# The Control of Protein Production Related to Lung Fibrosis

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## Abstract

Pulmonary lung fibrosis is a serious disease that may be caused by many particulate or fiber exposures, including exposure to asbestos. Thus far, there has not been a treatment discovered that completely cures the disease, therefore it is necessary we find one rather than merely using treatments to improve the quality of life. Previously, our lab identified SPARC, a matricellular protein involved in tissue repair, extracellular matrix (ECM) regulation, cellular proliferation, and cellular adhesion, as a candidate for involvement in the fibrosis that occurs after asbestos exposure. Through our studies with wild type and SPARC knockout mice we found that expression of both SPARC and collagen (a major component of the ECM) is increased in asbestos treated mice compared to control and that SPARC knockout mice did not produce as much collagen in response to asbestos. Studies this summer were designed to see if we could control the expression of SPARC after asbestos exposure with RNA interference (RNAi) and thus decrease the amount of collagen produced. As a first step in these studies, we packaged the sequences coding against SPARC into lentivirus, which was then transduced into C57Bl/6 primary mouse lung fibroblast cells. We then tested for SPARC and collagen production by western blot protein assay.

## Introduction

Millions of people around the world are affected by pulmonary lung fibrosis. The number of people who have this disease as well as the severity of it both contribute to the importance of finding a cure. Many people who have this disease have been exposed to asbestos through inhalation as shipyard workers, miners, and many other jobs. Because asbestos is trapped in the lungs after inhalation, cells react by producing excess collagen, which causes a buildup of scar tissue. Normally collagen is made and turned over as needed, but when it is trapped in the lungs, it does not degrade and therefore causes collagen production to accumulate beyond what is necessary. With all this excess collagen, scar tissue forms and causes negative physiological effects such as shortened breath, fatigue, discomfort, weight loss, and shortened life expectancy.

There has been a protein found that is linked to the production of collagen. This protein, SPARC, is found in tissue areas with high cellular turnover, or in wound or disease sites. It is found specifically in the extracellular matrix (ECM). Because lung fibroblast cells produce collagen, SPARC is also found in lung tissue.

RNAi is a method that works to silence the expression of a specific gene. This technique holds a promise in targeting and curing many diseases that thus far do not have a treatment. RNAi works when a specific sequence of RNA is introduced into the cell as double stranded RNA then cleaved by a cytoplasmic ribonuclease called DICER. DICER cleaves the RNA into smaller strands called small interfering RNA (siRNA). These siRNA unwind inside of an RNA-induced silencing complex (RISC). One of the two strands is then used to recognize a complimentary mRNA. When the complimentary mRNA strand is found and matched with the siRNA, it leaves the RISC and degrades therefore preventing the translation of that specific protein. In our experiment, we used short hairpin RNA (shRNA) that have a short (5-10 bp) loop at one end. When the shRNA enter the cell, they are cleaved at the loop by DICER and then continue on to the RISC as siRNA.

We introduced the shRNA coding against SPARC into the cell by way of a viral vector. We used a lentivirus, which is unique in the fact that it integrates itself into the host cells' genome. This is useful for long-term expression (or knockdown) of a gene. The shRNA sequences were contained in pre-designed plasmid DNA within bacterial stocks. By growing up those bacterial stocks and isolating the plasmid DNA, we were able to package the sequence into lentivirus vectors. The plasmid DNA in addition to a ViraPower packaging mix was transfected to a human embryonic kidney 293T cell line where the virus was produced. We then harvested the virus and transduced it to the cells to allow for a protein assay to determine the amount of SPARC and collagen produced thereafter.

The protein assay used is known as a western blot. A western blot is a technique used to determine the amount of protein in a certain sample. We first use a Bradford assay to quantify the protein samples. The first part in a western blot involves gel electrophoresis where the samples are separated by molecular mass. The acrylamide in the gels serves as a sieve that separates the proteins as they move towards the positive end of the gel by size (measured in kilodaltons, kDa). Then the proteins are moved from the gel to a membrane that makes them accessible to antibody detection. Then through chemiluminescent detection, protein is analyzed and quantified.

## Experimental Design

• **Lung fibroblast cells:** cells were isolated from C57Bl/6 wild type mice and cultured in complete DMEM in cell culture flasks at 37°C and 5% $CO_2$.

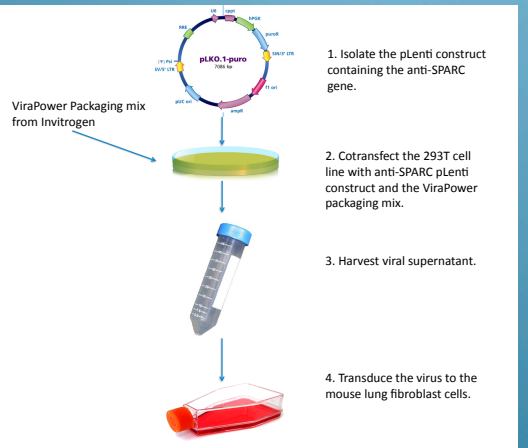
• **Lentivirus generation:** Bacterial stocks containing plasmids encoding SPARC siRNA sequences were grown up overnight then plasmid DNA isolated following the MidiPrep protocol from Qiagen. The lentivirus containing SPARC siRNA was made following the ViraPower Lentiviral Expression Systems protocol from Invitrogen (production of lentivirus figure).

• **Asbestos:** Crocidolite asbestos was weighed, sterilized by exposure to UV light then suspended in PBS and triturated through a a 22g needle before addition to cell culture.

• **Lung fibroblast cell treatment**: Confluent fibroblast cell cultures were exposed to crocidolite asbestos at a concentration of 5 ug/cm² for 12 hours. After washing, the media were replaced with media to which lentivirus was added. 18 hours later, the virus-containing media were removed and the cells grown for a further 24 hours before protein was isolated.

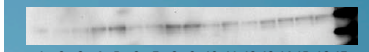
• **Western Blotting:** Using 4-12% BisTris Criterion gels, the protein samples were separated by electrophoresis according to size. They were then transferred to a PVDF membrane where we were able to compare SPARC expression using chemiluminescence.

## Production of Lentivirus




## Western Results

SPARC




Collagen


| Lane # | Sample | SPARC | Collagen |
|---|---|---|---|
| 1 | N Control | 1.0 | 1.0 |
| 2 | N + Asbestos | 1.2 | 0.7 |
| 3 | N + Asbestos + 350 hi | 2.1 | 0.9 |
| 4 | N + 350 hi | 5.2 | 1.0 |
| 5 | N + 350 low | 8.2 | lost |
| 6 | N + Asbestos + 350 low | 2.5 | 0.6 |
| 7 | N + Asbestos + 349 low | 4.3 | 1.1 |
| 8 | N + 349 low | 10.7 | 1.5 |
| 9 | N + 349 high | 9.1 | 1.4 |
| 10 | N + Asbestos + 349 high | 2.5 | 1.5 |
| 11 | O Control | 1.0 | 1.0 |
| 12 | O + Asbestos | 0.5 | 1.0 |
| 13 | O + Asbestos + 349 high | 0.98 | 1.3 |
| 14 | O + 349 high | 1.0 | 0.6 |
| 15 | O + 349 low | 1.1 | 0.8 |
| 16 | O + Asbestos + 349 low | 1.6 | 1.1 |
| 17 | MW marker | | |

Table 1. Density values were normalized to appropriate control values.

## SPARC-specific siRNA sequences

| MISSION™ TRC No. | TRC shRNA Target Set Sequence |
|---|---|
| TRCN0000080349 | CCGGCCTAGACAACGACAAGTACATCTCGAGATGTACTTGTCGTTGTCTAGGTTTTTG |
| TRCN0000080350 | CCGGGAAGGTATGCAGCAATGACAACTCGAGTTGTCATTGCTGCATACCTTCTTTTTG |

## Results

• We did not obtain the expected results. It appears that the fibroblasts reacted to the virus transduction by increasing SPARC protein production in the less confluent cell cultures.
• In general, collagen levels were less responsive to virus than SPARC levels.
• As always, there were exceptions. 349 virus induced collagen production in less confluent cultures, but not in more confluent cultures.

## Accomplishments

- Learned sterile techniques and successfully established primary lung fibroblast cultures from C57Bl/6 mice.
- Learned bacterial culture techniques and isolated high quality plasmid DNA.
- Successfully transfected plasmid DNA into 293T mammalian cells and harvested lentivirus from the media.
- Transduced primary mouse lung fibroblasts with lentivirus.
- Learned to harvest and quantify protein.
- Learned to perform protein electrophoresis and western transfer.
- Learned data analysis and the patience required for scientific research.

## Conclusions

• Experiments should be done more than once since it is difficult to come to a conclusion based on one experiment.
• The virus appeared to induce SPARC protein production instead of inhibiting it.
• Virus titers should be verified before use in experiments.
• Culture conditions should be consistent with previous experiments in order to properly replicate them.

## Future Directions

• Quantify the lentivirus.
• Include western blot analysis on beta actin to act as a loading control.
• This work can be used to further study the results of SPARC RNA interference in vivo. A safe and effective delivery system for human clinical trials can be researched and designed to enable successful treatment for pulmonary lung fibrosis.

## Acknowledgements

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