

Final Progress Report for Research Projects Funded by Health Research Grants

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

1. **Grantee Institution:** The Pennsylvania State University
2. **Reporting Period (start and end date of grant award period):** 1/1/2009- 12/31/2012
3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** John Anthony, MPA
4. **Grant Contact Person's Telephone Number:** 814-935-1081
5. **Grant SAP Number:** 4100047645
6. **Project Number and Title of Research Project:** 10 - IRES-mediated Synthesis of Proteins Integral to Adaptation to Hyperoxia
7. **Start and End Date of Research Project:** 03/26/2009 – 06/30/2010
8. **Name of Principal Investigator for the Research Project:** Jeffrey S. Shenberger, MD
9. **Research Project Expenses.**

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

\$43,443

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

Last Name	Position Title	% of Effort on Project	Cost
Zhang	Research Associate	25%	\$18,140

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

Last Name	Position Title	% of Effort on Project
Shenberger	Principal Investigator	10%

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

Type of Scientific Equipment	Value Derived	Cost
None		

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

Yes _____ No X

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

11. Leveraging of Additional Funds

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

Yes _____ No X

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

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If

you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	<input type="checkbox"/> NIH <input type="checkbox"/> Other federal (specify: _____) <input type="checkbox"/> Nonfederal source (specify: _____)		\$	

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

Yes X No _____

If yes, please describe your plans:

We have started investigation of the FGF2 IRES and its regulation during oxygen-induced retinopathy. This will be the subject of an R21 submission we are currently preparing.

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

We plan to finish the BiP studies within the next 6 months once additional funds are available. That should complete the project in terms of a publishable study.

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

Yes _____ No X

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

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

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

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

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

Yes _____ No X

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

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

Yes _____ No X

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

16. Collaboration, business and community involvement.

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

Yes _____ No X

If yes, please describe the collaborations:

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

Yes _____ No X

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

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

Yes _____ No X

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

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

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

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

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

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

Planned Project Overview

Exposure of the lung to hyperoxia generates reactive oxygen species capable of modulating the translational control of gene expression. Although nearly all eukaryotic mRNAs are translated by a 5'-cap-dependent process under basal conditions, a select group of transcripts containing internal ribosome entry sites (IRES) within 5'-UTR (untranslated region) may be preferentially translated in a cap-independent manner during periods of stress. In this project, we hypothesized that hyperoxia would acutely depress cap-dependent mRNA translation in the parenchymal epithelium of the lung and augment the expression of select proteins integral to O₂-tolerance translated by cap-independent mechanisms. Specifically, using dual-luciferase, dicistronic constructs containing the 5'-UTR of GADD45 α and p53 mRNAs, we sought to determine the changes in the relative translational efficiency of cap-dependent and IRES-mediated translation of each transcript in human lung epithelial cell lines. One primary goal of this project was to provide additional evidence for an R01 investigating the role of translational regulation of protein synthesis in the architectural alterations induced by hyperoxia.

We anticipated that exposing human lung cells to high concentrations of oxygen would alter the mechanisms used to synthesize proteins. Specifically, we hypothesized that high oxygen levels would enhance the production of proteins the cells need to repair oxygen-mediated injury. This information was used as part of a National Institutes of Health R01 grant proposal investigating how oxygen-mediated changes in protein synthesis modify the expression of factors essential to normal human growth and development. The overall objective of the study was to provide basic science findings with the potential to lead to innovative therapies promoting normal lung development in premature infants requiring oxygen therapy for treatment of surfactant deficiency.

Original Specific Aim: Determine that hyperoxia increases the protein expression of GADD45 α and p53 through cap-independent activation of IRES sequences. We hypothesized that hyperoxia would enhance the translation of GADD45 α and p53 mRNA into protein through a relative increase in IRES activity. Dual-luciferase, dicistronic constructs containing the 5'-UTR of GADD45 α and p53 were studied and correlated with protein expression and mRNA content in human lung epithelial cell lines.

Modification of Objectives and Specific Aim:

After completing initial experiments using dicistronic plasmid constructs for HIF-1 α , hSP-A, and p53, we concluded that accurately determining the effect of hyperoxia on IRES-mediated translation required a modified approach. First, we selected cells with increased transfection efficiencies, HEK293 and HeLa cells, rather than the proposed A549 cells, which attained transfection efficiencies of only 20-30%. In addition, we recognized that HIF-1 α and VEGF were not likely to be highly translated in the presence of hyperoxia as numerous studies had illustrated that gene expression is markedly suppressed in the presence of high O₂ concentrations. We also decided to study a gene with established oxidant-induced differences in protein expression, but not in mRNA expression, indicative of IRES-mediated upregulation. In this case, we chose BiP/GRP78 (immunoglobulin binding protein), an ER-resident chaperone whose protein expression has been shown to be modified by hyperoxia (Gewandter, et al. *Free Rad Biol Med*, 2010) and is involved in the unfolded protein response. The 5'-UTR of BiP, like p53, contains a putative IRES capable of regulating translation. We also used deletion mutagenesis to

isolate the effects of the plasmid, minus the IRES-containing 5'-UTR sequence, on luciferase activity. Finally, we performed a detailed dose-response curve using easily transfected HEK293 and HeLa cells with the intent on establishing the optimal times for hyperoxia exposure. The project is complete within the context of the funding provided, but investigation in this area continues in the laboratory.

Methods:

Dicistronic Constructs. The dicistronic reporter construct, pRL-HCV-FL (a kind gift from Dr. Fei Chen), contains a RL reporter gene upstream of the 5'-UTR of hepatitis C virus (HCV) and a FL reporter gene downstream. A dicistronic reporter plasmid for p53 was a gift from Dr. Barsanjit Mazumber at Cleveland State. The 5'-UTR (+39) of wild-type p53 was cloned into the pRF vector containing an upstream RL and downstream FL reporter gene (8, 9). The pRLuc BiP IRES Fluc dicistronic plasmid was a gift from Dr. Celeste Simon at the University of Pennsylvania. Dicistronic plasmids were also obtained containing the 5'-UTR of HIF-1 α , VEGF, and the ABD 5'-UTR variant of the human SP-A gene (hSP-A)(Wang 2005; 2009). At the completion of the exposure period, cell lysates will be analyzed for the activities of Firefly (FL) and *Renilla* (RL) luciferase.

Cell culture. A549 cells were grown in Ham's F12 medium; H441 cells in RPMI 1640; and HEK293 and HeLa cells in DMEM all containing 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Transfected cells were cultured in their respective medium at 37°C in 5% CO₂ atmosphere until use.

Transient transfection. Cell-specific protocols were utilized to transfect each of the four cell lines used in experiments. Electroporation was chosen for the more difficult to transfect A549 cells. Chemical transfection protocols currently employed by collaborators were used for H441, HEK293, and HeLa cells.

1. *Electroporation of A549 cells* - A549 cells were grown to 80% confluency, trypsinized, centrifuged, and suspended in Nucleofector T solution (1x10⁶ cells/100 μ l; Amaxa). Cell suspensions in serum-free medium were transfected in the Nucleofector I electroporation unit according to the manufacturer's protocol. Immediately following completion of the transfection protocol, cells were removed from the cuvet, replenished with serum-containing medium, and seeded into wells.

2. *Chemical Transfection of H441 cells* - Cells were grown to 80-90% confluence in flask and subcultured into six-well culture plates with 1x10⁶ cells/well (~80% confluence) 24 hrs before transfection. The transfection procedure was performed with the Lipofectamine Plus reagent kit (Invitrogen). Four hours before transfection, the RPMI 1640 medium with 10% FBS was replaced with DMEM without FBS and antibiotics. One μ g of experimental plasmid DNAs were diluted into DMEM medium without serum without antibiotics total 100 μ l), and then 6 μ l of PLUS Reagent was added to the mixture. The DNA and PLUS reagents were incubated for 15 min at room temperature. LIPOFECTAMINE Reagent (4 μ l) was diluted 100 μ l of DMEM without serum in a second tube. The pre-complex DNA with PLUS and diluted LIPOFECTAMINE Reagent was combined and incubated for 15 min at room temperature. The DNA-PLUS-LIPOFECTAMINE Reagent complex was put into cell-containing well of plate. Four hours after transfection, DMEM with 10% FBS was added to each well to the original normal volume. Transfected cells were cultured at 37°C in 5% CO₂ atmosphere until use.

3. *Chemical Transfection of HEK293 and HeLa cells* - Cells were seeded into 6-well plates at densities of 50k/cm² and 18k/cm², respectively. Twenty four hours later, dicistronic constructs were transfected into both cell types using Effectene Transfection Reagent (QIAGEN). Briefly, 0.4 µg plasmid or negative control construct DNA were diluted in buffer EC to a total volume of 100 µl prior to adding 3.2 µl enhancer and incubating for 4 min at room temperature. Ten µl of Effectene transfection reagent was added to the DNA-Enhancer mixture and the complete transfection solution added to cells in 600 µl of fresh medium.

Hyperoxic exposure. Cells were exposed to 95% O₂ + 5% CO₂ for 6-24 hrs using a computerized control system (BioSpherix Ltd, Redfield NY) housed in the Shenberger laboratory. This unit continually monitors O₂ and CO₂ concentrations and adjusts inlet flow to maintain preset levels. The chamber was humidified and housed inside a standard water-jacketed incubator maintained at 37°C.

Deletion mutagenesis. To generate a negative control plasmid for the BiP construct, we deleted the BiP 5'-UTR sequence by deletion mutation. In brief, two oligonucleotides (sense: 5' GATACCGTCGACCTCGAATCGTTGGTAAAGCCACCATGGAA3', antisense: 5' GATTCGAGGTCGACGGTATC3') were synthesized by the core facilities at Penn State University College of Medicine (PSUCOM). To eliminate the BiP-IRES fragment from the construct, both oligos were used for PCR amplification with the construct pRLuc BiP IRES Fluc as a template according to the instructions in the Site-directed mutagenesis kit (Stratagene, La Jolla, CA). After PCR and digestion with Dpn I, the DNAs were transformed into *E. coli* cells

using standard methods, three positive clones selected, and the mutated plasmid DNA confirmed by DNA sequencing.

Luciferase assay. A dual-luciferase assay was performed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). In brief, transfected cells were harvested 3-32 hrs after transfection. The wells were washed once with PBS and lysed in 500 μ l of passive lysis buffer (Promega) with gentle rocking for 15 min at room temperature. Collected cell lysates were cleared by centrifuging and 20 μ l of lysate combined with 100 μ l of luciferase reagent I. Tubes were placed into an FB12 luminometer (Zylux, Maryville, TN) and 100 μ l of Stop & Glow reagent added to each tube. Firefly (FL) and *Renilla* (RL) luciferase luminosities were recorded and the FL:RL ratio calculated. Three trials for each clone were performed with each trial measured in duplicate.

Results:

Unsuitability of p53 IRES construct and electroporation.

Initial experiments using the p53 construct transfected into A549 cells found that exposure to 95% O₂ for 3 and 9 hours after transfection (4 hr recovery) lead to little change in Luc FL:RL ratio. Inspection of the individual luciferase activities revealed that while FL activity was similar at 3 and 9 hours,

that RL activity declined by 10-15% in both room air and O₂-treated cells. Accordingly, control studies were performed to determine the stability of luciferase activities of the p53 construct over time. As shown in Figure 1, FL activity peaked between 6-9 hours post-transfection and remained more than 2-fold greater than baseline transfected cells for at least 21 hours. In contrast, the RL was greatest immediately following transfection, declining progressively over time to values about 20% of baseline by 18 hours. These studies indicate that construct is

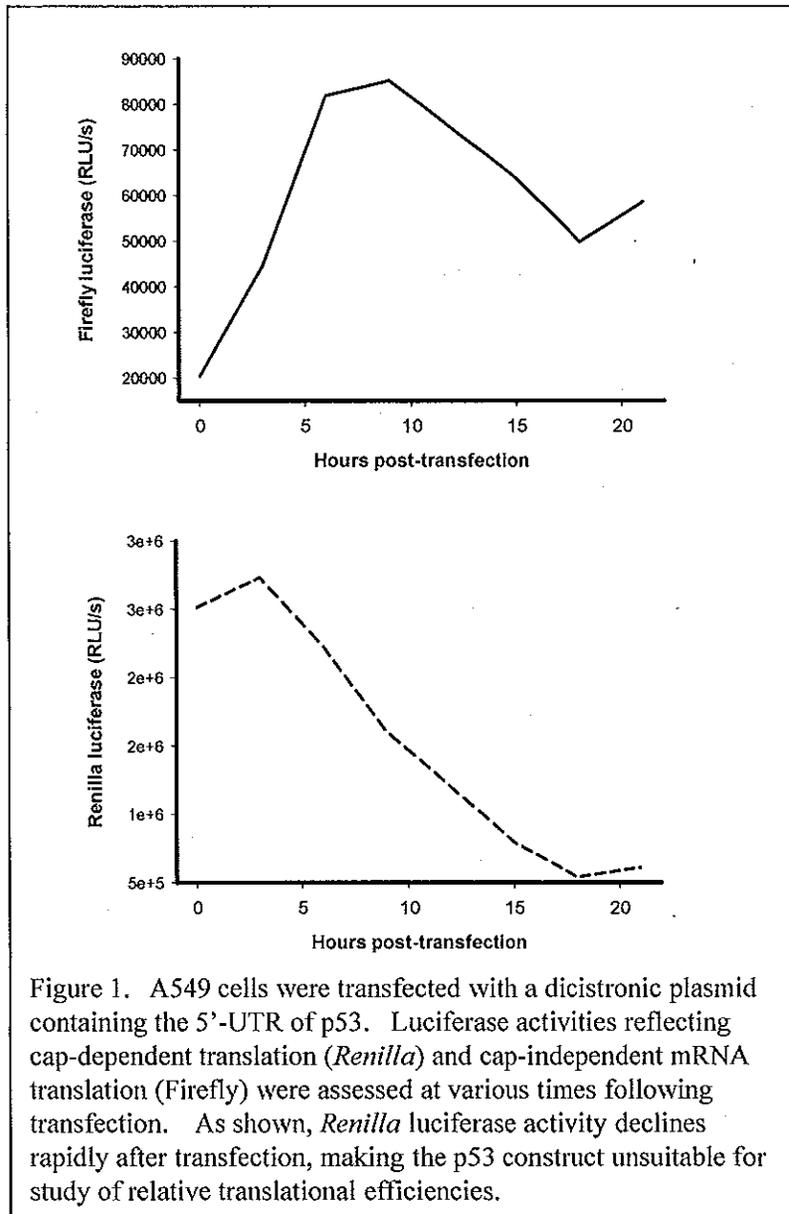


Figure 1. A549 cells were transfected with a dicistronic plasmid containing the 5'-UTR of p53. Luciferase activities reflecting cap-dependent translation (*Renilla*) and cap-independent mRNA translation (Firefly) were assessed at various times following transfection. As shown, *Renilla* luciferase activity declines rapidly after transfection, making the p53 construct unsuitable for study of relative translational efficiencies.

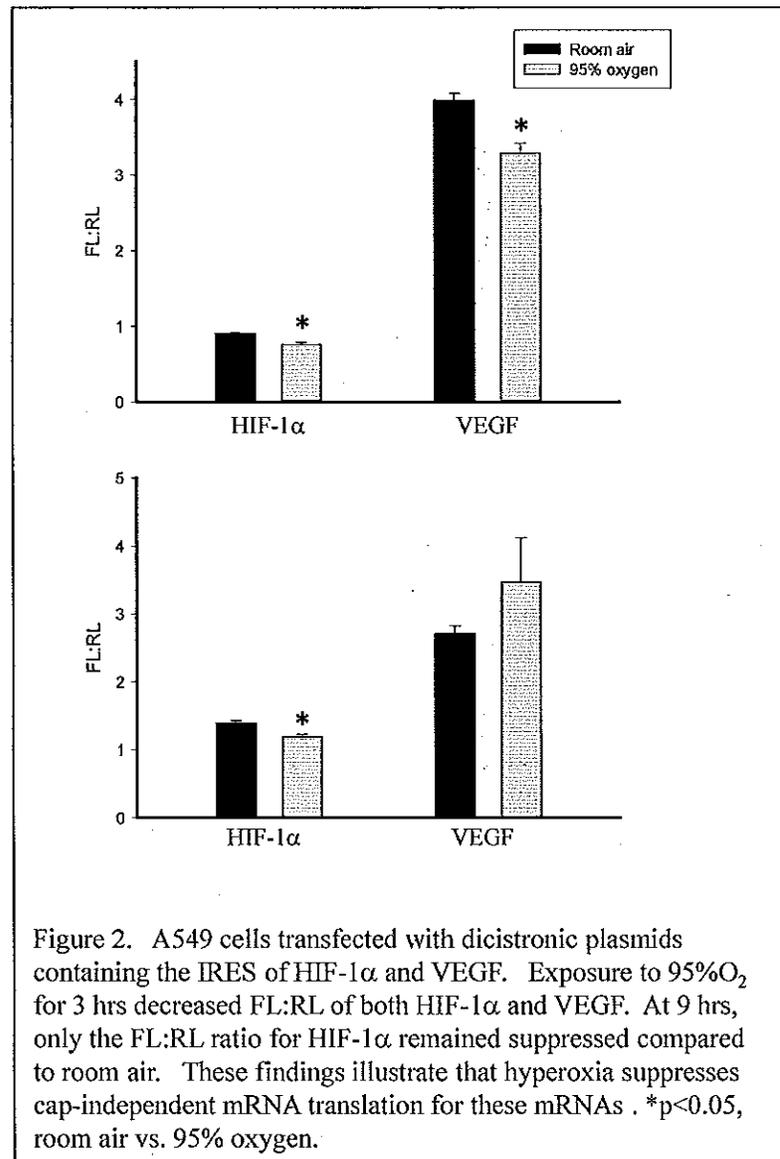
unlikely to be capable of resolving alterations in translation efficiency which could be reliably attributed to the effects of O₂ alone. Accordingly, we abandoned the use of the p53 construct.

Effect of hyperoxia on the cap-dependent and -independent translational efficiencies of HIF-1 α , hSP-A (ABD), and VEGF.

A preliminary experiment exposed electroporated A549 cells to hyperoxia just 4 hours after transfection in an attempt to maximize the duration of O₂ exposure. Twenty hours after exposure, 36% of the cells in O₂ had died as determined by the MTT viability assay. Thereafter, electroporated cells were studied 8-12 hours after transfection, thereby reducing cell loss to <20%. Using the 12-hour recovery period, cells transfected with the VEGF construct demonstrated a modest (15-18%) decrease in FL:RL ratio after 6 and 18 hours in 95%O₂. Little change was observed for cells transfected with either HIF-1 α or hSP-A (ABD). Due to very low luciferase activity in the hSP-A (ABD) construct in A549 cells, we omitted this plasmid from further use in A549 cells.

Next, we exposed transfected A549 cells to 3 and 9 hours of

hyperoxia following an 8-hour post-transfection recovery period. The shorter recovery time was selected to maximize luciferase activity. By using this strategy, we obtained significant decrements in FL:RL ratios of both HIF-1 α and VEGF after just 3 hours of O₂ exposure (Fig. 2). Hyperoxia induced no change in the relative luciferase activity in the control plasmid lacking an intervening IRES. Interestingly, after 9 hours of exposure, the FL:RL ratio for HIF-1 α remained depressed relative to room air, while the ratio for VEGF had normalized. These studies indicate that hyperoxia hinders cap-independent mRNA translation relative to cap-dependent mRNA translation, at least for HIF-1 α and VEGF.



Although the previous studies show that hyperoxia decreases cap-independent mRNA translation relative to cap-dependent, they cannot discern absolute changes in either translational process. Accordingly, we conducted the subsequent experiments with the inclusion of protein concentration of the various lysates in order to be able to make direct comparison of cap-dependent and – independent translational efficiencies. To perform this, we used the lung adenocarcinoma cell line H441, a line which had been used by collaborators to study the translational efficiencies of hSP-A splice variants. Exposure to H441 cells transfected with hSP-A (ABD), HIF-1 α , and a control construct lacking an IRES sequence are depicted in Figure 3. The control or negative plasmid (Neg) should have constitutively translated RL activity but little or no FL activity as the intervening IRES is absent. As illustrated, hyperoxia decreased RL and

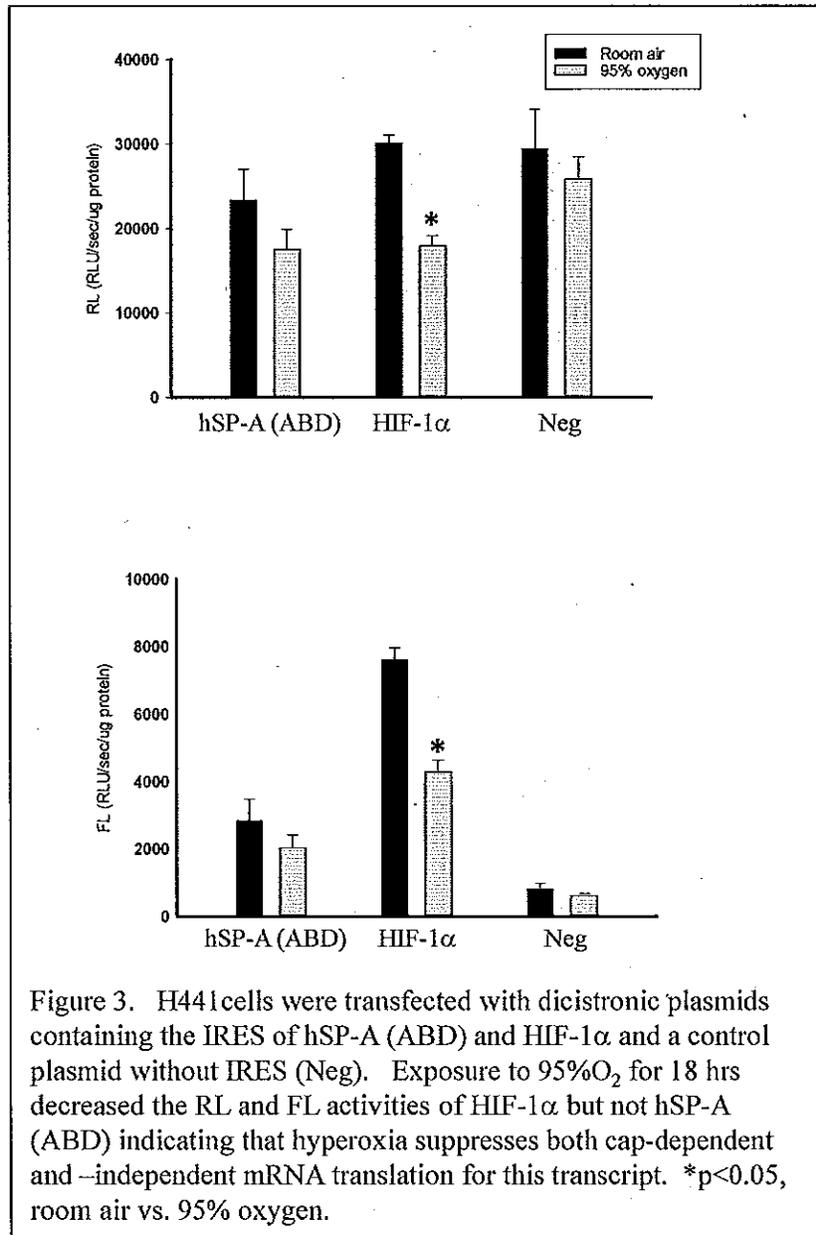


Figure 3. H441 cells were transfected with dicistronic plasmids containing the IRES of hSP-A (ABD) and HIF-1 α and a control plasmid without IRES (Neg). Exposure to 95%O₂ for 18 hrs decreased the RL and FL activities of HIF-1 α but not hSP-A (ABD) indicating that hyperoxia suppresses both cap-dependent and –independent mRNA translation for this transcript. *p<0.05, room air vs. 95% oxygen.

FL activities of HIF-1 α thereby demonstrating that while cap-independent mRNA translational efficiency is somewhat more sensitive to O₂, both processes are suppressed in contrast to our original hypothesis.

Translational efficiency of BiP. After our initial experiments, it became clear that hyperoxia was unlikely to activate IRES activity in HIF-1 α and VEGF mRNA, given that hyperoxia also suppresses transcription of these genes. Accordingly, we decided to study a gene with established hyperoxia-induced differences in protein expression, but not in mRNA expression. In this case, we chose BiP/GRP78 (immunoglobulin binding protein), an ER-resident chaperone whose protein expression was recently shown to be reduced by hyperoxia (Gewandter, et al.

Free Rad Biol Med, 2010). Our laboratory has observed time-dependent, hyperoxia-induced alterations in BiP protein expression in A549, H441, and HeLa cells (not shown). In order to construct negative control plasmids for the BiP IRES construct, we sequenced the portion of the BiP plasmid from the SV40 promoter to the *Renilla* luciferase reporter. By matching the published BiP IRES sequence to the plasmid sequence, we were able to remove the BiP 5'-UTR from the plasmid using mutagenesis. Eighteen ampicillin-resistance *E. Coli* colonies were selected and mutated DNA amplified by PCR. Six of the eighteen colonies showing appropriate DNA sizes on agarose gel electrophoresis were sequenced, of which 3 showed exact matches to the devised plasmid deletion. To confirm the absence of luciferase activity, the 3 negative control plasmids were transfected into HEK293 cells along with the native BiP plasmid. As shown in Figure 4, all three negative controls displayed little IRES activity (Firefly luciferase, low FL:RL ratio). The BiP 5'-UTR, on the other hand, possessed nearly 100-fold greater activity for the IRES than the negative control plasmids under basal conditions (48 hrs after transfection).

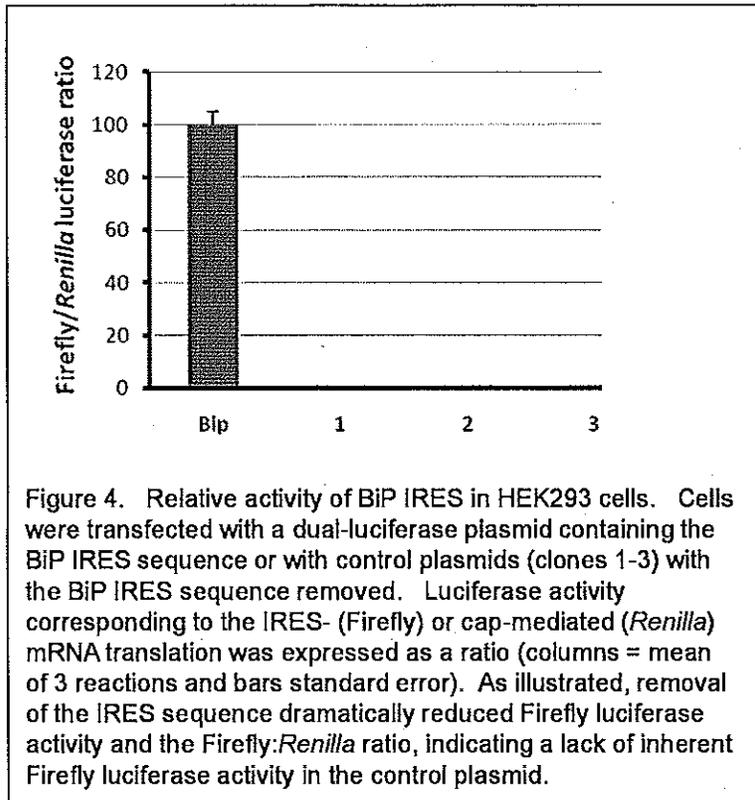
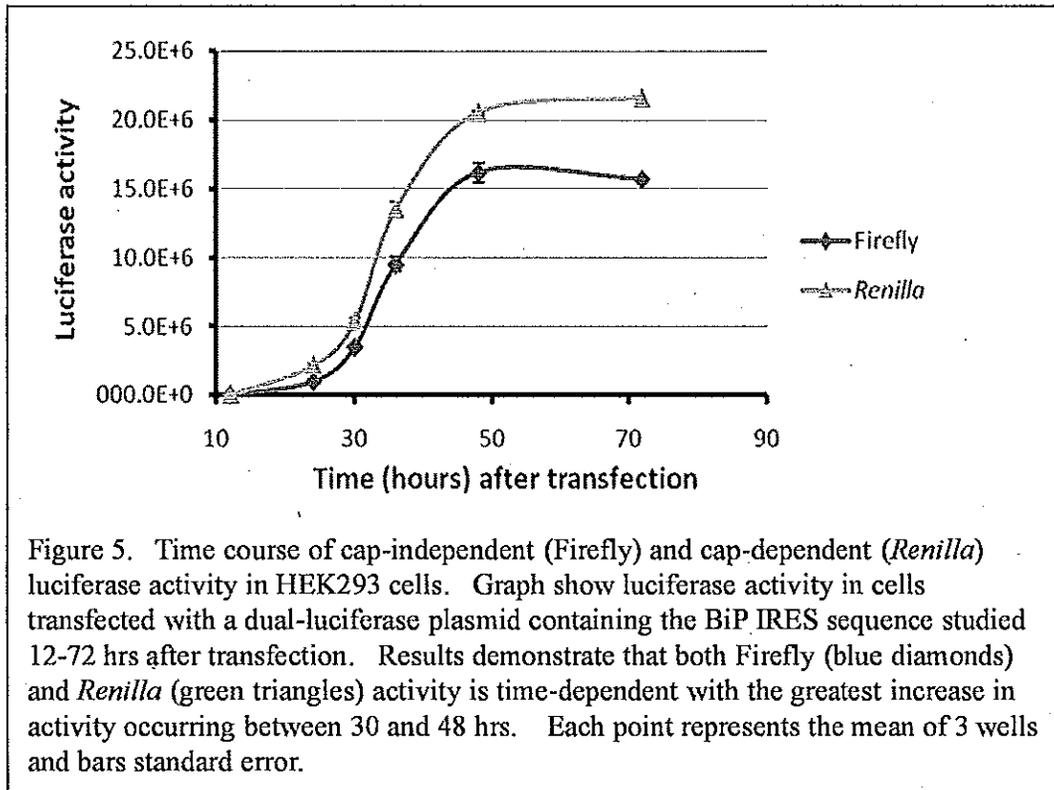


Figure 4. Relative activity of BiP IRES in HEK293 cells. Cells were transfected with a dual-luciferase plasmid containing the BiP IRES sequence or with control plasmids (clones 1-3) with the BiP IRES sequence removed. Luciferase activity corresponding to the IRES- (Firefly) or cap-mediated (*Renilla*) mRNA translation was expressed as a ratio (columns = mean of 3 reactions and bars standard error). As illustrated, removal of the IRES sequence dramatically reduced Firefly luciferase activity and the Firefly:*Renilla* ratio, indicating a lack of inherent Firefly luciferase activity in the control plasmid.

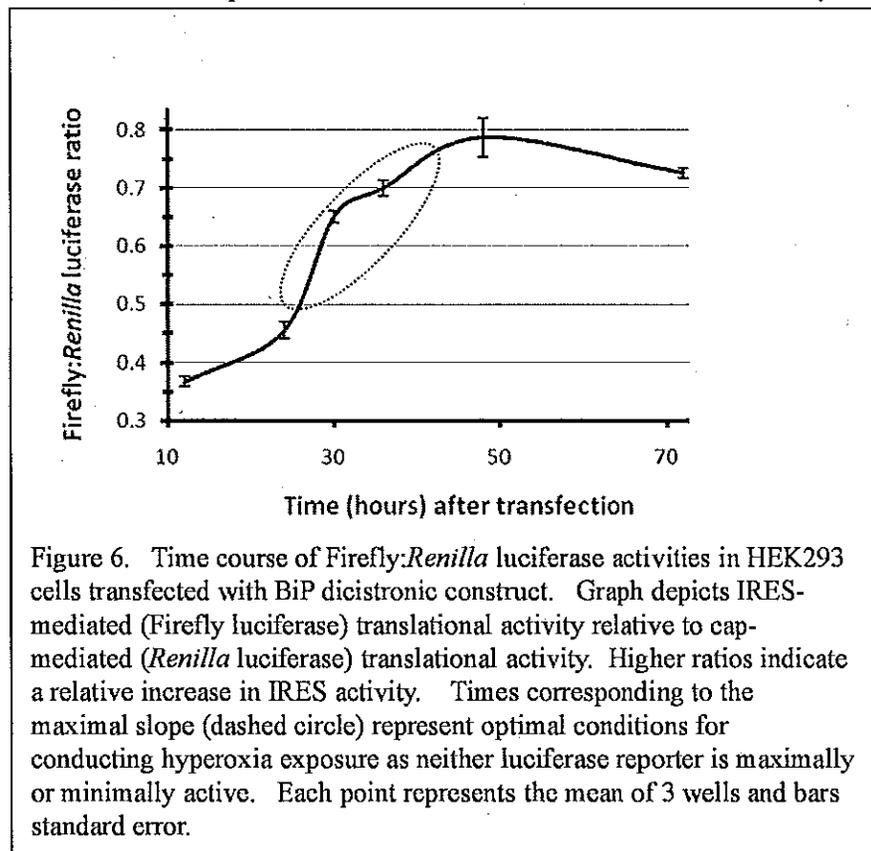
We next performed time course studies to identify the conditions at which changes in both Firefly and *Renilla* could be detected. Figure 5 illustrates that the transfected HEK293 cells lead to time-dependent increases in both FL and RL activities. From 30-48 hrs post-transfection maximal rates of change in both luciferase activities were observed. Importantly, this time frame corresponds to the period when it is possible to detect relative changes in IRES activity (FL:RL ratios) in either a positive or negative direction (Fig 6). These studies indicate that finishing hyperoxic exposure between 28-42 hrs post-transfection in HEK293 cells provides the best conditions for the identification of either an increase or decrease in IRES activity.



We also performed an identical time course in HeLa cells. Although the experiment produced a similar curve for Firefly luciferase, time-dependent alterations in *Renilla* luciferase activity were markedly less dramatic (Fig 7), resulting in a “flat” FL:RL curve.

This suggests that HeLa cells are less optimal for conducting hyperoxia experiments due to the low basal *Renilla* activity. In addition, transfection efficiency is much greater in HEK293 cells than in HeLa cells.

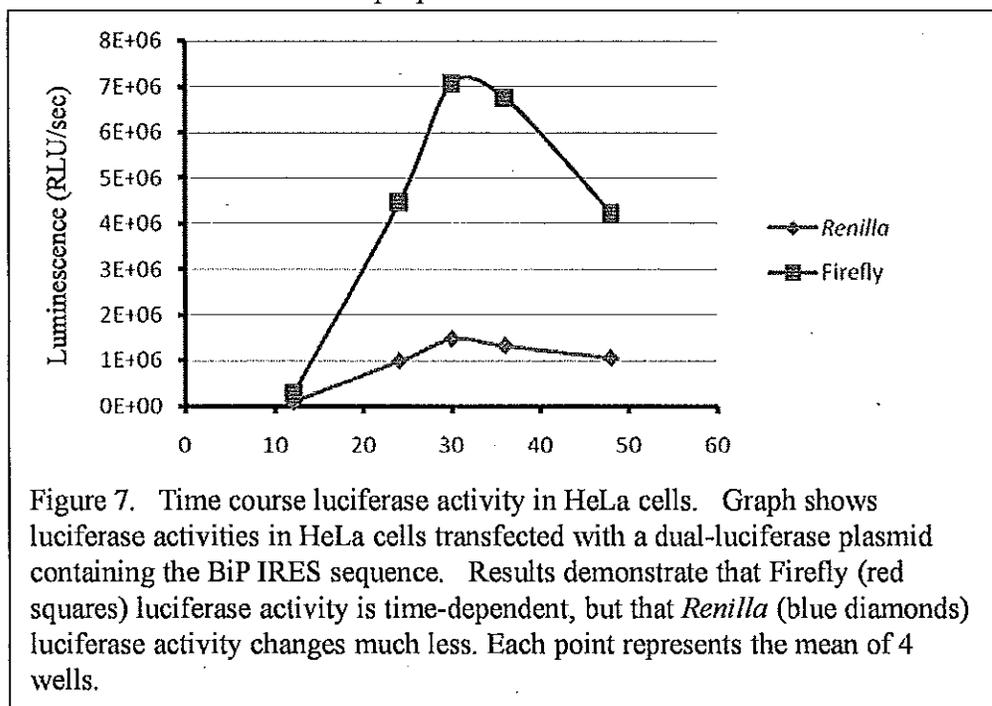
Preliminary studies indicate that exposure of A549 and HeLa cells to 95% O₂ for 6 hours transiently increases the expression of BiP protein. Similar studies in non-



transfected HEK293 cells will be used to identify exposure times correlating altered BiP protein expression. These time periods will then be utilized to for investigations of transfected HEK293 cells when additional funds are available. Treatment of A549 cells with thapsigargin (100 μ M) has also been shown to dramatically increase BiP protein expression after 3-6 hours (not shown). We plan to perform these same trials in HEK293 cells to provide a suitable positive control for our transfection studies.

Summary of Project Findings

These studies illustrate that, for the transcripts studied, hyperoxia suppresses both cap-dependent and cap-independent mRNA translation as rapidly as 3-6 hours. Previous work in our laboratory found that hyperoxia decreases global protein synthesis in cultured cells within 24 hours and, in the lungs of newborn rat pups, within 72 hours (Shenberger et al, *Free Rad Biol Med*, 2007; Konsavage et al, *Am J Physiol Lung Cell Mol Physiol*, 2010). The translation of specific transcripts, therefore, may be more sensitive to hyperoxia than previously appreciated. In addition, in contrast to our original hypothesis, this work illustrates that IRES-mediated mRNA translation is at least as sensitive to hyperoxia-induced suppression as cap-mediated translation. Within the context of the transcripts studied, there is no indication that hyperoxia activates IRES activity. It must be pointed out, however, that IRES-containing transcripts whose protein expression is enhanced by hyperoxia will need to be studied to ascertain the general applicability of these early findings. Once additional funding is secured, we anticipate completing the studies on the BiP construct to serve this purpose.



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

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

Yes
 No

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

Yes
 No

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

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

_____ Number of hospital and health care professionals involved in the research project

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

_____ Number of subjects originally targeted to be included in the study
_____ Number of subjects enrolled in the study

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

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

Gender:

_____ Males
_____ Females
_____ Unknown

Ethnicity:

_____ Latinos or Hispanics
_____ Not Latinos or Hispanics
_____ Unknown

Race:

- American Indian or Alaska Native
 Asian
 Blacks or African American
 Native Hawaiian or Other Pacific Islander
 White
 Other, specify: _____
 Unknown

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

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

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

- Yes
 No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?

- Yes
 No

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

20. Articles Submitted to Peer-Reviewed Publications.

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

Cancer” research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI

Project 1 – Smith – Publication 2 – Cognition and MRI

Project 3 – Zhang – Publication 1 – Lung Cancer

Project 3 – Zhang – Publication 2 – Lung Cancer

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

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

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
None				<input type="checkbox"/> Submitted <input type="checkbox"/> Accepted <input type="checkbox"/> Published

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

Yes X No

If yes, please describe your plans:

We plan on submitting our findings in the near future.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project.

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

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment.

Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert “None”; do not use “Not applicable.”

Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes _____ No X

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

a. Title of Invention:

b. Name of Inventor(s):

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

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

Yes _____ No _____

If yes, indicate date patent was filed:

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

Yes _____ No _____

If yes, indicate number of patent, title and date issued:

Patent number:

Title of patent:

Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes _____ No _____

If yes, how many licenses were granted? _____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes _____ No _____

If yes, describe the commercial development activities:

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

Yes _____ No X

If yes, please describe your plans:

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

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME Jeffrey S. Shenberger, M.D.		POSITION TITLE Associate Professor of Pediatrics and Cellular & Molecular Physiology	
eRA COMMONS USER NAME JSHENBERGER			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Franklin and Marshall College, Lancaster, PA	B.A.	1984	Chemistry
Pennsylvania State University, Hershey, PA	M.D.	1989	Medicine
Univ. of Minnesota Hospitals, Minneapolis, MN	Residency	1992	Pediatrics
Univ. of Minnesota Hospitals, Minneapolis, MN	Fellowship	1995	Newborn Medicine

A. Positions and Honors

Positions and Employment:

- 1995-1999 Staff Neonatologist, Wilford Hall USAF Medical Center, Lackland AFB, TX (San Antonio)
Staff Neonatologist, Darnall Army Community Hospital, Fort Hood, TX (Killeen)
- 1996-1999 Director, Neonatal Research, Wilford Hall USAF Medical Center, Lackland AFB, TX (San Antonio)
- 1998-1999 Clinical Assistant Professor, Dept. of Pediatrics, Div. of Neonatology, University of Texas San Antonio School of Medicine, San Antonio, TX
- 1999-2000 Assistant Director of Extracorporeal Membrane Oxygenation Program, Dept. of Pediatrics, Div. of Neonatology, University of Kentucky Medical School, Lexington, KY
- 1999-2001 Assistant Professor, Dept. of Pediatrics, Div. of Neonatology, University of Kentucky Medical School, Lexington, KY
- 2000-2001 Staff Neonatologist, St. Joseph's East Hospital, Lexington, KY
- 2000-2001 Director of Extracorporeal Membrane Oxygenation Program, Dept. of Pediatrics, Div. of Neonatology, University of Kentucky Medical School, Lexington, KY
- 2001-2002 Assistant Professor, Dept. of Pediatrics, Div. of Neonatology, Dartmouth Medical School, Hanover, NH
- 2002-2006 Associate Professor, Dept. of Pediatrics, Div. of Neonatology, Dartmouth Medical School, Hanover, NH
- 2006-2009 Physician-Scientist Program, Pennsylvania State University College of Medicine, Hershey, PA
- 2006- Associate Professor, Dept. of Pediatrics, Div. of Newborn Medicine, Dept. of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, PA
- 2006- MD/PhD Training Program Faculty member, Pennsylvania State University College of Medicine, Hershey, PA
- 2009- Director of Neonatal Extracorporeal Membrane Oxygenation, Div. of Neonatology, The Milton S. Hershey Medical Center, Hershey, PA

Other Experience and Professional Memberships

- 1995- Member, American Academy of Pediatrics
1999- Member, American Thoracic Society
2001- Member, Society for Free Radical Biology and Medicine

Honors and Awards:

- 1995-1999 Military Indoctrination for Medical Officers, Top Performer (1995), Ogden Bruton Award for Basic Science Research, Winner (1997)
Meritorious Achievement Medal (1997)
Ogden Bruton Award for Basic Science Research, Semi-finalist (1998),
Meritorious Achievement Medal, First Oak Leaf Cluster (1998)
Ogden Bruton Award for Basic Science Research, Finalist, (1999)
Meritorious Achievement Medal, Second Oak Leaf Cluster, (1999) - United States Air Force.

B. Selected Peer-reviewed publications (Out of 19 total publications)

Most relevant to current application

1. Shenberger JS, Adams MH, and SG Zimmer. Oxidant-induced hypertrophy of A549 cells is accompanied by alterations in eukaryotic translation initiation factor 4E and 4E-binding protein. *Am J Respir Cell Mol Biol* 27:250-256, 2002.
2. Shenberger JS, Myers JL, Zimmer SG, Powell RJ, and A Barchowsky. Hyperoxia alters the phosphorylation and binding of multiple factors regulating translation initiation. *Am J Physiol Lung Cell Mol Physiol* 288:L442-L449 2005 (PMC2675186).
3. Shenberger JS, Zhang L, Hughlock M, Ueda T, Watanabe-Fukunaga R, and R Fukunaga. Roles of mitogen-activated protein kinase signal-integrating kinases 1 and 2 in oxidant-mediated eIF4E phosphorylation and translational repression. *Int J Biochem Cell Biol* 39(10):1828-42, 2007 (PMC2001257).
4. Shenberger JS, Zhang L, Powell R, and A Barchowsky. Hyperoxia enhances VEGF release from A549 cells via post-transcriptional processes. *Free Radic Biol Med* 43(5):844-52, 2007 (PMC1959513).
5. Zhang L, Kimball SR, Jefferson LS, and JS Shenberger. Hydrogen peroxide impairs insulin-stimulated assembly of mTORC1. *Free Radic Biol Med* 46(11):1500-9, 2009 (PMC2677139).
6. Konsavage W, Zhang L, Vary T, and JS Shenberger. Hyperoxia Inhibits Protein Synthesis and Increases eIF2 α Phosphorylation in the Newborn Rat Lung. *Am J Physiol Lung Cell Mol Physiol*, 298:678-686, 2010 (PMC journal; In Process).

Additional publications

1. Shenberger JS, Prophet SA, Waldhausen JA, Davidson WR, and Sinoway LI. Left subclavian flap aortoplasty for coarctation of the aorta: effects on forearm vascular function and growth. *J Am Coll Cardiol* 14: 953-959, 1989.
2. Baily RG, Prophet SA, Shenberger JS, Zelis R, and Sinoway LI. Direct neurohumoral evidence of isolated sympathetic nervous system activation to skeletal muscle in response to cardiopulmonary baroreceptor unloading. *Circ Res* 66: 1720-1728, 1990.
3. Sinoway LI, Shenberger J, Leaman GJ, Prophet SA, Gray K, Baily RG, and Leuenberger U: The generalized attenuation of the sympathetic nervous system response to submaximal exercise following localized conditioning. *Journal of Applied Physiology* 81(4):1778-1784, 1996.

4. Shenberger JS, Shew RL, Johnson DE, and Kannan MS. Relaxation of porcine tracheal smooth muscle by parathyroid hormone-related protein. *Respiration Physiol* 107:59-66, 1997.
5. Shenberger JS, Shew RL, and DE Johnson. Hyperoxia-induced airway remodeling and neuroendocrine cell hyperplasia in the weanling rat. *Pediatr Res* 42(4):539-544, 1997.
6. Shenberger JS and PS Dixon. Oxygen induces S phase growth arrest and increases p53 and p21^{WAF1/CIP1} expression in human bronchial smooth muscle cells. *Am J Respir Cell Mol Biol* 21:395-403, 1999.
7. Shenberger JS, Dixon PS, Choate J, Helal K, Shew RL, and W Barth. Pregnancy and labor increase the capacity of human myometrial cells to secrete parathyroid hormone-related protein. *Life Sciences* 68:1557-66, 2001.
8. McAdams RM, Mustafa SB, Shenberger JS, Dixon PS, and RJ DiGeronimo. Cyclic stretch attenuates the effects of hyperoxia on cell proliferation and viability in human alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 291:L166-174, 2006 (PMC2683386).

C. Research Support

Completed

K08HL071905, NHLBI

Shenberger (PI)

2003-2009

"Regulation of eIF4E activity during oxidant stress"

The major goals of this project were to determine the translational activity of eIF4E during exposure to oxidants and hyperoxia and to identify if alterations in activity altered cell survival.