Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Magee-Womens Research Institute and Foundation

2. **Reporting Period (start and end date of grant award period):** 01/01/10 – 12/31/10

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Cheryl A. Richards, MBA

4. **Grant Contact Person's Telephone Number:** 412-641-8932

5. **Grant SAP Number:** 4100050901

6. **Project Number and Title of Research Project: Project**: 4 **-** The role of microRNA-424 in the differentiation and function of placental trophoblasts

7. **Start and End Date of Research Project:** 01/01/10-12/31/10

8. **Name of Principal Investigator for the Research Project:** Jean-Francois Mouillet, PhD

9. **Research Project Expenses**.

   9(A) Please provide the amount of health research grant funds spent on this project for the entire duration of the grant, including any interest earned that was spent:

   $ 264,688.00

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Mouillet	PI	50%	$63,923.23
Jennings	Technician	100%	$26,731.44
Sadovsky, Y	Collaborator	10%	$24,414.15
Sadovsky, E	Technician	100%	$2,644.00

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No___X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No___X_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

Several lines of experimental evidence point to a role of the miR-424 cluster in the biology of the placenta. We collected valuable information on these miRNAs during the year of funding of this project, yet the experiments conducted did not fully capture the full spectrum of activities of miR-424 cluster in placental trophoblasts. Because of their potential in shaping trophoblast physiology we will further interrogate the role of the miR-424 miRNA cluster using our molecular tools.

12. Future of Research Project. What are the future plans for this research project?

In the future we plan to develop two complementary approaches that will help us decipher the role of miR-424 cluster in placental trophoblasts. First, we will advance our effort to alter miR-424 levels in primary human trophoblasts. Our recent study has shown that miR-424 could be effectively neutralized using morpholino antisense oligonucleotides. This technology will be adapted for our primary trophoblast system. To complete this approach we plan to overexpress miR-424 using a lentiviral vector and analyze the consequences in trophoblasts. Second, we plan to produce a mouse model with a targeted deletion of miR-424. Gene targeting will allow us to completely remove miR-424 and therefore determine the developmental consequences of miR-424 deficiency.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____ No___X_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown				

Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other				
Unknown				
Total				

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No___X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes___X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

Our research enhanced technologies that address the epigenomic role of miRNAs in cell differentiation. As other researchers in our institute seek to utilize these techniques, our approaches will be instrumental for their pursuits.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X_____ No_____

If yes, please describe the collaborations:

Research in the field of placental miRNAs has bolstered a new collaboration with Carolyn Coyne from the Department of Microbiology and Molecular Genetics at the University of Pittsburgh.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No___X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No___X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant application's strategic plan). Summarize the progress made in achieving these goals, objectives and aims for the entire grant award period. Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Introduction

The small non-coding RNAs, referred to as microRNAs (miRNAs), have emerged as critical regulators of development and homeostasis. Their abundance and diversity in the human placenta, and particularly in the trophoblast layer, has been confirmed in a number of reports. The role of miRNAs in the placenta remains unknown, yet several lines of experimental evidence suggest that they are essential for its development and function. The goal of this project was to generate insight into the biology of miRNAs in placental trophoblasts, specifically into the role of MicroRNA-424 (miR-424), one of the predominant miRNA species in the placenta.

MiRNA-424 was recently identified in a microarray-based screen of miRNAs that were differentially regulated in primary trophoblasts cultured in hypoxia, a condition frequently associated with placental insufficiency and feto-placental injury. Microarray data revealed that miR-424 is one of the very few miRNA species whose expression is reduced in primary trophoblasts that are exposed to hypoxic conditions. We hypothesized that decreased levels of miR-424 are likely to cause de-repression of molecular targets, and may have functional consequences in trophoblasts. Consistent with this hypothesis we observed that two predicted miR-424 target genes, FGFR1 and MAP2K1, were upregulated in hypoxic trophoblasts.
In order to further investigate the role of miR-424 in the function of placental trophoblasts we designed the following specific aims.

> Aim 1: Analyze the impact of miR-424 on the cellular functions of primary trophoblasts
>
> Aim 2: Examine the expression of miR-424 and associated species in the human and mouse placenta

Summary of Research Completed

During the past year we have analyzed the expression and function of miR-424 in the placenta. Marked progress was made in advancing our knowledge related to the two specific aims.

Aim 1: Analyze the impact of miR-424 on the cellular functions of primary human trophoblasts

We first sought to validate two potential miR-424 target genes that were originally identified in a screen combining miRNA expression profiling data and in silico prediction of miRNA targets. Both FGFR1 and MAP2K1 contain miR-424 target sites within their 3'UTR, and the expression level of their corresponding transcripts were increased in hypoxic trophoblast. The presence of miR-424 target sites and the inverse relationship between the expression of the miRNA and the transcript designated these two genes as potential miR-424 targets.

Three miR-424 target sites within the 3'UTR of FGFR1 were predicted by the publicly accessible database TargetScan. The two 5'-proximal sites overlap, and were counted as one site in the experiments described herein (Fig. 1). In order to determine whether these elements mediate silencing by miR-424, we introduced inactivating mutations by PCR mutagenesis in the FGFR1-reporter construct and monitored the resulting luciferase activity.

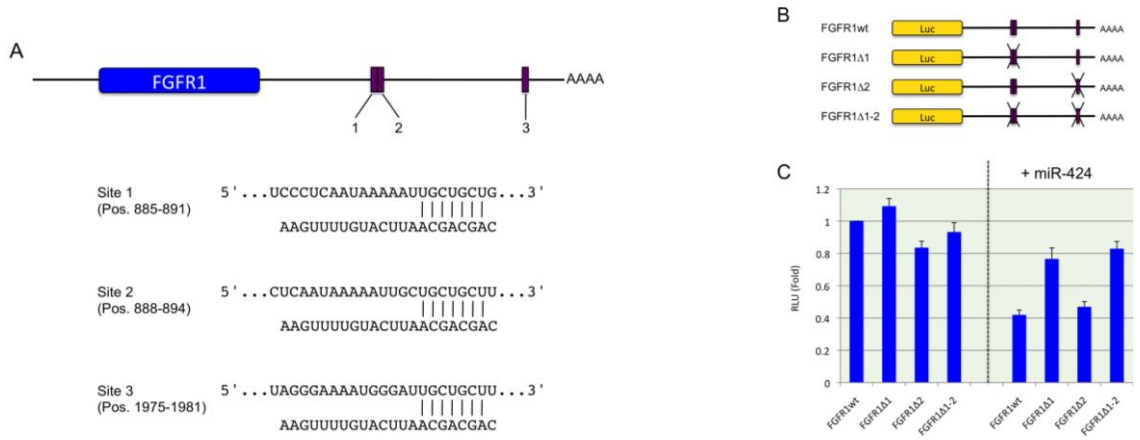

Fig. 1: A conserved target site mediates the influence of miR-424 on FGFR1 3'UTR. A) Schematic representation of the predicted miR-424 target sites within the 3'UTR of FGFR1 mRNA. B) Diagram of the mutations introduced in FGFR1 3'UTR and tested in our reporter assay. C) HTR8/SV cells were transfected with the wild type FGFR1 3'UTR reporter (wt) and with mutations in the proximal site (Δ1), distal site (Δ2), or a combination of the two elements (Δ1-2). Relative luciferase unit (RLU) activity was determined 48 h later. Data represent the mean of three independent experiments, performed in duplicate.

In our assay, inactivation of the proximal conserved binding site abolished the silencing by miR-424 while mutation in the 3'end non-conserved element did not affect the luciferase activity of the reporter (Fig.1). These data confirm the functional interaction between FGFR1 and miR-424, and indicate that the silencing of FGFR1 by miR-424 is entirely mediated by the conserved element, at least in the context of human trophoblasts.

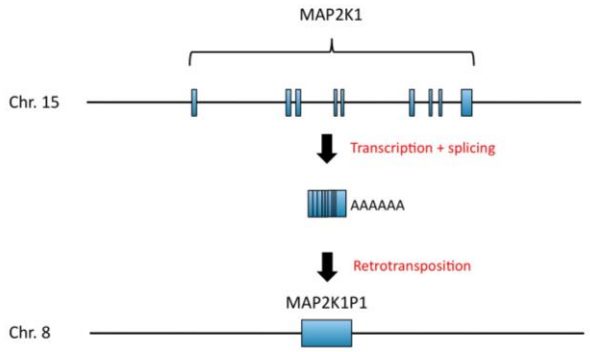

Fig. 2: Schematic representation of a model of MAP2K1 retrotransposition

Only one target site was predicted within the 3'UTR of MAP2K1 by TargetScan. Surprisingly, deletion of this site in our reporter construct did not suppress silencing by miR-424, hinting at the possibility of another yet unidentified target site. However, upon furether inspection of the cloned sequence we found several differences between the sequence of our clone and the published sequence of MAP2K1. A BLAST search showed a perfect match of the cloned sequence with MAP2K1P1, a pseudogene that is thought to have arisen by retrotransposition of a Map2k1 transcript (Fig. 2). The cloned sequence was obtained after PCR amplification using genomic DNA as template. Because of the extensive similarity between the two sequences, our PCR products likely contained DNA fragments derived from the two genes. Sequencing of additional clones confirmed this scenario and led to the identification of several true MAP2K1 clones. Curiously, despite the presence of a conserved target site, we did not detect any silencing by miR-424 in our reporter construct containing the correct MAP2K1 sequence. In contrast, the MAP2K1P1 reporter was effectively silenced by miR-424. The MAP2K1P1 sequence contains a discrete mutation in the first nucleotide corresponding to the miR-424 seed match, which is probably non-functional (Fig. 3). However, two other mutations in a downstream location recreate a perfect miR-424 seed match that mediates silencing in our reporter assay. This observation confirms the fact that a perfect seed match is essential for a functional target site but it also firmly demonstrates the importance of the sequence environment. It is also interesting to note that conservation across species is one of the criteria used in most algorithms for the prediction of miRNA target sites. Yet here we identified a highly conserved predicted target site, that manifests no evidence of functionality and a non-conserved site within a pseudogene carrying functional activity.

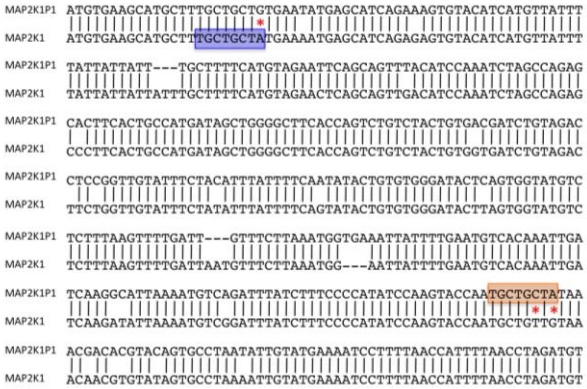

Fig. 3: Alignment between the sequences of selected MAP2K1P1 and MAP2K1 3'UTR regions. The blue box represents the miR-424 seed-match identified by TargetScan. The orange box shows the seed match we identified in MAP2K1P1. Stars highlight differences between miR-424 seed match s from the two genes.

As expression of a transcript from this pseudogene could potentially reduce the availability of miR-424 by acting as a decoy or a miR-424 sink, we sought to determine whether it is expressed in trophoblasts. Sequence-specific primers were designed and used in RT-PCR experiments to detect the expression of the two genes. Using this approach, we confirmed the presence of MAP2K1 transcripts in primary trophoblasts but failed to detect MAP2K1P1 (Fig. 4). We therefore did not further study MAP2K1.

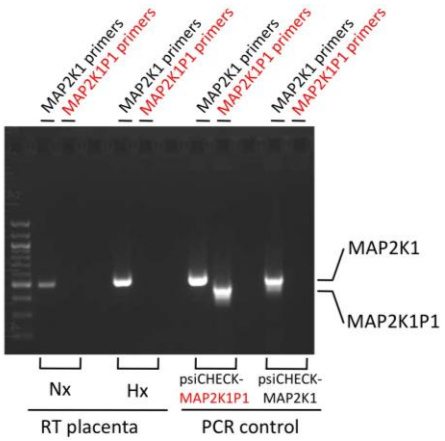

Fig 4: RT-PCR detection of MAP2K1 and MAP2K1P1 in trophoblasts cultured in normoxia (Nx) and hypoxia (Hx). Total RNA were reverse-transcribed using a poly-(dT) primer and PCR amplification was performed with sequence-specific primers as indicated. As a control for the specificity of the primers we used as templates our reporter constructs containing either MAP2K1P1 3'UTR or MAP2K1 3'UTR.

After validating the interaction between miR-424 and FGFR1, we sought to analyze the functional consequences of this interaction in trophoblast cells. We first assessed the influence of hypoxia on FGFR1 expression in primary trophoblasts. As predicted by the downregulation of miR-424 in hypoxic conditions, hypoxia led to a major increase in the accumulation of FGFR1 transcripts as determined by Real-time qPCR (Fig. 5A). Similarly, we observed a two-fold increase of FGFR1 at the protein level in western blot analysis (Fig. 5B). These observations show that FGFR1 is specifically upregulated in hypoxic trophoblasts and suggest that this is mediated, at least in part, by the down-regulation of miR-424.

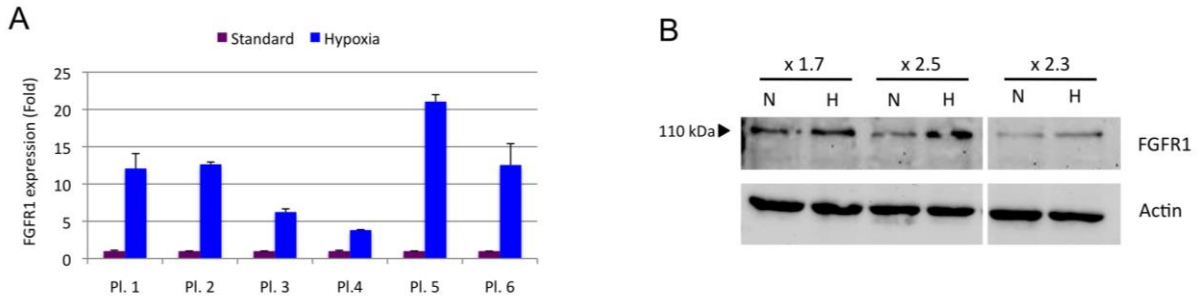

Fig. 5: Expression of FGFR1 in hypoxic trophoblasts. A) Real-time qPCR analysis of FGFR1 mRNA levels in primary trophoblasts from 6 different placentas (Pl.1 to Pl.6). B) Western blot analysis of FGFR1 (top panel) and actin (bottom panel) in primary trophoblasts isolated from three different placentas. Numbers above represent fold difference between band intensities, analyzed by densitometry.

Having uncovered a link between the decrease of miR-424 expression in hypoxic trophoblasts and the concomitant upregulation of one of its targets, FGFR1, we sought to assess the physiological relevance of this observation, and demonstrate that miR-424 is directly involved in the control of FGFR1 protein output in primary trophoblasts. We therefore elected to alter miR-424 activity in trophoblast using antisense inhibitor oligonucleotides. We first attempted to set up the system in the HTR8/SV cell line, which is easier to manipulate compared to primary trophoblasts. Increasing concentrations of an anti-miR-424 inhibitor from Dharmacon were

transfected along with the FGFR1 luciferase reporter. Upon transfection of the antisense oligonucleotide we observed a modest increase of the luciferase activity (Fig. 6), as predicted.

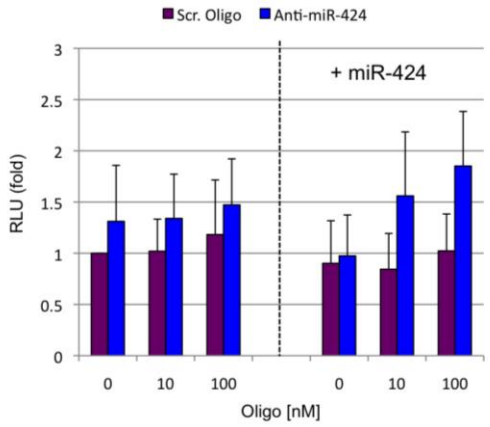

Fig. 6: Knockdown of miR-424 activity release silencing of FGFR1 reporter. HTR8/SV cells were transfected with the FGFR1 3'UTR reporter and an anti-miR-424 inhibitor or a scrambled oligonucleotide control (Scr. Oligo). Relative luciferase unit (RLU) activity was determined 48 h later. Data represent the mean of three independent experiments, performed in duplicate.

Perhaps not surprisingly, considering the low level of inhibition in a cell line, these inhibitors failed to significantly alter miR-424 levels in primary trophoblasts. Because modified antisense oligonucleotides form highly stable sequence-specific duplexes with their target miRNA sequences, they interfere with miRNA detection and cause a reduction of the signal intensity in RT-qPCR. However, we did not see any reduction of miR-424 signal by RT-qPCR, suggesting that endogenous miR-424 was not significantly impacted by the approach. Furthermore, miR-424 target, FGFR1 mRNA was also unaffected. We also tried LNA-based miRNA inhibitors in a new formulation (miRCURY LNA™ microRNA Power Inhibitors, Exiqon) for which we obtained a significant inhibition of several miRNAs in primary trophoblasts. However, miR-424 again appeared refractory to inhibition using this type of inhibitors.

Finally, we undertook an approach based on the use of miRNA sponges. The sponge strategy is based on the overexpression of a transcript containing multiples copies of a given miRNA target site that sequesters the targeted miRNA and neutralizes its activity. To assemble the sponge, two antisense oligonucleotides containing two miR-424 target sites with a bulge at position 9-12 (to prevent cleavage of the sponge transcript), were annealed and cloned into the pIS0 vector. Using a cloning strategy based on the compatible cohesive ends SpeI/XbaI, we sequentially entered six more sites into the vector. The sponge cassette containing eight miR-424 target sites was PCR-amplified and cloned downstream of a GFP reporter gene of the pCMV-d2eGFP vector. Although ultimately designed for expression from a lentiviral vector, we first decided to test the miRNA sponge efficiency using transient transfection in the HTR8/SV cell line. The sponge construct was transfected in HTR8/SV cells along with either a miR-424 sensor construct containing three perfectly complementary target sites, or the FGFR1 3'UTR reporter. Again, we observed consistent release of silencing with both reporter constructs although the effect was relatively modest, particularly with the FGFR1 3'UTR construct (Fig.7).

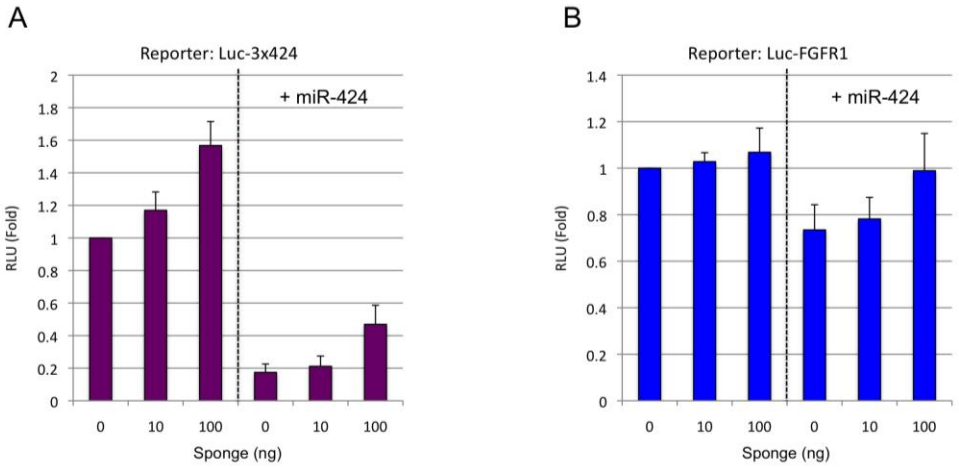

Fig. 7: Effect of the miRNA sponge on the activity of miR-424-mediated silencing of a luciferase reporter. HTR8/SV cells were transfected with a miR-424 sensor construct (A) or the FGFR1 3'UTR reporter (B) along with various amount of a plasmid expressing the miR-424 sponge. Relative luciferase unit (RLU) activity was determined 48 h later. Data represent the mean of three independent experiments, performed in duplicate.

Using higher amounts of transfected sponge resulted only in minor potentiation of the inhibition but also led to a drop of overall luciferase activity. This is likely a result of transcriptional interference due to the accumulation of high amounts of plasmid carrying strong viral promoters in transiently transfected cells. The apparent low level of miR-424 knockdown observed in the HTR8/SV cell line is problematic because it portends an even lower miR-424 suppression in primary cells. Without efficient neutralization of miR-424 it will be difficult to obtain a measurable phenotype and interrogate the function of miR-424 in trophoblasts. We have recently cloned the sponge into a lentiviral vector (pLemiR, Open Biosysems), and obtained very high transduction efficiency of primary trophoblasts using this system (over 90%), we have not tested it yet in the context of miR-424 inhibition. Lentiviral vectors can efficiently deliver transgenes to "difficult-to-transfect" cells, however they usually produce lower doses of transcript as a result of lower copy numbers compared to transient transfection. Therefore it is not clear whether or not lentiviral-mediated delivery of sponge constructs would efficiently neutralize miR-424 in primary trophoblasts. These experiments will be performed in the next several weeks, once we complete the optimization of our lentivirus system.

As an alternative, we plan to try another approach based on the use of Peptide Nucleic Acid-based miRNA inhibitors (PNA inhibitors). PNA are DNA mimics with repeating N-(2-aminoethyl)-glycine units linked by peptide bonds as a backbone. They bind to complementary DNA and RNA with high affinity and are resistant to nucleases. PNA oligonucleotides can be modified by the addition of a cell-penetrating peptide that allows efficient cell entry, even in primary cells. Recent studies have shown that these PNA-based miRNA inhibitors combine effective miRNA neutralization and low cell toxicity, even at high concentrations (Oh SY, Ju Y, Kim S, Park H, PNA-based antisense oligonucleotides for micrornas inhibition in the absence of a transfection reagent. Oligonucleotides. 2010;20:225-30).

Aim 2: Examine the expression of miR-424 and associated species in the human and mouse placenta.

The miR-424 gene is located on mammalian chromosome X in a region that contains other miRNA genes that span a short stretch of genomic DNA (Fig. 8).

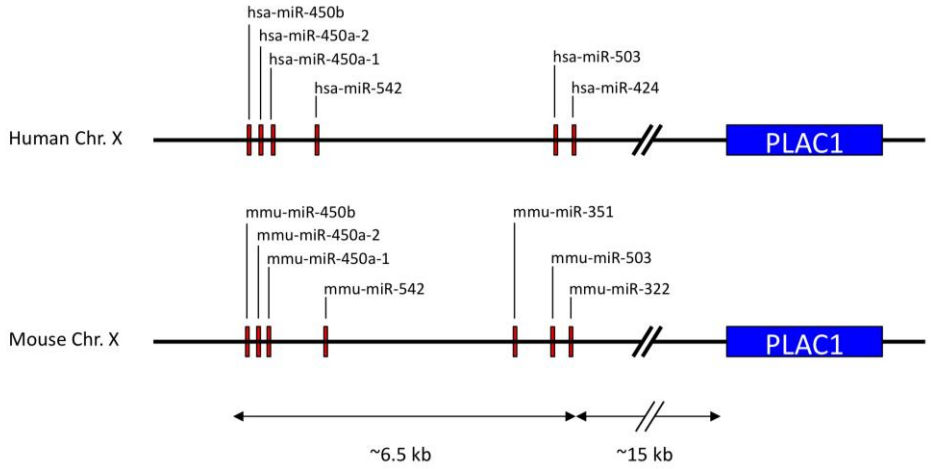

Fig. 8: Schematic representation of the genomic organization of the human and mouse miR-424 cluster depicting the relative position of the miR-424 cluster member genes as well as the placenta-specific gene 1 (PLAC1).

Expression patterns reported for miR-424 and the nearby miRNAs indicate that they are primarily expressed in the placenta. This observation suggests that they may belong to a cluster of co-regulated miRNAs. In humans, six miRNA genes have been identified within a 6.5 kb region of DNA. Several stem loop precursors code for two mature miRNAs (miR-424, miR-542, and miR-450b), while miR-450a-1 and miR-450a-2 produce the same mature miRNA. Therefore, this cluster potentially generates a total of eight potential mature miRNAs.

We used real-time qPCR to analyze the relative expression of the different miRNA species from this region in primary human trophoblasts cultured for 48 h in standard conditions (FiO_2=20%) or in hypoxia (FiO_2=1%). As expected, we observed a reduction of miR-424 levels in trophoblasts exposed to hypoxic conditions. Interestingly, we also detected lower levels of the associated miRNAs, strongly suggesting that they might be co-regulated (Fig. 9A). The assay also revealed that there is apparently a gradient in the expression of these miRNAs with miR-424 being the most abundant and the miR-450 species being the least abundant (Fig. 9B). This pattern of expression suggests the presence of a regulatory element located in the proximity of miR-424, whose influence fades as the regulated genes are further downstream. However, at this stage we do not know whether these miRNAs derive from distinct transcripts under the influence of a unique promoter element, or they are processed with variable efficiency from a unique primary transcript.

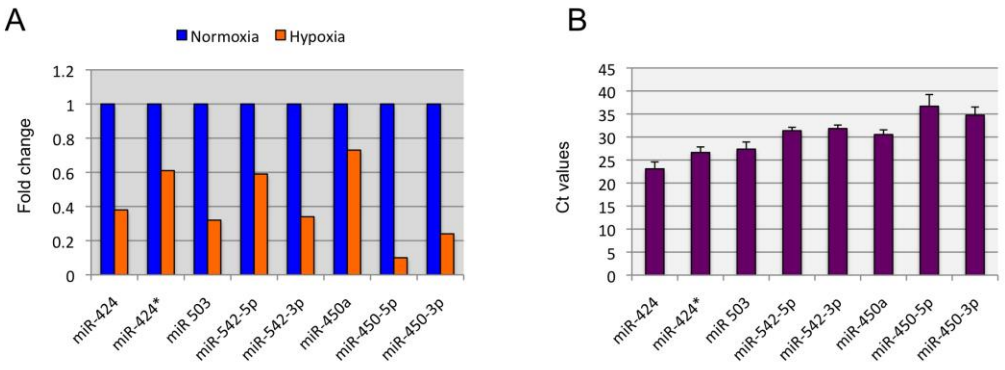

Fig. 9: Analysis of miR-424 cluster expression by real-time PCR. A) Downregulation of miR-424 cluster in hypoxia. Primary trophoblasts were cultured for 48 h in normoxia (20% O2) or hypoxia (1% O2). Expression level of individual miRNA was determined by real-time PCR using the miScript PCR System (Qiagen) and normalized with RNU6B. ΔΔCt method was used to analyse RT–qPCR data. Bars represent the mean value of four experiments. B) Relative expression levels of the different miRNA species from the miR-424 cluster. Bars represent the mean Ct value of four experiments.

It is important to note that although miR-424 downregulation is observed in hypoxia, it may not have a direct effect on the miR-424 gene. In fact, it is plausible that reduction of miR-424 levels in hypoxia is an indirect consequence of reduced oxygen levels. For example, time course analysis of miR-424 expression in primary trophoblasts show an effect only 48 h after cells were exposed to hypoxia (Fig. 10). A direct effect of hypoxia on miR-424 transcript may occur in a shorter time. Interestingly, 48 h is also when miR-424 expression increases in trophoblasts cultured in standard conditions. Primary trophoblast isolated from term third trimester placentas are mostly mononuclear cytotrophoblasts that differentiate into syncytiotrophoblasts in the presence of 20% oxygen. When cultured in low oxygen conditions the differentiation is severely impaired and most cells do not form syncytiotrophoblasts. This observation suggests that miR-424 might be associated with trophoblast differentiation. In these conditions, it would be expected to see a reduction of miR-424 expression in non-differentiating hypoxic trophoblasts. However, because miR-424 expression in hypoxia falls below basal levels, other mechanisms likely contribute to repressed miR-424 expression.

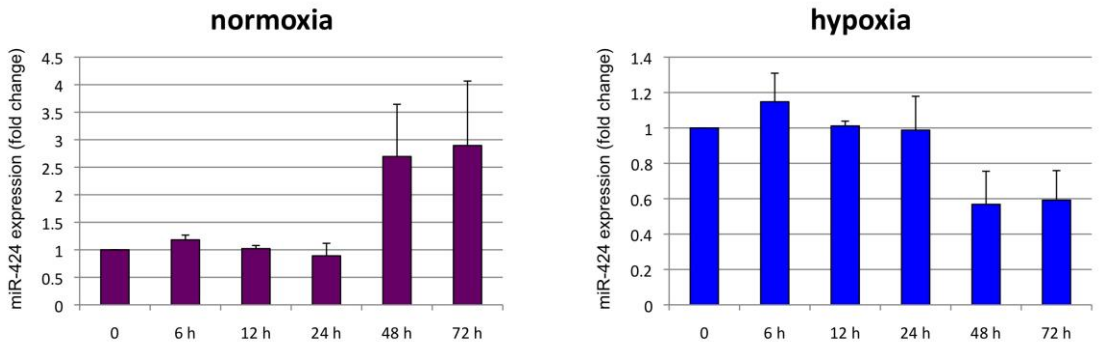

Fig. 10: Real-time PCR analysis of miR-424. Primary human trophoblasts were cultured for the indicated time in normoxia (FiO2=20%) or hypoxia (FiO2=1%). ΔΔCt method was used to analyse RT–qPCR data. The analysis was performed in duplicate on cDNA from trophoblasts from three different placentas using the miScript PCR System (Qiagen) and normalized with RNU6B.

MicroRNA-424 is highly conserved across mammals and, interestingly, the genomic organization of this region is conserved as well. Although the mouse homolog of miR-424 is referred to as miR-322, it differs from human miR-424 by a single nucleotide at position 21. The mouse could constitute a valuable model for the study of the function of these miRNAs. We therefore investigated their expression in diverse mouse tissues. Northern blot experiments performed using probes corresponding to several miRNAs from this cluster revealed that they are primarily expressed in the placenta although we also detected high levels in the mouse ovary (Fig. 11). Interestingly, the three miRNAs analyzed exhibit the same pattern of expression across tissues. This data further support the hypothesis that these miRNAs are co-regulated, and likely belong to a unique cluster.

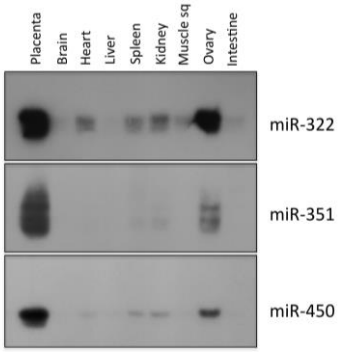

Fig. 11: Northern blot analysis of miR-424 miRNA cluster member expression in mouse tissues. Twenty micrograms of total RNAs were resolved on 15% denaturing poly-acrylamide gels, and transferred to nylon membranes. The blots were hybridized with ^{32}P-labeled probe complementary to the miRNAs indicated.

In order to examine whether hypoxic conditions can influence the expression of the mouse miR-322 cluster similarly to its human miR-424 counterpart we exposed pregnant mice to reduced oxygen and analyzed placental miRNA by real-time qPCR. In these experiments, pregnant mice were exposed to low oxygen (FiO2=12%) in a hypoxia chamber between gestational day 11.5 and 17.5. In this model of hypoxia, mouse fetuses are growth-restricted (by 15-25%). We

analyzed placental miR-322 and miR-503, which are the two most abundant miRNAs expressed from this region, which may facilitate the detection of small variations. We also examined expression of miR-210, a miRNA that is characteristically strongly induced in hypoxia and serves here as a marker of hypoxia. As expected, we observed a small change in the expression of miR-210 (Fig. 12) although the difference was statistically insignificant (p= 0.09, paired t-test). In contrast, we observed a significant increase of the expression of both miR-322 (p= 0.01) and miR-503 (p=0.008).

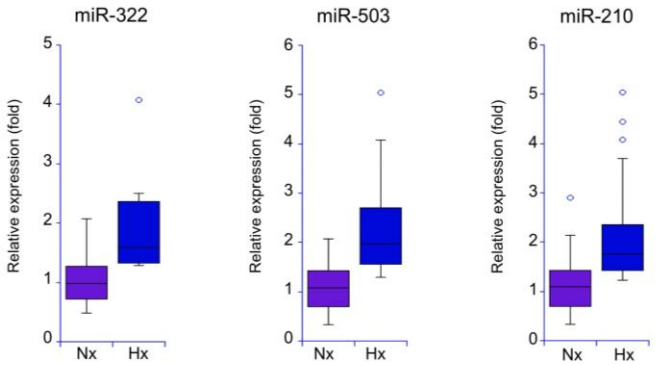

Fig. 12: Box plot comparing the expression of two miR-424 cluster members and miR-210 in placentas of pregnant mice exposed to low oxygen (Hx, n=10) relative to mice housed in standard conditions (Nx, n=11). The bottom and the top of the box indicate the 25 and 75 percentiles, respectively; the line in the box indicates the median value, and the whiskers indicate the minimum and maximum data values.

The effect of hypoxia on miR-424 cluster expression was somewhat surprising, but may reflect the profound differences between isolated cells and a complex organism in which many cellular circuits are inter-connected. In particular, miR-424 is known to be specifically upregulated by hypoxia in endothelial cells. In contrast to primary trophoblast cultures, the intact placenta contains many cell types, including endothelial cells in its rich vasculature. Therefore it is possible that miR-424 is down-regulated in mouse trophoblasts but this effect may be masked by the simultaneous increase of miR-424 in neighboring endothelial cells. Admittedly, we cannot rule out the possibility that despite the similarities between the human and mouse clusters, individual miRNAs may respond differently to various environmental cues.

Finally we performed *in situ* hybridization (ISH) to better delineate miRNA expression in the mouse placenta. Whole mouse placentas were collected on embryonic day 17.5 and pre-fixed in 4% paraformaldehyde (PFA) in PBS overnight, then in sucrose in PBS for an hour before mounting in Tissue-Tek OCT. Frozen sections (10 μM) were probed using (DIG)-labeled LNA-modified oligonucleotides (Exiqon) specific to miR-424/322. We also used probes against miR-205 and miR-335 as positive controls and a scrambled oligonucleotide as a negative control. Figure 13 shows representative photographs of mouse placenta sections stained with the different miRNA probes. These pictures reveal a weak staining for miR-322/424 compared to the staining observed with miR-205 and miR-335 probes, which show the same range of expression levels in the placenta. We currently pursue the significance of these findings.

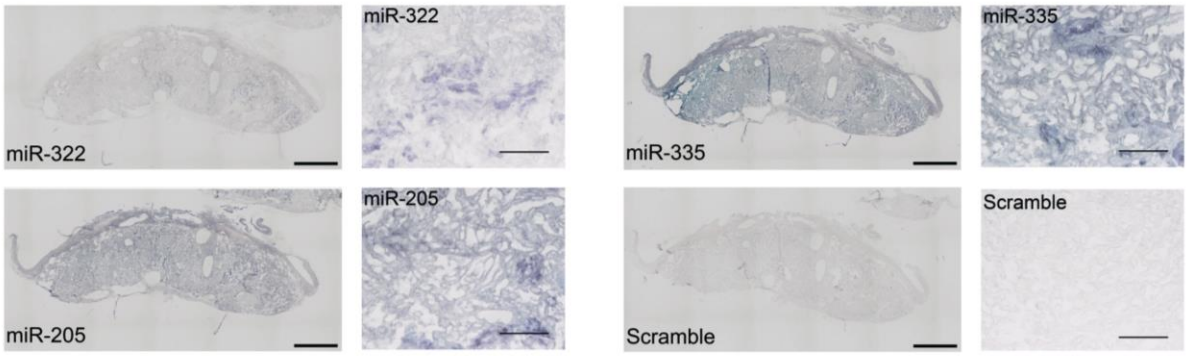

Fig.13: *In situ* hybridization of miRNA in the mouse placenta. In situ hybridization analyses using 5' DIG-conjugated, LNA-modified antisense probes for miR-322, miR-205, and miR-335 and a scrambled oligonucleotide control. After washing the slides were incubated with alkaline phosphatase conjugated anti-DIG antibody and detected using BM Purple Substrate. For each miRNA we show a picture at low magnification (40x, scale bar = 1 mm) and high magnification (200x, scale bar = 100 μm).

Conclusion:

We showed that expression of the miR-424 cluster is altered in trophoblasts exposed to a hypoxic environment. Interestingly, the discrete species located in the vicinity of miR-424 exhibit a similar pattern of expression. This strongly suggests a common mechanism of regulation and co-regulation of the same cellular targets. Although miR-424 seems to be the most abundant, the coordinated expression of the other miRNAs could have a significant impact for the modulation of molecular pathways involved in response of trophoblasts to environmental cues. As miR-424 expression levels are also clearly correlated with the differentiation status of trophoblasts, it is tempting to postulate that miR-424 is involved in the regulation of this key cellular event. To verify this possibility, we tried to neutralize miR-424 activity and monitor the effect on trophoblast differentiation. Despite our efforts using a variety of strategies we were not able to significantly alter endogenous miR-424 levels in primary trophoblasts, and therefore achieve a level of inhibition necessary to observe potential perturbations in trophoblasts. Admittedly, efficient neutralization of a given miRNA is rather difficult as miR-424 is highly expressed. It is also intriguing that ISH of miR-424 also turned to be the most challenging despite its abundance. Both ISH and knockdown techniques rely on the hybridization *in vivo* of an antisense oligonucleotide. Therefore it is possible that *in vivo*, miR-424 is rendered inaccessible to Watson-Crick base-pairing with a complementary nucleic acid due to its subcellular localization or binding with a putative RNA-binding factor. Although highly speculative, this would explain why detection of miR-424 poses no problem by Northern blot, RT-PCR, and microarray, techniques that are also based on probe hybridization but not on purified RNA. Although the knockdown approach should be amenable to improvement by optimizing several parameters or the use of alternative chemistry (PNA, or morpholino oligonucleotides), an interesting option would be to create mutant mice in which miR-322/424

levels are genetically altered. This could be achieved by generating a conditional, placenta-specific knockout of miR-322/424 in mice. We could also produce mice with a placenta-specific overexpression of miR-322/424 using lentiviral transduction of mouse blastocysts in order to restrict the transgene expression to the trophoblast lineage. These experiments may shed light on the contribution of this cluster in the development and function of the placenta.

We identified FGFR1 as a potential target gene of miR-424 in the placental trophoblast. We unambiguously identified the sequence within FGFR1-3'UTR that mediates its silencing by miR-424. We also demonstrated that targeting miR-424 results in increased stability of a mRNA controlled by the FGFR1-3'UTR. While the function of FGFR1 in the placenta remains unclear, preliminary experiments using siRNA against FGFR1 caused differentiation impairment with a significant decrease of hCG production. Therefore, FGFR1 might represent a pathway by which miR-424 influences trophoblast biology. Further assessment of miR-424 function will likely illuminate these possibilities.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X__No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication, listed in the table, in a PDF version 5.0.5 format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.				☐Submitted ☐Accepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X__ No_____

If yes, please describe your plans:

We plan the submission of a new manuscript based on our analysis of the miR-424 and its cluster of miRNAs. The funding from the Pennsylvania Department of Health will be appropriately acknowledged in any future publication arising from this work.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?

Yes_____ No___

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__ No___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No___X__

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Jean-Francois Mouillet, PhD	Instructor of OBGYN and Reproductive Sciences
eRA COMMONS USER NAME	
jfmouillet	

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Universite de Bourgogne, Dijon, France	BS	1988	Cellular biology
Universite de Bourgogne, Dijon, France	MS	1989	Animal physiology
Universite de Bourgogne, Dijon, France	PhD	1996	Molecular biology

Swiss Federal Institute of Technology, Zurich, Switzerland	Postdoctorate	1997-1999	Molecular biology
Washington University School of Medicine	Postdoctorate	1999-2005	Reproductive biology

A. Personal statement

Dr. Mouillet is a research scientist skilled in molecular biology, particularly in the area of regulation of gene expression. He has several years of experience in research on placental microRNAs. He also has knowledge and experience in generation of mouse mutant models, including "knockout" mice and transgenic mice using modified BAC plasmids.

B. Positions and honors

2005-2007	Staff Scientist, Department of OBGYN, Washington University
2007-2009	Research Instructor, Department of OBGYN and Reproductive Sciences, Univ of Pittsburgh
2009-Present	Instructor, Department of OBGYN and Reproductive Sciences, Univ of Pittsburgh

C. Selected publications

1. Lezzi M, Bergman T, Mouillet JF, Henrich VC. The ecdysone receptor puzzle. *Arch Insect Biochem Physiol* 1999;41:99-106.
2. Mouillet JF, Bousquet F, Sedano N, Alabouvette J, Nicolai M, Zelus D, Laudet V, Delachambre J. Cloning and characterization of new orphan nuclear receptors and their developmental profiles during *Tenebrio* metamorphosis. *Eur J Biochem* 1999;265:972-81.
3. Ou Q, Mouillet JF, Yan X, Dorn C, Crawford PA, Sadovsky Y. Steroidogenic factor 1 is a monomeric orphan, but does not work alone. *Endocr Res* 2000;26:1003-4.
4. Ou Q, Mouillet JF, Yan X, Dorn C, Crawford PA, Sadovsky Y. The DEAD box protein DP103 is a regulator of steroidogenic factor-1. *Mol Endocrinol* 2001;15:69-79.
5. Mouillet JF, Henrich VC, Lezzi M, Vogtli M. Differential control of gene activity by isoforms A, B1 and B2 of the Drosophila ecdysone receptor. *Eur J Biochem* 2001;268:1811-9.
6. Yan X, Mouillet JF, Ou Q, Sadovsky Y. A novel domain within the DEAD-box protein DP103 is essential for transcriptional repression and helicase activity. *Mol Cell Biol* 2003;23:414-23.
7. Mouillet JF, Sonnenberg-Hirche C, Yan X, Sadovsky Y. p300 regulates the synergy of steroidogenic factor-1 and early growth response-1 in activating luteinizing hormone-□ subunit gene. *J Biol Chem* 2004;279:7832-39.
8. Dorn C, Mouillet JF, Yan X, Ou Q, Sadovsky Y. Insulin enhances the transcription of luteinizing hormone-□ gene. *Am J Obstet Gynecol* 2004;191:132-7.
9. Donker RB, Mouillet JF, Nelson DM, Sadovsky Y. The expression of Argonaute2 and related microRNA biogenesis proteins in normal and hypoxic trophoblasts. *Mol Hum Reprod*, 2007;13:273-9.
10. Mouillet JF, Yan X, Ou Q, Jin L, Muglia L, Crawford PA, Sadovsky Y. DP103 (Ddx20) is essential for early embryonic development and modulates ovarian morphology and function. *Endocrinology* 2008;149:2168-75. PMC2329271
11. Mouillet JF, Chu T, Nelson DM, Mishima T, Sadovsky Y. miR-205 silences MED1 in hypoxic primary human trophoblasts. *FASEB J*, 2010;4:2030-9.
12. Mouillet JF, Chu T, Hubel CA, Nelson DM, Parks WT, and Sadovsky Y. The levels of hypoxia-regulated microRNAs in plasma of pregnant women with fetal growth restriction. Placenta 2010;31:781-4.
13. Mouillet JF, Chu T, and Sadovsky Y (2011) Expression patterns of placental microRNAs. Expression patterns of placental microRNAs. Birth Defects Research (Part A). In press.

D. Research support

Ongoing

Pennsylvania Department of Health (PI: Mouillet) 1/1/11–12/31/11
(Research Formula Funds)
Functional analysis of the C19MC microRNAS in trophoblasts
In this project we investigate the function of the placenta-specific, highly expressed microRNAs from the C19MC region.

NIH/NICHD R01 HD065893 (PI: Sadovsky, Co-Inv: Mouillet) 7/1/10–6/30/15
Placental injury and microRNA
The main objective of this proposal is to mechanistically decipher the action of miRNAs that are regulated during trophoblast adaptation to injury.

Completed

NIH/NICHD R21 HD053878 (PI: Sadovsky, Co-Inv: Mouillet) 8/1/06–7/31/08
The role of microRNAs in placental differentiation
This grant assesses the expression of microRNAs in human placental trophoblasts under basal conditions and in response to cellular stress.

Pennsylvania Department of Health (PI: JF Mouillet) 1/1/09–12/31/09
(Research Formula Funds)
Post-Transcriptional Regulation of Fstl1 mRNA in Human Trophoblasts
The focus of this project is to investigate the influence of hypoxia on the post-transcriptional regulation of Fstl1 in the human placenta, furthering our understanding of how cellular stress influences placental function, with its consequences on the developing fetus.

Pennsylvania Department of Health (PI: Mouillet) 1/1/10–12/31/10
(Research Formula Funds)
The role of microRNA-424 in differentiation of placental trophoblasts
Building upon our recent discovery that hypoxia regulates the expression of several microRNAs, this grant interrogates the expression and the role of miR-424 cluster in placental trophoblasts.