

METHODS PAGE

Highly Efficient siRNA Delivery into Primary Cultures of Rat

Pancreatic Stellate Cells

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Introduction

RNA interference (RNAi) was discovered about 10 years ago and has become a standard widely used method for silencing the expression of any target gene of interest (3). Target gene silencing is mediated by small interfering RNAs (siRNAs; double stranded RNA molecules 19-25 nucleotides in length), which trigger a catalytic mechanism for mRNA degradation. siRNA is unstable and has poor cellular uptake due to its physicochemical and biological properties. To increase the stability of siRNA and improve its uptake into cells, scientific companies have designed siRNA modifications and/or developed a variety of in vitro delivery agents.

Pancreatic stellate cells (PSCs) are now considered therapeutic targets in pancreatic cancer and chronic pancreatitis. Delivery of siRNA into PSCs provides a valuable tool for selectively silencing genes to examine their cellular function. Several factors can influence the degree to which a target gene is silenced in vitro including: 1) transfection efficiency; 2) transcription rate of the target gene; 3) protein stability and turnover; 4)

efficacy of the siRNA sequence; and 5) growth characteristics of the cells. Primary cultures of cells can be difficult to transfect. The following method has taken all these factors into account and has been optimised for highly efficient delivery of siRNA into primary cultures of PSCs. The example which will be illustrated is transfection of rat PSCs with HSP90 specific siRNA using lipofectamine™ 2000.

1. Materials/Reagents/Equipment

1. Primary rat pancreatic stellate cells
2. siRNA for Target Gene; rat HSP90 siRNA (ON-TARGET plus™ siRNA, Dharmacon, Thermo Fisher Scientific)
3. ON-TARGET plus Non-targeting siRNA Pool (Cat # D-001810-10-20; Dharmacon, Thermo Fisher Scientific)
4. AllStars Negative Control siRNA + Alexa fluor-488 (Cat # 1027284; Qiagen)
5. Lipofectamine™ 2000 (Cat # 11668-019; Invitrogen)
6. Opti-MEMI Reduced Serum Medium, pre-

- warmed (Cat # 31985-062; Invitrogen)
7. 6-well tissue culture plate
 8. RNeasy Plus Mini Kit (cat # 74134; Qiagen)
 9. QIAshredder (cat # 79654; Qiagen)
 10. Nanodrop spectrophotometer (Thermo)
 11. High Capacity cDNA kit (Cat # 4368814, Applied Biosystems)
 12. Quantifast SYBR Green PCR kit (Cat # 204054; Qiagen)
 13. Quantitect Primers for target gene (HSP90) and housekeeping gene (18S) (Qiagen)
 14. Light Cycler 480 Real-Time PCR machine (Roche Diagnostics Australia Pty Ltd)
 15. 1X siRNA buffer (diluted from 5X siRNA buffer; Cat # B-002000-UB-100, Dharmacon Thermo Fisher Scientific)
 16. Antibody to detect target gene; mouse anti-HSP90 (Stressgen, Sapphire bioscience)
 17. GAPDH antibody, loading control (cat # ab8245; Abcam)

2. Methods

2.1 Isolation of rat pancreatic stellate cells

Isolation of rat PSCs is performed by density gradient centrifugation using a method developed by Apte et al (2). Briefly, excised pancreatic tissue is digested with proteases and DNase and the digest centrifuged through a 11.4 % w/v Nycodenz gradient for 20 minutes at 1400 g. Stellate cells are harvested and resuspended in IMDM containing 10% FBS, 4 mM glutamine and penicillin / streptomycin 1% v/v. Cells are cultured at 37°C in a humidified 5% CO₂ / air atmosphere. Purity of freshly isolated PSCs is assessed by dual staining of cells for the presence of lipid droplets in the cytoplasm and for glial fibrillary acidic protein (GFAP). Upon culture on plastic, activated PSCs were identified by immunostaining

for α -smooth muscle actin (α -SMA) as described previously (2). Transfection of activated PSCs is performed with siRNA as described below (Section 2.3).

2.2 siRNA resuspension

ON-TARGET plus siRNA pool is used because it contains a pool of four individual siRNA sequences for the target gene (Note 1). The main advantages of using a pool of siRNA are 1) lower concentration of siRNA can be used, reducing off target effects; and 2) at least one of the 4 siRNA sequences is guaranteed to provide silencing of the target gene. To prepare siRNA use the following steps:

1. Briefly centrifuge the tube containing the siRNA to ensure that the siRNA pellet is at the base of the tube.
2. Resuspend siRNA (20nmol) with 1000 μ l of 1X siRNA buffer to yield a final concentration of 20 μ M siRNA. Make sure siRNA buffer is sterile and resuspension of siRNA is performed in a biosafety cabinet. Use pipette tips and tubes which are RNase/DNase free.
3. Mix solution by pipetting and place solution on an orbital mixer/shaker for 30 minutes at room temperature. Aliquot siRNA (50 μ l) and store at -20°C until use.

2.3 Transfection of PSCs with siRNA

1. The day before transfection, seed PSCs into 6-well plates (Note 2) at a density of 50,000 cells / well. *Transfect cells when they are between 30-50% confluent depending on your downstream functional assay. Gene silencing and functional assays are usually assessed at 24h-72h post transfection and it is preferable that cells are not too confluent at the endpoint of an assay. Note: Do not add antibiotics to media during transfection as this will result in cell death.*
2. For the calculation of the transfection mix, all volumes are increased by 10% to allow for pipetting error. On the day of transfection, a

- 10x stock solution (corresponding to a 1:50 dilution in Opti-MEM of the original stock of 1mg/ml) of Lipofectamine™ 2000 (L2K) is prepared. 2.2µl of L2K is added to 107.8µl of Opti-MEM (per well) or 660 µl of this mix is prepared for a 6 well plate. Mix the solution gently using a pipette and incubate for 5 minutes at room temperature.
- A 10x siRNA or non-silencing RNA stock solution (1µM of siRNA or non-silencing RNA) is then prepared by mixing 5.5µl of siRNA (20µM stock) with 104.5µl of Opti-MEM and mix carefully.
 - Add 110µl of the 10x L2K stock (step 2) to 110µl of the 10x siRNA or non-silencing RNA solution (Step 3). Mix gently with a pipette and incubate for 20 minutes at room temperature. *Note: Make sure to pipette gently when complexing the siRNA with lipofectamine. Harsh pipetting directly results in poor cell uptake.*
 - During this incubation, remove the cell medium containing 10% serum from the cells and wash the cells with 1x Opti-MEM followed by addition of 1ml of Opti-MEM and incubation for 20 minutes.
 - Add 880µl of Opti-MEM to the siRNA/non-silencing RNA + L2K solutions (Final concentration is 100nM, Note 3), mix well with a pipette and add 1ml of the appropriate transfection complex into the dedicated well.
- For each transfection, a mock no transfection control (L2K only) is included (Note 4).
- Incubate cells with the transfection complex for 5 hours.
 - At the end of this period, replace medium with fresh IMDM containing 10% serum (without antibiotics).
 - mRNA and protein levels are assessed after 72 hours (Note 5) using real-time PCR and western blotting.

2.4 Uptake of siRNA labelled with Alexa Fluor-488

Rat PSCs were treated as above (section 2.3) with lipofectamine alone, siRNA alone (100nM; Alexa Fluor-488) or lipofectamine + siRNA (100nM; Alexa Fluor-488) for 5 hours and cellular uptake is assessed after 24 hours by flow cytometry and confocal microscopy.

With this protocol, siRNA (Alexa fluor-488) uptake using lipofectamine as the transfection vehicle was 67.2% (Fig 1A and 1B). Confocal microscopy also demonstrated delivery of siRNA to perinuclear regions of the cytoplasm which is typical for siRNA transfection. Once inside the cell siRNA is first localised to the cytoplasm and then released from L2K to enter the RNAi machinery to enable the silencing of its target gene (Fig 1C). Note 6.

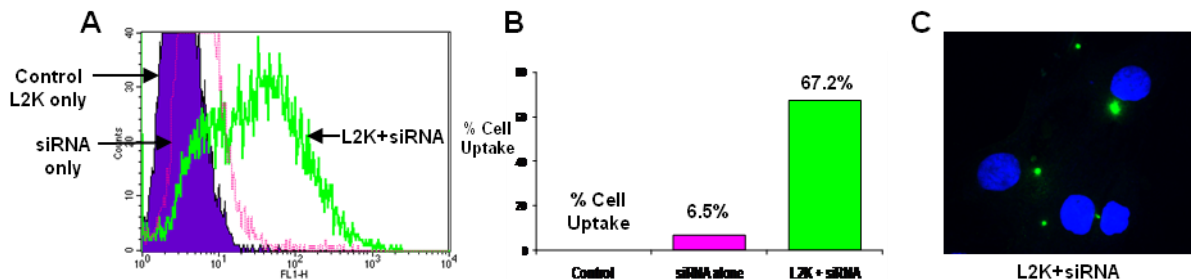


Figure 1. Uptake of siRNA labelled with Alexa Fluor-488. Rat PSCs were treated with lipofectamine (L2K) alone, siRNA alone or lipofectamine+siRNA for 5 hours and uptake assessed 24 hours post-transfection by flow cytometry (A and B) and confocal microscopy (C; blue stained nuclei [DAPI] and green siRNA in the cytosol).

2.5 RNA extraction from transfected PSCs

1. Recover PSCs from the 6-well plate (72 hours post-transfection) using trypsin. The resulting cell pellet is disrupted by addition of 350µl of Buffer RLT Plus containing β-mercaptoethanol.
2. Lysates are then added onto a QIA shredder spin column which is placed into a 2ml collection tube and centrifuged for 2 minutes at >8000g.
3. The homogenised lysate in the collection tube is then transferred to a genomic DNA eliminator spin column in a 2 ml collection tube and centrifuged for at >8000g for 30 seconds.
4. RNA is then extracted using the RNeasy Plus Mini Kit as per the manufacturers instructions (Qiagen).
5. RNA content is then estimated using a Nanodrop spectrophotometer. RNA samples are stored at -80°C until required.

2.6 Real-time PCR to assess mRNA silencing capacity of HSP90 siRNA

1. RNA (0.2µg) is reverse transcribed using the High Capacity cDNA synthesis kit from Applied Biosystem's as per the manufacturer's instructions.
2. Real-time PCR is performed using the

QuantiFast Sybr Green PCR kit (Qiagen) and Quantitect primers (HSP90 and 18S rRNA) according to the manufacturer's instructions.

3. Analyze samples using the Light Cycler 480 real-time PCR machine (Roche Diagnostics).
4. After completion of the reaction, analysis of data is performed using the Roche Light Cycler software. The relative value of each sample is normalized to the relative value of the housekeeping gene 18S rRNA. The relative values of the siRNA treated samples is then normalized to control values (i.e. mock transfected cells).

Rat PSCs (n=3) were transfected with either L2K alone (mock), non-silencing siRNA (100nM) or HSP90 specific siRNA (100nM) and HSP90 mRNA levels were assessed at 72 hours post-transfection. Figure 2A demonstrates that transfection of rat PSCs with HSP90 specific siRNA resulted in >75% silencing of HSP90 mRNA levels at 72 hours post-transfection. Similar results were obtained as early as 48 hours after transfection (data not shown). We have demonstrated significant silencing using this technique with more than ten specific siRNA sequences, including TLR4 (4).

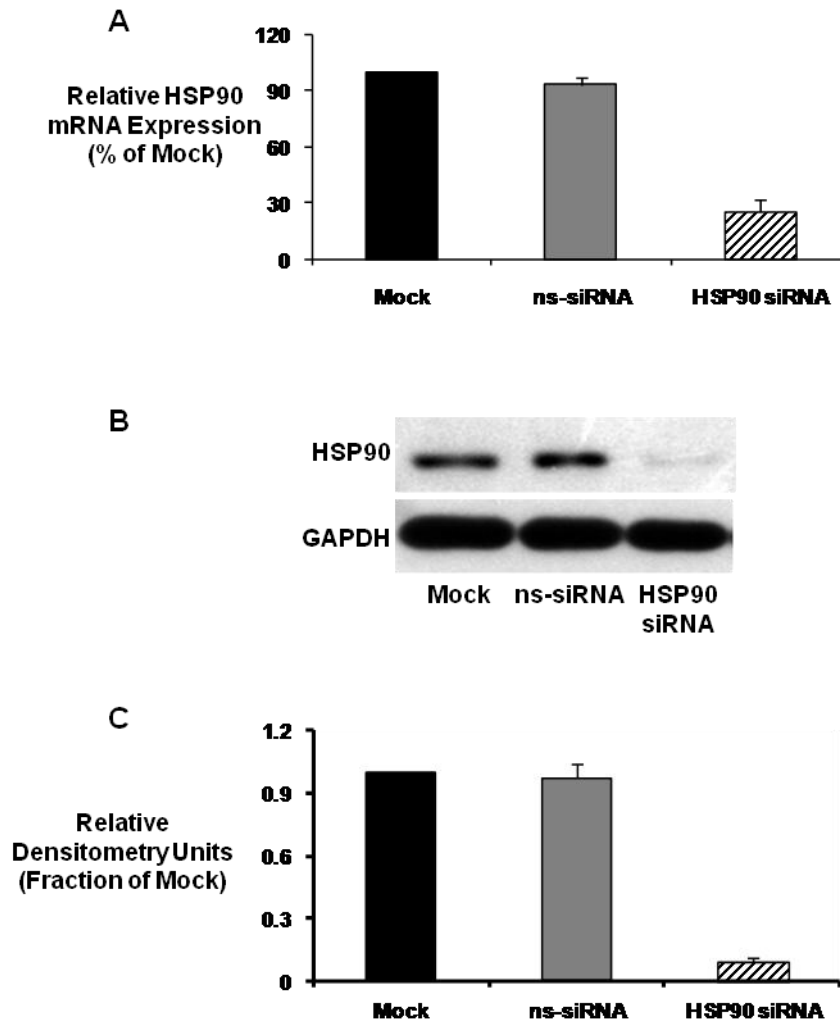


Figure 2. Effect of transfection of rat PSCs with siRNA for HSP90 on HSP90 mRNA and protein expression. A) Transfection of rat PSCs with siRNA for HSP90 significantly decreased HSP90 mRNA expression after 72 hours (* $p < 0.05$ vs mock and non-silencing siRNA [ns-siRNA], $n = 3$ separate PSC preparations). B) Representative western blots for HSP90 and GAPDH 72 hours post-transfection. C) HSP90 siRNA significantly reduced HSP90 protein levels as indicated by the graph of densitometry data of western blots (C; * $p < 0.05$ vs mock and ns-siRNA, $n = 3$ separate PSC preparations). In contrast GAPDH was not affected by the HSP90 siRNA.

2.7 Western blotting to assess protein silencing capacity of HSP90 siRNA

1. Expression of HSP90 protein in siRNA treated cells (72 hours post-transfection) is examined by western blotting of cell lysates prepared by resuspending cells in lysis buffer. Composition of lysis buffer: 65 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet P40, 1% sodium deoxycholate, 1 $\mu\text{g}/\text{mL}$ aprotinin, and 100 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride. Protein samples are collected and stored at -80°C .
2. Total protein concentration is determined using the Pierce bicinchoninic acid assay.
3. Proteins (5 μg) are separated on a 10% (w/v) sodium dodecyl sulfate (SDS) polyacrylamide gel by electrophoresis.
4. Protein standards of known molecular weight are run alongside the samples. Separated proteins are electrophoretically transferred onto nitrocellulose membranes using a

commercial semi-dry blotting apparatus (Biorad).

5. Incubate membranes for 1h at room temperature in blocking buffer [Tris-HCl (pH 7.6), skim milk 5%, Tween-20 0.1%].
6. Incubate membranes overnight at 4°C with the anti-mouse HSP90 antibody (1:1000 in 0.1% TTBS containing 5% skim milk) or the anti-mouse GAPDH antibody (1:50,000 in TBS containing 0.1% tween).
7. Wash membranes 3 times for 10 minutes in 0.1% TTBS (pH 7.5) and incubate with the secondary antibody (HRP-labelled anti-mouse IgG, 1:2000 in 0.1% TTBS containing 5% skim milk) for one hour at room temperature.
8. Wash membranes 3 times as described above. Relevant bands are detected using the Amersham Enhanced Chemiluminescence kit.

Rat PSCs (n=3) were transfected with either L2K alone (mock), non-silencing siRNA (100nM) or HSP90 specific siRNA (100nM) and HSP90 protein expression was assessed at 72 hours post-transfection. Figure 2B demonstrates that HSP90 siRNA significantly decreased HSP90 protein expression by rat PSCs by more than 90% (Fig 2C). GAPDH was unaffected by the siRNA treatment.

3. Notes

1. I prefer to use Dharmacon's ON-TARGET plus siRNA, however, there are several other reputable suppliers of siRNA including Qiagen and Invitrogen.
2. This siRNA transfection protocol has been optimised for cells cultured in a 6-well plate.

Acknowledgments

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However, post-transfection the cells can be harvested and reseeded into other plate-formats (i.e. 96-well plates, migration chambers etc) for functional studies.

3. In most cases, the optimal dose of siRNA should be between 25-100nM. Each new siRNA sequence should be titrated and gene silencing assessed. All future experiments should use the lowest dose of siRNA which provides the maximal knockdown of the target gene.
4. It is important to always include a mock no transfection control in each assay (i.e. cells transfected with lipofectamine alone) and a non-silencing siRNA as an additional control. Make sure that if you are using a pool of siRNA (i.e. 4 siRNA sequences, as above) to use the appropriate pooled non-silencing control.
5. It is important to perform a time course study when initially testing a new target siRNA sequence. Test gene silencing capacity at 24h, 48h, 72h and 96h post-transfection. This will provide essential data on the time point which corresponds to maximal knockdown of the target gene and also the duration of knockdown.
6. This method has also been successfully used to transfect human cancer-associated PSCs with siRNA, as well as several pancreatic cancer cell lines (MiaPaCa-2, Panc-1, HPAF-II and ASPC-1) (1).

4. References

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