

Response Form for the Final Performance Review Report— Magee Womens Health Institute 2009F*

1. Name of Grantee: Magee Womens Health Institute

2. Year of Grant: 2009 Formula Grant

A. For the overall grant, briefly describe your grant oversight process. How will you ensure that future health research grants and projects are completed and required reports (Annual Reports, Final Progress Reports, Audit Reports, etc.) are submitted to the Department in accordance with Grant Agreements? If any of the research projects contained in the grant received an “unfavorable” rating, please describe how you will ensure the Principal Investigator is more closely monitored (or not funded) when conducting future formula funded health research.

We are cognizant of, and truly grateful, for the impact that the Formula Research Funds have had upon our research at Magee-Womens Research Institute. In 2009, program funds supported seven projects that spanned diverse issues in reproductive biology and women’s health, ranging from infectious disease in pregnancy, to developmental biology, and cancer. We provide the following general comments regarding our management of the program award to MWRI, and specific comments regarding the 2009 projects.

MWRI has established a system for oversight to insure success, efficiency, and responsible conduct of research with the Formula Research Funds. This system begins with careful identification of projects that, while ambitious and in their early stages of development, have real potential to launch successful research trajectories. Each investigator’s proposal is discussed with and reviewed by the Director for scientific merit, and is refined as needed. Post award, the Director conducts progress meetings with all investigators prior to the six-month and one-year marks. Special attention is given to junior investigators, who meet regularly with mentorship teams to monitor all aspects of research and professional development. Additionally, we have instituted requirements for internal review of all NIH-level grant applications, as well as grant-in-progress (GRIP) sessions in which established investigators provide key feedback on proposals in development. Administratively, Ms. Cheryl Richards, MWRI’s Director of Grants and Contracts, provides oversight of our compliance with the program. She assists investigators with all aspects of budget preparation, monitors regulatory compliance in the responsible conduct of research, and insures all reporting is completed in a timely and thorough manner. Furthermore, she maintains detailed records of all investigators and projects funded during the life of the program, and assists the Director in identifying candidates for funding. The success of these policies is evident that, in the past four years, none of our reviewed projects received “unfavorable” scores.

We are grateful to the program reviewers for their thorough evaluation of the 2009 projects, which received six “Favorable” scores and one “Outstanding” score. Each investigator has provided a detailed response to the reviewers’ critiques in the attached document, and values their input in the research process. We highlight that, given the “seeding” focus of the one-year

* Please note that for grants ending on or after July 1, 2007, grantees’ Final Performance Review Reports, Response Forms, and Final Progress Reports ***will be made publicly available on the CURE Program’s Web site.***

awards and the germination time of new lines of biomedical research, reporting measurable outcomes of success at the conclusion of the one-year term is very difficult. We are now happy to report, within our responses to the reviewers, that the projects have generated important data that have since been presented in national forums, published in quality, relevant journals and buttressed applications for new funding.

We thank the Pennsylvania Department of Health for its support of the Health Research Award program, which has been integral to MWRI's status as the top-funded research institute in the United States in the field of reproductive sciences and women's health. The program has afforded our investigators the opportunity to creatively address key health issues and promote cutting edge-scientific discovery under a mantle of conscientious oversight. We look forward to furthering this research to improve the health of women and their infants, in Pennsylvania and beyond.

For each research project contained in the grant, please provide a response to items B-D as listed on the following page(s). When submitting your response please include the responses for all projects in one document. The report cannot be submitted as a ZIP file, because the Department's exchange server will remove it from the email. If the report exceeds 2MB, please contact the Health Research Program for transmittal procedures: 717-783-2548.

Project Number: 0990101
Project Title: Refinement of the Appropriate Animal Model for
Respiratory Infection with Influenza in Pregnancy
Investigator: Beigi, Richard

B. Briefly describe your plans to address each specific weakness and recommendation in Section B using the following format. As you prepare your response please be aware that the Final Performance Review Report, this Response Form, and the Final Progress Report will be made publicly available on the CURE Program's Web site.

Reviewer 1:

1. The data for the examination of aerosol characteristics of novel 2009 H1N1 influenza were not provided, although the experiment was said to be done.

Response:

We did perform this experiment. It was an experiment with a range of virus concentrations to determine the spray factor (the relationship between aerosol concentration of the virus and the concentration of virus in the aerosol generator). The H1N1 behaved exactly like the other two flu viruses we have aerosolized, and therefore we saw no justification to pursue further. The data are included here:

Aerobiology	Spray Factor Determination					
Aerosol Date:	1-Oct-10			Collison:	B	
Species:	Ferret			# of Jets	3	
Exposure Chamber:	Ferret Whole-Body					
Run #	[Nebulizer]	[AGI]	AGI Vol	Time	[Aerosol]	SF
1	1.00E+04		10	10		
2	1.00E+04	4.22E+01	10	10	7.0E+00	7.0E-07
3	1.00E+04	1.47E+02	10	10	2.4E+01	2.4E-06
4	3.16E+04	1.65E+02	10	10	2.8E+01	8.7E-07
5	3.16E+04	6.81E+01	10	10	1.1E+01	3.6E-07
6	3.16E+04	1.00E+02	10	10	1.7E+01	5.3E-07
7	5.58E+05	1.47E+02	10	10	2.4E+01	4.4E-08
8	5.58E+05	1.00E+03	10	10	1.7E+02	3.0E-07
9	5.58E+05	3.16E+03	10	10	5.3E+02	9.5E-07
					Average	7.7E-07
					St Dev	7.4E-07
					95% CL	6.5E-07

2. In the experimental analysis (Figures 1 and 2), no statistical data are presented. Also, the PI proposed to perform reverse transcription polymerase chain reaction (RT-PCR) to measure the virus titer in upper respiratory tract and in blood and tissues, but this was not done.

Response:

Given the small sample size that we used (N=3 per group), we felt that it was impractical to perform statistical analysis on such small numbers in each group. We obtained a refund on the original ferret order, and did subsequently perform another challenge experiment in which we did have the ability to quantify the virus present in serial sacrificed ferrets after exposure, and do very gross pathological evaluation. Further immunological and more specific pathologic evaluation is pending funding given the expense of the pregnant ferrets.

The design of this second study was: eight pregnant and eight non-pregnant ferrets for exposure. All ferrets in each group were exposed on day 0, and then two were sacrificed on day 0, day 3, day 5 and the final two on day 7. This was done for both pregnant and non-pregnant ferrets.

The main findings from these experiments were:

- a. We were able to quantify viral infection by RT-PCR in both groups in the lungs as early as 2 hours after challenge and in the brain tissues by day 3. There were some differences in titers when comparing pregnant vs. non-pregnant (in both brain and in the lungs), however, given n=2, these numbers were too small for any proper statistical comparison.
- b. We were able to document viral presence in the placentas of the pregnant ferrets by day 3, and definitively in the kits themselves by day 7.
- c. Gross pathologic, as well as H&E evaluation of the tissues, demonstrated pathologic findings indicative of influenza infection in the lung tissues of both groups, and no major differences were detected.

Given these findings above, our plan is to seek and obtain larger-scale funding to replicate these experiments with sufficient numbers to allow for more thorough comparison, complete immunologic evaluation (increasing reagent availability noted recently for ferret investigations) and a more detailed pathologic/virologic investigation.

3. It is unfortunate that the PI could not perform the second experiment that involves multiple important virologic and immunological assays. The results need to be provided.

Response:

We did perform this work. Please see above response to question #2.

Reviewer 2:

1. Design of experiment 1: The investigators did not test for virus shedding or test for seroconversion in inoculated ferrets. Without this, it is impossible to confirm infection; and the differences in pregnant and non-pregnant ferrets may be unrelated to influenza exposure. This data needs to be collected for all future experiments, otherwise they will not have shown they have a model for influenza infection in ferrets (pregnant or otherwise). This is a major weakness, although they still have funding to complete a second experiment where they can test their hypothesis.

Response:

We do have virologic verification of infection as well as pathologic verification of infection in the lung tissues in subsequent investigations described above. These serve to validate our experiment's ability to cause infection in the ferrets. We also have frozen tissue samples from both experiments that can allow (in concert with future investigations) for future serologic and virologic confirmation of infection, and this is our intent, pending funds availability.

2. Presentation of data from experiment 1: The investigators did an insufficient job of presenting experimental data from the sole ferret experiment. The ferret infection model provides a variety of opportunities to measure and describe disease including, but not limited to, activity, fever, virus shedding (nasal, rectal), and seroconversion. The investigators showed percent weight only and did not provide statistical analysis of those data. This was a major weakness of the report, since there was variability in the weights and no direct evidence of infection presented. The data could be improved by providing data for individual animals and/or statistical analysis of the groups.

Response:

Please see response above.

3. Failure to describe goals of future grant submissions: The investigators need to describe briefly, the long-term goals and aims of proposed grants. While the assertion of planned proposals is comforting, they failed to provide clear direction for the work. This would be similar to the one-page summary provided to NIH where one describes the problem, the goal of the proposal and the aims to address the goal.

Response:

With the experiments performed subsequent to this report due date that has been delineated above, we have solidified documentation of infection with virus present in multiple tissue compartments. We are currently preparing a manuscript detailing these findings, and plan to submit a NIH R21 grant application using this model of influenza in pregnancy to validate our findings with much larger groups of ferrets that will permit meaningful comparisons.

Subsequent to validation experiments, we will then propose to use this model to study disease pathogenesis and response to countermeasures, such as antiviral drugs, at different time points in the infection cycle.

4. Data generation for a manuscript: This is related to Recommendations 1 and 2, but more specifically, the investigators should not attempt to address Aim 3 until they confirm the infection model in Aim 2. Also, tools for immune response analysis in ferrets are very limited, so the investigator should pose a very specific question as opposed to proposing gene expression microarrays.

Response:

As noted above, we have validated the presence of virus in multiple tissues and do plan to move forward. We agree with the assessment of relative low amount of immune response analysis tools in ferrets, but have been encouraged recently with more availability of some reagents.

Your points are most helpful and our future experiments will be designed with those points in mind.

5. Delineation of Aims 2 and 3: The original proposal states that viral loads and necropsy will be assessed in Aim 2, however the investigators propose this is the goal of Aim 3 and state that Aim 2 is completed. As mentioned, the single experiment does not complete Aim 2. Also, there is no mention of the proposed optimization of the aerosol chamber. Was this done? The dose of viable virus aerosolized is a key component of the model. These points need to be addressed and Aims 2 and 3 clearly defined.

Response:

Please see responses to the queries above addressing these points.

Reviewer 3:

1. To address the issue with obtaining animals, it is possible to challenge animals that were seropositive for H1N1 pandemic influenza with another influenza subtype to salvage a very limited resource (pregnant female ferrets).

Response:

This is correct, however, we sent the H1N1 seropositive ferrets back, received a refund, and were able to perform subsequent experiments with seronegative ferrets obtained from an alternate vendor. These experiments have been detailed above in response to reviewers 1 and 2.

2. To address the clinical score, virology, and immune parameters samples could have been collected for virus load (nasal aspirates) and immune characterization serum and nasal wash Ab or cytokine production by PCR.

Response:

As detailed above, we collected tissue and ran PCR to confirm viral presence, and performed pathologic evaluation on the tissues obtained as funding permitted.

3. Issues with the aerosol challenge system (including sham animals) must be addressed for these studies to proceed. Perhaps more detailed spray factor studies would be helpful for this deficiency.

Response:

We have provided that spray factor data above. We do not believe we have major issues with the aerosol challenge system, but are always concerned with quality control of our system and validation of our findings. It is our impression that the sham exposure pregnant animals became sick and died as a result of the stress from the procedures (daily handling to collect weights & temps, the exposure, and alike). Pregnant ferrets are very sensitive to stress. When ferrets get stressed they stop eating, which may spiral them into general deterioration, and even shock. Going forward, we have included additional acclimation (handling and collection of weights/temps before exposure) and we've changed the food and water given to the ferrets to insure they eat and drink properly to minimize the risk of repeat problems.

Generic Recommendations for Magee Womens Research Institute and Foundation

Reviewer 2:

Clarify the goals of the final progress report to provide instructions on the description of future (proposed) grants and provide recommendations for data reporting.

Response:

Please see earlier comments.

Reviewer 3:

An important aspect of this project is influenza pathogenesis using a rational model. Additional expertise with influenza in the ferret may have helped with the overall design and endpoint collection. There is influenza expertise at the institution (Dr. Ted Ross), and collaboration with that laboratory may be helpful in further experiments, if planned.

Response:

We have already met with Dr. Ross prior to receiving this report, and intend to initiate research collaborations.

ADDITIONAL COMMENTS

Reviewer 3:

The project did not characterize aerosols prior to challenge despite that having been a goal in the beginning of the program. There was a lack of complete endpoints for the few animals that were used in the project, and no virus loads were measured in animals through the time after challenge. No immune parameters were measured in these animals. Sham challenged animals were lost in this study, indicating issues with the aerosol challenge or some other husbandry practice for these animals.

Response:

Please see earlier comments.

C. If the research project received an “unfavorable” rating, please indicate the steps that you intend to take to address the criteria that the project failed to meet and to modify research project oversight so that future projects will not receive “unfavorable” ratings.

Response: N/A

D. Additional comments in response to the Final Performance Review Report (OPTIONAL):

Response:

We wish to express our appreciation to the State of Pennsylvania for its support of our research. While the project reporting period has concluded, we are pleased to confirm that the work initiated is ongoing, and we look forward to our future investigations and collaborations.

Project Number: 0990102

Project Title: Relationships among PPAR gamma Activity, Hypoxia, and Differentiation in Human Placenta

Investigator: Chu, Tianjiao

B. Briefly describe your plans to address each specific weakness and recommendation in Section B using the following format. As you prepare your response please be aware that the Final Performance Review Report, this Response Form, and the Final Progress Report will be made publicly available on the CURE Program's Web site.

Reviewer 1:

1. A careful sample size and power analysis should be conducted before a study begins. For microarray experiments, there are available tools for this type of study planning.
2. A research team should be multidisciplinary, including clinicians, who can interpret and understand the meaning of data analysis results.
3. The home institution should provide more guidance and support to a junior investigator and include the individual project in its overall organizational research effort.

Response:

1. We agree that the use of historical data limited the sample size and the overall experimental design. Upon the completion of this project, we designed and performed several new experiments to validate and expand our finding from this study. These experiments include a large scale PCR experiment, and an integrated experiment employing miRNA, mRNA microarrays and mass spectrometry to study the effects of hypoxia on human primary trophoblast cells.
2. This project was conducted jointly with our co-investigator Dr. Sadovsky, who is an OBGYN clinician, a perinatologist, and an expert in the molecular mechanisms of placental development and differentiation. He not only provided the experimental data, but also helped interpret the finding of this study. Our multidisciplinary team is one of the main strengths of our approach.
3. I am grateful to the support from the Magee Womens Research Institute during this project. This project is part of the ongoing effort to study placental development and injury, which is one of the research areas that Magee-Womens Research Institute excels in.

Reviewer 2:

Pay greater attention to the gestational age of the specimens used from FGR pregnancies and placentas used to prepare cultures of trophoblast. It is challenging to obtain sufficient numbers of human placentas from pregnancies of first or second trimester; nonetheless, many of the features of FGR, including hypoxia and/or preeclampsia and other placental insufficiencies, occur prior to the third trimester (the time when cultures were prepared in this project). If this cannot be done easily at the PI's home institution, perhaps there are collaborative opportunities or some well-annotated rationale for the use of term cultures in studies such as this one.

Response:

We agree that a study of the placentas at an earlier gestational age may offer additional relevant insights into the cause of diverse types of placental injuries. As trophoblast injury can occur

throughout pregnancy, we believe that our findings are pertinent and important. Notably, we also plan to use a pregnant mouse model in order to study key aspects of placenta development/injury at earlier gestational ages.

Reviewer 3:

If researchers undertake stated plans to study micro RNA and protein levels, I would encourage them to consider generating better data sets. Certainly this will cost more than the current project, but there are ways to make progress without too much expense. For example, one well-replicated data set from a well-designed experiment would be much better than four data sets that have minimal or no replication. Better high-throughput data would give better gene candidates for follow-up studies.

Response:

We certainly agree with the reviewer's excellent suggestion. As a matter of fact, one specific aim of our 2010 NIH R01 grant application was the study of miRNA and protein expression pattern in PHT cells under normoxia and hypoxia conditions. We were able to systematically generate data from four-time course for the expression level of miRNA, mRNA, and proteins in primary human trophoblasts cultured in either normoxic or hypoxic conditions. This high quality data set allows us to perform a more thorough and comprehensive analyses on the impact of hypoxia on primary human trophoblasts.

ADDITIONAL COMMENTS

Reviewer 1:

The strengths of the project include: 1) the PI and the research team possess adequate knowledge and skills to carry out the proposed data analysis; and, 2) all the research aims were explored and analysis results produced. The weaknesses of the project include: 1) there is a lack of sample size and power consideration during the study design phase; 2) there is a lack of careful interpretation of the results and discussion of the meaning and impact of the results; and, 3) this project is weak on all other metrics, such as impact on health research and clinical application, improvement of organizational infrastructure, and staff education.

Response:

Please see our earlier response. We regret that we failed to provide sufficient data to the reviewers explaining the multidisciplinary nature of our group, and the key impact of our findings on clinical diseases associated with trophoblast hypoperfusion and hypoxia, such as fetal growth restriction. We hope that our comments here are helpful. We are confident that results from our findings will serve to shed light not only on trophoblast biology, and but also on clinical diseases.

Reviewer 2:

This was an interesting and potentially important bioinformatics study of gene expression patterns in placental development and differentiation, especially in the setting of hypoxia. The PI used historical data from trophoblast cultures grown under various conditions, that is, hypoxia (0% O₂ vs. 8 or 20% O₂) and peroxisome proliferator-activated receptor (PPAR) gamma agonists. Then, hypoxia *in vitro* was compared to FGR placentas. It was interesting that the gene

expression patterns for cells grown in hypoxia were dissimilar from the patterns noted in FGR placentas. Although the PI did not specifically address the implications of this finding, this may indicate that we have been misinterpreting the devastating role of relative hypoxia (i.e., short term, oscillating) in placental differentiation. This could have important implications for future studies and for our understanding of the role of oxygen in feto-placental development.

Response:

We fully agree with the reviewer's comments. Following this project, we conducted a large scale PCR experiment to study the effects of various patterns of hypoxia on primary human trophoblasts, and compare the exposed cells with normal and FGR placentas. These studies yielded intriguing findings that served to characterize trophoblast response to hypoxia, which were published earlier this year (Oh et al, Placenta 2011;32:1004-9).

C. If the research project received an “unfavorable” rating, please indicate the steps that you intend to take to address the criteria that the project failed to meet and to modify research project oversight so that future projects will not receive “unfavorable” ratings.

Response: N/A

D. Additional comments in response to the Final Performance Review Report (OPTIONAL):

Response: We are grateful to the Pennsylvania Department of Health for its support. This award helped us to advance our studies and formulate the next set of questions to inform our understanding of placental development and injury.

Project Number: 0990103

Project Title: Hypoxia Inducible miR-210 as a Potential Therapeutic Target in Renal Cell Carcinoma

Investigator: Huang, Xin

B. Briefly describe your plans to address each specific weakness and recommendation in Section B using the following format. As you prepare your response please be aware that the Final Performance Review Report, this Response Form, and the Final Progress Report will be made publicly available on the CURE Program's Web site.

Reviewer 1:

None.

Response:

N/A.

Reviewer 2:

There are many miRs that would conceivably get altered as part of VHL loss, and miR-210 is certainly a leading one, but unlikely the only one. As designed in this project, the deep sequencing approach from VHL-positive versus VHL-negative cells will yield targets that are not only miR-210-specific. Multiple HIF-regulated miRs seem to exist, and there are also HIF-independent effects of VHL loss (which may certainly involve other miRs).

A more miR210-specific approach should be added to complement the forthcoming data. Overexpression of miR-210 in VHL-positive cells for example, followed by Ago IP and deep sequencing may be valuable. I realize how costly this approach is, and additional funds would certainly be required. Therefore an additional grant, such as an R01, that the applicant states he will pursue, would be paramount.

The applicant needs to consider carefully how he will identify the miR-210-specific targets versus targets of other miRs that will likely be present in the RNA-induced silencing complex (RISC).

Response:

Our preliminary results from next generation sequencing completely agree with this reviewer's comments. We not only found enrichment of miR-210 in the RISC complex, but also found many other miRNAs. We have since performed the AGO2-IP and sequencing in both RCC4 and RCC4/VHL cells, which partially addressed the reviewer's second concern, and anticipate that the final results will be extremely interesting. We expect to complete the data analysis in the next two months.

We are currently deploying a combination of computational prediction and sequencing results to identify miR-210 targets.

Reviewer 3:

1. Expand the research to include more tumor cell lines, and modulate miR-210 by additional means. Move into an animal model. Obtain clinical samples to increase the relevance of findings.
2. Identify targets of miR-210 to uncover the mechanism.

Response:

Currently, we have collected seven additional RCC cell lines and are in the process of analyzing miR-210 function in them. We are also planning to over express miR-210 in RCC cell lines introduced with wild-type VHL to examine the phenotypes. We certainly plan to proceed with an animal model once the mechanism of miR-210 function is elucidated in vitro. We also established collaborations with a medical oncologist, Dr. Jodi Maranchie, in the Urology Department at the University of Pittsburgh Medical Center (UPMC). She will provide access to the primary RCC tumors and will serve as a co-investigator in our future grant submissions. As we stated, we are in the process of analyzing miR-210 AGO2-IP next generation sequencing data, inform? miR-210 targets.

C. If the research project received an “unfavorable” rating, please indicate the steps that you intend to take to address the criteria that the project failed to meet and to modify research project oversight so that future projects will not receive “unfavorable” ratings.

Response: N/A.

D. Additional comments in response to the Final Performance Review Report (OPTIONAL):

Response: As a new investigator, funds from the State of Pennsylvania Formula Research have been crucial in establishing my laboratory’s research program. I am sincerely grateful for this support and am excited to incorporate this data and new collaborations into my research.

Project Number: 0990104

Project Title: Role of miR-424 in the Differentiation and Function of Placental Trophoblasts

Investigator: Mouillet, Jean-Francois

B. Briefly describe your plans to address each specific weakness and recommendation in Section B using the following format. As you prepare your response please be aware that the Final Performance Review Report, this Response Form, and the Final Progress Report will be made publicly available on the CURE Program's Web site.

Reviewer 1:

1. Might it be possible to perform placenta-specific knockdown in the mouse model using the lentiviral strategy which confines vector expression to the placenta? Would an shRNAmir for miR-424 or other targets prove useful?

Response:

We agree, and are actually developing this approach in our laboratory. So far we managed to produce placenta-specific transgenes in the mouse by transducing embryos at the blastocyst stage using a lentiviral vector. Our next step is to engineer efficient miRNA decoys that can be expressed from our lentiviral system and that will be used to specifically deplete miR-424/322 in mouse placental trophoblasts. Although use of miRNA inhibitors based on the method commonly referred to as a miRNA "sponge" is not very efficient in our system, we recognize the importance of inhibiting endogenous miRNAs in order to investigate their biological function and are actively pursuing the design of better inhibitors. To this end we are working on a new type of miRNA decoy termed TuD ("tough decoys") that will hopefully allow us to efficiently inhibit miR-424 function in trophoblasts.

2. Laser capture microdissection might have been appropriate in the mouse model to distinguish between the responses in different cell types.

Response:

We agree with the reviewer that laser capture micro-dissection (LCM) would clarify the ambiguity regarding the effect of hypoxia on miR-424/322 levels at the cellular level in the mouse placenta. However, this technology is more involved and we thought it would be premature to invest in this approach before we validate the role of this miRNA in mouse placental function. LCM remains a potentially useful option that we will plan to carry out when we further demonstrate the relevance of the mouse model to study the function of miR-424 in placental trophoblasts.

3. The investigators have indicated that they have a recent study in which miR-424 was neutralized using morpholino antisense oligonucleotides, addressing the major weakness in the current study.

Response:

Several studies have now reported the successful use of morpholino antisense oligonucleotides in miRNA knockdown experiments and this approach remains a valid option for the future.

However, because these inhibitors are also delivered to cells via transfection we are concerned over the possibility that our primary human trophoblasts would be insufficiently permeable to the delivery of these morpholinos. Instead, as mentioned earlier, we decided to further develop and optimize a strategy based on the use of miRNAs decoys that we know can be effectively introduced in primary trophoblasts. If successful, this approach can be easily adapted to diverse cell types, including mouse models.

Reviewer 2:

The proposal is solid, overall, and helps us understand the nature of miR-424 expression in human trophoblast. However, little attention is paid to the functional role of its proposed targets (i.e., FGFR1 and MAP2K1). The proposal already suggested that although in silico target scanning revealed a miR-424 binding site in MAP2K1, this did not yield meaningful functional consequences in trophoblasts. Moreover, inasmuch as target scanning did reveal a potentially interesting site for FGFR1, nonetheless, the PI and his team did not demonstrate or attempt to demonstrate directly, an effect of miR-424 on FGFR1 function in the trophoblast. This should be addressed in the future to make relevant the observations in the proposal.

Response:

We agree with the reviewer that our initial finding showing the targeting of FGFR1 by miR-424 must be completed with a study of the relevance of FGFR1 to the biology of human placental trophoblasts. These experiments are currently being conducted in our laboratory. Indeed, we recently observed a substantial reduction in the release of human chorionic gonadotropin (hCG) by BeWo cells after an experimental reduction of FGFR1 levels produced by siRNA. This effect was specifically observed in cells transfected with siRNAs directed against FGFR1 but not when cells were exposed to the same amount of control non-targeting siRNA. Interestingly, exposure of BeWo cells to recombinant FGF-basic, which signals through FGFR1, led to a stimulation of the production of hCG. These results suggest a role for miR-424/FGFR1 signaling in the differentiation of trophoblasts.

Reviewer 3:

The investigators have made tremendous progress in identifying roles for miRs in trophoblast cells and placentation. They have established the foundation for a novel experimental approach and thus significant momentum in their research. There is a high probability that the research will be rewarded with NIH support; however, in the interim it is imperative that this research project continue to receive financial support.

Response:

We thank the reviewer for the encouraging comments. Although important questions remain to be addressed, we have made a number of interesting observations that reveal the potential of miRNA to influence key aspects of placental biology. As our research progresses we remain strongly committed to deciphering some of the mechanisms by which these miRNAs, and miR-424 in particular, are connected to the regulatory networks that govern placental development and homeostasis. In this pilot project we have shown that environmental cues can affect miR-424 levels and in this manner can alter important signaling pathways. In addition, the experimental techniques developed for this work will certainly become useful for future studies on placental miRNAs.

C. If the research project received an “unfavorable” rating, please indicate the steps that you intend to take to address the criteria that the project failed to meet and to modify research project oversight so that future projects will not receive “unfavorable” ratings.

Response: N/A

D. Additional comments in response to the Final Performance Review Report (OPTIONAL):

Response:

We thank the referees for their insightful comments and thorough review. As the reviewers likely know, a one year plan cannot always be completed as anticipated. Yet, we are pleased that the reviewers appreciated our work. We have carried on additional experiments, and are pleased to report that our work supported by this award led to a successful NIH grant application entitled Placental injury and microRNA (R01-HD065893), funded in August 2010 (PI, Y Sadovsky; Co-I, JF Mouillet).

Project Number: 0990105
Project Title: Epigenetic Analysis of Human Aneuploidy
Investigator: Peters, David

B. Briefly describe your plans to address each specific weakness and recommendation in Section B using the following format. As you prepare your response please be aware that the Final Performance Review Report, this Response Form, and the Final Progress Report will be made publicly available on the CURE Program's Web site.

Reviewer 1:

1. The model: This study requires a model system where only the differences in chromosome numbers can be tested. For example, identical twins, cloned mice or pigs, etc.

Response:

The reviewer is correct to state that a model system would be helpful. In practice, however, this is challenging considering our interest in human aneuploidy. Specifically, identical human twins with aneuploidy are rare and clearly the study of placental tissue from such pregnancies would be extremely difficult due to the scarcity of the tissue. Recent studies have shown that it is possible to identify methylation based differences in out-bred human populations that are secondary to aneuploidy:

(Kerker K et al. (2010). Altered DNA methylation in leukocytes with trisomy 21. PLoS Genet. 2010 Nov 18;6(11):e1001212.).

Based upon these examples in the literature it would appear that a larger sample size would certainly help overcome likely inter-individuals differences in the epigenotype.

A major positive outcome of the program of research is that we have been able to refine our hypotheses and work towards the development of a non twin-based human model system. Discussion of the details of this model system in this forum would be inappropriate but we believe this new direction will prove to be productive and this will become the focus of a major grant submission in the near future.

2. The method: There are more sensitive methods becoming available for the study of DNA methylation.

Response:

We are currently using Illumina next generation sequencing technology (HiSeq2000) to carry out whole genome bisulfite sequencing and Reduced Representation Bisulfite Sequencing (RRBS). Unfortunately, these methods were not available to us in 2009, at which time we felt the most reliable and accessible method was the Illumina microarray platform.

3. Data analyses: Because of the extensive amounts of data produced, a computational biologist and a systems biologist would improve the proposal.

Response:

We were well supported by Dr Tianjiao Chu, an expert in computational biology. Published examples of studies in which his expertise has been invaluable are listed below.

Chu T, Bunce K, Hogge WA, Peters DG*. Statistical considerations for digital approaches to non-invasive fetal genotyping. *Bioinformatics*. 2010;26(22):2863-6.

Chu T, Burke B, Bunce K, Surti U, Hogge WA, Peters DG*. A microarray-based approach for the identification of epigenetic biomarkers for the noninvasive diagnosis of fetal disease. *Prenat Diagn*. 2009;29(11):1020-30.

Chu T, Bunce K, Hogge WA, Peters DG*. Statistical model for whole genome sequencing and its application to minimally invasive diagnosis of fetal genetic disease. *Bioinformatics*. 2009;25(10):1244-50.

4. Sample size: Larger sample sizes would improve the proposal.

Response:

This is a very valid point, with which we fully agree (see above).

Reviewer 2:

None.

Reviewer 3:

1. The overall weakness was the failure to reach the objective or provide meaningful advancement in the field. The main failure was in the genomics technique used. It is difficult to determine if this was inherent in the technique or part of the application of the technique in the PI's laboratory. The PI has since moved to deep sequencing based technologies, but reports he is developing them himself when these are already available from vendors. I would encourage them to use those already available and use his expertise in the biology to ensure success and that an important biological question is answered.

Response:

We agree that published and commercially available methods are favorable. We are currently using Illumina next generation sequencing technology (HiSeq2000) to carry out whole genome bisulfite sequencing and Reduced Representation Bisulfite Sequencing (RRBS). Unfortunately, these methods were not available to us in 2009, at which time we felt the most reliable and accessible method was the Illumina microarray platform.

2. The weakness of the study was that no additional funds were garnered. The researcher does plan on applying for an R21 grant in the future. If an R21 grant is still planned, I would concentrate on attaining preliminary data that shows you can effectively produce reliable deep sequencing data.

Response:

We agree with this comment and work is currently underway to generate these data. Our ongoing work will become the focus of a new major grant submission within the next 3 months.

3. A weakness of this study is that no definitive measures of success, such as peer-reviewed publications, licenses, patents or commercial development opportunities, were garnered.

This is largely a result of the failure in the experiments. Getting more advice from experts in the field may have prevented this failure.

Response:

We are currently communicating with a number of groups that have expertise in the use of next generation DNA sequencing for the analysis of DNA methylation. We believe that the technology has matured rapidly in this area and we remain optimistic that this area of focus will prove to be fruitful in the medium term. Specifically, we believe that a major problem is the likely significant inter-individual variation in DNA methylation that is not necessarily secondary to aneuploidy. We believe we have now resolved this problem by developing a very strong experimental model system that has relevance in the human context.

4. The PI states no enhancement of research at the institution was made. This is a weakness. Enhancement of the skills of the PI in carrying out these experiments could be listed.

Response:

The reviewer is correct to point out the valuable learning opportunity provided by the program of work. The research process and the outcomes were an essential component in the refinement of our program and have greatly assisted in the formation of new hypotheses in this realm.

5. A weakness of the study is that no collaboration outside the institution was involved. I think enlisting skilled investigators in the techniques would have enhanced the probability of success in this case. A mentor with expertise in genomic-scale analysis would have been a good plan.

Response:

We have indeed initiated such communication with outside collaborators and these have, thus far, proved to be very helpful.

C. If the research project received an “unfavorable” rating, please indicate the steps that you intend to take to address the criteria that the project failed to meet and to modify research project oversight so that future projects will not receive “unfavorable” ratings.

Response: No response required

D. Additional comments in response to the Final Performance Review Report (OPTIONAL):

Response: We wish to thank the State of Pennsylvania for its support of our research. Although the funding period has concluded, the tools we have refined in the project continue to serve our discoveries.

Project Number: 0990106
Project Title: Antigen Expression and Adaptive Immunity in Human
Endometriosis and Endometriosis-Related Ovarian Cancers
Investigator: Vlad, Anda

B. Briefly describe your plans to address each specific weakness and recommendation in Section B using the following format. As you prepare your response please be aware that the Final Performance Review Report, this Response Form, and the Final Progress Report will be made publicly available on the CURE Program's Web site.

Reviewer 1:

1. Development of an immunoassay to measure the humoral immune response against MUC1 will improve the translational significance of the findings of the project.

Response:

We thank the reviewer for this advice. This tool might indeed become useful for studies we are conducting in mice and in human samples.

2. Findings of the project need to be presented and published in peer-reviewed journal(s) so that investigators working in the field will benefit.

Response:

We are pleased that the reviewer saw the merit of our work, and its value to other investigators. Work proposed under this grant and previous grants by the PA formula grants assisted us in a successful NIH grant application entitled "Muc1 roles in ovarian cancer pathogenesis and immune therapy" (1R01CA163462-01). Key aspects of the work were also published in our manuscript entitled "Soluble MUC1 and serum MUC1-specific antibodies are potential prognostic biomarkers for platinum-resistant ovarian cancer", published in *Cancer Immunol Immunother* 2011;60:975-84. Work toward additional publications is currently in progress.

Reviewer 2:

The results have not yet been published. My recommendation is to prioritize what needs to be done in order to publish. The publication of preliminary data in peer-reviewed journals will greatly enhance the competitiveness of this project for funding.

Response:

We thank the reviewer for recognizing the significance of our work. Please see our second comment to the first reviewer.

Reviewer 3:

1. Weakness: It is not clear based on the data presented whether the link between endometriosis and ovarian cancer is mediated by an enhanced inflammatory response. If this is not the case, then this type of research will not play a key role in identifying immune-based means of cancer prevention.

Recommendation: Strong preliminary evidence needs to be developed to support the major assumption in the proposal that a dysfunction in immune surveillance mechanisms contributes to disease initiation in endometriosis and its malignant transformation to ovarian cancer. It would be interesting to prove that endometriotic or cancer lesions can in fact be eliminated by an intact immune system or prevented by vaccine-activated immune effectors in animal models recapitulating human disease.

Response:

We agree with the reviewer, and note that ovarian tumors in our recently developed mouse model for ovarian cancer [triple Tg MUC1^{+/-}LSLKrasG12D/+PtenloxP/loxP (MUC1KrasPten)], which develop MUC1⁺ ovarian tumors, is characterized by increased accumulation of immune suppressive T cells (Tregs), as well as an inflammatory environment, dominated by VEGF expression, over expression of COX2, and accumulation of inflammatory prostaglandin (PGE2). Examining these pathways may assist us in deciphering defects in the underlying immune surveillance in these tumors.

2. Weakness: Although considerable progress has been made towards the original scope of the application, it does not appear that the specific aims of the original application have been completed. The findings are too preliminary and lack detail.

Recommendation: The study needs to include a detailed analysis of all categories of immune effectors in order to provide a comprehensive picture of immune surveillance deficiencies in both affected tissues and peripheral blood in animal models and patients. The type and number of infiltrating lymphocytes also need to be analyzed. Antibodies for T cell and B cell markers can be used to identify the makeup of immune infiltrates in the lesion microenvironment as originally proposed by the investigators.

Response:

We thank the reviewer for the comment. As the reviewer knows, a one year grant may not be sufficient to complete all projects, yet is instrumental in achieving main goals and in setting the stage for future studies. See also our comments to reviewer 1.

3. Weakness: The RNA collected from FFPE was largely degraded and could not be used for a detailed and complete genetic/genomic analysis.

Recommendation: RNA needs to be collected from fresh samples to prevent RNA degradation. In addition, the significant RNA changes identified have to be confirmed at the protein level in both affected tissues and peripheral blood.

Response:

We are in the process of improving our RNA collection techniques, and assembling relevant fresh tissues.

4. Weakness: It is worth noting that although endometriosis is a precursor for certain subtypes of ovarian cancer, no mention is made whether the scientists attempted to collect only these cases.

Recommendation: The authors need to include the number and characteristics of endometrioid and clear cell ovarian tumors collected, respectively.

Response:

We are in the process of bolstering our ovarian cancer bio-repository, which will include specimens from other histological types of ovarian cancer. Data obtained through the current grant will guide us in studies using these samples.

5. Weakness: The role of MUC1 in ovarian cancer remains unclear.

Recommendation: The investigators proposed in the original application to study MUC1-specific antibody responses in endometriosis and ovarian cancer and measure the IgM and IgG anti-MUC1 antibodies in the sera of animal models and patients by ELISA. It is important that this study be completed in order to facilitate the development and investigate the efficacy of MUC1-based vaccines.

Response:

As noted to reviewer 1, we are pleased that the NIH has agreed to fund our work through an R01 grant, which started in the last few months. This will allow us to complete these studies and integrate data from human specimens and mouse models in order to advance our studies.

Generic Recommendations for Magee Womens Research Institute and Foundation

Reviewer 2:

While this project has not yet been published or acquired additional funding, the research has been productive in generating infrastructure needed to answer clinically relevant questions that could lead to new diagnostics, prevention and treatments for a significant health problem. My recommendation is to continue supporting projects such as this, and encourage them to prioritize what needs to be done to possibly patent and to publish their work, and then to share the infrastructure they developed with others at the institution and community.

Response:

We are grateful to the reviewer for this supportive comment and to the Pennsylvania Department of Health for providing us the opportunity to advance our studies and illuminate previously unrecognized pathways in the pathogenesis of ovarian cancer. We hope that our studies will assist in developing innovative MUC1-based vaccines for this devastating disease.

C. If the research project received an “unfavorable” rating, please indicate the steps that you intend to take to address the criteria that the project failed to meet and to modify research project oversight so that future projects will not receive “unfavorable” ratings.

Response: N/A.

D. Additional comments in response to the Final Performance Review Report (OPTIONAL):

Response: We are grateful to the State of Pennsylvania for its support of our efforts to discover novel means to diagnose and treat women's cancers.

Project Number: 0990107
Project Title: The Impact of Age on the Nematode Germline
Investigator: Yanowitz, Judith

B. Briefly describe your plans to address each specific weakness and recommendation in Section B using the following format. As you prepare your response please be aware that the Final Performance Review Report, this Response Form, and the Final Progress Report will be made publicly available on the CURE Program's Web site.

Reviewer 1:

A disconnect exists between the rationale originally proposed for project (to investigate factors relevant to germline aging and replicative capacity) and the final report (we acquired the microscope and have performed some preliminary studies that gave inconclusive results). Given the expenditures involved in both equipment and salaries, it would behoove the PI to double efforts on the former.

Response:

Although the specific studies funded by the grant were inconclusive, our work on germline aging and meiosis continues. Indeed, the confocal microscope has been essential to these studies as evidenced by our recently published study of the *C. elegans* meiotic gene, *him-5* (Meneely et al., Genetics 2012). *him-5*, like *xnd-1*, is required to establish the crossover landscape in *C. elegans* and our studies revealed an increase in nondisjunction with maternal age in these mutants. More recent studies reveal genetic interactions with the worm p53 homolog, *cep-1*, as well as *rfs-1*, the gene we showed to have a maternal aging component. In both cases, the primary defect appears to be caused by problems in DNA damage repair that are significantly enhanced with maternal age. These studies are being written up for publication and will become the basis of a government-sponsored grant proposal within the next year.

In addition to following up on studies of reproductive aging, we have continued to pursue examination of histone modifications in the germline and their connection with maternal age. In this regard, we applied for a March of Dimes Basil O'Connor award to study the histone acetyltransferase complex that is required to modify H2AK5Ac, one of the two marks that we observed were altered in older germlines. The preliminary studies from the Formula Research Funds award were instrumental for obtaining that funding.

Further, our more recent studies on *xnd-1* indicate that it may have a direct role in germline proliferation, raising the possibility that perhaps the changes observed during normal aging are driven by changes in *xnd-1* expression, a hypothesis that we are actively testing. To this end, we have embarked on genetic and biochemical studies to identify XND-1 protein partners and target genes. The studies funded by the Formula Research Funds contributed preliminary data for an NIH R01 grant that was recently submitted.

Reviewer 2:

1. The majority of the funding (some \$600K) went to the purchase of the confocal system. It would be helpful to provide some solid evidence (publications, new collaborations

with external labs who wish to use this, letters of collaboration etc) that the system has in fact improved the infrastructure.

2. The approach of using primarily antibody staining to look for age-related changes in histone modifications lacks the sensitivity required to address the hypothesis. Although the PI referred to this problem in the initial proposal, the experimental approach did not provide appropriate alternatives. It is recommended that Chip-on-chip or ChIP-Seq approaches are used to address this type of question.

Response:

To more fully address this project's impact across MWRI, we have collected letters of support from MWRI investigators who have benefitted from the Nikon A1 confocal microscope system purchased for Dr. Yanowitz's project.

We thank the reviewer for these comments and thoughtful consideration of the techniques we proposed. However, ChIP-Seq is not feasible for these studies for several reasons: First, the major difficulty is obtaining sufficient amounts of germline tissue for ChIP given that there is no large-scale method to separate the germline cells of *C. elegans* from somatic cells. Currently, it might be feasible to hand-dissect several hundred gonads per experiment (or about 25,000 nuclei), but this is still pushing the lower limit of reliability for ChIP-Seq studies. Second, although perceived as somewhat routine, ChIP-Seq and the validation of bioinformatic analysis is very difficult, particularly when it comes to quantitative rather than qualitative changes. Despite the limitations of immunofluorescence, it can be a robust method to detect changes in distribution or amounts of a protein in aging tissue. As the reviewer suggests, moving forward with more molecular techniques may indeed prove more fruitful, especially since over the last two years significant improvement to ChIP-Seq protocols have been made and it is now even becoming feasible to extract information from single cells. To this end, we are using the data that we obtained on H2AK5 acetylation differences in young and old worms as a part of the preliminary data for an R01 grant that will be resubmitted this July which will look more intensely at the precise location of this modification in germline tissues.

Reviewer 3:

1. Questionable grantee commitment to the project: this could be minimized if the grantee had made an effort to: 1) prepare the findings for peer-reviewed publication; 2) leverage the project to obtain longer term funding for the work (which would reflect the grantee's commitment to this line of investigation); and 3) give some indication that this line of study will be continued after completion of this project, with details as to next steps (immediate and long term).

Response:

At the writing of the final report, it was not clear whether these studies would be continued. However, in combination with the other work in the lab, the studies performed under this grant have become the basis of two grant submissions and significant further effort (see responses and details described above).

The lab is committed to gaining an understanding both of H2A lysine 5 acetylation and its function in vivo as well as mechanisms that control germ cell proliferation and how that relates to aging and cessation of the meiotic program. Our recent publication on the *him-5* locus reveals in part how these studies helped shape our thinking about this gene with regard to maternal age and ongoing work in the lab continues to try to dissect the relationship between maternal age, stress, nutrition and germline function.

2. Questionable need for such a large investment in infrastructure improvement: one of the main advantages of the use of *C. elegans* as a model is related to its cost effectiveness for performing complex genetic studies (compared with, for example, mice); however, that advantage was completely lost by purchase of a \$600,000.00 confocal image analysis set-up that was not really necessary for this project to be pursued. While the use of this system might facilitate some of the work, none of the work was dependent on its use.

Response:

C. elegans does indeed generally provide a cost-effective advantage for genetic studies. The work that our lab performs could in theory be performed on a high end wide field scope, but requires post-acquisition processing (deconvolution) that can be fraught with difficulties. This project, and the continued work in our lab, relies heavily on the use of this microscope to analyze sub-nuclear structures in a rapid and robust manner.

The microscope also adds significantly to the reproductive sciences work at MWRI with at least five other labs using the confocal on a regular basis. These now include time-lapse imaging and multi-point quantitation that are greater facilitated by the configuration we purchased with these funds. For our lab alone, the data obtained using the confocal has been incorporated into two NIH proposals, two external proposals, one published manuscript and another in preparation. If we include the other researchers at MWRI, the numbers are even more convincing that this apparatus is essential for the progress of our studies on reproductive health.

3. Lack of current or future beneficial impact: although the grantee's experiments to did produce the outcomes expected, (and thus there is no clear current beneficial impact), some indication that the grantee will take the information obtained and continue to build a research program around this line of study would lend some degree of confidence that, in the future, the project will have a beneficial impact. As things stand now, the grantee has viewed the "negative" outcomes as a reason to simply abandon the work and not pursue either future grant funding or experiments to build on the results obtained.

Response:

As described above, the studies did help for the basis of several grants and publications but those studies only came to fruition after the completion of the final report. The mis-segregation of chromosomes during meiosis is the major cause of human congenital abnormalities and is the leading cause of birth defects, including Down's Syndrome. As women delay child-bearing, the effect of maternal age on embryonic outcomes become more pronounced. Recent studies have highlighted the epigenetic impact of maternal health on offspring health outcomes and suggested that inherited chromatin architecture states may have a profound effect on the next generation. Studies such as ours, using model organisms, set the stage for analyses of these complex

problems in higher organisms and humans. Although, we did not conclusively find the holy grail of an epigenetic landscape changes, our future studies aimed at understanding the roles of specific meiotic genes in crossover control, DNA damage repair, and fertility are poised to shed light on these critical problems.

C. If the research project received an “unfavorable” rating, please indicate the steps that you intend to take to address the criteria that the project failed to meet and to modify research project oversight so that future projects will not receive “unfavorable” ratings.

Response: No response required

D. Additional comments in response to the Final Performance Review Report (OPTIONAL):

Response: We are grateful to the State of Pennsylvania for its support of our investigations, through which we hope to gain important insight into the effects of aging on reproductive biology.