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TITLE: Anti-scar Treatment for Deep Partial-thickness Burn Wounds

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14. ABSTRACT

The FDA-approved drug pirfenidone is an anti-inflammatory/anti-fibrosis drug indicated for pulmonary fibrosis that we hypothesize can diminish scarring when applied topically to deep partial-thickness burn wounds in two animal models. The long-term objective is to learn to effectively use pirfenidone with regard to dosage, formulation and timing of treatment of burn wounds, such that animal studies will likely translate to the clinic. The objective of this proposal is to evaluate pirfenidone for efficacy in reducing fibrosis and scarring parameters in mouse and porcine models of deep partial-thickness burn wounds. The dosage formulation and schedule of treatment will be optimized and molecular markers of inflammation, angiogenesis, wound healing, and fibrosis will be correlated with scar reduction.

15. SUBJECT TERMS

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INTRODUCTION

Deep Partial-thickness (DPT) burns frequently result in hypertrophic scars that can lead to severe functional impairment, psychological morbidity, and costly long-term healthcare. Current treatment options lack effectiveness. The purpose of this research is to identify dosage formulations and treatment schedules for the FDA-approved drug Pf to evaluate it for use as a topical prophylactic and treatment against fibrotic scarring of DPT-burn wounds. The scope of the research is to evaluate pirfenidone for efficacy in reducing fibrosis and scarring parameters in mouse and porcine models of deep partial-thickness burn wounds. The dosage formulation and schedule of treatment will be optimized, and molecular markers of inflammation, angiogenesis, wound healing, and fibrosis will be correlated with scar reduction.

KEYWORDS

Deep Partial-Thickness Burn; Pirfenidone; Hypertrophic Scar; Fibrosis; Formulations; mouse Burn Model; Porcine Burn Model; Topical; Inflammation; Granulation; Proliferation

ACCOMPLISHMENTS

What were the major goals of the project?

1. Identification of topical formulations and doses that effectively deliver Pf to the dermis of DPT-burn wounds at each phase of healing and mitigate fibrosis of the closed wounds.
2. Optimization of the schedule of topical applications and uses this optimized schedule to determine detailed molecular changes in healing wounds resulting from Pf treatment.
3. Validation of the efficacy of Pf to reduce hypertrophic scarring in the Duroc porcine DPT-burn model.

What was accomplished under these goals?

The followings summarize the major activities and accomplishments associated with the goals described above for Year 4:

Major accomplishments of Goals #1 and #2:

- Previously, we demonstrated that Pirfenidone (Pf) inhibited normal human dermal fibroblasts (NHDF) transdifferentiation to myofibroblasts, decreased collagen deposition and fibrosis-related gene expression, and reduced p38 MAPK activation in TGF-β1 stimulated human dermal fibroblasts *in vitro* (1). In this reporting period, we determined that Pf also altered the profibrotic contractile phenotype of the stimulated myofibroblasts (2) (**Figure 1**). We also demonstrated that Pf not only acted as a prophylactic measure against expression of profibrotic phenotype, Pf was able to mitigate the profibrotic phenotype of stimulated and differentiated myofibroblasts, suggesting the Pf could treat existing scar (**Figure 2**).

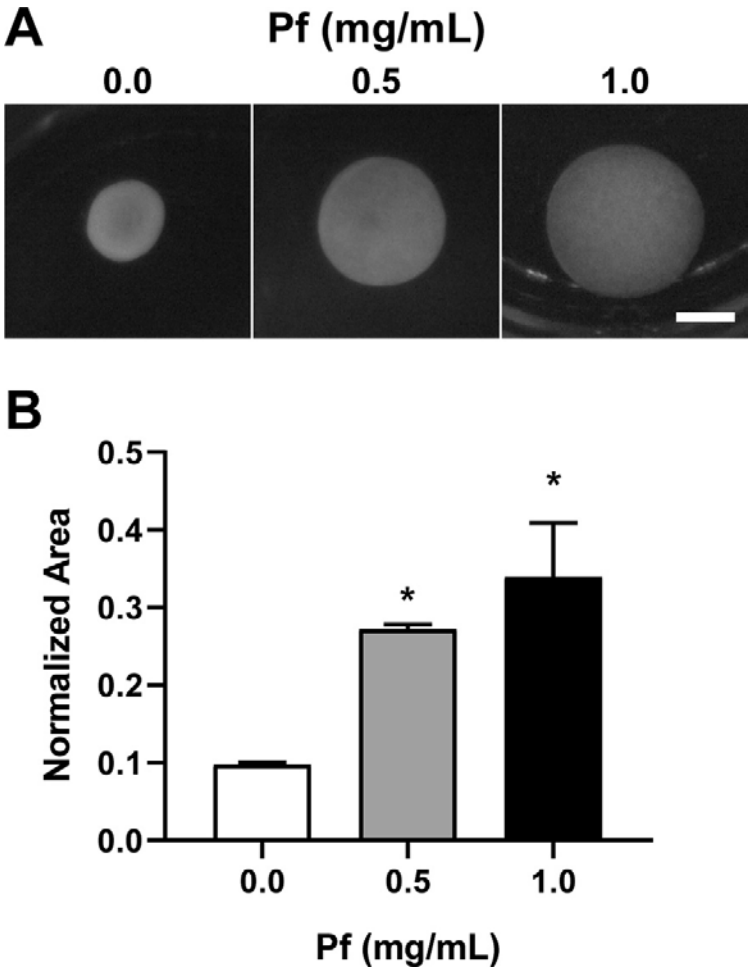

Figure 1. **Pf inhibited cell contractility in myofibroblast-populated collagen lattices.** NHDF were seeded into collagen lattices and cultured in complete media with 1 ng/mL TGF-b1, remaining attached to the culture plate for 2 days. Stressed lattices were released from the plate after 1 h treatment with complete media ± Pf at 0.5 or 1.0 mg/mL. (A) Images shown of representative lattices taken 1 day after Pf treatment and release, scale bar 1.5 mm. Gel area measured and normalized to pre-release area is plotted in (B). Data represents normalized area, average ± standard deviation for 2 replicate gels per treatment group from one representative experiment of three. *P < 0.05 with one-way ANOVA and Bonferonni post-test comparing each to SFM treated control (0.0 Pf).

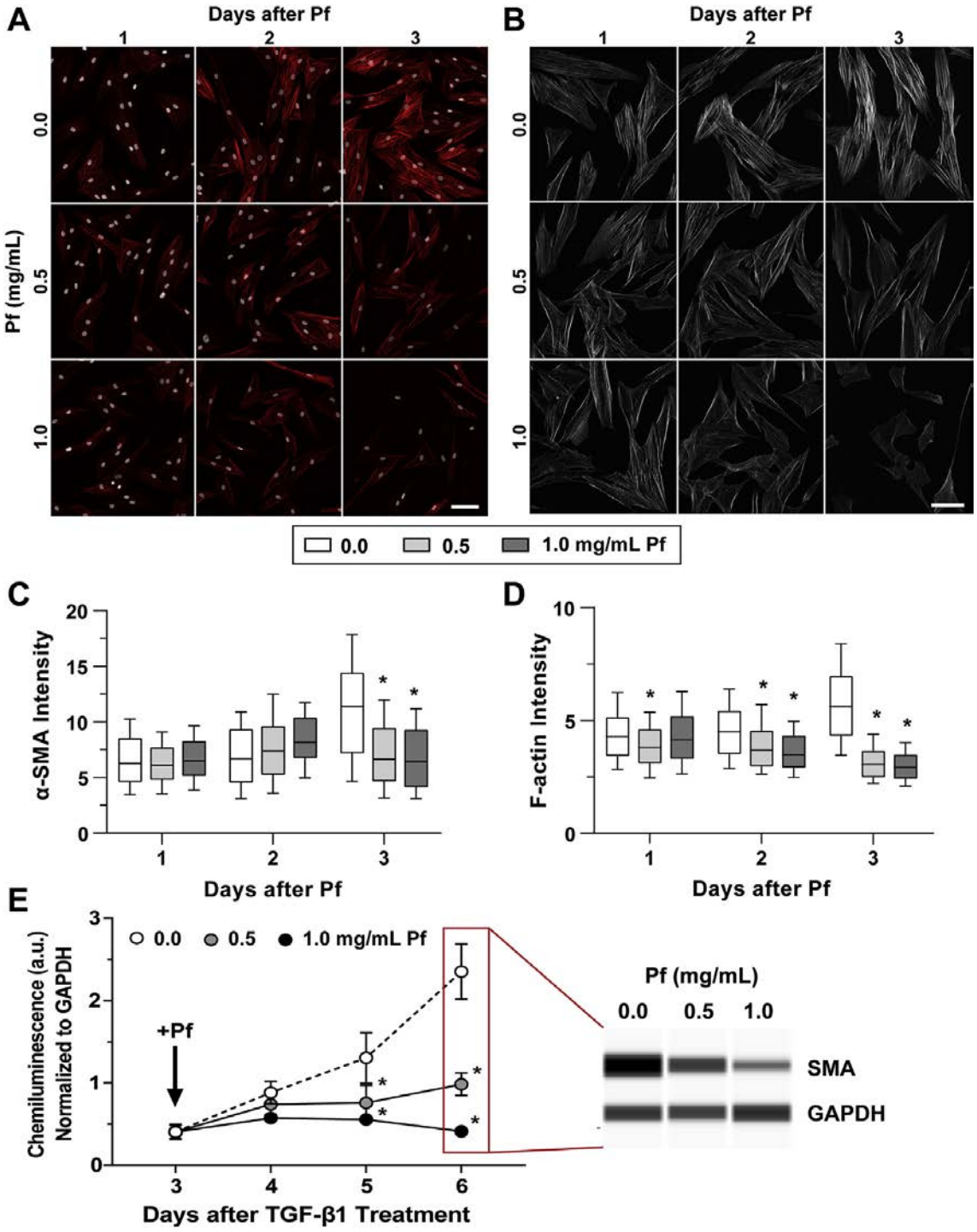

Figure 2. Pf treatment prevented further a-SMA protein expression and reduced F-actin stress fibers. NHDF stimulated with TGF-b1 for 3 days were treated with SFM ± Pf at 0.5 or 1.0 mg/mL and then fixed after 1, 2, or 3 days. (A) Representative confocal images for each treatment and time point immunostained for a-SMA (red) with a nuclear counterstain (Hoechst, white). (B) Representative confocal images from the same experimental conditions as in (A) stained for F-actin with TRITC-labeled phalloidin. Scale bars are 100 mm. (C, D) Mean cell intensity for a-SMA and F-actin measured with CellProfiler. Box plots depict median and inner quartile range with 10-90th percentaile error bars for all cells captured in three images per condition (see Supplemental Table S1 for number of cells per measurement). Plots reflect data from same experiment in corresponding image panel. *P < 0.01 with Kruskal-Wallis non-parametric test and Dunn's post-test comparing each sample to TGF-b1-stimulated, SFM alone treated control sample at the matching time point (white box). Each panel/plot from one representative experiment of four. (E) a-SMA protein expression measured in whole cell lysates via SimpleWes capillary electrophoresis system. NHDF

cells treated as for immunocytochemistry with SFM ± Pf after 3 days of TGF-b1 stimulation. Plot of a-SMA band chemiluminescence quantified with Compass software is shown normalized to that of GAPDH measured within the same capillary. Data represents average normalized chemiluminescence ± standard deviation from three independent experiments. *P < 0.01 with two-way ANOVA and Bonferonni post-test comparing Pf-treated samples to time-matched, TGF-b1-stimulted, SFM treated control samples (white circles). For final time point (outlined in red), images of a-SMA and GAPDH bands for single representative experiment shown on right.

- We have demonstrated the dose that effectively deliver Pf to the dermis of DPT-burn wounds to be at 6.5% (w/w) Pf. The most optimal treatment schedule of topical applications occurred during the inflammatory and/or remodeling phase but not the proliferative phase (3, 4).

- We have demonstrated that Pf ointment diminished early inflammation (reduced the pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-13, G-CSF, and MIP-1α) and later fibrotic indicators (α-SMA and collagens) in mouse DPT wounds (3, 5). Additionally, to expand the anti-inflammatory properties of Pf, during this reporting period we found that Pf reduced chemotaxis of lipopolysaccharide (LPS)-activated human neutrophils to fMLP, LTB4, and IL08 (**Figure 3**), and decreased their production of pro-inflammatory reactive oxygen species (ROS) (**Figure 4**) and cytokines (TNF-α, IL-1β, and IL-6) (**Figure 5**). Furthermore, Pf decreased LPS-activated neutrophil degranulation (**Figure 6**). Collectively, we have established that Pf possesses both anti-fibrotic and anti-inflammatory properties.

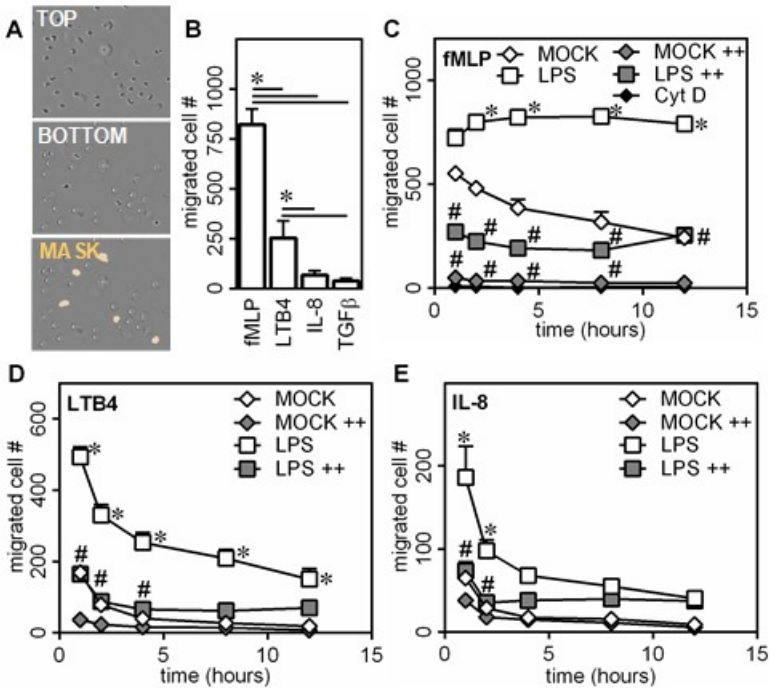

Figure 3. **Pf reduces chemotactic migration of neutrophils.** Pf/LPS treated neutrophil migration towards chemo-attractants fMLP (300nM), LTB4 (100nM), IL-8 (100ng/ml) and TGF-β (100ng/ml) were measured in a trans-well assay system by Incucyte S3 live cell imaging system. (A) Representative image with cell masks of +LPS+Pf treated neutrophils. (B) Graph representing LPS treated neutrophil migration in response to different chemotactic agents at t = 4 hours. (C-E) Graphs representing time course of migrated neutrophil number (normalized) to bottom well with chemotactic agents: fMLP (C), LTB4 (D), IL-8 (E). Data representing mean ± SEM values from 6 donors with □ and □□representing *p*-value < 0.05 (Two-way ANOVA) between groups.

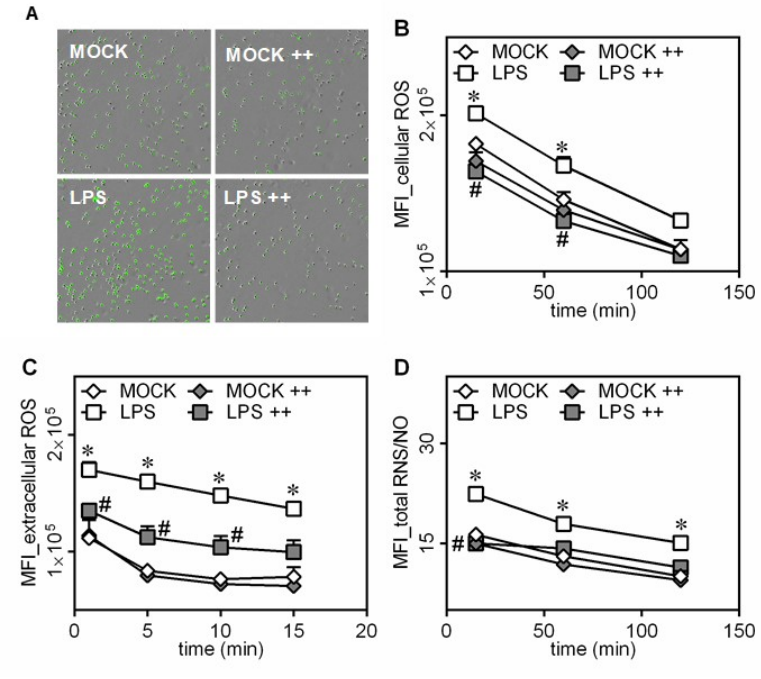

Figure 4. **Pf reduces neutrophil oxidative burst.** CellROX Green (cellular ROS), OxyBURST Green H2HFF BSA (extracellular ROS), and DAF-FM Diacetate (total RNS /NO) dyes were used to measure free radicle quantification. Cellular ROS and total RNS/NO was measured by fluorescent live cell imaging and fluorescent intensity measurements respectively, using Incucyte S3 system. Extracellular ROS was measured by fluorescent intensities of wells obtained from Cytation5 readings. (A) Representative overlay images (bright field & fluorescent) of neutrophils with ±Pf ±LPS showing cellular ROS production at 15 min post treatment. Graphs showing mean fluorescence intensity units (MFI) of cellular ROS (B), extra cellular ROS (C) and total RNS/NO (D) obtained from mean ± SEM values from 6 donors. □ and □□represent significant difference with p-value < 0.05 (Two-way ANOVA) between groups.

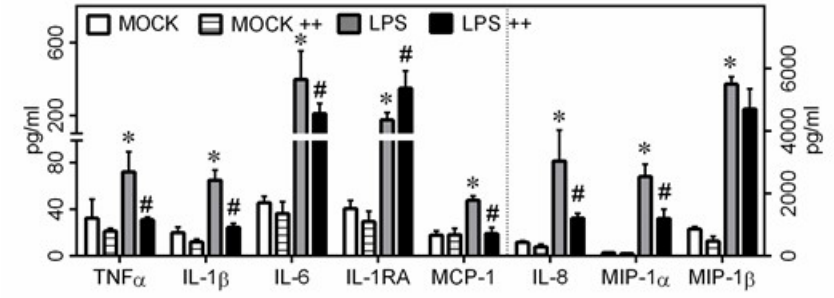

Figure 5. Pf modulates neutrophil inflammation. Neutrophils were treated with ±Pf [0.5mg/ml] ±LPS [20ng/ml] and incubation for 4 hours. Supernatants were collected and analyzed for quantity of inflammatory mediators secreted by neutrophils using Procarta multiplex assay. The graphs were plotted using data of mean ± SEM values from 6 donors with □ and □□representing p-value < 0.05 (Two-way ANOVA) between groups.

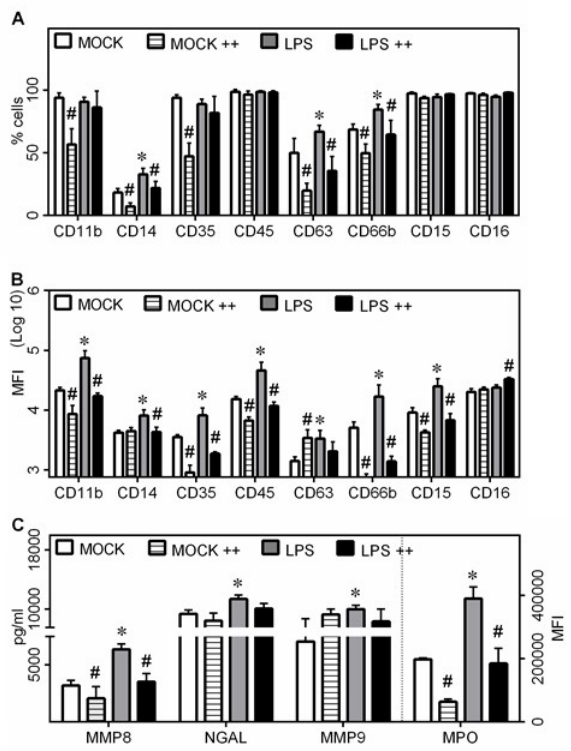

Figure 6. Pf decreases neutrophil degranulation. Neutrophils were treated with ±Pf [0.5mg/ml] ±LPS [20ng/ml] and incubation for 4 hours. Cells and supernatants were collected and analyzed for degranulation markers/proteins. (A, B) Cells were stained for degranulation markers by fluorescent antibodies and quantified by flow cytometry for % cells (A) and mean fluorescence intensity (MFI) (B). (C) Supernatants were analyzed for MPO and quantity of degranulation proteins (MMP8, MMP9, and NGAL) by fluorescent MPO assay and Procarta-Multiplex Immunoassay respectively. The graphs were plotted as mean ± SEM values from 6 donors with and # representing p value < 0.05 (Two-way ANOVA) between groups.

References:

1. Hall *et al*. 2018. Lab. Invest. 98(5):640-655. PMID: 29497173.
2. Wells AR and Leung KP. 2019. Biochem Biophys Res Commun. 2019 Oct 31. pii: S0006-291X(19)32087-X. doi: 10.1016/j.bbrc.2019.10.177. PMID: 31679692.
3. Dorati *et al*. 2018. AAPS PharmSciTech. 19:2264. PMID: 29790019
4. Medina *et al*. 2018. Int. J. Burns Trauma. 8:26. PMID: 29755839
5. Medina *et al*. 2019. Inflammation. 42(1):45-53. PMID: 30120654

Major accomplishments of Goal #3:

To accomplish the Goal #3, we have established a hypertrophic scarring model using the red Duroc DPT-burn model for testing the Pf treatment efficacy in reducing hypertrophic scarring. We have established the primary (scar thickness) and secondary endpoint (wound contraction) as the endpoints for the study. Unfortunately, we have run into contract issue for purchasing healthy red Duroc pigs. The request for a contract occurred in November 2018. Up to now (Nov 5, 2019), the purchase contract has not been finalized. The evaluation of Pf treatment efficacy in reducing hypertrophic scarring is the last study for this proposal.

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

Nothing to Report.

How were the results disseminated to communities of interest?

We intend to publish the findings in a peer-reviewed journal as a means to disseminate the results to reach the members of research communities who are interested in developing therapeutic solutions to reduce fibrosis and scarring. Recently, we have submitted 2 manuscripts describing the anti-fibrotic and anti-inflammatory effects of Pf. One of the 2 manuscripts (anti-fibrotic study) has been published in Biochemical Biophysical Rapid Communication (see below for authors and title of the submitted manuscripts).

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

We plan to do the following in the next reporting period:

1. Test Pf treatment efficacy in reducing DPT burn wound induced hypertrophic scarring in red Duroc DPT burn wound scarring model. Continue to characterize the shallow and DPT burn wounds in red Duroc pigs.

IMPACT

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

From the studies conducted, we have established that Pf possesses both anti-fibrotic and anti-inflammatory properties.

The development of the porcine burn model establishes the clinically relevant endpoints for assessing Pf treatment efficacy in reducing hypertrophic scarring.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

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

Delays in having a contract for purchasing Duroc pigs caused significant delays to study Pf treatment efficacy in reducing burn-induced hypertrophic scarring.

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

Nothing to report.

PRODUCTS

Publications, conference papers, and presentations

Journal publication.

Wells AR and **Leung KP**. Pirfenidone attenuates the profibrotic contractile phenotype of human dermal myofibroblasts. 2019. Biochem. Biophys. Res. Commun. Oct 31. pii: S0006-291X(19)32087-X. doi: 10.1016/j.bbrc.2019.10.177. PMID:31679692.

Evani SJ, Rajasekhar Karna SL, Seshu J, and **Leung KP**. Pirfenidone regulates LPS mediated hyper-activation of neutrophils. Submitted. Lab. Invest.

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

Nothing to report.

Other publications, conference papers, and presentations.

Invited speaker (Leung)
"Pirfenidone as a Candidate Preventative for Hypertrophic Scarring"
Wound Healing Society Meeting, May 7, 2019. San Antonio, TX

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Provisional Patent Application
Title: Tri-Layer Laminated Pirfenidone-Containing Film for Scar Treatment and Prevention
Filing Date: July 30, 2019
Application Number: 62880396

Other Products

Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Kai Leung
Project Role:	PI
Nearest person month worked:	3
Contribution to Project:	Dr. Leung is responsible for insuring compliance with all regulatory requirements. He has chosen the following personnel to assist him in the proposed studies because of their expertise in animal surgery and in the field of molecular biology, histology, histochemistry, and immunohistochemistry, PCR array analysis, as well as wound healing analysis.

Name:	Li-Wu Qian
Project Role:	Research Associate
Nearest person month worked:	10.8
Contribution to Project:	Dr. Qian is responsible for the animal surgical procedures and will plan and execute the animal model required for this proposed research. He will be responsible for the animals for insuring compliance with all regulatory requirements for this project and our institution.

Name:	Andrea Fourcaudot
Project Role:	Research Associate
Nearest person month worked:	9.6
Contribution to Project:	Ms. Fourcaudot assists in the development of the porcine DPT burn wound model. She also does the tissue processing and sectioning.

Name:	Kishan Evani
Project Role:	Post-Doctoral Fellow
Nearest person month worked:	9.6
Contribution to Project:	Dr. Evani works on the anti-inflammatory properties of Pf.

Name:	Adrienne Wells
Project Role:	Post-Doctoral Fellow
Nearest person month worked:	9.6
Contribution to Project:	Dr. Wells works on the anti-fibrotic properties of Pf.

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

Nothing to report.

What other organizations were involved as partners?

Not applicable

Provide the following information for each partnership:

Organization Name:

Not applicable.

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

SPECIAL REPORTING REQUIREMENTS

Quad Chart: Attached.

APPENDICES

Quad Chart is included.