Magee-Womens Research Institute and Foundation

Annual Progress Report: 2010 Formula Grant

Reporting Period

July 1, 2011 – June 30, 2012

Formula Grant Overview

The Magee Womens Research Institute and Foundation received $1,209,415 in formula funds for the grant award period January 1, 2011, through June 30, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Analysis of Small RNAs in the Fetal Placental Maternal Interface - Small RNAs are present in the fetal and maternal circulations, and within the placenta. We recently found that maternal plasma microRNAs (miRNAs) inversely correlate with placental miRNAs. We surmised that small RNAs serve as intercellular and systemic signals between the maternal and feto-placental compartments. To test the hypothesis that discrete miRNA species are transported across the maternal-placental-fetal interface, we use next generation sequencing technologies to comprehensively define the expression of miRNAs and other small RNAs in the maternal plasma, placenta, and fetal blood, and deploy novel statistical-computational tools to interrogate the dynamic communication patterns of miRNA during human gestation.

Duration of Project

1/1/2011 - 6/30/2012

Project Overview

Development of the eutherian embryo is utterly dependent on the placenta, which governs the exchange as well as communication between the blood of two organisms, taking place through a hemochorial layer of epithelial trophoblast and fetal endothelial cells. The expression of mRNAs and proteins are regulated by microRNAs (miRNAs) and other small RNAs in distinct spatial patterns. Although unique types of miRNAs have been validated in the human placenta, the contribution of these miRNAs to maternal plasma RNAs during pregnancy is unknown. Moreover, the expression and function of fetal miRNAs and small RNAs are unknown. We posited that exquisite expression patterns of miRNAs and small RNAs are germane for intact embryonic development and growth. We first defined the expression profiles of all miRNAs and small RNAs in the placenta and interfacing maternal and fetal blood during human pregnancy. We then used novel statistical analyses to interrogate dynamic changes in microRNA expression during human gestation prior to delivery, and search for the patterns of miRNAs and small RNA communication among maternal blood, placenta, and fetal blood. Information gleaned from our
data may not only illuminate epigenomic pathways that influence human embryonic
development, but may also suggest innovative, clinically-relevant biomarkers, as well as
preventive interventions designed to decrease the likelihood of injury before birth, thereby
reducing the incidence of postnatal and adult diseases.

Specific Aims:

Aim 1: Define the expression profile of all miRNA and small RNA species in the maternal
blood, placenta, and fetal blood at the end of human pregnancy using next generation sequencing
technology.
Aim 2: Develop and apply novel statistical/computational tools to interrogate dynamic,
coordinated patterns of miRNA and small RNA expression.

Project outcomes are instrumental in suggesting a possible role for small RNA molecules in
communication patterns among the maternal plasma, placenta, and the fetal blood.

Principal Investigator

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Other Participating Researchers

Yoel Sadovsky, MD - University of Pittsburgh and Magee-Womens Research Institute
Chunming Zhu, PhD, Ilya Goldin - employed by Magee-Womens Research Institute

Expected Research Outcomes and Benefits

After completing this project, we will define the microRNA expression profiles in the three
compartments during human gestation prior to delivery, and establish the pattern of
communication of microRNAs among maternal plasma, placenta, and fetal blood. Information
gleaned from our data may not only illuminate epigenomic pathways that influence human
embryonic development, but also suggest novel biomarkers and therapeutic approaches to
obstetrical diseases stemming from placental dysfunction, which affect more than 10% of the
annual four million deliveries nationwide.

It is also expected that results from the proposed studies will fuel additional research and
submission of new grant proposals that center on the targets and regulation of miRNA and small
RNAs in the placenta, fetal and maternal plasma.
Summary of Research Completed

In our original proposal we described our goal to deep-sequence all miRNAs from the placenta, maternal pre-delivery blood and fetal cord blood from 20 pregnant women, for a total of 60 libraries. Because of technical difficulties in obtaining reliable sequencing libraries for plasma miRNA, we generated only 27 miRNA sequencing libraries, and compensated for the reduced sequencing libraries with a large number of microarray analyses of the relevant compartments, all described below.

Overall, we collected specimens and extracted RNA from more than 100 participants. The RNA was extracted from placental biopsies, maternal blood before delivery (early in labor), maternal blood within 24 h after delivery, and fetal cord blood at delivery. Because of a number of technical issues (patient’s refusal of an additional blood test, sample not collected and alike), post partum maternal blood was not collected from a fraction of the participants, resulting in 83 incomplete sets (all RNA samples except for post partum collection). There are also sets we will have to exclude because one or more of plasma samples was hemolyzed (which happened in 24 of the “complete” sets).

Our main challenge was the low level of miRNA in the plasma samples which resulted in inadequate miRNA libraries from our initial preparations, performed at Expression Analysis, a commercial laboratory in Durham, NC. After repeatedly failing to obtain reliable libraries, we contracted Ocean Ridge Bioscience, which uses a customized approach to library preparation. As described below, Ocean Ridge managed to construct and sequence several libraries, which provided useful information on miRNA expression levels at the desired compartments.

Sequencing data analysis
We obtained eight high quality miRNA sequencing libraries, generated by Ocean Ridge Bioscience, for placental tissue, pre-delivery maternal plasma, cord blood plasma, and post-delivery plasma samples from two patients. Before the Ocean Ridge libraries, we also obtained another 19 miRNA sequencing libraries generated by Expression Analysis. The data were processed as described in our prior half-year report. The processed miRNA sequencing libraries then were normalized by the library sizes to generate the reads per million (RPM) data. We noticed that the quality of the plasma libraries generated by Expression Analysis was not satisfactory —the size of some the plasma libraries were less than 30,000 reads before alignment. Therefore, we decided to focus our analysis on the eight libraries by Ocean Ridge. The information about these eight libraries is provided in Table 1. Using hierarchical clustering, we can see that the placenta samples form a tight group, while the plasma samples form another tight group. Within the plasma group, the maternal plasma were grouped together, and divided into subgroups according to if the samples were collected before or after the delivery (Figure 1).

Our original study plan was to test the correlation of the expression levels for each miRNA between placenta samples and pre-delivery plasma samples, and between placenta and cord blood plasma samples. However, with data for only two patients, this approach would not be feasible. Instead, we used a modified model:
Equation 1
\[ X_{B,i,j} = \left( k_{A,B,i} + k_{A,B,j} \right) X_{A,i,j} + W_{B,i} + W_{B,j} + \varepsilon'_{B,i,j} \]
where \( X_{B,i,j} \) and \( X_{A,i,j} \) are the measured expression level of miRNA \( i \) in compartments \( B \) and \( A \) respectively from the individual \( j \), \( k_{A,B,i} \) and \( k_{A,B,j} \) are miRNA/individual specific transport rate from \( A \) to \( B \), \( W_{B,i} \) and \( W_{B,j} \) are miRNA/individual specific factor for expression of miRNA from other source (including native expression), and \( \varepsilon'_{B,i,j} \) is an error term. If miRNA could be transported to compartment \( B \) from both compartment \( A \) and compartment \( C \), then our model would be:

Equation 2
\[ X_{B,i,j} = \left( k_{A,B,i} + k_{A,B,j} \right) X_{A,i,j} + \left( k_{C,B,i} + k_{C,B,j} \right) X_{C,i,j} + W_{B,i} + W_{B,j} + W_{C,i} + W_{C,j} + \varepsilon'_{B,i,j} \]

With data from eight libraries (two patients), we could not fit the above model specified in Equations 1 and 2. We decided to take the model selection approach, where we select the best unsaturated submodel of the model specified in Equations 1 and 2 using the Akaike Information Criterion (AIC).

Next we selected the lists of miRNAs to be included in our regression analysis. We examined the expression profile of the miRNAs in non-pregnant adult plasma and cultured PHT cells. In the first half-year of this project, we already produced two miRNA libraries from cultured PHT cells under either normal or hypoxia conditions. We also located a miRNA sequencing library from a pooled normal male and non-pregnant female plasma samples and a library from pooled lung cancer patient sera.

For the study of the transport of miRNA from placenta to either maternal blood or cord blood, we selected those miRNAs abundant in PHT cells and the placenta (RPM ≥ 1500) but almost absent from the non-pregnant normal serum and the cancer serum (RPM ≤ 30). To reduce the effect of heteroscedasticity, we further required that these miRNAs have moderate expression in pre-delivery maternal plasma (average RMP ≥ 100). For the study of the transport of miRNAs from either maternal blood or fetal blood to placenta, we selected those miRNAs abundant in non-pregnant normal serum (RPM ≥ 750) but almost absent from PHT cells (RPM ≤ 15). In total we identified 10 miRNAs for the study of miRNA transport from placenta to plasma, and five for the study of miRNA transport from plasma to placenta, as shown in Table 2.

Applying the AIC score based model selection procedure to the sequencing data of the selected miRNA to compare the sets of all unsaturated models, we identified, for each direction of transportation, the best unsaturated model, as shown in Table 3. Note that instead of compartments \( A, B, C \) in the general models, here we use \( P \) to represent placenta, \( M \) maternal plasma, \( F \) fetal plasma (cord blood), and \( MF \) the average of maternal and fetal plasma.

The interpretation of the model selection results is straightforward. For the transport of miRNA from placenta to pre-delivery maternal plasma, the only non-zero parameter in the best model is \( k_{P,M,i} \), which suggests that the selected miRNAs in maternal plasma come entirely from the placenta, and the rate of transport may vary from miRNA to miRNA.
In the model for the miRNA transport from placenta to cord blood, the presence of $k_{F,P,j}$ suggests that these miRNAs are transported from placenta to cord blood, and that the rate varies slightly from patient to patient. The absence of $k_{F,P,i}$ may imply the transport rate does not vary much from miRNA to miRNA. The presence of $W_{F,i}$ is interesting. While none of the parameters associated with this term are significant, they suggest that miRNAs may be also transported between cord blood and other fetal tissues.

In the model for the transport of miRNA from maternal/fetal blood to placenta, the term $X_{MF,i,j}$ represents the average expression level of the miRNAs in the pre-delivery maternal plasma and the cord blood plasma. This model attains a better AIC score than any model that uses only the expression of miRNAs in pre-delivery maternal plasma, or the expression of miRNAs in cord blood plasma. The presence of $k_{MF,P,j}$ and $k_{MF,P,j}$ implies that the transport of miRNA from maternal and fetal blood to placenta varies from miRNA to miRNA, and from patient to patient. The parameter $W_{P,j}$ could represent a normalization artifact.

Array Data Analysis
We used the Ocean Ridge miRNA microarray to measure the miRNA levels in maternal/fetal plasma and placenta samples from 37 patients, out which the data from 35 patients passed the quality control. The Ocean Ridge miRNA microarray has 3 probes for each target miRNA. We applied a modified two-step RMA normalization/summary procedure to the placenta samples and plasma samples separately. 147 samples which passed the quality control were divided into two groups, plasma group (109 samples) and placenta group (38 samples). The array data in each group were pre-processed first by quantile normalization. Next, the expression values of each miRNA were estimated by fitting the normalized intensities of three probes for the miRNA to a linear model using the robust median polish (MP) algorithm.

We studied the 16 miRNAs in Table 2 using the same method as we did for the sequencing data, and identified the best model for each direction of the transport of miRNA between placenta and maternal/fetal plasma, as presented in Table 4. These models show strong similarity to the models we obtained for the sequencing data or the Taqman data. For those miRNAs abundant in PHT cell but absent in normal non-pregnant plasma, the model from the microarray data suggests that their expression in maternal plasma comes almost entirely from the placenta, as suggested by the models from the sequencing data and the Taqman data. For the expression of these miRNAs in fetal plasma, the model from the microarray data suggested that they largely come from the placenta. For those miRNAs abundant in non-pregnant normal plasma but absent in PHT cells, the model from the microarray data suggested that they were transported both between placenta and fetal plasma, and between fetal plasma and other tissues.

Discussion
Using the plasma miRNA samples from pre-delivery maternal blood and cord blood, as well as placenta miRNA samples, utilizing different high throughput platforms, include Illumina sequencing and Ocean Ridge miRNA microarray, we investigated, for a selection of 16 miRNAs, their pattern of transport between maternal blood, placenta, and fetal blood. Through a comprehensive information score based model selection procedure, we found that the data confirmed the transport of miRNA. Our analyses were limited by the small number of sequencing libraries and high noise of microarrays. We plan to obtain more sequencing libraries,
and explore other platforms, including Taqman RT-PCR and Nanostring miRNA assay. The new data would enable us to better study the mechanism of miRNA transport in the fetal placental maternal interface.

Figure 1. Clustering of the 8 plasma miRNA libraries
### Table 1. Size of miRNA sequencing libraries

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sample</th>
<th>Library Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>122</td>
<td>Pre-Delivery</td>
<td>1907871</td>
</tr>
<tr>
<td>122</td>
<td>Cord Blood</td>
<td>1506999</td>
</tr>
<tr>
<td>122</td>
<td>Post-Delivery</td>
<td>1835196</td>
</tr>
<tr>
<td>123</td>
<td>Pre-Delivery</td>
<td>2436866</td>
</tr>
<tr>
<td>123</td>
<td>Cord Blood</td>
<td>2277488</td>
</tr>
<tr>
<td>123</td>
<td>Post-Delivery</td>
<td>3619539</td>
</tr>
<tr>
<td>122</td>
<td>Placenta</td>
<td>2142379</td>
</tr>
<tr>
<td>123</td>
<td>Placenta</td>
<td>3219145</td>
</tr>
</tbody>
</table>

### Table 2. Select miRNAs and Their Expression (RPM)

<table>
<thead>
<tr>
<th></th>
<th>Pre-D 122</th>
<th>CB 122</th>
<th>Post-D 122</th>
<th>Pre-D 123</th>
<th>CB 123</th>
<th>Post-D 123</th>
<th>Plac 122</th>
<th>Plac 123</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-1323</td>
<td>1718</td>
<td>1038</td>
<td>275</td>
<td>1463</td>
<td>1120</td>
<td>64</td>
<td>44482</td>
<td>42028</td>
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<tr>
<td>hsa-miR-141-3p</td>
<td>83</td>
<td>53</td>
<td>83</td>
<td>124</td>
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<td>24</td>
<td>5546</td>
<td>5748</td>
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<tr>
<td>hsa-miR-516b-5p</td>
<td>173</td>
<td>359</td>
<td>141</td>
<td>169</td>
<td>300</td>
<td>18</td>
<td>18086</td>
<td>13728</td>
</tr>
<tr>
<td>hsa-miR-517b-3p</td>
<td>258</td>
<td>229</td>
<td>84</td>
<td>293</td>
<td>234</td>
<td>36</td>
<td>47698</td>
<td>43056</td>
</tr>
<tr>
<td>hsa-miR-520a-3p</td>
<td>78</td>
<td>51</td>
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<td>12</td>
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<td>3854</td>
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<tr>
<td>hsa-miR-524-5p</td>
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<td>475</td>
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<td>27</td>
<td>5352</td>
<td>5102</td>
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<tr>
<td>hsa-miR-525-5p</td>
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<tr>
<td>hsa-miR-517c-3p</td>
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<tr>
<td>hsa-miR-517a-3p</td>
<td>258</td>
<td>229</td>
<td>84</td>
<td>293</td>
<td>234</td>
<td>36</td>
<td>47698</td>
<td>43056</td>
</tr>
<tr>
<td>hsa-miR-512-3p</td>
<td>84</td>
<td>83</td>
<td>34</td>
<td>140</td>
<td>100</td>
<td>13</td>
<td>5462</td>
<td>5368</td>
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<tr>
<td>hsa-miR-106a-5p</td>
<td>311</td>
<td>284</td>
<td>721</td>
<td>152</td>
<td>219</td>
<td>253</td>
<td>36</td>
<td>46</td>
</tr>
<tr>
<td>hsa-miR-144-3p</td>
<td>33197</td>
<td>21727</td>
<td>14229</td>
<td>46550</td>
<td>99168</td>
<td>47869</td>
<td>10243</td>
<td>22464</td>
</tr>
<tr>
<td>hsa-miR-185-5p</td>
<td>3199</td>
<td>4144</td>
<td>3205</td>
<td>3079</td>
<td>7468</td>
<td>2532</td>
<td>236</td>
<td>348</td>
</tr>
<tr>
<td>hsa-miR-20b-5p</td>
<td>872</td>
<td>1811</td>
<td>1520</td>
<td>806</td>
<td>1484</td>
<td>807</td>
<td>91</td>
<td>135</td>
</tr>
<tr>
<td>hsa-miR-363-3p</td>
<td>2346</td>
<td>1237</td>
<td>994</td>
<td>3517</td>
<td>3214</td>
<td>2380</td>
<td>266</td>
<td>215</td>
</tr>
<tr>
<td>hsa-miR-7-5p</td>
<td>533</td>
<td>1311</td>
<td>1178</td>
<td>544</td>
<td>1219</td>
<td>582</td>
<td>135</td>
<td>164</td>
</tr>
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</table>
**Table 3. Best Models for miRNA Transport (Based on Sequencing Data)**

<table>
<thead>
<tr>
<th>Direction of Transport</th>
<th>Best Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta to Pre-Delivery Blood</td>
<td>$X_{M,ij} = k_{P,M,i} X_{P,ij} + \epsilon'_{M,ij}$</td>
</tr>
<tr>
<td>Placenta to Cord Blood</td>
<td>$X_{F,ij} = k_{P,F,j} X_{P,ij} + W_{F,j} + \epsilon'_{F,ij}$</td>
</tr>
<tr>
<td>Maternal/Fetal Blood to Placenta</td>
<td>$X_{P,ij} = (k_{MF,P,i} + k_{MF,P,j}) X_{MF,ij} + W_{P,j} + \epsilon'_{P,ij}$</td>
</tr>
</tbody>
</table>

**Table 4. Best Models for miRNA Transport (Based on Microarray Data)**

<table>
<thead>
<tr>
<th>Direction of Transport</th>
<th>Best Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta to Pre-Delivery Blood</td>
<td>$X_{M,ij} = (k_{P,M,i} + k_{P,M,j}) X_{P,ij} + \epsilon'_{M,ij}$</td>
</tr>
<tr>
<td>Placenta to Cord Blood</td>
<td>$X_{F,ij} = (k_{P,F,j} + k_{P,F,j}) X_{P,ij} + \epsilon'_{F,ij}$</td>
</tr>
<tr>
<td>Maternal/Fetal Blood to Placenta</td>
<td>$X_{P,ij} = (k_{MF,P,i} + k_{MF,P,j}) X_{MF,ij} + (k_{F,P,i} + k_{F,P,j})$</td>
</tr>
<tr>
<td></td>
<td>$X_{F,ij} + W_{P,i} + \epsilon'_{P,ij}$</td>
</tr>
</tbody>
</table>

**Research Project 2: Project Title and Purpose**

*miR-210 Regulation of Mitochondria Function* - We plan to characterize the function of a hypoxia inducible microRNA, miR-210, in ovarian cancer oncogenesis, especially the mechanism of miR-210 regulating mitochondria metabolism and its contribution to ovarian cancer initiation and progression. By completing this study, we hope to establish miR-210’s role in ovarian cancer and identify novel miR-210 target genes as potential targets for ovarian cancer therapy.

**Duration of Project**

1/1/2011 - 12/31/2011

**Project Overview**

The main objective of this project is to illustrate the biological function of miR-210 in ovarian cancer and its potential for ovarian cancer therapy. We propose a series of mechanistic studies to investigate miR-210’s function in ovarian cancer, especially its contribution to ovarian cancer energy metabolism under hypoxic conditions. Specifically, we will examine miR-210’s copy number in a variety of ovarian cancer cell lines using fluorescence in situ hybridization (FISH) and corresponding miR-210 expression in these cell lines to correlate its copy number with gene expression level. Subsequently, we will ectopically express miR-210 in at least two ovarian cancer cell lines to examine miR-210’s expression on ovarian cancer cell survival, proliferation, apoptosis, and metabolism. In addition, we will verify our computationally predicted miR-210 target genes in mitochondria metabolism pathways by Argonaute 2 immunoprecipitation followed by real-time PCR assays and by Western Blotting. We will also investigate the function...
of miR-210 target genes on mitochondria function by overexpressing and knocking down the genes on energy metabolism, oxygen consumption, and cell survival and proliferation. Finally, we will examine the expression of miR-210 and its target gene in banked clinical specimens to correlate its expression with tumor hypoxia and ovarian cancer patient clinical outcomes. In summary, this project is intended to provide mechanistic insights on miR-210’s function in ovarian cancer and to explore the possibility to target miR-210 pathway for ovarian cancer therapy.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death in women in the United States and currently, there is no effective therapy for late stage EOC. Our project will address the function of miR-210 in EOC cells. We expect to identify the role miR-210 plays in ovarian cancer cell energy metabolism by genetic and biochemical approaches and verify its target gene in clinical specimens by immunohistochemistry (IHC), real-time PCR, and Western blotting. miR-210 and its target gene identified in this study may provide novel therapeutic targets for EOC treatment.

Summary of Research Completed

In the second half of the project, we have determined the function of miR-210 on ovarian cancer cell respiration and demonstrated that depletion of glucose could lead to death of ovarian cancer cells with NDUFA4 knockdown when compared to controls. We also found that miR-210 could regulate HIF1α expression and thus, enhance aerobic glycolysis. Our data suggest that miR-210 may be a master regulator of cellular energy metabolism and a potential therapeutic target in ovarian cancer. Here, we summarize our findings:

miR-210/NDUFA4 regulate mitochondrial respiration

We have identified NDUFA4 as a miR-210 target. We next performed immunofluorescence staining in SKOV3 cells exposed under normoxia and 0.5% oxygen to examine cellular localization of NDUFA4 and its response to hypoxia treatment in situ. A mitochondrial localized GFP protein construct was transected into the cells as a control. As expected, NDUFA4 is
exclusively localized to mitochondria, but strikingly, NDUFA4 protein is completely repressed under hypoxia (Fig. 1). Because NDUFA4 is a subunit of mitochondrial ETC Complex I, we expected that oxygen consumption will be inhibited when miR-210 is overexpressed or NDUFA4 is knocked down by siRNA. As predicted, SKOV3 cells that overexpress miR-210 or were transfected with siRNA targeting NDUFA4 demonstrated reduced oxygen consumption compared to parental cells (Fig. 2), indicating attenuated mitochondrial respiration.

miR-210/NDUFA4 regulate aerobic glycolysis

When we observed attenuated mitochondrial respiration, a direct prediction is that cellular ATP level will be affected since the majority of ATP is produced through oxidative phosphorylation (OXPHOS). We compared cellular ATP levels in parental cells and cells expressing miR-210 (210E) or with NDUFA4 knockdown (shNDU). As expected, ATP level is lower in 210E and shNDU cells when compared to parental controls (Fig. 3). We then reasoned that when cellular ATP level is decreased, cells may try to compensate for the lost ATP level by other means, one of which is glycolysis. We then measured cellular lactate production, the final product of glycolysis. Interestingly, lactate levels in both 210E and shNDU cells are increased (Fig. 4), suggesting that miR-210 regulates a feedback mechanism that can down-regulate OXPHOS while enhancing aerobic glycolysis. Currently, we are investigating the mechanism of this enhanced aerobic glycolysis.

miR-210 expression in primary ovarian tumors

The Cancer Genome Atlas (TCGA) project is intended to comprehensively analyze tumor samples using multiple platforms, such as gene expression, miRNA expression, promoter methylation, DNA copy number, etc. We analyzed the ovarian cancer data that include tumors from 506 high grade serous ovarian cancer patients. Despite the fact that the chromosome 11p15.5 region, where miR-210 is located, is frequently lost, miR-210 expression level is still comparable to that of normal ovary controls (Fig. 5A), suggesting that miR-210 likely resides in a genomic location of heterozygous loss, and that other mechanisms may up-regulate miR-210 to compensate for the loss of miR-210 copy number. This also suggests that maintaining cellular miR-210 levels may be important for EOC development. Since miR-210 is robustly induced under hypoxic conditions, we examined the correlation between miR-210 expression and a hypoxic gene expression signature, consisting of 9 well-characterized hypoxia-responsive genes (Fig. 5B). The highly significant correlation of miR-210 expression with that of classic HIF1α target genes, such as genes in the glycolytic pathway, SLA2AI (GLUT1) and LDHA (Fig. 5C; p=0 for both genes), strongly supports the notion that miR-210 expression in primary ovarian tumors is regulated by tumor hypoxia and tumor hypoxia may be the mechanism responsible for upregulating miR-210 expression in EOC to compensate for its copy number loss. We then analyzed NDUFA4 expression in primary ovarian cancer tumors using a tissue microarray (TMA) consisting of 60 primary tumor samples by immunohistochemistry (IHC). Interestingly, NDUFA4 is found localized exclusively with a classic tumor hypoxia marker, CAIX (Fig. 6), further confirming our analysis from ovarian cancer TCGA data, that miR-210/NDUFA4 is regulated by tumor hypoxia in vivo.
**Figures**

**Figure 1.** Immunofluorescence staining of NDUFA4. NDUFA4 is located in mitochondria where its expression is completely abolished when treated under hypoxia. Mito-GFP, a GFP protein that is localized in mitochondria, is used as a control.

![Immunofluorescence staining of NDUFA4](image1)

**Figure 2.** Ectopic expression of miR-210 (210E) and knockdown of NDUFA4 (shNDU) in SKOV3 cells lead to decreased oxygen consumption when compared to parental control cells.

![Relative O2 Consumption](image2)

**Figure 3.** Ectopic expression of miR-210 (210E) and knockdown of NDUFA4 (shNDU) in SKOV3 cells lead to decreased cellular ATP levels when compared to parental control cells.

![ATP Assay](image3)
Figure 4. Ectopic expression of miR-210 (210E) and knockdown of NDUFA4 (shNDU) in SKOV3 cells lead to increased lactate production when compared to parental control cells, indicating enhanced glycolysis.

Figure 5. miR-210 in primary EOC. A) miR-210 expression in TCGA ovarian cancer and normal control samples. B) In 506 TCGA samples, miR-210 expression is clustered with that of classic hypoxia inducible genes; each column represents one patient. C) miR-210 expression is highly correlated with that of SLC2A1 and LDHA. Left panel, SLC2A1; right panel, LDHA.
Figure 6. NDUFA4 is localized outside of hypoxic regions in primary EOC tumors. Left panel, H&E staining; Middle panel, CAIX staining; Right panel, NDUFA4 staining. Serial sections were used to ensure the same tumor areas are stained. The top panel is a low magnitude (5X) view of the staining. The bottom panel shows the enlarged areas of the top panel at a higher magnitude (40X).

Research Project 3: Project Title and Purpose

Functional Analysis of the C19MC MicroRNAs in Trophoblasts - Our goal is to better understand placental physiology and the causes of placental insufficiency leading to gestational diseases. MicroRNAs have emerged as critical regulators of virtually every biological processes and their altered expression is increasingly found associated with pathological states. Recently, it was found that the placenta is the exclusive source of a large family of miRNAs originating from a unique cluster located on chromosome 19 (C19MC). While the expression of these miRNAs is normally restricted to placental trophoblasts, their aberrant expression in other cell types is often associated with malignant conditions. However, the relevant biological function of these miRNAs in the placenta remains poorly understood. In this project, we propose to investigate the function of the C19MC miRNAs in trophoblast cells.

Duration of Project

1/1/2011 – 12/31/2011

Project Overview

Placental insufficiency is one of the main complications of pregnancy and is associated with compromised fetal development and poor outcome. These pathologies are thought to stem from of inadequate placental development but the molecular mechanisms underlying placental insufficiency remain poorly understood. The discovery of miRNAs and the realization that
miRNAs are abundant in the human placenta offer new perspectives on placental development and pathophysiology. Consistent with this, mutations that disable the miRNA biogenesis machinery lead to impaired placental development. In addition, miRNA expression profiling experiments performed in placentas from patients with preeclampsia have shown anomalies in miRNA expression patterns compared to uncomplicated pregnancies.

One of the most notable observations about placental miRNAs is the abundance of highly related miRNAs that originate from a unique large cluster termed C19MC encoding at least 48 mature miRNAs. Added to the fact that each miRNA can possibly target several hundred transcripts, the combined regulatory potential of these miRNAs is huge. The biological role of these abundant miRNAs is unknown but several reports indicate that their aberrant expression can have dramatic cellular consequences such as cancer.

The overall aim of this project is to shed light on the role of the C19MC miRNAs in trophoblasts as they could be instrumental for placental morphogenesis and function. To test this hypothesis, we will first use a loss-of-function approach in primary trophoblasts and investigate the cellular consequences of suppressing the function of several members of these miRNAs. In the second specific aim we will focus on the molecular targets of these miRNAs and use microarrays to determine the impact of C19MC miRNAs on gene expression and identify mRNA targets in a trophoblast cell line.

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Expected Research Outcomes and Benefits

Our long-term goal is to understand the response of the human placenta to injury, and in particular to low oxygen (hypoxic) stress. Low placental oxygenation is one of the main stress conditions in the human placenta and is believed to play a role in the development of pathologies such as intrauterine growth restriction (IUGR) and hypertension of pregnancy. These conditions are associated with increased perinatal illnesses and mortality. Newborns surviving these insults are at risk for lifelong complications, including developmental diseases and a greater incidence of the adult metabolic syndrome (type-2 diabetes, high lipids, hypertension, obesity and related illnesses). While these complications often originate from impaired placenta function, the
molecular mechanisms involved remain elusive. A prerequisite to a better understanding of these gestational pathologies is the identification of the genes that play a role in the morphogenesis and function of the placenta. The recent recognition of microRNAs (miRNAs) as critical regulators of development and homeostasis and the discovery of miRNA abundance in the placenta have stimulated research routes that target the molecular underpinnings of placental insufficiency. We recently showed that miRNA expression was altered in placental trophoblasts that are exposed to hypoxic stress, which often leads to placental injury. We also demonstrated that the dysregulation of these miRNAs had the potential to affect cardinal regulators of placental development. Additionally, a family of placenta-specific miRNAs that originate from a single large cluster of miRNA genes located on human chromosome 19 (C19MC) was recently described. While highly expressed in trophoblasts, the role of miRNAs from the C19MC cluster and their impact on cellular function is unknown. The analysis of C19MC miRNA function will illuminate molecular pathways governing trophoblast differentiation and function. Furthermore, conclusions from our studies may uncover important mechanisms that contribute to placental dysfunction.

Summary of Research Completed

The purpose of this project is to decipher the role of a unique family of miRNAs expressed in placental trophoblasts, and referred to as C19MC miRNAs. During the first funding period we tried to inhibit discrete species in primary trophoblasts and analyzed the cellular impact. We also generated stably transfected cell lines with a modified BAC vector containing the entire C19MC locus. In this second period of the project we analyzed the cellular consequences of forced expression of the C19MC miRNAs in the HTR8 cell line. This cell line does not naturally express these miRNAs.

Aim 1: Inhibit selected C19MC miRNAs and analyze functional consequences in primary human trophoblasts.

As reported in the first progress report, we transfected primary human trophoblasts with a series of anti-sense miRNA inhibitors (miRCURY LNA™ microRNA Power Inhibitors Exiqon). To monitor knockdown efficiency, cells were co-transfected with a sensor construct carrying a Renilla luciferase reporter gene, which includes artificial miRNA target sites. We generated these sensors by cloning oligonucleotides bearing three perfectly complementary miRNA binding sites downstream of the luciferase reporter gene in the plasmid psiCHECK2 (Promega). Increased luciferase activity was detected upon transfection of each of the antisense miRNA inhibitors, indicating inhibition of the targeted miRNAs. However, we did not observe any phenotypic alterations of the recipient trophoblasts. Considering that the cells received only one miRNA inhibitor at a time it is possible that this selective inhibition was too limited to visibly affect the phenotype, especially in the context of a family of highly related miRNAs.

In order to increase the likelihood of detecting differences, we performed microarray analyses and compared gene expression between cells that received the LNA-inhibitors and cells that were transfected with a non-targeting scramble oligonucleotide. Total RNAs were extracted from transfected primary trophoblasts and were hybridized to a SurePrint G3 Human GE 8x60k microarray (Agilent Technologies). Analysis of the data revealed that only a relatively small
number of differentially expressed genes reached statistical significance although none of them were confirmed by subsequent real-time PCR. Therefore, we did not further pursue this approach and instead focused on the second approach described in the second specific aim.

Aim 2: Analyze gene expression in cells that ectopically express the C19MC miRNAs.

The recombined BAC containing the entire C19MC locus and tagged with GFP was transfected into HTR8-Sv/Neo cells using a combination of polyethyleneimine (PEI) and Virofect as described in the grant proposal. Stable transfectants were selected using Zeocin (200 \( \mu \)g/ml) over a period of two weeks and individual clones were picked and further expanded in separate dishes. Five different HTR8-C19MC clones (Clone #1 to clone #5) were further studied for their expression of the transgene. We also derived a line consisting of a mixed population of stably transfected cells and a line that was stably transfected with a modified BAC carrying a deletion of the entire sequence encoding the C19MC miRNAs (BAC-C19-Del-GFP). These cells were also maintained as a mixed population of Zeocin-resistant cells.

Examination of the live transfected cells by fluorescent microscopy showed that 100% of the cells expressed the protein GFP, which confirms their transfection status. We analyzed the expression of C19MC miRNAs using real-time PCR and Northern-blot assay. Real-time PCR was performed starting with 1 \( \mu \)g of RNA that was reverse transcribed and amplified using the miScript PCR System (Qiagen). The expression level of five different C19MC species was assessed: miR-517a, miR-517c, miR-518a-5p, miR-518e, and miR-520c-3p. These miRNAs were chosen on the basis of their location across the cluster as well as their high expression in primary trophoblasts. Important differences in C19MC miRNA expression levels were observed between the different cell lines (Fig. 1). For Northern blot experiments RNA (20 \( \mu \)g) was resolved on a 15% acrylamide/urea gel, electrotransferred onto a nylon membrane (Hybond N+, Amersham/GE), and hybridized at 37ºC using an oligo-DNA probe labeled with \( ^{32}P \) using the StarFire system (IDT). As a positive control we used RNA sample from primary human trophoblasts that are known to express high levels of miRNAs from the C19MC locus. Figure 2 shows a northern blot hybridized with a probe for miR-517a. Although the differences in expression level between the different lines were less obvious than by PCR, we note that real-time PCR is clearly more quantitative than northern analysis. Therefore, we retained clone #1 for most of our further experiments.

Interestingly, a simple examination of the cells appearance under the microscope revealed a strikingly different morphology between the cells expressing the C19MC miRNAs and the cells that do not (Fig. 2). These cells appeared less elongated and present fewer cellular extensions. In contrast, cells transfected with the deleted BAC did not display this phenotype. The phenotype was less conspicuous in the stably transfected clones expressing lower levels of C19MC miRNAs.

Ectopic expression of C19MC miRNAs has been associated with abnormal cell proliferation and cancer. Therefore, we compared the cell viability and proliferation capacity of the HTR8 WT and C19MC miRNA-expressing cells using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Cells were seeded in a 96-well plate and incubated overnight at 37ºC. The day after plating the MTS/PMS solution was added to the culture medium
and the cells were further incubated at 37°C for 3 h. The absorbance at 490 nm was then recorded using an automatic microplate reader. Unexpectedly, the cells expressing the C19MC miRNAs showed a lower rate of proliferation compared to the wild-type cells. We also analyzed the response of the cells treated with Cobalt chloride (CoCl), an agent that stabilizes HIF-1α and mimics hypoxic conditions. No significant difference was observed between the hypoxia-challenged cells and the cells cultured in standard conditions.

Finally, to capture the changes in gene expression we used microarrays to compare the gene expression profiles in the parental cells with the transfected cells with the BAC WT and the deleted BAC lacking the C19MC miRNA genes. Total RNA from the different lines was purified using the miRNeasy kit (Qiagen), labeled using the Agilent Low RNA Input Linear Amplification Kit in the presence of cyanine 3-CTP, and hybridized to Agilent SurePrint G3 Human GE 8x60K microarray. After hybridization and washing, the microarrays were processed with an Agilent scanner. A total of eight arrays corresponding to three biological replicate per transfected lines and two replicates of the parental cells were hybridized. After normalization using the cyclic loess algorithm, we selected genes that were differentially expressed in the different settings. These genes were selected based on false discovery rate controlled at 5%. We then removed the genes that differed between the cells that were not transfected and the cells that were transfected with the trimmed BAC as they most likely correspond to genes that are influenced by the transfection process rather than the expression of the C19MC miRNAs. Finally, the selected genes were ranked according to their fold change as evaluated by the log-ratio of the signal between the two groups (BAC-C19-Del and BAC-C19-WT). 589 genes were found significantly upregulated by more than two-fold while 601 were downregulated between the two groups.

Microarray experiments were validated using real-time PCR. Total RNA were reverse transcribed using the High Capacity RNA-to-cDNA Master mix (Applied Biosystems) and PCR-amplified using Power SYBR Green mix (Applied Biosystems). A set of ten genes was selected for validation independently from their targeting by C19MC miRNAs. For comparison we assessed the expression level in parental cells that were not transfected at all, in cells transfected with the deleted BAC and in two different clones of cells ectopically expressing the C19MC miRNAs at high level or low level (Fig. 4). Several of the selected genes showed the expected trend as determined by the microarray data. For example CXCL12, IGFBP3, MAGEH1, and WNT5A show a substantial reduction in cells expressing the C19MC miRNAs. We also confirmed the trend for three positively regulated genes (Fig. 4).

We sought to identify potential C19MC miRNA targets by combining microarray expression data with miRNAs target predictions. From a total of 59 mature miRNAs originating from the C19MC locus we identified 29 of them (table 1) that account for more than 95% of the C19MC miRNAs in trophoblasts and predicted their target using 3 algorithms: TargetScan, miRDB, and microrna.org. A total of 4419 genes were predicted by the 3 databases, of which 1030 were downregulated in C19MC miRNA-transfected cells.
Tables, Figures and Legend

**Figure 1.** Analysis of C19MC miRNA expression in stably transfected HTR8-SV/Neo cell lines (C: non-transfected cells, 1 to 5: stable clones, M: stable mixed cells). Relative expression level of individual miRNA was determined by real-time PCR using the miScript PCR System (Qiagen) and normalized with RNU6B. ΔΔCt method was used to analyze RT–qPCR data. Bars represent the mean value ±SD of three experiments.

**Figure 2.** Northern blot analysis of miR-517a expression in stably transfected HTR8-SV/Neo and primary human trophoblasts (PHT). The lower panel shows ethidium bromide-stained gel before transfer.
**Figure 3.** Phase contrast micrographs of non-transfected cells (HTR8 WT), cells stably transfected with the deleted BAC (HTR8 BAC Del), and cells stably transfected with the full length BAC driving the ectopic expression of C19MC miRNAs (HTR8 BAC C19). Original magnification: x 100.

**Figure 4.** Cell proliferation in HTR8-SV/Neo cells stably transfected with the deleted BAC (BAC-Del) or the full length BAC (BAC-C19). Cells were cultured in medium alone or in the presence of 500 mM CoCl to mimic hypoxia. After 24 h, cell proliferation was measured using MTS assay. The results represent the mean of 6 replicated wells (*P < 0.001).
Table 1: List of the C19MC miRNAs retained for target predictions. These miRNAs were determined to represent more than 95% of the total C19MC miRNAs expressed in primary trophoblasts based on by deep sequencing data.

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Figure 5. Validation of microarray data. Real-time PCR was used to validate the down- (Blue) or up-regulation (Orange) of 10 selected genes. The fold change was calculated by determining the expression of each gene in different transfected cell lines (Del: transfection with deleted BAC, #1: clone 1 high transgene expression, #5: clone 5- low transgene expression) relative to the expression in non-transfected cells (WT). Bars represent the mean value ±SD of three experiments.
**Research Project 4: Project Title and Purpose**

*Microtubule Post-Translational Modifications and Centrosome Dynamics During Mitosis in Normal and Cancerous Cells* - Microtubule defects and centrosome aberrations cause cancers and birth defects, since they induce chromosome aneuploidies after mitosis in both somatic and embryonic cells. They are responsible for inherited disorders and their functioning is essential for brain activities. Consequently, their activities span life from conception through death. While cell biologists a century ago were as familiar with the centrosome (‘the cell’s central body’) as they were with chromosomes (‘the cell’s colored bodies’ because they bind cytological stains), progress in characterizing the molecular constituents and mechanisms responsible for functional activities has paled when comparing centrosome molecular biology with chromosome molecular biology. Nevertheless, the essential roles of the centrosome for normal cell function are now incontrovertible and a panoply of diseases and disorders result from microtubule and centrosome dysfunctions or ‘centrosomopathies.’ In this project we will characterize the vital permanent molecules in the mitotic centrosome and discover which reside temporarily at the centrosome, along with the post-translational modifications of microtubules which occur during normal and cancerous cell cycles.

**Duration of Project**

1/1/2011 - 12/31/2011

**Project Overview**

In this project, we will examine the following specific aims with the objective to first define the basic molecular targets and later explore inhibitors which might uniquely target cancerous cells.

1. Do cancer cells display differences in the delta-2 post-translational modification of alpha-tubulin as compared with noncancerous cells?
2. Using dynamic confocal imaging with a living marker for centrioles, does GFP-centrin behave aberrantly in cancer cells as compared with controls?
3. Do the molecules which reside at the centrosome differ between cancer cells and controls?
4. Do the anticancer drugs currently used target these posttranslational modifications of alpha tubule, centrioles and/or these centrosomal molecular and can new drugs be discovered.

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Expected Research Outcomes and Benefits

Understanding the dynamics and molecular composition of the cell’s spindle poles, the centrosomes, in normal and cancerous cells, as well as the post-translational modifications of the microtubules, affords new targets for designing chemotherapeutic strategies. This may well translate into innovative cancer treatments. This also is of keen importance for understanding the basic biology of every cell during division.

Summary of Research Completed

The Aims of this research projection have not been changed or significantly modified during this reporting period.

Aim 1. Do cancer cells display differences in the delta-2 post-translational modification of alpha-tubulin as compared with noncancerous cells? This aim was completed in the first reporting period and no additional research investigations were performed on this specific Aim.

Aim 2. Using dynamic confocal imaging with a living marker for centrioles, does GFP-centrin behave aberrantly in cancer cells as compared with controls? In this reporting period, we successfully prepared our GFP-centrin (pEGFP-CETN2) construct (donated generously by Dr. Jeffrey Salisbury, Mayo Clinic, Mn). We used the ViraPower™ Lentiviral Packaging Mix (Invitrogen) to produce lentiviral particles and successfully transduced the WI 38 control lung fibroblast line as well as both NCI H292 and MCF7 cancer cell lines for GFP expression of centrin. All cell lines were grown in T-25 cell culture flasks. When cells were 30-40% confluent a solution of 60µls of concentrated centrin-GFP lentivirus and 6mg/mL of polybrene (Sigma) in complete culture media was added to the cells and incubated overnight at 37°C. Expression of GFP centrin was determined by inverted fluorescent microscopy. All lines were determined to be stably transduced based on GFP centrin detection following cell passaging as well as following cryopreservation and thawing for reconstituting cell lines in vitro (Fig. 1). We were able to detect cells with both normal centriole numbers (Fig. 1A-C: double arrows) as well as cells with over-duplicated centrioles (Fig. 1A-C: quad arrows) and at different stages of the cell cycle.

To explore centrosome behaviors between cancerous and non-cancerous lines, we next plated each cell line independently on 35 mm sterile iBidi dishes (Research Products International, Mt Prospect, IL) coated with 0.1% gelatin and permitted attachment and growth for 24-48 hrs at 37°C in a 5% CO2 incubator. Time-lapse video microscopy (TLVM; n= 9) was next performed on a T1 90 Nikon inverted microscope equipped with Perfect Focus to prevent Z-axis drifting during prolonged live-imaging. Exposure to fluorescent light during imaging was attenuated by using combined ND4 and ND8 filters to reduce photodamage. Image capture was accomplished using a Plan Fluor oil x100 objective (NA=1.3) using an Andor high resolution camera and Nikon Elements software for archival of data. Cells were placed in a Tokai stage incubator to
maintain temperature at 37°C and gas at 5% CO₂ throughout live cell imaging. Representatives of 9 TLVM trials are shown in Figure 2 and 3. Our experiments permitted us to image Centrin-GFP in cancer and non-cancer cells for extended times (up to 24 hrs) to observe centrin-GFP localization to centrosomes and how they functioned during a cell cycle, including mitosis and cell division. Figure 2 represents normal mitosis in a MCF7 cancer cell, showing GFP-centrin expression at single centrioles localized to each metaphase pole (Fig. 2A, arrowheads; double arrows: chromosome position). Subsequent frames over the next ~1.5 hr show this cell undergoing chromosome separation (Fig. 2B-F, arrows) and cell division (Fig. 2F, * denotes cleavage furrow). In Figure 3, we followed the fate of an interphase stage MCF7 cell with over-duplicated centrioles (Fig. 3A: arrowheads, pair of centrioles) during cell cycle progression. We observed all four centrioles first congressing within the cytoplasm (Fig. 3B: arrowheads) by 2 hrs after the start of TLVM imaging, followed by a rapid separation of centriole pairs (Fig. 3C-E: arrowheads) as this cell entered into mitosis within the next ~60 minutes (Fig. 3F). However, this cell failed to undergo cytokinesis even after 3.5 hours of further imaging and despite using attenuated light exposure (Fig. 3G-H). Eventually, this cell re-entered interphase and the centrioles became more closely aligned. We concluded that GFP-centrin was expressed properly at the centrioles and that these labeled cells could be followed dynamically for extended imaging periods to gain insight into centriole behavior during cell cycle progression.

To explore centrosome dynamics between cancerous and non-cancerous lines, we next performed Fluorescence Recovery After Photobleaching (FRAP) experiments using centrin-GFP expressing cells under live-cell laser confocal microscopy using a 4 laser point scanning Nikon A1 confocal microscope and Elements software. We typically used the UV (405 nm) laser line to photoablate single or pairs of centrioles at both interphase and mitosis, monitoring recovery of fluorescence (i.e., the migration of new centrin-GFP into the centrioles) over 5-10 minutes. Although the live cell DNA dye Hoechst 33342 was sometimes used to verify chromatin status, this compound was toxic to cell cycle progression after laser photoablation and thus we did not routinely employ this dye after initial experiments. For GFP-centrin labeled cells, we performed 11 Frap experiments with WI38 cell line (all interphase), 15 FRAP experiments with NCIH292 (8 interphase;7 mitotic), and 17 FRAP experiments with MCF7 (10 interphase: 7 mitotic). We also performed another 27 FRAP experiments with mCherry Tubulin labeled MCF7 (5 interphase:15 mitotic) and NCIH292 (4 interphase: 3 mitotic) cancer cells in this reporting period (data not shown). Figure 4 summarizes our findings from FRAP analysis of GFP centrin expressing cell lines. WI-38 GFP centrin labeled centrioles ablated with the 405 laser line generally saw full fluorescence recovery at centrioles within 10 minutes (Fig. 4A-D; n=4/11). Conversely, centrioles tagged with GFP centrin in cancer cells showed remarkably different dynamics, with no cells showing full GFP centrin fluorescence recovery in the centrioles between 5-10 minutes post-photoablation (NCI H292: Fig. 4E-H; n= 0/8 and MCF7: (Fig. 4I-L; n=0/10). These observations suggest that the centrosomes in cancer cells may be more unstable than noncancerous cells, with centrin localization easily altered during interphase and slow to recover when disrupted in the centriole lumen.

**Aim 3. Do the molecules which reside at the centrosome differ between cancer cells and controls?** In this reporting period, we continued our efforts to detect centrosome and centriole-specific affinity purified antibodies in WI-38, NCI H292, and MCF7 cell lines (Table 1). The overall goal of this aim has been to identify constituent proteins that are critical in centrosome...
assembly and function as well as to identify if any of these critical constituents vary between normal and cancerous cells. Pericentriolar material (PCM) components like γ-tubulin and pericentrin appear to be maintained in both noncancerous and cancerous cell lines. Here, we expanded our efforts by exploring if resident proteins involved in the initiation, elongation, and maturation of centriole assembly differ between normal and cancerous cell lines. Centrioles are microtubule-based organelles found at the core of the centrosome, which recruit microtubule nucleating factors that comprise the PCM and are critical to microtubule assembly. Although the exact role of the centrioles is still not fully known, recent work in the field of centriole biology has led to a number of discoveries on the mechanism of centriole assembly within duplicating human centrosomes. For this Aim, adhering cells on sterile coverslips were processed using one of three fixation methodologies: i. cold, absolute methanol for 8-10 mins; ii. 2% paraformaldehyde in warm culture media without serum for 15 min; and iii. permeabilization in a glycerol-based extraction buffer (Buffer M) containing 8% methanol and 1% Triton X-100 prior to absolute methanol fixation. All fixed cells were then blocked in phosphate-buffered saline (PBS) containing 150 mM glycine, 3 mg/ml bovine serum albumin, and 10% normal goat serum (PBS blocking solution) for a minimum of 1 hr before applying primary antibodies overnight at 4°C. After extensive PBS rinsing, appropriate fluorochrome-labeled secondary antibodies were applied overnight at 4°C. The penultimate rinse in PBS was performed in the presence of 10µg/ml Hoechst 33342 to detect the DNA prior to mounting coverslips in an antifade (Vectorshield, Vector Labs, Burlingame, Ca) and image analysis with the Nikon A1 confocal microscope. As summarized in Table 1, proteins involved in the assembly of the centrioles like Cep135 and HsSAS6 were strongly expressed in interphase and mitotic centrioles in all 3 cell lines tested. Likewise, proteins with known roles in microtubule (MT) capping, centriole anchoring, and scaffolding proteins for PCM proteins (ninein, cenexin1 and Cep 192) were largely conserved in noncancer and cancerous cell lines, although Cep 192 appeared more weakly detected in MCF7 breast cancer cells. Interestingly, we also observed that CP110, a protein involved in centriole duplication, was much more robustly detected in cancerous cell lines as opposed to control cell lines. Likewise, c-Nap1, involved in cohesion of parental centrioles, was not detected in either cancer cell line compared to the WI 38 control cell line. These observations are congruent with supernumerary centriole assembly and misalignment of centrioles found in many cancers cells. Collectively, these early observations suggest that specific centrosome molecules vary between noncancerous and cancerous cell lines and may provide interesting scientific avenues to investigate how defects arising in malignant cells contribute to mitotic errors and cancer onset.

Aim 4. Do the anticancer drugs currently used target these posttranslational modifications of alpha tubulin, centrioles and/or these centrosomal molecules and can new drugs be discovered? Novel targets for cancer therapy involving inhibition of histone deacetylation (HDACs) have recently been discovered. A unique member of the HDAC family, HDAC6, has been shown to target α-tubulin as its major substrate as opposed to histones, and thus it has been categorized as a tubulin deacetylase (TDAC). Reports showing over-expression of HDAC6 in a diverse group of tumors and cancer cell lines suggests that this protein has an important role in many cancers and drugs that target HDAC6 are now being investigated as new methods for cancer therapy. In this reporting period, we investigated a number of HDAC inhibitors (HDACi) on microtubule and centriole acetylation patterns, including Tubastatin A (TubA: 100nM-1µM), suberoylanilide hydroxamic acid (SAHA; 100nM-1µM), sodium butyrate (NaB; 100µM-1mM), and valproic acid (VPA; 500µM-1mM). All cell lines were treated for 48 hrs with various concentrations of...
HDACi’s prior to fixation in absolute methanol for 10 min. After rinsing, fixed cells were blocked in PBS blocking solution and immunostained with antibodies to Δ-2 tubulin, YOL 1/34, and acetylated α-tubulin as describe above. Analysis of all three cell lines revealed that HDACi did not affected Δ-2 tubulin expression at the centrioles (not shown) but did abolish centriole acetylated α-tubulin detection (Fig. 5). Conversely, only the known HDAC6 inhibitors TubA (Fig. 5D-F) and SAHA (not shown) increased spindle microtubule acetylation relative to untreated control cells while the non-HDAC6i NaB and VPA did not cause microtubule hyperacetylation (not shown). Collectively, these data suggest that inhibition of HDAC6 specifically affects centriole and microtubule acetylation patterns in cancer cells. Since microtubules and centrioles play significant roles in cell cycle divisions, HDAC6i may provide a novel mechanistic approach to understanding cancer onset at the cellular level.
Table 1. Detection of Centriole and Centrosome Markers in WI38, NCIH292 and MCF7 Cells.

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Figure 1. GFP centrin expression in control WI 38 lung fibroblast and two cancer cell lines, NCI H292 lung carcinoma and MCF7 breast cancer lines. A: WI 38 showing typical pair of centrioles as detected by GFP centrin construct expression (arrows). B: a pair of interphase NCI H292 cells showing a cell with a pair of centrioles (left image; double arrows) and another cell with four centrioles (right image; quad arrows). C: three interphase MCF7 breast cancer cells. The upper cell has four centrioles expressing GFP centrin (quad arrows), which are not tightly apposed in this cell. The far right hand cell has a pair of GFP centrin expressing centrioles tightly paired (dual arrows) while the bottom left cell has a pair of centrioles splitting apart (single arrows). This latter cell may be entering mitosis. Green: GFP centrin. Bars= 10µm.
Figure 2. *Time-lapse Video Microscopy (TLVM) of Mitotic GFP centrin MCF7 Breast Cancer Cell Line.* A-B: metaphase stage, with chromosomes aligned at cell center (double arrows) and GFP centrin-expressing centrioles (green) at each spindle pole (arrowheads). C-F: normal karyokinesis (arrows) ensues over the next ~38 mins, resulting in cell division (F: * denotes cleavage furrow) as GFP-centrin expressing centrioles (green) remain clearly visible at the spindle poles (arrowheads). Upper right of each panel: hours:minutes post imaging. Bar=1µm.

Figure 3. *TLVM of MCF7 Breast Cancer Cells Expressing 4 centrioles labeled with GFP centrin.* A: late interphase cell, with 4 GFP centrin expressing centrioles (green, arrowheads) visible in the cytoplasm. B: movement of the 4 centrioles in the cytoplasm occurs over the next 2 hrs. C-E: centriole splitting and migration (arrowheads) in the cytoplasm occurs rapidly just prior to mitosis onset (~26 min). F-G: mitosis following nuclear envelop breakdown. Note the centrioles pairs remain at their respective poles (arrowheads). H: cytokinesis failed after 2 hrs and the cell re-entered interphase. Note the centrioles pairs moved towards the cell center (arrowheads). Upper right of each panel: hours:minutes post imaging. Bar=1µm.
Figure 4. Dynamics of GFP Centrin Recovery following Fluorescent Recovery After Photobleaching (FRAP) in noncancerous and cancer cell lines. A-D: a WI38 noncancerous cell expressing GFP centrin at the centrioles (green; arrowhead). The centriole ablation region of interest (ROI) is shown in B (square box) while the full recovery of centriole GFP fluorescence over 10 min following laser ablation is depicted in C. The final appearance of GFP centrin-expressing centrioles after 10 min rescue is shown in D (arrowhead). E-F: NCI H292 cancer cell line with 4 centrioles observed by GFP centrin expression (E: arrowheads). After ablation of 1 centriole pair (F, circle), very little fluorescence recovery is observed over the following 10 minutes (G). H: centrioles expressing GFP centrin after 10 minutes rescue from ablation (arrowheads). Note that both centriole pairs demonstrate reduced GFP fluorescence suggesting cancer centrioles are less stable structures than controls. I-L: MCF7 breast cancer cell. Four centrioles are observed by GFP centrin fluorescence (I: arrowhead). All 4 centrioles were targeted for ablation (J, circle). Like the results for the NCI H292 cancer cell line, FRAP analysis indicated very little (<20%) rescue of GFP centrin in the centrioles over the subsequent 5 min (K). Final appearance of the GFP centrin centrioles post 5 min recovery is shown in L (arrowhead). M-N: a control WI 38 noncancerous cell expressing GFP centrin in the cytoplasm (M). A non-centriole region was selected for ablation (N: square box) and the full recovery of cytoplasmic fluorescence is shown employing the same settings as for WI 38 in the top panel (O; P: fluorescence image 5 min post ablation. N=nucleus. Bar=1µm.
Figure 5. HDAC6i Tub-A causes Loss of Centriolar Acetylation and Increases Spindle Microtubule Acetylation in Cancer Cells. A-C: non-drug treated mitotic metaphase cells showing acetylated alpha-tubulin localized to the centrioles (green, arrows). D-E: cells treated with 1µM Tubastatin A HDAC6i for 48 hrs prior to fixation. Acetylated alpha-tubulin is lost at the centrioles (arrows) and a dramatic increase in the spindle microtubule acetylation is observed (green) compared to drug-free controls. Insets: microtubules and DNA. Bar=1µm.