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AWARD NUMBER: W81XWH-16-1-0646

TITLE: Large Noncoding RNAs as Therapeutic Targets in IPF

PRINCIPAL INVESTIGATOR: Naftali Kaminski

RECIPIENT: Yale University
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REPORT DATE: October 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2018	Annual	15 Sep 2017 - 14 Sep 2018

4. TITLE AND SUBTITLE

Large Noncoding RNAs as Therapeutic Targets in IPF

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0646

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)

Yale University
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8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our proposal focuses on the role of FENDRR, a developmentally regulated lincRNA that controls gene expression by affecting chromatin remodeling in Pulmonary fibrosis (PF). PF is a condition in which the normal lung anatomy is replaced by a process of active remodeling, deposition of extracellular matrix (ECM) and accumulation of myofibroblasts. This condition can be idiopathic or secondary, but invariably associated with significant mortality and morbidity. In this project we test the hypothesis that FENDRR expression maintains fibroblasts differentiation status through its effects on chromatic organization, therefore when FENDRR expression is decreased, fibrosis is facilitated through persistence of myofibroblasts. We have made significant progress on our specific aims: **Specific aim 1:** To determine the mechanisms by which FENDRR regulates fibroblast phenotypes - We identified now more the key epigenetic modifications that FENDRR is affecting the promoters of target genes to prevent fibrosis; **Specific aim 2:** To determine the role of FENDRR in animal models of fibrosis - We have generated FENDRR knockout mice and demonstrate that they are more susceptible to fibrosis; **Specific Aim 3:** To determine the

15. SUBJECT TERMS

Pulmonary Fibrosis, Large Intergenic Non-coding RNA, RNA, FENDRR, Epigenetic, Myofibroblast, Extracellular Matrix, Knockout Mouse

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Our proposal focuses on the role of FENDRR, a developmentally regulated lincRNA that controls gene expression by affecting chromatin remodeling in Pulmonary fibrosis (PF). PF is a condition in which the normal lung anatomy is replaced by a process of active remodeling, deposition of extracellular matrix (ECM) and accumulation of myofibroblasts. PF can be idiopathic or secondary, but in either case, it is associated with significant mortality and morbidity.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Pulmonary Fibrosis, Large Intergenic Non-coding RNA, RNA, FENDRR, Epigenetic, Myofibroblast, Extracellular Matrix, Knockout Mouse

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

1. Identification of changes in chromatin remodeling leading to increased αSMA and stress fiber formation (90%)
2. Administration of Lentiviral FENDRR in animal models (100%)
3. Effects of Deletion of FENDRR on fibrosis (100%)
4. Reanalysis of LGRC dataset and identification changes in FENDRR compared to other lung disease and confirmation by nCounter (100%) analysis of correlation with clinical parameters (10%)
5. Determination of factors that regulate FENDRR (ECM scaffolds)

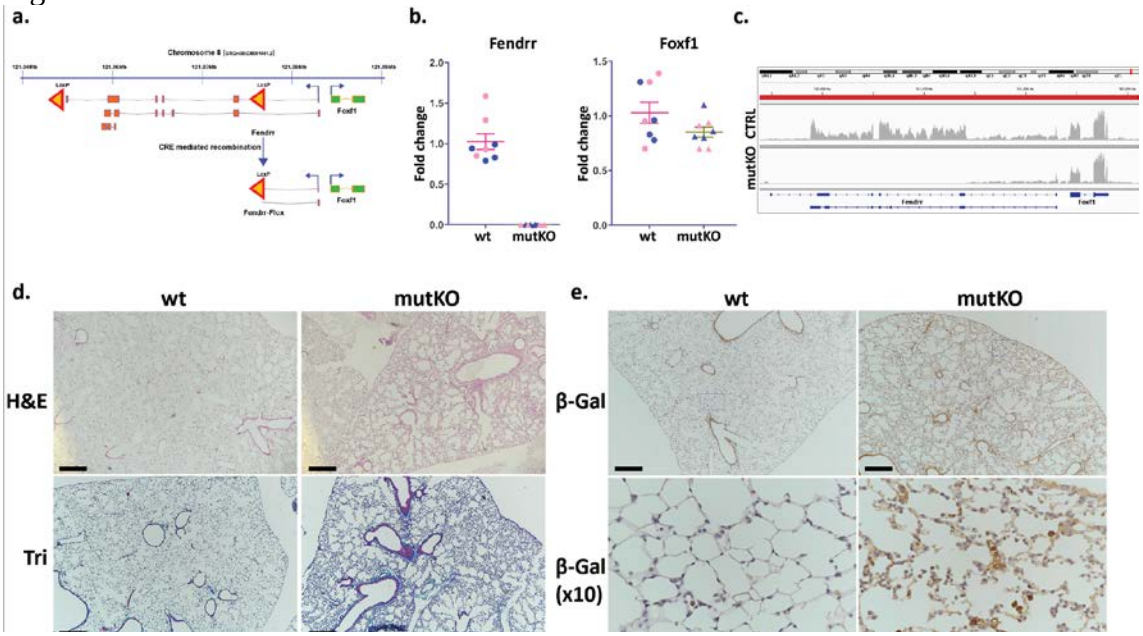
What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) *Major activities* – In the time since the grant was activated our major activities were focused on progress on the specific aims. We have worked in parallel on all aims a) Cell culture and in-vitro experiments: We used cell culture experiments to identify the molecular mechanisms by which FENDRR exerts its downstream effects on cellular phenotypes. b) Animal experiments – we have worked in parallel on two different paths, both developing the methods to introduce FENDRR gene by lentivirus and developing the reagents for a conditional knockout of FENDRR. c) Computational analysis of available human data to determine the behavior of FENDRR in human tissues.
2) *Specific objectives* – Our specific objectives over the second year included progress in all three aims including a) establishing the mechanisms by which FENDRR affected downstream gene expression, b) Assessing the potential to rescue mice by administering FENDRR, c) Testing the effects of knocking out FENDRR mice, d) Reanalyzing human data to determine the specificity of FENDRR expression.
3) *Significant new results* –

Loss of FENDRR increased susceptibility to lung fibrosis and senescence in mice

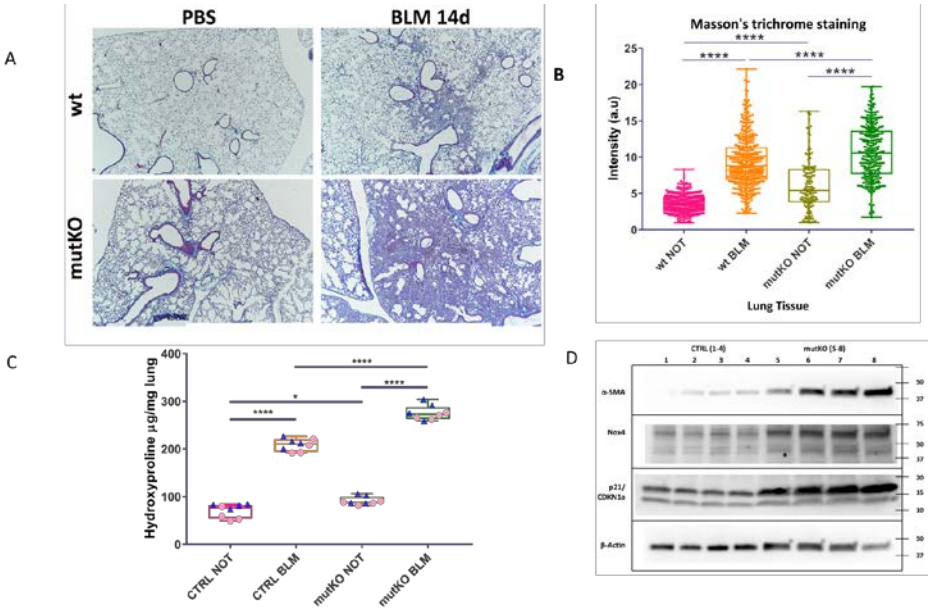
The significance of Fendrr in development has been suggested by two previous studies using genetic knockout mice. However, they showed conflicting results. Furthermore, its roles after development or in the context of lung disease have never been explored. In our bleomycin-induced lung fibrosis model, we observed reduced expression of Fendrr in fibrotic murine lungs. We aimed to elucidate the importance of FENDRR in lung fibrosis by generating a novel genetic deletion model. As shown in Fig. 1a, we generated a Fendrr flox allele with the background of C57BL/6J mice using CRISPR/Cas9 technology. We bred this strain with constitutively-active Cre-recombinase-positive strain to obtain mice with constitutional deletion of Fendrr genetic loci which lack the most exons without manipulating its promoter region shared with FOXF1. We harvested lungs from a resulting mouse (actb-Cre+/-, Fendrrfl/fl; hereby we call 'mutKO' mice) and confirmed Fendrr expression was completely lost, while FOXF1 expression was not disrupted (Fig. 1b). Specific deletion of Fendrr was also confirmed by genome-wide transcriptome analysis

Figure 1: FENDRR knockout structure and baseline effects.



in isolated lung fibroblasts (Fig. 1c). We first investigated lung tissue sections to ask whether Fendrr loss affects lung architecture. We did not observe any significant architectural distortion in sections stained with hematoxylin and eosin (H&E). However, Masson's trichrome staining revealed increased deposition of collagens in the mutKO mice lungs (Fig. 1d). Sections were also studied by anti- beta-Galactosidase staining. (Fig. 1e) Increased signals were observed in lung cells. Analysis of whole lung lysates exhibited increased expression in fibrotic markers including a-SMA and collagen. Meanwhile, isolated lung fibroblasts from mutKO mice revealed increased susceptibility to replicative senescence, as assessed by increased senescence associated-beta-Gal staining and by accelerated induction of p16 expression in the comparison with control fibroblasts from wild type. Lung fibroblasts from mutKO mice also increased cellular ROS levels further, replicating our findings from in vitro experiments using human lung fibroblasts. Next, we gave mutKO mice bleomycin intratracheally to develop lung fibrosis and assessed whether Fendrr loss affects the severity of lung fibrosis. In comparison with wildtype control, mutKO mice exhibited significantly increased fibrosis. As exhibited by collagen deposition in lungs 14 days after bleomycin treatment (Fig. 2a,b). Increased hydroxyproline (2c) and fibrotic markers (2D). Taken together, genetic deletion of Fendrr in mice altered the susceptibility of lung fibroblasts to senescence induction, and increased severity of fibrosis by bleomycin induction.

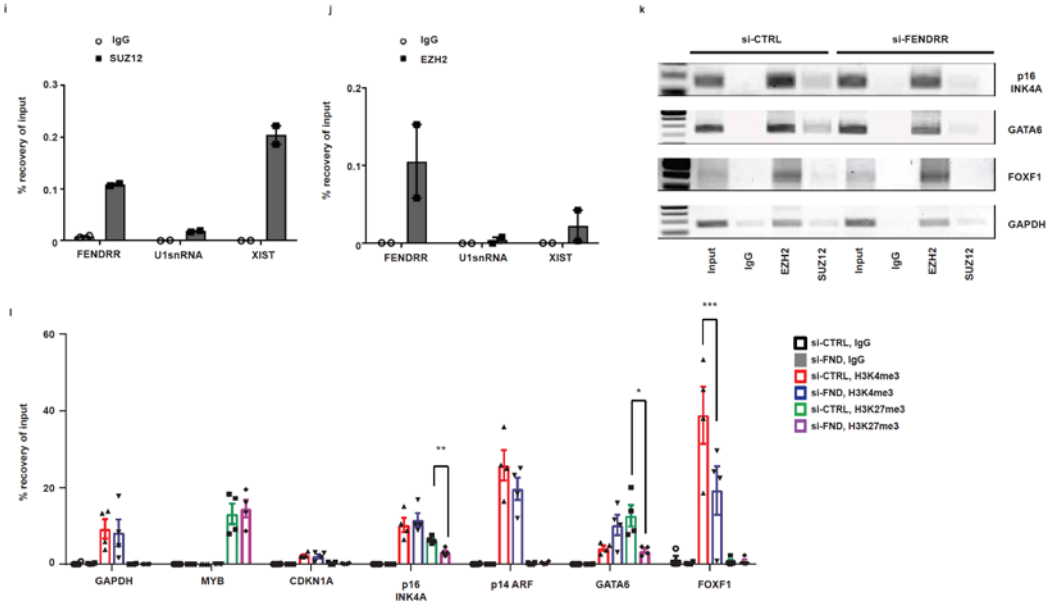
Figure 2: FENDRR knockout mice are more susceptible to bleomycin induced fibrosis



Effects of FENDRR on epigenetic regulators. In previous years we demonstrated that chemical inhibition of PRC2 and SUZ12 using 3-deazaneplanocin A, in NHLFs induced similar phenotypic changes as those seen in FENDRR knockdown cells including reduced cellular proliferation, as well as expression of markers of myofibroblastic differentiation. We also discovered that FENDRR regulated collagen gene expression but not of ASMA. We then studied whether FENDRR regulated p16INK4A. First we conducted si-RNA co-transfection with si-RNA against CDKN2A (which encodes $p16^{INK4A}$ protein) and FENDRR. Increased expression of ACTA2 and NOX4 by FENDRR knockdown were successfully reversed by addition of p16 knockdown. Further, we found collagen induction by FENDRR knockdown was reduced by co-inhibition of p16. We first assessed whether endogenous FENDRR was physically associated with PRC2 in human lung fibroblasts by RNA immunoprecipitation (RNA-IP) with an antibody against SUZ12,

a member of the PRC2 complex, and found FENDRR was 11.6-fold enriched relative to U1snRNA

Figure 2. FENDRR regulates the promoter of p16^{INK4A}.



negative control. This enrichment level is similar to Xist (12.6-fold) which is a lincRNA known to be associated with the PRC2 complex (Fig. 2i). We also found 9.3-fold association of FENDRR to EZH2, another component of PRC2 (Fig. 2j). We next explored whether FENDRR knockdown altered the association of PRC2 to the genomic loci of targets genes (Fig. 2k). Chromatin immunoprecipitation with SUZ12 and EZH2 antibodies suggested reduced association of PRC2 complex in the genomic region encoding GATA6 and p16^{INK4A}. Finally, we assessed the histone methylation patterns on the same genomic region to ask if reduced association of PRC2 resulted in changes in the histone methylation status of the genes (Fig. 4l). ChIP was conducted using antibodies for histone 3 lysine 27 trimethylation (H3K27me3), a repressive histone mark regulated by the PRC2 complex, and histone 3 lysine 4 trimethylation (H3K4me3), an active histone mark primarily regulated by the active histone modifier Trithorax/MLL complex. Consistent with the results of ChIP for PRC2 complex proteins, H3K27me3 mark on genomic loci on p16 and GATA6 decreased significantly in FENDRR knockdown cells, while active histone mark H3K4me3 showed a trend to increase on the same regions. Taken together, it is proposed that FENDRR knockdown specifically lowered the recruitment of histone modifier PRC2 to target regions, thus altering the histone methylation patterns resulting in a significant decrease in repressive marks on the promoters of GATA6 and p16. This result is consistent with the increased expression of p16 and GATA6 observed in NHLFs with FENDRR knockdown. Taken together, this suggests that FENDRR knockdown specifically lowered the recruitment of histone modifier PRC2 to target regions, thus altering the histone methylation patterns resulting in a significant decrease in repressive marks and trend towards increased active marks on the promoters of GATA6 and p16 leading to increased ECM.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

1. Complete first manuscript reporting the results.
2. Determine whether fibroblasts are the only cells affected in FENDRR knockout mice.
3. Assess effects of FENDRR inhibition in mice on fibrosis and whole animal phenotype.
4. Identify FENDRR targets in human cells – and characterize effects of FENDRR on aging/fibrosis mechanisms.
5. Assess effects of ECM on FENDRR expression.

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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our results identify a non-coding RNA that serves as an inhibitor of both aging and fibrosis – a concept very appealing because of the intersection of fibrosis and aging.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We have now determined that it is impossible to administer Lenti-viral FENDRR to wildtype mice or cells. We are working on FENDRR mimics. We also accelerated the use of knockout mice, originally aimed for year 3. We do not consider this a major change, as the aims remain the same, we are simply generating a resource earlier. We will try the FENDRR administration experiments using mimics later on in these mice.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

See above

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

As described, the major difficulty was administration of lentivirus to mice. We simply did not get any expression.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the

reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

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. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time*

conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

2017 Lung Development, Injury & Repair, 08/20/2017 - 08/25/2017,
Poster presentation: FENDDR lncRNA in pulmonary fibrosis

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

We generated FENDRR knockout mice.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: *Naftali Kaminski, MD*
Project Role: *Principal Investigator*
Researcher Identifier (e.g. ORCID ID): *n/a*
Nearest person month worked: *2*

Contribution to Project: *Dr. Kaminski is the principal investigator of this project and in charge of all aspects related to design and execution of all of the aims in the project.*

Funding Support: *N/A*

Name: *Matt Simon, PhD*
Project Role: *Co-Investigator*
Researcher Identifier (e.g. ORCID ID): *n/a*
Nearest person month worked: *1*

Contribution to Project: *Dr. Simon supervised the structural and function analysis of FENDRR in the context of fibrosis.*

Funding Support: *N/A*

Name: *Robert Homer, MD, PhD*
Project Role: *Co-Investigator*
Researcher Identifier (e.g. ORCID ID): *n/a*
Nearest person month worked: *1*

Contribution to Project: *Dr. Homer performed and oversaw the histologic evaluations of mouse lungs in this grant.*

Funding Support: *N/A*

Name: *Patty Lee, MD*
Project Role: *Co-Investigator*
Researcher Identifier (e.g. ORCID ID): *n/a*
Nearest person month worked: *1*

Contribution to Project: *Dr. Lee helped in all lenti-viral and transgenic experiments.*

Funding Support: *N/A*

Name:	*Norihito Omote, PhD*
Project Role:	*Postdoctoral Associate*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Omote performed cell culture and animal experiments as needed.*
Funding Support:	*N/A*

Name:	*Guoying Yu, PhD*
Project Role:	*Research Scientist*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Yu supervised the breeding and generation of FENDRR knockout mice as well lentiviral design and work.*
Funding Support:	*N/A*

Name:	*Gabriel Ibarra, MD*
Project Role:	*Postdoctoral Associate*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*7*
Contribution to Project:	*Dr. Ibarra performed all day-to-day animal handling, genotyping of transgenic mice as well as downstream molecular biology assays.*
Funding Support:	*N/A*

Name:	*Buqu Hu, MS*
Project Role:	*Biostatistician/Analyst*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*3*
Contribution to Project:	*Mr. Hu works with Dr. Kaminski on all statistical analyses.*
Funding Support:	*N/A*

Name:	*Giuseppe DeIuliis*
Project Role:	*Laboratory Manager*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*5*
Contribution to Project:	*Mr. DeIuliis is in charge of all orders requisitions, and supervises all of the needed orders for this project.*
Funding Support:	*N/A*

Name:	*Nikos Xylourgidis, PhD*
Project Role:	*Associate Research Scientist*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Xylourgidis led all research activities, mouse experimentation and breeding strategy, cell culture and mechanistic studies.*
Funding Support:	*N/A*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.