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TITLE: Molecular Targets in Endovascular Progenitors to Inhibit Lung and Skin Fibrosis in Scleroderma

PRINCIPAL INVESTIGATOR: Prof. Kiarash Khosrotehrani

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6. AUTHOR(S)

Prof. Kiarash Khosrotehrani

E-Mail: k.khosrotehrani@uq.edu.au

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

The University of Queensland
Diamantina Institute, Level 6,Translational Research Instiute,37 Kent Street,
Woolloongabba QLD 4102, Australia

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14. ABSTRACT

In this report we have provided updates on major works 1 and 2. Major work 3 as per schedule in SOW has not started.
We provide update on the set up of the project, on establisbing the models of scleroderma and pulmonary fibrosis.
All animal studies and animal work has been initiated as scheduled to track the fate of endothelial cells in situations of fibrosis and to examine the role of Sox9 in modulating skin and lung fibrosis.

15. SUBJECT TERMS
Endothelial cells, endothelial vascular progenitors (EVPs), systemic sclerosis (SSc),

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1. Introduction

This proposal addresses the FY19 Topic Areas of Scleroderma and Pulmonary fibrosis where we investigate molecular mechanisms and pathogenesis of scleroderma and pulmonary fibrosis, including the identification of novel and/or innovative therapeutic targets. Systemic sclerosis (SSc) and idiopathic pulmonary fibrosis are devastating diseases often resulting in mortality through loss of pulmonary function and a major reduction in quality of life through the progression of fibrosis across the skin and internal organs. Recent years have witnessed a renewed understanding of fibrotic mechanisms through endothelial to mesenchymal transition (EndMT). This might be extremely relevant to SSc as endothelial damage seems to be the initiating factor. Although there are indications that EndMT might be important in SSc, to date there is no definitive proof. The level of evidence required is best delivered by fate tracing experiments where endothelial cells in SSc scenarios are labelled genetically and followed over time to show the transition to myofibroblasts. Our project will demonstrate the importance of EndMT in SSc skin and lung fibrosis, identify a new target for therapy and propose a clinical translation solution. It therefore brings the following innovations to our understanding of SSc and pulmonary fibrosis. 1) We will for the first time show the importance of EndMT in SSc fibrosis. Furthermore, we will demonstrate that endothelial progenitors (EVPs) modulate their fate choice through the transcription factor Sox9. 2) Our study will demonstrate a paradigm shift in the therapy of SSc. Unlike current therapy treatment will not focus on immunosuppression or pulmonary hypertension. The proposed therapy in our project will shift to directly address the pathophysiology of the disease, EndMT. 3) If successful, our study will show that Sox9 is a target for therapy in skin and lung fibrosis. Targeting Sox9 will reduce EndMT and fibrosis and will be beneficial in SSc and pulmonary fibrosis. This novel therapeutic target has never been described before as opposed to TGFbeta or Wnt pathway inhibitors. 4) The possibility of endothelial-specific drug delivery is novel and is exploited here to translate our findings in a GMP compatible therapy for clinical trials. Indeed, microvesicles loaded with Sox9 siRNA if successful will allow a reduction of EndMT and fibrosis. Local intratracheal delivery is another innovative route with the potential to significantly reduce the toxicity of the treatment. Overall, we believe our proposal will open a new era of investigation and therapeutic innovation in SSc. It will validate a new mechanism of disease (EndMT), a new molecular target and deliver innovative solutions for a clinical trial.

2. KEYWORDS:

Endothelial cells, endothelial vascular progenitors (EVPs), systemic sclerosis (SSc), scleroderma, fibrosis, endothelial to mesenchymal transition (EndMT), Sox9, RBPJ, bleomycin.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

This project has three specific aims as following:

Aim 1: In fate tracing experiments, demonstrate endothelial to mesenchymal transition in endothelial cells in a bleomycin induced model of scleroderma. Rationale: Fate tracing experiments have shown in a variety of situations that endothelial cells undergo EndMT and contribute to fibrosis. There is ample indirect evidence that SSc and pulmonary fibrosis develop through EndMT, however a proper fate tracing experiment has never been conducted. Therefore, in this aim we develop a model of bleomycin induced skin and lung fibrosis and determine if endothelial cells undergo mesenchymal transition.

Aim 2: To examine the level of lung and skin fibrosis in mice with conditional and inducible deletion of Sox9 gene in the endothelium as compared to controls. Rationale: We have shown our preliminary data and previous publications that fibrosis can be modulated by Sox9 and RBPJ where depleting Sox9 from endothelium reduces fibrosis by allowing the EVPs to take endothelial fate and knocking out RPBJ increases fibrosis by allowing the EVPs to take mesenchymal fate. Therefore, in this aim we will now determine if EndMT during bleomycin induced fibrosis can be modulated by Sox9 and RBPJ.

Aim 3: To deliver an endothelial targeted systemic therapy using Sox9 siRNA to improve lung and skin fibrosis. Rationale: Sox9 is a gene involved in a variety of stem cell processes in the gut, brain, skin, cartilage etc. Although our findings point towards Sox9 as a target for therapy, it is likely that Sox9 inhibition has to be specific to the endothelium to alleviate side effects. Moreover, siRNA treatment of Sox9 is effective in reducing wound fibrosis but would be a large cost if delivered systemically and by restricting the delivery to the endothelium it is assumed that one can reduce both cost and toxicity of therapy. We have developed microvesicles from nanoemulsion that are coated with anti-VEcadherin antibodies. These vesicles in preliminary findings allow the targeting of the endothelium for drug delivery. In this aim we will deliver Sox9 siRNA to reduce bleomycin induced fibrosis.

What was accomplished under these goals?

Aim1:

Specific activities and objective: We have performed following experiments to determine if EndMT occurs in the bleomycin induced model of skin and lung fibrosis.

Adult and mix sex Cdh5Cre/ER ROSA-lsl-YFP mice were administrated with tamoxifen to genetically label all endothelial cells. Animals were then injected subcutaneously with 0.01 units of bleomycin five days per week, for a total of four weeks into a 1cm^2 patch of skin on the back to generate skin fibrosis (Figure 1A). A control group was injected with saline and after the final injection, skin was harvested from the mice. To model the pulmonary fibrosis seen in systemic sclerosis patients, we delivered 0.5mg of bleomycin sulfate or saline intranasally to Cdh5Cre/ER ROSA-lsl-YFP mice. After two weeks, lungs were collected from both bleomycin and saline treated control mice (Figure 3A). Both the skin and lungs were stained with hematoxylin and eosin (H+E), Masson Trichrome or Sirius red staining in order to determine histological changes and the level of skin and lung fibrosis. The tissues were also examined for EndMT by co-staining of YFP+ cells with endothelial (CD31) and mesenchymal markers (α-sma).

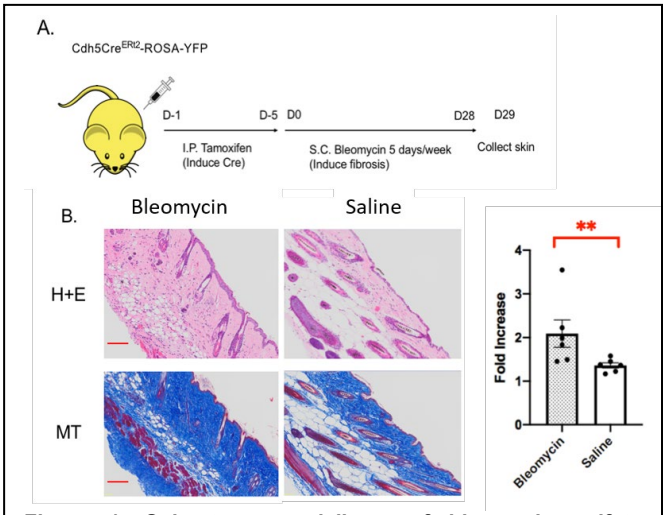

Figure 1. Subcutaneous delivery of bleomycin sulfate causes skin fibrosis. (A) Experimental design for inducing skin fibrosis using bleomycin. **(B)** Representative skin histology (H&E and Masson's Trichorme blue to show collagen deposition) and graph represents quantification of dermal thickness of bleomycin-treated mice compared to control mice treated with saline (**p<0.001, Welch's T-test, n=6). Scale bars are equal to 100μm. The graph is ± SEM.

Key Outcomes: Thickening of the dermal layer and a displacement of the hypodermis in the skin, consistent with a fibrotic phenotype was observed in bleomycin treated mice as compared to saline treated control mice. The fold increase in dermal thickness for the bleomycin treated mice was significantly higher than that of the saline treated mice (p<0.001 Welch's T-test), indicating the model successfully generates skin fibrosis (Figure 1C). To assess the presence of endothelial to mesenchymal

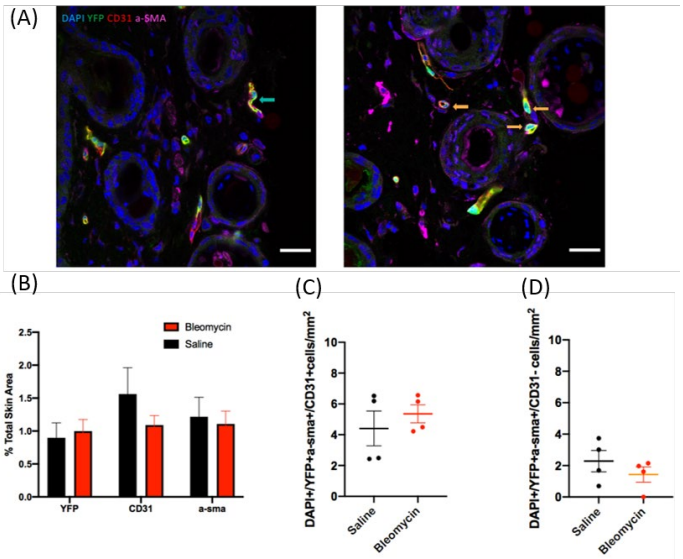

Figure 2. Endothelial to Mesenchymal transition (EndMT) in the skin of bleomycin treated mice. (A) Representative images of skin showing using mesenchymal and endothelial markers to identify EndMT. Orange arrows show DAPI$^+$YFP$^+$a-SMA$^+$CD31$^+$ cells and green arrows show DAPI$^+$YFP$^+$a-SMA$^+$CD31^{neg} cells. (B) Quantification of % of YFP$^+$ (VE-Cadherin), CD31 and a-smooth muscle actin (a-SMA) in the skin of saline and bleomycin treated mice. Numeration of (D) YFP$^+$a-SMA$^+$CD31$^+$ transitory cells and (E) YFP$^+$/a-SMA$^+$CD31^{neg} fully transitioned cells. Scale bars are 25 μm. Data was analyzed using Welch's t-test. All graphs are ± SEM.

transition (EndMT) or change in vasculature, bleomycin-treated skin sections and saline-treated skin sections were stained with anti-GFP (to boost our YFP endothelial trace for VE-Cadherin), anti-CD31 to show endothelial cells and anti-a-SMA to visualize mesenchymal cells (Figure 2A). There was no difference in the total coverage of YFP, CD31 or α-sma (Figure 2B) and there were no significant differences in EndMT (both transitory cells; DAPI^{+}YFP^{+}a-SMA^{+}CD31^{+} and fully transitioned cells; DAPI^{+}YFP^{+}a-SMA^{+}CD31^{neg}) (Figure 2C and D). This indicates that EndMT has either not occurred at this point or it was transitory and has resolved by this time point of bleomycin induced skin fibrosis.

Similarly, pulmonary fibrosis was achieved in the lungs with bleomycin as demonstrated by significant decrease in air space (Figure 3B) and a significant increase in collagen coverage in the lungs (Figure 3C). No

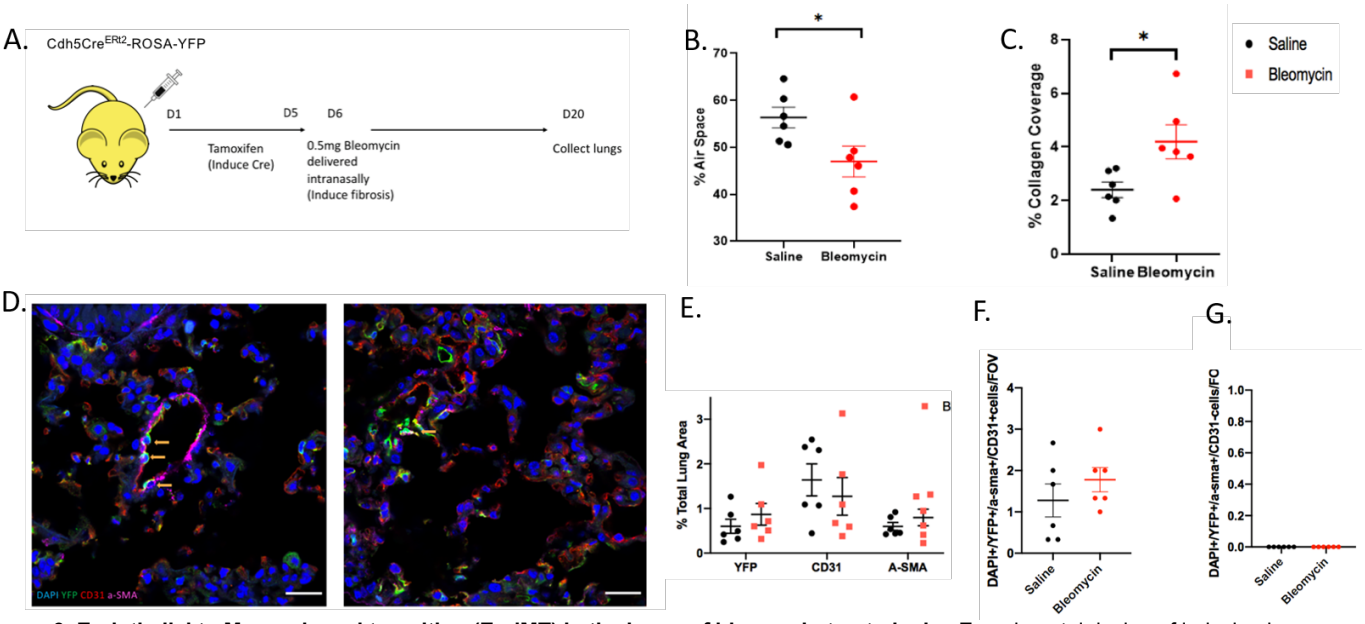

Figure 3 Endothelial to Mesenchymal transition (EndMT) in the lungs of bleomycin treated mice Experimental design of inducing lung fibrosis with bleomycin. Quantification of (B) percentage of air space and (C) collagen coverage in saline and bleomycin treated mice. (D) Representative images of lung section stained with CD31, α-SMA, YFP (VE-Cadherin) and a nuclear stain (DAPI). (E) Quantification of % of YFP^{+} (VE-Cadherin), CD31 and a-smooth muscle actin (a-SMA) in the lungs of saline and bleomycin treated mice. Numeration of (D) YFP^{+}a-SMA^{+}CD31^{+} transitory cells and (E) YFP^{+}/a-SMA^{+}CD31^{neg} fully transitioned cells. Scale bars are equal to 50μm. Data was analyzed using Welch's t test (n=6, *=p<0.05). All graphs are ± SEM.

change in % of CD31^{+}, YFP^{+} or α-sma was seen in bleomycin treated mice (Figure 3D and E) or number of YFP^{+}α-SMA^{+}CD31^{+} (orange arrows) transitory cells (Figure 3D and F). No YFP^{+}α-SMA^{+}CD31^{neg} fully transitioned cells undergone EndMT were seen in the lungs of either saline or bleomycin treated mice (Figure 3D and G) . Overall, it appears that EndMT is not a main driver of fibrosis in this model of lung fibrosis. Thus, this model may not be representative of a true systemic sclerosis phenotype as EndMT was not observed in this model.

Aim2:

Specific activities and objective: To examine if level of fibrosis can be modulated by conditionally depleting Sox 9 from endothelium. Cdh5Cre/ER ROSA-lsl-YFP Sox9lox/lox mice and control Cdh5Cre/ER ROSA-lsl-YFP were injected with tamoxifen to label and deplete Sox9 from endothelial cells. The mice were treated with bleomycin to generate skin and lung fibrosis as described in aim 1 (Figure 4A and 5A). The skin and lungs were collected to measure the degree of fibrosis by trichrome blue and Sirius red staining. We also performed immunofluorescence staining to identify YFP+ cells co expressing endothelial and/or mesenchymal markers.

Key Outcomes: Knocking out Sox9 from the endothelium did not change the dermal thickness of bleomycin induced fibrotic skin (Figure 4B and C). The lungs from Sox9KO bleomycin treated mice still had similar levels of fibrosis as CDh5 bleomycin treated mice which was reflected by no changes in collagen coverage or air space of lungs (Figure 5B-D). Infact, Sox9 KO bleomycin treated mice had poor survival rate as compared to CDh5 bleomycin treated mice (data not shown). It is possible that bleomycin delivered intranasal might be having more systemic effect which is aggravated in Sox9KO mice. Further experiments to determine change in vasculature or if there is any EndMT still needs to be performed.

Overall, bleomycin has not been able to promote EndMT in endothelial cells at the selected time point and is very likely not the right model to study EndMT which has been reported in patients with scleroderma. However, all the above experiments

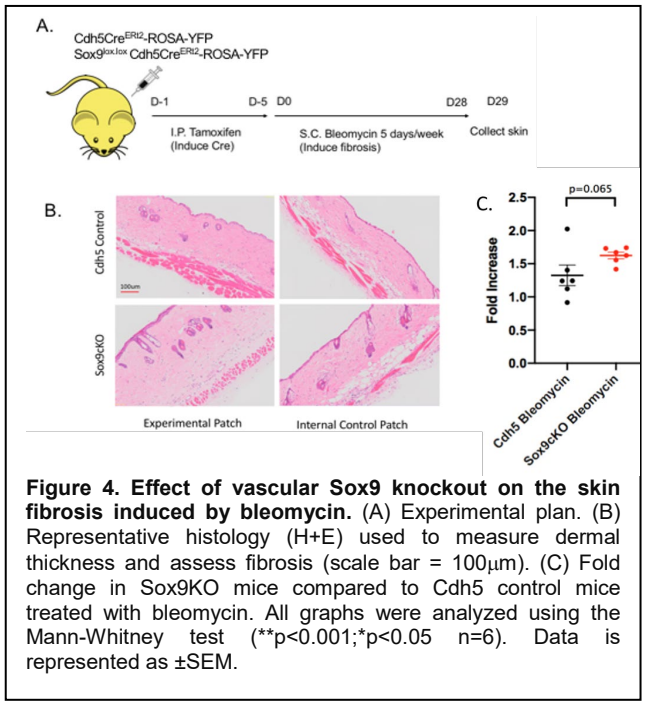

Figure 4. Effect of vascular Sox9 knockout on the skin fibrosis induced by bleomycin. (A) Experimental plan. (B) Representative histology (H+E) used to measure dermal thickness and assess fibrosis (scale bar = 100μm). (C) Fold change in Sox9KO mice compared to Cdh5 control mice treated with bleomycin. All graphs were analyzed using the Mann-Whitney test (**p<0.001;*p<0.05 n=6). Data is represented as ±SEM.

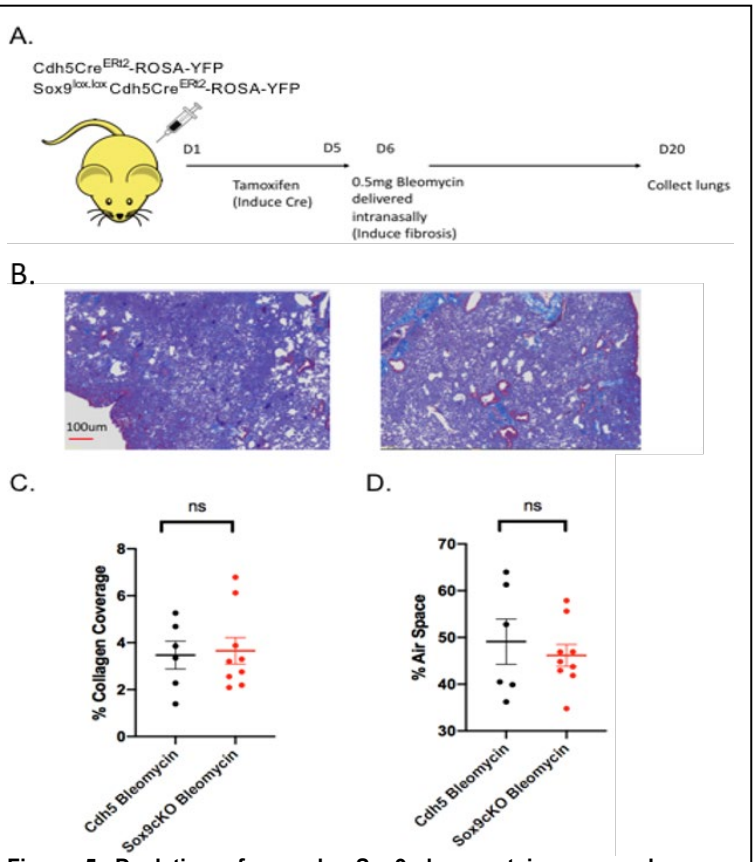

Figure 5. Depletion of vascular Sox9 does not improve pulmonary fibrosis induced by bleomycin. (A) Experimental design to induce lung fibrosis by injecting bleomycin. (B) Representative histology (Masson's Trichrome stain) used to analyze collagen coverage. Quantification of percentage of (C) collagen coverage and (D) air space in bleomycin and saline treated mice. Data is represented as ± SEM and was analyzed using Welch's T-test (n=6).

will be performed at early time points to determine if EndMT was transitory and has been resolved by later time point.

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

In addition to the post-doctoral fellow position, a PhD student has joined the team to manage and move forward the project and the key tasks include designing and performing the experiments, data analysis and interpretation, manuscript writing and data presentation at the conferences. This is enabling the student to learn various research, critical thinking, project management, leadership and written and oral communication skills which is allowing them to develop both academically and professionally.

How were the results disseminated to communities of interest?

The data from the project has been presented orally at Australian society for Dermatology Research conference in November 2020 by the PhD student who also won **the best oral presentation award**. We will continue to disseminate the outputs of the project to various future dermatology conferences along with publication of manuscript as and outcome of this project.

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

Early time points from Aim 1 and Aim 2 will be performed to determine if EndMT is transitory and resolves by D29 and D20 of skin and lung fibrosis, respectively. In aim 2, experiments will be performed on Cdh5Cre/ER ROSA-lsl-YFP RBPJ+/+ model which will act as a positive control of EndMT. We will also perform experiments from Aim3 determine if Sox9 siRNA can be used as endothelial targeted systemic therapy to improve lung and skin fibrosis.

4. IMPACT:

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

  Nothing to report

- **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.

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**

  Delays were incurred due to access restriction related to COVID lock-down at the start of the project. However at this stage we do not envision any change in the investigation plan.

- **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.

- **Significant changes in use or care of human subjects**

  Nothing to report.

- **Significant changes in use or care of vertebrate animals**

  Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

  Nothing to report.

6. **PRODUCTS:**

- **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 –** Oral Presentation by PhD student at the Australian Society of Dermatology Research, November, 2020. No published abstract
- **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:	*Prof Kiarash Khosrotehrani*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0002-6406-4076
Nearest person month worked:	1.8 months (15% FTE)
Contribution to Project:	*Prof Khosrotehrani has obtained all approvals for the project in terms of health and safety and ethics. Has established the accounting and management details.* *Has hired the personnel and instructed on the initiation of the research plan as per the approved project.* *Has supervised the progress of the project, examined results and interpreted findings for Major tasks 1 and 2 as per SOW*
Funding Support:	The National Health and Medical Research council of Australia, Career development fellowship 1125290

Name:	Prof Ranjeny Thomas
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	0002-0518-8386
Nearest person month worked:	1.2

Contribution to Project:	Contributed to establishing models of scleroderma (major task 1, subtask 2) Has helped examine results and interpret data
Funding Support:	The National Health and Medical Research council of Australia

Name:	Dr Meghna Talekar
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-6450-9055
Nearest person month worked:	0.3
Contribution to Project:	Dr Talekar has simply contributed in the study design and her main contribution is expected in the second year. As per SOW this is major task 3 to be started from 12 months
Funding Support:	The National Health and Medical Research council of Australia

Name:	Dr Edwige Roy
Project Role:	Post-doctoral research officer
Researcher Identifier (e.g. ORCID ID):	0000-0003-1991-5844
Nearest person month worked:	12
Contribution to Project:	Dr. Edwige Roy has contributed to all experimental parts of the project and has provided assistance in designing and conducting experiments and data analysis.
Funding Support:	DOD and completed by the National Health and Medical Research council of Australia

- **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?**

  Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** Nothing to report
   - **QUAD CHARTS:** Nothing to report
9. **APPENDICES:** None

ADDITIONAL NOTES:

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