

# Knockdown of Nox4 Gene in the Primary Human Macrophage

Will Yarberry, Mu Qiao, Wuqiong Ma, Reto Asmis

8-13-07

## Abstract

### *Objective*

Our goal was to evaluate the effectiveness of different small interfering RNA (siRNA) sequences in preventing the expression of NAD(P)H Oxidase Nox4 in the human monocyte-derived macrophage.

### *Approach*

Western blots, RT-PCR, and real-time PCR were used to measure the effective knockdown of Nox4 mRNA and protein levels using different siRNA directed against human Nox4. Human embryonic kidney (HEK) cells were used as a test in the RT-PCR and real-time PCR experiments, and human macrophages were used in the Western blot. Anti-human Nox4 scrambled siRNA was used as a control against the mere presence of siRNA in all experiments. siRNA tagged with Fluorescein Isothiocyanate (FITC) directed against GL2 luciferase was used both as a control and to determine and monitor transfection efficiency.

### *Results*

We tested five different anti-human Nox4 siRNA, labeled Old, #5, #6, #7, and #8. Table 1 gives the sequences for each siRNA. The RT-PCR results revealed that sequence #6 produced about a 60% knockdown

in mRNA levels in HEK cells, and the real time PCR confirmed this, also giving a 60% knockdown for sequence #6. The western blot showed no change in protein levels in human macrophages for any of the siRNA sequences.

### ***Conclusions***

Sequence #6 induced considerable knockdown in mRNA levels in HEK cells. However, no anti-human Nox4 sequence was effective at lowering protein levels in human macrophages after 72 h, leaving two possibilities. One possibility is that siRNA transfection simply did not take place. However, non-specific siRNA tagged with FITC revealed that the transfection reagent was effective, near 100% transfection, in delivering the siRNA into the cell through fluorescence microscopy. Another possibility is that Nox4 is a very stable protein with a long half-life. Future study will focus on increasing the concentration of siRNA, possibly performing a second transfection, and increasing incubation time to 96 h.

## **Background**

High cholesterol levels have been historically linked to heart disease, in particular to atherosclerosis<sup>1</sup>. However, recently the immune system, specifically the macrophage, has been recognized as a major contributing factor to the progression of this disease<sup>2</sup>. When a monocyte detects a buildup of density lipoprotein (LDL) inside the artery wall, it migrates from the blood into the wall and differentiates into a macrophage in an attempt to clean the LDL and atherogenic modified LDL from the area<sup>3</sup>. LDL accumulating in the vessel wall is exposed to reactive oxygen species and becomes oxidized, forming

oxidized LDL (OxLDL), which is cytotoxic to macrophages<sup>4</sup>. The macrophage would eventually die inside the artery wall, contributing to the formation of a necrotic core. Given enough time, this buildup develops into an atheroma, which in turn develops a fibrous cap. If this cap is torn off, the contents of the core can spill out, causing the blood to clot as a response to foreign matter in the artery, in turn causing a myocardial infarction. Dr. Asmis and his colleagues recently identified Nox4 as a new member of the NAD(P) oxidase family in macrophages, and discovered that OxLDL promotes the up-regulation in the levels of the Nox4 mRNA and protein, suggesting a possible link between OxLDL and macrophage cell injury. Whether this up regulation is protective or contributes to the cytotoxicity of OxLDL is unknown. To determine the role of Nox4 in OxLDL cytotoxicity we decided to develop an siRNA-based approach to specifically inhibit Nox4 activity in human macrophages. The aim of this project was to evaluate different anti-human Nox4 constructs and to determine the optimal conditions for specific Nox4 knockdown.

## **Materials and Methods**

### ***Cell culture and RNA extraction***

We obtained cells from human donor blood, and later aphaeresis filters. Several experiments were repeated to ensure that the cells from the filter were representative of macrophage cells found within the body. Cells were cultured in a 12-well plate on aclar film discs as a growing platform and RPMI as a medium. To perform the transfection, two sets of tubes were prepared, one set with the transfection reagent plus Opti-MEM, and the other set with siRNA dilute solution plus Opti-MEM plus the siRNA itself. The transfection reagent and the siRNA dilute solution were from GeneSilencer siRNA transfection kit

(Genlantis). After 5 minutes at room temperature, the contents of the two sets of tubes are mixed together and incubated for 20 minutes, again at room temperature. While incubating, the cells on the aclar are washed first in PBS, then in Opti-MEM for 20 minutes to rid the film of any non-coherent cells. After the incubation, parafilm is laid out in a dish, and the underside of the dish is marked to keep track of the samples. The aclar is then transferred to the parafilm. The contents of the tubes are then added to the aclar, using surface tension to keep the liquid on the film. The aclar is then incubated for 3 hours at 37°C. After this incubation, the liquid is removed and the film is transferred to new 12 well plates containing RPMI with 5% AB serum and incubated again at 37°C for 24 h. We used FITC-labeled siRNA in one well per dose group (300 ng siRNA, 600ng siRNA, and 900 ng siRNA) to monitor the effectiveness of the transfection reagent by fluorescence microscopy. Once the 24 h incubation was complete, we performed an RNA extraction using the Aurum total RNA mini-kit (Bio-Rad). The RNA samples were run through a spectrophotograph test to determine their concentration, then stored at -80°C.

### ***RT-PCR***

In order to determine the degree of knockdown of Nox4 mRNA by each of our anti-human Nox4 siRNAs, Reverse Transcriptase PCR (RT-PCR) was performed on EHK 293T cells. RT-PCR samples were run through PCR using the RETROscript kit (Ambion, Austin, TX). Table 2 shows the temperature and duration of cycles for Nox4 and Actin samples. Once the PCR was complete, samples were mixed with 8 uL of 6X blue/orange loading dye from Promega. 6 uL of Promega's 1Kb DNA Ladder was used as a marker for the samples. The gel used was 1% Agrose with 0.5 uL Ethidium Bromide for staining and was run for 20

minutes at 100 V. Images were taken and bands were quantified using Kodak's 4000mm Image Station.

### ***Real-Time PCR***

The real-time PCR experiment was performed using the ABI 7900 Real Time PCR System. Two rows of samples from HEK 293T cells were treated with 20X primer and probe (Taqman), one row with human Nox4 primer and probe and one with 18S primer and probe. All samples were loaded with 2X universal PCR master mixture (Taqman).

### ***Western Blot***

After mixing the samples with 5X loading buffer and adding Precision Plus Protein Standards (Bio-Rad) marker, samples were run in a 10% acrylamide running gel at 80 volts for 30 min, then at 140 V on ice for 1 hour and 30 minutes in 1X TRIS/GLY/SDS running buffer. After this, the protein was transferred to an Immobolin Membrane (Millipore) by running it in transfer buffer at 100 V for 2 h, then it was cut to separate the Nox4, actin, and p22 bands. Once this was complete, nonspecific sites were blocked with 5% non-fat dry milk for 1 h. The primary antibody was then added and incubated overnight in 0.5% non-fat milk. After washing the membrane 3 times in 1X TBS-T for 10 minutes, the secondary antibody was added in 0.5% non-fat milk and 1X TBS/TWEEN-20 buffer for 1 h. After the chemiluminescence's substrate detection reagent was added, images were taken and bands were quantified using Kodak's 4000MM Image Station.

### ***siRNA***

We used various anti-human Nox4 siRNA from Dharmacon RNA Technologies in 1x siRNA buffer. Table 1 gives the sequences for each

siRNA. All siRNA were 20 uM, with a concentration of 0.266 ug/uL. We referred to the different siRNA by their number, with J-010194-05 as #5, J-010194-06 as #6, J-010194-07 as #7, and J-010194-08 as #8. We also had siRNA used in previous experiments, also from Dharmacon, which we labeled Old (D-001210-01). Anti-human Nox4 Scramble siRNA was used as a control, and FITC siRNA was used both as a control and for its fluorescent properties.

## **Results**

### ***RT-PCR***

RT-PCR was run on RNA from EHK 293T cells treated with #5, #6, #7, FITC siRNA, and anti-human Nox4 scrambled siRNA. Figure 1 shows the resulting bands, and table 3 shows the resulting ratios between Nox4 and actin. This table indicates a significant knockdown in mRNA levels in macrophages treated with siRNA sequence #6. When quantified, sequence #6 induced a 50% knockdown in mRNA levels.

### ***Real-Time PCR***

Our real-time PCR experiment involved testing Nox4 mRNA levels against that of 18S. Figure 2 shows the ratios between Nox4 and actin standardized by RPMI, and table 4 shows the quantification. Anti-human Nox4 siRNA #6 induced a considerable knockdown of Nox4 mRNA levels, about 60% when quantified.

### ***Western Blot***

A Western blot was performed in which we transfected cells with different concentrations of siRNA. Human macrophage cells were transfected using 300, 600, and 900 ng of sequence #5, #6, #7, #8,

anti-human Nox4 scrambled siRNA, and non-specific FITC tagged siRNA. Included were a control group which received no transfection reagent and a control group with transfection reagent but without siRNA. Figure 3 shows FITC fluorescing under ultraviolet light according to the dose administered, indicating a successful transfection. After a 72 hour incubation and RNA extraction, a Western blot was performed on each group to determine Nox4 protein levels. Figure 4 shows the 900 ng siRNA dose group, with all protein levels being within 20% of RPMI.

## **Discussion**

RT-PCR and real-time PCR experiments showed that knockdown of Nox4 mRNA in HEK 293T cells was achieved through anti-human Nox4 siRNA sequence #6, with the strongest knockdown being 60% on the real time PCR. Nox4 protein levels, however, remained unchanged 72 h after transfection in human macrophages. One explanation is that transfection was ineffective in human macrophages. However, the pictures of FITC taken by fluorescence microscopy indicate that transfection did take place and affected nearly 100% of the cells. An alternate and more likely explanation is that Nox4 is an extremely stable protein with a very long half life, so even when mRNA is eliminated and Nox4 production ceases, levels of Nox4 remain constant. Nox4 knockdown the protein level could be improved on by increasing the concentration of siRNA in the initial transfection, increasing incubation time to 96 h, and possibly performing a second transfection.

Table 1: anti-human Nox4 sequences #5, #6, #7, #8, and Old

|     |   |
|-----|---|
| #5  | A C U A U G A U A U C U U C U G G U A U U |
| #6  | G A A A U U A U C C C A A G C U G U A U U |
| #7  | G G G C U A G G A U U G U G U C U A A U U |
| #8  | G A U C A C A G C C U C U A C A U A U U U |
| Old | G A G A A C A G A C C U G A C U A U G U U |

Table 2: Temperature and duration of cycles for Nox4 and Actin samples

| <b>Actin</b> |             |              | <b>Nox4</b> |             |              |
|--------------|-------------|--------------|-------------|-------------|--------------|
| <b>Temp</b>  | <b>Time</b> | <b>Cycle</b> | <b>Temp</b> | <b>Time</b> | <b>Cycle</b> |
| 95           | 5'          | 1            | 95          | 5'          | 1            |
| 95           | 30"         |              | 95          | 30"         |              |
| 55           | 45"         | 27           | 60          | 30"         | 30           |
| 72           | 45"         |              | 72          | 45"         |              |
| 72           | 5'          | 1            | 72          | 5'          | 1            |
| 4            | KEEP        |              | 4           | KEEP        |              |

Table 3: Ratios between Nox4 and actin mRNA levels for RT-PCR on EHK 293T cells

| <b>Ratio (Nox4/Actin) Standardized by RPMI</b> |      |
|--|------|
| RPMI   | 1.00 |
| #5   | 1.07 |
| #6   | 0.54 |
| #7   | 0.70 |
| Scr  | 1.21 |

Table 4: Ratios between Nox4 and actin mRNA levels for real-time PCR on EHK 293T cells

**Ratio (Nox4/Actin) Standardized  
by RPMI**

|      |      |
|------|------|
| RPMI | 1.00 |
| #5   | 1.29 |
| #6   | 0.41 |
| #7   | 1.24 |
| Scr  | 1.73 |

Figure 1: Nox4 and actin mRNA levels from RT-PCR

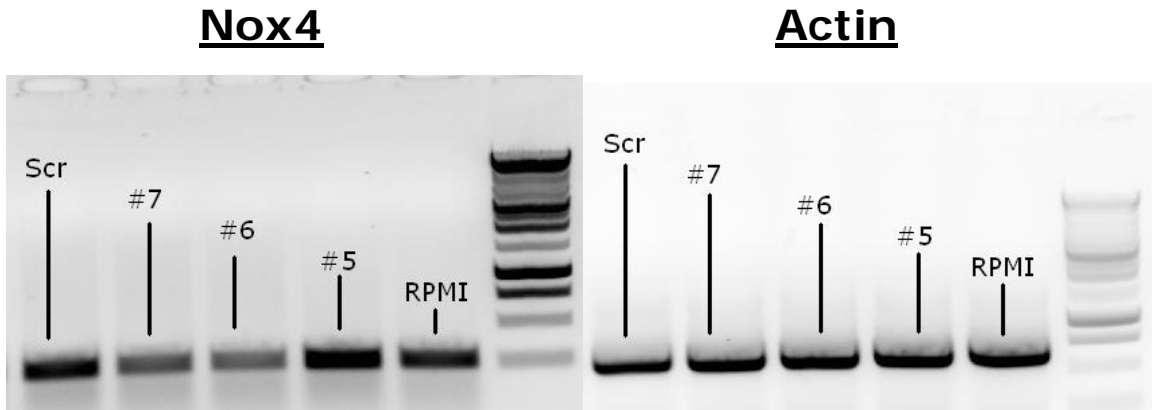


Figure 2: Nox4 and actin mRNA levels from real-time PCR

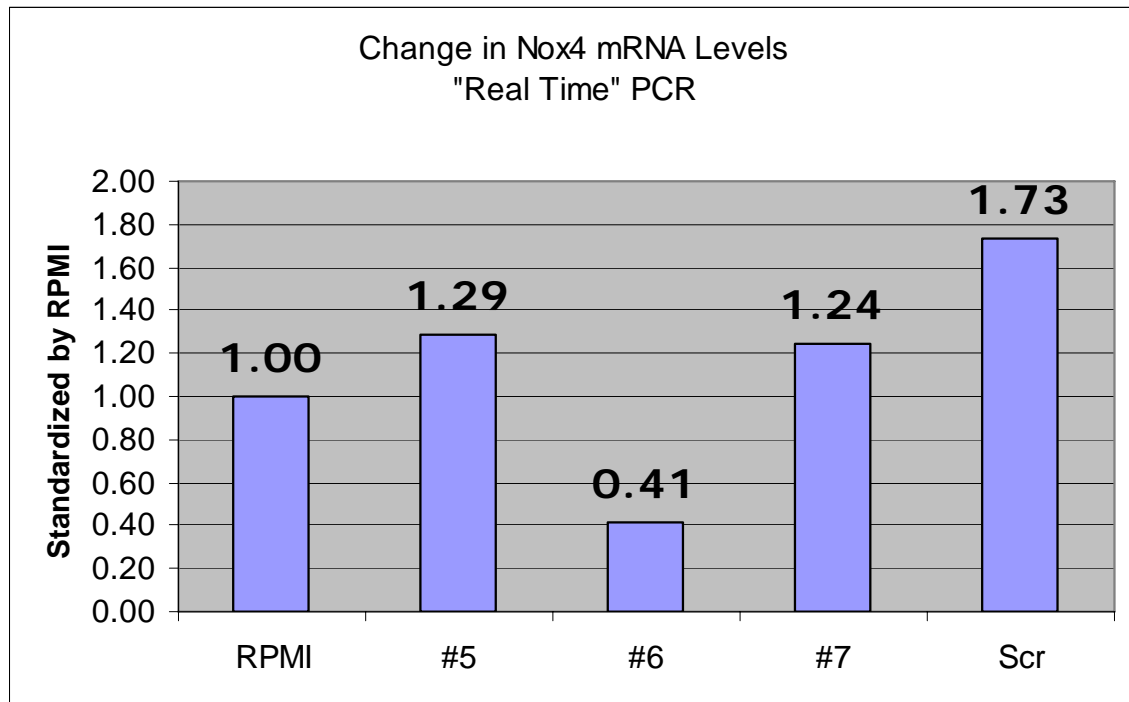


Figure 3: 0, 300, 600, and 900 ng non-specific FITC tagged siRNA under the fluorescence microscope

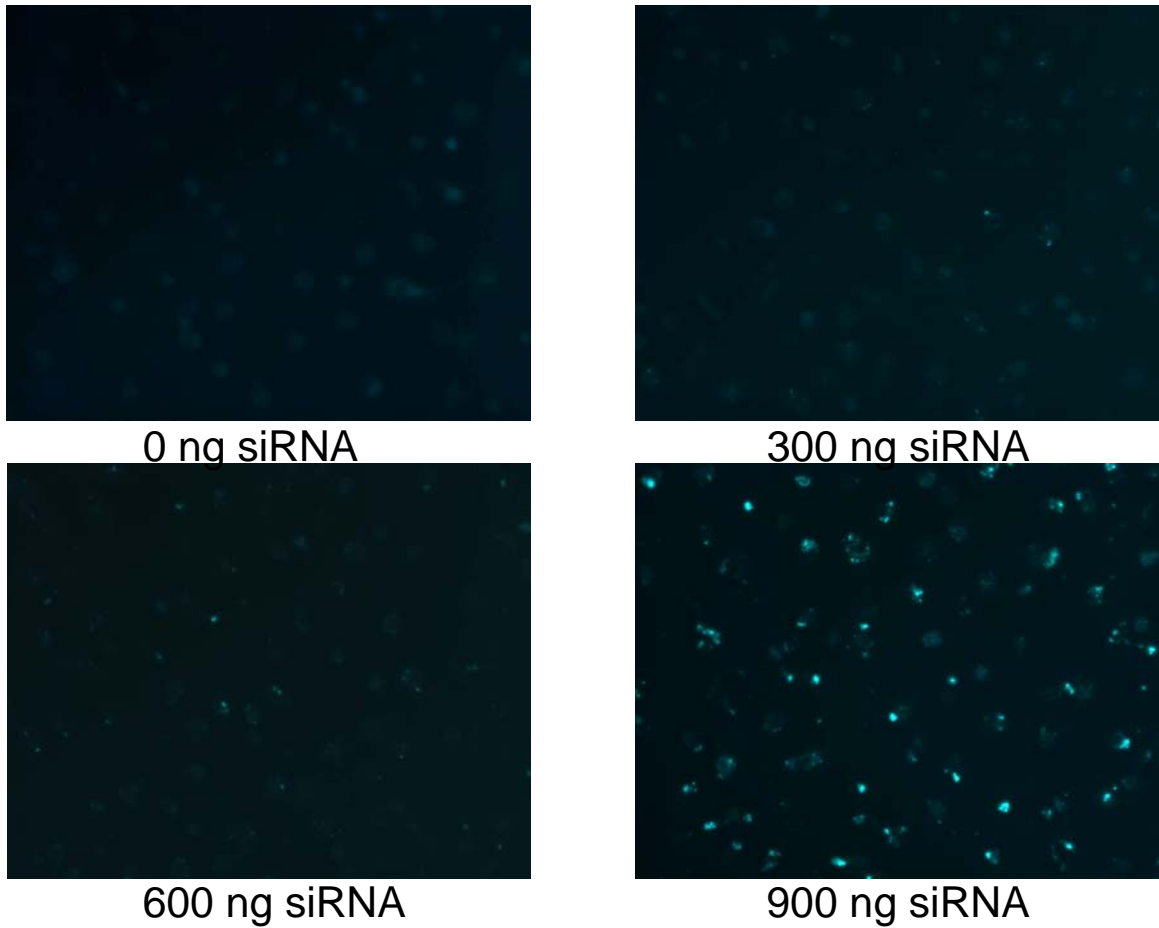
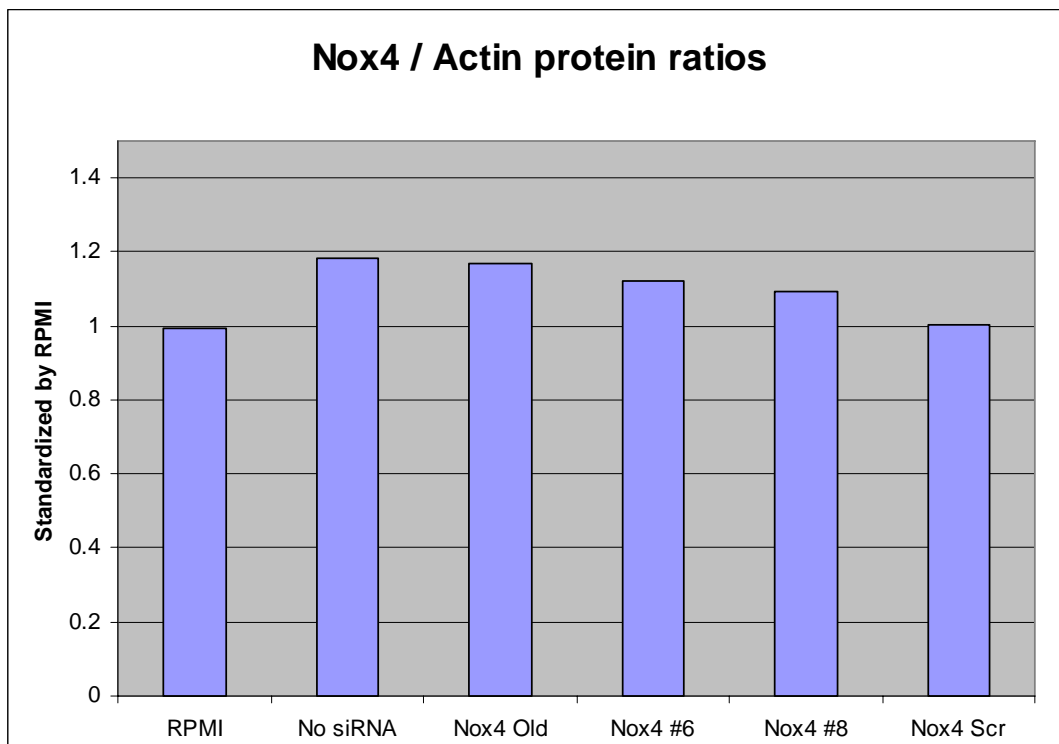


Figure 4: 900 ng siRNA dose group protein levels from Western blot



## References

1. Steinberg D., *Hypercholesterolemia and inflammation in atherogenesis: two sides of the same coin*. Mol Nutr Food Res. 2005 Nov; **49**(11): 995-8.
2. Liang CP, Han S, Senokuchi T, Tall AR., *The macrophage at the crossroads of insulin resistance and atherosclerosis*. Circ Res. 2007 Jun 8; **100**(11): 1546-55.
3. Libby P., *Inflammation in atherosclerosis*. Nature. 2002 Dec 19-26; **420**(6917): 868-74.
4. Piccoli C, Ria R, Scrima R, Cela O, D'Aprile A, Boffoli D, Falzetti F, Tabilio A, Capitanio N. *Characterization of mitochondrial and extra-mitochondrial oxygen consuming reactions in human hematopoietic stem cells. Novel evidence of the occurrence of NAD(P)H oxidase activity*. J Biol Chem. 2005 Jul 15; **280**(28):26467-76. Epub 2005 May 9.

# Acknowledgements

I would like to thank Dr. Merry Lindsay, Dr. Bruce J. Nicholson, and Anna Uriegas for their support and outstanding administration of the B-SURE program. I would also like to thank Mu Qiao, Wuqiong Ma, and Reto Asmis for their patience and leadership in the lab.