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):** 01/01/2011- 6/30/2012

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:** 4100054859

6. **Project Number and Title of Research Project:** 3 - Functional Analysis of the C19MC MicroRNAs in Trophoblasts

7. **Start and End Date of Research Project:** 01/01/2011-12/31/2011

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:

   $ 208,290

   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	100%	$41,708.34
Jennings	Technician	100%	$11,344.59
Sun	Technician	50%	$9,673.40
Sadovsky, E	Technician	50%	$14,140.50

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
Sadovsky, Y	Co-investigator	As needed
Chu	Consultant	As needed

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
None		

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:
None	☐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:

Promising results were obtained during this year of funding. We obtained experimental evidence that the C19MC miRNAs are involved in several aspects critical to the biology of placental trophoblasts. We also made recently a fundamental discovery about how these miRNAs can protect the placenta from viral infection. This finding could have direct implications in the clinic but further experiments are required to fully establish its therapeutic potential.

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

With 46 genes producing highly related miRNAs, the C19MC cluster is a complex genetic cluster. Therefore, multiple approaches will be necessary to capture the extent of its activities. We started by developing a cellular model that is more amenable to experimentation compared to primary cells. We need to further analyze these cells to better characterize and buttress our conclusions. A mouse transgenic model was also recently established and is being analyzed in detail. We plan to use these models to carefully dissect the function of the different units from this family of miRNAs.

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__X__ No_____

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

    Recently, we recruited a postdoctoral researcher to pursue the research initiated with this grant. Dr. Lan Xie obtained her PhD from Qing-Hua University in China.

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 period that the project was funded (i.e., from project start date through end date). 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 discovery of regulatory, small, non-coding RNAs during the past decade has contributed to a deeper understanding of the complex mechanisms involved in gene regulation and cellular physiology. Among small RNAs, the family of microRNAs (miRNAs) is probably the most diverse and the best characterized. Recent studies have unraveled some of the mechanisms by which miRNAs exert their function, and have shed light on the critical role they play in physiology and pathology. Despite these advances, the biological functions of many miRNAs remain poorly understood.

The placenta is the major source of a family of miRNAs originating from the largest human cluster of miRNA genes. This cluster, located on chromosome 19 and referred to as C19MC, encodes 59 mature miRNAs and produces the most abundant set of miRNA molecules in human placental trophoblasts. Although primarily expressed in trophoblasts, experimental evidence indicates that they are also expressed in certain types of cancers, where they promote cell proliferation and invasion.

The diversity and abundance of the C19MC miRNAs suggest that they harbor an important regulatory role, however, their biological function in the placenta remains completely unknown. Our goal was to explore the function of this family of miRNAs in the context of trophoblast biology. Two specific aims were designed to investigate the role of C19MC miRNAs in placental trophoblasts.

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

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

Summary of Research Completed

During this funding period we followed two independent strategies to probe the biological function of the C19MC miRNAs. In the first strategy we used chemically modified antisense oligonucleotides to deplete discrete members of this family of miRNAs, and examined the consequences of this inhibition on the function of primary human trophoblasts. As a part of the second strategy, we established HTR-8/SVneo cell lines stably transfected with a modified BAC plasmid that contained the entire C19MC miRNAs. Although HTR-8/SVneo cells are derived from human placental extravillous trophoblasts, they do not express these miRNAs endogenously. We also created a HTR-8/SVneo line transfected with a BAC in which the sequence encoding the C19MC miRNA genes was removed by recombineering. Finally, we investigated the impact of the C19MC miRNAs on the transfected cell transcriptome by comparing gene expression profiles, using microarrays.

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

As outlined in our research plan, the initial strategy to suppress the function of C19MC miRNAs was to use specific decoys, termed “miRNA sponges”. These inhibitors are based on the expression of transcripts containing multiple antisense miRNA binding sites, designed to titrate

off endogenous miRNAs, resulting in the inhibition of their silencing activity. To this end, we produced a series of constructs harboring up to 16 copies of antisense binding sites for several C19MC miRNAs. Although initially engineered for expression from a lentiviral vector, we first tested our sponge after cloning it into a pcDNA3 expression plasmid that could be tested in transient transfection along with a luciferase reporter construct. The luciferase reporter (or sensor) was produced by cloning an oligonucleotide containing three reverse complementary sequences to a given miRNA into psiCHECK2 (Promega). We were able to observe a small (20%) increase of the reporter activity in the presence of the sponge, which was insufficient for a substantial reduction of miRNA silencing activity in primary trophoblasts. While the cause for this low inhibiting activity is unknown, it may reflect an insufficient miRNA-binding affinity. We note that it is difficult to assess the real affinity of miRNAs for a particular sponge and a suboptimal design may not permit the sponge to efficiently sequester these abundant miRNAs.

We next tried to suppress the C19MC miRNAs using direct transfection of synthetic antisense oligonucleotides. Similar to miRNAs sponges, these antisense oligonucleotides [termed anti-microRNA Oligonucleotides (AMOs) or antimiRs] bind to cognate miRNAs and sequester them away from their natural targets, leading to target derepression. One of the advantages offered by the antisense technology resides in the chemical modifications that render these oligonucleotides extremely resistant to nuclease activity while increasing their binding affinities. A variety of antimiR oligonucleotides based on different chemical modifications are now commercially available. For our study, we used the miRCURY LNA™ microRNA Power Inhibitors from Exiqon that have yielded good performance in many cellular systems. We selected several miRNAs, chosen to originate from different locations on the C19MC locus and that are among the most highly expressed, as they are likely to have the strongest impact on gene expression. We obtained inhibitors for the following set of miRNAs: miR-525-5p, miR-520c-3p, miR-519d, miR-518b, miR-518a-5, miR-517a, and miR-512-5p. These antimiRs were transfected into primary human trophoblasts using the transfection reagent DharmaFect 1 from Dharmacon. Cells were also transfected with a scrambled oligonucleotide as a control (miRCURY LNA™ microRNA Inhibitor Negative Control A). To assess miRNA inhibition we first measured miRNA level changes by RT-qPCR. Although the results showed impressive reduction of the levels of the targeted miRNAs (more than 80% in cells exposed to antimiRs), this might have been due to high stability and high affinity of residual LNA-oligonucleotides, interfering with the detection step of the RT-qPCR. To better quantify miRNA inhibition we used a functional assay based on the co-transfection of a miRNA sensor construct along with a specific miRNA inhibitor. Results of luciferase assays performed using primary trophoblasts show a significant increase of the luciferase activity in cells that received the miRNA inhibitors. However, despite the major derepression of the reporter gene, we did not observe any phenotypic changes (differentiation, hCG production) in the primary trophoblasts. We also challenged the cells by culturing them in hypoxic conditions (O_2 <1%) for 48 hours, but again no difference was observed between cells that were transfected using a specific anti-miR or a control oligonucleotide.

In the absence of an altered phenotype in C19MC miRNA-blocked trophoblasts we used microarrays to interrogate gene expression that might reveal discrete changes that do not necessarily translate into phenotypic variation. We performed several microarray analyses and detected a small number of differentially expressed genes but none of them were confirmed by

subsequent analyses. In addition, we observed large experimental variation between biological replicates, which likely contributed to the difficulty of identifying potential targets for these miRNAs. One of the serious limitations of this approach resides in the difficulty of transfecting primary trophoblasts with high efficiency. The luciferase assay performed on antimiR-transfected cells indicated a potent inhibition, suggested by the robust increase of luciferase activity. However, these miRNA-depleted cells represent only a fraction of the cells in each well as our estimation of transfection efficiency indicates that less than 10% of the cells were transfected. This observation, combined with the high natural variability observed in any primary cells, suggests that unless suppression of a single C19MC miRNA can severely alter the phenotype it would be difficult to detect more subtle differences. These observations emphasize the need to optimize the miRNAs sponges that can be delivered to primary cells with high efficiency using a lentiviral vector. We are currently in the process of developing a better miRNA sponge that can be used to interrogate the function of the C19MC miRNAs in primary trophoblasts.

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

In this second aim we sought to complement the knock-down of discrete C19MC species, and investigate the impact of the whole C19MC miRNA cluster, transfected into cells. We used the HTR-8/SVneo cell line instead of primary cells. This line was derived from extra-villous placental trophoblasts that were immortalized via SV40 virus transformation (a gift from Professor Charles Graham, Toronto, Canada). In contrast to most trophoblast-derived cells, including JEG3, BeWo, and JAR, HTR-8/SVneo cells do not express the C19MC miRNAs endogenously. We took advantage of this feature, and created new lines expressing the C19MC miRNAs by stable transfection. We then analyzed the impact of the C19MC miRNAs by comparing cells that express these miRNAs to cells that were transfected with a BAC lacking the C19MC miRNA genes (C19-Del).

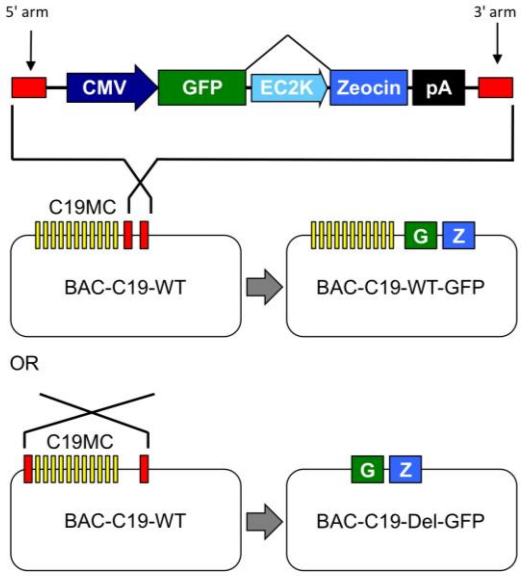

Fig. 1: Schematic representation of the BAC and the recombineering strategy. The upper panel depicts the approach to insert the tagging cassette into the C19MC miRNA expression BAC. The lower panel shows the BAC ”trimming” that permits the insertion of the tagging cassette while removing the sequence coding the C19MC miRNAs.

From the BACPAC Resource Center at the Children's Hospital Oakland Research Institute in Oakland, California, USA (http://bacpac.chori.org), we obtained a BAC plasmid (RP11-1055O17) containing the entire C19MC miRNA cluster spanning around 100 kb and an additional 60 kb of flanking sequences. The BAC was modified by recombineering to insert a tagging GFP reporter as well as the Zeocin resistance gene for stable transfection (Fig. 1). A GFP::zeocin cassette from the pSELECT-GFPzeo-mcs plasmid (InvivoGen, CA), was PCR amplified and cloned into pBluescript II SK(+) (pBS-SK). BAC specific homology arms of ~500

bp each were PCR amplified and cloned into pBS-SK using the restriction sites flanking the GFP::zeocin cassette. The entire targeting cassette was then PCR amplified and electroporated into the recombinogenic SW106 bacterial strain containing the recipient BAC. In addition, we created a BAC with a deletion of the sequence encoding all the miRNAs from the cluster, and used it as control in our transfection experiments. The control construct is referred to as BAC-C19-Del-GFP and contained ~60 kb of genomic DNA flanking the GFP::zeocin cassette. Bacteria harboring the BAC with the desired alteration were recovered on selective medium containing chloramphenicol and zeocin. We further confirmed that the modified BAC contained the intended recombination by restriction mapping and PCR. The correct BAC constructs were transformed back into DH10B bacterial cells for propagation. BAC DNA for transfection was prepared using the PhasePrep BAC DNA kit following the recommendations from the manufacturer (Sigma).

We used transient transfections to verify that the modified BAC can drive the expression of the C19MC miRNAs in HTR-8/SVneo cells. Delivery of BAC DNA was performed using a combination of polyethylenimine (PEI) and defective adenovirus particles, known to enhance transfection of large BAC plasmids into cells. After 48 h of incubation we isolated the RNA and used RT-PCR to detect a small region upstream of the first C19MC miRNA that corresponds to a segment of the pri-miRNA transcript (Fig. 2). We then generated stably transfected cell lines that were selected using Zeocin.

Stably transfected HTR-8/SVneo cells were established as described in the research plan. Briefly, cells were transfected using a mixture of PEI and Virofect and selection was carried out in 10-cm dishes for two weeks with 200 μg/ml Zeocin. Resistant clones were selected and expanded. We also generated stably transfected cells that were kept as a mixed population. We used qRT-PCR to determine the expression of several C19MC miRNA species from across the C19MC locus, and that are expressed at various levels. The results indicated an obvious difference in expression level of transgenic miRNAs among the different clones. Except for miR-517a, which showed a strong signal in the mixed population, all others indicated that clone # 1 exhibit the highest ectopic expression of C19MC miRNAs (Fig. 3).

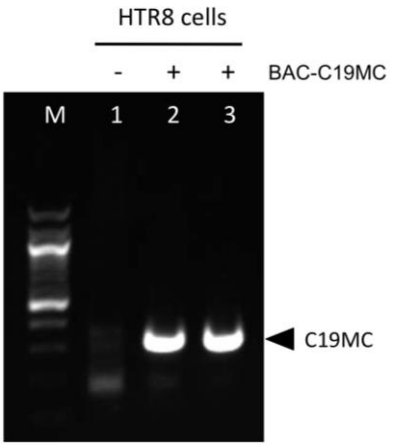

Fig. 2: RT-PCR analysis of parental HTR-8Sv/Neo cells and cells transfected with the modified BAC plasmid. Lane 1 corresponds to mock-transfected cells. In lanes 2 and 3, the cells were transfected with the BAC containing the intact C19MC locus.

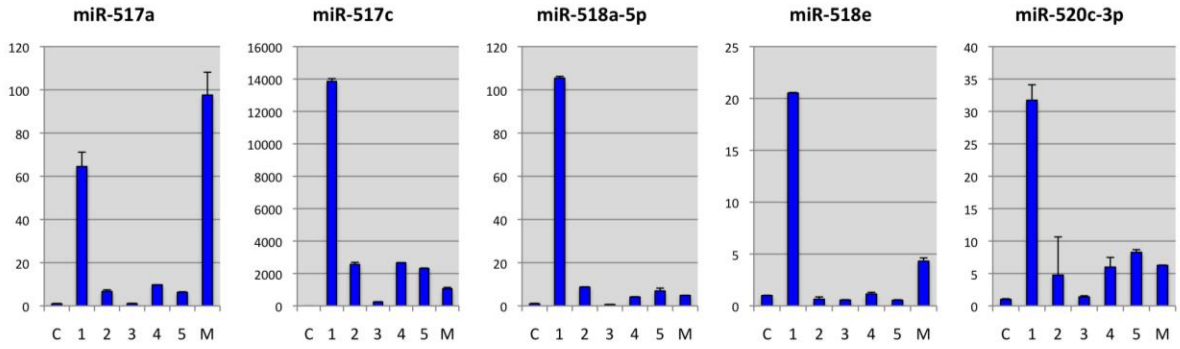

Fig. 3: RT-qPCR analysis of C19MC expression miRNAs in stably transfected HTR-8/SVneo cell lines (C: non-transfected cells, 1 to 5: stable clones, M: stable mixed cells). Relative expression level of individual miRNA was determined using the miScript PCR System (Qiagen) and normalized with RNU6B. Bars represent the mean value ±SD of three experiments.

Northern blot analysis confirmed the appropriate expression of miR-517a in the correct clones, but the differences were not as pronounced as those seen with the quantitative PCR analysis, (Fig. 4). Based on the qPCR data we selected clone # 1 for further experiments. We noted that the level of C19MC miRNAs in transfected HTR-8/SVneo cells was lower than the level typically observed in primary trophoblasts. The molecular basis for these differences might reflect (a) the integration of the transgene in an unfavorable genomic environment or subject to epigenetic silencing, (b) an inadequate concentration of specific cellular factor required for the expression of the C19MC miRNAs, (c) suboptimal processing of the precursors into mature miRNAs, or (d) inappropriate selection of transfected cells based upon their resistance to the selective drug rather than the expression of the transgene.

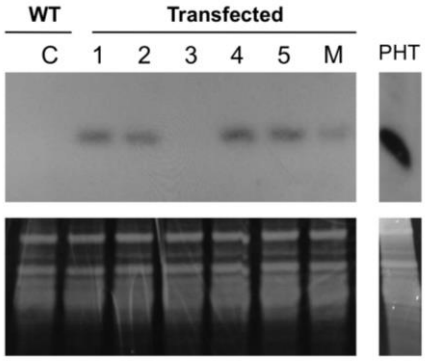

Fig. 4: Northern blot analysis of miR-517a expression in stably transfected HTR-8/SVneo and primary human trophoblasts (PHT). The lower panel shows ethidium bromide-stained gel before transfer.

Interestingly, we detected a change in the morphology of C19MC-expressing cells when compared to either the parental wild-type cells or the cells transfected with the deleted BAC (Fig. 5). The cells expressing the C19MC miRNAs appeared more compact and exhibited fewer cellular protrusions, particularly the cells located at the edge of a cell cluster. The cells at the periphery tended also to be smaller compared to the same subpopulation of cells in control dishes. These observations suggest that cell migration or other characteristics might be altered in cells expressing the C19MC miRNAs. We currently investigate migration and invasion processes using *in vitro* wound closure assay and Matrigel invasion assays respectively. Lastly, we noted a

longer doubling time of the cells expressing the C19MC miRNAs. This observation was confirmed using a MTS assay (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega). Further studies are required to clarify these observations as cell proliferation can be influenced by many factors.

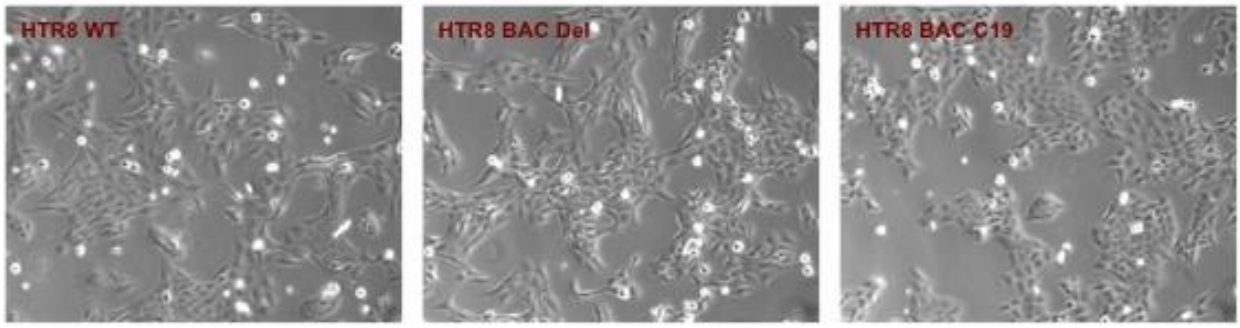

Fig. 5: Phase contrast micrographs of non-transfected cells (HTR8 WT), cells stably transfected with the deleted BAC (HTR8 BAC Del), and cells stably transfected with the full length BAC driving the ectopic expression of C19MC miRNAs (HTR8 BAC C19). Original magnification: x 100.

To better characterize the changes induced by ectopic expression of the C19MC miRNAs at the molecular level we analyzed gene expression profiles using microarrays (n=3). Non-transfected parental cells and cells transfected with the BAC-C19-Del were used as control. After selection for genes that were differentially expressed in cells expressing the C19MC miRNAs, we identified a set of 601 downregulated genes and 589 genes that were upregulated. Even prior to considering which of these might be a direct miRNA target, we examined miRNAs that were differentially expressed between the two conditions (BAC-C19-Del vs BAC-C19-WT). Using the Ingenuity Pathway Analysis, the top Bio function identified in the category diseases and disorders was “Cancer” (p-value = 6×10^{-7}). Similarly, the first class of Molecular and Cellular Functions associated with the downregulated genes was “Cellular Movement” (p-value = 9.9×10^{-9}). Thus, the result of this analysis seems compatible with the previous observation, suggesting an anti-proliferative function of the C19MC miRNAs in this cellular context. It is also not consistent with previous suggestion that ectopic expression of C19MC miRNAs is associated with higher proliferation rates and cancerous states. However, high C19MC miRNA expression is not always associated with a tumorigenic state as placental trophoblasts, including syncytiotrophoblasts, express large amounts of these miRNAs although they are terminally differentiated and do not proliferate.

We selected a set of ten genes to conduct a small-scale validation of the microarray data using RT-qPCR. Among these, six were chosen among the downregulated transcripts and four among the upregulated genes in C19MC miRNA-expressing cells.

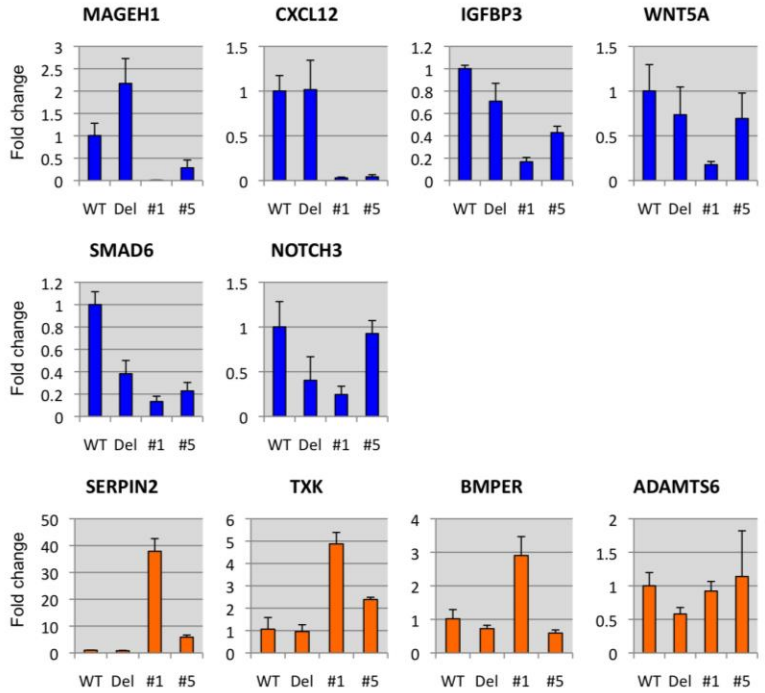

Fig. 8: Validation of microarray data. RT-qPCR was used to validate the down- (blue) or up-regulation (orange) of ten selected genes. The fold change was calculated by determining the expression of each gene in different transfected cell lines (Del: transfection with deleted BAC, #1: clone 1 high transgene 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.

Four of the six repressed genes (MAGEH1, CXCL12, IGFBP3, and WNT5A) showed the expected expression change by RT-qPCR (Fig. 8). Interestingly, in each case, the trend was more pronounced in clone #1 compared to clone #5. No clear pattern emerged from these experiments for the two other genes (SMAD6 and NOTCH3), as they were also significantly reduced in cells with the deleted BAC. The first gene in the list of clearly repressed genes, MAGEH1 (Melanoma antigen family H, 1), was originally identified in tumors and the placenta, yet several members of the MAGE family have been found in non-tumorigenic tissues. Function is not fully characterized; it may be involved in apoptosis. Interestingly, CXCL12 and IGFBP3 are highly expressed in extravillous trophoblasts or cancer cells, but were substantially reduced in HTR8 cells transfected with the BAC-C19-WT construct. CXC12 (Chemokine (C-X-C motif) ligand-12) is a cytokine that is highly expressed by invasive trophoblasts, but not in cytotrophoblasts or syncytiotrophoblasts. Multiple functions have been assigned to CXCL12 in the placenta including cytotrophoblast invasion, differentiation, survival, and recruitment of NK cells. In cancer, CXCL12 promotes tumor cells migration and invasion. We also confirmed the upregulation of three genes among those that were significantly upregulated in C19MC miRNA-expressing cells (SERPINE2, TXK, and BMPER). In particular SERPINE2 was dramatically induced. SERPINE2 is a member of the superfamily of serine protease inhibitors that is highly expressed in the human placenta. It has been shown to be important for the invasion and remodeling capabilities of extravillous trophoblasts. Yet, the role of SERPINE2 in syncytiotrophoblasts is unknown. Our results suggest that ectopic expression of the C19MC miRNAs could interfere with the invasiveness of extravillous trophoblasts.

We next sought to identify the genes that are targeted by transgenic C19MC miRNAs. Analysis of the miRNA landscape of placental trophoblasts by deep sequencing revealed that only a subset of the 59 C19MC miRNAs is expressed at a significant level. Based on these data we included in our analysis 29 miRNA species that account for more than 95% of total C19MC miRNA

expression in trophoblasts. This allowed us to eliminate 32 miRNA that likely had a minimal contribution to silencing. We identified the predicted targets of these 29 miRNAs using three algorithms: Target Scan 6.0, miRDB 4.0, and microrna.org (August 2010 release). While miRDB and microrna.org have predictions for all 29 miRNAs, four out of the 29 miRNAs were not included in TargetScan. We also noticed that three of these four miRNAs (miR-518e-5p, miR-522-5p, and miR-523-5p) have seeds identical to other C19MC miRNA families, and decided to use the TargetScan prediction of the corresponding miRNA families for these three miRNAs. We then identified, for each miRNA, the list of target genes that are predicted by all three algorithms. The only exception is miR-517-5p, for which we used the prediction from miRDB and microrna.org only, because TargetScan does not include this miRNA. Finally, a total of 4419 predicted targets for the C19MC miRNAs were identified using this approach. Among these, we identified 1030 genes that were significantly downregulated in cells expressing the transgenic miRNAs.

Interestingly, MAGEH1 and CXCL12, which were among the selected genes for the validation of microarray data, are also found among the predicted targets of the C19MC miRNAs. TargetScan indicates the presence of several potential C19MC miRNA target sites within the 3'UTR of these transcripts (Fig. 9).

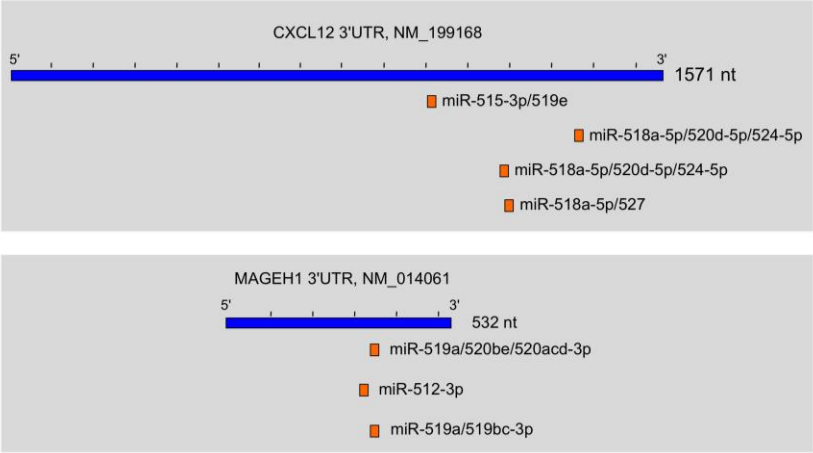

Fig. 9: Schematic illustration of predicted C19MC miRNAs targeting the 3'UTR of CXCL12 and MAGEH1 (Adapted from TargetScan).

Conclusion

During this funding period we have successfully created a cell-based model that showed great promise in our search of the function of C19MC miRNAs in placental trophoblasts. Our system of HTR-8/SVneo cells, stably transfected with a BAC containing the entire cluster of miRNAs, led to very exciting findings regarding the role of this large family of miRNAs in cell invasion and proliferation, both in terms of gene expression and functional assays. The similar invasive pattern of the human placenta and tumors is well established, and many placental genes are commonly expressed in a number of tumors. Unlike cancer, the proliferation and invasion properties of trophoblasts are under tight control. The nature of the molecular mechanisms deployed to exert this regulation on trophoblasts is not known. Our observations raise the

exciting possibility that the C19MC miRNAs may have evolved the ability to regulate the proliferation and invasion of trophoblasts during primate and human pregnancy.

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

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

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 or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) 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. None				☐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 function of the C19MC 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__X____ No_____

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: Primary human trophoblasts and the transfer of viral resistance

b. Name of Inventor(s): Yoel Sadovsky and Carolyn Coyne

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

   Our data show that primary human placental trophoblasts are capable of transferring viral resistance to non-placental recipient cells via miRNA-mediated enhancement of xenophagy. These studies constitute a new paradigm in the study of host-pathogen interactions at the maternal-fetal interface and represent the novel use of placental-derived miRNAs as possible antimicrobial therapeutics in non-placental tissues.

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 __X__ No____

   If yes, indicate date patent was filed: 03/07/2012

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 _____- *Application review ongoing at this time.*
   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_X

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_X_

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:

Given the recent filing of the invention disclosure and patent application it is premature to address whether we plan to pursue any licenses, patents or other development opportunities at this time.

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, 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. PMC3204658
12. **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. PMC2874470
13. **Mouillet JF**, Chu T, and Sadovsky Y. Expression patterns of placental microRNAs. Expression patterns of placental microRNAs. Birth Defects Res A Clin Mol Teratol 2011;91:737-43.
14. Donker RB, **Mouillet JF**, Chu T, Hubel CA, Stolz DB, Morelli AE, Sadovsky Y. The expression profile of C19MC microRNAs in primary human trophoblast cells and exosomes. Mol Hum Reprod. 2012;18:417-24. PMC3389496.

D. Research support

Ongoing

Pennsylvania Department of Health (PI: Mouillet) 1/1/12–12/31/12
(Research Formula Funds)
In vivo analysis of human C19MC microRNAs in a transgenic mouse model
The goal of this study is to develop a transgenic mouse model that will allow us to investigate the function of the placenta-specific C19MC miRNAs.

NIH/NICHD R21 HD071707 (PI: Sadovsky, Co-I: Mouillet) 12/1/11 – 12/31/13
Small RNAs at the placental maternal communication interface
This project will use next generation deep sequencing technology to comprehensively interrogate maternal-placental-fetal trafficking of microRNAs, and analyze dynamic changes in microRNA in pregnancies complicated by clinically relevant placental injury.

NIH/NICHD R01 HD065893 (PI: Sadovsky, Co-I: Mouillet) 8/15/10–5/31/15
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.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Yoel Sadovsky, MD	Director, Magee-Womens Research Institute, Professor of OBGYN and Reproductive Sciences, and Microbiology and Molecular Genetics and CTSI

TRAINING INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Hebrew University, Jerusalem Israel	BS	1981	Biomedical sciences
Hebrew University, Jerusalem Israel	MD	1985	Medicine
Washington University, St. Louis		1986-90	Resident, OBGYN
University of California, San Francisco	Fellow	1990-92	Maternal-Fetal Medicine
University of California, San Francisco	Postdoctorate	1992-93	Gene regulation

A. Personal statement

Dr. Sadovsky is a physician-scientist whose research centers on feto-placental development and trophoblast function. He seeks to decipher the molecular mechanisms underlying placental dysfunction, which impacts pregnancy and impedes fetal growth. Using primary human trophoblasts, human placental biopsies, and genetically altered mice, his lab examines the homeostatic adaption of trophoblasts to cellular stressors. Employing computation-intensive, high-throughput transcriptomic (RNAseq and arrays) and proteomic methodologies, Dr. Sadovsky examines the role of microRNAs and other small RNAs in placental pathways that govern the adaption of human trophoblasts to altered micro-environment.

B. Professional experience and appointments

1993	Assistant Professor, Maternal-Fetal Medicine, OBGYN, Washington Univ
1997-2007	Joint Appointment: Department of Cell Biology and Physiology, Washington Univ
1997-2007	Director, Maternal-Fetal Medicine Fellowship Training Program, Washington Univ
1998-2007	Member, Division of Biology and Biomedical Sciences, Washington Univ
1999	Associate Professor (tenure), OBGYN, Cell Biology and Physiology, Washington Univ
1999-2007	Director, Division of Maternal-Fetal Medicine and Ultrasound, Washington Univ
2004-2007	Professor (tenure), OBGYN, and Cell Biology and Physiology, Washington Univ
2007-Present	Scientific Director, Magee-Womens Research Institute Univ of Pittsburgh
2007-Present	Professor (tenure) and Vice Chair (research), OBGYN, Univ of Pittsburgh
2007-Present	Elsie Hilliard Hillman Chair of Women's Health Research, Univ of Pittsburgh
2009-Present	Professor of Microbiology and Molecular Genetics, and CTSI, Univ of Pittsburgh

Honors, awards, and national service

1995, 1997-2000, 2002, 2004, 2005	Excellence in Teaching Award, Department of OBGYN, Washington Univ
1997	Excellence in Teaching Award, Assoc of Professors of Gynecology and Obstetrics (APGO)
1998, 1999, 2000	NIH-NICHD study sections (*ad hoc* member)
1999, 2000, 2003	Teacher of the Year Award, Washington Univ
2002-2006	NIH-NICHD Maternal and Child Health Research Study Section
2005-2006	Chair, NIH-NICHD Obstetrics and Maternal-Fetal Biology Research Study Section
2004	Alpha Omega Alpha Honor Society
2004	Society for Gynecologic Investigation (SGI) President's Achievement Award

2005-2010	Steering Comm Chair, NICHD Genomic and Proteomic Network for Preterm Birth Research
2007-2008	President, Perinatal Research Society
2009-Present	Council Member, Society for Gynecologic Investigation
2009-2013	NICHD Council Member, National Advisory Child Health and Human Development
2011-2012	NICHD Council Subcommittee on Planning and Policy member
2011-present	March of Dimes Prematurity Research Study Section chair
2012-present	University of Colorado Center for Women's Health Research's Advisory Board
2012-present	Editor, *Placenta*
2012-present	NICHD Division of Intramural Research Review Panel
2012-present	March of Dimes Scientific Advisory Council

C. Selected publications (from a list of 124)

1. **Sadovsky Y**, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, Simburger K, Milbrandt J. Mice deficient in the orphan receptor SF-1 lack adrenal glands and gonads, but express P450scc enzyme in the placenta, and have normal embryonic serum levels of corticosteroids. Proc Natl Acad Sci USA 1995;92:10939-43.
2. Lee SL, **Sadovsky Y**, Swirnoff AH, Polish JA, Goda P, Gavrilina G, Milbrandt J. Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). Science 1996;273:1219-21.
3. Dorn C, Ou Q, Svaren J, Crawford PA, **Sadovsky Y**. Activation of luteinizing hormone-□eta gene by gonadotropin releasing hormone requires the synergy of early growth response-1 and steroidogenic factor-1 J Biol Chem 1999;274:13870-6
4. Mariani TJ, Budhraja V, Mecham BH, Gu CC, Watson MA, **Sadovsky Y**. A variable fold-change threshold determines significance for expression microarrays. FASEB J 2002;10.109.
5. Budhraja V, Spitznagel E, Schaiff WT, **Sadovsky Y**. Incorporation of gene-specific variability improves expression analysis using high-density DNA microarrays. BMC Biol 2003;1:1.
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;28:414-23.
7. Mouillet JF, Sonnenberg-Hirche C, Yan X, **Sadovsky Y**. p300 regulates the synergy of SF-1 and Egr-1 in activating LH□eta gene. J Biol Chem 2004;279:7832-9.
8. Chen BS, Nelson DM, **Sadovsky Y**. NDRG1 modulates the response of term human trophoblasts to hypoxic injury. J Biol Chem 2006;281:2764-72.
9. Mouillet JF, Chu T, Nelson DM, Mishima T, **Sadovsky Y**. miR-205 silences MED1 in hypoxic primary human trophoblasts. FASEB J 2010;24:230-9.
10. Mouillet JF, Chu T, Hubel CA, Nelson DM, Parks WA, **Sadovsky Y**. The expression of hypoxia-regulated miRNAs in plasma of pregnant women with fetal growth restriction. Placenta 2010;31:781-4.
11. Donker RB, Mouillet JF, Chu T, Hubel CA, Stolz DB, Morelli AE, **Sadovsky Y**. The expression profile of C19MC microRNAs in primary human trophoblast cells and exosomes. Mol Hum Reprod, 2012;18:417-24.

D. Ongoing research support

- NIH/NICHD P01HD069316: Molecular and Cellular Controls of Placental Metabolism (PD: Sadovsky), 5/15/12–3/31/17

- NIH/NICHD R01HD065893: Placental Injury and microRNA (PI: Sadovsky), 7/1/10–6/30/15
- NIH/NIEHS R01ES11597: Regulation of Trophoblast Differentiation and Function (PI: Sadovsky), 9/22/01–5/31/13 (NCE)
- NIH/NICHD R21HD071707: Small RNAs at the Placental Maternal Communication Interface (PI: Sadovsky), 12/1/11-11/30/13NIH/NICHD K12HD063087: Magee-Womens Basic and Translational Reproductive Health Training Program (Res Director: Sadovsky, PI: Hogge), 9/30/09–8/31/14
- NIH/NICHD K12HD043441: Building Interdisciplinary Research Careers in Women's Health (BIRCWH) in Pittsburgh (Co-PI: Sadovsky, Roberts), 9/1/12 – 6/30/17
- NIH/NIAID U19AI068021: The influence of ionizing radiation on trophoblast differentiation and function (PD: Greenberger, Proj Co-PI: Sadovsky), 11/1/10–10/31/12

Recently completed research support

- Penn. Dept. of Health: Analysis of Small RNAs in the Fetal Placental Maternal Interface (PI: Chu, Co-I: Sadovsky), 1/1/11–6/31/12
- Penn. Dept. of Health: The role of microRNA-424 in Differentiation of Placental Trophoblasts (PI: Mouillet; Co-I: Sadovsky), 1/1/10–12/31/10
- Penn. Dept of Health: Analysis of Functional Domains within NDRG1 (PI: Sadovsky), 1/1/09–12/31/09
- Penn. Dept of Health: Post-transcriptional Regulation of *FSTL1* mRNA in Human Trophoblasts (PI: Mouillet, Co-I: Sadovsky), 1/1/09–12/31/09