

Response Form for the Final Performance Review Report— Magee-Womens Research Institute and Foundation 2008F*

1. Name of Grantee: Magee-Womens Research Institute and Foundation
2. Year of Grant: 2008 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.

Response:

We are grateful to the Pennsylvania Department of Health for this funding opportunity. The funds provided through the 2008 allocations have been used to fund eight important projects, which received scores between Favorable to Outstanding, with no Unfavorable scores. The projects supported by the Pennsylvania Department of Health generated very important results that have been instrumental in pursuing new ideas and advanced knowledge that is directly relevant to women’s health. As noted within the documents, some of these projects played a central role in the attainment of new grant funding, which directly contributed to the status of Magee-Womens Research Institute as the top-funded research institute in the United States in the field of reproductive sciences and women’s health.

We also thank the reviewers for their comments and constructive criticisms. With the critiques, our projects have improved, with tighter approaches, thorough analysis, and closer monitoring of progress and attainment of goals. As noted by the reviewers, we often composed fairly ambitious projects. This reflects our understanding that a key goal of the one-year support is to plant seeds for long-term pursuits that exceed the limited one-year research plan. We therefore believe that some of the reviewers’ comments stem from the possibility that the studies may require more time to mature, a common practice during the initiation of new projects. It is also not surprising that at the end of a one-year grant an investigator might not be able to demonstrate the full impact of the research (e.g., publications might not be ready after 12 months of initiating a new line of research). We therefore expect that the report will reflect the achievements and their long-term impact on the researcher’s career.

Over the past two years, we deployed a new process for selection of researchers and projects, monitoring of progress, preparation of summary reports and the development of new initiatives that build on the Department of Health’s grants. We were extremely pleased that our scores have improved in recent years, and that none of the projects reviewed in the past three years received an “unfavorable” score. As MWRIF’s Director, Dr. Sadovsky carefully assesses applicants for Department of Health grants. He meets with them to discuss the research plan prior to

submission of the proposal. As noted above, a key principle is a long-term plan that builds on the one-year project, with projected research expansion, and recognition of educational and commercial prospects. We have also implemented a number of steps designed to improve and monitor the quality of the research protocols. These include (a) mentorship process, where the Director and established members of MWRIF are in direct contact with less experienced grant recipients, (b) meetings between Dr. Sadovsky and all program researchers near the six-month mark to ensure that the research is progressing according to plan, and (c) greater attention to progress and timely reports.

We also highlight that Cheryl Richards, MWRIF's Director of Grants and Contracts, is responsible for assuring that MWRIF is in full administrative compliance with the Health Research Formula Grant Program. She assists the investigators in preparation of the budget and proposals according to Program guidelines, and oversees completion of all administrative reporting in a timely manner. Additionally, she maintains a record of all investigators who have been funded through the program and their research reviews. Ms. Richards and MWRIF's Director, Dr. Yoel Sadovsky, oversee the program and identify investigators that are suitable for the support. We believe that our efforts have been successful in avoiding any (unfavorable) reviews in recent years.

We again thank the Pennsylvania Department of Health for supporting our efforts to promote women's health to the forefront of science, and to translate the knowledge to better care for women and their infants. With the review process, we believe that our research becomes more profound, important, creative, and meticulous. The support of the Pennsylvania Department of Health Tobacco Settlement Act Grant is vital to our mission. We look forward to furthering this research for the health of women and their infants.

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: 0863901

Project Title: Roles of the Nuclear Receptor Cofactor LCoR in Placental Development and Gene Expression

Investigator: Barak, Yaacov

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:

None.

Reviewer 2:

The investigators should work to complete the studies outlined in Aims 2 and 3.

Response:

We indeed continued to work on both aims, and have successfully leveraged this project into a full R01 size component of a Program Project grant with Drs Sadovsky and Chaillet at the institute (P01-HD069316), whose 5-yr funding is slated to start on April 1st, 2012. We thank the reviewer for their encouragement.

Reviewer 3:

1. As written, it is presently unclear that Muc 1 (a central model used in these studies) plays a crucial role during placental development. A more extensive or convincing rationale would have been helpful for why the analysis of Muc 1, as a unique and novel target of PPAR γ anNCOR molecular mechanisms, is necessary for our understanding of placental function.

Response:

At this juncture, we have identified the glucagon receptor gene, *Gcgr*, as a new PPAR γ -LCoR target gene whose physiological relevance is clearer both generally and in specific reference to the LCoR-null phenotype. We have already identified the minimally PPAR γ /LCoR-responsive promoter fragment of *Gcgr* and are continuing the same line of work, alongside the less physiological understood Muc1, which still provides an outstanding template for nuclear receptor-cofactor biochemistry.

2. A more comprehensive description of the use of statistical analyses in these studies would have added strength to the outcomes described.

Response:

All studies presented in the progress report used two-tailed Student's T-tests with P=0.05 as a cutoff for significance. Sample sizes for qPCR reactions were 3-4 placenta pools per point. For placenta and fetus sizes, the data represent 24 WT and 30 null embryos collected from 10 (ten) different litters of LCoR \pm parents at E18.5.

3. The histological analyses of the important finding of changes in population of glycogen cells within the spongiotrophoblast layer of the mouse placenta, is relatively poorly characterized. Additional analyses using immunohistological approaches would be more convincing to determine the identity of this lineage and how it may affect placental function.

Response:

I completely agree. These more in-depth analyses are currently in the works in pursuit of a more accurate understanding of the phenotype. The material presented in the progress report should not be misconstrued for a final assessment, as it is only an interim summary of a one year, \$100K project.

4. In Aim 3, good progress is recognized with the analysis of the Muc 1 gene promoter; however, the use of many different promoter fragments including very small segments of the Muc-1 gene promoter leave open to argument how to interpret these data. This reviewer believes there is important information in these studies. A more systematic approach to how these reporter gene studies are carried out and the data interpreted would be of great help.

Response:

Agreed. Interpretation of these studies is relatively complicated, and we are currently trying to carry them on not only with *Muc1*, but also with *Gcgr*, which on first glance, seems to both share some similarities with and possess some differences from Muc1. We hope that as this type of study teases out individual components of the overall transcriptional response, it will culminate in detailed understanding of the intimate mechanics of general and placental gene regulation by transcription complexes.

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:

Not applicable.

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

Response:

I am thankful to the PA Dept of Health for this funding opportunity. Without it, I doubt that we would have seriously carried out this project, which turned out very important results that start to change the way we understand placental metabolism and physiology in late pregnancy. It is even more reassuring that preliminary data obtained with the help of this grant were instrumental in obtaining >\$1M in federal funding for this investigator alone, which was in turn critical in landing the approximately \$6M for the group of investigators involved in the awarded P01 grant.

Project Number: 0863902

Project Title: Establishment of an Animal Model for Respiratory Infection with Influenza during Pregnancy

Investigator: Beigi, Richard H.

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. One of the major potential confounders in whole-body exposure experiments is an animal behavior called "preening," where rodents lick each others fur during and after exposure. Thus, the virus may be ingested as well as inhaled in these experiments, and allowance for the ingested virus must be made in the dosimetry and deposition experiments. There is no indication that the investigators have accounted for this phenomenon, which may be contributing to (or responsible for) their conclusion that the virus has a lower LD50 (by 10-fold) by the aerosol challenge than the prior literature values estimated for the LD50 by intranasal inoculation. This putative additional route of exposure should be controlled for in subsequent studies of virus infectivity. A nose-only exposure study to test whether this phenomenon of preening is affecting the apparent LD50 for the virus would be a simple approach.

Response:

This is an interesting point and we have considered this. In previous experiments (done by the co-investigators on this proposal) with similarly transmitted pathogens, there was no evidence that preening and subsequent potential ingestion of virus via the the GI tract produced any demonstrable difference. Performing nose-only experiments does not completely control for this given that much of what is given also ends up in the GI tract of the animals. The difference we noted in LD50s is so large that it is not believed to have been from this potential issue, but we recognize the importance and concept of the suggestion to direct comparison. Given that after intranasal inoculations, much virus is exhaled, this potential issue is hard to control for regardless of exact approach for challenge.

2. In addition, the comparison of viral load (and/or viral titers), by one route of administration, aerosol exposure , with literature values for another route of exposure (intranasal instillation) previously published by other laboratories, is subject to many potential errors. The investigators should calibrate their conclusion by determining whether the comparative parameters for intranasal instillation performed in their laboratory with their viral strain and their animals agree with the cited values from the literature.

Response:

We agree, and will certainly recognize this in any publications that result from this work (currently in progress). We would prefer to do intranasal inoculations as well on similar batches of animals for direct comparisons, however, funds did not allow for those experiments.

3. The assumption that the inert microspheres behave identically to live virus in aerosol exposures should also be tested experimentally in future work.

Response:

We agree, and will consider as we plan future experiments.

4. Viral deposition and retention should be evaluated in tissues other than the lung after whole-body exposures, especially in the gastrointestinal tract, liver, uterus, and the fetuses themselves. Virus particles may be swallowed after preening or after mucus clearance from the lung and nasopharynx. Dosimetry calculations should account for these alternative routes of exposure and loci of distribution.

Response:

As this work requires additional funding, it was carried out in subsequent experiments that were performed with ferrets from additional and subsequent funding (and are reported in subsequent funding reports). We agree, and have frozen these tissue samples from these mice. This would require additional funding to perform assays, which is our goal.

5. The basic maxim of toxicology as attributed to Paracelsus is “the dose makes the poison.” That adage may also apply here; very high viral loads (LD50-LD99) are being used for these experiments. This might be part of the reason for the obvious fetal losses at the highest dose of virus used. The relevance of these findings for clinical use is questionable. Careful attention to appropriate dose-response experiments as this project proceeds is highly recommended.

Response:

Agreed, and we are cognizant of this as we are interpreting and writing our results.

6. The investigators identified an issue of non-pregnant mice among the animals purchased as timed pregnant and allocated to the pregnant group. They have shifted the dates of exposure to allow themselves to better ascertain pregnancy prior to virus exposure, which seems to be an appropriate response. The researchers also suggest the possibility of creating their own breeding colony to better control the overall process. They do not discuss the possibility of stress-related abortion in the mice that should have been pregnant, and should better monitor their animals in order to be able to rule out this possibility. They also mention cannibalization of dead or moribund fetuses as a possible reason for smaller litter size in the infected animals, which suggests less than optimal monitoring of the mice might be occurring during critical experimental periods.

Response:

We understand these concerns. It is highly challenging in reality to monitor mice 24/7, as they will often cannibalize at all hours and quite quickly. We monitored the animals 4-5 times per day when they were sick and after the pups are born. So it is doubtful that we missed stress-related abortions. We have designed future studies to include pregnant non-exposed animals (sham exposures) to attempt to control for stress factors related to transit and acclimation to a new environment as well as the experimental procedures themselves. Most of our subsequent work

has been with ferrets, which have a longer gestation, thus providing more time for pregnancy verification, acclimation after travel, and monitoring after exposure to virus.

Reviewer 2:

1. Provide a better description of additional methods to measure the effects of flu infection during pregnancy and change the challenge virus to the pandemic H1N1 strain.

Response:

In subsequent experiments, we have used ferrets, and also used a 2009 H1N1 influenza strain to 1) better match clinical relevance, and 2) not have to mouse-adapt the influenza virus strain for animal infection. We have started using implanted chip technology for some clinical data capture, have included pregnant sham-exposed animals to attempt to control for pregnancy specific outcomes, and have done necropsies of pregnant and non-pregnant ferrets for comparison of various different tissue penetrations of virus, pathological differences between pregnant and non-pregnant animals, etc. The start-up funding in this current cycle was instrumental in coalescing our collaboration and allowing us to really begin to refine our pregnant animal challenge studies.

Reviewer 3:

2. Weakness: Use of 1mm latex beads for Aim 3 instead of live virus.

Recommendation: Complete experiments as proposed with aerosolized virus in the whole-body and nose-only aerosol devices. Measure virus deposition (titration and immunohistochemistry) at 2, 24, 48 and 72 hours post infection. Compare to topical delivery. Do not compare pregnant to non-pregnant as you first need a baseline in normal mice. If you see differences, you may decide to focus your pregnant to non-pregnant in only one delivery model based upon those differences. If they are identical, then just use one delivery. Since the topical will not be identical to aerosol, you should compare this to aerosol in your studies.

Response:

We appreciate your comments on the model presented. The funding procured was instrumental in initiating our experiments. We have done subsequent challenges using ferrets, but have retained the pregnant to non-pregnant aerosol approach due to the focus of the project being developing an animal model during pregnancy. We are considering comparing aerosol approaches to nose-only as you suggest, pending funding. We have also done serial sacrifices, as has been suggested above, in ferrets in subsequent studies.

3. Weakness: Omission of tissue titers and pathology.

Recommendation: Include these endpoints in all studies. These are established endpoints in the murine model of influenza infection; without them, you have not established the baseline of a murine model, especially when these endpoints are critical for aerosol models.

Response:

We have included tissue titers and pathology in subsequent studies with additional funding, and do have some tissue samples frozen from these experiments that we will consider going back to should we be successful in obtaining funding.

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:

Not applicable.

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

Response:

We are grateful to the PA Dept of Health for this support. It has been extremely helpful in terms of starting this exciting project and setting the stage to an important research direction.

Project Number: 0863903

Project Title: Immune responses to herpes simplex virus type 2 and Chlamydia muridarum in a murine model of co-infection

Investigator: Cherpes, Thomas L.

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:

Clarify if CD25(+) cells that were transferred (or eGFP(+) cells from the special Foxp3-eGFP mice) were actually true Treg or just activated CD4 T cells. There is some evidence that CD4 T cells can non-specifically turn on FoxP3 after activation. The PI needs to work out whether the beneficial adoptively transferred CD4 T cells are really Treg or just activated day seven splenocytes from infected mice that could represent antigen-specific regular CD4s or bystander activated regular CD4s.

Response:

As stated by this reviewer, mice transferred of CD4⁺CD25⁺ splenocytes demonstrated less genital pathology and were more likely to survive infection long-term than controls or mice that received CD4⁺CD25⁻ splenocytes. As transfer of CD4⁺CD25⁻ cells also conferred some protection, compared to control mice, against development of genital pathology and death, it was possible these findings were due to the inclusion of small numbers of Foxp3⁺ Tregs in the CD4⁺CD25⁻ transfer population. To test this hypothesis, we repeated the experiment using Foxp3⁺GFP mice as donors, in order to isolate only Foxp3⁺ cells from splenocytes of infected donors using flow cytometric cell sorting. We saw that CD4⁺Foxp3⁺GFP donor cells were capable of preventing tissue damage and mortality, but CD4⁺Foxp3⁻ cells did not change the course of the infection, as compared to untransferred controls.

Clarify if CD4 T cells and/or Treg-like cells from vaginally infected donor mice behaved differently from similar adoptively transferred cells from ocularly infected mice.

Response:

Because of the possibility that anti-viral effects exerted by HSV-induced Tregs might result from non-classical immune responses that are unique to the corneal route of infection, we compared the ability of HSV-induced Tregs generated from donor mice infected vaginally or ocularly to rescue recipient mice. We found that both HSV-induced Treg populations were able to confer indistinguishable protection to recipient mice vaginally infected with typically lethal doses of HSV-2.

Do IL-17R KO mice have a difference in HSV pathogenesis?

Response:

We have not been able to explore the phenotype of IL-17R KO mice that are vaginally infected with HSV-2. IL-17 produced by CD4 T cells is implicated in immunopathological host responses to multiple viral pathogens, and we have studied if neutralization of IL-17 with a blocking monoclonal antibody can prevent the morbidities seen in mice vaginally infected with HSV-2. Interestingly, inhibition of IL-17 prevented genital pathology and increased survival. Although we have also not tested viral replication in IL-17 treated mice, we speculate that IL-17 blockade induces responses capable of preventing HSV-2 invasion of the CNS, since viral replication in these tissues precedes encephalopathy and death. Furthermore, we also observed that IL-17 treatment was associated with reduced neutrophilic infiltrates and increased numbers of inflammatory monocytes and NK cells recruited into infected vaginal tissue. IL-17 is key host defense element, and can recruit neutrophils to infected tissue to limit pathogen replication. However, in our experimental model it seems that IL-17 elicits host responses that cause tissue damage but also cannot effectively control HSV-2 replication and CNS spread.

Reviewer 2:

The main weakness surrounds the lack of data presented to indicate the PI and team evaluated the latency/reactivation potential in the long-term survivors created through the adoptive transfer studies. There are indications that the latent viral DNA burden was evaluated, but too little information is provided to indicate this was pursued effectively. The focus was clearly on the immunological outcomes, but the virology is crucial to support the utility of the enhancements in the model.

Response:

Because transfer of CD4⁺CD25⁺ T cells was shown to confer protection to mice vaginally infected with HSV-2, we also determined if this intervention altered the kinetics of genital tract viral clearance and CNS dissemination. To do this, we used rt-PCR to compare the HSV-2 burden in vaginal lavages and spinal cord specimens between controls and mice receiving CD4⁺CD25⁺ splenocytes concomitant with infection. Despite finding previously observed decreases in genital pathology, no detectable differences in viral burden were seen among transferred mice, compared to controls. However, spinal cord invasion by HSV-2 was greatly reduced among transferred mice. These results indicate that Tregs from HSV-2 infected donors induce complex modulation of the host responses to HSV-2 infection that both prevent tissue damage and control viral invasiveness. However, our results also indicate that our model cannot be used to explore HSV latency/reactivation.

Another weakness is the lack of publications reporting the intriguing findings. The PI is strongly encouraged to publish the data because it has value for the field and could lead to opportunities for additional collaboration.

Response:

We agree, and regret our work has not advanced sufficiently to permit a meaningful publication. We hope to build on our findings, advance our data, and publish soon.

Reviewer 3:

The use of adoptively transferred CD4⁺ T cells is not practical as a treatment of HSV-2 infection. How can these results be used for more translational applications and the design of therapies for HSV-2 infection?

Response:

Treg responses can preserve host tissue by limiting immunopathologic damage elicited by anti-pathogen immune responses and facilitate pathogen persistence by subverting protective host responses. Therefore, increased understanding of Treg activation and migration, the nature of the antigens Tregs recognize, and mechanisms by which Tregs regulate host immune responses to viral infection are logical next steps for the sculpting of novel therapeutic approaches that create acceptable balances between pathology and protection during infection. A number of clinically important infections (e.g., influenza and hemorrhagic viruses) are caused by pathogens that elicit immunopathological host responses, but lack suitable experimental models or require high level biocontainment laboratories. Possibly because it evolved with humans, vaginal HSV-2 infection of mice is not characterized by the establishment of life-long latency. Instead, murine vaginal HSV-2 infection elicits exuberant inflammation and extensive collateral host damage. The preliminary data we have collected suggests that vaginal HSV-2 infection of mice will prove to be an ideal model for in vivo exploration of IL-17-mediated inflammatory responses and mechanisms by which adaptive Tregs can prevent development of IL-17-mediated immunopathology. Our model may be of particular utility for investigation of the host immune responses achieving a homeostatic balance between protection and pathology as HSV-2 can also be used in lower level biocontainment facilities. Our data also shows that transfer of Treg populations from infected to naïve mice at the time of HSV-2 vaginal infection suppresses genital pathology and prevents viral dissemination. Therefore, elucidation of Treg-regulated immune responses that prevent tissue damage and permit viral clearance may also inform design of therapeutic approaches against clinically relevant invasive pathogens.

The antigen-specificity of the protective CD4⁺ and CD4⁺CD25⁺ regulatory T cells should be interrogated. This seems important for designing preventative and curative therapies for HSV-2 infection.

Response:

We agree, and we currently seek further funding to explore this idea. Tregs are induced by nearly all pathogens that cause persistent infection, but definitive confirmation of Treg antigen specificity requires generation of antigen-specific T cell clones, antigen specific proliferation, or ex vivo intracellular cytokine staining of antigen-stimulated T cells. Our preliminary data show that the transfer of CD4⁺CD25⁺ splenocytes from HSV-2 infected donors into naïve mice concomitant with vaginal HSV-2 infection was associated with decreased genital pathology, increased survival, and significantly lower concentrations of several important inflammatory cytokines in the lower genital tract. Our preliminary data also indicates that HSV-2 infection is critical for the induction of the protecting CD4⁺CD25⁺ cell population, since naïve or non-activated natural Tregs were not associated with increased protection. However, naturally occurring CD4⁺CD25⁺Foxp3⁺ T have been shown to play an important role in preventing immunopathology caused by autoimmunity, and so it remains

possible that protection seen in our studies was conferred by transfer of natural Tregs that were activated by vaginal HSV-2 infection. Therefore, we need to test the hypothesis that activation, rather than antigen specificity, is more integral for the protection conferred by HSV-induced CD4⁺CD25⁺ splenocytes against murine vaginal HSV-2 infection.

Use of CD25 to identify regulatory T cells is problematic because the most activated T cells will also express high levels of this marker. Instead, additional markers Foxp3⁺, CTLA-4, etc. that more specifically identify true regulatory T cells among CD25⁺CD4⁺ T cells would strengthen the interpretation of the results presented for Aim 2.

Response:

We agree (and please see our first response to reviewer #1). To ensure contaminating T_H cell lineages did not contribute to the observed diminution of genital pathology, more recently we have performed transfer experiments with highly purified HSV-induced Treg populations that were generated in C57Bl/6 Foxp3⁺GFP infected mice.

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:

Not applicable.

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

Response:

We thank the Pennsylvania Department of Health for this support. With our results and the reviewer's comments we are hopeful to carry out additional research and gain additional knowledge in this important field of female viral infections and immunity.

Project Number: 0863904

Project Title: Integration Study of the Target Genes of PPAR gamma in Human and Mouse 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. The proposed ChIP on chip studies were not performed. These studies will provide data to further validate the targets identified.
2. There was no functional analysis of the target genes identified.

Response:

1. During the one year time-frame of the studies, we were not able to complete data validation. Since the completion of the one year funding period were able to validate key targets (such as Plin2 in humans, and shed much more light on the important associations found in our study. We have also markedly advanced our analysis in mouse placentas. A manuscript detailing our findings has been completed, and is now under review.
2. Further analysis of the identified target genes is ongoing. A detailed analysis of Plin2 is currently prepared for publication. Additional analyses of murine targets for PPAR gamma has been accomplished, and described in a submitted manuscript entitled, "Placental PPAR gamma regulates spatiotemporally diverse genes and a unique metabolic program". Detailing our transcriptional profiling of PPAR gamma targets, this manuscript demonstrates a widespread role for the coactivator NCOA6/AIB3, but not MED1/PBP, in PPAR gamma-dependent gene expression. In addition, spatial and temporal expression analyses revealed that PPARgamma regulates genes in diverse trophoblast lineages and during different stages of differentiation.

Reviewer 2:

1. The study was superficial and the gene expression analysis used only gene ontology descriptions, which are a reasonable starting place, but current bioinformatics capabilities allow for far more comprehensive analyses of these datasets.
2. There was no attempt to corroborate the microarray data with follow-up quantitative RT-PCR analysis of selected genes. This is a minimum requirement for subsequent publication.
3. While the aims outlined in the proposal were addressed by the analysis in the project, there was no clearly defined hypothesis tested.

Response:

1. As noted by the reviewer, we have taken the preliminary studies to a much greater depth, where we integrated data from mouse development and human trophoblast differentiation. As noted in the previous set of comments (reviewer 1), our results using mouse tissues have been submitted for publication. Our results using human trophoblasts are currently in final stages of analysis, and selected targets are described in a new paper we are completing.
2. We conducted detailed PCR validation of key targets, with an analysis that follows the time course detailed in our initial array assays. PCR-based validation of mouse targets has been completed. For example, we further analyzed the *Ldhd* gene, which encodes the H isoform of lactate dehydrogenase, as a robust trophoblast-specific PPAR gamma target. These results are detailed in a submitted manuscript entitled, "Placental PPAR gamma regulates spatiotemporally diverse genes and a unique metabolic program" (see above). In human tissues this was completed for selected genes (publication pending).
3. The data derived from our analysis served as a platform for a number of hypotheses, which have been tested and continue to support on-going studies.

Reviewer 3:

1. One important weakness was the lack of some preliminary validation of the array analyses using qPCR and or western blot analysis. In my view, this was a missed opportunity, particularly, for example, the single gene that demonstrated the overlapping expression pattern between mouse and human placental material.
2. Caution is suggested when assuming the pharmacological drugs used have high selectivity in their actions. Off-target effects are always an important caveat to these types of studies and how alternative actions could alter interpretation of the results.
3. This reviewer fully appreciates that populations of human TSC are highly precious; however, studies examining a single experimental replicate is very difficult to describe in terms other than highly preliminary. I am certain the PI and this research group seek to add more replicates to these analyses, which will strengthen interpretation of the human TSC data markedly.

Response:

1. As noted above, we have taken the preliminary studies to a much greater depth, which include a comparative genomics analysis of mouse and human tissues. These data are based on events that take place during mouse development and human trophoblast differentiation, and served as a basis for submitted papers, described in the comments above.
2. We fully agree, and therefore combined developmental and pharmacological approaches to define "real targets".

3. We agree. In follow up studies we have been able to perform additional replicates, and support our results.

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:

Not applicable.

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

Response:

I thank the Pennsylvania Department of Health for this grant. Exploratory data from our work was extremely useful in generating new research directions.

Project Number: 0863905
Project Title: Soluble KIT Receptor in the Pathogenesis of Preeclampsia
Investigator: Hubel, Carl A.

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:

Aim 3 is interesting and should have been tested.

Reviewer 2:

1. I would encourage the authors to evaluate the biological consequence of the altered c-kit in future studies. For example, is the low c-kit responsible for the insulin resistance of preeclampsia?
2. The authors should develop an ELISA kit that measures free SCF levels. It appears that the current kit only measures total SCF levels and therefore does not correlate with clinical disease.
3. The source of c-kit alterations and nature of regulation of c-kit should be explored in future studies.

Reviewer 3:

There is a central question that needs to be answered (placenta source of sc-kit?) and one that was proposed as a specific aim (Aim 2) was not attempted. The techniques and samples are in hand, so it was disappointing not to see at least some initial attempts. This is a relatively minor weakness in an otherwise well done project.

Response to Reviewer 1:

The goal of Aim 3 was to 1) compare expression levels of *c-kit* and SCF in third trimester archived placental biopsies from preeclamptic and normal pregnancies, 2) to determine if third trimester preeclampsia placental villous tissue in fresh explant culture show significant reductions in SCF and *kit* protein mass, and secrete less SCF and *sc-kit* into the culture medium compared to normal pregnancy placental tissue, and 3) to determine whether concentrations of *kit* in villous explant culture and explant conditioned media are reduced under low oxygen partial pressure (2% oxygen) compared to standard culture conditions (21% oxygen).

We have now obtained sufficient quantities of conditioned media from placental villous tissue in explant culture from preeclampsia patients and controls. The explants were cultured for either 24 or 48 h under 2%, 21% and 8% oxygen, the latter approximating "normoxic" condition for villous trophoblast. Some of this work was accomplished by a summer college intern from Washington and Jefferson College, with funding from the Merck Internship for Excellence in Science. However, there was an approximate one year lag in the time from delivery (procurement of fresh placenta samples during the summer of 2010) to the committee diagnosis

of pregnancy outcome. We now have sufficient preeclampsia samples. We have optimized techniques for placental tissue immunostaining and measurement of another soluble receptor, syndecan-1, and plan to probe for both syndecan-1 and sc-kit in our samples.

Reviewer 2:

1. I would encourage the authors to evaluate the biological consequence of the altered c-kit in future studies. For example, is the low c-kit responsible for the insulin resistance of preeclampsia?

Response: Most of the plasma samples we obtain are non-fasting. However, we can obtain information on the time interval from last meal to veni-puncture and thus assess this question indirectly by measuring insulin and sc-kit in tandem.

2. The authors should develop an ELISA kit that measures free SCF levels. It appears that the current ELISA only measures total SCF levels and therefore does not correlate with clinical disease.

Response: This appears to be correct given that sc-kit at high levels did not affect SCF readings from the ELISA. This would require collaboration with investigators familiar with ELISA development.

3. The source of c-kit alterations and nature of regulation of c-kit should be explored in future studies.

Response: We tried to make progress on this Aim by first measuring sc-kit in conditioned media from HMC-1 cells in culture as these transformed mast cells reportedly produce large quantities of sc-Kit. Despite months of attempts, however, we were unable to detect sc-kit secretion from these cells either under basal or phorbol ester-stimulated conditions, using either ELISA or Western blot. We thus agree with Reviewer 2 recommendation to “establish collaboration with investigators in the stem cell field who have expertise in c-kit signaling pathways”.

Reviewer 3:

There is a central question that needs to be answered (placenta source of sc-kit?) and one that was proposed as a specific aim (Aim 2) was not attempted. The techniques and samples are in hand, so it was disappointing not to see at least some initial attempts. This is a relatively minor weakness in an otherwise well done project.

Response: Soluble c-kit concentrations declined over the course of normal or preeclamptic pregnancy. Significant decreases occurred at the 16-19-, 27-30- and 40-42-week of normal pregnancy compared to the 5-10 week interval, and between the 16-19 and 40-42 weeks' gestation intervals. Soluble c-kit concentrations were significantly lower at 40-42 weeks (ng/mL median [IQR]: 8.0 [6.2 – 8.7]) compared to values in 23 non-pregnant/nulligravid women of similar age who were not on oral contraceptives (10.5 [8.8 – 12.1]; $P < 0.05$). Non-pregnancy and 5-10 week gestation levels did not differ. Data obtained since the Final Progress report shows that soluble c-kit levels rebound by 4-10 weeks postpartum (10.3 [8.9 - 10.3]) compared to term levels (6.7 [5.3 – 7.7]). The steady decline in sc-kit over gestation with rebound

postpartum is indirectly suggestive of a placental inhibitor of sc-kit leading to reduced levels in the maternal circulation.

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:

Not applicable.

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

Response:

We thank the reviewers for their comments, and the PA Department of Health for this support.

Project Number: 0863906

Project Title: Post-Transcriptional Regulation of Fstl1 mRNA in Human 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. The seemingly contradictory data shown in the Final Report must be clarified. This will be critical for the project's funding success at the national level.

Response: We acknowledge that the original data presented in the report were puzzling. On the one hand a Northern blot analysis of RNA isolated from primary trophoblasts revealed a signal for FSTL1 but on the other hand both immunochemistry and in situ hybridization experiments failed to detect the presence of FSTL1 in villous trophoblasts. Using an optimized immunohistochemical staining protocol we recently detected FSTL1 in villous trophoblasts. However, the staining in these cells was clearly weaker than in extravillous trophoblasts (EVT). Moreover, these new data also indicate that the FSTL1 signal is mostly restricted to the cytotrophoblast subpopulation of villous trophoblasts while the syncytiotrophoblast was almost uniformly negative. This observation is consistent with our Northern blot experiments because primary trophoblasts isolated from term placenta are almost exclusively cytotrophoblasts early in culture. Together, we believe these new data address the apparent discrepancies from the original report.

2. The mRNA turnover studies were inadequately performed. The PI saw no substantive decay of the Fst1 mRNA during the chase period. This could have been due to the lack of sufficient transcriptional suppression. A pilot experiment to assess the percent of transcription inhibition must be performed to clarify the half-life calculations.

Response: We agree with the reviewer that the mRNA stability assay requires further optimization. For confirmation of transcription inhibition, we will test the ability of a transcriptional inhibitor to prevent the induction of NDRG1 mRNA upon exposure to hypoxia. We have shown in our laboratory that NDRG1 is strongly and rapidly induced in trophoblasts following exposure to hypoxic conditions. Suppression of hypoxia-mediated induction of NDRG1 will constitute a clear indication that de novo transcription is efficiently suppressed. Upon treatment with a transcription inhibitor and exposure to hypoxia, cells will be collected at different time points for RNA isolation and NDRG1 levels will be assessed by real-time PCR. Moreover, in addition to actinomycin D, we also plan to use triptolide, a new type of transcription inhibitor, which exhibit a potent inhibition of RNA polymerase II-mediated transcription and present a superior specificity compared to other inhibitors. Upon establishing the adequate conditions for efficiently inhibiting transcription we will further assess FSTL1 mRNA stability in normoxic and hypoxic conditions.

3. The primary trophoblast cultures represent principally the villous component of the trophoblast of the placenta. Yet, HTR-8/SVneo cells represent the extravillous component of the placenta. The work alludes to the notion that perhaps it is EVT and not villous trophoblast that expresses Fst1. Thus, the two models are inconsistent. This should be addressed by the PI.

Response: While we acknowledge the limitations of our cell model we would like to point out that HTR-8/SVneo cells were the only trophoblast-derived cell line in which we detected the expression of FSTL1. Other common placenta cell lines including JEG3, BeWo, and Jar did not show any signal for FSTL1 in Northern analysis. Also, while we originally failed to detect the expression of FSTL1 in villous trophoblasts, we recently identified FSTL1 in these cells by immunohistochemistry using an optimized protocol. We are now confident that FSTL1 is expressed in the extravillous compartment as well as the villous compartment of the human placenta. It is possible that FSTL1 plays different roles in the different compartments. Furthermore, because FSTL1 is a secreted protein, it is very likely that it acts on other cell types than trophoblasts. Targeted cells could be other subpopulations of trophoblasts, various immune cells or decidua cells. Therefore, we believe that investigation of the function of FSTL1 in the placenta will require the use of several cellular models.

Reviewer 2:

None.

Response:

None.

Reviewer 3:

1. Analyses of the Fst1 3'UTR reporter constructs should be revisited with comparisons made between similar length regions (native vs. mutant).

Response: We agree with the reviewer that creating large truncations of the 3'UTR may alter the overall structure of this region and may render interpretation of the results more difficult. However, considering the large size of FSTL1 3'UTR (almost 3 kb) and the abundance of potential regulatory elements, it was difficult to select a specific site for introducing inactivating mutation. This pilot experiment was undertaken in order to identify a subregion carrying fewer regulatory sites that could be mutated individually. In addition, microRNA target site prediction remains very inaccurate despite progress made in the design of target prediction algorithms. To date we have no clear experimental evidence that the AU-rich elements identified in the 3'UTR of FSTL1 regulate the stability of the FSTL1 transcript, although computational tools give a high score for the prediction of its destabilization. Upon demonstration that the 3'UTR of FSTL1 determine the stability of the mRNA (see below) we will be ready to create a series a mutation in each of these AU-elements to assess their impact on the mRNA stability.

2. Whether stability of Fst1 is affected by its 3'UTR at all, needs to be determined before launching into more detailed and specific mapping experiments.

Response: We hypothesized that FSTL1 mRNA was targeted for decay based on the observation that FSTL1 signal was reduced in hypoxia when analyzed by Northern blot and by the identification of numerous potential destabilizing motifs within the 3'UTR of its mRNA. Our preliminary experiments described in the report did not produce conclusive evidence showing accelerated decay of the FSTL1 mRNA in hypoxia. However, as pointed out by reviewer 1 (point 2), the assay aiming at determining the turnover of the FSTL1 mRNAs needs to be optimized. We described our strategy to address this concern in our response to reviewer 1.

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:
Not applicable.

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

Response:
We thank the reviewers and the PA Department of Health for the support. With this support we are able to carry out additional studies that build on those reported here, and advance our understanding of human placental dysfunction.

Project Number: 0863907
Project Title: Analysis of Functional Domains within NDRG1
Investigator: Sadovsky, Yoel

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. Freshly isolated primary term trophoblast cells do not proliferate. How about using trophoblast cells from *the* first trimester.
2. Although domain mapping of the NDRG1 protein is necessary, the studies may be better designed to focus on first trimester trophoblast cell endovascular differentiation.

Response:

1. While we understand the significance of first trimester trophoblasts, we elected not to use these cells for our experiments for the following reasons: (a) our preliminary data were performed in cells from late pregnancy. The biology of trophoblasts from the latter part of pregnancy is markedly different from that of first trimester cells (b) we were interested in hypoxic trophoblast injury, which characteristically occurs after the first trimester. In fact, first trimester trophoblasts naturally grow in a hypoxic environment. Thus, it is likely that the action of NDRG1 is markedly different in these cells.
2. See comment #1. The reviewer may recall that hypoxia is a normal part of physiology of first trimester invasive extravillous trophoblasts. Our experiments are designed to test the function and mechanism of action of injured cells, where NDRG1 is up-regulated. Performing these experiments in first trimester cells would not be optimal.

Reviewer 2:

1. The primary weakness that reduced enthusiasm for this grant was a lack of progress on structure function mutants developed in Aim 1, and how quickly the studies progressed within Aim 2. This is also reflected in the lack of publishable material from this application to date given such a well-established laboratory and the relatively large group of individuals supported on these funds. This may clearly reflect technical challenges overcome during the course of these studies.
2. Careful consideration of technical aspects of the structure function mutants is necessary since loss of function of these mutants may reflect an inability of a given mutant to fold properly within the cell. Analysis at this level may require the addition of biochemical expertise currently not present within this research group.

Response:

1. We sincerely hope that the delay in establishing the NDRG1 mutants and in analyzing their function is perceived as a part of a normal experimental conduct, where results sometimes require the generation of additional tools or necessitates a change in experimental plan, as dictated by new results.

2. As we advanced our experiments we have clearly validated that one of our NDRG1 mutants, del128-143, which deletes the phosphopantetheine attachment site (PPAS, detailed in our proposal), impacts NDRG1's function. Upon silencing endogenous NDRG1 and concomitantly over-expressing mutant NDRG1, we found that this mutant does not localize to the nucleus or cell membrane, as we observed with wild type NDRG1. Although expression of the PPAS mutant, and even point mutations within this domain, resulted in reduced protein expression level, we were able to restore adequate expression level using the proteasome inhibitor MG-132. Lastly, we used sub-cellular fractionation to show that deletion of the C-or N-terminus (using the mutants described in our report) markedly diminished NDRG1's translocation into the nucleus in response to hypoxic injury.

Reviewer 3:

None

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:

Not applicable.

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

Response:

We thank the reviewers for their comments and their thorough review. We are grateful to the reviewers for appreciating that we have presented important and compelling evidence for the role of NDRG1 in trophoblast cell function. We also thank the reviewers for realizing that a one-year plan cannot always be completed as anticipated. As noted by the reviewers, our original research plan was fairly ambitious, and included the need to overcome several technical hurdles during the funded year. This was predictable, as the proposed project was designed to create a platform for a larger, long-term pursuit.

While the results regarding cell proliferation were incomplete, we have developed a series of relevant measurements, including assays for apoptosis and measurements of trophoblast differentiation. After the one-year project was completed, we expanded our experimental tools or investigations to include assays for protein localization by imaging and fractionation, as well as apolipoprotein and cholesterol analysis.

Our studies have been incorporated into a new grant submission by a physician-scientist trainee in our lab, who is currently submitting a new NIH grant that builds on our findings and interrogates the function of NDRG1 in placental mobilization of cholesterol and its derivatives.

Project Number: 0863908

Project Title: Immunity to MUC1 Tumor Antigen in Conditional and Transplantable
in vivo Models for Ovarian Cancer

Investigator: Vlad, Anda M.

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:

None

Reviewer 2:

The project has not generated any publications so far. Thus, the productivity over the past few years appears to be limited. It is recommended that the investigators publish the results in peer-reviewed journals and cite the Pennsylvania Department of Health in the acknowledgements.

Response:

Results from this work were incorporated in two manuscripts, both currently under review at Oncogene and Cancer Immunology and Immunotherapy, respectively, (revisions requested, final decisions pending). In both manuscripts Pennsylvania Department of Health is appropriately acknowledged.

- Budiu R, Elishaev E, Brozick, J, Lee, MJ, Edwards R, Kalinski P, **Vlad, AM**. Immunobiology of human mucin 1 (MUC1) in a preclinical ovarian tumor model. Oncogene. 2012.
- Zhang L, **Vlad AM**, Milcarek C and Finn. Human mucin MUC1 RNA undergoes different types of alternative splicing resulting in multiple isoforms. Cancer Immunol Immunother. 2012.

Reviewer 3:

1. I feel the progress of the PI and associates was truly outstanding. I would recommend, however, once additional funding is obtained that the PI clearly evaluate CTL responses as well as the potential role of MDSC in immune suppression. Since levels of Tregs did not meet expected changes in the murine model, it is possible that MDSC have an increased role. In addition, since the PI has such a strong murine model developed with a defined antigen, evaluation of circulating tumor cells utilizing MUC1 expression could be very helpful to the field. This data could be easily translatable to human translational studies, and could provide an avenue for additional NIH funding through cancer prevention and cancer detection study sections, as well as SBIR funding if appropriate collaborations within business were initiated.

Response:

We agree that MDSC are likely involved in loco-regional immune suppression in ovarian cancer and are currently evaluating their roles in our ongoing projects. In addition, the applicant is happy to report her recent success in securing NIH R01 funding. This newly funded R01 grant will enable us to expand our efforts in identifying MUC1 roles in ovarian cancer progression, loco-regional inflammation and immune suppression (Treg and/or MDSC-mediated) and to explore the translational potential of new and improved MUC1 vaccines, as suggested.

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:

Not applicable

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

Response:

The applicant is grateful to all reviewers for their detailed, insightful comments and overall favorable review.