Extracellular RNAs as potential biomarkers for placental dysfunction

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EXTRACELLULAR RNAs AS POTENTIAL BIOMARKERS FOR PLACENTAL DYSFUNCTION

by

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EXTRACELLULAR RNAs AS POTENTIAL BIOMARKERS FOR PLACENTAL DYSFUNCTION

TREVOR R. LEONARDO

ABSTRACT

Placental dysfunction affects approximately 1 in 10 pregnant women in both the developed and developing worlds. Most commonly, it is manifested as preeclampsia or fetal growth restriction. Over the past two decades, an increasing body of research into the developmental biology of the placenta has been amassed, which points to defects in the differentiation of the trophoblast cell lineage as a key player in the pathophysiology of placental dysfunction. A number of clinical parameters are known to be associated with an elevated risk of placental dysfunction. These include maternal risk factors (such as chronic hypertension, renal disease, and lupus), history of placental dysfunction in a prior pregnancy, abnormalities in the levels of certain proteins in the maternal blood that are commonly used to estimate the risk of fetal genetic defects, and abnormalities in uterine artery Doppler waveforms. These current methods have significant drawbacks, including low specificity and sensitivity, high cost, lack of widespread availability, and lack of validity early in pregnancy. In order to provide a more cost-effective and reliable method to detect an elevated risk for placental dysfunction early in pregnancy, we explored the potential for extracellular RNAs (exRNA) in the maternal serum to be used as biomarkers. In our study, we used next generation sequencing technologies to compare extracellular microRNA (miRNA) levels in serum samples of pregnant women of different gestational ages, nonpregnant women, and placental tissue samples. We
discovered that the large majority of microRNAs that were present at higher levels in pregnant serum samples than nonpregnant serum samples and were likely of placental origin. We also found that these pregnancy-specific miRNAs were enriched for miRNAs encoded on chromosomes (Chr) 14 and 19, with changes in the relative expression of these two groups of miRNAs throughout pregnancy. Moreover, the miRNA signatures of late gestational pregnant samples correlated more closely with placental tissue samples than those of early pregnant samples, which could be related to the increasing impact of a larger placenta on the maternal serum exRNA profile. Our results demonstrate the potential utility of next generation sequencing technologies in regards to differentiating between different conditions using clinical samples.
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<thead>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cds</td>
<td>Coding sequences</td>
</tr>
<tr>
<td>Chr</td>
<td>Chromosome</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EP</td>
<td>Early Pregnancy</td>
</tr>
<tr>
<td>exRNA</td>
<td>Extracellular ribonucleic acid</td>
</tr>
<tr>
<td>lincRNA</td>
<td>large intergenic non-coding ribonucleic acid</td>
</tr>
<tr>
<td>LP</td>
<td>Late Pregnancy</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>NP</td>
<td>Nonpregnant</td>
</tr>
<tr>
<td>piRNA</td>
<td>Piwi-interacting ribonucleic acid</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>snoRNA</td>
<td>Small nucleolar ribonucleic acid</td>
</tr>
<tr>
<td>snRNA</td>
<td>Small nuclear ribonucleic acid</td>
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</table>
INTRODUCTION

Every person on this planet has implanted their blastocyst-stage-self into the endometrial lining of their mother’s uterus, and a subset of the trophoblast cells in their placenta has invaded deep into the uterine wall in order to gain access to crucial nutrients, water, and oxygen to grow and develop. For most people, this process happens smoothly and development continues normally. However, when implantation and invasion of the uterus go awry, many problems arise that can be detrimental to the health of both the mother and the developing fetus. Placental dysfunction, also known as placental insufficiency, is a complication that occurs when the placenta cannot provide adequate support to exchange nutrients and waste products between the fetus and the mother (Benirschke, Kaufmann, & Baergen, 2006). Placental dysfunction can result in a constellation of pregnancy complications, including preeclampsia, intrauterine growth restriction, and placental abruption. While the precise mechanisms leading to placental dysfunction are not entirely clear, there are a few commonly accepted ideas regarding the general causes of this condition during development. Furthermore, there are specific risk factors that are used clinically to identify patients at elevated risk for developing placental dysfunction, as well as specific criteria for diagnosis of its various manifestations.

Clinical Aspect of Placental Dysfunction and Related Complications

In 2007, 16.7, 6.1, and 3.9 percent of infant deaths were caused by complications related to low birthweight, newborns affected by maternal complications of pregnancy,
and by abnormalities of the placenta, cord, and membranes, respectively (Xu, Kochanek, Murphy, & Tejada-Vera, 2010). Placental dysfunction is typically defined as an inability of the placenta to supply a sufficient amount of nutrients and oxygen to the fetus, making it unable to provide full support to the developing baby. This can occur due to mechanical problems with the placenta or umbilical cord, improper development of the placenta in early-mid pregnancy leading to small placental size, or other pregnancy complications. Many clinical studies have been conducted in an attempt to characterize the incidence and severity of placental dysfunction and related complications, which have led to the present tools used for diagnosis and management of these complications. Maternal risk factors for placental dysfunction include diabetes, hypertension, conditions that increase blood clotting risk, smoking, drug use, and certain medications (“A.D.A.M. Medical Encyclopedia,” 2014). There is also an association with an increased risk for placental dysfunction if the patient has a history of two or more miscarriages prior to the current pregnancy (Gunnarsdottir, Stephansson, Cnattingius, Åkerud, & Wikström, 2014). In a study observing neonates that were diagnosed with fetal growth restriction due to placental dysfunction, gestational age was the major determining factor in survival, where reaching a higher gestational age before birth significantly improved the rate of survival of the neonate (Baschat et al., 2007).

Intrauterine growth restriction is a term used to define a fetus with an estimated weight that is below the 3rd percentile when compared to all fetuses at the same gestational age and is typically the result of a problem during development of the fetus and/or the placenta (Table 1) (Lausman, McCarthy, & Walker, 2012). Intrauterine growth
restriction has been shown to increase the morbidity and mortality of premature neonates between gestational ages of 25-32 weeks, including an increased risk of necrotizing enterocolitis and need for prolonged respiratory support (Bernstein, Horbar, Badger, Ohlsson, & Golan, 2000; Garite, Clark, & Thorp, 2004). There has also been an association with an increasing risk of spontaneous preterm birth if the fetus has a low estimated weight for its gestational age (Lackman, Capewell, Richardson, daSilva, & Gagnon, 2001). Intrauterine growth restriction is thought to be more closely associated with placental dysfunction, compared to small for gestational age fetuses (between the 3rd and 10th percentiles for growth), which is more likely to be caused by factors such as the genetic contributions of the parents.

Table 1. Fetal Weight Classification. Fetuses are placed in specific categories based on their weight for their gestational age. This reference is commonly used in the clinic to help clinicians determine whether or not further action should be taken when considering potential complications due to a small or larger than normal fetus.

<table>
<thead>
<tr>
<th>Fetal Weight for Gestational Age</th>
<th>Term used to describe the Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 3rd percentile</td>
<td>Intrauterine Growth Restriction</td>
</tr>
<tr>
<td>&lt; 10th percentile</td>
<td>Small for Gestational Age</td>
</tr>
<tr>
<td>11-89th percentile</td>
<td>Normal</td>
</tr>
<tr>
<td>&gt;90th percentile</td>
<td>Large for Gestational Age</td>
</tr>
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</table>
Preeclampsia is a pregnancy complication clinically diagnosed by the presence of both pregnancy-specific hypertension (maternal blood pressure that is $\geq 140$ mm Hg systolic or $\geq 90$ mm Hg diastolic in a woman who was normotensive prior to pregnancy), and proteinuria ($\geq 0.3$ grams of protein in a 24 hour urine collection) (Sibai, 2003). It is possible that preeclampsia arises from two distinct origins: reduced placental perfusion due to a placental pathology, and preexisting maternal disorders including hypertension, renal disease, being overweight, and diabetes (Ness & Roberts, 1996). It is estimated that preeclampsia affects 6-8% of all pregnancies, and is a factor in many cases of decreased birth weight (most significantly for preterm births) when compared to normotensive pregnancies (Xiong, Demianczuk, Saunders, Wang, & Fraser, 2002). A population-based study done in Norway looking at preeclamptic pregnancies over the past 35 years showed a nearly 20% increase in induced births from 1991-2003, and while stillbirth rates have declined due to this, neonatal death rate after a preeclamptic pregnancy has not changed significantly (Basso et al., 2006). Taking these observations together, while risk of neonatal death post-delivery has not changed in preeclamptic pregnancies, inducing pregnancy in preeclamptic patients results in an overall increase in fetal survival.

The following is a brief introduction to common mechanical problems of the placenta and umbilical cord. Velamentous cord insertion occurs when the umbilical cord is inserted into the fetal membranes instead of the body of the placenta. This improper insertion leaves the vessels that provide nutrients to the fetus unprotected, making them vulnerable to compression and even rupturing (Benirschke et al., 2006). The compression of these vessels during development can cause intrauterine growth restriction, and can
lead to placental dysfunction and fetal morbidity if it is not carefully monitored throughout the pregnancy.

Another mechanical cause of placental dysfunction can occur when there is a placental infarction. Placental infarctions are thought to occur partly due to poor fetal blood flow, or due to the occlusion of maternal spiral arteries during pregnancy, both of which will cause a portion of the placental tissue to undergo ischemia induced necrosis over time (McDermott & Gillan, 1995). While minor placental infarctions are relatively common and tend not to have a negative effect on the fetus, larger, more significant placental infarctions can severely limit exchange between the fetus and the mother, causing placental dysfunction.

**Developmental Based Etiology of Placental Dysfunction**

Upon fertilization of a female ovum by a sperm, the newly created zygote undergoes multiple cellular divisions as it travels from the fallopian tubes towards the uterus. Approximately five to six days after fertilization occurs, the mass of cells is now organized into what is known as a blastocyst, and is ready to undergo implantation. Outer trophoblast cells lining the blastocyst differentiate into mitotically active cytotrophoblasts, which then differentiate into a separate group of trophoblast cells called syncytiotrophoblast cells. The syncytiotrophoblast layer is a group of cells that fuse together to form a syncytium, which is the primary cellular interface between the maternal and fetal circulations (Junqueira & Mescher, 2013). Syncytiotrophoblast cells are also responsible for the secretion of human chorionic gonadotropin, a hormone that is critical in maintaining the function of the corpus luteum in early pregnancy (Junqueira &
During placental invasion of the maternal endometrium and myometrium, column-like structures form, lined by syncytiotrophoblasts with cytotrophoblasts remaining inside (Chaddha, Viero, Huppertz, & Kingdom, 2004). These columns develop from the primary villi into the tertiary villi over the course of the first three weeks of gestation, enzymatically breaking down endometrial glands and forming lacunae within the syncytiotrophoblasts.

Cytotrophoblasts that invade into the endometrium are termed extravillous cytotrophoblasts. This group of cells are responsible for two main processes: colonization of the decidua, and remodeling the ends of the spiral arteries into a low pressure system with highly dilated vessels, creating and enabling a permanent source of maternal blood flow. A subset of extravillous trophoblast cells, called endovascular trophoblast cells, specialize in occluding the spiral arteries and inevitably prevent maternal blood from reaching the intervillous space (Kaufmann, 2003). It is currently thought that this action of the endovascular trophoblast cells early in the first trimester of pregnancy induces a hypoxic placental environment relative to the mother, which in turn promotes the angiogenesis of the tertiary placental villi (Rodesch, Simon, Donner, & Jauniaux, 1992). Once angiogenesis has taken place by the end of the first trimester, the endovascular trophoblasts no longer block blood flow from the spiral arteries and maternal blood via the newly established remodeling begins to oxygenate the placenta at around twelve weeks gestation (Burton, Jauniaux, & Watson, 1999). The coordination of these two cell types appears to dictate the level of angiogenesis of the placental villi and in turn the size of the placenta, which is critical for proper growth of the fetus.
A problem with any step in the process of trophoblast differentiation and development can potentially contribute to poor placental development in the first trimester of pregnancy, leading to intrauterine growth restriction, preeclampsia, or placental dysfunction. Specifically, any aberrations in the differentiation of, or interplay between the extravillous cytotrophoblasts and endovascular trophoblasts can potentially lead to placental dysfunction as well. While it appears that these two cell types are partially responsible for the genesis of complications that can lead to placental dysfunction as early as the first trimester, as of now specifics have not been elucidated.

There is histologic evidence supporting the premise that preeclampsia, intrauterine growth restriction, and placental abruption are manifestations of placental dysfunction. In preeclamptic patients, it has been observed that the extravillous trophoblasts do not invade past the endometrial-myometrial border as far as in control patients, and the endovascular trophoblasts are seen at a much lower density in the endometrium of preeclamptic patients when compared to control patients (Kadyrov, Schmitz, Black, Kaufmann, & Huppertz, 2003). A related study has also confirmed the first observation, reporting a lower percentage of trophoblast cells in the decidua of severely hypertensive patients when compared to normotensive patients, while also showing a lack of invasion of the myometrial spiral arteries, which are normally remodeled and occupied by endovascular trophoblast cells during early pregnancy (Naicker, Khedun, Moodley, & Pijnenborg, 2003). One explanation could be due to the fact that these trophoblast cells in preeclamptic patients do not express certain cell adhesion molecules seen in normal pregnancies during the invasion and remodeling of
the uterine spiral arteries, including integrin, cadherin, and Ig superfamily members (Zhou, Damsky, & Fisher, 1997). These are thought to be critical for both invasion and for the trophoblasts to mimic a vascular phenotype when remodeling the spiral arteries. More recent reports have correlated the impaired angiogenesis of placentas in preeclamptic patients with elevated serum levels of a soluble vascular endothelial growth factor receptor-1, which is thought to competitively inhibit binding of VEGF in the placenta and has been shown to inhibit cell migration and tube formation of placental villous explants in vitro (Ahmad, 2004).

**Extracellular RNAs as Tools for the Diagnosis of Placental Dysfunction**

The quest for finding biomarkers that have diagnostic and prognostic value is more significant than ever in today's technocentric world. Currently, many of the common biomarkers used for diagnostic testing in the clinic include the measurements of analytes such as metabolites or proteins in the blood and urine (Etheridge, Lee, Hood, Galas, & Wang, 2011). With the advent of new technologies, such as next generation sequencing and more sensitive molecular quantification assays, extracellular nucleic acids, including deoxyribonucleic acid (DNA) and different classes of ribonucleic acids (RNA), now present a new avenue for biomarker discovery. Nucleic acids were first detected in serum in 1952 (DeFilippo & Versari, 1952). It wasn't until 1997 that fetal DNA was first discovered and extracted from cell free extracts of maternal blood (Y. M. Lo et al., 1997). While fetal DNA has been shown to be potentially useful as a prenatal diagnostic tool for complications such as fetal aneuploidy, the fact that every cell in a given individual contains essentially the same complement of DNA means that the study
of circulating DNA lacks the ability to detect the current functional state of the placenta and the fetus at a given point in time (Fan, Blumenfeld, Chitkara, Hudgins, & Quake, 2008). However, since RNAs are more short-lived than DNA and the RNA content of cells can vary widely according to cell type and cell status, it is possible that an examination of the different types of RNAs present in the maternal circulation may yield a snapshot of the current interactions occurring between the mother and the fetus. Since the placenta is in constant contact with the maternal blood flow, it follows that shedding of placental debris and extracellular vesicles could provide a glimpse into the placental transcriptome. By studying the various RNAs in the blood of pregnant women at various points in time, different patterns in RNA levels could yield insight to the current state and potential complications of the placenta and therefore the fetus throughout pregnancy.

The first study demonstrating the presence of endogenous exRNAs in the blood was that of tumor-associated RNAs in cell-free plasma samples (K.-W. Lo et al., 1999). Within a year of this initial report, the field of maternal fetal medicine converged with the new field of exRNAs when fetal RNA was found in the plasma of pregnant women carrying male fetuses using probe designed to detect a male specific RNA (Poon, Leung, Lau, & Lo, 2000). This study not only provided evidence of exRNAs of fetal origin in the maternal circulation, but also opened the door to the idea of fetal signaling to the mother through these RNAs. In fact, placental messenger RNAs that code for both hormones and transcription factors have been found in maternal plasma, the former of which were found at varying levels depending on gestational age (Go et al., 2004; Ng et al., 2003). It is also interesting to note that a majority of the fetal RNAs normally detected in the maternal
plasma were shown to be cleared within two hours of delivery. Thus, without a placenta that is continuously releasing transcripts into the maternal circulation, the signaling between the placenta and the mother is rapidly lost due to RNA degradation in the blood. This rapid RNA turnover in the maternal blood presents a technical challenge to profiling of circulating RNAs in the maternal blood, and it is possible that there are many RNAs in the blood that have not been discovered yet. A good candidate biomarker displays a unique profile in cases compared to normal controls, and can be reliably measured in all subjects; it is possible that instability of circulating RNAs may prevent them from being good biomarkers.

In 2008, microRNAs were not only observed in human plasma, but were also shown to be stable over long periods of time at room temperature, as well as over multiple freeze thaw cycles in collected plasma (Mitchell et al., 2008). Their reported stability in plasma after collection and the development of sensitive and robust detection assays make them good biomarker candidates. microRNAs are short, approximately 22 nucleotide long non-coding RNAs that possess the ability to regulate gene expression at the post-transcriptional level by degrading or destabilizing messenger RNAs (Bartel, 2004). Upon post-transcriptional processing into their mature form, individual microRNAs are loaded into the RNA Induced Silencing Complex, where they can bind to and regulate a broad range of target mRNAs in the cytoplasm in a sequence-dependent fashion (Selbach et al., 2008). The target specificity of the miRNAs lies largely within nucleotides 2-8 on the 5’ end of the mature miRNA, which are responsible for the binding of miRNAs to their target mRNAs through Watson-Crick base pairing interactions.
(Lewis, Burge, & Bartel, 2005). These “seed” sequences have been shown to be shared among microRNA families, which are groups of miRNAs, sometimes encoded at distant sites in the genome, that are coordinately expressed across different tissue types (Laurent et al., 2008). In recent years, specific miRNA families have been shown to modulate important cell states in development, including the pluripotent stem cell state and the cell cycle (Leonardo, Schultheisz, Loring, & Laurent, 2012). microRNAs have been a topic of great interest in regard to biomarker discovery and are currently being tested for diagnostic potential in many different systems, including different cancer types and drug-induced liver injury, to name a few (Starkey Lewis et al., 2012; Wittmann & Jäck, 2010).

Currently, the methods used to detect elevated risk of placental dysfunction in pregnancies include evaluation of maternal risk factors, asking about history of placental dysfunction in a previous pregnancy, review of the results of certain analyte levels in the maternal blood, evaluation of fetal growth, and interrogation of uterine artery Doppler waveforms (“Placental Insufficiency,” 2014). These methods lack both sensitivity and specificity, can be costly, and are not available to many pregnant women. Finding an effective and inexpensive way to identify pregnancies at risk for placental dysfunction that can be applied early in pregnancy would improve the ability of the clinician to reassure low-risk patients and to monitor high-risk patients appropriately. Specifically, if a sensitive and specific serum biomarker for elevated risk for placental dysfunction is discovered, this test could become a routine test offered to all pregnant women. Since the study of extracellular miRNAs in maternal blood is in its infancy, it is not yet known whether placental miRNAs can be reliably detected in maternal blood, and whether the
miRNA profile changes across gestation. In this study, we sought to answer these fundamental questions.
Specific Aims

The objective of the present study is to look at the relationship between miRNA profiles in placental samples, nonpregnant women and pregnant women at different gestational ages. Specifically, we performed:

1. Collection of serum samples from nonpregnant women, and pregnant women in the first and third trimesters of pregnancy (termed “early” and “late” pregnancy samples, respectively). Placental tissue samples were from a bank of previously collected tissue.

2. Isolation of total RNA from the collected serum samples followed by quantification and quality control assays.

3. Next generation sequencing library construction using the NEBNext Small RNA Library prep kit followed by sequencing using the Illumina HiSeq 2000. Data was collected, processed, and normalized for further analysis.

4. Statistical analysis of the overall data and comparisons between the following groups were done: pregnant versus nonpregnant women; early pregnant versus late pregnant; and a multi-group comparison between nonpregnant, early pregnant and late pregnant groups.

These studies were aimed at identifying miRNAs unique to pregnant patients, and determining which of them were of likely placental origin. We also sought to discover whether there were miRNAs that were unique to early or late pregnancy. We hope these studies will shed light on the interactions between the placenta and the mother, and that
the techniques we develop will be used in future studies to find biomarkers unique to patients at elevated risk of placental dysfunction.
METHODS

Patient Samples

Serum samples were obtained from nonpregnant and pregnant women presenting for pregnancy care at UCSD clinics and hospitals. Approximately 10cc of whole blood was collected as a separate venipuncture or during a routine clinical blood draw into sterile red top vacutainer tubes (BD). The tubes were then inverted 5-10 times and then placed upright for 10 minutes to allow for clotting to occur. Samples were processed within two hours of the initial blood collection. Once clot formation occurred, tubes were centrifuged at 1200xg for 10 minutes and then the serum was pipetted in 500 uL aliquots into labeled 2.0 mL sterile conical screw cap tubes (Fisherbrand). Samples were then placed immediately into a secure -80° C freezer for storage.

RNA Isolation from Placental Tissue

Approximately 100mg of placental tissue was first homogenized in lysis buffer using a Mini-Beadbeater-24 (Biospec) for one minute. The homogenate was then collected into a new 1.5mL microfuge tube and further processed using the mirVana miRNA Isolation Kit (Ambion) according to manufacturer’s instructions and eluted in 50uL of nuclease free water. RNA was quantified using the Qubit RNA Broad Range Assay (Life Technoloigies), and quality was assessed using the RNA 6000 Nano Kit (Agilent).

RNA Isolation from Serum

Total RNA was isolated from 500uL serum samples from five nonpregnant, four first trimester and four third trimester females. First, serum samples were homogenized
by adding TRIzol LS Reagent (Ambion) at a 3:1 ratio of TRIzol to sample volume, followed by vortexing. Chloroform was then added in a ratio of 0.2mL per 0.75mL TRIzol and samples were mixed by vigorously shaking for 15 seconds. Samples were incubated at room temperature for five minutes and then centrifuged at 12,000xg for 15 minutes at 4°C. The resulting aqueous phase was collected and further processed using the column-based purification with the miRNeasy kit (Qiagen). Samples were eluted in 30uL of water and concentrated using a SpeedVac (Thermo Scientific) until there were approximately 10uL left. Two microliters of each eluted sample were used to quantify both the concentration total RNA using the Quant-iT RiboGreen RNA Reagent and Kit (Life Technologies) on the Infinite M200 Pro microplate reader (Tecan), and the sample quality using the RNA 6000 Pico Kit (Agilent) on the 2100 Bioanalyzer (Agilent).

**cDNA Library Preparation and Sequencing**

Complementary DNA (cDNA) libraries were prepared using the NEBNext Small RNA Kit according to manufacturer’s instructions (NEB). Briefly, Isolated RNA from serum (up to 6uL) or 50 nanograms of placental RNA was ligated to the 3’ adaptor for one hour. Following ligation, a reverse transcription primer was hybridized to bind to all excess 3’ adaptors in solution to prevent adaptor-dimer formation. RNA was then ligated to the 5’ adaptor for one hour followed by reverse transcription. Once the RNA was reverse transcribed into cDNA, a PCR amplification step was done for 15 cycles. Amplified cDNA was then purified by size selection using AMPure XP Beads according to manufacturer’s instructions and eluted in a final volume of 15uL of nuclease free water. Two microliters of each eluted sample were used to quantify both the
concentration of the cDNA libraries using the Quant-iT High Sensitivity DNA Kit (Life Technologies) on the Infinite M200 Pro microplate reader (Tecan), and the sample quality using the High Sensitivity DNA Kit (Agilent) on the 2100 Bioanalyzer (Agilent). Indexed cDNA libraries were then pooled to yield a final concentration of two nanomolar. Samples were submitted to the UCSD Genomics Core and run on the Illumina HiSeq 2000 using 100 base pair, paired end reads according to manufacturer’s instructions (Illumina).

Data Analyses

Demultiplexed fastq files were assessed for quality using FastQC (“FastQC,” 2014). The reads were first trimmed using the FASTX-Toolkit (“FASTX-Toolkit,” 2014) to 26 base pairs. The reads were additionally trimmed using Trim Galore (“Trim Galore,” 2014) to remove reads with bases whose quality scores were below 20 and read lengths below 18 base pairs. The reads were then collapsed into a read-count format, which lists the sequence followed by the number of times the sequence appeared in the trimmed fastq file, using a custom in-house perl script. The read-counts were then mapped to the reference genome assembly version hg19 (“UCSC Genome Bioinformatics,” 2014) using miRanalyzer with default parameters (Hackenberg, Rodriguez-Ezteleta, & Aransay, 2011). Analysis of the mapped reads was performed in Qlucore Omics Explorer 3.0 (“Qlucore,” 2014).
RESULTS

Sample Statistics

Next generation sequencing was conducted on serum samples from five nonpregnant, four first trimester pregnant, and four third trimester pregnant patients, along with four placental tissue samples for a total of 17 samples. An average of 2.6 million total reads per serum sample was recorded, with an average of 5.5 million total reads for the placental tissue samples. Approximately 9% of total reads were successfully mapped to known miRNAs in the serum samples, with 12% of total reads from placental samples mapped to miRNAs (Table 2). In a separate analysis, a subset of the sequenced data was used to analyze the distribution of mapped reads among all small RNA types (Figure 1). Interestingly, the percentage of miRNAs, long noncoding intergenic RNAs (lincRNA) and coding sequences (cds) remained the same between groups, with miRNA averaging close to 17% of the total mapped reads between all sample groups.
Figure 1: small RNA Profiles of Nonpregnant, Pregnant and Placental Samples. The mapped data for a subset of the sequenced samples was taken to analyze the distribution of RNAs within each group. Each pie graph represents 100% of the total mapped reads for that group, with the percentage of mapped reads to each RNA type labeled above.

Table 2. Sequencing Read Alignment. Table showing the total number of reads, the number of mapped reads to microRNAs, and percentage of total reads that aligned to miRNAs using the reference genome hg19 for each sample.

<table>
<thead>
<tr>
<th>SAMPLE NAME</th>
<th>Total Reads</th>
<th>Total Aligned Reads to miRNA</th>
<th>Number Aligned 1 Time</th>
<th>Number Aligned &gt;1 time</th>
<th>Percentage Aligned to miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Pregnant 1</td>
<td>816397</td>
<td>76758</td>
<td>60917</td>
<td>15841</td>
<td>0.09</td>
</tr>
<tr>
<td>Early Pregnant 2</td>
<td>1097392</td>
<td>85000</td>
<td>67571</td>
<td>17429</td>
<td>0.08</td>
</tr>
<tr>
<td>Early Pregnant 3</td>
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Early and Late Pregnancy Samples Differ in miRNA Profiles

In order to detect potential differences in miRNA expression profiles between different gestational ages, a two-tailed t-test with a level of significance of 5% was conducted between the early pregnancy (EP) and late pregnancy (LP) subgroups (Figure 2). Of the 1391 starting miRNAs, 94 were identified as statistically significant (Figure 2). First, the data for the EP and LP samples only were visualized, and revealed that miRNAs from two large clusters on Chr 14 and Chr 19 were expressed at significantly higher levels in LP samples (Figure 3A). Subsequently, the data for these 94 selected miRNAs was shown for all 17 samples to examine their expression levels in the placental and nonpregnant (NP) samples (Figure 3B).
Figure 2: Early vs. Late Pregnancy Comparison. Venn diagram showing the experimental setup comparing late and early pregnancy samples. miRNAs, which were either more highly expressed in the early pregnancy group when compared to the late pregnancy group or vice versa were found performing one two-tailed t-test.
Figure 3: Chromosomal Distribution of Early vs. Late Pregnancy miRNAs. Heatmaps displaying the 94 miRNAs differentially expressed between early and late pregnancy, with unbiased hierarchical clustering of the samples. miRNAs are arranged based on chromosome number followed by chromosomal location. A) Data for EP and LP samples only. B) Data for placental, EP, LP, and NP samples. Data was log
transformed. Expression level legend is to the right, with high expression in yellow and low expression in blue.

**Pregnancy Specific miRNAs**

Early and late pregnancy samples were each compared to the nonpregnant samples by conducting one-way t-tests with a p-value cutoff of <0.05, thus revealing miRNAs that were more highly expressed in either EP or LP compared to NP (Figure 4). This resulted in 46 of the 1391 miRNAs showing significantly higher expression in EP when compared to NP, while 152 of the 1391 miRNAs showed higher expression in LP compared to NP (data not shown). A subset of 20 miRNAs was significantly expressed at higher levels in both EP and LP compared to NP, distinguishing them as pregnancy-specific miRNAs (Figure 5). A total of 196 of the 1391 miRNAs were found to be more highly expressed in either EP, LP, or both compared to NP. This set of miRNAs was enriched in miRNAs located on chromosomes 14 and 19, with 66 of the 196 miRNAs located on one of the two chromosomes, as can been seen in Figure 6.
Figure 4: Pregnant vs. Nonpregnant Comparison. Venn diagram showing the comparisons between each gestational age group and the nonpregnant group. Intersect is the number of miRNAs that were overexpressed in both groups. All miRNAs that were either highly expressed in LP or EP when compared to NP individually and were kept for further analysis.
Figure 5: Pregnancy Specific microRNAs. Expression of microRNAs that were highly expressed in both pregnancy groups when compared to nonpregnant groups is shown in the heatmap above. miRNAs are arranged by chromosome number followed by chromosomal location, with samples grouped by unbiased hierarchical clustering.
Figure 6: Chr14 and Chr19 Expression in Pregnancy. Chromosomal expression of miRNAs that were found upregulated in EP, LP, or both compared to NP. miRNAs are arranged by chromosome number followed by chromosomal location, with samples grouped by unbiased hierarchical clustering. Data was log transformed.

microRNAs Unique to Nonpregnant, Early Pregnancy, and Late Pregnancy

Three independent one-tailed t-tests with a p-value cutoff of <0.05 were performed between EP and LP, EP and NP, and NP and LP. miRNAs that showed significantly higher in expression in each group compared to both of the other groups were considered specific to that group. This comparison yielded a total of 74 microRNAs, with two unique to NP, four unique to EP, and 68 unique to LP (Figure 7). The data for these miRNA probes for all four sample types were displayed after a log transformation to look for any correlation to placental expression (Figure 8). This data was then plotted on a principal component analysis plot in order to compare the relationship of placental expression of these miRNAs to their expression in the EP, LP, and NP groups (Figure 8).
Figure 7: Early, Late and Nonpregnant Comparison. Venn diagrams show the comparisons between each group. miRNAs that were uniquely more highly expressed in one group when compared to the other two were considered unique to that group and are shown as the intersects of each Venn diagram.
**Figure 8: Expression Profiles of Group-Specific miRNAs.** A miRNA expression profile of all samples for the microRNAs unique to each sample group. These groups are separated by white spaces and are coded on the right of the figure. The data was log transformed, followed by unbiased hierarchical clustering of the samples with the miRNAs in chromosomal order followed by chromosome location.
Figure 9: PCA Plot of Group Specific miRNAs. The following is a principal component analysis plot showing the relationship of every sample within each group based on 3-dimensional spatial arrangement. miRNAs uniquely expressed in either early pregnant, late pregnant, or nonpregnant groups were used in this analysis.
DISCUSSION

Rigorous profiling of exRNAs in maternal serum has the potential to yield diagnostic and prognostic biomarkers for a variety of pregnancy complications as well as to identify miRNAs that may mediate fetal-maternal communication. It has become apparent that cells of the placenta release miRNAs into the circulation via apoptotic placental shedding, packaging these miRNAs into exosomes or microvesicles before releasing them, or transporting them into the maternal circulation by some other unknown mechanism (Ouyang, Mouillet, Coyne, & Sadovsky, 2014). Our results indicate not only that miRNA of placental origin are specifically found in the serum of pregnant women, but that the abundance of these placental miRNAs varies across gestational age. Examining the early and late gestation serum miRNA profiles in the context of published data on trophoblast miRNA expression reveals that the differences in the serum miRNA profiles reflects changes in placental miRNA expression with gestational age (Morales-Prieto et al., 2012). Examining the overall relationships among the four groups of samples in our study (placenta, nonpregnant, early pregnant, and late pregnant), it is clear that the late pregnant profile resembles the placental profile, while the early pregnant profile resembles the nonpregnant profile more closely (Figure 3).

In particular, a large group of Chromosome 14 miRNAs was identified as specifically expressed in the late pregnant group compared to the nonpregnant and early pregnant groups (Figure 6). The Chromosome 19 miRNA cluster was expressed in both the early and late pregnant groups, but more highly in the late pregnant group (Figure 6).
One possible reason for the similarity between the late pregnant and placenta profiles is the growth of the placenta. The placenta has been shown to grow linearly throughout pregnancy, with RNA levels increasing linearly with placental weight until the pregnancy reaches term (Winick, Coscia, & Noble, 1967). Due to the increasing size and ability to produce larger quantities of RNA over time, the placenta could be producing much more significant amounts of miRNA to be released than the smaller placenta in the first trimester, inevitably having a larger influence on the miRNA expression profile in the maternal circulation.

In a recent report, blood samples from pregnant patients were sequenced and compared to nonpregnant blood samples, in the hopes of finding a placental microRNA signature. This paper identified members of the miR-498 cluster as highly abundant in placental tissue and maternal and fetal plasma (Williams et al., 2013). Our data correlated with this finding, and out of the 20 miRNAs determined to be "pregnancy specific" (i.e. expressed at significantly higher levels in both early and late pregnant compared to nonpregnant), all of the miRNAs from chromosome 19 fell within the miR-498 cluster (Figure 5). This not only demonstrates reproducibility within the sequencing of extracellular RNAs between independent lab groups, but also shows a consistent group of miRNAs that are highly expressed in pregnant samples.

In an analysis to identify microRNAs specific to either the early pregnant, late pregnant, or nonpregnant groups, the EP and NP groups clustered quite closely together (Figure 9), while the LP group was clearly distinct. This is consistent with the finding that the late pregnancy group had a much more significant miRNA signature when
compared to the others, with 68/74 of the "group-specific" miRNAs falling within the LP group (Figure 8). Only 6/74 probes identified were specific to either the EP group or NP group, leading to weak nonpregnant and early pregnant microRNA signatures. This result could be due to the lack of a large sample size or the lack of depth of sequencing for each individual sample.

In this study, we developed an effective method to obtain high quality small RNA sequencing data from the exRNA of serum samples and also total RNA from placental tissue samples. Our efforts to explore the miRNA profiles across pregnant samples of different gestational ages and nonpregnant samples confirmed observations that have been previously seen in the field, including a pregnancy-specific microRNA signature. We also identified two chromosomes as being enriched in pregnancy related microRNAs, chromosomes 14 and 19. A novel finding of this study is the dramatic difference between the exRNA signatures of serum samples from early and late pregnancy, highlighting the need for gestational age-matched controls for our future studies aimed at identifying miRNA signatures indicative of pregnancy complications.

In future studies, we will also examine other RNA types in maternal serum. This can be done using RNA isolation kits that capture total RNA, rather than enriching for small RNAs. An interesting approach in finding biomarkers for placental dysfunction would be to utilize both small RNA sequencing and total RNA library preparation kits to gain a more comprehensive picture of the exRNAs that vary across gestation age and between normal and affected pregnancies. Overall, our results provide a promising start
to the analysis of exRNA levels in the maternal circulation, and will be used as a backbone for subsequent studies to come.
REFERENCES


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CURRICULUM VITAE

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Born 1987

OBJECTIVE
Matriculation into a competitive M.D. or M.D./PhD program

EDUCATION
Boston University School of Medicine
Master of Science in Medical Sciences
September 2012 – present

University of California, Davis
Bachelor of Science, June 2009
Major: Cell Biology

EXPERIENCE
University of California, San Diego | La Jolla, CA September 2013 – Present
Department of Reproductive Medicine, Staff Research Associate
  § Process blood samples collected from patients presenting at UCSD clinics and hospitals to collect and store isolated serum.  
  § Isolate RNA from serum samples, testing various methods for isolation using quality and quantification controls, following RNA library preparations for sequencing  
  § Perform next generation sequencing on selected samples followed by data analysis using various downstream applications.

Massachusetts General Hospital | Boston, MA February 2013 – July 2013
Patient Escort, Volunteer Opportunity
  § Provide support and compassionate service to patients, families, visitors, and staff in a caring and respectful manner  
  § Take patients from various parts of the hospital along with their belongings to their next destination, whether it is discharging the patient, ensuring they make it to their next appointment, or whatever they may need.  
  § Navigate through the MGH hospital to ensure timely and comfortable assistance and delivery of patients, and assist the patients until they no longer require assistance.  
  § Assist new patient inquiries with answers to common questions, direct complicated questions to the Information Associates, and maintain a welcoming and friendly environment in the main entrance of the hospital.
The Scripps Research Institute | La Jolla, CA

December 2009 – August 2012

Department of Chemical Physiology, Research Technician

- Responsible for reprogramming human dermal fibroblasts (HDFs) from multiple patient and disease-specific cell lines to induced pluripotent stem cells (iPSCs) using viral-mediated overexpression of transcription factors or non-integrative methods.
- Investigated novel methods of reprogramming HDFs into iPSCs including using microRNAs, mRNAs, Sendai virus and other small molecules. Performed microarray analysis on HDFs during different time points of the reprogramming process and explore the roles of specific miRNAs in reprogramming.
- Long-term culture of multiple iPSC lines to compare methods of reprogramming and their effects on genetic integrity of the cells over time.
- Maintenance of an extensive sample database that tracks samples from collaborators globally and locally for subsequent microarray analysis including microRNA Expression, mRNA Gene Expression, SNP Genotyping, and Methylation arrays.

University of California, San Diego, School of Medicine

January 2012

Department of Trauma Surgery, Volunteer Shadowing Opportunity (40 hours)

- Spent time shadowing Vishal Bansal, MD, in the Trauma Surgery department during SICU patient rounds with residents and medical students.
- Scrubbed down for tracheotomy and percutaneous endoscopic gastronomy surgical procedures performed by different medical residents with Dr. Bansal as the Attending Surgeon.
- Gained insight on meaningful patient-doctor interaction, was able to speak with patients for support during multiple wound dressing changes and others on a daily basis.
- Spent quality one on one time with multiple medical students, medical residents, and practicing physicians to fully understand the lifestyle and the rigorous demands required on the road to becoming a doctor.

University of California, Davis | Sacramento, CA

January 2008 – December 2008

Institute for Pediatric Regenerative Medicine, Volunteer Internship

- Used molecular cloning techniques to modify multiple lentiviral constructs for transduction into Human Embryonic Stem Cells (hESCs) to differentiate into specific cardiac cell fates.
- Performed basic cell culture techniques with 293T cell line including multiple rounds of passaging and transfections of created constructs.
- Assisted fellow lab members with multiple different projects simultaneously while working in the laboratory ranging from plasmid construction to PCR amplification, and shadowing patch-clamping electrophysiology.
- Gained experience with project development, group collaboration and meetings, and independent lab work.
PUBLICATIONS AND PRESENTATIONS

**Leonardo TR**, Schell JP, Nickey KS, Tran HT. “Culturing Human Pluripotent Stem Cells on a Feeder Layer”

**Leonardo TR**, Tran HT, Schell JP, Peterson SE. “Preparation of Mouse Embryonic Fibroblast Feeder Cells”

**Leonardo TR**. “Cryopreservation of Human Embryonic Stem Cells”

Tran HT, **Leonardo TR**, Peterson SE. “Generation of Human Pluripotent Stem Cell-Derived Teratomas”


Peterson SE, Tran HT, Garitaonandia I, Han S, Nickey KS, **Leonardo T**, Laurent LC, Loring JF.


**Leonardo, TR**, Laurent, LC, and Loring, JF. “Stem Cells: From Cell Therapy and Drug Screening to Saving Endangered Species”. BMES Lab Fair, February 2011 (3rd Place Outstanding Poster Award)