

## siRNA-Mediated Inhibition of Thrombospondin 2 Gene Expression

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**Abstract:** *Thrombospondin 2 (TSP2) is a matricellular protein that inhibits angiogenesis in response to wound healing and upon implantation of a biomaterial in the body. TSP2 down-regulation by antisense cDNA has been demonstrated to increase local angiogenesis and offers the potential to increase the efficiency and life span of implanted materials and devices. In this work, siRNA (small interfering RNA)-based down-regulation of TSP2 was investigated as an alternative and possibly more potent approach for TSP2 knockdown. RNA interference (RNAi) is an effective approach to sequence-specific gene knockdown, and the delivery of siRNA into cells has been shown to significantly alter protein levels. DNA sequences encoding TSP2 siRNA were inserted into a plasmid for in vitro delivery, and TSP2 protein expression was evaluated by real-time PCR.*

### 1. INTRODUCTION

The ability to inhibit gene expression through the cell's own mechanisms is a compelling technique for specific gene targeting. RNA interference (RNAi) is considered, to date, to be the most potent approach to accomplish this sequence-specific knockdown of expression [10]. The delivery of siRNA (small interfering RNA) into cells *in vitro* has been shown to clearly inhibit gene expression; however, the *in vivo* delivery of siRNA is limited by several problems such as poor stability, low cellular uptake, and toxicity. Overcoming these barriers will be important in advancing this technique for use in medical applications.

Current barriers to siRNA delivery include poor cell uptake as well as the rapid rate of siRNA degradation. More specifically, nonviral siRNA delivery faces obstacles such as inadequate cell membrane penetration, endosomal escape, and unpackaging of the nucleic acid before it reaches its intended target site. To overcome these barriers, systems using or mimicking viral approaches of inserting genetic material into cells have been explored. Viral vectors have, as yet, been unmatched in delivery efficiency; however they cause unwanted immunogenic side effects which prevent prevalent use of these viral systems for clinical applications. Non-viral delivery systems have proven to be less immunogenic, but their efficiency needs to be improved before they can be actively pursued for therapeutic applications in humans.

Thrombospondin 2 is an extracellular matrix (ECM) protein shown to affect the inhibition of angiogenesis during wound healing by

influencing the migration and proliferation of endothelial cells during the granulation tissue stage of wound healing [3]. Current research exploring therapeutic uses of TSP2 include the suppression of tumor growth [9], the restoration of cardiac function after infarction [4], and wound healing applications [1]. TSP2 down-regulation by antisense cDNA has been demonstrated to increase local angiogenesis and offers the potential to increase the efficiency and life span of implanted materials and devices [7]. Existing methods for implanted sensors have faced many obstacles in long-term efficiency. Constant interaction with the surrounding tissue is essential to maintaining a clear reading of the targeted molecule. However, integrating foreign materials into the body is no easy feat as the human body is a worthy foe to foreign invaders. The key is to outsmart the host's defense systems by either making it recognize the foreign material as its own or to shut down molecular pathways that lead to the destruction or rejection of the material. Biomaterial implants, in particular, elicit a specific foreign body response that forms a fibrous, avascular capsule around the implant which limits interactions with the surrounding tissue [2].

The goal of this work was to develop a method to down-regulate TSP2 gene expression. Our approach was to incorporate a DNA sequence into a plasmid that is processed intracellularly. Advantages of plasmid-based delivery of interfering RNA include higher stability of the DNA materials and the ability to amplify and produce siRNA *in situ*.

## 2. MATERIALS AND METHODS

### 2.1 siRNA Delivery to HeLa Cells

A 21-nt duplexed siRNA sequence designed against GFP (Green Fluorescent Protein) and a scrambled (control) sequence of the GFP siRNA were purchased from IDT. HeLa cells were purchased from ATCC and HeLa cells that stably express GFP were donated by Mark Davis (Caltech). Both cell lines were maintained in Modified Eagle Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% AbAm (Gibco). Transfections were performed by plating 40000 cells/well in 24-well plates 24 h before transfection. Prior to transfection cells were rinsed twice with phosphate buffered saline (PBS). Oligofectamine (Invitrogen) – DNA complexes were formed by mixing equal volumes of DNA solution (2 µg /well) with Oligofectamine. Complexes were incubated at room temperature for 20 min before adding them to 200 µl of OptiMEM (Invitrogen). The complexes were then added to the wells. The cells were incubated for 5 h and then the cells were rinsed twice with PBS and the media was replaced with 500 µl of fresh MEM. The cells were rinsed with PBS and the media changed again 24 h post-transfection. The fluorescence of cell lysates was measured with a plate-reader at 24, 48, 72, and 96 h post-transfection and normalized to protein content as measured by BCA (Pierce).

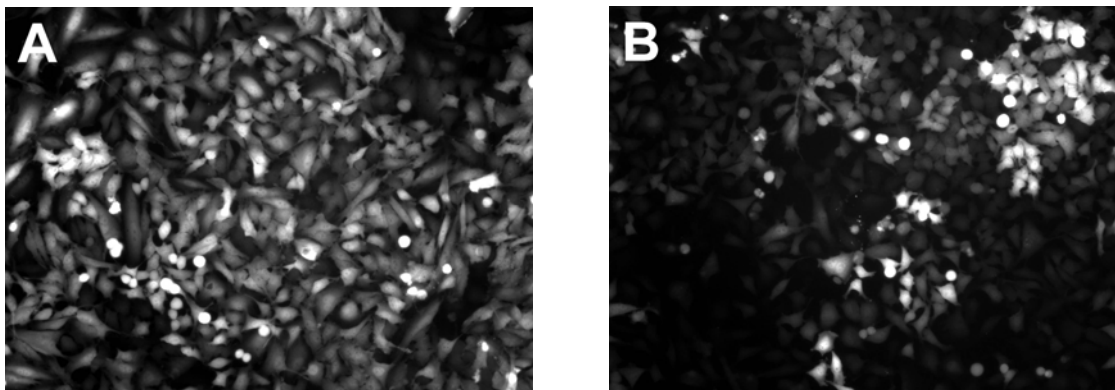
### 2.2 TSP2 siRNA DNA Plasmid Synthesis

The two TSP2 siRNA target sequences were determined by Tom Robey and Azin Agah (named TSP2 plasmid 1 and TSP2 plasmid 2, respectively). 63-nt DNA sequences that encode for the TSP2 siRNA used in this experiment were generated using Ambion's Insert Design Tool for its pSilencer vectors. The TSP2 plasmid sequences are as follows, Plasmid 1: sense 5'-GATCCGCATTAACCGGAAGACCATCTTC AAGAGAGATGGTCTTCCGGTTAATGTTTT TTGAAA-3' and antisense 5'-AGCTTTTCCAAAA AACATTAACCGGAAGACCATC TCTCTGAAGATGGTCTTCCGGTTAATG CG-3' and Plasmid 2: sense 5'-GATCCACACACCAGGTCACGTGAATTCA AGAGATTCACGTGACCTGGTGTGTTTTT

TGAAA-3' and antisense 5'-AGCTTTTCCAAAAACACACCAGGTCA CGTGAATCTCTTGAATTCACGTGACCTGG TGTGT G-3'. The sense and antisense DNA oligonucleotides were annealed and the resulting dsDNA was inserted into the BamHI-HindIII site in the pSilencer 2.1-U6 neo vector kit (Ambion). A pSilencer vector with no insert and a pUC19 vector with the TSP2 dsDNA inserts were used as negative and positive controls, respectively. *Escherichia coli* DH5α competent cells were transformed with the plasmids. For each cloned plasmid, a single colony from a freshly streaked selective plate was picked and inoculated in a starter culture of 5 ml LB medium containing an ampicillin antibiotic selective marker and incubated at 37°C for 8 h with vigorous shaking. Bacterial cells were harvested by centrifugation at 6000xg for 15 min 4°C. A DNA maxiprep was run on the harvested bacterial cells using Qiagen's Maxiprep Kit. Isolated DNA plasmids were digested with BAMH I and Hind III to confirm that the template insert was present. DNA sequencing was also run on the resulting plasmids to confirm sequence accuracy. Restriction enzyme digestion was performed on the plasmids and the resulting fragments run on a 3% agarose gel.

### 2.3 Fibroblast Transfection with Plasmids

NIH 3T3 mouse fibroblasts purchased from ATCC were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 1% AbAm (Gibco). The synthesized TSP2 siRNA DNA plasmids and a pEGFP-C1 plasmid (ClonTech) were used for the fibroblast transfections. DNA plasmids (1 µg/well) were mixed with an equal volume of Lipofectamine transfection reagent (Invitrogen) and incubated at room temperature for 25 min. Immediately prior to transfection, cells were washed twice with PBS. DMEM was added to lipoplexes to bring total volume to 1 ml. PBS was aspirated and 1 ml of lipoplex solution was added to each well. The cells were incubated for 5 h and then the cells were rinsed twice with PBS and the media was replaced with 1 ml of fresh DMEM. The cells were rinsed with PBS and the media changed again 24 h post-transfection.



**Figure 1** GFP (Green Fluorescent Protein)-expressing HeLa cells were transfected with an siRNA sequence designed to inhibit GFP protein production. Images taken at 72 h post transfection. (A) Cells transfected with a scrambled siRNA sequence. (B) Successful siRNA transfection of a GFP-specific sequence shows a decrease in fluorescence compared to cells transfected with the scrambled siRNA sequence.

#### 2.4 Quantitative Real-Time PCR (qPCR) Analysis

mRNA was collected from transfected cells 48 h after transfection using the RNeasy Mini Kit (Qiagen). The collected mRNA was converted to cDNA using Omniscript Reverse Transcriptase (Qiagen). A TaqMan Gene Expression Assay (Applied Biosystems) for the detection of TSP2 mRNA sequences was used to quantify the amount of TSP2 mRNA produced by the fibroblasts. A mouse GAPDH TaqMan assay was used to normalize for differences in mRNA collection. The qPCR was run in 96-well plates on an Applied Biosystems 7300 Real-Time PCR System.

### 3. RESULTS

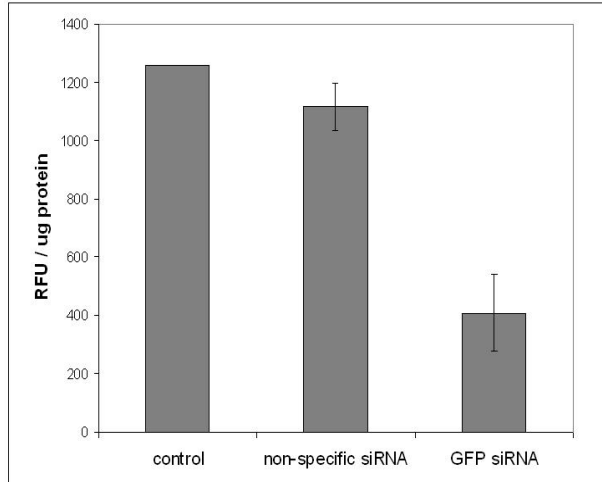
#### 3.1 siRNA Delivery to HeLa Cells

siRNA delivery was optimized by delivery of GFP siRNA to cells constitutively expressing GFP. This system was selected because GFP expression can be rapidly screened by fluorescence detection in a 96 well plate reader. Optimum siRNA knockdown was detected by fluorescence analysis of cell lysates normalized for protein content. Several delivery formulations using Lipofectamine 2000, PEI (Polyethylenimine), and Oligofectamine (Invitrogen) were tested at various delivery agents to siRNA ratios. Figure 1 shows HeLa cells that stably express GFP transfected with a control siRNA (A) and with a GFP-specific

siRNA (B) 72 h post transfection. A noticeable decrease in fluorescence is seen in the cells transfected with the GFP siRNA. Figure 2 shows a comparison of normalized total GFP fluorescence between untransfected cells (control), cells transfected with non-specific siRNA and cells transfected with GFP siRNA at 72 h post transfection. There is a 65% decrease in fluorescence in the GFP siRNA transfected cells, as compared to cells transfected with a non-specific siRNA sequence.

#### 3.2 Construction of Plasmids

The TSP2 targeted DNA sequences were generated using the guidelines from Ui-Tei *et al.* [11]. Both sequences had GC contents below 50% and were Blast searched to ensure that non-targeted genes would not be affected by the siRNA produced. The pSilencer vector uses a U6 promoter to direct the synthesis of hairpin dsRNA that will later be cleaved by Dicer to generate the target 21-nt siRNA. This siRNA should bind to TSP2 mRNA in the cell and, as a result, inhibit TSP2 production. Plasmids isolated from selected clones were digested and products of digestion were run on an agarose gel. All inserts were shown to be ~63 nucleotides in length thus verifying that the DNA sequences were successfully incorporated into the plasmid. DNA sequencing confirmed that the inserted DNA sequences were correct.



**Figure 2** Normalized GFP expression in HeLa cells.

### 3.3 Delivery of TSP2-siRNA plasmids to 3T3 fibroblasts

3T3 fibroblasts were selected as the model cell type because they are known to produce TSP2 and they are more easily transfected than other fibroblast cell lines (data not shown). The TSP2 siRNA plasmids described in Section 3.2 were delivered to NIH 3T3 cells following optimized delivery protocols described in Section 3.1. Efficient plasmid delivery was confirmed by PCR run on DNA collected from TSP2-siRNA plasmid transfected 3T3 cells. An agarose gel (Figure 3) was also run to determine if there were detectable amounts of TSP2 in fibroblasts. Although the intensity of the TSP2 band is much lighter in the lane with the untransfected 3T3 cells (lane 7) repeated experiments have shown comparable intensities of the untransfected 3T3 cells to the other transfected cells.

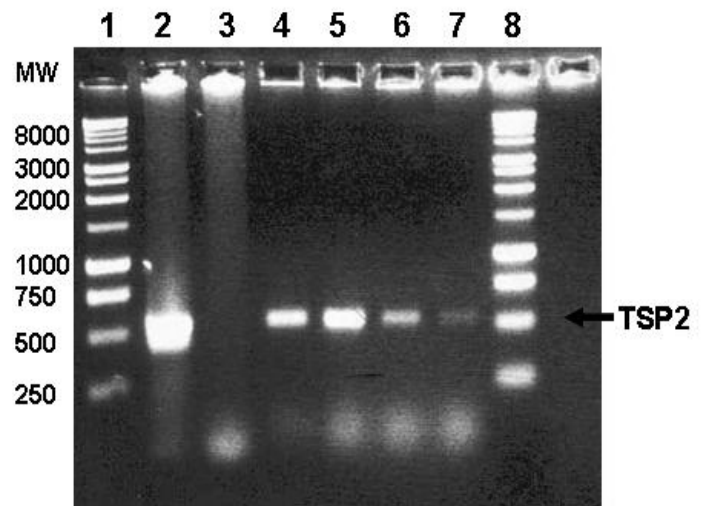
### 3.4 Quantitative Real-Time PCR of TSP2 mRNA

Quantitative real-time PCR (qPCR) was used to assess the plasmid's ability to inhibit TSP2 expression. It is a versatile technique that can simultaneously quantify and amplify DNA allowing for the determination of a specific nucleotide sequence. When combined with RT-PCR, relative gene expression can be determined from the initial mRNA present in a cell. The standard curve for TSP2 mRNA isolated from 3T3 fibroblasts is shown in Figure 4. The higher the cycle number in which the curve begins its linear growth the lower the concentration of the target mRNA. The mean fold change in TSP2

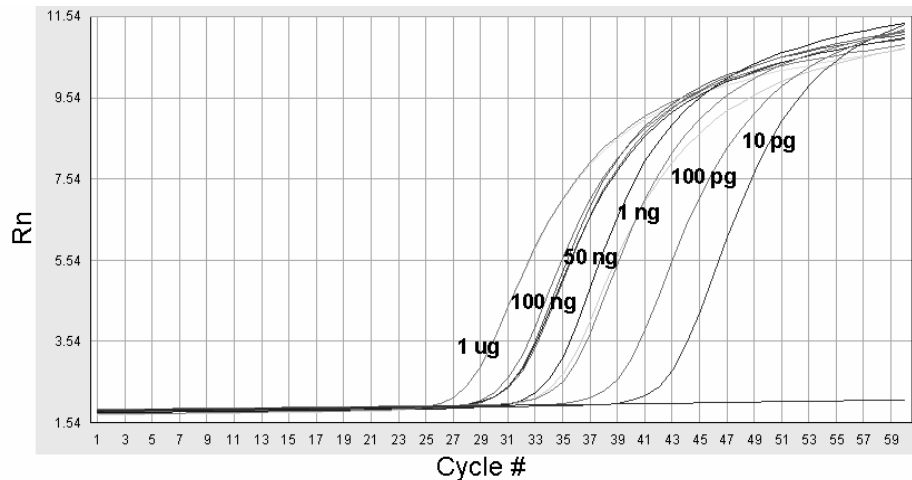
expression of the transfected cells compared to the untransfected cells was found using the  $2^{-\Delta\Delta C_T}$  method as described by Livak and Schmittgen [8]. Although there was a decrease in TSP2 expression in the transfected cells, the GFP transfected cells had TSP2 levels similar to those of the cells transfected with the TSP2 siRNA DNA plasmid (Figure 5).

## 4. DISCUSSION

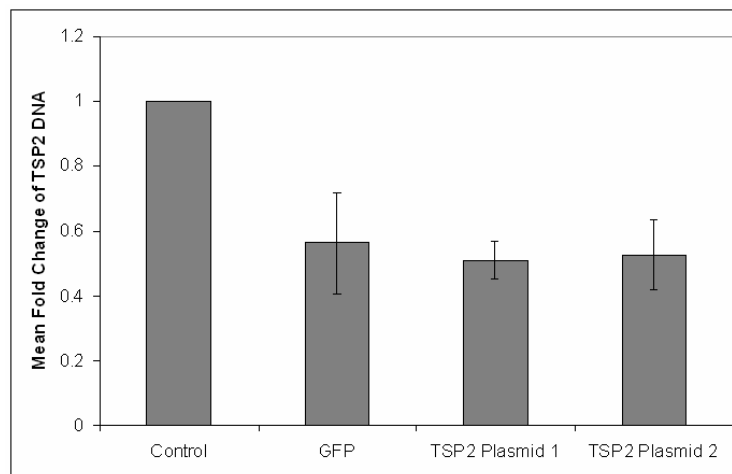
This study attempts to utilize siRNA's potential by incorporating an siRNA encoding a mouse TSP2 cDNA sequence into a plasmid to knockdown TSP2 gene expression. Delivery of siRNA directly into the cell has shown significant gene down-regulation of the targeted mRNA. Unlike DNA, which needs to be delivered to the nucleus for therapeutic activity, siRNA needs only to be delivered to the cytoplasm to cause silencing. However, siRNA is more prone to degradation due to its sensitivity to RNases and is more limited in its ability to be taken up by a cell. Plasmid delivery increases the stability and uptake of the DNA and can produce the intended siRNA intracellularly. Therefore, we explored the use of DNA plasmid encoding the siRNA sequence.



**Figure 3** Detection of TSP2 DNA from transfected 3T3 cells. Positive and negative controls of DNA obtained by PCR from TSP2-positive mice and TSP2-negative mice (Lanes 2 and 3), respectively. TSP2 DNA produced by PCR was detected in all transfected cells: pEGFP plasmid transfected (Lane 4), TSP2 plasmid 1 (Lane 5), TSP2 plasmid 2 (Lane 6), untransfected 3T3 cells (Lane 7).



**Figure 4** Standard Curve for TSP2 mRNA qPCR in 3T3 Fibroblasts plotted as Rn (Raw Fluorescence) vs. Cycle Number. Different amounts of synthesized cDNA from isolated TSP2 mRNA from transfected cells were run with real-time RT-PCR to create a standard curve.



**Figure 5** Mean Fold Change in TSP2 Expression. Control: untransfected cells, GFP: Cells transfected with eGFP plasmid, TSP2 Plasmid 1 and 2: Cells transfected with two different TSP2 siRNA plasmids.

We verified that, *in vitro*, NIH 3T3 fibroblasts both produce and secrete TSP2 into the surrounding media. However, introduction of plasmids coding for TSP2 RNA results in similar TSP2 levels as delivery of control plasmids. The lack of TSP2 knockdown may be due to several reasons. Because the amount of TSP2 mRNA produced may be low in 3T3 cells the mRNA collection procedure as well as the synthesis of cDNA must be extremely sensitive. Also, it has been shown that the introduction of siRNA into cells *in vitro* caused both nonspecific downregulation and upregulation of proteins [5]. This may explain why there was no

difference in the mean fold change of TSP2 expression between the GFP plasmid transfected cells and the TSP2 plasmid transfected cells.

The delivery of the pEGFP plasmid into 3T3 fibroblasts shows that they can take in and express the target protein as the transfection efficiency, determined by fluorescence microscopy, is well over 70%. Therefore, it is most likely not a matter of the TSP2 plasmids' inability to enter into the nucleus of the cell. One possibility that is currently being investigated is that the siRNA sequences currently encoded by the plasmids may not target TSP2. On average, only 1 in 5 siRNAs chosen for a specific gene

are efficient in silencing the target [6]. A screen of several different siRNA sequences that target TSP2 is being explored. They will be delivered first in dsRNA form as described in Section 2.1 for high throughput screening. Promising sequences will be incorporated into the pSilencer vector for analysis of stable TSP2 knockdown. Once a TSP2-siRNA plasmid that efficiently down-regulates TSP2 expression is found, *in vivo* inhibition of TSP2 in a mouse model will be investigated.

### ACKNOWLEDGMENTS

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