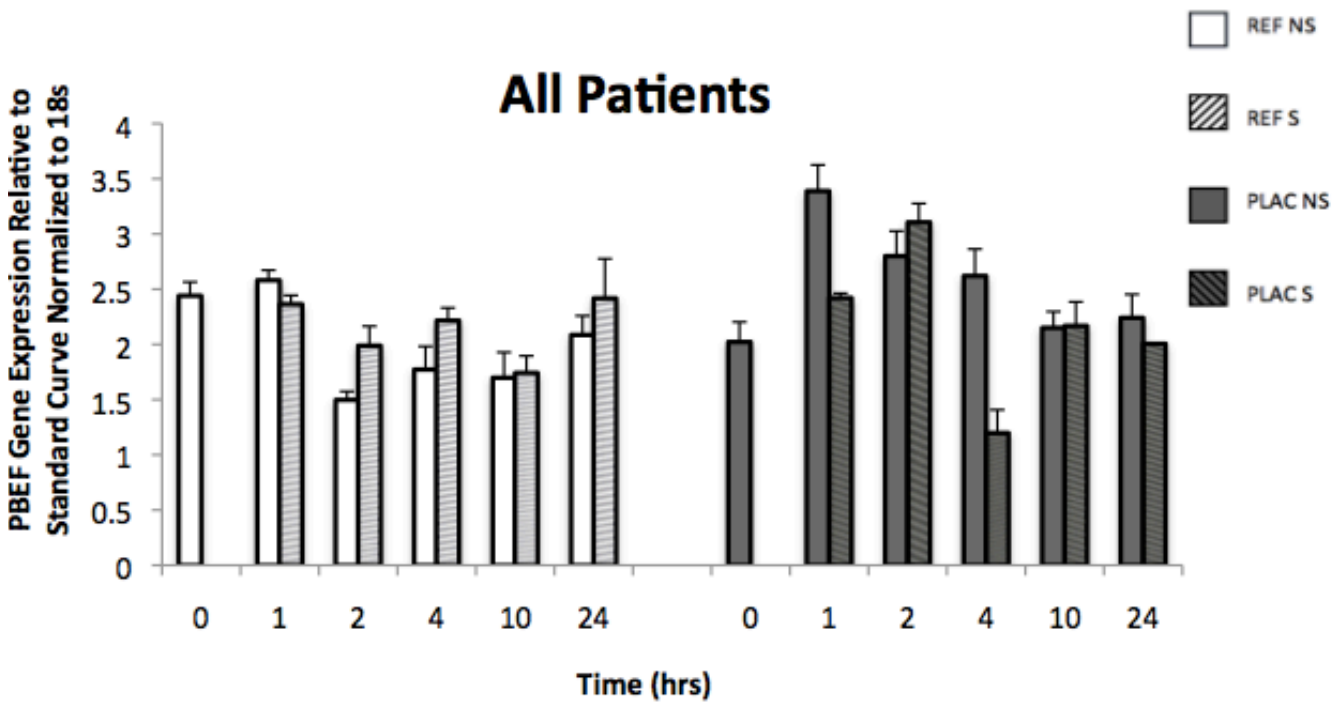



**Figure 3.** Pooled data from all patients (n=6) for PBEF qRT-PCR on AEC stretch experiment RNA normalized to 18s housekeeping gene. REF= reflected amnion, PLAC= placental amnion, NS= nonstretch, S= stretch. Bars indicate SEM.

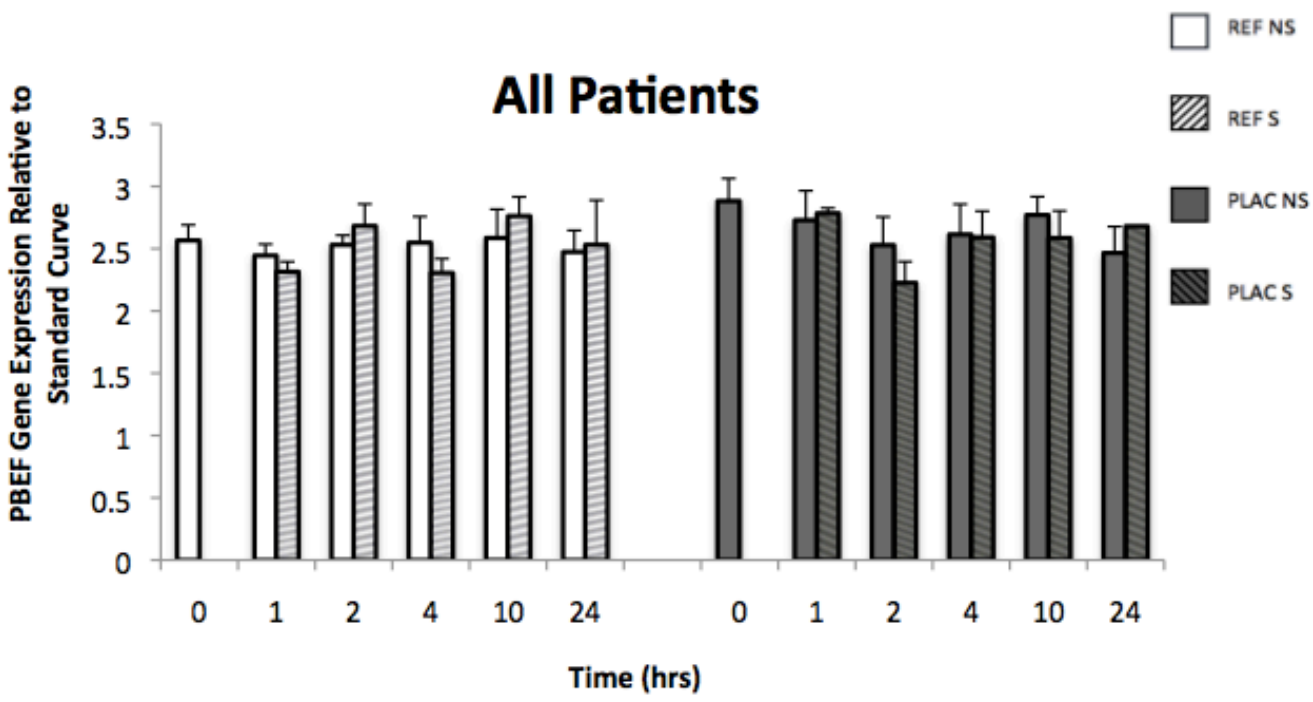



**Figure 4.** Pooled data of all patients (n=6) for PBEF qRT-PCR from AEC stretch experiment RNA not normalized to 18s housekeeping gene. REF= reflected amnion, PLAC= placental amnion, NS= nonstretch, S= stretch. Bars indicate SEM.

| | Relative Concentration to Standard Curve **Not** Normalized to 18s Housekeeping Gene | Relative Concentration to Standard Curve Normalized to 18s Housekeeping Gene |
|---|---|---|
| Real-Time PCR Plate-to-Plate Standard Deviation | 0.122 | 10.47 |
| Real-Time PCR Sample-to-Sample Standard Deviation | 0.365781806 | 8.165462667 |

**Table 1.** qRT-PCR plate-to-plate and sample-to-sample variation comparison with and without 18s house-keeping gene. Plate-to-plate variation was measured by the variation in the standard deviation of all 10 plates. Sample-to-sample variation was determined by the standard deviation of the time zero untreated controls of all patients. A greater variation is apparent in relative concentration of the PBEF gene when normalized to 18s housekeeping gene.

| Experiment | Region of Amnion | Patient-to-Patient Variation (Standard Deviation) |
|---|---|---|
| qRT-PCR | Reflected | 0.280 |
| | Placental | 0.476 |
| Western Blot (intracellular) | Reflected | 0.155 |
| | Placental | 0.403 |
| Western Blot (Secreted) | Reflected | 0.154 |
| | Placental | 0.262 |
| EIA | Reflected | 11.030 |
| | Placental | 6.802 |

**Table 2.** Patient-to-patient variation from the reflected and placental regions for four downstream experiments. Patient-to-patient variation for qRT-PCR and EIA data was calculated as the standard deviation between time zero untreated controls for all patients. Patient-to-patient variation for western blot data was calculated as the standard deviation between nonstretched data points for all patients.

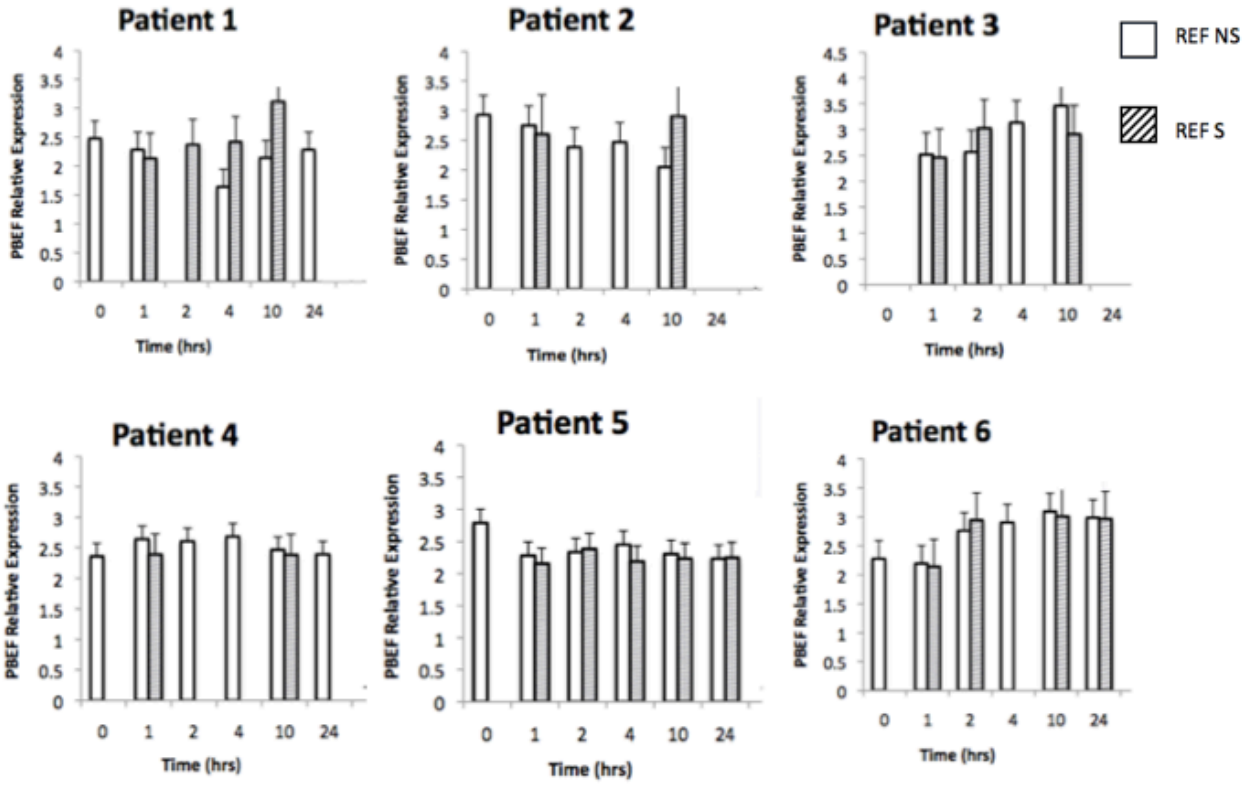



**Figure 5.** Individual patient (n=6) qRT-PCR relative PBEF expression data in cells from the reflected amnion. REF= reflected amnion, NS= nonstretch, S= stretch. Bars indicate SEM.

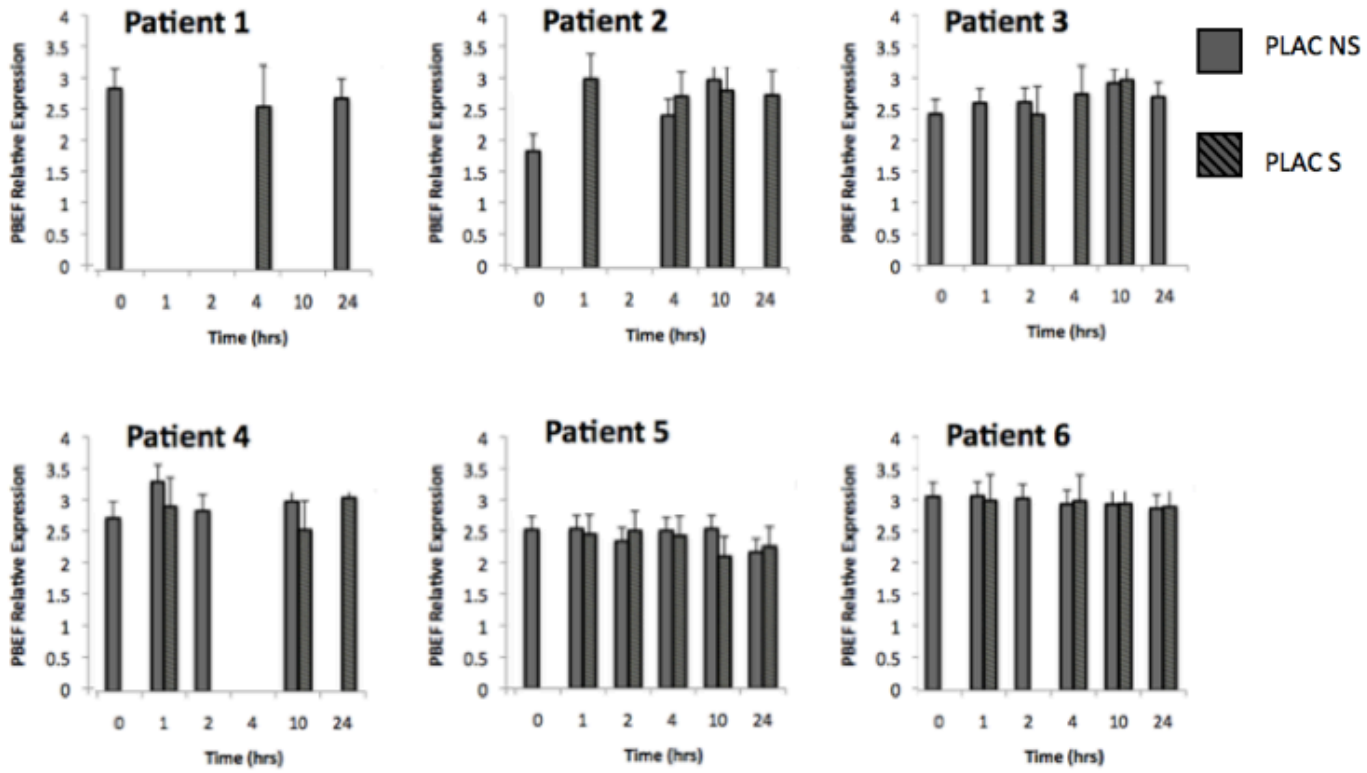



**Figure 6.** Individual patient (n=6) qRT-PCR relative PBEF expression data from cells from the placental amnion. PLAC= placental amnion, NS= nonstretch, S= stretch. Bars indicate SEM.

| | | Mean Difference | 95% CI | P-value |
|---|---|---|---|---|
| All Patients | Placenta NS - Reflected NS | 0.10 | (-0.07, 0.26) | 0.252 |
| | Placenta NS - Placenta S | 0.02 | (-0.16, 0.20) | 0.820 |
| | Reflected NS - Reflected S | -0.06 | (-0.23, 0.10) | 0.445 |

**Table 3.** Summary of qRT-PCR analysis of PBEF gene expression in stretch experiments. Note that analysis implicitly assumes that mean differences do not vary across time. Mean differences account for the overall effect of time and the correlation between repeated measurements on the same patient. NS = non stretch. S= stretch.

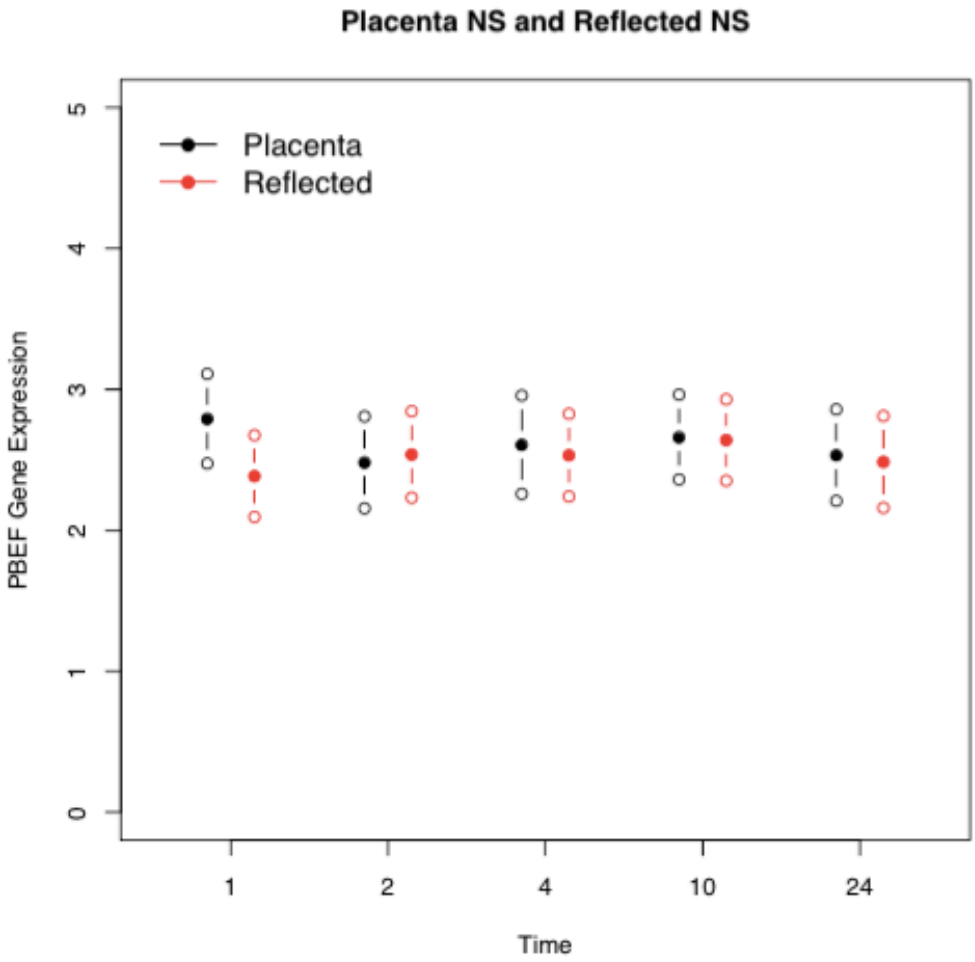



**Figure 7.** Mixed-model analyses of mean qRT-PCR relative expression data at 5 time points. The central point is the mean measurement of relative gene expression for all patients and the extended hollow points indicate the 95% confidence interval for the mean. NS= nonstretch, S= stretch.

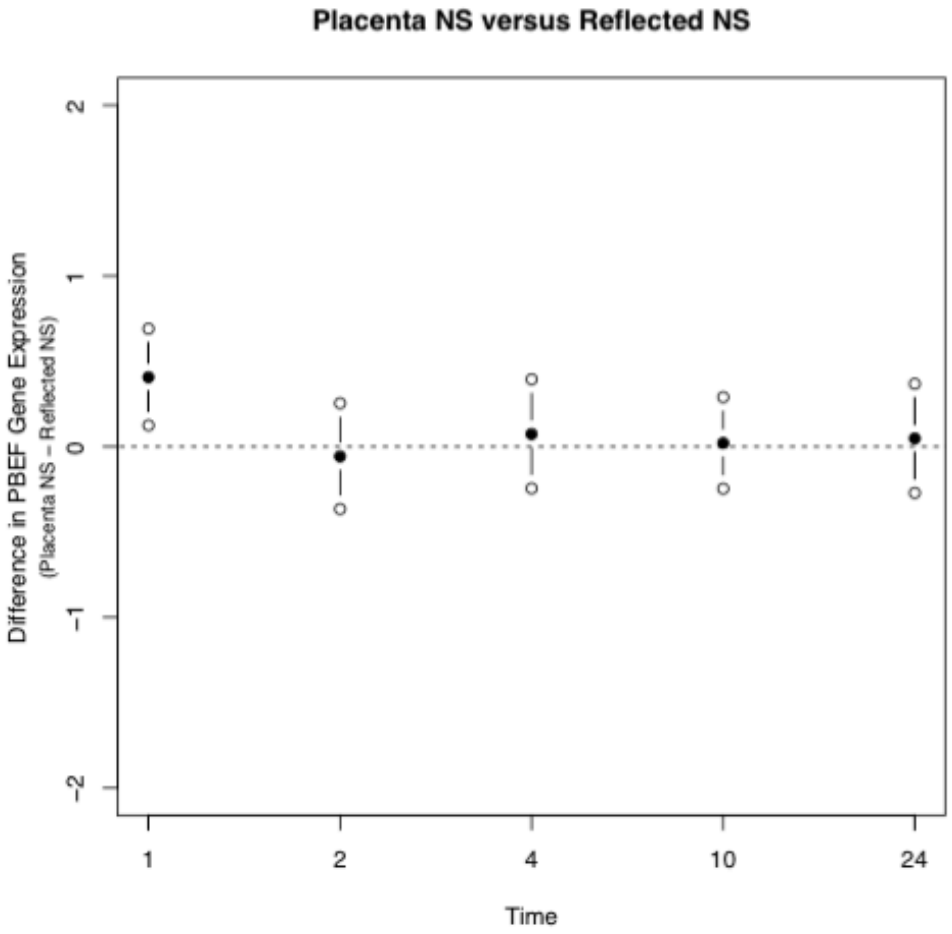


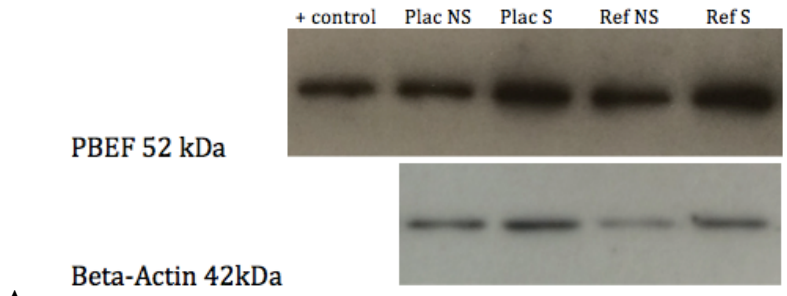

**Figure 8.** Mixed-model analysis of difference between mean placental nonstretch versus reflected nonstretch qRT-PCR relative expression data on PBEF gene. The central point is the difference in mean measurement of relative gene expression for all patients and the extended hollow points indicate the 95% confidence interval for the mean. NS= nonstretch, S= stretch.

| Comparison | Time 1 | Time 2 | Time 4 | Time 10 | Time 24 |
|---|---|---|---|---|---|
| Placenta NS versus Reflected NS | 0.41 (0.12, 0.69) | -0.06 (-0.37, 0.25) | 0.07 (-0.25, 0.39) | 0.02 (-0.25, 0.29) | 0.05 (-0.27, 0.37) |
| Placenta NS versus Placenta S | 0.13 (-0.16, 0.42) | 0.00 (-0.33, 0.34) | 0.03 (-0.29, 0.35) | 0.05 (-0.22, 0.32) | -0.08 (-0.40, 0.25) |
| Reflected NS versus Reflected S | 0.01 (-0.28, 0.30) | -0.11 (-0.43, 0.20) | -0.09 (-0.43, 0.25) | -0.07 (-0.33, 0.20) | -0.20 (-0.54, 0.14) |

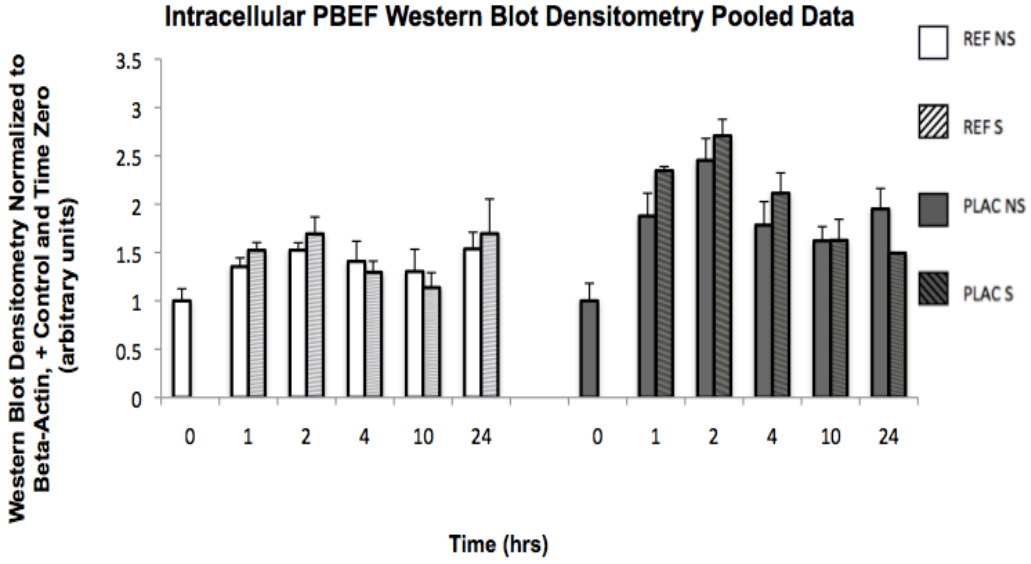
**Table 4.** Mixed-model analysis of difference between mean comparisons in qRT-PCR relative expression data on PBEF gene. NS= nonstretch, S= stretch. In parentheses indicates the 95% confidence interval. Time 1 = 1hr, Time 2 = 2hr, Time 4 = 4hr, Time 10 = 10hr, Time 24 = 24hr.

| Experiment | Comparison of Interest | Time Point | Effect | Standard Deviation | Within-Patient Correlation | Required Sample Size |
|---|---|---|---|---|---|---|
| pCR | Placenta NS - Reflected NS | 4 Hours | 0.066 | 0.459 | 0.45 | Too Large |
| | Placenta NS - Placenta S | 4 Hours | 0.023 | 0.367 | 0.45 | Too Large |
| | Reflected NS - Reflected S | 4 Hours | 0.244 | 0.454 | 0.45 | 32* |
| ELISA | Placenta NS - Reflected NS | 24 Hours | -0.740 | 1.459 | 0.526 | 31 |
| | Placenta NS - Placenta S | 24 Hours | 0.799 | 1.304 | 0.526 | 22 |
| | Reflected NS - Reflected S | 24 Hours | 0.614 | 1.345 | 0.526 | 38 |
| Western Blot (Intracellular) | Placenta NS - Reflected NS | 4 Hours | 0.376 | 0.675 | 0.245 | 41 |
| | Placenta NS - Placenta S | 4 Hours | -0.33 | 0.78 | 0.245 | 69 |
| | Reflected NS - Reflected S | 4 Hours | 0.112 | 0.391 | 0.245 | Too Large |
| Western Blot (Secreted) | Placenta NS - Reflected NS | 24 Hours | 0.013 | 0.541 | 0.162 | Too Large |
| | Placenta NS - Placenta S | 24 Hours | -0.153 | 0.621 | 0.162 | Too Large |
| | Reflected NS - Reflected S | 24 Hours | -0.333 | 0.613 | 0.162 | 47 |

**Table 5.** Power analysis for the various downstream analyses at specified time points. The 'Required Sample Size' refers to the sample size necessary to achieve at least 80% power. A required sample size of 'Too Large' corresponds to a required sample size over 100. *Many missing observations; likely an unreliable sample size estimate.




**A.**




**B.**

**Figure 9.** Western blotting and quantitation of intracellular PBEF protein expression.
A. Representative intracellular PBEF and Beta-Actin Western Blots.
B. Pooled intracellular PBEF protein (n=6) quantification by Western Blot densitometry. Densitometry of western blot bands were normalized to beta-actin loading control, and expressed relative to time zero of each experiment. REF= reflected amnion, PLAC= placental amnion, NS= nonstretch, S= stretch. Bars indicate SEM.

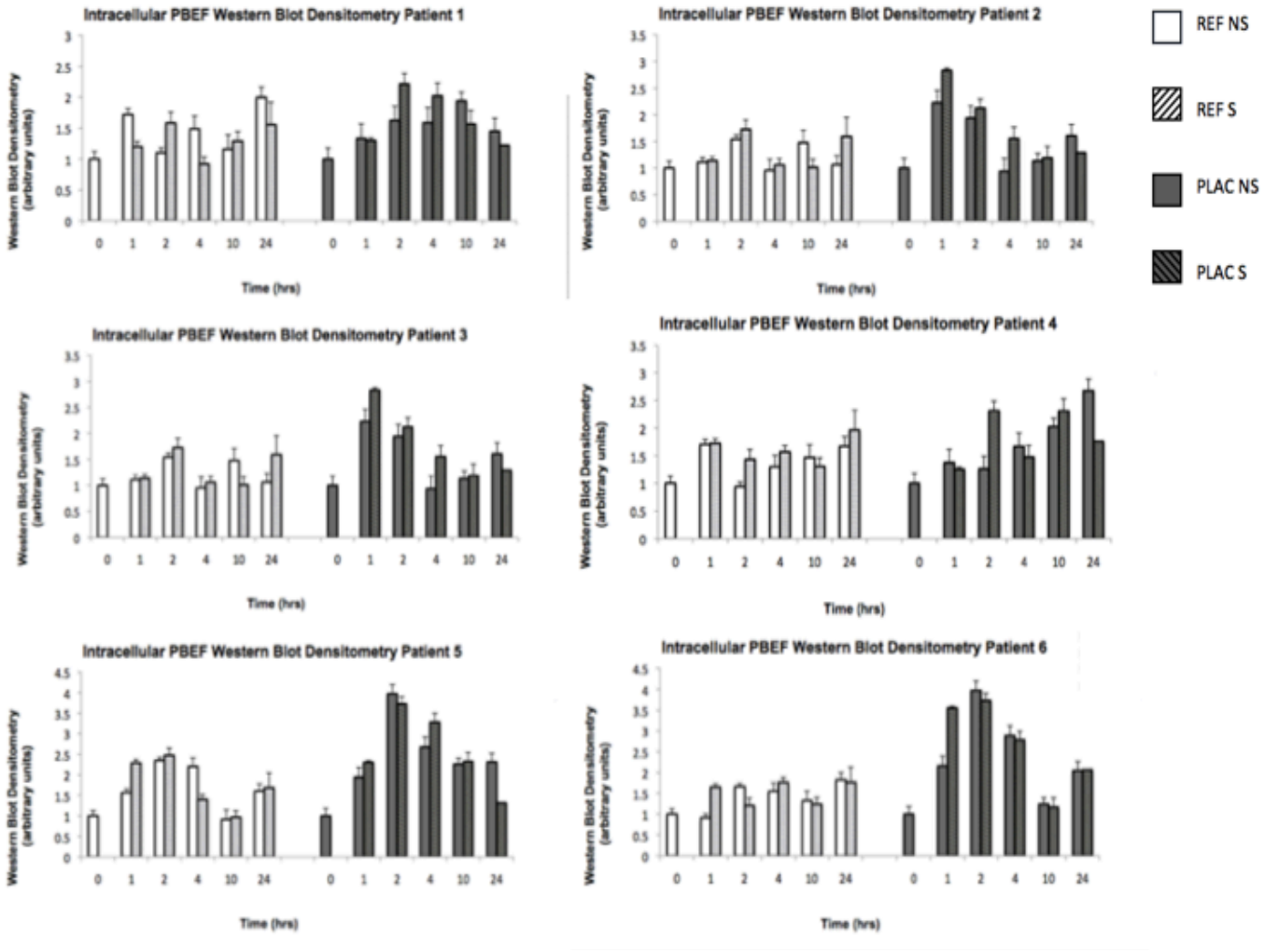
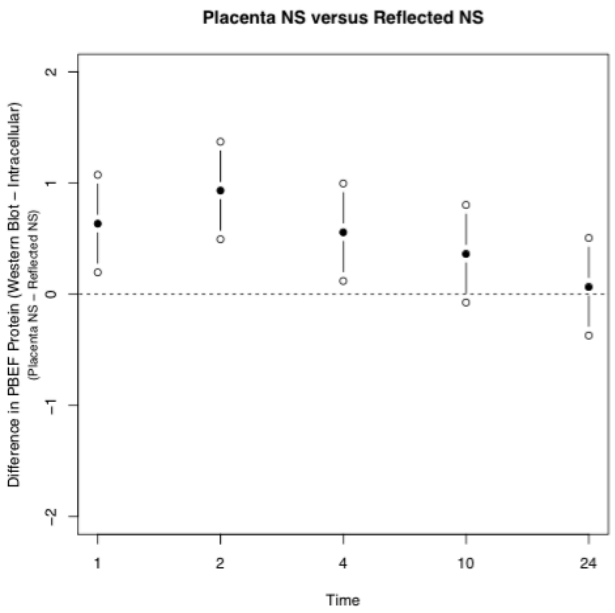
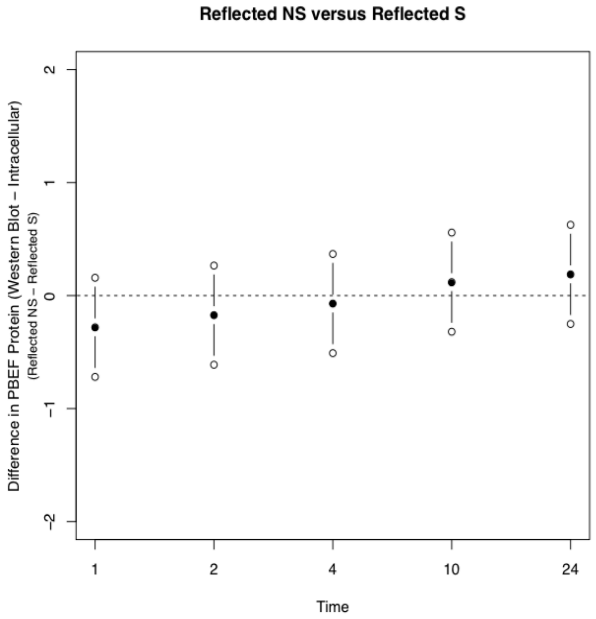



**Figure 10.** Individual patient data of intracellular PBEF protein quantification by western blot densitometry. Western blotting was performed on cell lysates from epithelial cells from all samples from the stretch experiments. REF= reflected amnion, PLAC= placental amnion, NS= nonstretch, S= stretch. Bars indicate SEM.




A.

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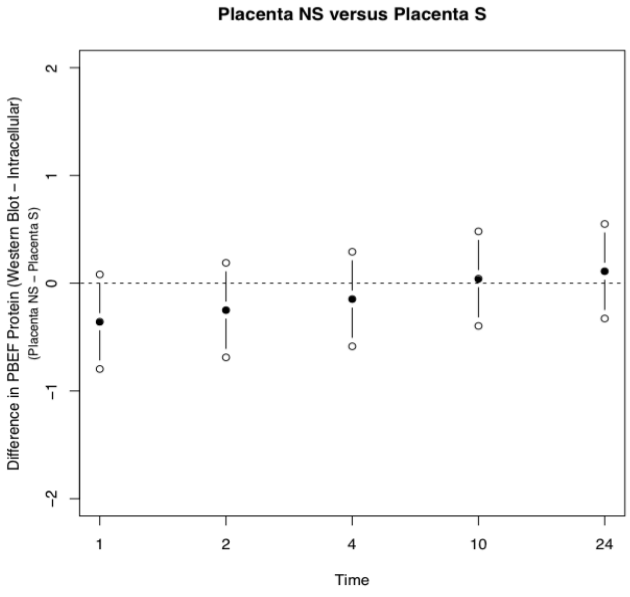
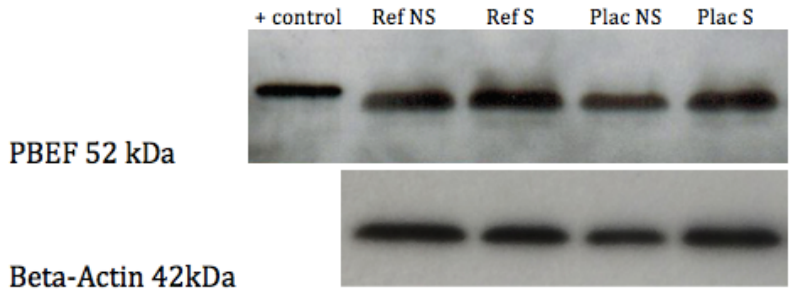



C.

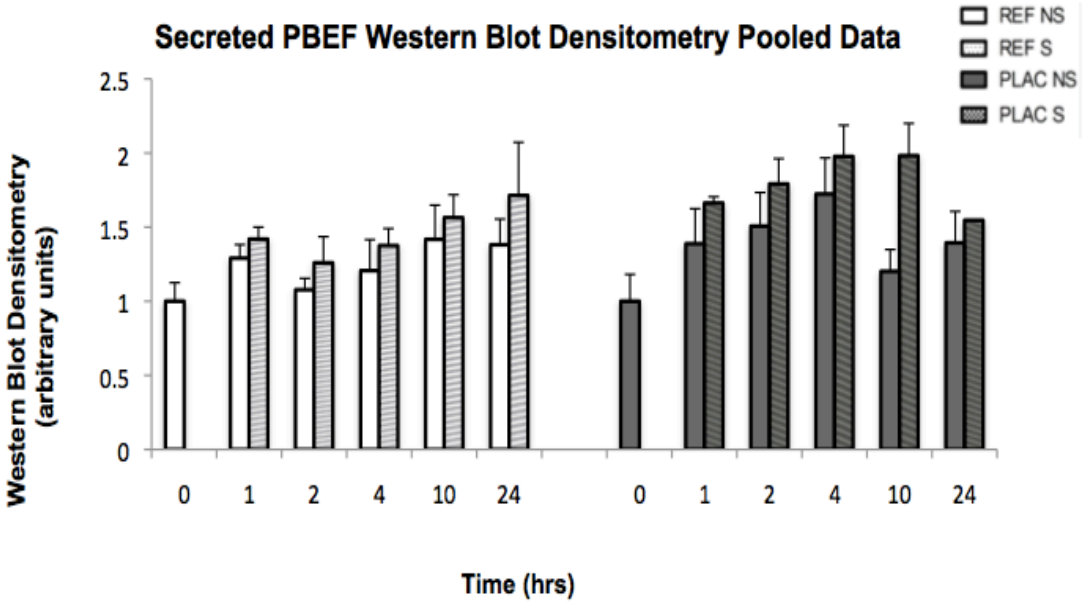
**Figure 11.** Mixed-model analysis of difference between means in intracellular PBEF protein quantification by western blot densitometry. The central point is the difference in mean measurement of relative gene expression for all patients and the extended hollow points indicate the 95% confidence interval for the mean. NS= nonstretch, S= stretch. A. Placental nonstretch minus reflected nonstretch. B. Reflected nonstretch minus reflected stretch. C. Placental nonstretch minus placental stretch.

| Comparison | Time 1 | Time 2 | Time 4 | Time 10 | Time 24 |
|---|---|---|---|---|---|
| Placenta NS versus Reflected NS | 0.64 (0.20, 1.07) | 0.93 (0.50, 1.37) | 0.56 (0.12, 1.00) | 0.37 (-0.07, 0.80) | 0.07 (-0.37, 0.51) |
| Placenta NS versus Placenta S | -0.36 (-0.80, 0.08) | -0.25 (-0.69, 0.19) | -0.15 (-0.59, 0.29) | 0.04 (-0.40, 0.48) | 0.11 (-0.33, 0.55) |
| Reflected NS versus Reflected S | -0.28 (-0.72, 0.16) | -0.17 (-0.61, 0.27) | -0.07 (-0.51, 0.37) | 0.12 (-0.32, 0.56) | 0.19 (-0.25, 0.63) |

**Table 6.** Mixed-model analysis of difference between mean comparisons in intracellular PBEF protein expression between certain comparisons after 1 to 24 hours. NS= nonstretch, S= stretch. In parentheses indicates the 95% confidence interval. Time 1 = 1hr, Time 2 = 2hr, Time 4 = 4hr, Time 10 = 10hr, Time 24 = 24hr.




A.




B.

**Figure 12.** Western blotting of secreted PBEF protein expression after stretching placental and reflected amnion epithelial cells (n=6). A. Representative PBEF and Beta-Actin Western Blots from a single patient's cell lysates. B. Pooled patient data of secreted PBEF protein quantification by Western Blot densitometry. Western blotting was performed on cell lysates from all samples from the stretch experiments (n=6). Densitometry of western blot bands were normalized to beta-actin loading control, and expressed relative to time zero for each experiment. REF= reflected amnion, PLAC= placental amnion, NS= nonstretch, S= stretch. Bars indicate SEM.

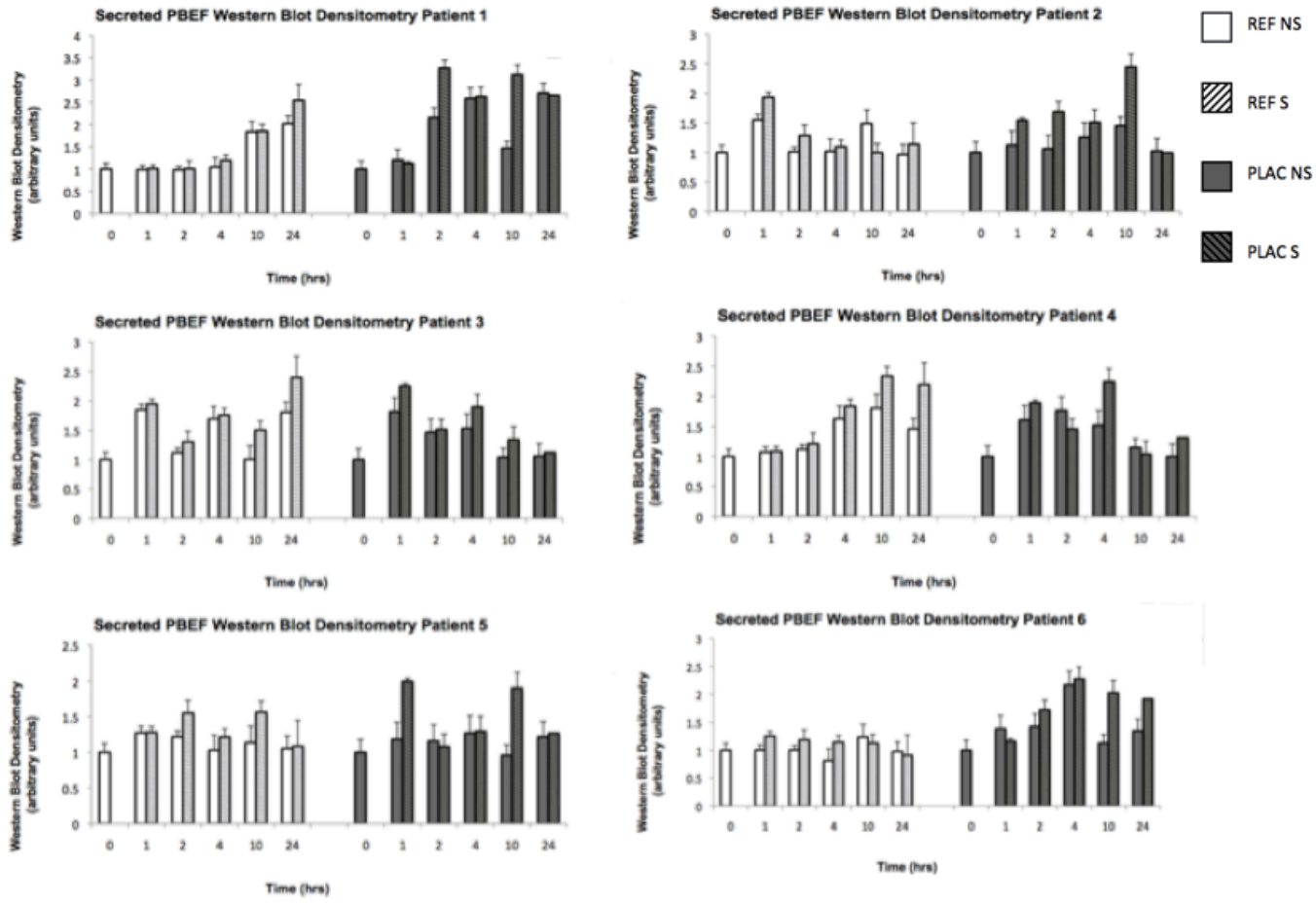



**Figure 13.** Individual patient data of secreted PBEF protein quantification by western blot densitometry. Western blot was performed on media from all epithelial cell samples from the stretch experiments. REF= reflected amnion, PLAC= placental amnion, NS= nonstretch, S= stretch. Bars indicate SEM.

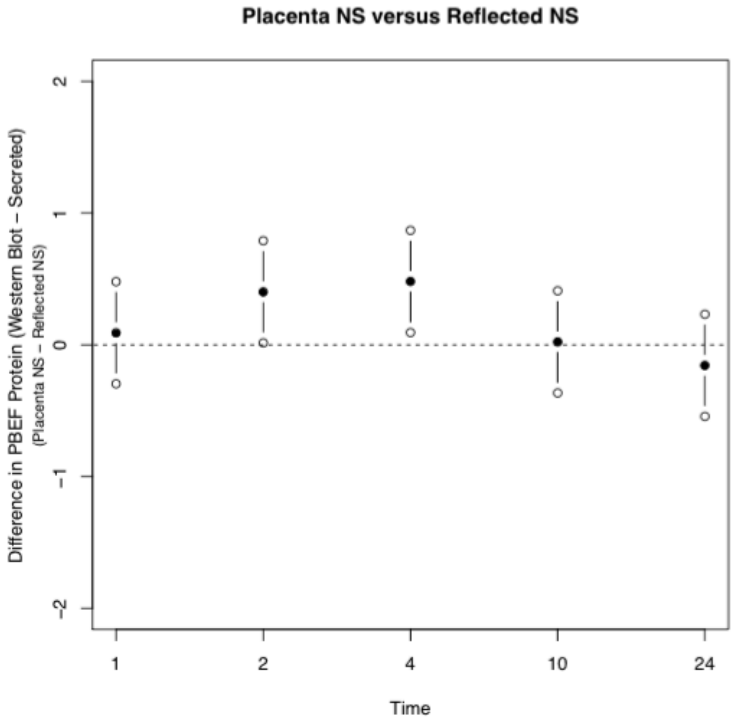



**Figure 14.** Mixed-model analysis of mean differences in Western Blot secreted PBEF protein expression data of placental nonstretch minus reflected nonstretch. The central point is the difference in mean measurement (reflected nonstretched mean subtracted from placental nonstretched mean) of relative gene expression for all patients and the extended hollow points indicate the 95% confidence interval for the mean. NS= nonstretch, S= stretch.

| Comparison | Time 1 | Time 2 | Time 4 | Time 10 | Time 24 |
|---|---|---|---|---|---|
| Placenta NS versus Reflected NS | 0.09 (-0.30, 0.48) | 0.40 (0.01, 0.79) | 0.48 (0.09, 0.87) | 0.02 (-0.37, 0.41) | -0.16 (-0.54, 0.23) |
| Placenta NS versus Placenta S | -0.28 (-0.67, 0.11) | -0.31 (-0.70, 0.08) | -0.29 (-0.68, 0.10) | -0.54 (-0.93, -0.15) | -0.32 (-0.71, 0.07) |
| Reflected NS versus Reflected S | -0.12 (-0.51, 0.26) | -0.16 (-0.54, 0.23) | -0.13 (-0.52, 0.26) | -0.38 (-0.77, 0.00) | -0.16 (-0.55, 0.22) |

**Table 7.** Mixed-model analysis of mean differences in Western Blot secreted PBEF protein expression data between certain comparisons of mean after 1 to 24 hours. Table with values for the central point and 95% confidence interval. NS= nonstretch, S= stretch. Time 1 = 1hr, Time 2 = 2hr, Time 4 = 4hr, Time 10 = 10hr, Time 24 = 24hr.

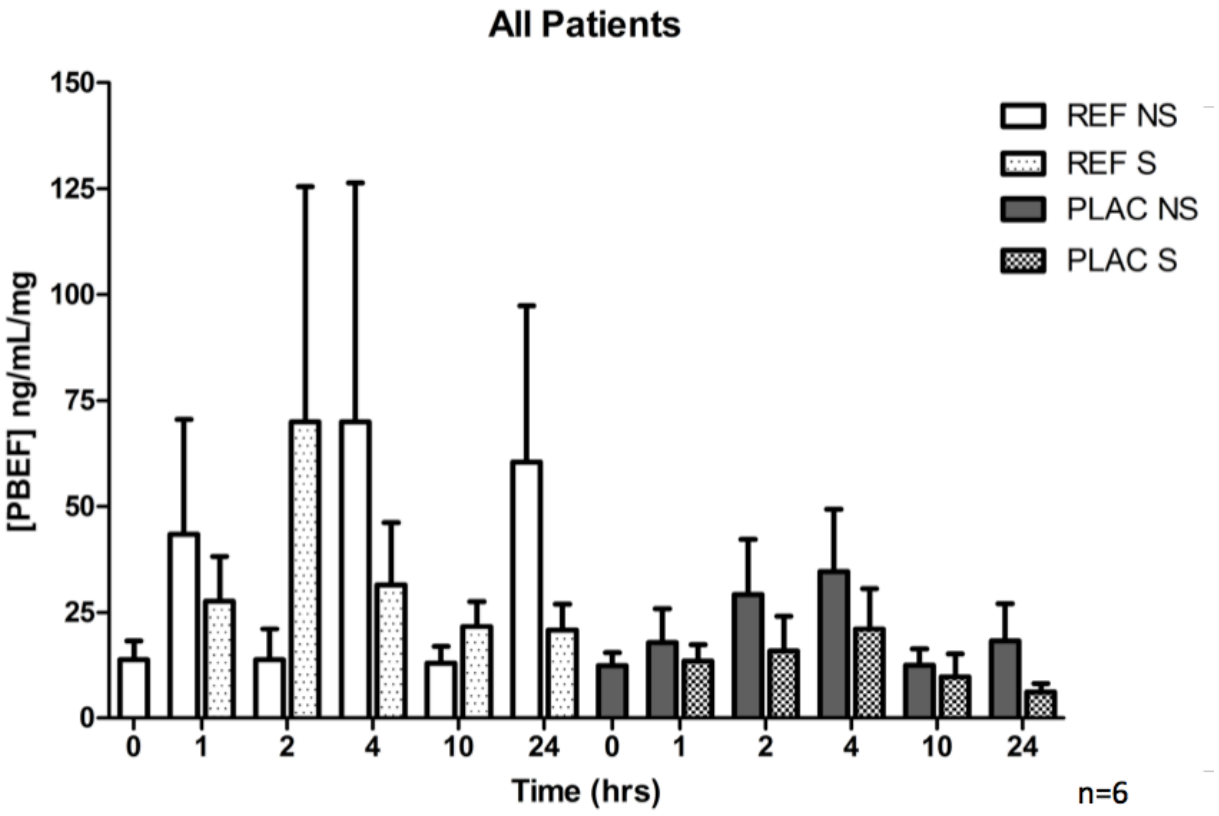


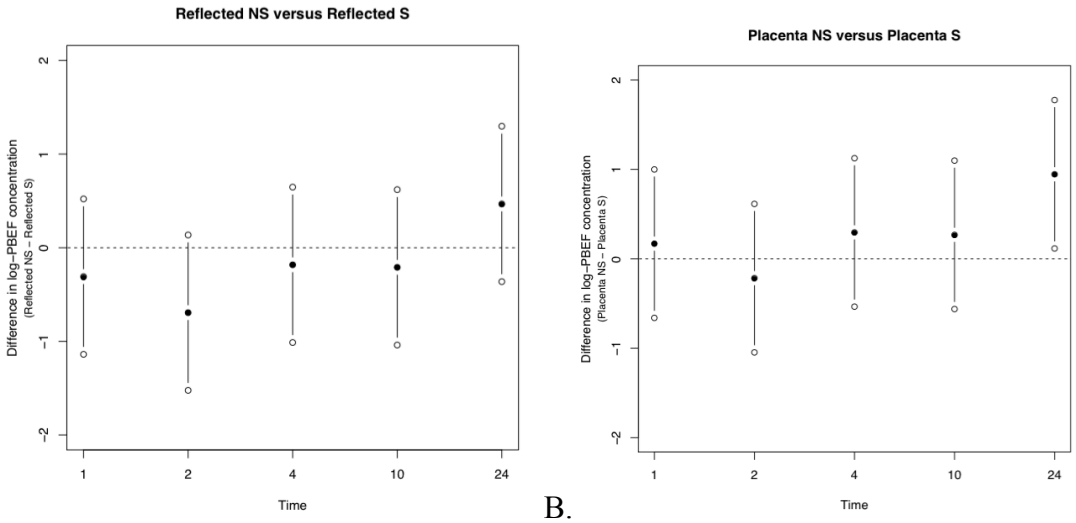

**Figure 15.** Pooled patient EIA Data (n=6) performed on conditioned media to measure secreted PBEF protein. REF= reflected amnion, PLAC= placental amnion, NS= nonstretch, S= stretch. Bars indicate SEM. Bars indicate SEM.

| | Average of Reflected Samples | Average of Placental Samples |
|---|---|---|
| Patient 1 | 2.578893669 | 3.111448547 |
| Patient 2 | 20.98970244 | 9.330713666 |
| Patient 3 | 14.16619625 | 14.65331299 |
| Patient 4 | 9.764188335 | 2.02431559 |
| Patient 5 | 105.6898758 | 35.54145904 |
| Patient 6 | 22.86730989 | 23.09979957 |

**Table 8.** Average of EIA data for secreted PBEF in the reflected and placental regions. Patient 5 is outlier due to its 750% increase when compared to the values in the reflected region of other patients and 340% increase in the placental region.

| | | Mean Difference | 95% CI | P-value |
|---|---|---|---|---|
| All Patients | Placenta NS - Reflected NS | -0.15 | (-0.64, 0.33) | 0.533 |
| | Placenta NS - Placenta S | 0.29 | (-0.19, 0.78) | 0.238 |
| | Reflected NS - Reflected S | -0.19 | (-0.67, 0.30) | 0.455 |
| Without Patient 5 | Placenta NS - Reflected NS | -0.10 | (-0.63, 0.43) | 0.722 |
| | Placenta NS - Placenta S | 0.07 | (-0.46, 0.60) | 0.785 |
| | Reflected NS - Reflected S | -0.25 | (-0.78, 0.28) | 0.363 |

**Table 9.** Summary of EIA analysis of secreted PBEF protein expression in stretch experiments. Mean differences account for the overall effect of time and the correlation between repeated measurements on the same patient.




A. B.

**Figure 16.** Mixed-model analysis of mean differences in EIA secreted PBEF protein expression data. The central point is the difference in mean measurement (reflected nonstretched mean subtracted from placental nonstretched mean) of relative gene expression for all patients and the extended hollow points indicate the 95% confidence interval for the mean. NS= nonstretch, S= stretch. A. Reflected nonstretch versus reflected stretch. B. Placental nonstretch versus placental stretch.

| Comparison | Time 1 | Time 2 | Time 4 | Time 10 | Time 24 |
|---|---|---|---|---|---|
| Placenta NS versus Reflected NS | -0.71 (-1.54, 0.12) | 0.47 (-0.36, 1.30) | 0.01 (-0.82, 0.84) | 0.04 (-0.79, 0.87) | -0.59 (-1.42, 0.24) |
| Placenta NS versus Placenta S | 0.17 (-0.66, 1.00) | -0.22 (-1.05, 0.61) | 0.30 (-0.53, 1.13) | 0.27 (-0.56, 1.10) | 0.95 (0.12, 1.78) |
| Reflected NS versus Reflected S | -0.31 (-1.14, 0.52) | -0.69 (-1.52, 0.14) | -0.18 (-1.01, 0.65) | -0.21 (-1.04, 0.62) | 0.47 (-0.36, 1.30) |

**Table 10**. Mixed-model analysis of mean differences of certain comparisons in EIA secreted PBEF protein expression data. Table with values for the central point and 95% confidence interval. NS= nonstretch, S= stretch. Time 1 = 1hr, Time 2 = 2hr, Time 4 = 4hr, Time 10 = 10hr, Time 24 = 24hr.

## 2.4 DISCUSSION

The stretch experiments performed on the amniotic epithelial cells yielded some results that support previous findings (Kendal-Wright 2010; Kendal-Wright *et al*. 2008; Sood *et al*. 2006). An expected result was that the reflected region would be more stretch-responsive with regards to PBEF gene expression than the placental region (Kendal-Wright 2010). This result supports the premise, which is based on the assumption that the reflected portion of the amnion stretches more than the placental *in-vivo* as seen by the visible expansion of the reflected amnion with progression of pregnancy, that the reflected amnion exhibits stretch-induced PBEF gene and protein expression while the placental region may not stretch *in-vivo* (Kendal-Wright 2010; Kendal-Wright *et al*. 2008). The data presented here also suggest that while the reflected amnion responds more to stretch, the placental amnion more robustly expresses the PBEF gene at baseline. This is also supported by the western blotting data of intracellular PBEF levels since baseline intracellular PBEF protein expression was higher in the placental region.

Together the PBEF gene expression data combined with the intracellular protein expression data from the cells of the stretch experiments demonstrates an apparent distinction in the stretch-response of cells from differing regions of the amnion. This is an important result because although variations in placental gene expression have been linked to abnormalities in maternal and fetal health, regional gene expression in the amnion in response to stretch has as yet, not been explored, despite the precedent that the regions function differently (Sood *et al*. 2006).

Secretion of the PBEF protein has been shown to be stimulated by the stretch in the reflected region of the amnion, but until this study the stretch response of the placental region had not been studied. The two methods of secreted protein analysis, western blot and EIA,

proved to have contrasting results. While the western blot data indicated a statistically significant placental stretch response and higher placental baseline PBEF secretion, the EIA showed the opposite trend, with a stretch response in the reflected region and higher baseline secretion in the reflected than the placental. This unexpected change in expression over time in the nonstretched placental controls may indicate a culture effect in which cells taken out of their *in-vivo* environments produce a stress response over time in culture (Halliwell 2003). In addition, previous studies using the same EIA kit, discrepancies in the order of magnitude of results between data from western blotting and EIA assays on intracellular and secreted PBEF has been seen and has been attributed to lower sensitivity in the EIA (Chua *et al*. 2005; Kendal-Wright *et al*. 2008; Nusken *et al*. 2007). Given that variation between patient nonstretched values was greater in the EIA (standard deviation of 11.030 and 6.802 in the EIA versus 0.154 and 0.262 in the western blots) (Table 2, 10), together with previous indications of the EIA's insensitivity provides the rationale for more confidence in the results from western blotting data over the EIA data.

The western blot data performed on secreted PBEF indicates a stretch response in the placental region and a higher baseline in the placental region. Putting this together with the gene and intracellular protein results, a cohesive hypothesis of the mechanism of PBEF expression with distension of the amnion may be constructed. With distension, the reflected amnion is prompted to increase PBEF transcription and *de novo* protein synthesis, while the placental amnion, inherently has a higher baseline level of the PBEF gene and protein, which, upon distension induces the secretion of this protein.

In terms of time points of peak response for PBEF expression, these varied greatly between patients regardless of the methodology implemented. Variation in human subject

research on fetal membranes is common, even when tissues are collected in the same way, as in this investigation (Penaherrera *et al*. 2012; Miller and Loch-Caruso 2010; Salafia *et al*. 2009; Poggi *et al*. 2009; Micera *et al*. 2014). Compounding this issue is that studies involving human tissues, especially when investigating immune molecules, exhibit large patient-to-patient variation due to individual environment and history of exposure to microbes (Micera *et al*. 2014). This intrinsic variation between individual human subjects, as opposed to animal studies in which variation between animals is limited by genetic cloning and controlled breeding environments, means that it is pertinent that careful attention is paid to individual trends. In the case of fetal membrane research, a patient's specific immune response, gestational age and time of tissue collection in proximity to labor all factor in to the tissue's response to experimental manipulation.

As far as regional variation is concerned, the amniotic epithelial cells from the placental region exhibited greater patient-to-patient variation in the gene expression assay, western blot for intracellular protein and western blot for secreted protein. Higher variation in the placenta was expected since its role is so crucial to fetal development and physiology, and it adjusts to provide and react to its environment, whether it is the physiological needs of the fetus or of the mother (Sood *et al*. 2006). The results from this study shed light on the diverse expression profiles of tissues of the fetal membrane between patients and between regions of fetal tissue.

In future experiments it will be imperative to use the 18s gene as a control in the qRT-PCR. In the analysis of PBEF gene production in response to stretch, the data were initially normalized to the housekeeping gene 18s, however, due to high sample-to-sample and plate-to-plate variation, 18s was deemed an invalid endogenous control. This level of high variation may have been due to discrepancies in the pipetting of the very small volume, 1.5ul, allowing for

large variations between samples in pipetting error. When 18s was eliminated as a control, the gene expression data provided increased confidence due to a reduction in within and between patient sample-to-sample variations. In the future in order to publish the data, 18s must be used to normalize the data, and using a larger sample size and increasing the volume of cDNA to pipette is expected to increase confidence in the 18s gene expression data. With regards to the samples that could not be used for gene analysis, it will be imperative in future experiments to collect a greater number of cells to obtain sufficient RNA to run every sample in the qRT-PCR.

Statistical analysis for this investigation was conducted using the Mixed Model method that assumed no change over the course of time, which is biologically suitable since the peak response times between patients varied drastically. The power analysis data provides useful information for experimental design. Although statistically significant results were found with the western blot on intracellular and secreted protein in this study, the power analysis suggests a larger sample size. This is because for the secreted western blot, the time points of interest chosen for the power analysis were different from the significant time points found on the experimental analyses. Although the time points selected for power analyses herein were chosen based on peak response times in previous studies, further power analyses on the time points at which trends were observed would be informative (qRT-PCR reflected 10hr, qRT-PCR placental 10hr, western blot intracellular reflected 2hr, western blot intracellular placental 4hr, western blot secreted reflected 24hr, western blot secreted placental 10hr, EIA reflected 10hr, EIA placental displayed no trend at any time point) (Kendal-Wright *et al*. 2008; Ognjanovic and Bryant-Greenwood 2002).

In conclusion the data from Aim 1 supports the hypothesis that the physiological stimulus of stretch has an effect on the amnion epithelial cells, and also more specifically the theory that

the reflected and placental regions differ in their response to stretch. The 3 sub-aims were achieved and provide answers, however under-powered in the case of the gene expression assay and EIA secreted protein assay, about the difference between the two regions of the amnion in response to stretch. It can also be concluded from the results that the placental amniotic epithelial cells possesses a higher PBEF gene and protein level within the cells, then with the stimulus of stretch they secrete a high level of the protein into the amniotic fluid space. Contrastingly the reflected amniotic epithelial cells contain a lower baseline level of the PBEF gene and intracellular protein, however, their levels of these intracellular contents are stretch-responsive, while their secretion of PBEF is less stretch responsive than in cells from the placental amnion.

# CHAPTER 3. SPECIFIC AIM 2

## 3.1 INTRODUCTION

In order to understand the role of the placental human amnion in amniotic fluid volume regulation, it is important to study the potential regulation of down stream pathways that may be activated by stretch to ultimately regulate fluid absorption across the amnion. One potential pathway within the fetal membranes that has indirectly been shown to be activated by stretch *in vitro* is one in which Pre-B-cell-colony-enhancing factor (PBEF) promotes vascular endothelial growth factor (VEGF) synthesis and secretion, also up-regulates the expression of its receptor, VEGF receptor 2, leading to increased permeability across the placental amnion (Astern *et al*. 2013).

The pathway of VEGF system up regulation by PBEF (Figure 17) is a working hypothesis that provides the basis for further research. In the experiments performed to generate this hypothesis, PBEF was added exogenously to the human amnion cells from both the reflected and placental regions to assess for VEGF secretion (Astern *et al*. 2013). It was found that this PBEF treatment led to increased VEGF secretion from both regions of the amnion. However, PBEF treatment also increased VEGF Receptor 2 expression and decreased VEGF receptor 1 expression from the placental amnion but not the reflected amnion. When the combination of PBEF and VEGF prime the placental amnion for increased intramembranous absorption, whether the permeability resulted from an interaction between VEGF and its VEGF Receptor 2 is unknown. This pathway elucidated a step-by-step mechanism for a possible way to increase the permeability across the human placental amnion *in vivo*.

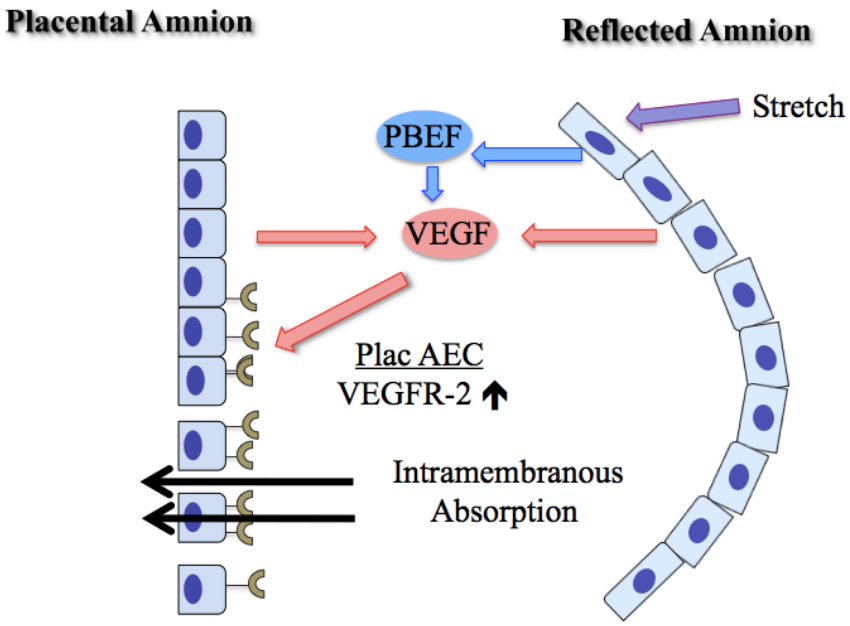



**Figure 17.** Working hypothesis of intramembranous absorption stimulation by PBEF and VEGF. Stretch of the reflected amnion induces PBEF production in reflected amnion. Exogenous PBEF induces VEGF production by both the reflected and placental regions of the amnion, although the reflected is significantly more, while also increasing the production of VEGF Receptor 2 on the placental amnion. These events lead to increased intramembranous absorption through the placental amnion. (Figure adapted from Figure 5, Astern, J. M., Collier, A. C., & Kendal-Wright, C. E. (2013). Pre-B cell colony enhancing factor (PBEF/NAMPT/Visfatin) and vascular endothelial growth factor (VEGF) cooperate to increase the permeability of the human placental amnion. *Placenta*, *34*(1), 42-49.)

To further understand the interaction of molecules in this proposed pathway, in both normal and disease states, it was assumed that known inhibitory molecules of this pathway may also have a role in regulating amniotic fluid. A well-known molecule that has been shown to recruit free-floating VEGF molecules is Sflt-1, a splice variant of VEGFR1 (Nevo *et al*. 2006). Sflt-1 is produced by a variety of tissues and binds VEGF, as it shares the ligand-binding domain of the wild-type VEGFR1 but lacks the membrane-spanning and C-terminal domains. Sflt-1 has been heavily studied in preeclampsia due to its elevated circulating levels in patients with the condition (Nevo *et al*. 2006). In preeclampsia, the placenta does not implant properly causing

maternal hypertension and an imbalance of angiogenic and anti-angiogenic markers such as VEGF and Sflt-1 (Beall *et al*. 2007). Thus in this condition, serum levels of Sflt-1 are increased, leading to an inhibition of angiogenesis, hypertension and ischemia (Jung *et al*. 2012). While serum Sflt-1 levels have been studied in preeclampsia patients, the protein's levels in amniotic fluid have also been shown to be elevated in preeclampsia (Vuorela *et al*. 2000; Park *et al*. 2005). However, the protein's role in permeability of the fetal membranes to amniotic fluid over the placenta has not been studied. Since increased Sflt-1 levels indicate a lower availability of VEGF to bind its receptors, it would be expected that increases in Sflt-1 would ultimately lead to a decrease in amnion permeability.

Thus, Aim 2 of this thesis was designed to measure the inhibitory effect of Sflt-1 on VEGF Receptor 2 activation. It is proposed that this could explain the modulation of the permeability pathway in the placental amnion *in vivo* during oligohydramnios with decreased Sflt-1 to allow for excessive fluid transport out of the fetal membranes and polyhydramnios with increased Sflt-1 to inhibit the release of amniotic fluid out of the fetal membranes. The hypothesis for Aim 2 is therefore that increased Sflt-1 in the amniotic fluid inhibits the PBEF/VEGFR-2 permeability pathway, specifically the activation of VEGFR-2 by its ligand.

While Aim 1 of this proposal focused on the affect of stretch on PBEF gene and protein expression, the subsequent effect of PBEF on permeability of the amnion was examined in Aim 2. Therefore, the second hypothesis of this thesis focuses on understanding the mechanisms behind transmembrane absorption pathway regulation through the placental amnion into the placental circulation.

### 3.2 AIM 2

**To test the hypothesis that Sflt-1 causes a decrease in VEGFR-2 activation in the placental amnion**.

Rationale: By showing that Sflt-1 causes a decrease in VEGFR-2 activation in the placental amnion, the potential inhibitory effect of Sflt-1 on permeability of the placental amnion would be tested. There are several signaling pathways that are activated after the VEGF ligand binds to VEGFR-2. Three of the many signaling molecules c-Src, PLC-y and PI3K were used demonstrate the activation of this receptor as they are activated by different pathways downstream of this receptor and produce different outcomes such as migration, vascular permeability, survival and proliferation (Figure 18). The activation of VEGFR-2 signaling was expected to decrease with increased exogenous Sflt-1 application. A combination of decreased activation of three signaling molecules downstream of VEGFR-2 provides powerful evidence for the potential of Sflt-1 to inhibit the regulation amniotic fluid over the placenta.

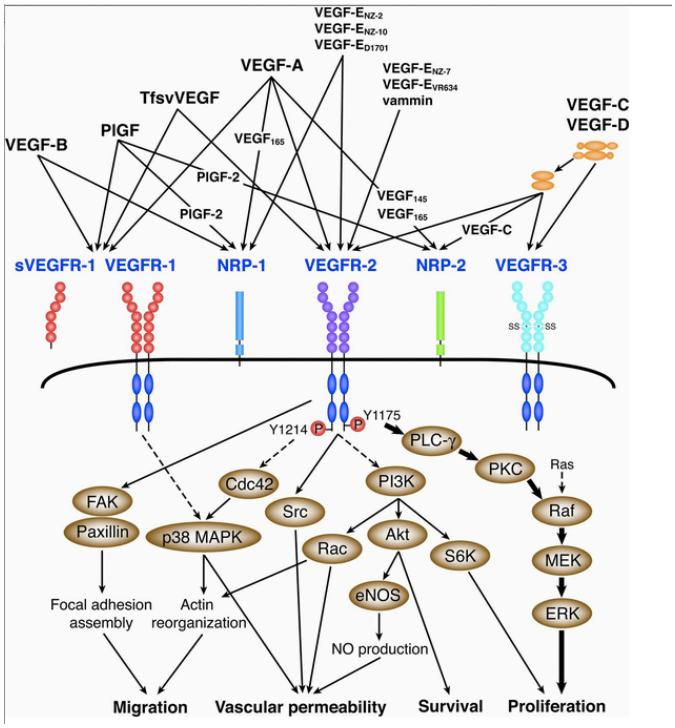



**Figure 18**. Schematic diagram describing the receptor-binding specificity of VEGF family members and the VEGFR-2 signaling pathways. The VEGF family of ligands and their receptor-binding patterns are shown in the top half of the figure. The downstream VEGFR signaling pathways focusing on VEGFR-2, with their cellular effect, are shown in the bottom half of the figure. Tyr1175 (Y1175) and Tyr1214 (Y1214) are the two major autophosphorylation sites in VEGFR-2. PLC-g binds to Y1175, leading to the phosphorylation and activation of this protein. Y1214 appears to be required to trigger the sequential activation of Cdc42 and p38 MAPK. Many proteins are activated by VEGFR-2 through an unknown

mechanism, including FAK, PI3K and Src. The activation of downstream signal transduction molecules leads to several different endothelial cell functions such as migration, vascular permeability, survival and proliferation. (Figure adapted from H. Takahashi, M. Shibuya. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. Clin. Sci.: 2005, 109(3);227-41.)

### 3.3 MATERIALS AND METHODS

**Explant Collection:** Placental amnion tissue explants would be collected from 6 patients at repeat Cesarean-section performed at Kapiolani Women and Children's Hospital (Honolulu, HI, USA). The collection of this tissue was approved by the IRB as an exempt protocol. 12 mm punch biopsies (Acuderm Punch, FL, USA) would be taken at random locations determined with a statistical model from each explant of placental amnion and placed into 6 well tissue culture plates in triplicate for each treatment condition. These explants would be allowed to rest in culture for 4 hours, the media changed, then they will be serum starved in 0.05% FBS and DMEM/F12 overnight. The next day the treatments would be applied as follows; a negative control sample with no treatment, a VEGF alone equivalent to endogenous basal level (30 pg/mL) (recombinant VEGF, Peprotech Inc., NJ, USA) and further samples would be treated with the VEGF basal level treatment plus exogenous Sflt-1 (recombinant Sflt-1, PlexBio, CA, USA) at doses of 0, 2, 6, 20 ng/mL and the samples collected at 0, 10, 30 and 60 minute intervals.

**Tissue lysate preparation:** Once the explants have undergone treatment, the tissue would be washed with cold 1X PBS while kept on ice, and put into a Sonifier (Model 102C, Branson Untrasonics Co., CT, USA) for 30 minutes in 500 mL of RIPA buffer. Then the tissue would be sonicated on ice for 4 minutes at 180 watts, in increments of 10 seconds of sonication and 10 seconds of rest. Then the broken tissue would be centrifuged at 9614 g for 20 minutes at 4˚C to

pellet the cellular debris. The supernatant would be transferred to a fresh tube and would be further centrifuged at 9614 g for 1 hour at 4˚C. This supernatant would be transferred to a new tube, then the protein concentration will be determined using a BCA protein assay. Then the samples would be frozen at -80˚C.

**Western Blotting:** Western blotting would be conducted to measure the activation of c-Src, PLC-y and PI3K proteins. The protocol for western blot would be completed as per Aim 1.2. 10ug of protein (determined by BCA assay) would be loaded in each lane and the resultant blots probed with; anti-Src antibody (1:1000, mouse monoclonal, Abcam, MA, USA), anti-SRC activated antibody (1:2000, mouse monoclonal, Life Technologies), anti-PLCγ antibody (1:10000, mouse monoclonal, Abcam, MA, USA), anti-PLCG2 activated antibody (1:1000, mouse monoclonal, abcam), anti-P13K antibody (1:1000, mouse monoclonal, Abcam, MA, USA) or anti-P13K activated antibody (phospho Y607) (1:1000, mouse monoclonal, Abcam, MA, USA). The secondary HRP-conjugated sheep anti-mouse monoclonal (1:5000, Abcam, MA, USA). The VEGF alone treatment would serve as a positive control to demonstrate the activation of these signaling molecules in the explants. Each of the western blots would be reprobed with a beta-actin antibody at a 1:300,000 dilution to serve as a loading control. The density of the bands will be analyzed by ImageJ as per Aim 1.2.

**Statistical Analysis:** Statistical analyses would be conducted in collaboration with the Biostatistics Core at University of Hawaii John A. Burns School of Medicine. Statistical analyses on the data would be completed using the Random Intercepts Mixed Model to allow each patient trajectory to begin at a different intercept, and to include change over time as a

question of interest. A power analysis using a t-test will also conducted for achieving 80% power on each of the downstream assays.

### 3.4 DISCUSSION OF POSSIBLE RESULTS

**Scenario 1: Sflt-1 treatment decreases VEGFR2 activation in all three signaling pathways**

The Sflt-1 treated samples were expected to decrease VEGFR2 activation over time and with increased Sflt-1 dosage. This was to be measured with the proxy of three signaling proteins activated by VEGFR2. If the activation of these proteins were inhibited by Sflt-1 addition, this would indicate that Sflt-1 disrupts VEGF receptor signaling. With western blot analysis on the activated form of the three proteins c-Src, PLC-y and PI3K, decreased intensity in the bands (quantified by Image J analysis) for the three proteins would be expected as relative to the control exposed to basal levels of VEGF. Increased Sflt-1 dosage treatment would be expected to be associated with decreased western blot band intensity for the three proteins.

In the case that the activation of the downstream signaling protein c-Src decreased with the increase of Sflt-1 treatment, it would indicate that VEGFR2-mediated c-Src activation was inhibited by Sflt-1. A previous study suggests that the activation of c-Src, through the binding of VEGF to VEGFR2, initiates transcytosis of amniotic fluid through caveloae in amniotic epithelial cells, specialized membrane micro-domains (Cheung *et al*. 2010). In essence the activation of c-Src leads to permeability of the amnion via caveloae, small (50-100 nm) specialized invaginations of the plasma membrane thought to play a role in endocytosis and uptake of bacteria and viruses formed by the protein caveolin. The study found that through the activation of the VEGF pathway and subsequently c-Src signaling pathway, caveolae production via caveolin-1 was upregulated. This study found that by inhibiting c-Src activation, the

downstream effects of VEGFR2 activation such as amniotic fluid transport across the amnion via caveolae were halted. Therefore in our investigation if Sflt-1 decreases c-Src activation, a consequent decrease in amniotic fluid transport through caveolae is expected. This becomes a fascinating study linking Sflt-1 levels in amniotic fluid to the eventual intramembranous absorption of the fluid through the amnion.

While neither PLC-γ nor PI3K inhibition have been studied in the fetal membranes, studies in endothelial cells shed light upon their possible responses in the amnion. In a study that inhibited the phosphorylation of PLC-γ via blockage of VEGF binding to VEGFR2, the most inhibition was seen at times greater than 5min and phosphorylation was not changed at 2 minutes, highlighting the momentous precision by which signaling molecules are phosphorylated (Halliwell 2003). In Aim 2 of this study the peak response times for VEGFR2 activation in the controls are expected to be seen at similar time points between patients since the specific cellular timing of signaling events is conserved between individuals. In another study when endothelial cells were pre-incubated with a PI3K inhibitor, the effect of VEGF was revoked, leading to increased apoptosis. This blockage of the PI3K signaling pathway has been studied for its benefits in reducing tumor growth (Elayppan *et al*. 2011). The FDA approved PI3K pathway inhibitors as a therapy against tumor growth and survival, demonstrating the significance of the PI3K signaling pathway for cell survival, and that by blocking its activation, cell death may be observed. Thus in Aim 2 of this investigation, the inhibition of VEGFR2 pathway may result in cell death of PI3K pathway is also blocked.

If exogenous Sflt-1 treatment decreased VEGFR2 activation, it would suggest that, for example, in preeclampsia when serum levels of Sflt-1 rise, VEGFR2 activation on the placental amnion would decrease, leading to decreased intramembranous absorption across the placental

amnion. This would cause a disruption in amniotic fluid volume regulation among other potential problems. In a previous report in mice it was found that intra-amniotic Sflt-1 treatment suppressed the activation of VEGFR2 in the fetal lung.[64] This study suggests that the pre-eclampsic fetal environment with heightened Sflt-1 can cause disruption of the VEGFR2 activation pathway in the fetus, an interaction between amniotic fluid composition and fetal organ development which is not the focus of this thesis but provides incentive to better understand the effect of increased Sflt-1 levels in amniotic fluid.

**Scenario 2: Sflt-1 treatment increases VEGFR2 activation**

The unexpected result for Aim 2 is if Sflt-1 treatment increased the activation of VEGFR2. Assuming that Sflt-1 binds the VEGF ligand, this result would indicate that while the Sflt-1-VEGF complex blocks the VEGF site for VEGFR2 attachment, another region on VEGF is still available to bind other receptors, such as VEGFR3, that promote the activation of the same downstream signaling molecules as VEGFR2. While VEGF may be binding to other receptors, such as VEGFR3, which also activates PI3K (Figure 19), it is unknown whether Sflt-1 bound VEGF can conformationally still bind to its receptor 3 (Sase *et al*. 2009). If the assumption that Sflt-1 binds the VEGF ligand is incorrect, this would indicate that the exogenous addition of basal levels of VEGF progressively increased VEGFR2 activation. The cellular mechanisms controlling the regulation of Sflt-1 are not clear, but a study in endothelial cells showed that the cell has an external stress sensing mechanism that regulates VEGF ligand availability and modulates Slft-1 and VEGFR2 levels, to guarantee that cell survival takes priority (Latham *et al*. 2012). Thus in Scenario 2, it is possible that due to the stresses imparted

upon the cells by the culture effect, the cells upregulate the VEGFR2 pathway to ensure that cell survival is maintained.

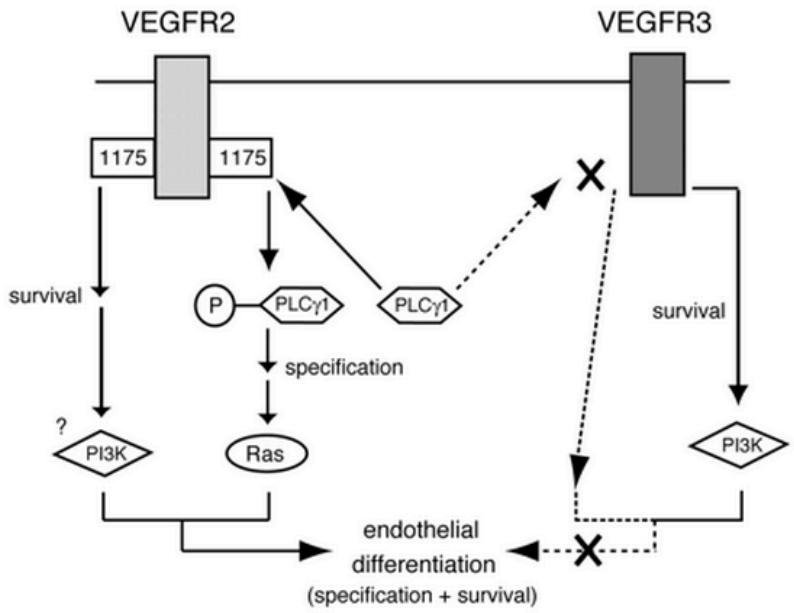



**Figure 19.** Theoretical model of endothelial differentiation of cells with VEGFR2 and VEGFR3 receptors. PLCγ1 is phosphorylated through interaction with Y1175-*P* of VEGFR2 in response to VEGF-A stimulation. The VEGFR2-PLCγ1 signaling axis might lead to activation of Ras, one of the pivotal regulators of endothelial specification. PI3K might also be activated via VEGFR2 Y1175-*P* to maintain survival of ECs. VEGFR3 does not induce endothelial specification of $VEGFR2^{+}$ cells, presumably because VEGFR3 fails to activate PLCγ1, although VEGFR3 has the potential to maintain the survival of ECs via PI3K. (Figure adapted from Sase, H., Watabe, T., Kawasaki, K., Miyazono, K., & Miyazawa, K. (2009). VEGFR2-PLCγ1 axis is essential for endothelial specification of VEGFR2+ vascular progenitor cells. *Journal of cell science*, *122*(18), 3303-3311.)

**Scenario 3: Sflt-1 treatment causes no change in VEGFR activation**

If Sflt-1 treatment causes no change in VEGFR2 activation, meaning the control and treatment samples exhibit the same level of VEGFR2 activation, it would signify that Sflt-1 is not affecting the availability of VEGF to its receptor 2. This indicates that perhaps a higher dosage of Sflt-1 is needed to see an effect or that Sflt-1 may not have an effect at all. With the progression of pregnancy there is an increase in Sflt-1 levels in the amniotic fluid, with a mid-trimester level of 10 ng/mL, which this study mimicked with the dosages (0-20 ng/mL), but since this is an *in-vitro* study, more Sflt-1 may be needed to incur an effect in VEGFR2 activation, although no precedent has been set on this (Xu *et al*. 2009).

Also the source of Sflt-1 in the fetal membranes *in-vivo* is still under debate, and a simple exogenous supply of the protein on random explants from the placental region of the amnion may not incur an effect on VEGFR2 activation, although it may *in vivo*. It has been shown that the placenta is a robust source of Sflt-1 to the amniotic fluid, and it is possible that *in vivo* the path that Sflt-1 takes to move from the placenta to the amniotic fluid enhances its capacity to bind VEGF (Clark *et al*. 1998).

A previous study showed that stretch induced the activation of PLC-γ in endothelial cells, and its downstream affect of exocytosis was lessened by VEGFR2 knockdown, thus demonstrating the complex and even reversible outcomes in the VEGFR2 activation pathway (Hao *et al*. 2009).

# CHAPTER 4. DISCUSSION

While the fetus controls the majority of the regulation of amniotic fluid via swallowing and urination, fluid absorption through the amnion is less well understood. In addition, it seems apparent that although the amnion is a continuous layer of the fetal membranes, its regions function differently, and the specific behavior of placental and reflected parts of the amnion with respect to fluid regulation, also appear different but remain to be completely defined.

This thesis set out to investigate two hypotheses centered on the regulation of human amniotic fluid through the amnion layer of the human fetal membranes. These studies were conducted to better understand the role of the fetal membranes, specifically the amnion, in amniotic fluid volume regulation, by looking at the effect of stretch as a potential stimulus of fluid regulation as well as the effect of an exogenous molecule on the partially defined mechanistic pathway for intramembranous fluid absorption by this tissue.

The first hypothesis tested in Aim 1 was whether stretch would induce PBEF expression in both the placental and reflected amnion. A previous study documented that stretch-activated PBEF expression in the reflected amniotic epithelial cells, but the effect of stretch on PBEF expression in placental amniotic epithelial cells had not been done. The results from this study indicate a regionally distinct stretch-induced PBEF expression patterns in the amnion, which expand on the previously described working hypothesis (Figure 2). The data from this research support the idea that the placental amnion naturally produces a higher level of PBEF gene and intracellular protein, while the reflected amnion has a greater stretch-responsive production of the PBEF gene and intracellular protein, indicating *de novo* synthesis of this protein within the cells of the reflected amnion. PBEF's secretion pattern is also distinctive between the two regions of the amnion, with a significantly greater stretch-induced secretion in the placental

region, as measured by western blot. Although the EIA results were dismissed, the pattern of a stretch-responsive PBEF secretion, especially in the reflected region, is quite noticeable. In future studies a greater sample size would provide more discernment in evaluating the EIA results. The higher secretion pattern of the placental amnion begs the question of PBEF's role within the amniotic fluid. As pregnancy progresses the fetal membranes stretch and this distension can lead to fetal membrane weakening and eventual rupture (Kendal-Wright *et al*. 2008). Since the fetal membranes have been shown to demonstrate a stretch-responsive production of PBEF, a protein linked to cell survival and the increased gene expression of which parallels an increase in a cell survival gene SIRT1 and a decrease in an inducer of cell death p53. Thus, it is plausible that the secreted PBEF from the placental region serves as a protector to the placental AEC, as described previously (Kendal-Wright *et al*. 2008).

In Aim 2, the hypothesis was that Sflt-1 would inhibit VEGFR2 activation, disrupting downstream permeability pathways of VEGFR2 activation. The affect of Sflt-1 on VEGFR2 activation in amniotic epithelial cells has not yet been looked at, although its expression in the amniotic fluid has been shown to inhibit VEGFR2 downstream pathways in endothelial cells. It is valuable to understand the effect of Sflt-1 on amniotic fluid volume regulation since Sflt-1 has been documented to rise in the pregnancy disorder preeclampsia (Park *et al*. 2005). Since the amount of Sflt-1 needed to shut down the effect of VEGF is unknown, the results of this study are imperative to determining the effective amount. The level of Sflt-1 in the amniotic fluid of pre-eclampsic patients rises to levels of 0.73 ng/mL, which falls into the range of doses chosen for the experiment from 0-20 ng/mL. Thus the implications of this study would be beneficial to increase the understanding the role of Sflt-1 in the eventual intramembranous absorption pathway through the fetal membranes.

Combining the implications derived from Aim 1 of regional distinction within the amnion in response to stretch with the idea in Aim 2 of a protein potentially capable of inhibiting the effects of a VEGF/PBEF-primed amnion to VEGFR2-mediated intramembranous absorption of amniotic fluid, a complex network of cause and effect surfaces. Therapeutically this thesis indicates the benefits of monitoring distension and AFI during pregnancy as a trigger for the misregulation of amniotic fluid volume regulation that may indicate risk for polyhydramnios or oligohydramnios. Potential for the establishment of therapeutic interventions, first in model organisms before use in humans, that augment pieces of the stretch-induced permeability pathway (Figure 17) is eminent, once the pathway has been solidified. Once a step-by-step, causative and linear mechanistic pathway is determined between stretch, PBEF, VEGF, VEFGR2 and its downstream signaling molecules and eventual intramembranous absorption whether transcellular or paracellular, a single step may be augmented to affect the volume of amniotic fluid during pregnancy. In the case of polyhydramnios, an intervention method may be established utilizing the principle of a VEGF/PBEF-primed amnion to activate a permeability pathway by increasing the level of PBEF in the amniotic fluid, whether by introducing PBEF into amniotic fluid or by increasing distension of the amnion. However care must be taken to ensure that inflammation is not increased in the tissues of the amnion by overdistension, in which case preterm birth becomes an imminent risk. On the other hand, in oligohydramnios where a decrease in the out flux of amniotic fluid is necessary, an intervention method that may be proposed is to administer Sflt-1 to the amniotic fluid to inhibit further loss of amniotic fluid. Both of these proposed novel therapies require substantial advancement in the understanding of the direction and mechanism of amniotic fluid permeability through the amnion before they could come to fruition, as this has not been explored in depth. With the work done in this thesis

along with investigations in amniotic fluid volume regulation across the scientific community in both the human and ovine models, we aspire to press forward in exploring the underlying mechanisms orchestrating fluid flux in hopes to establish measures of predicting and preventing preterm birth in the coming generations.

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