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14. ABSTRACT

IPF is a fatal fibrotic lung disease associated with aberrant activation of fibroblasts and progressive fibrosis. The pirfenidone therapy for IPF has been shown to slow the rate of decline in lung function but do not halt ongoing fibrosis. This application will test whether inhibition of HSP90 activity, coupled with anti-fibrotic therapy of pirfenidone, will attenuate WT1-driven fibroblast activation. The proposed studies will provide robust, highly novel observations to rapidly pursue the long-term goal: Translational exploration of the molecular mechanisms by which HSP90 activity positively regulates IPF pathogenesis in order to devise novel preventive and therapeutic strategies for IPF

15. SUBJECT TERMS

NONE LISTED

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TABLE OF CONTENTS

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

IPF is a fatal fibrotic lung disease associated with aberrant activation of fibroblasts and progressive fibrosis. The pirfenidone therapy for IPF has been shown to slow the rate of decline in lung function but do not halt ongoing fibrosis. This application will test whether inhibition of HSP90 activity, coupled with anti-fibrotic therapy of pirfenidone, will attenuate WT1-driven fibroblast activation. The proposed studies will provide robust, highly novel observations to rapidly pursue the *long-term* goal: Translational exploration of the molecular mechanisms by which HSP90 activity positively regulates IPF pathogenesis in order to devise novel preventive and therapeutic strategies for IPF.

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

Idiopathic Pulmonary fibrosis; Fibroblasts; Heat shock protein 90; Wilms' tumor 1; lung; collagens

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.

Specific Aim 1: To determine the role of HSP90 in fibroblast activation in IPF
The major task is to determine which HSP90 isoform is involved in fibroblast activation.

Subtask 1: Obtain regulatory approvals: ACURO and HRPO review (Duration: 3-6 months)
Subtask 2: Develop lentiviral plasmids to overexpress HSP90AA or HSP90AB isoforms to assess changes in fibroblasts activation. (Duration: 1-12 months)
Subtask 3: Develop lentiviral plasmids to knockdown HSP90AA or HSP90AB isoforms to assess changes in fibroblast activation (Duration: 7-24 months)
Subtask 4: Develop lentiviral plasmids to overexpress wild type and D88N-HSP90 (a dominant negative mutant of HSP90) to assess changes in fibroblast activation (Duration: 12-24 months)

Specific Aim 2: Define the physiological and functional relevance of HSP90-WT1 interaction in fibroblast activation
The major task is to identify contributions of HSP90 and WT1 on fibroblast activation.

Subtask 1: Knockdown HSP90AB, WT1 or both to assess changes in fibroblast-specific gene networks and function. (Duration: 1-24 months)
Subtask 2: Overexpress HSP90AB, WT1 or both to assess changes in fibroblast-specific gene networks and function (Duration: 12-24 months)
Subtask 3: Overexpress D88N-HSP90 and WT1 alone or together to assess changes in fibroblast-specific gene networks and function (Duration: 12-36 months)

Specific Aim 3: Determine whether combined therapy using pirfenidone and 17-AAG is effective to reverse established and ongoing pulmonary fibrosis

The major task is to determine if combined therapy of 17-AAG and Pirfenidone attenuates fibrosis in vivo.

Subtask 1: Utilize a mouse model of TGFα-induced pulmonary fibrosis to test if combined therapy of 17-AAG and Pirfenidone attenuates fibrosis (Duration: 6-24 months)

Subtask 2: Utilize a mouse model of bleomycin-induced pulmonary fibrosis to test if combined therapy of 17-AAG and Pirfenidone attenuates fibrosis (Duration: 16-36 months)

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.

Major activities: The first 8-10 months of funding for this project have been focused upon several key start up areas, including i) protocol and manual operation development, ii) obtaining all regulatory approvals including ACURO and HRPO (100% Completed), and iii) initiated studies to breed up mice and develop lentiviral plasmids to overexpress or knockdown WT1, D88N-HSP90, HSP90AA, HSP90AB isoforms to assess changes in fibroblasts activation. (80-90% completed).

Specific objectives: The objectives of this are captured in the specific aims described above, but can be succinctly summarized in three aims: Aim 1) To determine the role of HSP90 in fibroblast activation in IPF; Aim 2) Define the physiological and functional relevance of HSP90-WT1 interaction in fibroblast activation, and Aim 3) Determine whether combined therapy using pirfenidone and 17-AAG is effective to reverse established and ongoing pulmonary fibrosis.

Significant results:

Aim 1: To determine the role of HSP90 in fibroblast activation in IPF. We obtained regulatory approvals needed from ACURO and HRPO.

Subaim 1A: To determine which HSP90 isoform involved in fibroblast activation. In this subaim, we will use normal fibroblasts and IPF fibroblasts to assess the effect of HSP90 isoforms and mutant overexpression. We completed cloning and overexpression of dominant negative isoform of HSP90AB (D88N-HSP90) and wildtype HSP90AB **(Figs 1 & 2)**.

Subaim 1B: To determine if the loss of HSP90 attenuates fibroblast activation in IPF (Duration: 7-24 months). In mammalian cells, Hsp90AA and Hsp90AB are the two major isoforms involved in Hsp90-driven functions. We developed the efficient and isoform-specific knockdown method for inhibiting the expression of HSP90AA and HSP90AB isoforms using stealth silencer RNA method (**Fig 3**). Although both isoforms bind similarly to their intracellular co-chaperones and display ATPase activity, in some cases Hsp90AA and Hsp90AB have differential effects on substrate interactions.

To assess the relative contributions of Hsp90 isoforms on fibroblast migration, we measured migration of lung fibroblasts that were treated with siRNA specific to either Hsp90AA or Hsp90AB mRNA, as well as control siRNA. Treatment of fibroblasts with Hsp90AA or Hsp90AB

siRNA specifically knocked down the corresponding isoform expression compared to control siRNA (**Fig 3**). Fibroblast migration was significantly attenuated with the deficiency of either Hsp90AA or Hsp90AB isoform compared to control siRNA treatment (data not shown). Also, we used IPF fibroblasts for HSP90AA or HSP90AB knockdown experiments. IPF fibroblasts were transfected with HSP90AA- and HSP90AB-specific siRNA for 72 hrs. Notably, the genetic knockdown of Hsp90AA was sufficient to attenuate both the proliferation and migration of IPF fibroblasts (**Fig 4**). Further, combined inhibition of both Hsp90AA and HSP90AB had additive effect on proliferation and migration in IPF fibroblasts (**Fig 4**). These findings clearly establish that Hsp90AB and HSP90AA may function as positive regulators of fibroproliferation and migration in IPF. Also, we analyzed HSP90AA or HSP90AB-driven gene transcripts using next generation RNA-seq method. As shown in **Fig 5**, we compared the respective gene expression profiles of HSP90 isoforms to that of IPF and identified multiple transcripts that were either up- or down-regulated in IPF lungs. We performed an enrichment analysis of these negatively correlated gene sets (i.e., genes upregulated in IPF, but downregulated by HSP90AA or HSP90AB knockdown and *vice versa*) using the ToppFun application of the ToppGene Suite. Notably, among the top enriched biological processes were fibroblast motility, proliferation, growth and ECM production (**Fig 6**).

Subaim 1C. To determine if overexpression of a dominant negative mutant of HSP90 attenuates fibroblast activation in IPF. We developed lentiviral plasmids to overexpress wild type and D88N-HSP90 (a dominant negative mutant of HSP90) to assess changes in fibroblast activation. This subaim will determine the effect of wild type and D88N-HSP90 forced expression on activation of IPF fibroblasts, including proliferation, invasion, and apoptosis. Earlier structural studies have demonstrated that Asp93 in HSP90 makes a direct hydrogen bond to the exocyclic N6 group of adenine of bound ATP/ADP nucleotide and site-directed mutagenesis of Asp88 to asparagine (D88N) in HSP90AB resulted in the loss of ATP binding and ATPase activity. We completed the cloning of human-HA-N-Hsp90βDN in pLJMI using standard cloning methods (Addgene Plasmid #22480). Clones were confirmed by DNA sequencing. To determine the effect of D88N-HSP90, we transfected HEK293 cells with control and D88N-HSP90 and measured D88N-HSP90 protein expression and effect on migration of HEK293 cells. In support to our hypothesis, we observed a significant increase in the expression of D88N-HSP90 that resulted in decrease in the migration of HEK293 cells compared to control cells (**Fig 7**). To overexpress D88N-HSP90 using lentivirus, we used pLenti vector (pLJM1-EGFP) to clone HA-N-Hsp90βDN. Agarose gel analysis of pLento-HA-N-Hsp90βDN digestion with restriction enzymes Nhe1 and EcoR1 confirms the generation of lentiviral clone of D88N-HSP90. To assess the effect of Hsp90βDN, we transduced IPF fibrobalsts with either control or Hsp90βDN overexpressing lentiviruses. Overexpression of Hsp90βDN was sufficient to attenuate the expression of Hsp90-driven ECM genes with no effect of endogenous HSP90AA or HSP90AB isoforms (**Fig 8**). However, we observed no significant changes in the proliferation or migration of IPF fibroblasts with the overexpression of Hsp90βDN compared to controls (**Fig 9**).

Aim 2: To define the physiological and functional relevance of HSP90-WT1 interaction in fibroblast activation.

Subaim2A: Knockdown HSP90AB, WT1 or both to assess changes in fibroblast-specific gene networks and function. We obtained regulatory approval for primary cell culture and performed the knockdown HSP90AA and HSP90AB to determine fibroblast specific gene networks (**Fig 5 and 6**).

To determine whether the loss of Hsp90AB, WT1 or both alter ECM production, lung fibroblasts were treated with control, Hsp90AB- , WT1- or both siRNA for 72h. Notably, the loss of Hsp90AB or WT1 was sufficient to attenuate the expression of ECM genes, such as Col1α, and Col3α compared to control siRNA (**Fig 10**). Also, combined inhibition Hsp90AB- and WT1 had no additive effect on the ECM gene expression compared to Hsp90AB- or WT1-specific siRNA treatment. Similarly, we observed no significant changes in the proliferation and invasiveness of IPF fibroblasts treated siRNA for Hsp90AB-, WT1- or both (data not shown).

Subaim 2B: Overexpress HSP90AB, WT1 or both to assess changes in fibroblast-specific gene networks and function. We overexpress WT1 in human fibroblasts using adenovirus and observed a significant increase ECM gene expression (**Fig 11**). Also, we generated HSP90AB lentivirus and validated upregulation of HSP90AB. To determine mechanisms underlying WT1-driven fibroproliferation, we performed the knockdown or overexpression of WT1 in fibrobalsts and assessed changes in the expression of mitotic kinase B (AURKB) involved in fibroproliferation. We performed the knocked down of WT1 using WT1-specific siRNA in fibroblasts isolated from IPF lungs or TGFα mice on Dox for 4 wks. Compared to control siRNA, knockdown of WT1 was sufficient to attenuate the expression of AURKB in lung fibroblasts isolated from both IPF and a mouse model of TGFα-induced pulmonary fibrosis (**Fig 12A & 12B**). We next assessed the effect of adenovirus-mediated overexpression of WT1 on AURKB levels in fibroblasts. Overexpressing WT1 in fibroblasts led to a significant increase in both transcripts and protein of AURKB (**Fig 12C**). Knockdown of WT1 in fibroblasts also abolished the ability of TGFα to increase AURKB expression (Fig 12D). To determine if WT1 is a transcription factor that directly binds to *AURKB*, we performed a computational analysis of the promoter region of human and mouse *AURKB* and found the presence of conserved WT1 binding sites (data not shown). We performed chromatin immunoprecipitation (ChIP) analysis using primary lung-resident fibroblasts isolated from TGFα mice on Dox for 8 wks. ChIP analysis using WT1-specific antibody compared with control isotype antibody revealed that WT1 bound to the *AURKB* gene, suggesting the role of WT1 in AURKB expression (**Fig 12E**). We next overexpressed WT1 in HEK293 cells co-transfected with AURKB-promoter fused with luciferase reporter and observed increased luciferase activity compared to cells transfected with control plasmids (**Fig 12F**). These results support that WT1 functions as a positive regulator of AURKB by directly binding to the AURKB promoter in fibroblasts. The part of these finding is now published in *EMBO molecular medicine* 2020.
Subaim 2C: Overexpress D88N-HSP90 and WT1 alone or together to assess changes in fibroblast-specific gene networks and function. We completed the overexpression of WT1 in primary fibroblasts and identified WT1-driven gene networks in fibroblasts. We demonstrated the effects of D88N-HSP90 overexpression on fibroblast activation. These new findings are now published in *JCI Insight 2018*.

Aim3: To determine whether combined therapy using pirfenidone and 17-AAG is effective to reverse established and ongoing pulmonary fibrosis. To determine whether combined therapy using pirfenidone and 17-AAG is effective to reverse established and ongoing pulmonary fibrosis. To determine in vivo doses of 17-AAG and pirfenidone, we evaluated the potential benefits of 17-AAG and pirfenidone in pulmonary fibrosis using a mouse model of TGFα-induced pulmonary fibrosis. Our previous studies have demonstrated that conditional overexpression of TGFα induces progressive fibrosis in the adventitia, parenchyma, and subpleural areas of the lung with histologic features and genomic profiles similar to human IPF.

To determine whether in vivo doses of pirfenidone and 17-AAG that influences the progression of established fibrosis, following three wk of Dox treatment when fibrosis is already manifest, TGFα mice were administered 17-AAG or pirfenidone while remaining on Dox for an additional three wk (6 wks total) (**Fig 13A**). The total lung hydroxyproline levels were increased in TGFα mice treated with either vehicle or pirfenidone (**Fig 13B**). However, pirfenidone therapy had no effect on this increase in the lung hydroxyproline levels in TGFα mice treated with pirfenidone compared to vehicle-treated TGFα mice (**Fig 13B**). However, H&E stained lung sections show significant improvement in alveolar size in TGFα mice treated with pirfenidone compared to vehicle-treated TGFα mice (**Fig 13C**). Therefore, we evaluated mean alveolar chord length to determine the potential benefit of pirfenidone therapy on lung alveolar integrity during TGFα-induced pulmonary fibrosis. The mean alveolar chord length was increased in doxycycline induced TGFα mice compared to control mice treated with doxycycline for six weeks (**Fig 13D**). This increase in alveolar chord length was attenuated in TGFα mice treated with pirfenidone when compared to vehicle-treated TGFα mice. Notably, the total lung hydroxyproline levels were decreased in TGFα mice treated with 17-AAG compared to vehicle (**Fig 14A**). However, 17-AAG therapy had no effect on alveolar size or chord length in TGFα mice treated with 17-AAG compared to Vehicle (**Fig 14B**). Thus, our in vivo data suggest that Hsp90 plays a critical role in pulmonary fibrosis and inhibition of Hsp90 using 17-AAG attenuates TGFα-induced progressive subpleural thickening and pulmonary fibrosis. Studies are in progress to treat TGFα mice with the combination of 17-AAG and pirfenidone to testreversal of established and ongoing pulmonary fibrosis. To determine whether 17-AAG attenuates TGFβ-induced ECM gene expression, human fibroblasts were treated with TGFβ in the presence and absence of 17-AAG and transcripts of ECM genes were quantified by RT-PCR. As predicted, TGFβ induced the expression of COL1α and FN1 (**Fig 15**). Notably, treatment with 17-AAG was sufficient to attenuate TGFβ-driven expression of COL1α and FN1. Together, our in vitro and in vivo results establish that either inhibition of Hsp90 ATPase activity or Hsp90AB expression was sufficient to attenuate myofibroblast transformation and ECM gene expression.

Significant results: As noted above, we have completed the key studies and the major findings are now published in high-impact journal (*JCI insight 2018 and EMBO Mol Med 2020*). We have completed cloning of D88N-HSP90 and HSP90 isoforms and validated expression using HEK293 cells. Also, we completed the generation of lentiviruses to overexpress human and mouse WT1 (**JCI insight 2018; Figure 3**). The effects of WT1 overexpression on fibroblast activation are now completed and the findings were published in ***JCI insight 2018*** **(Figures 3-4)**. This study also describes the knockdown effects of WT1 on fibroblast activation. Similarly, we evaluated the specific effects of Hsp90AA or Hsp90AB isoforms on fibroblast activation. In particular, our knockdown studies have identified potential differences between Hsp90AA and Hsp90AB isoforms on fibroblast activation. For Aim3, we completed to set-up breeders to generate wild type and TGFα transgenic mice. Initial studies are completed to evaluate the effects of 17-AAG and pirfenidone in the pathogenesis of TGFα-induced pulmonary fibrosis. In support to our hypothesis, pharmacological inhibition of Hsp90 activity or the genetic loss of WT1 has attenuated fibroblast activation and pulmonary fibrosis. Currently, studies are in progress to evaluate whether inhibition of Hsp90 with 17-AAG in a mouse model of bleomycin-induced pulmonary fibrosis will also have similar effects observed with mouse model of TGFα-induced pulmonary fibrosis. Breeding of TGFα mice is completed to test the effect of combination therapy.

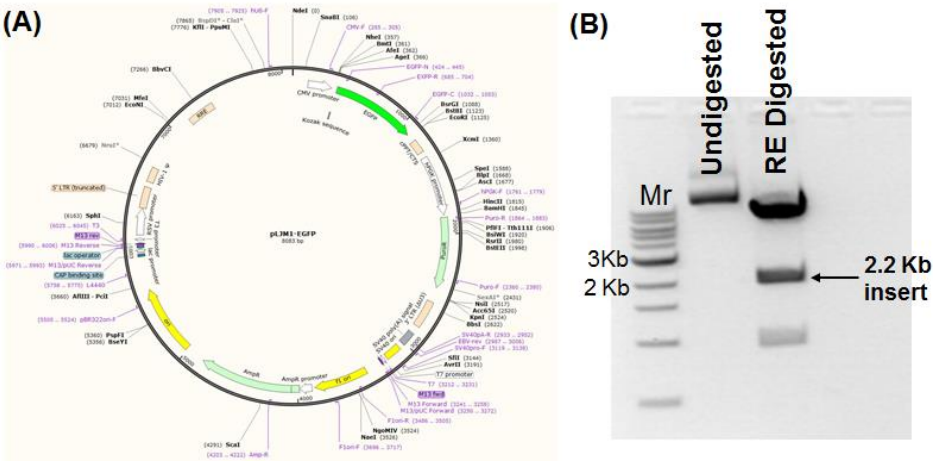

Figure 1. (A) The map of pLJMI-EGFP vector and D88N-HSP90 gene inserted using restriction enzymes, Nhe1 and Age1. (B) Agarose gel showing the right insertion and orientation of D88N-HSP90 in Lenti vector using restriction enzyme digestion (Age1 and EcoR1).

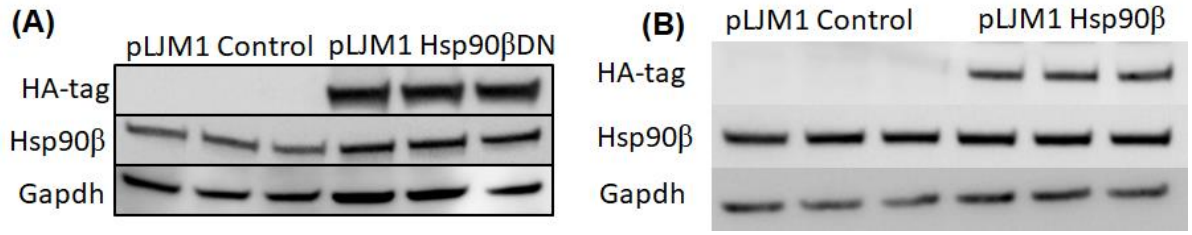

Figure 2. (A) HEK293 cells transfected with control or Hsp90βDN lentivirus for 72hrs and immunoblotted with antibodies against HA-tag, Hsp90β and GAPDH (B) HEK293 cells transfected with control or Hsp90β lentivirus for 72hrs and immunoblotted with antibodies against HA-tag, Hsp90β and GAPDH.

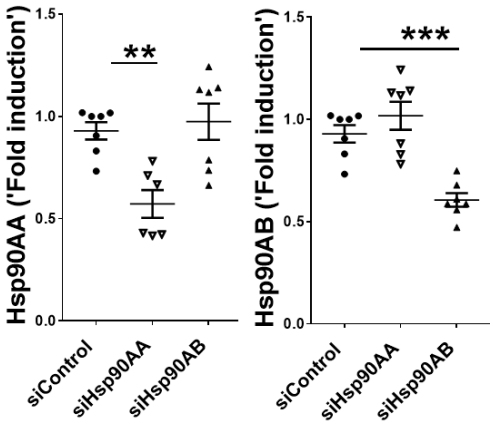

Figure 3: Primary Lung resident fibroblasts ($CD45^{-}Col1^{+}$) were isolated from lung cultures of TGF-α mice which was placed on Dox for 4wk using anti-CD45 magnetic beads and transfected with either control or Hsp90 isoform specific siRNA for 72 hr. The transcripts for Hsp90AA and Hsp90AB isoform are shown as the fold induced by normalizing to hypoxanthine guanine phosphoribosyl transferase (HPRT) control. Data shown are mean + SEM values (n = 7). Statistical significance between groups was measured using an ANOVA. **p<0.005 and ****p<0.00005

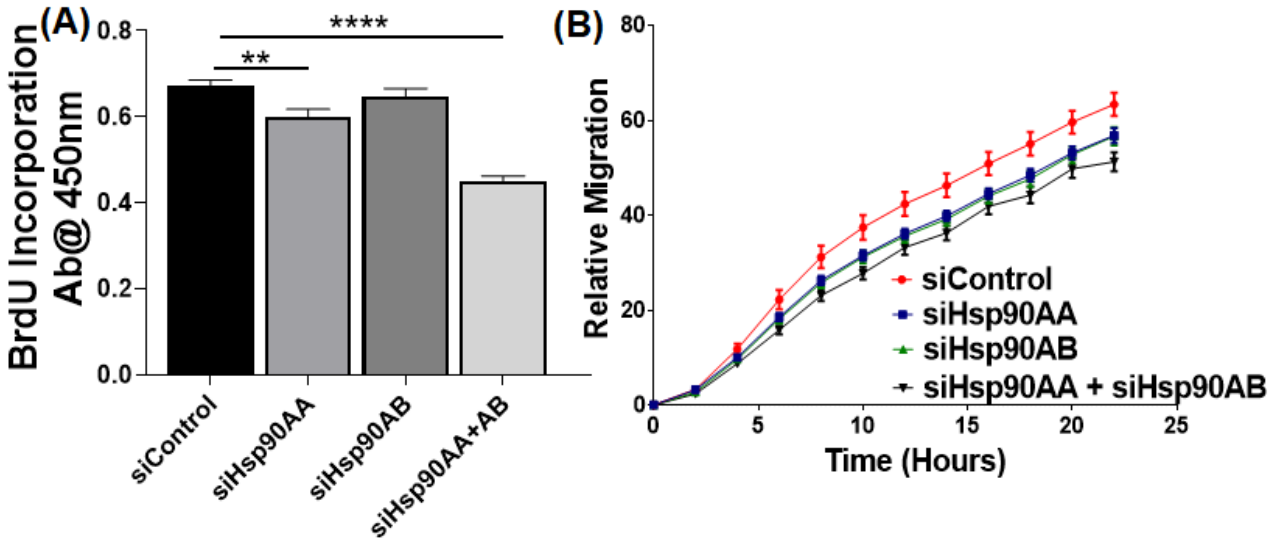

Figure 4. (A) Quantitation of proliferation using the Brdu incorporation assay in lung fibroblasts isolated from IPF lung cultures. Fibroblasts treated with HSP90AA, HSPAB or both for 72hr. ***P*<0.005, *****P*<0.00005. (B) Quantitation of migration in lung fibroblasts isolated from IPF lung cultures. Fibroblasts treated with HSP90AA, HSPAB or both for 72hr.

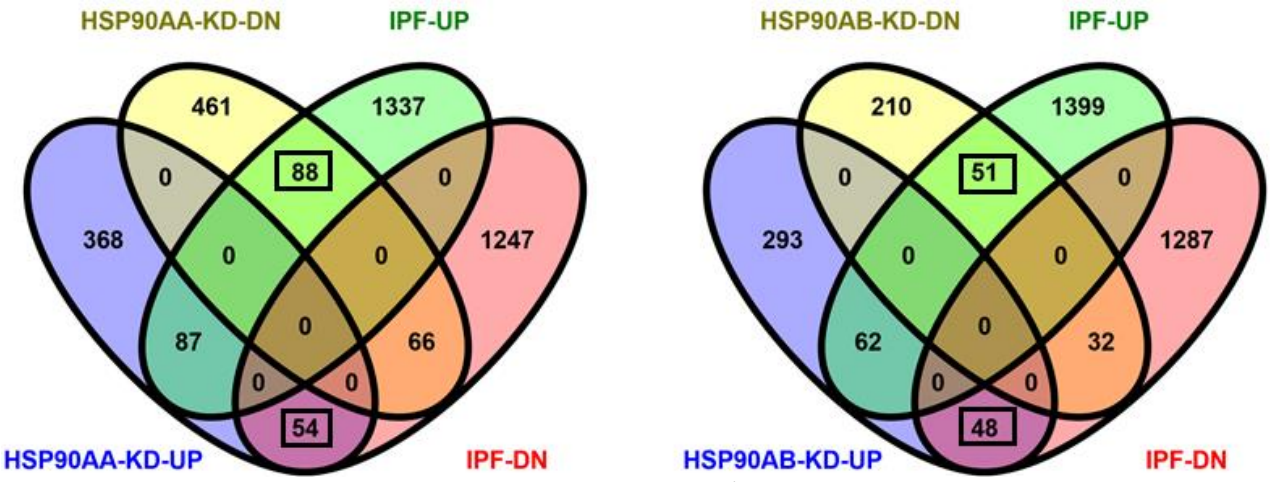

Figure 5: Primary Lung resident fibroblasts ($CD45^{-}Col1^{+}$) were isolated from IPF lung cultures using anti-CD45 magnetic beads and transfected with either control or Hsp90 isoform specific siRNA for 72 hr. Differentially expressed genes in IPF lungs were compared with HSP90AA- and HSP90AB-driven genes in IPF fibroblasts. The overlapping genes that are up or down regulated in IPF or HSP90 isoform deficient fibroblasts are indicated with a box.

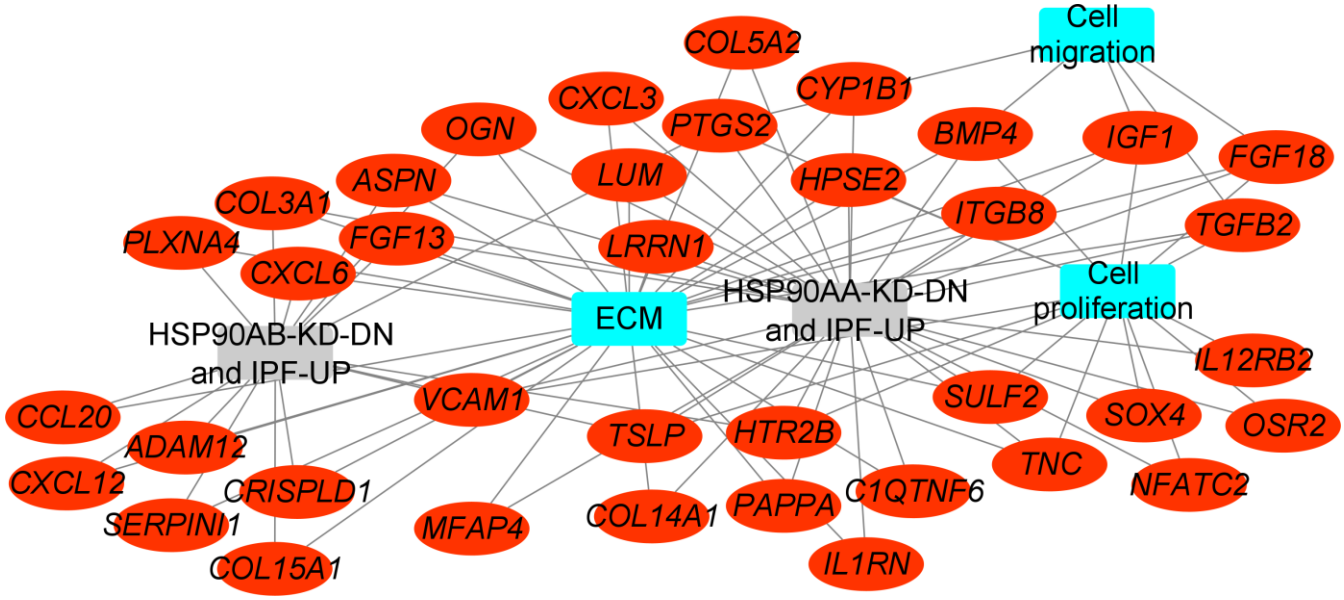

Figure 6: HSP90AA- or HSP90AB-driven genes activated in IPF were analyzed using ToppFun and visualized using Cytoscape. Red-colored circles represent genes that are upregulated in IPF lungs. The turquoise-colored squares represent enriched biological processes for the inversely correlated genes between HSP90AA or HSP90AB siRNA knockdown and IPF.

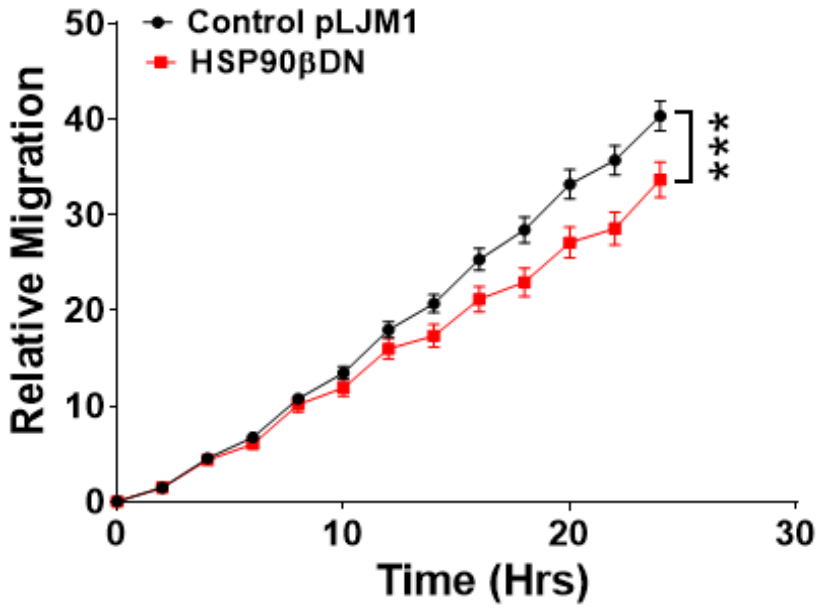

Figure 7. Scratch wound migration assay was performed on HEK293 cells transfected with control or Hsp90βDN overexpressing plasmid for 24hr. Quantitation of migration represented as relative migration over time. Data shown are mean ± SEM values. Statistical significance between groups was measured using ANOVA. ***p<0.0005.

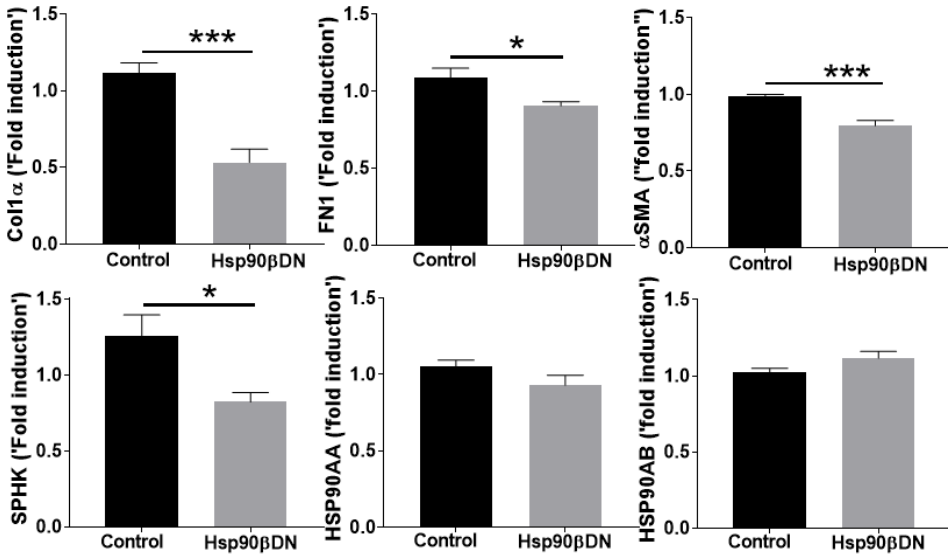

Figure 8. The effect of overexpression of HSP90AB DN on Hsp90-driven ECM gene expression. Primary lung fibroblasts ($CD45^{-}Col1^{+}$) were isolated from IPF lung cultures using anti-CD45 magnetic beads and transduced with either control- or Hsp90AB DN lentivirus for 48 hrs. The levels of Col1a, FN1, αSMA, SPHK, HSP90AA, and HSP90AB gene transcripts relative to HPRT are quantified using qRT-PCR. Data shown are mean ± SEM values (N = 3/group).

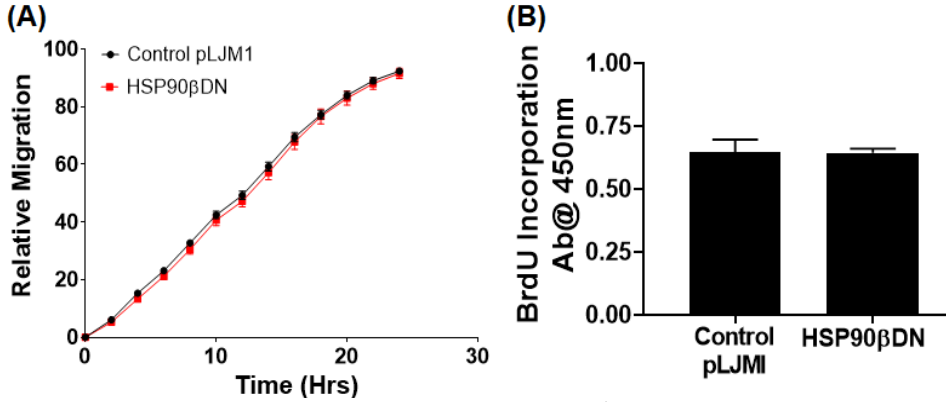

Figure 9. Primary lung fibroblasts ($CD45^{-}Col1^{+}$) were isolated from IPF lung cultures using anti-CD45 magnetic beads and transduced with either control- or Hsp90AB DN lentivirus for 48 hrs. (A) Scratch wound migration assay was performed and quantitation of migration represented as relative migration over time. (B) Quantitation of proliferation using the Brdu incorporation assay.

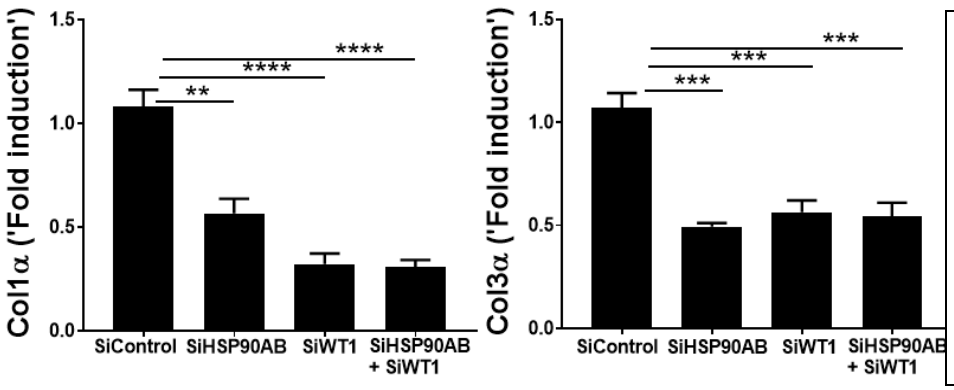

Figure 10. IPF fibroblasts were transfected with either control, Hsp90AB, WT1 or both siRNA for 72 hr. The levels of Col1α and Col3α gene transcripts relative to HPRT are quantified using qRT-PCR.

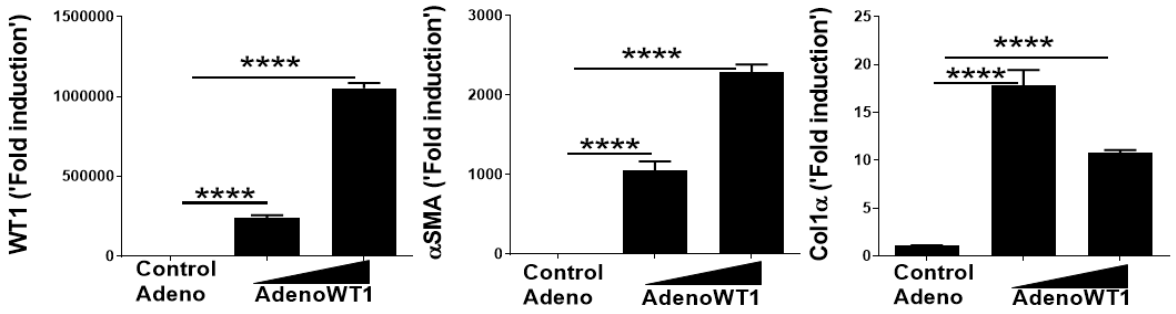

Figure 11. Human lung firboblasts were transduced with either control- or WT1 adenovirus for 72 hrs. The levels of WT1, αSMA, and Colα gene transcripts relative to HPRT are quantified using qRT-PCR.

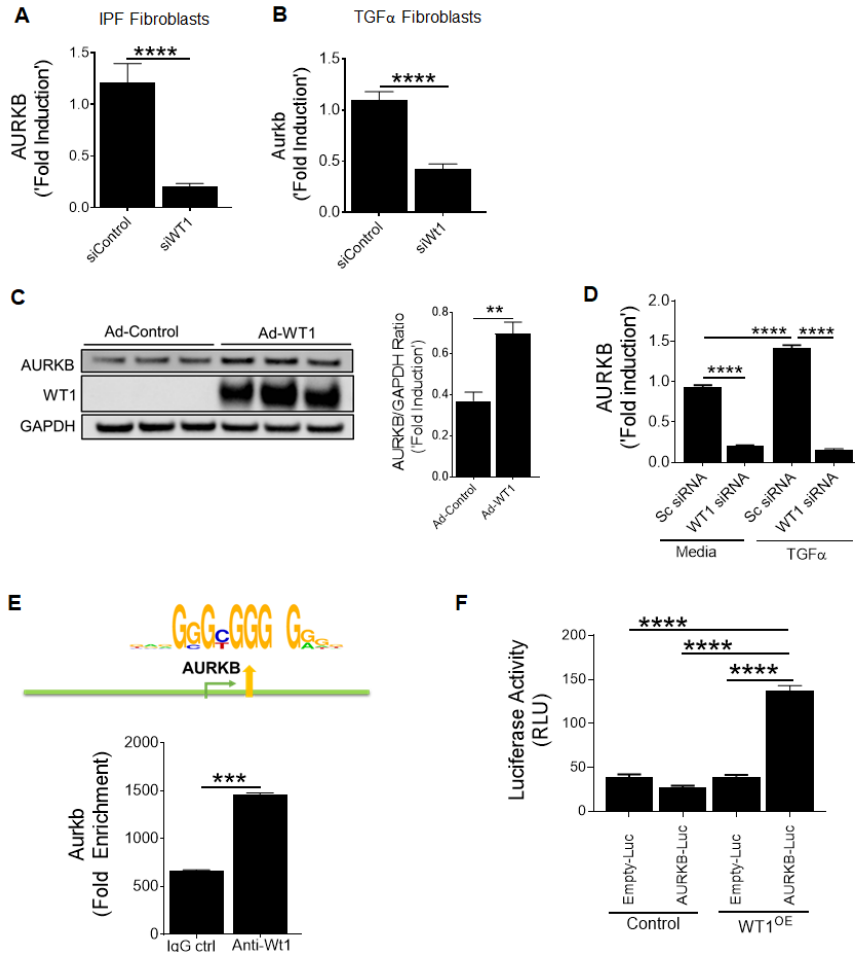

Figure 12. **WT1 induces fibroproliferation by altering AURKB expression. (A)** IPF lung fibroblasts were transiently transfected with control or WT1 siRNA for 72hrs and AURKB transcripts were quantified. **(B)** Lung-resident fibroblasts from TGFα mice on Dox for 4 wks were transiently transfected with control or WT1 siRNA for 72hrs and AURKB transcripts were quantified. **(C)** Immunoblot analysis of AURKB and WT1 in the lysates of non-IPF fibroblasts transduced with control or WT1-adenoviral particles for 72hrs. **(D)** Quantification of AURKB transcripts in primary fibroblasts from IPF lung treated with either control or WT1 siRNA and stimulated with media or TGFα (100ng/mL) for 16hrs. **(E)** The ChIP assay was performed with anti-WT1 antibody or normal rabbit IgG as a negative control. Non-immunoprecipitated DNA is represented as input DNA (product size, 104 bp). **(F)** AURKB-promoter luciferase activity was measured in HEK293 cells transfected with control or WT1 overexpressing (OE) vector.

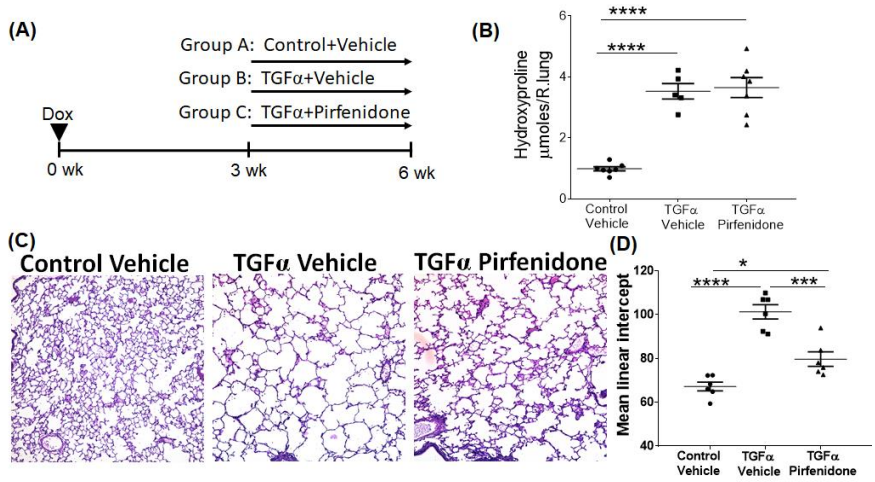

Figure 13. (A) Schematic representation of pirfenidone treatment protocol. Control mice and TGFα mice were placed on Dox for 3 weeks then treated with vehicle or pirfenidone (300 mg/kg; twice a day) for 3 weeks while continued on Dox for a total of 6 weeks. (B) Quantitation of hydroxyproline levels of mice treated with vehicle or pirfenidone. (C) Alveolar regions of H&E stained lung sections from all the groups. Images are representative of each group with N=6-7 per group. (D) Mean linear intercept was quantified in the lung sections of all mice stained with H&E. One-way ANNOVA, *p<0.05, ***p<0.0005 and ****p<0.00005.

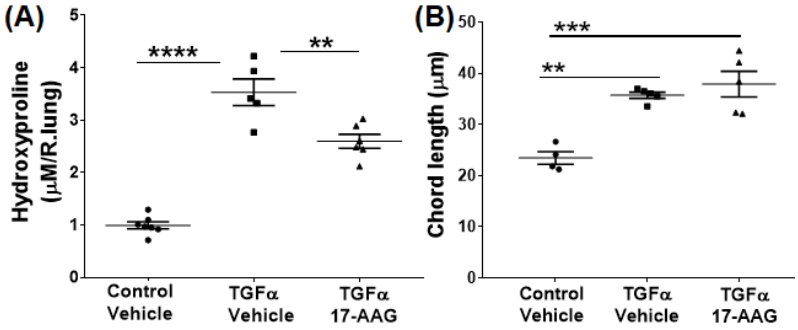

Figure 14. (A) Quantitation of hydroxyproline levels of mice treated with vehicle or 17-AAG. Control mice and TGFα mice were placed on Dox for 3 weeks then treated with vehicle or 17-AAG (15 mg/kg; once a day) for 3 weeks while continued on Dox for a total of 6 weeks. (B) Chord length was quantified in the lung sections of all mice stained with H&E. One-way ANNOVA , **p<0.005, ***p<0.0005 and ****p<0.00005.

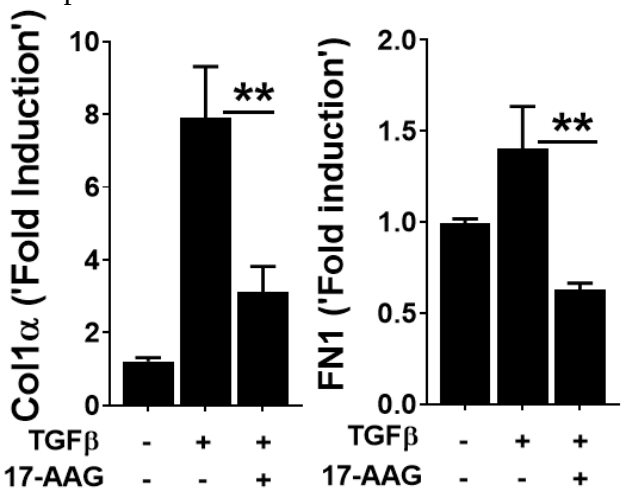

Figure 15. Hsp90 inhibition attenuates TGFβ induced ECM gene expression. Human lung resident fibroblasts ($CD45^{-}Col1^{+}$) were isolated from non-IPF lung fibroblast cultures and treated with TGFβ in the presence and absence of 17-AAG for 24h. Quantified transcripts of ECM genes, COL1α and FN1 using qRT-PCR and shown as the fold induced gene transcripts relative to GAPDH. . $^{**}P < 0.005$, one-way-ANOVA.

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

How were the results disseminated to communities of interest?
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.

Over the next year we will continue our studies using in vivo mouse models of pulmonary fibrosis to assess changes in fibroblasts activation and therapeutic benefits of HSP90 inhibition. In particular, we will continue to test if combined therapy of 17-AAG and Pirfenidone attenuates fibrosis using mouse models of TGFα- and bleomycin-induced pulmonary fibrosis.

4. **IMPACT:** D*escribe 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).

IPF is a chronic and ultimately fatal disease in which tissue deep in the lungs becomes increasingly thick and stiff, or scarred, over time, making breathing more and more difficult. With the funding support from the DoD, our team identified that in IPF patients, activity of a particular protein, Hsp90 (Heat shock protein 90), is elevated in fibroblasts. Fibroblasts are cells in connective tissues that produce collagen and other fibers that have not yet progressed to form scar tissue. We also identified Hsp90 inhibitors as a potential effective therapy to stop or possibly even reverse the disease.

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.

Our new findings demonstrate that Hsp90 is a positive regulator of fibroblast activation and ECM production. Increased Hsp90 activity and fibroblast activation has been shown in multiple organs undergoing fibrotic remodeling. Thus, the proposed studies will not only highlight the role of HSP90 activity in initiation and maintenance of pulmonary fibrosis, but also are essential to the development of novel therapeutics for the treatment of fibrosis in the lung and perhaps other organs. Nearly 45% of all deaths in the developed world are attributed to chronic fibroproliferative diseases, emphasizing the potential impact that an anti-fibrotic therapy would provide. Therefore, successful treatment that either reverses fibrosis or prevents disease progression would have an immediate and profound impact on healthcare.

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.*

CCHMC has filed a US provisional patent application (D14-0129) on the repurposing of 17AAG for IPF. The Center for Technology Commercialization (CTC) at CCHMC is in the process of initiating discussions with industry partners for joint development of this project after obtaining results of this project.

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:*

Nothing to report

Remember that significant changes in objectives and scope require prior approval of the agency.

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.

Due to the Covid19 lockdown, our ongoing studies to generate TGFα mice and testing of HSP90 inhibitor combined with pirfenidone were delayed. However, we completed the generation of the majority of mice needed for in vivo testing and we hope to complete studies proposed in Aim3 in the next six to twelve months. Our studies on the role of WT1 in fibroblast activation were completed and accepted for publication in *EMBO Molecular Medicine*.

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.

Nothing to report

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

No changes to report

Significant changes in use or care of vertebrate animals

No changes to report

Significant changes in use of biohazards and/or select agents

No changes 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).*

Total four manuscripts have been accepted or under consideration for publication.

1. Vadde SR, **Madala SK** and Geereddy BR (2018) Extracellular small heat shock proteins: Exosomal biogenesis and function. ***Cell Stress & Chaperones*** 23(3):441-454. PMID: 29086335
2. Sontake V, Kasam RK, Debora S, Korfhagen TR, Geereddy BR, White ES, Jegga AG and **Madala SK** (2018) Wilms' Tumor 1 Drives Fibroproliferation and Myofibroblast Transformation in Severe Fibrotic Lung Disease. ***JCI insight*** 3(16) PMID: 30135315
3. Sontake V, Gajjala P, Kasam RK, and **Madala SK** (2018) New therapeutics based on emerging concepts in pulmonary fibrosis. ***Expert Opinion On Therapeutic Targets*** 23(1):69-81 PMID: 30468628
4. Kasam RK, Ghandikota S, Soundarajan, D, Reddy GB, Huang SK, Jegga AG, and **Madala SK** (2020) Inhibition of Aurora Kinase B attenuates fibroblast activation and pulmonary fibrosis. ***EMBO Molecular Medicine.*** (in press)

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.*

1. Sontake V, Wang Y, Kasam RK, McCormack FK, Debora S, Geereddy BR, Naren AP, White ES, Jegga AG and **Madala SK** (2017) Hsp90 Regulation of Fibroblast Activation in Pulmonary Fibrosis. ***JCI insight*** 2: e91454. PMID: 28239659
2. Kasam RK, Reddy GB, Jegga AG, and **Madala SK** (2019) Dysregulation of mesenchymal cell survival pathways in severe fibrotic lung disease: The effect of nintedanib therapy. ***Front. Pharmacol.*** 10:532 PMID: 31156440
3. Yombo JKD, Mentink-Kane MM, Wilson MS, Wynn TA, and **Madala SK** (2019) Heat Shock Protein 70 is a positive regulator of airway inflammation and goblet cell hyperplasia in a mouse model of allergic airway inflammation. ***Journal of Biological Chemistry*** (In press)
4. Abstract titled **"Inhibition of fibroblast activation by integrating multiomics data and preclinical models of severe fibrotic lung disease"** presented at 2nd Annual IPF summit, San Francisco, August 20-22, 2018.
5. Kasam RK, Reddy GB, Jegga AG, and **Madala SK** (2019) Dysregulation of mesenchymal cell survival pathways in severe fibrotic lung disease: The effect of nintedanib therapy. ***Front. Pharmacol.*** 10:532 PMID: 31156440
6. Yombo JKD, Mentink-Kane MM, Wilson MS, Wynn TA, and **Madala SK** (2019) Heat Shock Protein 70 is a positive regulator of airway inflammation and goblet cell hyperplasia in a mouse model of allergic airway inflammation. ***Journal of Biological Chemistry*** 294 (41): 15082-94. PMID: 31431507
7. Kasam RK, Gajjala P, Courtney J, Kramer EL, Randell S, Clancy JP, and **Madala SK** (2020) Subacute TGF exposure drives Airway Hyperresponsiveness in CF mice through the PI3K pathway. ***J. Cystic Fibrosis*** (in press)

- **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.

  News article titled "Computational Analyses Identify New Therapeutic Targets For Idiopathic Pulmonary Fibrosis"
  https://www.cincinnatichildrens.org/research/divisions/b/bmi/news/2017/2-27-computational-analyses-identify-new-therapeutic-targets-for-idiopathic-pulmonary-fibrosis

- *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.*

  Nothing to report

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

Funding Support:
Name: *Satish Madala – "No Change"*

Funding Support:
Name: *Anil Jegga – "No Change"*

Funding Support:
Name: *Prathibha Gajjala*
Project Role: *Research Fellow*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *12*
Contribution to Project: *Dr. Gajjala has performed biochemical analysis of Hsp90 and in vivo studies.*

Funding Support:
Name: *Rajesh Kasam*
Project Role: *Graduate Assistant*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*
Contribution to Project: *Dr. Kasam has performed in vitro studies using primary fibroblasts*

Funding Support:
Name: *Divyalakshmi Soundararajan*
Project Role: *Research Assisatant*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Sudhir has performed gene expression analysis under Dr. Jegga's supervision*

Funding Support:

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

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "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.*

Organization Name: University of Cincinnati
Location of Organization: (if foreign location list country) 51 Goodman Drive, Cincinnati, OH
Contribution to Project: Dr. McCormack has provided human samples and clinical perspective on IPF. The project staff have helped in collection and distribution of clinical samples.

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.