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TITLE: Simultaneous Expression from Both the Sense and Antisense Strand of the Erythropoietin Receptor Gene Mitigates Acute Lung Injury

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14. ABSTRACT

Natural bidirectional transcription of the erythropoietin receptor (EpoR) gene produces a sense (EpoR) and a complementary antisense (RopE) transcript that have distinct mechanisms of action; EpoR promotes cell growth and angiogenesis while RopE repairs DNA strand breaks via homologous recombination repair. We hypothesized that balanced EpoR-RopE expression mitigates injury and facilitates repair/regeneration. The goal of this project is to determine the therapeutic efficacy of locally manipulating the EpoR–RopE transcript system for the prevention and treatment of acute lung injury. In this reporting period, the investigators accomplished several essential steps towards this goal: a) generation and characterization of novel specific monoclonal antibodies to EpoR and RopE polypeptides, b) characterization of the expression patterns of EpoR and RopE polypeptides during normal pre- and post-natal lung development, c) establishment of proof-of-concept efficacy that increasing EpoR or RopE expression by cDNA delivery to lung cells in vitro enhances cytoprotection against hyperoxia-induced injury, and d) demonstration that simultaneous expression of both EpoR and RopE offers additive protection compared to expression of each individually. These results support a role for EpoR-RopE synergism in cytoprotection against oxidative injury.

15. SUBJECT TERMS
Acute lung injury, hyperoxia, oxidative damage, cell culture, rodent model, inhalation cDNA delivery, sense and antisense erythropoietin receptor transcripts

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. INTRODUCTION:

Test the therapeutic efficacy of locally increasing the sense erythropoietin receptor (EpoR) and/or its complementary antisense (RopE) transcript system on the prevention and treatment of acute lung injury.

2. KEYWORDS:

Acute lung injury, hyperoxia, oxidative damage, lung cell culture, rodent model, airway instillation, inhalation cDNA delivery, EpoR, RopE

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Project Activities	**SOW Timeline** (Months)	**Completion Status** Date Completed or Percentage of Completion
Specific Aim: Test the therapeutic efficacy of locally increasing the EpoR (sense)–RopE (antisense) transcript system (either singly or in combination) on the prevention and treatment of acute lung injury.		
Major Task 1: Test the therapeutic efficacy of locally increasing the EpoR-RopE sense-antisense transcript system (either singly or in combination) on the prevention of acute lung injury.	1-6	50%
Subtask 1: Prepare plasmid cDNA of EpoR and RopE in nanoparticle formulation.	1	Completed 06.2017
Subtask 2: Simulate uni- or bi-directional gene transcription by delivering via inhalation of nanoparticles containing: **1.** Control (empty vector), **2.** EpoR (sense), **3.** RopE (antisense), or **4.** EpoR + RopE (both) cDNA's. At peak gene expression, create acute lung injury via hyperoxia exposure.	2-4	50%
Subtask 3: Perform analysis to compare the extent of injury (and protection) in different exposure groups.	4-6	50%
Milestone(s) Achieved: Determine the efficacy of intervention on prevention of acute lung injury.	6	50%
Local IACUC Approval.	3	5/9/2016; APN Renewal Approved 5/9/2017

Milestone Achieved: HRPO/ACURO Approval.	4	9/15/2016; ACURO approval for approved APN Renewal is pending.
Major Task 2: Test the therapeutic efficacy of locally increasing the EpoR-RopE sense-antisense transcript system (either singly or in combination) on early post-exposure outcome following acute lung injury.		
Subtask 1: Prepare plasmid cDNA of EpoR and RopE in nanoparticle formulation.	7	Completed 06.2017
Subtask 2: Create acute lung injury via hyperoxia exposure (90% O2 x 3 days). Following exposure, simulate bi-directional EpoR gene transcription by delivering via inhalation of nanoparticles containing: **1.** Control (empty vector), **2.** EpoR (sense), **3.** RopE (antisense), or **4.** EpoR + RopE (both) cDNA.	8-11	0%
Subtask 3: Analyze and compare the extent of injury and recovery 1 week after treatment.	10-12	0%
Milestone(s) Achieved: Determine the efficacy of post-exposure treatment on early outcome.	12	0%
Major Task 3: Test the therapeutic efficacy of locally increasing the EpoR-RopE sense-antisense transcript system (either singly or in combination) on long-term post-exposure outcome following acute lung injury.		
Subtask 1: Prepare plasmid cDNA of EpoR and RopE in nanoparticle formulation.	13	Completed 06.2017
Subtask 2: Create acute lung injury via hyperoxia exposure. Following exposure, simulate bi-directional gene transcription by delivering via inhalation of nanoparticles containing: **1.** Control (empty vector), **2.** EpoR (sense), **3.** RopE (antisense), or **4.** EpoR + RopE (both) cDNA.	14-16	0%
Subtask 3: Analyze and compare the extent of injury and recovery 4 weeks after treatment.	17-18	0%
Milestone(s) Achieved: Determine the efficacy of post-exposure treatment on long-term outcome.	18	0%

What was accomplished under these goals?

1) Major activities in this reporting period have accomplished the essential validation steps including a) development and characterization of the essential monoclonal antibodies to EpoR and RopE, and b) establishing proof-of-concept efficacy of the reagents on lung cell models. These steps are detailed below:

Purify RopE peptide:

In silico analysis of the antisense EpoR (i.e., RopE) transcript revealed one open reading frame (termed ORF1) is conserved across several mammalian species in terms of start codon, exon-intron boundary, and the primary sequence of putative translated protein (**Fig. 1A**). In contrast, other ORF's in the RopE transcript sequence are either very short or not conserved across species for the same parameters. In fact, ORF1 is so well conserved that it has been annotated during genome mining as a hypothetical protein (LOC61130). ORF1 predicts a cytoplasmic protein with putative ATP/GTP binding and clathrin binding motifs (**Fig. 1A**).

We inserted epitope (FLAG) tags into the C-termini of the two antisense ORF's that codes for putative polypeptides of >10 amino acids just before the stop codons, transfected the cDNA into cells and looked for FLAG expression. Data for the two largest ORF's (ORF1 and 2) are shown. Only ORF1 gave rise to an expressed polypeptide and mutation of the start codon ATG abolished the expression (**Fig. 1B**). This data strongly suggests that this ORF1 is translated into an "antisense" peptide, i.e., RopE peptide.

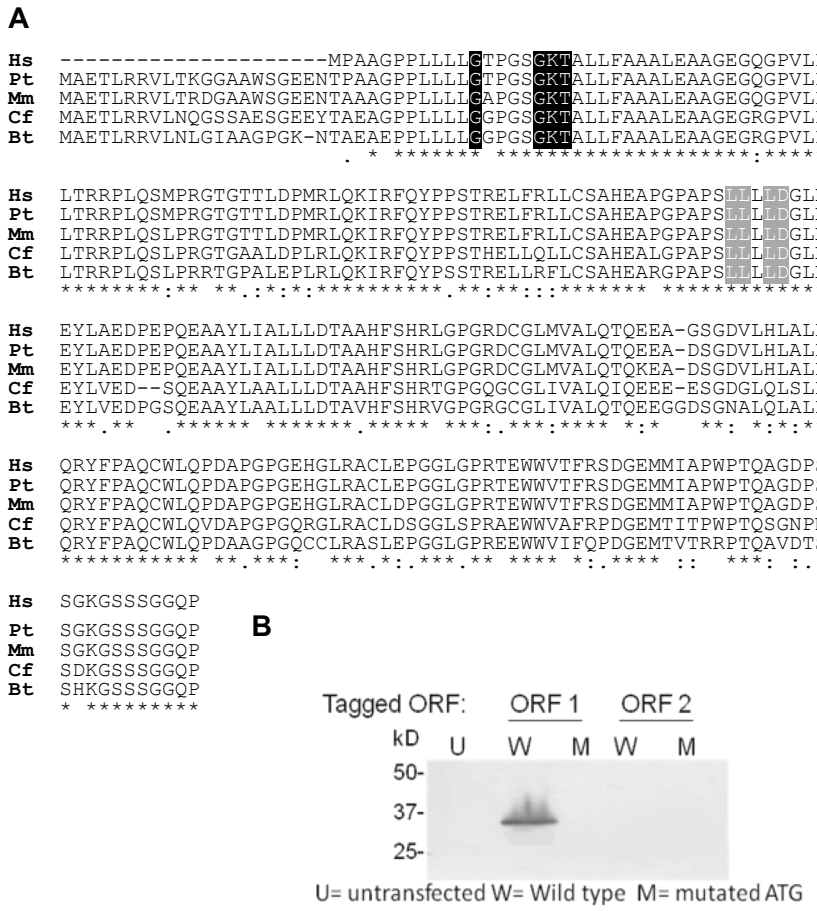


Figure 1: A. **Sequence alignment of the putative proteins coded by ORF1 in 5 mammalian species** (Hs, *Homo sapiens* LOC126074; Pt, *Pan troglodytes* LOC455727; Mm, *Macaca mulatta* LOC716529; Cf, *Canis lupus familiaris* LOC611130; Bt, *Bos taurus* LOC507152). Asterisks (*), identical amino acids in all sequences. Colons (:), conserved substitutions. Periods (.), semi-conserved substitutions. The putative ATP/GTP binding P-loop (Walker A) motif is highlighted in black; the putative clathrin binding motif is in gray. Based on European Bioinformatics Institute (EBI) InterPro integrated sequence analysis service for protein families [1]. **B**. Lysates from cells transfected with FLAG-tagged RopE cDNA probed with anti-FLAG on immunoblot.

Develop specific monoclonal antibodies to EpoR and RopE peptides

One important obstacle hindering EpoR and RopE research is the lack of specific commercial antibodies. Of the available polyclonal anti-EpoR, only one is suitable for EpoR detection by immunoblot; none is suitable for immunohistochemistry[3] and one recognizes mostly heat shock protein-70 instead of EpoR [34, 5]. These commercial EpoR antibodies revealed positive staining in tissues where EpoR mRNA was absent. None of these antibodies immuno-precipitates EpoR, and none reliably detects EpoR in tissue. We previously developed a synthetic anti-EpoR in collaboration with Sachdev Sidhu (University of Toronto) which immunoprecipitates EpoR but works poorly in immunoblots and not at in immunohistochemistry (Hu et al., Kidney Int. 2013 Sep;84(3):468-81. doi: 10.1038/ki.2013.149. Epub 2013 May 1). Therefore, we still have a major obstacle to surmount. Furthermore, there is no commercial antibody to RopE. Therefore, our essential first steps are to remedy this deficiency and secure reliable reagents. Recombinant protein is available for EpoR but not RopE. We purified RopE protein, then collaborated with Philip Streeter, PhD (Oregon Stem Cell Center) to develop monoclonal antibodies (MAbs) against human RopE (**Fig. 2**). We also used commercial EpoR peptide to generate MAbs against human EpoR (*data not shown*).

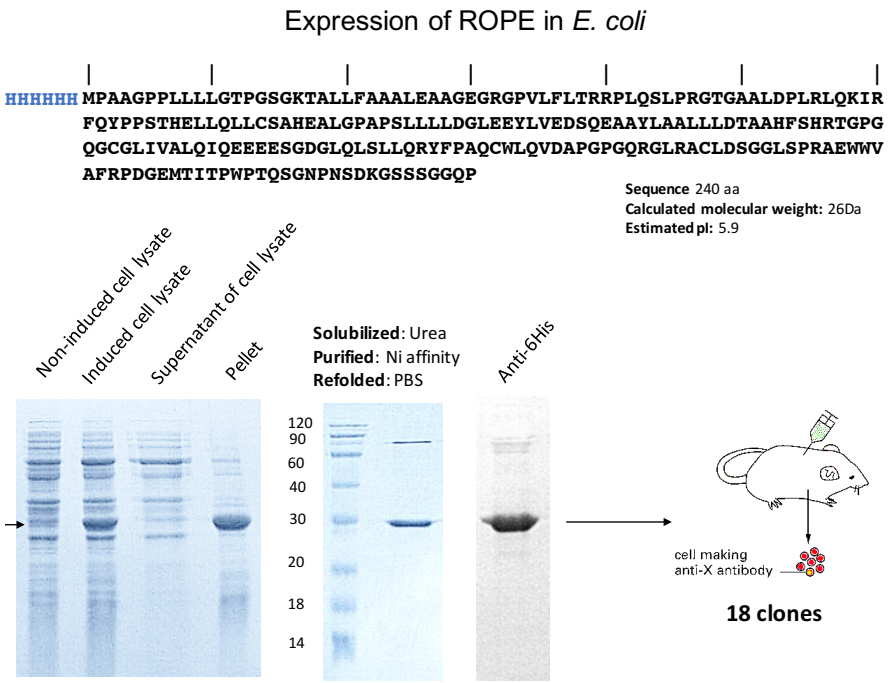


Figure 2. **Generation of MAbs to EpoR and RopE polypeptide.** Peptide epitopes were selected from the predicted primary sequence in conserved regions and MAbs generated using standard protocols. Proteins were expressed in Sf9 cells with a cleavable poly-histidine affinity tag, purified, and used as immunogen for MAb production. Hybridomas were produced and screened for reactivity against and immunogens using ELISA. Positive clones were further characterized.

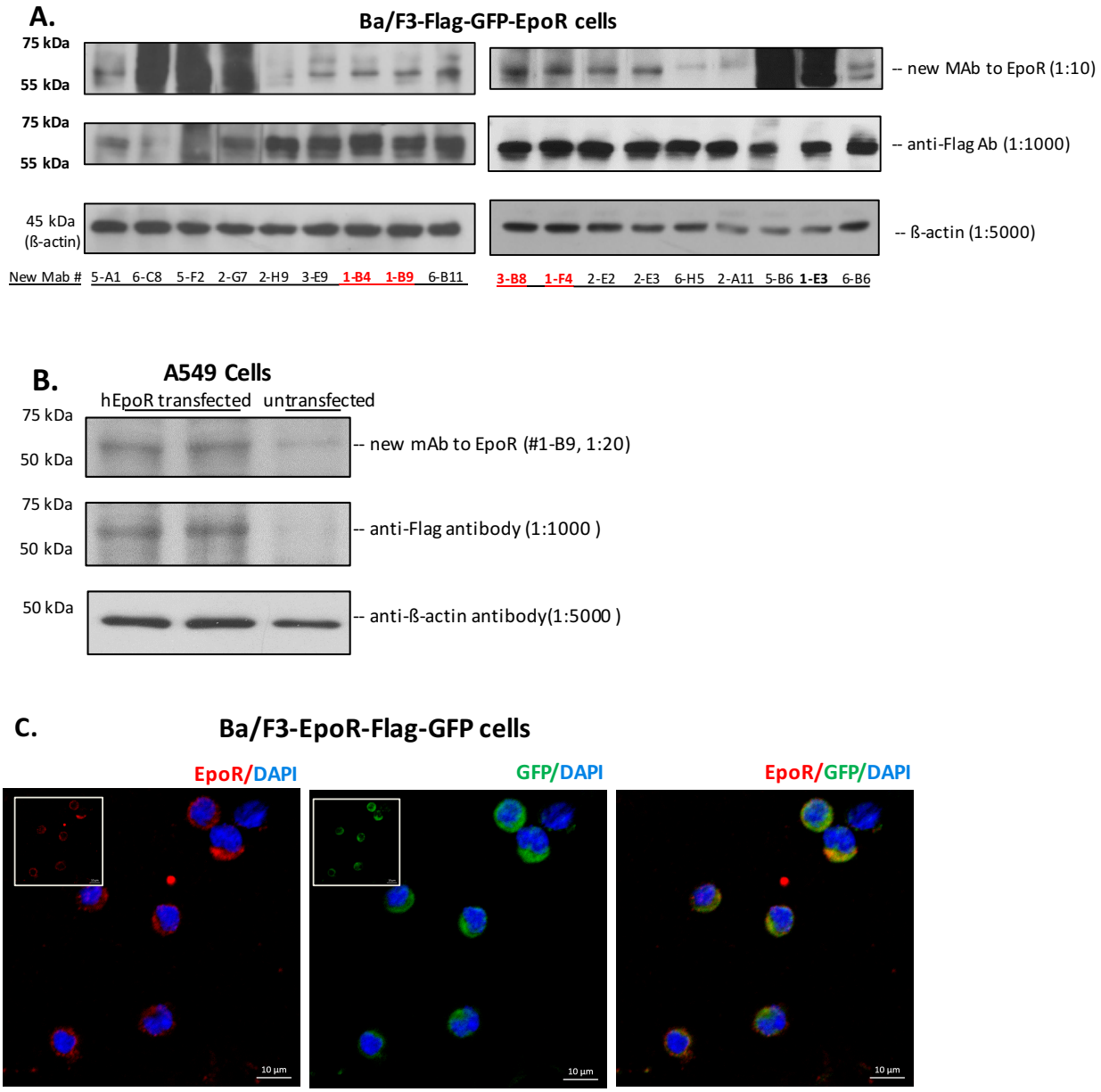


Figure 3. Screening of new MAb clones to human EpoR. Ba/F3 cells that are naturally without EpoR expression were transfected with human EpoR cDNA, tagged Flag and GFP and used to probe each of the multiple MAb clones against human EpoR. Several clones (indicated in red) show specific labeling (**A**) and were used to probe native EpoR expression in A549 lung epithelial cells by immunoblot (**B**, #1-B9 is shown), and the Ba/F3 cells transfected with EpoR-Flag-GFP by immunocytochemistry (**C**). DAPI stains the nuclei.

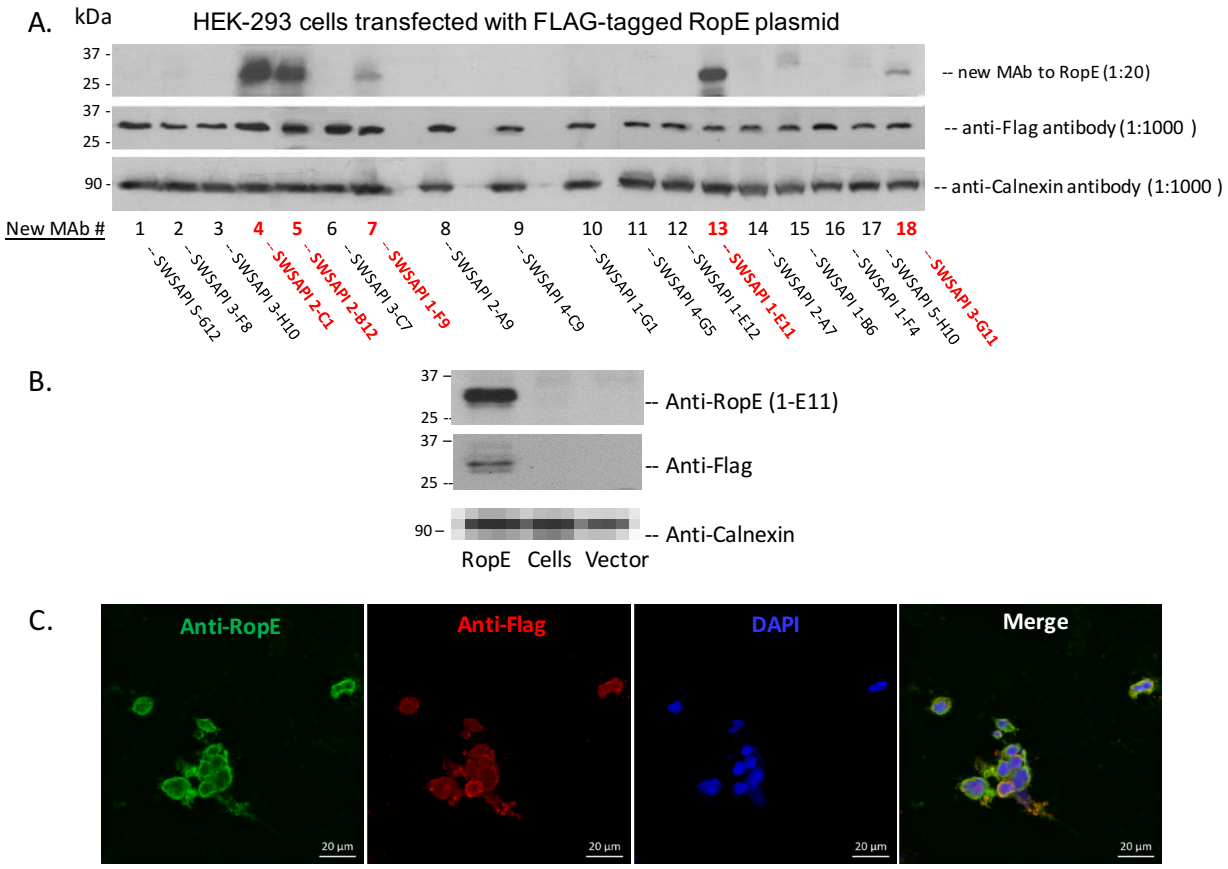


Figure 4. Screening the new MAbs to human RopE. Human embryonic kidney-293 (HEK-293) cells were transfected with Flag-tagged RopE plasmid and probed by each of the 18 clones of MAbs. Several clones (indicated in red) show promising labeling (**A**). The 1-E11 MAb showed specific labeling of recombinant RopE polypeptide by immunoblot (**B**), and the cells transfected with RopE cDNA by immunocytochemistry (**C**). DAPI stains the nuclei.

Localization of native EpoR and RopE expression in lungs by immunohistochemistry

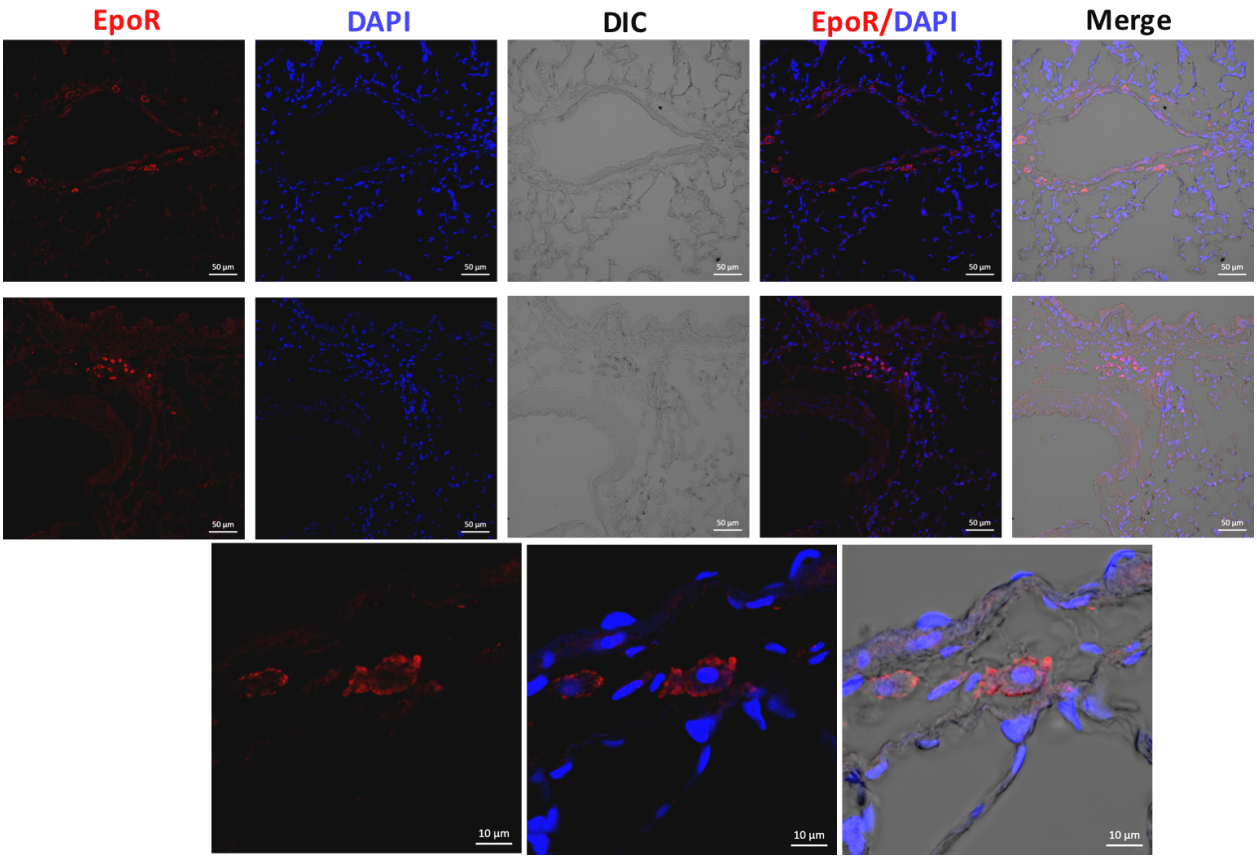


Figure 5. localization of EpoR expression in paraformaldehyde-fixed rat lung tissue by immunohistochemistry using the newly developed MAb. EpoR expression is seen along bronchiolar walls and scattered within alveolar septa. DAPI stains the nuclei. DIC: differential interference contrast.

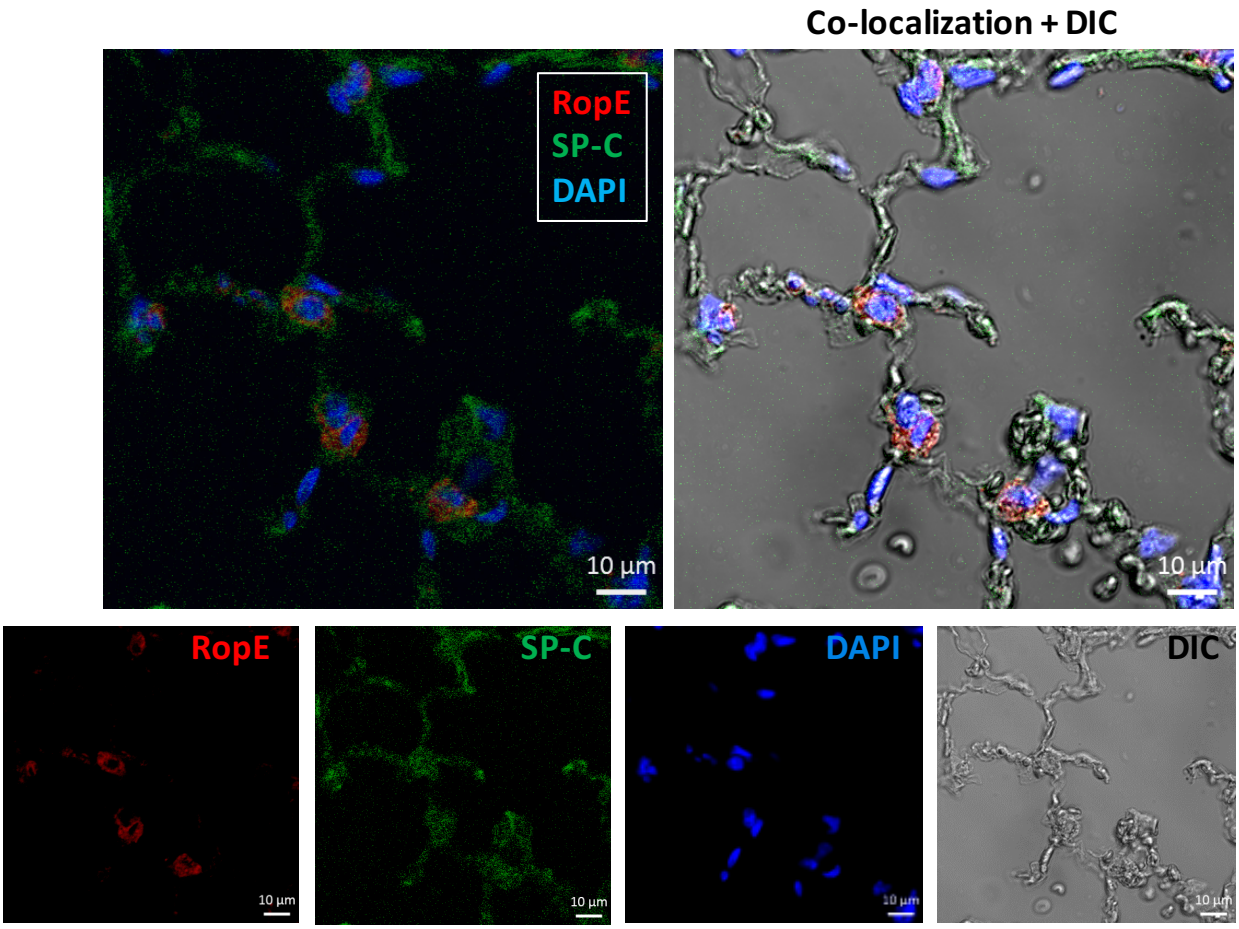


Figure 6. Immunohistochemical localization of RopE expression in paraformaldehyde-fixed murine lung tissue using the newly developed MAb. RopE expression co-localized with that of SP-C, a marker of alveolar type-II epithelium. DAPI stains the nuclei. DIC: differential interference contrast.

Characterization of the ontogeny of EpoR and RopE expression

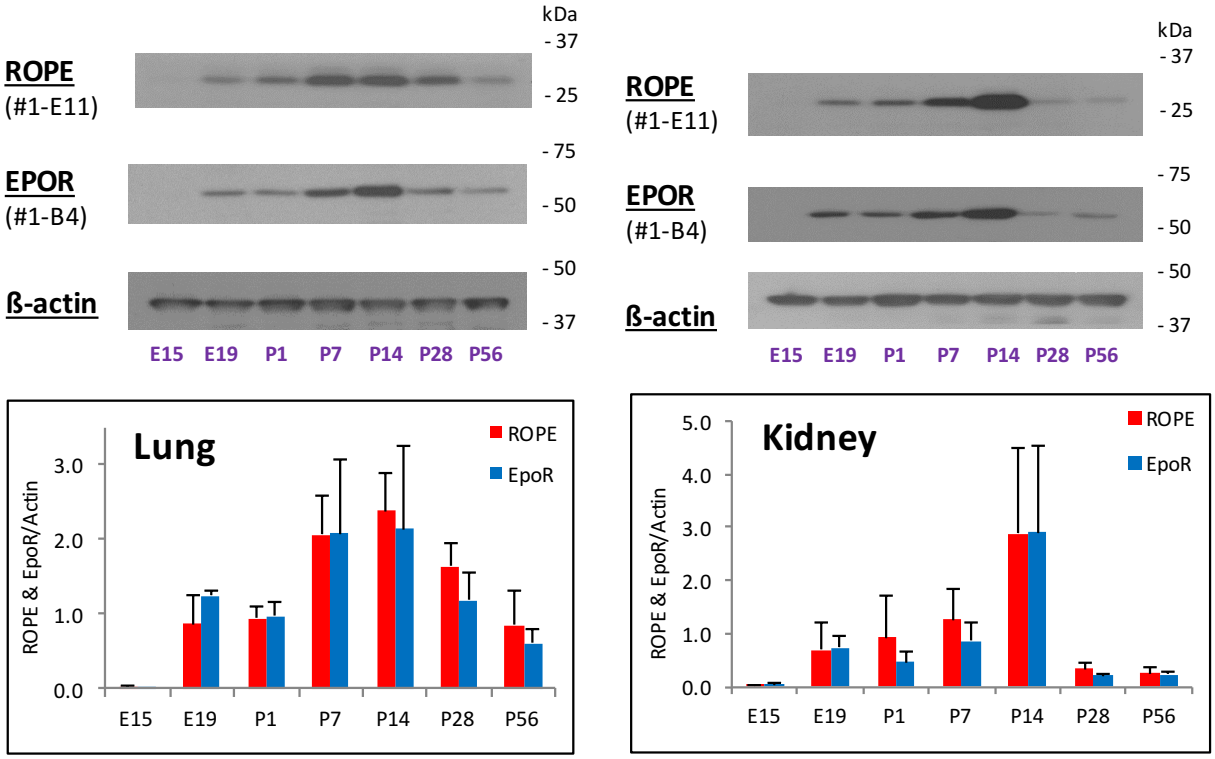
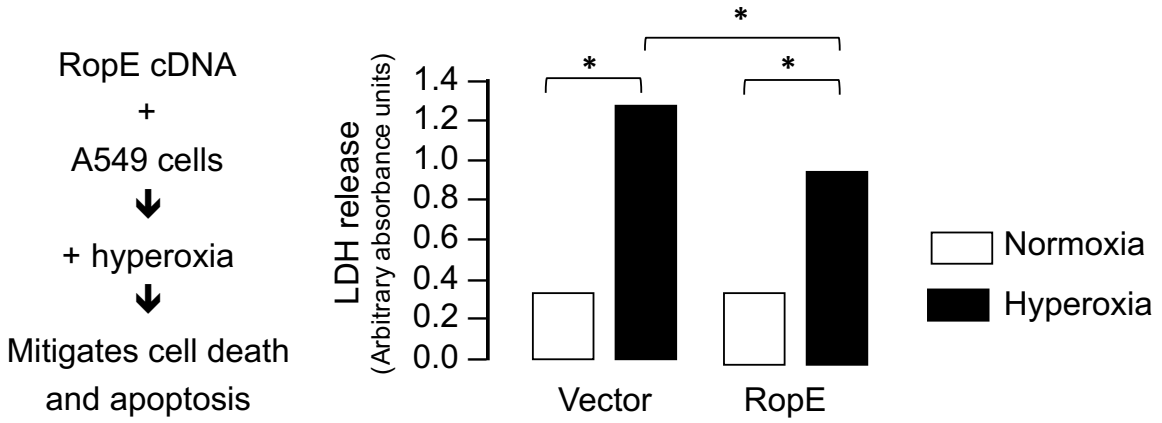


Figure 7. Concordant RopE and EpoR expression was observed in the lung (left) and the kidney (right) that increase with age during embryonic (E15, E19 days) and early postnatal life (P7, P14, P28, P56 days), peaking by P7 to P14 days, then declining with age (P28 and P56 days). Mean±SD. Triplicate assays used independent lung samples.

Establishing the protective effects of EpoR and/or RopE in lung cells exposed to hyperoxia

A.



B.

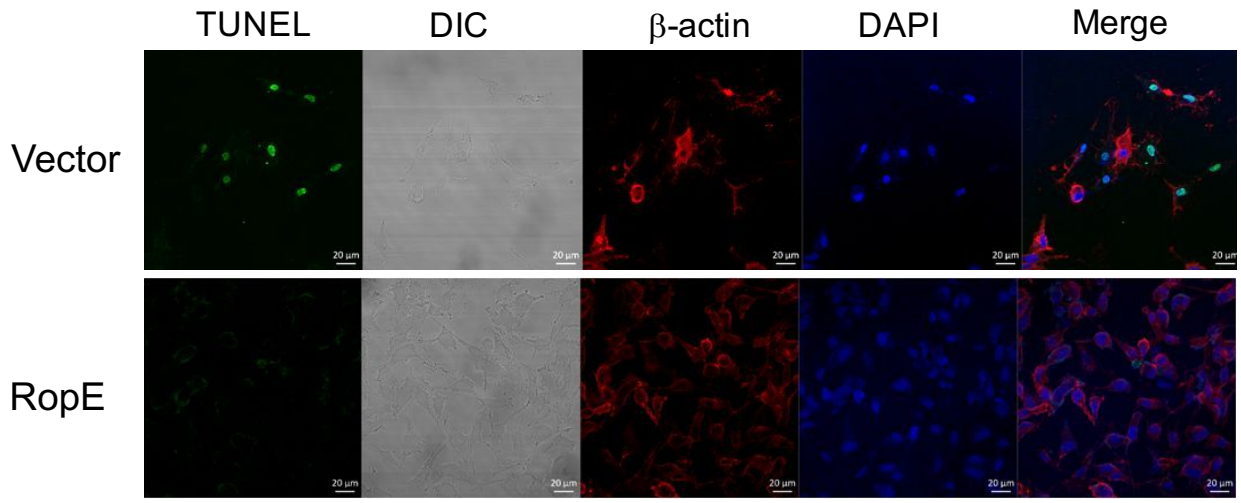


Figure 8. **RopE transfection protects lung cells against hyperoxia-induced injury.** A549 lung epithelial cells were transfected with RopE cDNA or vector (control), then exposed to normoxia (21% O_2) or hyperoxia (95% O_2) x 24 hr. Cell death measured by lactate dehydrogenase (LDH) release (A) and apoptotic DNA fragmentation measured by Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay (B) were attenuated in RopE-treated cells. *$p<0.05$ by analysis of variance. DIC: differential interference contrast. DAPI stains the nuclei.

Comparison of cytoprotection conferred by EpoR and RopE transfection, and their combined action, in lung cells

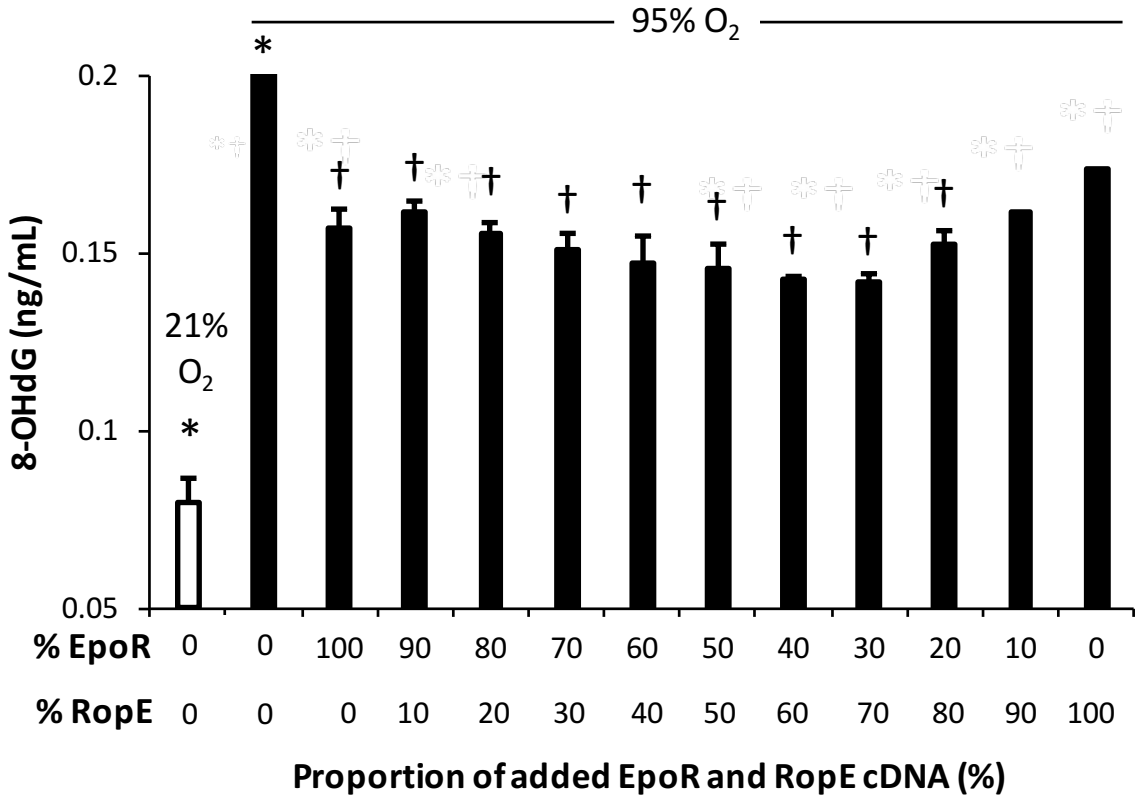


Figure 9. Combined transfection of EpoR and RopE offers additive cytoprotection against hyperoxia-induced DNA damage *in vitro*. A549 lung epithelial cells were transfected with EpoR and/or RopE cDNA in different proportions, and exposed to hyperoxia (95% O_2) for 24 hr. Control cells were exposed to hyperoxia or normoxia (21% O_2) without any EpoR or RopE cDNAs. Oxidative damage to DNA was measured by 8-hydroxy-2'-deoxyguanosine (8-OHdG) level. Mean±SD of duplicate experiments. P<0.05: * vs. all other intervention groups; † vs. (0% EpoR, 100% RopE) by analysis of variance.

Characterize EpoR and RopE cDNAs in nanoparticles

The standard emulsion method of incorporating EpoR cDNA into poly-lactic-co-glycolic acid (PLGA) nanoparticles is well established (Ravikumar et al, Nanomedicine. 2016;12:811-821). The same methodology was used to prepare nanoparticles containing RopE cDNA. We plan to improve upon this methodology by testing a new microfluidics platform to achieve more rapid manufacture and higher incorporation efficiency.

Assess baseline oxidative damage and total antioxidant capacity in murine lungs

We compared oxidative DNA damage (8-OHdG) marker and total antioxidant capacity (copper reducing capacity) at baseline (normoxia) in the lungs of the widely used C57/BL6 laboratory strain (n=6) with that in the deer mice (*peromyscus m. sonoriensis*) that originated at high altitude but has been bred at sea level for 81 generations and used in a separate protocol (n=13). The 8-OHdG level was similar in the two strains (C57/BL6 0.082±0.010 and deer mice 0.083±0.008 ng/mL, mean±SD, p=0.67). However, endogenous total antioxidant capacity was significantly lower in C57/BL6 than deer mice (7.44±0.80 vs. 8.57±1.10 μM/μg protein, p=0.04). Thus, there are strain-related differences in total antioxidant capacity that suggest differential susceptibility to induced lung injury.

2) Specific objectives

- Produce and validate essential reagents needed to advance EpoR and RopE research
- Characterize the pattern and ontogeny of EpoR and RopE expression in the lung
- Test the efficacy of EpoR and/or RopE in protecting lung cells against oxidative injury

3) Significant results

- We produced and validated novel highly specific monoclonal antibodies to EpoR and RopE that are essential reagents for further investigation in the field.
- We characterized the pattern of parallel EpoR and RopE expression in the lung during fetal and postnatal development, in comparison with that in the kidney.
- We demonstrated the cytoprotective effect of EpoR and RopE cDNA transfection in cultured lung cells.
- We demonstrated the additive protective effects of combined EpoR and RopE cDNA transfection against oxidative lung cell injury.
- We demonstrated significant differences in baseline endogenous total antioxidant capacity in the lung between two mouse strains, suggesting differential susceptibility to oxidative stress damage that impact on the design of animal models of lung injury.

What opportunities for training and professional development has the project provided?

Priya Ravikumar participated in a Young Investigator Workshop at the NHLBI Lung Repair and Regeneration Consortium Sept. 15-16, 2016, Bethesda, MD.

Connie Hsia is active in training students and research scientists in her laboratory. She co-chaired a symposium "State-of-the-art on thoracic imaging and radiographic metrics in the quantification of lung disease". American Thoracic Society International Conference. Washington, DC. May 23, 2017.

Orson Moe is active in training scientists, students and post-doctoral fellows in his own laboratory, he teaches a translational medicine course in the UT Southwestern Graduate School called Human Biology and Disease and Renal Physiology for first year medical students. At the national level, he is a faculty member on the annual American Society of Nephrology Board Review and Update, and a hands-on course in Mount Dessert Island Biologic Laboratory called Origins of Renal Physiology for Nephrology Fellows.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

All the reagents have been developed and verified. These are the most important preparations prior to launching the testing.

1. We have begun inhalational delivery studies in animals and will continue with these experiments.
2. We have manufactured the necessary nanoparticles, and are testing a newer more efficient microfluidics method of manufacturing nanoparticles.
3. A manuscript is under preparation on the development of specific MAbs to EpoR and RopE, and the characterization of EpoR and RopE expression in the lung.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

The availability of highly specific and well characterized monoclonal antibodies to EpoR and RopE will facilitate progress in basic and applied research in the fields of cell growth and differentiation, signal transduction and cancer biology.

The finding of concordant EpoR and RopE expression and additive protective effects against induced injury in lung cells support our hypothesis of their synergism and translational potential.

What was the impact on other disciplines?

We found expression of EpoR and RopE transcripts and proteins in multiple tissues. Although our focus is on the lung, findings from this project could direct further studies of EpoR-RopE interaction in cytoprotection of other organs.

What was the impact on technology transfer?

The monoclonal antibodies to EpoR and RopE have potential for intellectual property transfer.

What was the impact on society beyond science and technology?

Nothing to report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

The objective and approach of the project have not changed.

Actual or anticipated problems or delays and actions or plans to resolve them

The progress of the project has experienced delay due to unforeseen factors:

a) The development of highly specific monoclonal antibodies required a longer time than anticipated.
b) The unexpected departure of Dr. Priya Ravikumar who for personal reasons moved to another city at the end of October, 2016. The recruitment and training of her replacement caused a delay in this project.

These issues have been resolved and the experiments can proceed rapidly.

Changes that had a significant impact on expenditures

Delay in replacement of Priya Ravikumar slowed expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

  Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Monoclonal antibodies to EpoR and RopE. These may be shared via a materials transfer agreement.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Status:
Connie CW Hsia	Principal Investigator No change
Orson Moe	Co-investigator No change
Priya Ravikumar	Assistant Professor 2 person months Dr. Ravikumar prepared nanoparticles, performed in vitro and in vivo experiments and various assays. Funding support: DoD, NHLBI
Jianfeng Ye	Sr. Research Associate 5 person months Dr. Ye performed in vitro and in vivo experiments and various assays. Funding support: DoD, NHLBI
Veena Naik	Research Scientist 3 person months Dr. Naik prepared cDNA's, performed in vitro and in vivo experiments and various assays. Funding support: NHLBI

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Connie CW Hsia received the following new R01 grant from NIH:

1 R01 HL134373-01A1	Hsia (PI)	01/01/2017-11/30/2020
NIH/NHLBI	Annual Direct: $454,474	1.2 calendar months

Sara Lin
National Institutes of Health (NIH), 9000 Rockville Pike, Bethesda, Maryland 20892
Title: Structural Plasticity in Compensatory Lung Growth and Remodeling
Goal: To use state-of-the-art imaging techniques and nanotechnology to test a novel treatment strategy that reducing oxidative stress enhances endogenous regenerative response and improves lung function.
Specific Aims: 1) Test the hypothesis that αKlotho interacts with erythropoietin axis to mitigate oxidative stress in the lung. 2) Test the hypothesis that αKlotho augments erythropoietin-stimulated angiogenesis and acinar remodeling in compensatory lung growth to facilitate translation of structural growth into functional gain.
Overlap: There is no budgetary or scientific overlap.

Dr. Orson Moe is on the above listed award as a Co-Investigator with 0.7 calendar months.

Dr. Priya Ravikumar departed UT Southwestern on 10/31/2016. Her activity on funded awards previously reported to the Department of Defense came to a close on this date.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not Applicable.

QUAD CHARTS:

Not Applicable.

9. APPENDICES: N/A