2015

Characterizing the role of kisspeptin in placental invasion

https://hdl.handle.net/2144/16262

Boston University
CHARACTERIZING THE ROLE OF KISSPEPTIN IN PLACENTAL INVASION

by

ZAHRAH MASHEEB

B.A., Princeton University, 2010

Submitted in partial fulfillment of the requirements for the degree of

Master of Science

2015
ACKNOWLEDGMENTS

I would first like to thank my inspiring and supportive mentor, Dr. Wendy Kuohung for her tireless dedication to teaching and clinical discovery. Many thanks to Dr. Sandy Michaud of the Schepens Eye Institute, as well as the rest of the Gipson Lab, for their generosity and guidance in the ongoing pursuit of success in laser capture microdissection, which has proven to be a worthy foe. I would also like to acknowledge Dr. Jeffrey Pudney, and the rest of Anderson Lab at Boston University School of Medicine for their sage advice on tissue preparation. Finally I would like to thank Dr. Victor Navarro for his expertise as my second reader, and the rest of the Kaiser Lab at Brigham and Women’s for their help with troubleshooting.

This research was funded through 1R01AI101088-01, under Principal Investigator Dr. Robin Ingalls.
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ABSTRACT

Preeclampsia is an increasingly prevalent disorder of placentation that has very limited options for treatment. The disease is characterized by aberrant invasion of placental trophoblasts into the decidualized maternal endometrium. In order to identify pathways of therapeutic interest during placentation, we are focusing on the pathway of the neuropeptide kisspeptin and its receptor KISS1R, both highly expressed in the human placenta. Early functional studies of the ligand-receptor system identified a role for kisspeptin in the inhibition of cancer metastasis. Parallels exist between cancer and placentation, suggesting the possibility of an inhibitory role for kisspeptin during pregnancy as well.

Existing functional data supports kisspeptin’s inhibitory influence on cellular invasion, but the mechanism remains unknown. Evidence for the localization of the KISS1R receptor in the current literature was established via a nonspecific antibody and requires further investigation. Current literature suggests involvement of the ERK (extracellular signal-regulated kinase) pathway as well. Our work aims to solidify the localization of kisspeptin and KISS1R, avoiding the use of KISS1R antibodies. Using immunohistochemistry for protein localization of kisspeptin and placental fractionation followed by quantitative PCR analysis for gene expression, we provide evidence of kisspeptin's restriction to the syncytiotrophoblast layer of the placenta, and KISS1R gene expression limited to the villous cytotrophoblast layer. This distribution of ligand and
receptor suggests a paracrine mechanism for kisspeptin action, with syncytiotrophoblasts secreting kisspeptin to act on its receptor on the villous cytotrophoblast layer, and thus restricting cytotrophoblast invasion.

We further attempt to support these data with the use of laser capture microdissection of placental tissue to isolate the different layers, followed by quantitative PCR. This technique introduced a particularly challenging aspect of working with the placenta: maintaining tissue morphology while also preserving RNA integrity. This thesis outlines our troubleshooting process for that technique and introduces alternatives for future work. We also employed Western blot analysis of ERK activation to establish the mechanism of kisspeptin's inhibitory effect on fractionated trophoblasts.
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GSK-3 .................................................................glycogen synthase kinase 3
HAI-1.............................................................hepatocyte growth factor activator inhibitor 1
hCG...............................................................human chorionic gonadotropin
HPL..............................................................human placental lactogen
HRP..............................................................horseradish peroxidase
HTR8/SVneo .................................................transformed extravillous cytotrophoblast cell line
IRB ...............................................................Institutional Review Board
JEG-3 .............................................................human choriocarcinoma cell line
KISS1.............................................................human kisspeptin gene
Kiss1 ............................................................murine kisspeptin gene
KISS1R..........................................................human kisspeptin receptor protein
KISS1R..........................................................human kisspeptin receptor gene
Kiss1R ..........................................................murine kisspeptin receptor gene
Kp.................................................................kisspeptin
LCM.............................................................laser capture microdissection
LH...............................................................luteinizing hormone
MAP kinase..................................................mitogen-activated protein kinase
MDA-MB-435..............................................melanoma cell line
mRNA........................................................messenger ribonucleic acid
OCT............................................................optimal cutting temperature embedding medium
PBS............................................................phosphate-buffered saline
PCR.............................................................polymerase chain reaction
PE.................................................................preeclampsia
PEN.................................................................polyethylene naphthalate
p-ERK.......................................................phosphorylated extracellular signal-related kinase
PIP_2.................................................................phosphatidylinositol 4,5-bisphosphate
PMA.................................................................phorbol 12-myristate 13-acetate
PMSF......................................................phenylmethanesulfonylfluoride
PVDF.................................................................polyvinylidene difluoride
qPCR...............................................................quantitative polymerase chain reaction
RIPA...............................................................radioimmunoprecipitation assay
RNA...............................................................ribonucleic acid
RNase...............................................................ribonuclease
RPMMI...............................................................cell media developed at Roswell Park Memorial Institute
RT.................................................................reverse transcriptase
SDS-PAGE......................................................sodium dodecyl sulfate polyacrylamide gel electrophoresis
STBs...............................................................syncytiotrophoblasts
T1........................................................................Trypsin step 1
T2........................................................................Trypsin step 2
T3........................................................................Trypsin step 3
TBS...................................................................Tris-buffered saline
TBST................................................................Tris-buffered saline with Tween 20
TLR3................................................................Toll-like receptor 3
UV........................................................................ultraviolet
INTRODUCTION

During pregnancy, the placenta is the interface of gas exchange, nutrient delivery, and waste disposal between the fetus and the mother. During placental development, the branching network of chorionic villi grows and embeds itself within the decidualized maternal endometrium, culminating in a surface area of approximately 12-14 square meters (Jauniaux, Poston, & Burton, 2006). Normal placental differentiation involves the formation of two types of placental villi: floating villi and anchoring villi (Caniggia et al 2000). Floating villi are designed for nutrient and gas exchange and are made up of villous cytotrophoblasts (CTBs) that differentiate and fuse to create the syncytiotrophoblast (STB) layer. The layer of STBs forms an epithelial barrier between the villi and the mother’s blood, mediating nutrient and gas exchange and secreting a number of hormones, including human chorionic gonadotropin (hCG) and human placental lactogen (HPL). CTBs in the anchoring villi (pictured in Figure 1) differentiate to either form the STB layer or to break through it as extravillous cytotrophoblasts (EVTs) that assemble at the interface and secure the placenta to the uterine decidua. These EVT migrate into the decidua as deep as the first third of the myometrium, circling or invading the vasculature to replace the vascular endothelium. This invasion triggers the transition of the mother’s spiral arteries, narrow vessels that support a hypoxic environment characteristic of the early placenta, into the wider, low-resistance uteroplacental arteries that promote oxygen exchange later in gestation.
**Abnormal placentation: clinical relevance**

The hypertensive disorders of pregnancy, including preeclampsia, stem from a lack of conversion from spiral to uteroplacental arteries (pictured in Figure 2). This defect is due to insufficient invasion of the EVTs that are pathologically localized in the basal plate rather than extending past this superficial layer to the myometrium. Clinical findings include high blood pressure in the mother and often with proteinuria. For the fetus, this means a diminished capacity for nutrient exchange, hindering its growth and development and sometimes resulting in infant death. Although the relationship between impaired EVT invasion and preeclampsia is well established, the mechanism behind it is unknown.

Preeclampsia affects 6 to 8 percent of pregnant women in the United States. Paired with seizures, the illness is identified as the more severe eclampsia, the second leading cause of maternal death in the U.S.
Unfortunately, the prevalence of preeclampsia is on the rise, and the only cure is delivery of the fetus. Depending on the severity of symptoms, the risks of premature delivery are weighed against the risks of the disease. Further, management of mild preeclampsia involves the close monitoring of the mother’s blood pressure and organ function, significantly contributing to medical costs. Thus, uncovering the mechanisms underlying aberrant placentation is crucial to finding alternative treatment options for preeclampsia and related disorders, and to finding earlier indicators of the disease that can prevent its progression into more severe forms. Our investigation of the kisspeptin/KISS1R system aims to uncover a role for this ligand and receptor as an indicator or therapeutic target for disorders of placentation such as preeclampsia.

Figure 2. Abnormal trophoblast invasion characterizes preeclampsia. A) The normal morphology of a spiral artery during the first 6 weeks of placental invasion, and B) the normal progression of invasion at 20 weeks and beyond. Notice that in the normally developed placenta, EVTs invade the placental bed, altering the vascular morphology. C) In preeclampsia, EVTs are restricted to the superficial basal plate. Figure from Caniggia and Winter (2002).
**Kisspeptin: the basics**

The *KISS1* gene was initially described for its role in cancer metastasis, first in metastatic melanoma, and then in breast carcinoma. The first defining study showed that prior microcell-mediated transfer of a human chromosome into two different melanoma cell lines conferred a loss of metastatic activity (Welch et al., 1994). To determine the gene behind this change, they employed a modified subtractive hybridization method to identify mRNA that was uniquely upregulated in the non-metastatic cell line versus its metastatic parent, narrowing their scope from a full chromosome to a single gene, *KISS1* (Lee et al., 1996). Upon identifying *KISS1* as a candidate, their next study employed transfection of the gene into the breast carcinoma line MDA-MB-435, where they similarly observed a loss of metastatic activity without any loss of tumorigenicity (Lee & Welch, 1997). By 1998, this group localized and sequenced the *KISS1* gene and determined that its open-reading frame was 145 amino acids long. However, localization of the product’s multiple proteolytic cleavage sites and identification of its main 54-amino acid translated product after cleavage of the full transcript, kisspeptin-54, did not occur until 2001 (Ohtaki et al., 2001). In the initial years after its discovery, kisspeptin-54 was aptly named metastin for its inhibitory role in cancer metastasis.

A human G-protein-coupled receptor homologous to the rat orphan receptor GPR54 was found to be specifically activated by kisspeptin-54, and numerous shorter C-terminal fragment peptides were also identified. Based on studies of intracellular Ca\(^{2+}\) concentration, C-terminal fragments kisspeptin-14, kisspeptin-13, and kisspeptin-10 also activated GPR54 (Muir et al., 2001). Measurements of PIP\(_2\) hydrolysis, MAP kinase
phosphorylation, and ERK1/2 phosphorylation confirmed that the shortest sequence sufficient for receptor activation, the C-terminal decapeptide kisspeptin-10, was as potent as kisspeptin-54 and its other shorter fragments (pictured in Figure 3); all were comparable enough in their downstream effect on cells over-expressing GPR54 to be collectively termed the kisspeptins, and for the receptor to be referred to as KISS1R (Kotani et al., 2001).

**Figure 3. Kisspeptin gene product and post translational processing.** Adapted from M. Tena-Sempere (2006).

Aside from extensive study in the context of tumor metastasis, researchers were surprised to find that a loss-of-function mutation in the human receptor for kisspeptin (KISS1R) resulted in a clinical case of hypogonadotropic hypogonadism, pointing to an entirely new function for the ligand-receptor system in reproduction and puberty (de Roux et al., 2003; Seminara et al., 2003). A role in the hypothalamic-pituitary-gonadal axis was confirmed with studies showing that mutant mice deficient for the receptor had underdeveloped gonads and juvenile reproductive tract development and were responsive to exogenous gonadotropin and gonadotropin releasing hormone (GnRH) (Funes et al., 2003; Seminara et al., 2003). It was evident that in these mice lacking the *Kiss1R*, that
GnRH release was impaired by the lack of the receptor. Injection of kisspeptin directly into the rodent brain stimulated luteinizing hormone (LH) release via GnRH (Gottsch et al., 2004) that appeared to be controlled by negative feedback from the sex hormones (V. M. Navarro, Castellano, et al., 2004). Functional studies indicated that sustained, cyclical intracerebroventricular exposure to kisspeptin induced precocious puberty in female rodents and introduced a complex relationship with metabolism through leptin (V. M. Navarro, Fernández-Fernández, et al., 2004). The intricacy of this neuropeptide’s role in puberty and metabolism is still an active area of research (Víctor M. Navarro & Kaiser, 2013).

The discovery of high gene expression levels of both KISS1 and KISS1R in the placenta gave rise to an investigation of the system’s role in pregnancy (Ohtaki et al., 2001). Kisspeptin-54’s original identification as a metastasis inhibitor would support a similar function for the peptide in placental invasion, a process likened by many to the growth and spread of a tumor but distinguished from cancer by its precise regulation (Janneau et al., 2002; Strickland & Richards, 1992). *In vitro* studies of trophoblast migration activity after exogenous treatment with kisspeptin support kisspeptin’s inhibitory influence on trophoblast invasion. Treating trophoblasts with kisspeptin causes a measurable decrease in migration and outgrowth across multiple models: 1) primary trophoblasts isolated from first trimester human placental tissue (Francis, Abera, Matjila, Millar, & Katz, 2014), 2) first trimester human villous explants in culture (Bilban et al., 2004), and 3) a human extravillous cytotrophoblast-derived cell line HTR8/SVneo (Roseweir, Katz, & Millar, 2012; Taylor, Pampillo, Bhattacharya, & Babwah, 2014).
This inhibitory influence of kisspeptin on trophoblast migration seems to rely on a number of downstream effectors, diagrammed in Figure 4. Francis and Taylor both showed a resultant increase in ERK1/2 phosphorylation after kisspeptin treatment, while Roseweir et al.’s work suggests a link to a receptor-GSK3 beta-FAK feedback loop, a focal adhesion kinase involved in cytoskeletal interaction with the extracellular matrix.

**Figure 4.** Kisspeptin/KISS1R system and downstream effectors. Taken from Cvetković et al. (2013). Kisspeptin is the ligand for a G-protein coupled receptor, KISS1R, linked to phospholipase C and inositol triphosphate second messenger intracellular cascades.

To investigate a more physiologically relevant system that offers the freedom of manipulation that human subjects do not, animal models have been developed to study the kisspeptin system. With 83% amino acid homology between the rat kisspeptin receptor and human KISS1R, measures of kisspeptin levels and localization in the rat placenta showed that expression of the ligand and its receptor peak at day 12.5, corresponding with the rat placenta’s peak invasiveness (Terao et al., 2004). To study the
functional role of the ligand-receptor system in pregnancy, *Kiss1* and *Kiss1R* knockout mice were studied in the context of fetal birth weights and placental morphology (Herreboudt, Kyle, Lawrence, Doran, & Colledge, 2015). The study concluded that mutants of the kisspeptin signaling system produced normally functioning placentas in the mouse. Given that expression of *Kiss1* was much lower in mouse placenta than is seen in humans, the results do not suggest a role for kisspeptin in murine pregnancy but do not exclude a role in human pregnancy.

**Kisspeptin’s changing expression levels in the human placenta**

In human studies, levels of serum kisspeptin in the mother increase dramatically throughout pregnancy and drop abruptly postpartum, suggesting that the placenta is the source of this 7000-fold increase in protein (Horikoshi et al., 2003). However, linking the rise in circulating protein to an increase in ligand and receptor mRNA within placental tissue has proven difficult due to the lack of reliable antibody for localizing the receptor and the variability of placental sampling among researchers. One group has shown *KISS1* mRNA and kisspeptin protein to increase over the course of the pregnancy (Qiao, Cheng, Zhang, Wang, & Lin, 2005), as may be expected to correspond with the decreasing invasiveness of the developing placenta (Strickland & Richards, 1992). In contrast, some studies show decreasing levels of placental *KISS1* and *KISS1R* mRNA and protein in parallel, while others show the receptor decreasing as the ligand maintains consistent levels (Bilban et al., 2004; Janneau et al., 2002). These inconsistencies could be explained by sampling differences of the diverse population of trophoblast layers.
throughout the placenta. The variability of expression levels paired with a skewed representation of the trophoblast population in any given experiment could explain why some data shows an increase in placental kisspeptin over pregnancy while others report just the opposite.

The literature on the placental kisspeptin/KISS1R system in preeclampsia (PE) demonstrates differential expression compared to that of normal placertas but does little to substantiate one particular mechanism of action. Expression of ligand mRNA and protein was decreased in placentas collected from mothers with PE, as compared to term placentas in one study (Cartwright & Williams, 2012), but was increased in others (Qiao et al., 2005; Zhang et al., 2011). Most studies of kisspeptin and PE are limited by the lack of gestational age-matched controls for the PE placentas that were collected before term. Qiao et al. eventually overcame that challenge by finding premature, otherwise healthy age-matched controls for preterm deliveries and eventually confirmed an increase in KISS1 and its receptor in “early-onset preeclampsia” (2012). As determined through ultrasound evaluation of uterine arteries and measurement of angiogenic factors, preeclampsia established before 34 weeks of gestation is more likely to be due to a defect in trophoblast invasion than late-onset disease after 34 weeks, which appears to arise from preexisting vascular defects and genetic predisposition in the mother (Crispi et al., 2008; Oudejans & van Dijk, 2008). Decreased kisspeptin during PE versus increased kisspeptin in PE support two potential and distinct roles for the ligand. A decrease in peptide expression would contradict the idea that kisspeptin serves to inhibit placental invasion given that PE is a disease characterized by incomplete invasion of the spiral
arteries. However, Smets et al. (2008) postulated that the amount of kisspeptin present could be a function of that placenta’s inherent invasiveness and not necessarily a cause of the disruption, but rather a result of it. Still, the distinction between early- and late-onset preeclampsia may have been a confounding factor in the other studies, supporting the theory of the inhibitory tone of the kisspeptin system.

To complete the story about kisspeptin’s mechanism of action and potential relationship to the etiology of preeclampsia, a clearer picture of the system’s location in the placenta is required. The data for KISS1 localization suggest that the ligand is restricted to the STB compartment, but the data on KISS1R localization is more controversial. Many studies rely on immunohistochemistry to identify KISS1R, utilizing an antibody of questionable specificity against this G-protein-coupled receptor. A report from Bilban et al. that utilized KISS1R antibody showed ubiquitous KISS1R protein expression throughout the placental compartments (2004). Much of the subsequent literature has failed to address the confounding nature of the cross-reactive KISS1R antibody that was used, limiting a thorough discussion of kisspeptin’s therapeutic potential.

Our research aims to establish the precise localization of kisspeptin and KISS1R throughout the layers of the placenta. This would inform us about the direction of communication between the trophoblast layers and could shed light on the critical period of placental development when this interaction can lead to disease.
Objectives

Given the prevalence of maternal and fetal pathologies due to defective placental invasion, we aim to investigate the role of kisspeptin in regulating the migration of trophoblasts and their invasion of maternal tissues throughout pregnancy. Studies establishing levels of kisspeptin and its receptor are inconsistent in normal and diseased placenta, and localization of KISS1R has been limited by the use of an unreliable immunohistochemical antibody. The goal of our work is to address those inconsistencies and to apply that information toward elucidating kisspeptin’s mechanism of action on placenta invasion.

- We will analyze placental fractions for expression levels of KISS1 and KISS1R mRNA by real-time PCR as well as kisspeptin protein by immunohistochemistry in order to precisely localize them in the STB and CTB compartments.
- We aim to determine the mechanism by which kisspeptin modulates CTB invasiveness. Based on existing data, we hypothesize that kisspeptin inhibits trophoblast invasion through upregulation of the ERK1/2 pathway.

With this study, we aim to better understand kisspeptin’s role in placental invasion and how this could be manipulated clinically. Given that the only current treatment for preeclampsia is delivery of the fetus regardless of its stage of development, an improved comprehension of this pathway has valuable therapeutic potential.
METHODS

IRB-approved discarded human tissue collection

De-identified discarded first and second trimester human placental tissue was collected from pregnancy terminations at the Gynecological Procedure Unit at Boston Medical Center under IRB approved protocol H-26575.

Immunohistochemistry

Kisspeptin/HPL/HAI-1 staining

Placental tissue was flash frozen in liquid nitrogen after a 4 hour fixation in 4% paraformaldehyde and cryoprotection in 30% sucrose. 5 μm sections were incubated overnight at 4°C with antibodies to kisspeptin, hepatocyte growth factor activator inhibitor type I (HAI-1), and/or human placental lactogen (HPL) (ABCAM cat# AB11396). Following several washes, sections were incubated in fluorescently-labeled secondary antibodies for 1 hour at room temperature and counterstained with DAPI.

HPL/TLR3 staining

Placental tissue was cryoprotected using a 5-15% sucrose gradient (5% intervals, with 30 minutes at each step). Next, the tissue was mounted in rectangular plastic molds filled with Optimal Temperature Cutting (OCT) Compound (Tissue-Tek®). The mold was then frozen on a level block of dry ice, followed by storage at -80°C. Tissue blocks were removed from the molds and sectioned at 6 μm using a Tissue-Tek® Cryostat at -20°C.
The sections were mounted on Fisherbrand Superfrost Plus slides and allowed to dry overnight at room temperature. Slides were fixed for 15 minutes in 10% formaldehyde (Polysciences, Inc.). The slides were washed 5 times for 5 minutes each in TBST (1X TBS + 0.25% Triton), blocked for 30 minutes in 10% Horse Serum (Vector) and 4% Donkey Serum (Jackson Immunoresearch Laboratories) in TBST, and incubated at 4°C for 48 hours in primary antibodies: mouse-anti-HPL (ABCAM cat# AB11396) diluted 1:1000 and rabbit anti-TLR3 (Santa Cruz cat #sc-10740) diluted 1:50 in blocking solution. Slides were washed 3 times for 5 minutes each in TBST, and then incubated for one hour at room temperature in secondary antibodies from Invitrogen: CY3-anti rabbit and AlexaFluor 488-anti mouse, diluted 1:1000 in TBST. Slides were washed 3 times for 5 min each in TBST, dried of excess liquid, and coverslipped using Vectashield with DAPI (Vector Laboratories). Images were captured using an Olympus BH2 Microscope and an attached digital camera.

**RNA Extraction**

*From whole tissue*

At collection, tissue was minced with sterile scissors and stored in RNALater (Life Technologies) at 4°C for up to two weeks. For extraction, roughly 30 mg of tissue was transferred to a 2 mL sample tube (Qiagen cat #990381). 1.2 mL of Trizol (Life Technologies) and a sterile 5mm stainless steel bead (Qiagen cat # 69989) were added to each sample. Tissue was disrupted and homogenized using the TissueLyser LT (Qiagen) and the 12-tube adapter for 8 minutes at a speed of 40 Hz. The samples stood at room
temperature for 5 minutes before a 10 minute spin at 12,000 x g to pellet cell debris. The supernatant was transferred to a new 1.5 mL tube, and 250 μL of chloroform was added to each sample in a fume hood. After vortexing vigorously for 30 seconds, the samples stood at room temperature for 5 minutes before centrifugation at 10,000 x g for 10 minutes. The top aqueous layer was collected and transferred to another 1.5 mL tube. Next, the protocol for the RNeasy Mini Kit was followed to achieve RNA extraction via a spin column that binds RNA (pictured in Figure 5 below).

**Figure 5. RNeasy Mini Kit Protocol.** (QIAGEN RNeasy Mini Handbook, 2012, p. 9)
Briefly, 70% ethanol was added 1:1 to the sample (roughly 600 μL). The sample was spun to bind RNA to the column, followed by washes to clear unwanted contaminants. The membrane of the column was treated with RNase-free DNase I (Qiagen cat #79254) to degrade DNA contaminants, washed, and eluted in a volume of 30 μL RNase-free water. To improve RNA yield, the spin column was eluted for a second time with the same 30 μL eluent. RNA was quantified using Gen5 spectrophotometric nucleic acid quantification software on the Biotek Synergy HT Microplate Reader.

**From fractionated cells**

Pellet was disrupted and homogenized in a volume of Buffer RLT (Qiagen) specified in Table 1. The protocol above is resumed by adding ethanol and carrying out steps as described (pictured on the left column of Figure 5).

**Table 1. Qiagen handbook designations for lysis buffer volume based on number of cells.**

<table>
<thead>
<tr>
<th>Number of pelleted cells</th>
<th>Volume of Buffer RLT (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5×10^6</td>
<td>350</td>
</tr>
<tr>
<td>5×10^6 – 1×10^7</td>
<td>600</td>
</tr>
</tbody>
</table>

**Reverse Transcription**

To obtain stable DNA that is complementary to the previously extracted RNA, we used the SuperScript® III First-Strand Synthesis System (Life Technologies). The kit contains all the necessary reagents and buffers for optimum use of the provided reverse transcriptase enzyme. Oligo(dT)_{20} was used (see left pathway of Figure 6) as the primer for synthesis of cDNA. Negative controls, containing deionized water in the place of
reverse transcriptase, were included in the experiment. CDNA products were stored at -80°C before use in quantitative PCR.

Figure 6. SuperScript III Reverse Transcription Protocol. Diagram taken from page 1 of user manual (Life Technologies cat # 18080-051) ("SuperScript®III First-Strand Synthesis System for RT-PCR," 2013)

**Quantitative Polymerase Chain Reaction (qPCR)**

All reagents were thawed on ice to prevent degradation of samples and polymerases. 2 μL of cDNA (product of the reverse transcription) were used per reaction with TaqMan® Gene Expression Assays. Each assay contained primers that flanked either KISS1 (Genbank Accession #NM_002256.3), KISS1R (Genbank Accession #NM_032551.4), HPL (Genbank Accession # J00118.1), or GAPDH (Genbank Accession # JN613429.1), as a housekeeping gene for normalization. Reactions were performed in duplicate and mixed as shown in Table 2. Negative controls for each specific Gene Expression Assay were also included by using water in the place of cDNA in one well.
Table 2. PCR reaction mixture using Taqman primers.

<table>
<thead>
<tr>
<th>PCR reaction mix component</th>
<th>Volume per 20-uL reaction (µL)</th>
<th>Single reaction</th>
<th>Two replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X TaqMan® Gene Expression Assay</td>
<td>1.0</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>cDNA template + RNase-free water</td>
<td>9.0</td>
<td>18.9</td>
<td></td>
</tr>
<tr>
<td>2X TaqMan® Gene Expression Master Mix</td>
<td>10.0</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Total Volume</td>
<td>20.0</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

96 well-plates containing the reagents were sealed and placed in an Applied Biosystems 7500 Real-Time PCR System with the thermocycling program outlined in Table 3.

Table 3. Thermocycling program used.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>50</td>
<td>2 min</td>
</tr>
<tr>
<td>Hold</td>
<td>95</td>
<td>10 min</td>
</tr>
<tr>
<td>Cycle (40 Cycles)</td>
<td>95</td>
<td>15 sec</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1 min</td>
</tr>
</tbody>
</table>

Ct values were exported to Microsoft Excel for analysis. Ct values were then normalized to GAPDH expression and averaged between duplicates.

*Placental Fractionation*

Placentas collected for fractionation were immediately rinsed in sterile Phosphate Buffered Saline (PBS) containing antibiotics, and all visible blood clots were removed. Tissue was kept on ice in sterile media also containing antibiotics, while branched terminal villi were dissected free into solution using sterile tweezers and scissors over a
light box. Placental villi were pooled and spun down in a centrifuge (8 min at 1300 rpm). The supernatant was discarded, and tissue was weighed to determine the appropriate amount of enzyme to use for digestion (pictured in second column of Table 4). Solutions of collagenase and trypsin were made to accommodate multiple rounds of digestion in a 37°C shaking water bath. At each step, a drop of supernatant was examined under a microscope to identify the majority cell type that had come off of the villi and into solution.

<table>
<thead>
<tr>
<th>Enzyme Step</th>
<th>Amount (mL)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase 1 (C1)</td>
<td>Mass * 6</td>
<td>6 min</td>
</tr>
<tr>
<td>Trypsin 1 (T1)</td>
<td>Mass * 6</td>
<td>6 min</td>
</tr>
<tr>
<td>Trypsin 2 (T2)</td>
<td>Mass * 5</td>
<td>4.5 min</td>
</tr>
<tr>
<td>Trypsin 3 (T3), if needed</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Collagenase 2 (C2)</td>
<td>10 (&lt;20 grams)</td>
<td>2.5 min</td>
</tr>
<tr>
<td></td>
<td>20 (&gt;20 grams)</td>
<td></td>
</tr>
</tbody>
</table>

C1 was drawn out until some CTBs and some STBs were visually present under the microscope. T1 supernatant featured more CTBs than STBs, and T2 contained mainly CTBs and not fibroblasts, a connective tissue component that measures a similar density as CTBs and thus cannot be separated through density centrifugation. T3 was only collected if more CTBs could be salvaged without including fibroblast contamination. Supernatant with the appropriate composition was collected, quenched of enzymatic activity with 5 mL fetal bovine serum, and passed through a sterile gauze filter. The supernatants were spun (8 min at 1300 rpm, 4°C) and the pellet was digested with C2 to
separate clumps. Once again, cells were spun and the pellet was resuspended in serum free media to undergo Percoll gradient centrifugation. The cells in serum free media were slowly layered on top of the gradient without disturbing the strata and centrifuged for 25 min at 2700 x g, 4°C. CTB and STB layers were collected separately and washed in sterile media. Removal of CD45-positive leukocytes and CD9-positive fibroblasts is carried out by immunomagnetic separation with the EasySep Biotin Positive Selection Kit (StemCell). A portion of purified cells is subjected to staining with cytokeratin 7 antibody to assess final purity. Finally, cells were counted and kept on ice for use the next day for qPCR, Western blotting, or migration assays, or frozen in Recovery Cell Culture Freezing Medium (Life Technologies).

**Culture of JEG-3 Cells**

The choriocarcinoma JEG-3 immortalized cell line (a generous gift from Dr. Danny Schust) was cultured in a Sanyo CO₂ Incubator at 37°C in RPMI 1640 media (Fisher Scientific) with added 10% Fetal Bovine Serum (Life Science) and 1% Penicillin/Streptomycin (Life Technologies, 100X). Cells were passed when 70-90% confluent and grown in cell culture dishes (60 mm, BD Falcon).

**Laser-Capture Microdissection (LCM)**

At collection, placental tissue was rinsed of blood, immediately embedded in OCT, and frozen on dry ice. Tissue blocks were sectioned at 10 µm using a cryostat, and sections were mounted onto RNase-free plain glass slides. Slides were stained using an
abbreviated hematoxylin and eosin procedure adapted from an Arcturus protocol designed for LCM. The slides were dipped sequentially into autoclaved Coplin jars containing staining solution, washing solution, and increasing concentrations of ethanol (ETOH) for dehydration. All solutions were RNase-free. The full procedure is outlined in Table 5.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ETOH</td>
<td>30 seconds</td>
</tr>
<tr>
<td>dH₂O</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Hematoxylin</td>
<td>30 seconds</td>
</tr>
<tr>
<td>dH₂O</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Bluing reagent (dH₂O)</td>
<td>30 seconds</td>
</tr>
<tr>
<td>70% ETOH</td>
<td>30 seconds</td>
</tr>
<tr>
<td>95% ETOH</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Eosin</td>
<td>15 seconds</td>
</tr>
<tr>
<td>70% ETOH</td>
<td>30 seconds</td>
</tr>
<tr>
<td>95% ETOH</td>
<td>30 seconds</td>
</tr>
<tr>
<td>100% ETOH (fresh each time)</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Xylenes</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Air dry in a fume hood</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

After drying, slides were examined under a light microscope to assess tissue morphology and staining. They were stored with desiccant at -80°C until use for laser capture. Laser capture microdissection was first performed using an Arcturus Pixcell Ile at the Boston University School of Medicine Laser Capture Microdissection Core Facility, a microscope that employs an infrared laser beam directed by the user with a moveable stage and joystick. The laser contacts the special polymer surface of the CapSure LCM HS Cap (not the tissue) and to make the designated area adhesive. When
the cap is lowered onto the tissue, cells that contact the adhesive are pulled from their surroundings. At the end of the session, the caps (with all of the harvested cells attached) are washed in lysis buffer and processed for RNA extraction.

The LCM protocol was later modified as follows: the placental tissue was collected into sterile 5% sucrose as the first of a three-step dehydration gradient for protection of morphology during freezing (5% for 30 minutes, 10% for 30 minutes, 15% for 30 minutes). The dehydrated tissue was then embedded in OCT and flash frozen in liquid nitrogen. 10 μm sections were cut using a cryostat, and sections were mounted on polyethylene naphthalate (PEN) membrane glass slides (Arcturus) compatible with the UV-powered Leica laser capture system (Model AS LMD; Leica, Wetzlar, Germany) at the Schepens Eye Institute. Slides were stained using the protocol previously described (Guller et al., 2008). Briefly, slides were dipped sequentially in for 15 seconds each in 95% ETOH, 70% ETOH, and deionized water, followed by staining with Mayer’s Hematoxylin (Sigma) for 30 seconds. Slides were rinsed twice with deionized water for 15 seconds each, washed with 70% ETOH for 15 seconds, and counterstained with Eosin Y (Sigma) for 5 seconds or less. Finally, slides were dehydrated by placement into 95% ETOH twice for 15 seconds each, twice in 100% ETOH for 15 seconds each, and Xylenes for 60 seconds. The slides are allowed to air dry for two minutes before storage at -80°C in the presence of a desiccant, until they are thawed slowly (15 minutes at -20°C, 15 minutes at 4°C) for use on the LCM microscope at room temperature. The Leica LCM system employs a UV laser that contacts the tissue directly and detaches it from the slide by severing the PEN membrane from the glass. Microdissected cells were captured
into 1.5 mL microcentrifuge tube caps containing RLT buffer from the Qiagen RNeasy Micro Kit.

The final protocol modification for LCM involved collection of placental tissue directly into RNAlater. The tissue was placed in 15% sterile sucrose for 30 mins as an abbreviated cryoprotection method. Next, tissue was embedded in OCT and flash frozen using an ethanol bath in liquid nitrogen (-95°C) for the most rapid freezing to minimize ice crystal formation. Tissue was sectioned and stained using the Guller method described above, and RNA was extracted at multiple steps along the way to confirm presence of adequate levels of RNA. RNA was extracted using the RNeasy Micro Kit. Nucleic acid quantification was performed using a Biotek Synergy HT microplate reader and Gen5 spectrophotometry software.

**Invasion Assay**

250,000 cells were seeded on Matrigel (diluted 1:8) coated transwell membranes and allowed to invade towards decidua-conditioned media for 12 hours. Invasive trophoblast cells on the bottom side of the membrane were fixed in 4% paraformaldehyde and identified by cytokeratin-7 staining. The images were processed and counted by an automated thresholding algorithm.
Western Blot for p-ERK and ERK

10^6 cells (either JEG-3 or fractionated trophoblasts) were plated in each well of a 6-well plate, incubated overnight, and serum starved for 3 hours before kisspeptin treatment. Cells were treated in duplicate with vehicle, kisspeptin at 10^{-7} M for 5 minutes, or phorbol 12-myristate 13-acetate (PMA, a direct activator of protein kinase C) at 10^{-7} M for 20 min, all at 37°C. The plate was then placed on ice to end the reaction and washed twice with cold PBS before lysis with the following freshly prepared lysis solution:

RIPA buffer, 1X \hspace{1cm} 1 \text{mL}
PMSF \hspace{1cm} 5 \mu\text{L}
Sodium orthovanadate \hspace{1cm} 5 \mu\text{L}
Protease inhibitor cocktail \hspace{1cm} 10 \mu\text{L}

Cells were treated with 350 \mu\text{L} RIPA lysis solution per well for 30 min shaking at 4°C. Lysates were then collected into Eppendorf tubes and centrifuged at 12,000 rpm for 10 min at 4°C to pellet cell debris. Supernatants were transferred to fresh tubes and kept on ice during protein quantification. The Pierce BCA Protein Assay was used to measure protein concentrations with a microplate reader after reaction with a colorimetric substrate, alongside a standard curve of bovine serum albumin (BSA). Standards were made by performing six serial 1:2 dilutions of the BSA solution included in the kit (with a starting concentration of 2 mg/mL) with deionized water. 25 \mu\text{L} of each standard was arranged sequentially in the wells of a clear flat-bottom 96-well plate. 25 \mu\text{L} of each protein lysate was also added to the plate, plus one well of RIPA lysis solution as a blank.
Next, 4 ml of BCA Solution A was mixed with 80 uL of BCA Solution B, and 200 μL of the mixture was added to each sample, standard, and blank in the plate. The plate was covered with Parafilm ® M and incubated for 30 minutes at 37°C. Absorptions were recorded after excitation with 589 nm light.

Samples were diluted 3:1 using 4X Laemmli Sample Buffer (BIO-RAD) for SDS-PAGE and 10:1 with β-mercaptoethanol in a final volume of 30 μL, and then boiled at 95°C for 5 minutes to denature the proteins. Samples and a protein ladder were loaded onto a 4–20% Mini-PROTEAN® TGX™ Gel in a BIO-RAD electrophoresis chamber filled with 1X Running Buffer (Tris/Glycine/SDS). The gel was run for 1 hour at 100V in a stirred ice bath. Proteins were then transferred to a nitrocellulose membrane using the BIO-RAD transfer chamber attachments, run for 1.5 hours at 70V in a stirred ice bath.

The membrane was washed with 1X TBST, blocked for one hour in 4% nonfat dry milk, and incubated with mouse anti-p-ERK antibody (Santa Cruz, sc-7383) at 1:10,000 in TBST + 4% milk overnight at 4°C. The membrane was washed for 30 min and incubated with a horseradish peroxidase-anti mouse antibody (Santa Cruz) at 1:1000 for one hour at room temperature. The Amersham ECL Western Blotting Detection Reagent kit was used for chemiluminescent detection of the protein bands, and the image was exposed onto film. The membrane was then washed with Restore Western Blot Stripping Buffer and reprobed with goat anti-ERK antibody to serve as a loading control followed by HRP-anti goat secondary antibody and the same development process. Band intensity was compared after quantification with ImageJ.
Statistical analysis

Analysis of the qPCR and Western blotting results was performed when the number of values was equal to or greater than three. All statistical analyses were performed with ANOVA using Instat 3.0 (GraphPad Software) and using a two-tailed t-test (P < 0.05).
RESULTS

Preliminary Data

*Immunofluorescence of placental villi confirm KISS1 localization in the STB compartment*

As a proof of concept, we aimed to confirm the localization of kisspeptin protein in the syncytiotrophoblast compartment, as previously reported by Bilban et al. (2004). Utilizing an antibody against human placental lactogen (HPL) as a positive control for STB staining and an antibody against hepatocyte growth factor activator inhibitor-1 (HAI-1) as a positive control for the villous CTB compartment, Upadhyay et al. (2012) were able to show overlap between kisspeptin and HPL staining in the STB layer in both first and second trimester placentas. Figure 7 features a pattern representative across both trimesters. HAI-1 staining did not overlap with kisspeptin, signifying that kisspeptin expression was limited to the STB and not the CTB layer. These results are in line with the current literature, suggesting that the STBs may be responsible for the secretion of the ligand.

Subsequent staining experiments attempting to utilize the HAI-1 antibody revealed that it was not a reliable marker for the CTB compartment and sometimes stains STBs. As a result, it was necessary to seek out an alternative marker for CTBs (see Current Findings).
Figure 7. Kisspeptin protein expression overlaps with a staining for an STB-specific protein, HPL. A,D: HPL stains the STB compartment. B: HAI-1, an identifier of the CTB compartment stains differently than kisspeptin (E). C,F: While HPL and HAI-1 do not overlap, HPL and kisspeptin do, indicating that kisspeptin is expressed primarily in the STB compartment. Taken from Upadhyay et al. (2012).

*KISS1 mRNA expression predominantly found in STBs, while KISS1R only in CTBs*

After fractionating each placenta and isolating the STB and CTB cell fractions, RNA was extracted from those fractions and subjected to real-time quantitative PCR (RT-qPCR) analysis with primers for *KISS1*, *KISS1R*, *HPL*, and *GAPDH* (a housekeeping gene), as shown in Figure 8. HPL was used as a positive identifier of the STBs. Data were pooled from 11-17 week gestational age placentas and normalized to GAPDH expression, resulting in 25.2±7.7 fold higher *KISS1* mRNA levels in STBs than CTBs (p<0.05) and 23.1±6.9 fold higher *KISS1R* mRNA levels in CTBs than STBs (p<0.05) (Upadhyay et al., 2012). These results support the immunofluorescence data, placing the KISS1 transcript in the same compartment as the kisspeptin protein.
Figure 8. KISS1R was found to be predominantly expressed in the purified cytotrophoblast population, while KISS1 expression was primarily found in the syncytiotrophoblast. Via qPCR of fractionated trophoblasts, KISS1R mRNA levels were 23.1±6.9 fold higher in CTB relative to STB from the same placenta. In contrast, KISS1 mRNA levels were 25.2±7.7 fold higher in STB. Human placental lactogen (HPL) was used to identify STBs and mRNA levels were normalized to GAPDH. n=5; *p<0.05
Taken from Upadhyay et al. (2012).

Kisspeptin-10 inhibits trophoblast invasion

To address our second aim, Upadhyay et al. (2012) performed invasion assays to determine the effects of kisspeptin treatment on the migratory behavior of primary trophoblasts (see Figure 9). Cells treated with 100 pM of kisspeptin-10 had a significantly lower invasion index than the control (p<0.05). Inhibition of migration increased in a dose-dependent manner, as summarized in Figure 9, as 100 nM of kisspeptin-10 significantly decreased invasion yet more than 100 pM kisspeptin-10 (p<0.01).
Figure 9. Treatment with kisspeptin-10 inhibits cytotrophoblast invasion in a dose-dependent manner. Invading CTBs were stained for cytokeratin-7 (green) and counterstained with DAPI (blue). Representative images are shown for transwell membranes with no treatment (A), 1 pM (B), 100 pM (C), and 100 nM kp-10 (D). Treatment with 100 pM and 100 nM kp-10 reduced invasion to 64±8% and 48±10% of untreated controls, respectively (E). Treatment with a lower dose of 1 pM kp-10 yielded no significant effect. n=5; *p<0.05; **p<0.01. Taken from Upadhyay et al. (2012).

Kisspeptin/KISS1R pathway still under investigation

Fractionated trophoblasts were treated for five minutes with kisspeptin-10, lysed, and probed for activation of ERK to phosphorylated ERK (p-ERK) (Figure 10). PMA was used as a positive control and increased band intensity by an average fold change of 2.07. Kisspeptin, on the other hand, did not produce a significant change in p-ERK protein. Additional time points and doses of kisspeptin will be assayed.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>1</td>
</tr>
<tr>
<td>Kisspeptin</td>
<td>0.97</td>
</tr>
<tr>
<td>PMA</td>
<td>2.07</td>
</tr>
</tbody>
</table>

**Figure 10. Western blot shows no increase in ERK activation after treatment with kp-10.** Left: p-ERK Western blot of lysates from primary trophoblasts shows an increase in band density (measured by ImageJ) for PMA treatment at 200 uM (positive control) but none for Kisspeptin-10 at 100 uM. Kiss = 5 minutes, PMA = 20 minutes.

**Current Findings for this Thesis**

**TLR3 as a marker for CTBs**

Immunofluorescence staining with TLR3, an immunological marker, has introduced a new possibility for identifying the CTB layer, as TLR3 appears to stain this layer more consistently than HAI-1. Figure 11, panel A shows TLR3 concentrated to the inner villous cytotrophoblast layer, while in panel B HPL staining identifies the outer syncytiotrophoblast layer. The combination filter on the epifluorescent microscope in panel C shows specific staining for the two layers that is shown more clearly in panel D, composed of merged images from panels A and B.
Figure 11 TLR3 Staining of the placenta. A) TLR3 selectively stains the CTB layer. B) HPL consistently stains the STB layer. C) Combination filter image shows that the two layers are distinctly stained, and D) Merged images A and B show no overlap between TLR3 and HPL staining.

Location of KISS1R by in situ hybridization

Given that the localization of KISS1R in trophoblast layers is controversial due to the lack of KISS1R antibody specificity, we wished to support our finding that KISS1R is restricted to the CTB compartment by confirming with another method of mRNA measurement. We had some difficulty finding a reliable positive control for the CTB layer in the qPCR experiments (HPL was reliable for the STB fractions). Thus, we desired secondary confirmation of our results through another experimental modality and
considered in situ hybridization for \textit{KISS1R}. In order to determine the feasibility of in situ hybridization to localize \textit{KISS1R} mRNA visually to the inner trophoblast layer, we first needed to determine the bulk levels of \textit{KISS1R} expression in the placenta. \textit{KISS1} levels were challenging to detect by in situ hybridization by our collaborators due to high background signal (data not shown), and thus levels of \textit{KISS1R} would need to be comparable to those of \textit{KISS1} for minimal visualization of the transcript above background noise. QPCR of unfractionated 8 week and 16 week placental samples normalized to GAPDH expression (Figure 12) showed that, when compared to the 8 week expression of \textit{KISS1R}, 8 week \textit{KISS1} mRNA expression is 800 times higher than \textit{KISS1R} and 16 week expression of \textit{KISS1} is even greater at roughly 1400 times higher expression. On the other hand, 16 week \textit{KISS1R} is even lower than it is at 8 weeks. This is in agreement with literature that suggests increasing levels of kisspeptin throughout gestation and decreasing levels of the receptor. The vast difference in mRNA expression of receptor and ligand suggests that reliable detection of the receptor by in situ hybridization may be difficult.
Figure 12. *KISS1* and *KISS1R* expression in unfractionated first and second trimester placentas. qPCR was performed in duplicate, but n=1 for each gestational age. HPL was used as a positive control.

*Laser capture microdissection: tissue morphology and RNA quantity*

Tissue preparation for LCM required extensive optimization to achieve suitable tissue morphology for trophoblast identification and RNA quantity sufficient for eventual qPCR analysis. With each attempt, we adjusted our methods of collection, freezing, staining, drying, and laser capture to improve cell morphology/identification and our final yield of RNA. Methods that improved cell morphology seemed to have detrimental effects on RNA preservation. As a result, optimization of this technique is ongoing. The following results represent our progress.

Our protocol was originally optimized for tissue morphology, and we determined that a UV-powered LCM system would be best to address our specific needs. We performed LCM to collect CTBs separately from STBs, and intended to use the enriched cell populations to perform qPCR analysis. After extracting RNA from the dissected samples, quantification revealed a negligible amount of RNA for both the CTBs and
STBs collected, summarized in Table 6. Further, the 260/280 absorbance ratios were much lower than the value of 2 that is considered a sign of RNA purity.

Table 6: RNA quantification after LCM of CTBs and STBs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>260/280</th>
<th>ng/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTB</td>
<td>0.5</td>
<td>-0.836</td>
</tr>
<tr>
<td>STB</td>
<td>1</td>
<td>-2.487</td>
</tr>
</tbody>
</table>

Given the lack of RNA yield, we next decided to sample the tissue at different steps in the preparation process to determine at which step(s) RNA loss occurred. The laser capture dissection process itself takes place for an extended period of time at room temperature, resulting in expected RNA degradation, so determining the yields before that step would indicate the maximum amount of RNA achievable under ideal conditions. We sampled tissue after being embedded and flash frozen in liquid nitrogen, and again after staining (with or without a one hour drying step). The results, summarized in Table 7, reflect the presence of RNA throughout the preparation process with a steep drop off between freezing and staining. However, a starting value of less than 40 ng/µL after freezing indicated a need for a closer investigation of RNA loss during the collection and freezing process. We needed to re-optimize the procedure, eliminating steps that could interfere with RNA integrity.
Table 7. RNA quantification of tissue sampled after A) embedding and flash freezing in liquid nitrogen, B) staining without a drying step, and C) staining with a drying time of one hour at room temperature.

<table>
<thead>
<tr>
<th>Sample</th>
<th>260/280</th>
<th>ng/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Frozen tissue block</td>
<td>1.679</td>
<td>39.298</td>
</tr>
<tr>
<td>B. Stained w/o drying</td>
<td>1.5</td>
<td>4.974</td>
</tr>
<tr>
<td>C. Stained, dried one hour (pictured in Figure 13A)</td>
<td>1.25</td>
<td>4.125</td>
</tr>
</tbody>
</table>

While snap freezing in liquid nitrogen can be the fastest method of freezing to avoid crystal formation (since it is the coldest at roughly -190°C), direct submersion in liquid nitrogen can cause bubbles to form around the tissue, cell rupture, and RNase release/activation as the nitrogen vaporizes. To avoid this issue, we switched to using an ethanol bath cooled to -95°C by liquid nitrogen. Some crystal formation in the placental tissue during freezing was unavoidable due to the large amount of water present in the tissue. Our best tissue morphology was achieved by putting the tissue through a three step 5-15% sucrose gradient for dehydration and cryoprotection, followed by an abbreviated staining procedure. However, to improve our yield of RNA, we tried collecting the tissue into RNAlater solution to protect the tissue from degradation by endogenous RNases. Unfortunately, tissue kept in RNAlater becomes dehydrated to the point that it is very difficult to cryosection. As a compromise, we employed an abbreviated sucrose gradient by taking the tissue from RNAlater and soaking it in sterile RNase-free 15% sucrose for 30 minutes. Again, we sampled tissue at various points throughout the preparation: directly from collection into RNA extraction solution (buffer RLT) to determine the absolute maximum yield, after RNAlater and 15% sucrose, after freezing in the ethanol
bath, after sectioning and mild fixation, and finally after staining. The results of this extraction, summarized in Table 8, indicate a vast improvement in the concentration of RNA after staining, by roughly a factor of 8 from the previous trial.

### Table 8. RNA quantification of tissue sampled
A) immediately at collection, B) after equilibration in RNAlater and 30 min in 15% sucrose, C) after embedding and freezing in an ethanol bath cooled with liquid nitrogen, D) after sectioning and acetone fixation, and E) after staining.

<table>
<thead>
<tr>
<th>Sample</th>
<th>260/280</th>
<th>ng/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Collected into RLT</td>
<td>1.926</td>
<td>391.304</td>
</tr>
<tr>
<td>B. Collected into RNAlater, 15% sucrose</td>
<td>1.967</td>
<td>147.906</td>
</tr>
<tr>
<td>C. Frozen tissue block (-95°C ETOH)</td>
<td>1.886</td>
<td>283.855</td>
</tr>
<tr>
<td>D. Cut + Fixed in acetone</td>
<td>2.091</td>
<td>18.631</td>
</tr>
<tr>
<td>E. Stained (comparable section shown in Figure 13B)</td>
<td>2.235</td>
<td>32.224</td>
</tr>
</tbody>
</table>

However, the improvement in RNA yield was accompanied by a loss of morphology. Figure 13A features a section of tissue from the previous RNA quantification trial, which underwent a full sucrose gradient. Figure 13B shows a representative section from a placental sample collected into RNAlater, processed the same way as the tissue from the second RNA quantification trial. The epithelium of the tissue with higher RNA yield is torn and crystallized, impossible for use in LCM.
Figure 13 Placental tissue processed and stained for LCM. A) Tissue was processed optimally to preserve morphology by undergoing a complete sucrose gradient and drying one hour at room temperature after staining. B) Tissue was processed to preserve RNA quality through collection into RNeAlater and use of a single 15% sucrose step to reestablish some natural morphology, without any drying at room temperature. The staining procedure was otherwise identical for both sections.
DISCUSSION

Confirming KISS1R localization

Preliminary qPCR data from fractionated samples suggests that KISS1R expression is restricted to villous cytotrophoblasts, contradicting the report from Bilban et al. in 2004 that observed receptor expression throughout the placental compartments using a nonspecific antibody against KISS1R. While this is a step toward establishing the separation of kisspeptin ligand and receptor in the placenta, our conclusion would be better supported if we could find a reliable and consistent marker that distinguishes CTBs. By verifying the purity of our enriched CTB sample after fractionation, there would be no question as to the localization of KISS1R in the villous cytotrophoblasts.

Our discovery of TLR3 expression by the CTB layer and not the STB layer in the placenta suggests that a marker may exist to solve this problem. However, the use of an immunological marker would allow no distinction between CTBs and any leukocytes that distributed throughout the tissue. In future work we plan to subject purified CTB and STB fractions to qPCR with KISS1, KISS1R, HPL, and TLR3 primers. TLR3 would be expected to serve as the reliable marker for the CTB fraction that was missing from our experiments previously. With secondary magnetic purification steps to remove contamination from leukocytes, we could avoid that confound and confirm the distribution that our current data suggests.


**Balancing specificity, morphology, and RNA quality for LCM**

After realizing that in situ hybridization was not a feasible alternative, we turned to laser capture microdissection to confirm the localization of KISS1R expression. Our goal for laser capture microdissection was to visually identify the placental cell layers with hematoxylin and eosin staining and then to separate out the villous CTB layer from the STB layer using a laser, ideally one equipped to dissect on the scale of a single cell layer. During our original attempt to microdissect tissue at the Boston University School of Medicine LCM Core Facility, it became clear that the early model LCM system there was too crude to separate one cell layer from another. In the Arcturus Pixcell IIe, the diameter of the laser beam exceeded the diameter of our layer of interest. Additionally, the use of a system with an infrared laser relied on adhesion of the tissue to an activated membrane on the LCM cap, without any contact between the laser and the tissue. While the force of adhesion to the cap is likely sufficient for pulling particular sections of tissue away from others (for example epithelia from parenchyma), separating two functionally-related and adjacent single-cell layers from one another requires much more precision that may be achieved through an ultraviolet laser LCM system. Based on our observations, it was nearly impossible to prevent connective tissue contamination using an IR laser.

The caveat to using a UV-powered system, however, is that direct contact between the cells and the UV light can cause a striking change in the gene expression profiles of those cells. In addition to the RNA degradation and gene expression shifts expected to result from the stress of a lengthy tissue preparation, preserving tissue
morphology while also maintaining RNA quality is particularly challenging in the placenta, given the high water content and high level of endogenous RNases. As a result, freezing artifact is difficult to prevent without proper fixation, which would damage the RNA. Additionally, the many RNases present in placental tissue contribute to an abbreviated timeline for RNA stability (Fajardy et al., 2009), shortened further by the RNase contamination from vaginal exposure during delivery/collection (Cindrova-Davies et al., 2007). Collecting the tissue directly into RNAlater after rinsing was one way to prevent tissue RNases from degrading the RNA (Fajardy et al., 2009), but the protective solution caused crystal formation that, in our experience, was very disruptive to tissue morphology. Thus, we have concluded that the extensive and delicate process required for LCM is not ideal to address our hypothesis. QPCR analysis of fractionated placenta (as described in Figure 8) has proven a far more reliable method of identifying distinct trophoblast layers and preserving the integrity of their RNA. This method avoids the need to optimize tissue morphology and will allow us to localize KISS1R expression levels more precisely, again stressing the importance of finding unique gene identifiers for each trophoblast layer in the placenta.

Model for kisspeptin activity in the developing placenta

Although we are still in the process of finding a way to confirm the localization of KISS1R, we can speculate that if our hypothesized localization of KISS1 to the syncytiotrophoblast and KISS1R to the villous cytotrophoblast holds true, it would support the idea of a paracrine mechanism with the syncytiotrophoblast directing KISS1R action in the villous cytotrophoblast, the invasive component of the placenta, with
production of *KISS1*. As shown in Figure 14, STBs release kisspeptin that diffuses to nearby CTBs that are differentiating into extravillous trophoblasts (EVT) that invade maternal tissue. Based on our invasion assay data, kisspeptin could be a regulator of EVT invasion, acting to inhibit invasive activity as gestation progresses. Further, kisspeptin has been previously shown to activate an ERK1/2 pathway, and although our Western blotting experiments have yet to confirm this connection, we intend to continue investigating this mechanism with experiments including different incubation times and kisspeptin doses.

**Figure 14 Proposed kisspeptin-KISS1R signaling loop in fetal villi.** Pink: KISS1 expressed in the syncytiotrophoblasts. Yellow: KISS1R expressed in villous cytotrophoblasts differentiating and invading the decidua as extravillous cytotrophoblasts. The black squares represent kisspeptin protein that is released from the syncytium, inhibiting the invasion of extravillous cytotrophoblasts.
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CURRICULUM VITAE

ZAHRAH MASHEEB
DOB: 1988
33 Harry Agganis Way, Box 9364 | Boston, MA 02215
201.686.5054
zmasheeb@bu.edu

EDUCATION

Boston University School of Medicine, Boston, MA
Master of Science in Medical Sciences, GPA: 4.0
Expected May 2015

The Rockefeller University, Center for Clinical and Translational Sci., New York, NY
Certificate in Clinical and Translational Science
May 2013
A two-semester course on the design and execution of human subjects research, the protection of health information, and the application of scientific techniques in translational research

Princeton University, Princeton, NJ
Bachelor of Arts in Chemistry, Minor in Neuroscience
June 2010

Academy for the Advancement of Science & Technology, Hackensack, NJ
High School Diploma
June 2006

LABORATORY EXPERIENCE

Department of Obstetrics and Gynecology, Boston University School of Medicine, Boston, MA
Graduate research assistant
March 2014-July 2015

McEwen Laboratory (Neuroimmunology & Inflammation Program), The Rockefeller University, New York, NY
Lab Manager
Research Assistant
July 2012-August 2013
Oct. 2010-July 2012
Designed and executed experiments to study phenotype, function, and migration of putative brain dendritic cells (bDCs) in the mouse during virally-induced encephalitis, in normal aging, and in models of neurodegenerative disease (Parkinson’s and Huntington’s)

E. Gould Laboratory, Dept. of Psychology, Princeton University, Princeton, NJ
Research Assistant
July 2009-June 2010
Completed a thesis exploring effects of rewarding experience on neurogenesis in the rat hippocampus
Fresco Laboratory, Dept. of Molecular Biology, **Princeton University**, Princeton, NJ
Research Assistant
Feb 2009- May 2009
Studied the use of 2-amino, 6-vinyl purines and nitrogen mustards as site-specific DNA mutagens acting through triplex-mediated cross-linking; employed column purification and PAGE to purify DNA constructs

**CLINICAL EXPERIENCE**

Memorial Hospital, **Memorial Sloan-Kettering Cancer Center**, New York, NY
Visiting Volunteer
April 2013-August 2013
Visited patients to encourage them to participate in sponsored activities and improve their mood

Anxiety Disorders Unit, **New York State Psychiatric Institute**, New York, NY
Student Volunteer
Summer 2008
Performed phone interviews for subject eligibility in a clinical trial on obsessive compulsive disorder

Institutional Review Board, **New York State Psychiatric Institute**, New York, NY
Intern
Summer 2008
Reviewed human subject medical and research records to evaluate compliance with psychiatric research protocols, IRB policies, and federal regulations; prepared reports of compliance review data to be presented to the IRB and department of psychiatry

Health Information Management, **Holy Name Hospital**, Teaneck, NJ
Chart Control Clerk
Summer and Winter Recesses 2006-2007
Audited medical records and ran an independent project to assess error rate in past record-keeping

**PUBLICATIONS AND POSTERS**

**Journal articles:**


Meeting abstracts: