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14. ABSTRACT
Chronic lung diseases such as idiopathic pulmonary fibrosis (IPF) are prevalent among veterans and U.S. military personnel. IPF is a progressive, irreversible, and lethal disease with no effective treatment save for lung transplantation. IPF arises from relentless and extensive fibroproliferative injury, which itself stems from the inability of normal repair processes in the lung to deactivate following stimuli. The disease is characterized by focal zones of fibroblast proliferation. Transforming growth factor-β (TGF-β) plays a crucial role in fibrosis development, mediating cell activation, migration, and invasion. Increased TGF-β mediates metabolic reprogramming in cells involved in IPF progression, shifting bioenergetics from oxidative phosphorylation (OXPHOS) towards glycolysis. In fibroblasts and myofibroblasts, TGF-β increases expression of glycolytic enzymes and glucose transporters, as well as lactate production. In alveolar epithelial type II (AE2) cells, genes involved in metabolism are downregulated, and there is increased lactate production. A more glycolytic phenotype is also observed in alveolar macrophages in IPF. Decreased ATP production occurs in fibroblasts and myofibroblasts, while in IPF AE2 cells, fibroblasts, and macrophages, decreased electron transport chain (ETC) complex activity and lower oxygen consumption rates (OCR) have been observed. This work aims to deliver polymer-functionalized mitochondria to fibroblasts, AE2 cells, and macrophages in IPF lungs with the goal of restoring a favorable metabolic phenotype that can attenuate or reverse the disease. Our findings highlight that TGF-β treatment of fibroblasts resulted in metabolic reprogramming towards glycolysis and a pro-fibrotic phenotype. We demonstrate that mitochondria coated with a Dextran-triphenlyphosphonium (Dextran-TPP) polymer were efficiently internalized by alveolar epithelial cells and fibroblasts. Our results show that Dextran-TPP coated mitochondria transplantation to fibroblasts shifted their bioenergetics away from glycolysis and corrected or prevented aberrant cellular dynamics in both fibroblasts and alveolar epithelial cells. Findings are significant because they highlight a novel therapeutic strategy (mitochondrial transplantation) targeting dysregulated metabolism in IPF.

15. SUBJECT TERMS
Idiopathic pulmonary fibrosis, pulmonary fibrosis, fibroblasts, bioenergetics, mitochondria, metabolism, transforming growth factor-beta, glycolysis, oxidative phosphorylation, alveolar epithelial cells, alveolar macrophages

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1. INTRODUCTION

Chronic lung disease is one of the most prevalent diseases among veterans and U.S. military personnel deployed to Iraq and Afghanistan. An increasing trend of incidence was found between interstitial lung diseases, including idiopathic pulmonary fibrosis (IPF), and deployment-related exposures such as dust and sandstorms, as well as industrial fires. IPF is a progressive, irreversible, and lethal disease, with patients dying within 2-5 years after diagnosis. At present, there is no effective treatment for IPF, save for lung transplantation. IPF arises from relentless and extensive fibroproliferative injury that occurs when normal repair processes in the lung are unable to deactivate following stimuli. One of the pathophysiological hallmarks of IPF is fibroblast foci, or focal zones of fibroblast proliferation. Transforming growth factor-β (TGF-β) plays a crucial role in development of fibrosis, mediating cell activation, migration, and invasion. Importantly, increased TGF-β mediates metabolic reprogramming in IPF lungs, with specific cell types exhibiting a metabolic shift from oxidative phosphorylation (OXPHOS), the highly efficient method of ATP production, towards a less efficient process of glycolysis, despite an adequate supply of oxygen. In fibroblasts and myofibroblasts, TGF-β increases expression of glycolytic enzymes and glucose transporters, with findings showing heightened lactate production as well. In alveolar epithelial type II (AE2) cells, genes involved in lipid synthesis and metabolism are downregulated, and there is increased lactate production. A more glycolytic phenotype is also observed in alveolar macrophages in IPF. Decreased ATP production occurs in fibroblasts and myofibroblasts, while in IPF AE2 cells, fibroblasts, and macrophages, decreased electron transport chain (ETC) complex activity and lower oxygen consumption rates (OCR) has been observed. The proposed work aims to deliver polymer-functionalized mitochondria to fibroblasts, AE2 cells, and macrophages in IPF lungs with the goal of restoring a favorable metabolic phenotype that can attenuate or reverse the disease.

2. KEYWORDS

Idiopathic pulmonary fibrosis, pulmonary fibrosis, fibroblasts, bioenergetics, mitochondria, metabolism, transforming growth factor-beta, glycolysis, oxidative phosphorylation, alveolar epithelial cells, alveolar macrophages

3. ACCOMPLISHMENTS

What were the major goals of the project?

SA 1 - Evaluate the capacity of bioengineered mitochondria to restore cellular energetics in IPF fibroblasts, AE2 cells, and macrophages

Major Task 1: Cellular uptake examination in cells

Milestone 1: Relative quantification of mitochondria uptake in the different cell lines compared to non-coated mitochondria
Proposed completion date: month 3
Percentage of completion: 75%

Milestone 2: IACUC approval for *in vivo* experiments
Proposed completion date: month 3
Percentage of completion: 100% (completion in month 7)

Major Task 2: Bioenergetic and mitochondrial functional analysis

Milestone: Improved bioenergetic changes following mitochondrial transplantation into different pulmonary cells
Proposed completion date: month 7
Percentage of completion: 30%

Major Task 3: Cell proliferation, migration, morphology examination
Milestone: Reduction in TGF-β stimulated proliferation and migration in cells following mitochondrial transplantation
Proposed completion date: month 9
Percentage of completion: 30%

Major Task 4: Expression of fibrotic markers
Milestone: Demonstration of reduced expression of pro-fibrotic genes in cells following treatment with mitochondria
Proposed completion date: month 10
Percentage of completion: 30%

SA 2 - Determine whether bioengineered mitochondrial transplantation can treat experimental IPF

Major Task 5: Biodistribution examination in BLM model of fibrosis in mice
Milestone: Increased uptake of polymer functionalized mitochondria in lungs compared to uncoated mitochondria; increased uptake of mitochondria in lungs in diseased vs healthy lungs
Proposed completion date: month 13
Percentage of completion: 90%

Major Task 6: Efficacy evaluation in BLM model of lung fibrosis in mice
Milestone: Efficacious attenuation of disease progression, markedly reduced pro-fibrotic mediators after intravenous administration of polymer functionalized mitochondria
Proposed completion date: month 18
Percentage of completion: 0%

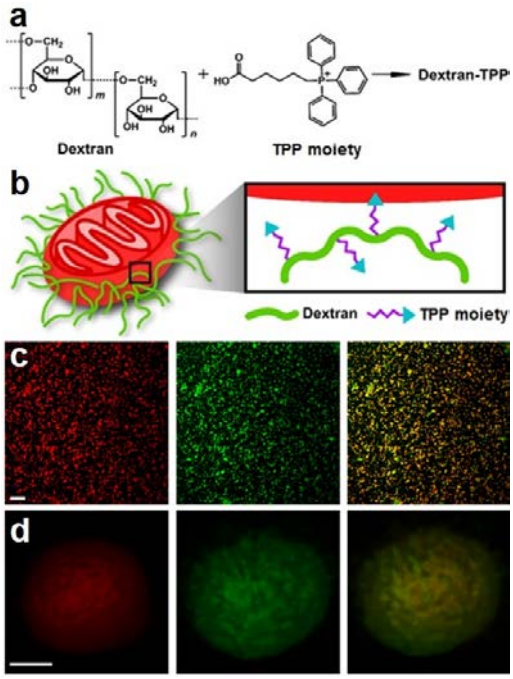

Figure 1. Dextran-TPP coating of isolated mitochondria. **a**) Chemical structures of dextran and TPP. **b**) Schematic of a Dextran-TPP coated mitochondrion, highlighting TPP incorporation into the mitochondrion. **c**) Confocal microscopy of Dextran-TPP coated mouse liver-derived mitochondria. Dextran-TPP was labelled with FITC (green) and mitochondria labelled with MitoTracker Deep Red (red). Scale bar = 20 μm. **d**) Magnification of coated mitochondrion. Scale bar = 0.5 μm.

What was accomplished under these goals?

Our overarching goal was to restore favorable metabolic phenotypes in IPF lungs. We hypothesized that mitochondrial delivery to fibroblasts, AE2 cells, and alveolar macrophages would properly regulate cellular bioenergetics, and prevent disease progression in a mouse model of IPF.

Previously, a dextran-triphenylphosphonium (TPP) (Dextran-TPP, **Fig. 1a**) conjugate was used as a polymer coating for isolated mitochondria (**Fig. 1b**). The goal was to biocompatibilize mitochondria for *in vivo* transplantation strategies to metabolically compromised cells. The

polymer conjugate was found to comprehensively coat isolated mitochondria (**Fig. 1c, d**). Dextran-TPP coated mitochondria had higher cellular uptake in cancer and cardiac cells compared to uncoated mitochondria. Importantly, transplantation of Dextran-TPP coated mitochondria triggered a bioenergetic switch in breast cancer cells and adult mouse cardiomyocytes. Upon examination of the average basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), a significant shift from a glycolytic to an OXPHOS phenotype occurred following mitochondrial transplantation into cells.

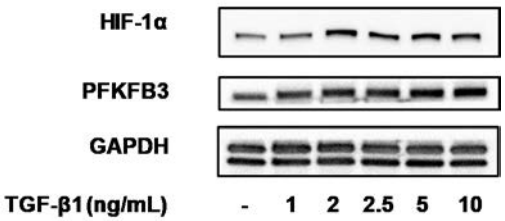

Figure 2. TGF-β effect on glycolytic proteins. Western blot of HIF-1α and PFKFB3. MRC-5 cells were incubated with TGF-β at different doses for 24 h.

Metabolic reprogramming away from glycolysis following mitochondrial transplantation was expected to prevent or offset aberrant cellular dynamics associated with dysregulated bioenergetics or mitochondrial dysfunction. As mentioned previously, TGF-β mediates metabolic reprogramming in IPF lungs towards a glycolytic phenotype that drives disease progression. Mitochondrial transplantation into IPF fibroblasts, AE2 cells, and macrophages was hypothesized to restore favorable bioenergetics and potentially treat IPF. Thus, our first aim involved evaluating the capacity of bioengineered mitochondria to restore cellular energetics in IPF fibroblasts, AE2 cells, and macrophages, while our second aim involved determining whether bioengineered mitochondrial transplantation can treat experimental IPF.

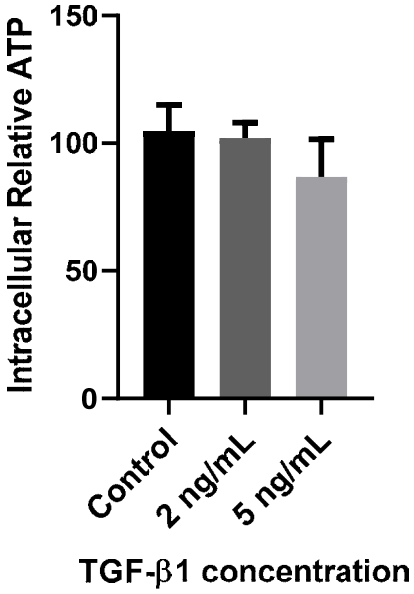

Figure 3. ATP production following TGF-β treatment of fibroblasts. ATPLite assay of relative intracellular ATP of MRC-5 cells incubated with different doses of TGF-β for 24 h.

Subtask 1 of Major Task 1 involved establishing conditions for TGF-β stimulation in cells. In the previous reporting period, work focused primarily on fibroblast cells, specifically the human lung fibroblast MRC-5 cell line. As mentioned previously, TGF-β is associated with mitochondrial dysfunction in fibroblasts, inducing metabolic reprogramming in fibroblasts through increased expression of glycolytic enzymes and enhanced glycolysis. Of note, IPF is associated with hypoxia, specifically the induction of HIF-1α in the tissue microenvironment. Stabilization of HIF-1α transactivates several genes involved in metabolic reprogramming towards glycolysis. Our results demonstrate that TGF-β treatment of MRC-5 fibroblasts increased the expression of HIF-1α, especially at doses higher than 2 ng/mL (**Fig. 2**). The expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), an enzyme responsible for increased rate of glycolysis, was also elevated in MRC-5 cells stimulated with TGF-β for 24 h in a dose dependent manner (**Fig. 2**).

TGF-β treatment of MRC-5 fibroblasts resulted in a dose-dependent decrease in ATP production (**Fig. 3**). This is significant because a metabolic shift towards glycolysis is associated with reduced

OXPHOS – the highly efficient method of ATP production. Moreover, an increase in glucose consumption was observed in MRC-5 cells stimulated with increasing doses of TGF-β (**Fig. 4**). Lastly, the effect of TGF-β stimulation on metabolic reprogramming in MRC-5 cells was examined via Seahorse XFe96 Analyzer, providing insights into the bioenergetic changes accompanying TGF-β treatment. Findings show that the ratio between ECAR and OCR increased following TGF-β treatment (**Fig. 5**), confirming the shift towards glycolysis that accompanies TGF-β stimulation.

Subsequently, MRC-5 cells were stimulated with TGF-β at different concentrations, and the effect on cell dynamics examined. TGF-β plays a crucial role in mediating cell activation, migration, and invasion, as well as cellular dynamics that drive fibrosis. It promotes myofibroblast differentiation, stimulates α-smooth muscle actin (α-SMA), and increases the synthesis of extracellular matrix (ECM) molecules such as ED-A fibronectin (ED-A-FN), which is expressed during wound healing and fibrosis. The intracellular proteins Smad transduce TGF-β signaling and are important for myofibroblast processes including collagen secretion, proliferation, and differentiation. Our results show that TGF-β treatment of cells led to upregulation of Smad signaling in fibroblasts (**Fig. 6**). TGF-β stimulation for 24 h also resulted in an increase in expression of ED-A-FN (**Fig. 6**). α-SMA is a protein indicative of activated fibroblasts. Following TGF-β stimulation of MRC-5 cells, α-SMA levels in these fibroblasts were elevated (**Fig. 6**). Fibronectin and α-SMA expression after TGF-β treatment in MRC-5 fibroblasts was also confirmed via confocal microscopy (**Fig. 7**).

Taken together, these data confirm that TGF-β stimulation of MRC-5 cells leads to a glycolytic reprogramming of cells and an activated fibroblast phenotype. This was the first Subtask in Major Task 1 of the proposed work. The goals we have not met were a detailed characterization of TGF-β stimulation in A549 cells and alveolar macrophages, both of which will be performed in the next reporting period.

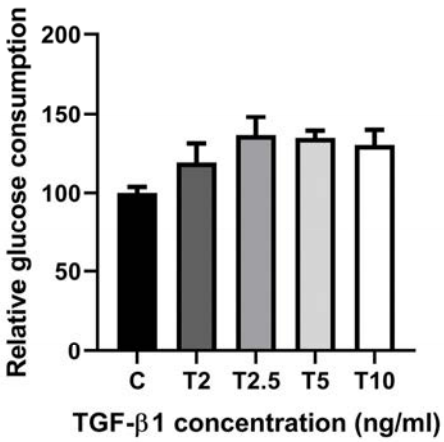

Figure 4. Glucose consumption following TGF-β treatment of fibroblasts. MRC-5 cells were incubated with different doses of TGF-β for 24 h and glucose determined via Glucose-Glo bioluminescent assay.

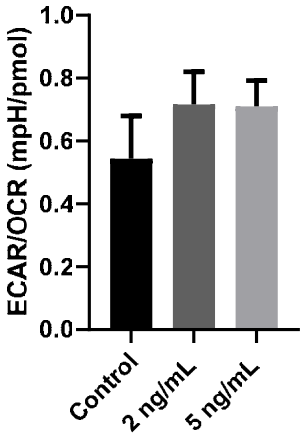

Figure 5. Glycolytic assessment of MRC-5 cells following TGF-β treatment. Ratio of basal extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of MRC-5 cells treated with different doses of TGF-β for 24 h.

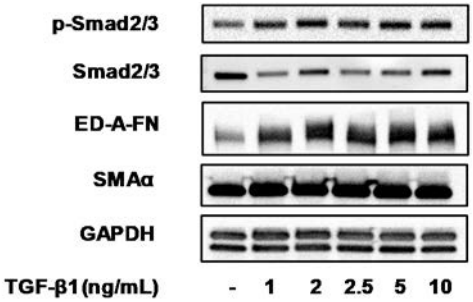

Figure 6. TGF-β effect on fibroblast cellular dynamics resulting in fibrosis and wound healing. Western blot of ED-A-FN, p-Smad2/3, Smad2/3 and α-SMA. MRC-5 cells were incubated with TGF-β at different doses for 24 h.

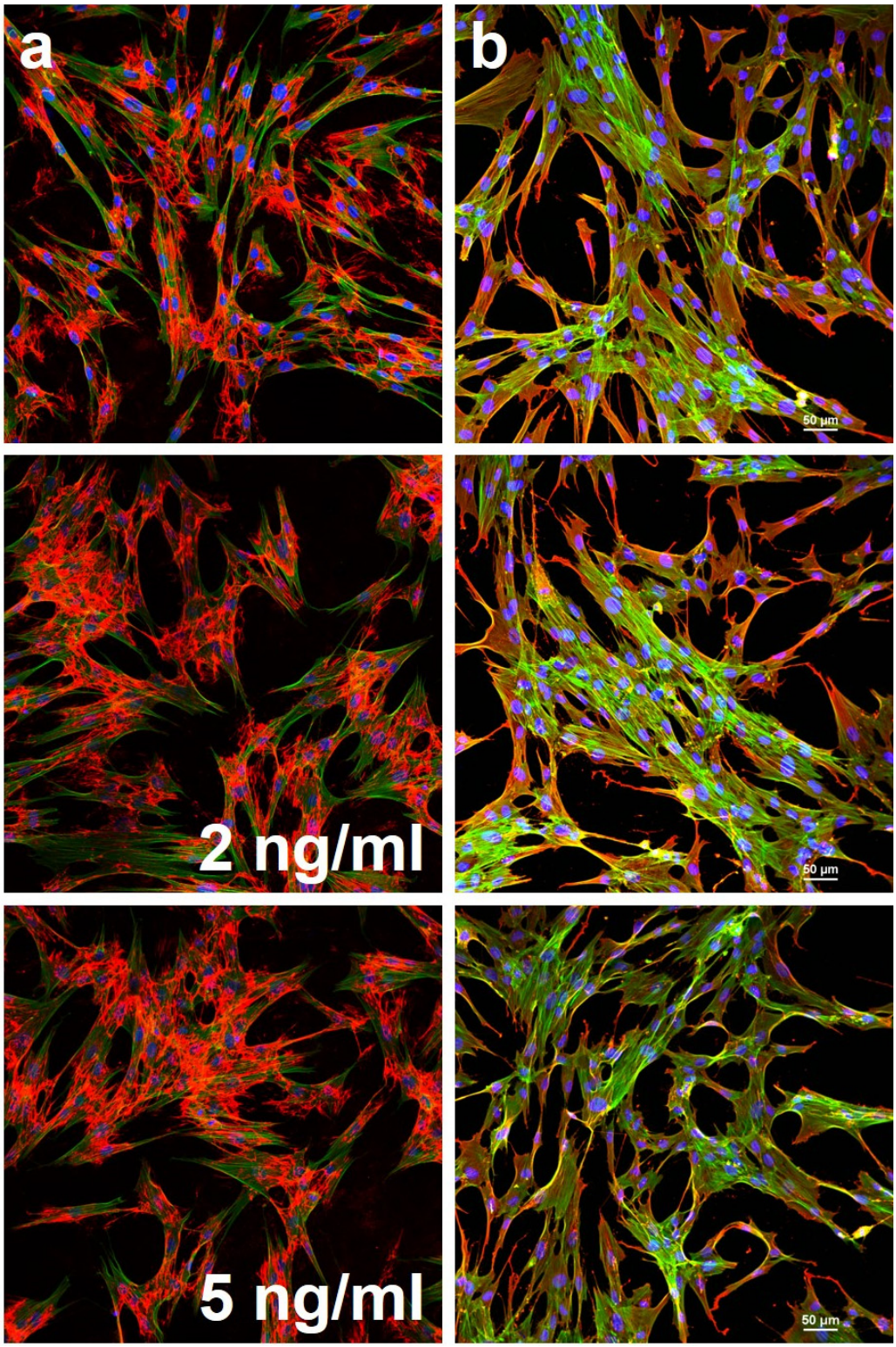

Figure 7. Immunofluorescent examination of fibronectin and α-SMA expression in fibroblasts following TGF-β treatment. MRC-5 cells were treated with TGF-β for 24 h and subsequently visualized via immunofluorescence with antibodies for fibronectin **(a)** and α-SMA **(b)**. In **(a)** blue color corresponds to DAPI nuclear staining, green color corresponds to F-actin staining, while red color is associated with fibronectin. In **(b)** blue color corresponds to DAPI nuclear staining, green color corresponds to α-SMA, while red color is associated with F-actin.

In the initial months of the work, IACUC approval from our institution was obtained and submitted for ACURO approval, which was necessary for all *in vivo* studies. This was Subtask 3 of Major Task 1.

Mitochondria were successfully isolated from livers of euthanized C57BL/6J mice and examined via OROBOROS Oxygraph-2k for high-resolution respirometry and ATP production assay. Results demonstrate that isolated mitochondria had high levels of ATP production (**Fig. 8a**). Findings also show that mitochondrial integrity and function was preserved following isolation, as evidenced by a high respiratory control ratio (RCR) (**Fig. 8b**).

Dextran (Mw 150 kDa) was conjugated to (5-Carboxypentyl) triphenylphosphonium bromide (TPP-COOH). For cell uptake and *in vivo* biodistribution, Cyanine5 carboxylic acid (Cy5), with an λ_{ex}=646 nm and an λ_{em}=662 nm, was conjugated onto the polymer at a molar ratio 1:3 Dextran-TPP:Cy5.

Cellular uptake following incubation with Cy5-Dextran-TPP coated mitochondria was examined via flow cytometry. Dextran-TPP coated mitochondria were administered to MRC-5 and epithelial A549 cells. A time- and dose-dependent increase in Dextran-TPP coated mitochondria internalization was observed in both cell lines (**Fig. 9a**). Dextran-TPP coated mitochondria internalization into MRC-5 fibroblasts was confirmed via

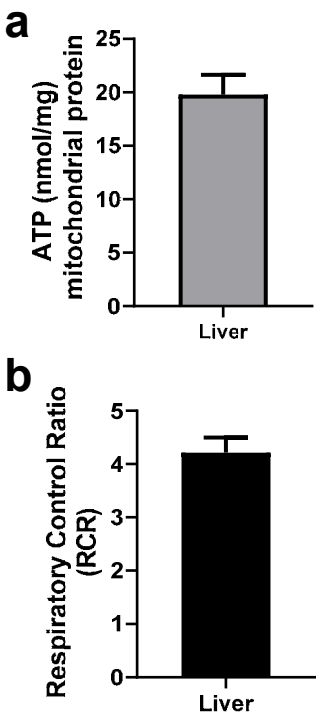

Figure 8. Mitochondrial integrity and function following isolation from mouse livers. a) ATP content as determined by ATPlite assay. **b)** Respiratory control ratio (RCR) of mitochondria isolated from mouse liver tissue as determined by Oroboros high-resolution respirometry, calculated as the ratio between the oxygen flux response before and after addition of ADP.

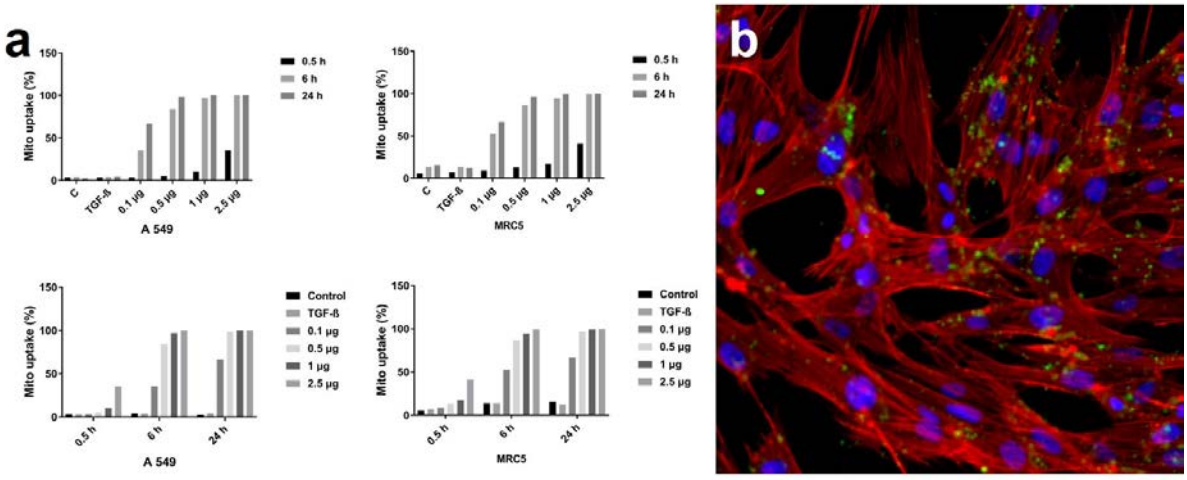

Figure 9. Mitochondrial uptake into fibroblasts and alveolar epithelial cells. a) Flow cytometry examination of time- and dose-dependent uptake of Dextran-TPP coated mitochondria in A549 epithelial cells and MRC-5 fibroblasts. **b)** Confocal microscopy image of Dextran-TPP coated mitochondria in MRC-5 cell. Blue color corresponds to DAPI nuclear staining, red color represents F-actin staining, and green is associated with Cy5-labeled Dextran-TPP mitochondria.

confocal microscopy (**Fig. 9b**) This work corresponded to Subtask 2 of Major Task 1. The goals that we did not meet were confocal microscopy examination of uptake and internalization of polymer coated mitochondria in alveolar macrophages. This will be performed in the next reporting period.

To examine the effect of Dextran-TPP coated mitochondria transplantation on cellular bioenergetics, TGF-β-stimulated MRC-5 cells were incubated with Dextran-TPP coated mitochondria, and 24 h after treatment, basal measurements of OCR were examined in MRC-5 cells via Seahorse XFe96 Analyzer. Findings highlight a dose-dependent increase in OCR following mitochondrial transplantation (**Fig. 10**). ATP production was also examined following Dextran-TPP coated mitochondria treatment of MRC-5 cells, with results highlighting a dose-dependent increasing trend in intracellular ATP (**Fig. 11**). Lastly, Dextran-TPP coated mitochondria treatment of MRC-5 cells resulted in a decrease in PFKFB3 (**Fig. 12**), signaling a decrease in the rate of glycolysis. Taken together, this data suggests that mitochondrial transplantation can metabolically reprogram TGF-β-stimulated cells with aberrant cellular bioenergetics and mitochondrial dysfunction. This work corresponds to Subtasks 4 and 5 in Major Task 2 of the project. The goals that we did not meet were examination of the effect of mitochondria transplantation in epithelial cells and alveolar macrophages. This will be performed in the next reporting period.

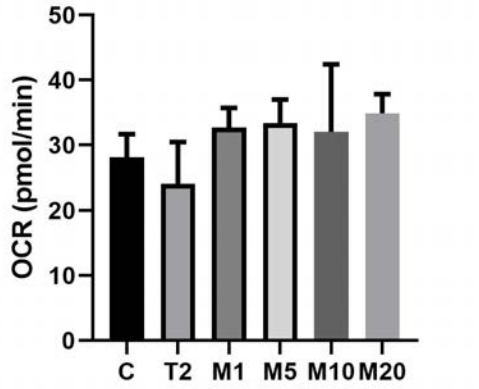

Figure 10. Effect of Dextran-TPP coated mitochondria transplantation on oxygen consumption rate in fibroblasts. Oxygen consumption rate (OCR) in MRC-5 cells treated with Dextran-TPP coated mitochondria and incubated with TGF-β for 24 h. C stands for non-treated MRC-5 cells, T2 for TGF-β (2 ng/ml) treated MRC-5 cells, and M1, M5, M10, and M20 for doses of 1, 5, 10, and 20 μg of mitochondria per 1.5 x 10^4 cells.

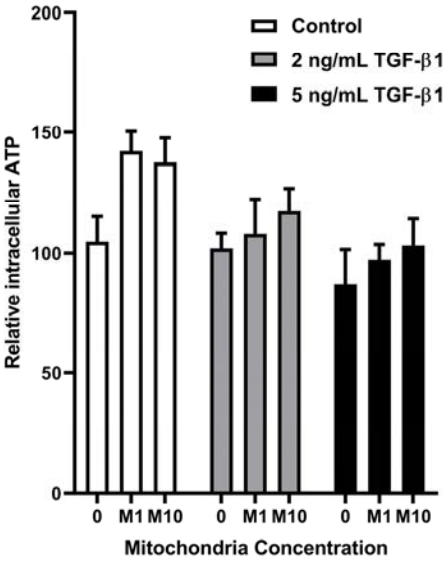

Figure 11. ATP production following mitochondria transplantation in TGF-β treated fibroblasts. ATPLite assay of relative intracellular ATP of TGF-β treated MRC-5 cells incubated with Dextran-TPP coated mitochondria for 24 h. M1 and M10 are doses of 1 and 10 μg of mitochondria per 1.5 x 10^4 cells.

The effect of Dextran-TPP coated mitochondria transplantation on morphological changes in TGF-β-stimulated A549 cells was examined. Epithelial-mesenchymal transition (EMT) is associated with metabolic reprogramming, particularly glycolytic induction and decreased oxygen consumption. TGF-β-induced EMT correlates with suppressed mitochondrial function, resulting in a bioenergetic shift towards glycolysis. Metabolic reprogramming can also feedback into EMT processes. Dextran-TPP coated mitochondrial transplantation into A549 cells impacted trans-differentiation. TGF-β-treated A549 cells showed a change from an epithelial shape towards the spindle-like morphology of fibroblasts (**Fig. 13**). Transplantation of Dextran-TPP coated mitochondria resulted in a loss of TGF-β-mediated spindle shape change (**Fig. 13**). This work corresponds to Subtask 6 in Major

Task 3. The goals we did not meet in the current reporting period were the evaluation of cell proliferation and the examination of migration specifically in fibroblasts. This will be performed in the next funding period.

Metabolic reprogramming in fibroblasts drives fibroblast-to-myofibroblast differentiation. Delivery of Dextran-TPP coated mitochondria to TGF-β-treated MRC-5 fibroblasts resulted in decreased expression of fibronectin and type I and IV collagens compared to TGF-β-treated MRC-5 fibroblasts (**Fig. 14**). Taken together, these results suggest that Dextran-TPP coated mitochondria transplantation are capable of offsetting and correcting cellular dynamics that play a major role in driving IPF progression. This work corresponds to Subtask 7 in Major Task 4. The goals we did not meet in the current reporting period involved examination of the effect of mitochondria transplantation on epithelial cell markers E-cadherin and mesenchymal markers (N-cadherin, vimentin, and α-SMA), p-Smad2/3 and Smad2/3 level examination in fibroblasts, and HIF-1α, plasminogen activator inhibitor-1 (PAI-1), TGF-β1, and prolyl-hydroxylase domain (PHD)-1, 2, and 3 protein expression in alveolar macrophages. This will be performed in the next funding period.

In this project, mitochondrial transplantation *in vivo* was explored in a bleomycin model in C57BL/6J mice. To determine *in vivo* localization following intratracheal (IT) administration to mice with BLM-induced fibrosis, Dextran-TPP polymer was conjugated with Cy5 and biodistribution of Dextran-TPP coated mitochondria in lungs and other major organs examined via epifluorescence using a Xenogen IVIS 200 system. Findings highlight substantial accumulation of Dextran-TPP coated mitochondria in lungs after IT administration, with a sustained presence over time (**Fig. 15**). Of note, minimal to no fluorescence signal was detected in other major organs at the different timepoints (**Fig. 15**). This work was associated with Subtask 8 in Major Task 5 of the proposed work. The goals we did not meet in the current reporting period was examination of co-localization of Dextran-TPP coated mitochondria with alveolar epithelial cells,

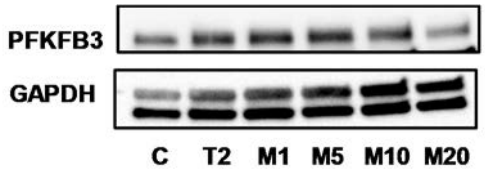

Figure 12. Dextran-TPP coated mitochondria effect on glycolysis in fibroblasts. Expression of PFKFB3 in TGF-β-treated MRC-5 cells. C stands for non-treated MRC-5 cells, T2 for TGF-β (2 ng/ml) treated MRC-5 cells, and M1, M5, M10, and M20 for doses of 1, 5, 10, and 20 μg of mitochondria per 1.5 x 10^4 cells.

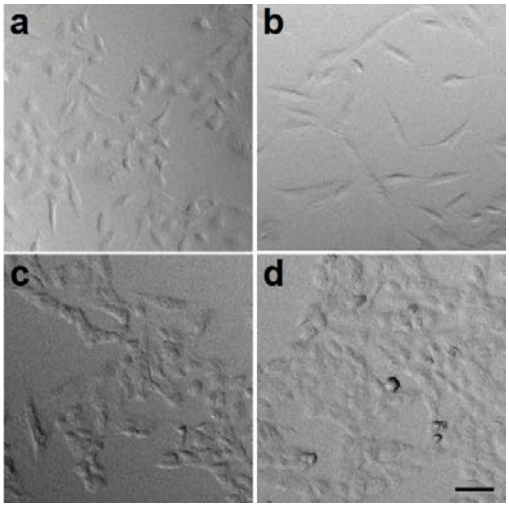

Figure 13. Dextran-TPP coated mitochondrial transplantation into epithelial cells prevented EMT. a) Non-treated A549 cells **b)** A549 cells 24 h after treatment with TGF-β. Transplantation of Dextran-TPP coated mitochondria inhibited TGF-β-induced spindle-like morphology in A549 cells at 24 **(c)** and 48 h **(d)**. Scale bar = 100 μm.

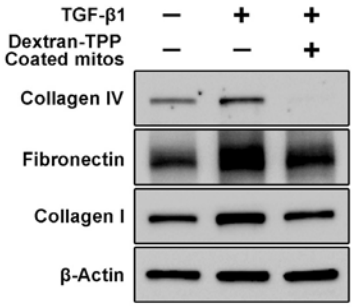

Figure 14. Effect of Dextran-TPP mitochondria transplantation on expression of fibronectin, collagen I, collagen IV on TGF-β-treated MRC-5 fibroblasts. MRC-5 fibroblasts were treated with either TGF-β or TGF-β and Dextran-TPP coated mitochondria for 24 h, after which protein expression was examined by western blotting.

alveolar macrophages, and fibroblasts via immunofluorescence analysis. This will be performed in the next funding period.

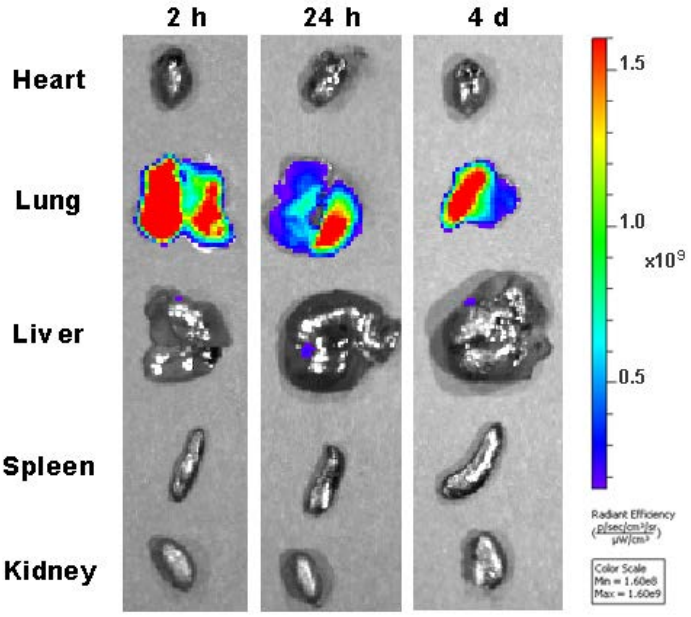

Figure 15. Examination of Dextran-TPP mitochondria localization in a murine model of IPF. Epifluorescence images of distinct organs from a bleomycin-induced mouse model of IPF. Dextran-TPP/Cy5 coated mitochondria were administered intratracheally and organs excised at timepoints of 2, 24, and 96 h.

To summarize, in this reporting period we have shown that Dextran-TPP coated mitochondria transplantation is capable of metabolically reprogramming cells with mitochondrial dysfunction and aberrant bioenergetics, as well as preventing cellular dynamics that contribute to IPF progression. Most of the work that was performed in this reporting period focused on fibroblast and A549 cells. In the next reporting period, we will conclude the evaluation of our mitochondrial transplantation strategy in fibroblasts and epithelial cells and begin evaluating the strategy in alveolar macrophages. Concomitantly, we will evaluate the efficacy of Dextran-TPP coated mitochondria treatment in a bleomycin-induced model of IPF.

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

The project has provided training and contributed towards the professional development of Dr. Suhong Wu, the Research Associate tasked with performing all experiments related to this grant

The project has provided opportunities for Dr. Suhong Wu to gain knowledge and skills in various fields. Dr. Wu is a polymer physicist by training with expertise in gene delivery. The project has enabled her to obtain knowledge in the areas of cellular metabolism/metabolic pathways and mitochondrial dynamics. She has learned new skills involving several metabolic assays and has become proficient in obtaining measurements of ECAR and OCR using Seahorse instrumentation. Moreover, this project has enabled Dr. Wu to gain more experience in molecular biology techniques including western blots. Lastly, Dr. Wu has attended workshops and seminars hosted by Agilent on subjects involving metabolic profiling of cells.

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?

In the next reporting period, we plan to focus heavily on the second major task of Specific Aim 2 involving efficacy evaluation of our strategy in the bleomycin model of lung fibrosis in mice.

Concomitantly, we will conclude all work related to Aim 1 of the project in alveolar epithelial cells and fibroblasts, and work towards determining the effect of mitochondrial transplantation in alveolar macrophages.

4. IMPACT

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

Findings from this reporting period will impact the base of knowledge, theory, and research in the principal disciplinary field of the project – pulmonary fibrosis. Results show the effect that TGF-β has on metabolic reprogramming in fibroblasts and alveolar epithelial cells – cells that play an important role in IPF progression. Upon TGF-β stimulation, cells undergo a transition towards glycolysis, potentiating pro-fibrotic effects such as fibroblast-to-myofibroblast differentiation. Our findings show that potential treatments for IPF should consider targeting the bioenergetic phenotype of these cells, principally reverting their energy handling towards oxidative phosphorylation. Given the modest responses observed with anti-inflammatory agents in IPF patients, novel treatment approaches may very well rely on mitochondrial transplantation, which is the focus of the current work, or the use of pharmacological therapies that impact aberrant metabolic signatures in cells involved in disease progression.

What was the impact on other disciplines?

Metabolic dysregulation and mitochondrial transplantation is rapidly being recognized as hallmarks in a variety of diseases, including but not limited to: pulmonary arterial hypertension (PAH), chronic obstructive pulmonary disease (COPD), atherosclerosis, and Alzheimer's disease. Our mitochondrial transplantation strategy has the potential to regulate aberrant cellular bioenergetics, preventing disease progression. In this period, we have shown that mitochondrial transplantation can alter cellular metabolism, resulting in a shift away from glycolysis. Moreover, we demonstrated that this shift towards favorable metabolic phenotypes abrogates cellular dynamics necessary for disease progression.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

Nothing to report

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

The project experienced delays. There was a delay in obtaining ACURO approval, which in turn postponed the start of several experiments. In the initial stages of the work, we struggled with ensuring viability and proper functioning of isolated mitochondria. A considerate amount of time

was also devoted to the characterization of the effects of TGF-β on fibroblasts in order to establish adequate conditions for treatment. Consequently, a delay in studies propagated throughout the funding period, resulting in later start dates for several tasks. These issues have all been resolved.

Changes that had a significant impact on expenditures

Nothing to report

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

This proposal aims to treat idiopathic pulmonary fibrosis by targeting fibroblasts, alveolar epithelial cells, and alveolar macrophages. We had originally proposed to administer Dextran-TPP coated mitochondria intravenously (IV). However, this route of delivery is better suited for targeting vascular and perivascular cells (e.g. endothelial cells, smooth muscle cells), and we realized that it would be very difficult to reach the cells of interest (i.e. fibroblasts, alveolar epithelial cells, and alveolar macrophages).

The use of intratracheal (IT) instillation was proposed as an Alternative Strategy in the original application to reach fibroblasts, alveolar epithelial cells, and alveolar macrophages. Importantly, this administration route was included in our IACUC protocol and was approved by ACURO (01/13/2020) prior to any *in vivo* experimentation. The positive data presented above regarding mitochondria accumulation and long-term presence in the lung following IT administration warrants further exploration and will be used as the route of mitochondria administration in subsequent efficacy analysis of our therapeutic strategy.

While not a significant change, we thought it best to mention at this time.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications.

Nothing to report

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

Nothing to report

Other publications, conference papers, and presentations.

Abstract # MHSRS-20-01601 “Mitochondrial replenishment of fibroblasts and alveolar epithelial cells in idiopathic pulmonary fibrosis” was accepted for an Oral Presentation in the scientific breakout session *Combating Long-Term Respiratory Consequences of Airborne Hazards on the Battlefield* at the 2020 Military Health System Research Symposium (MHSRS).

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

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:	**Elvin Blanco**
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-7683-3311
Nearest person month worked:	1.2
Contribution to Project:	PI of the proposed work. Oversees all aspects of the work and directly supervises the research staff.

Name:	**Dale Hamilton**
Project Role:	Co-Investigator
Nearest person month worked:	0.2
Contribution to Project:	Provides insights into bioenergetic pathways and cellular metabolism, provides resources in the form of Seahorse and Oroboros instrumentation.

Name:	**Harry Karmouty-Quintana**
Project Role:	Co-Investigator
Nearest person month worked:	0.6
Contribution to Project:	Provides mechanistic insights into pathology of idiopathic pulmonary fibrosis.

Name:	**Suhong Wu**
Project Role:	Research Associate
Nearest person month worked:	2.6
Contribution to Project:	Research associate assigned to the project. Performs all experiments involved in the project.

Name:	**Haoran Liu**
Project Role:	Postdoctoral Fellow
Nearest person month worked:	3.8
Contribution to Project:	Assists Dr. Wu in performing experiments.

Name:	**Scott Collum**
Project Role:	Postdoctoral Fellow
Nearest person month worked:	3
Contribution to Project:	Assists Dr. Wu with molecular biology assays.

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

Yes, the support for Dr. Blanco has changed.

What other organizations were involved as partners?

University of Texas Health Science Center at Houston

8. SPECIAL REPORTING REQUIREMENTS

Not applicable

9. APPENDICES

None

BLANCO, E.

CURRENT
Title: Mitochondrial transplantation: a novel therapy for lung fibrosis
Time Commitments: 12%
Supporting Agency: DOD/PRMRP
Performance Period: 6/1/2019-11/30/2020
Brief description of project's goals: The objective of this work is to restore favorable metabolic phenotypes in lungs undergoing idiopathic pulmonary fibrosis (IPF) through mitochondrial delivery to alveolar epithelial type II cells, fibroblasts, and macrophages.
Specific Aims: 1) Evaluate the capacity of bioengineered mitochondria to restore cellular energetics in IPF fibroblasts, AE2 cells, and macrophages; 2) Determine whether bioengineered mitochondrial transplantation can treat experimental IPF.
Role: PI

Title: Disrupting Six/Eya signaling as new therapy for lung fibrosis
Time Commitments: 5%
Supporting Agency: DOD/PRMRP
Performance Period: 5/15/2019-11/14/2020
Brief description of project's goals: The goal of this proposal is to determine whether Six1/Eya can be targeted pharmacologically or through gene therapy approaches.
Specific Aims: 1) Evaluate whether drugs targeting the Six1/EYA complex are able to treat experimental lung fibrosis; 2) Determine the capacity of gene therapy approaches to silence the Six1/EYA axis.
Role: Co-Investigator

(Change in Role)
Title: Nanoparticle-mediated hyperthermia to improve chemotherapeutic efficacy in HIPEC
Time Commitments: 10%
Supporting Agency: Cancer Prevention and Research Institute of Texas
Grants Officer: Israel Ramirez, 6565 Fannin, MGJ4-023, Houston, TX 77030
Performance Period: 8/1/2018-7/31/2020
Brief description of project's goals: The goal of this proposal is to create a nanoparticle platform that exploits hyperthermia to provide precision drug delivery to peritoneal malignancies.
Specific Aims: 1) Develop an innovative hydrogel film optimized to generate controlled, mild hyperthermia and provide local drug delivery; and 2) Determine if hyperthermia generated by a gold nanorod-embedded hydrogelchemotherapy film increases vascular permeability and chemotherapeutic efficacy
Role: co-I (8/1/18-5/31/20), PI (6/1/20-7/31/20)

Title: mmRNA-Based Transdifferentiation of Fibroblasts to Inducible Vascular Endothelial Cells in Myocardial Infarction
Time Commitments: 3%
Supporting Agency: George and Angelina Kostas Research Center for Cardiovascular Nanomedicine - HMRI Office of Strategic Research Initiative
Grants Officer: TBD, StrategicResearchOSRI@houstonmethodist.org
Performance Period: 11/2017-12/2020
Brief description of project's goals: The goal of this project is to examine mmRNA-based nanotherapeutics to promote fibroblast transdifferentiation to endothelial cells (MEndoT) and the potential role for cardiovascular regeneration.
Role: PI

Title: Re-Energizing Failing Hearts through Systemic Transplantation of Polymer-Functionalized Mitochondria
Time Commitments: 2%

Supporting Agency: George and Angelina Kostas Research Center for Cardiovascular Nanomedicine - HMRI Office of Strategic Research Initiative
Grants Officer: TBD, StrategicResearchOSRI@houstonmethodist.org
Performance Period: 11/2017-12/2020
Brief description of project's goals: The goal of the proposed work is to examine the potential of mitochondrial transplantation to increase contractility of isolated cardiomyocytes and examine the effect of systemically administering functional mitochondria to mice undergoing heart failure.
Role: PI

OVERLAP
None

PENDING
None