

Experimental siRNA Targeting the 469-624bp Region of Renilla Luciferase is Ineffective in Reducing Expression in Mouse Embryonic Stem Cells

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Abstract

dsRNA is an increasingly well studied and widely used tool for the regulation of gene expression at the post-transcriptional level. In this experiment, we designed an siRNA intended to specifically target the *Renilla reniformis* luciferase gene in order to reduce the amount of luciferase produced by the gene. However, Luciferase assays of the lysates from mouse embryonic stem cells transfected with this siRNA and a plasmid containing the *Renilla* luciferase gene suggest that the designed siRNA is not able to reduce *Renilla* luciferase expression. Moreover, microarray analysis suggests that the siRNA has widespread off-target effects. Over 8,000 genes were differentially expressed between cells transfected with the luciferase-containing plasmid only, and those transfected with the experimental siRNA and the plasmid. There is no clear pattern between the genes that showed the greatest differential expression between the two samples. Thus, we have determined the experimental siRNA is not able to specifically reduce *Renilla* luciferase expression, and instead has many off-target effects. To make RNAi a more useful tool than traditional techniques like homologous recombination, further research must be done to improve siRNA design algorithms.

Introduction

In order to perform their specified functions, cells must constantly regulate the amount and types of proteins they produce. When a cell is no longer able to properly regulate protein production, cell death or defects in the tissue or organism often result. For example, cancer is often caused by decreased production of proteins that inhibit cell growth, or by over production of proteins that cause the cell to continuously divide. Healthy cells, however, are able to regulate their protein expression at many levels, including transcription, translation, and after transcription and translation.

One way in which cells regulate protein expression at the post-transcriptional level is by preventing the translation of the mRNA transcript in a process called RNA interference (RNAi). RNAi, which occurs in organisms as diverse as *C. elegans* (Fire 1998), planarians (Sánchez-Alvarado 1999), and humans (Elbashir 2001), uses a double-stranded piece of RNA (dsRNA) to prevent the translation of a target mRNA transcript. One strand of the dsRNA is complementary to a region of the target mRNA. This strand is incorporated into the RNA-induced silencing complex (RISC) by a process that is not well understood, but is believed to involve the Argonaute family of proteins (Song 2003). The RISC then binds to the complementary sequence on the mRNA target and cuts the mRNA transcript. The cleaved mRNA is degraded completely, and thus will not be translated into protein.

Understanding how the RNAi pathway works is beneficial not only because it explains a natural means of protein expression regulation, but also because the induction of this pathway in the laboratory provides a means of artificially regulating protein expression. siRNAs can be designed to target a specific mRNA transcript with the intent of reducing the amount of protein translated from the transcript. However, there are many ambiguities associated with the design of effective and specific siRNAs. For

example, it is unclear if targeting a specific region in the mRNA transcript results in stronger RNAi. Thus, it is a common practice to design several different siRNAs to target different regions of the mRNA transcript with the hope that at least one of them will be effective. Similarly, there is no single set of siRNA properties that guarantees the construct's success. Several different properties, including G/C content, melting temperature, and free energy, have been found to influence specificity and efficacy of the siRNA (Ambion Design URL), but there is no algorithm that can consistently produce an siRNA that is highly specific and effective for a given target. As a result, siRNA design is often hit-or-miss.

The addition of siRNA into a cell may also have more wide-reaching effects than regulating the translation of the target mRNA. siRNA can alter the translation levels of off-target mRNAs, often because of similarities in sequences between the mRNA transcripts. Moreover, the presence of dsRNA in a cell may be interpreted as a viral infection, and as a result, the anti-viral interferon pathway may be triggered upon treatment with siRNA. Thus, siRNA treatment can result in the altered regulation of many genes besides the intended target.

In order to validate and explore the effects of RNAi on the regulation of protein expression, DNA microarrays are particularly useful because they can assess the relative expression levels of tens of thousands of genes at one time. In this technique, a slide or other surface is coated with DNA from the genes of interest. RNA is then isolated from the sample cells, reverse transcribed, and labeled. The resulting cDNA is hybridized to the array, allowing labeled sample DNA to bind to complementary sequences on the array. Relative gene expression can then be determined by looking at the relative amount of label on each spot.

Assays based on the functions of the protein encoded by a target gene can also be useful for measuring gene expression levels. One protein that has readily-measured expression levels is Renilla reniformis luciferase. Renilla reniformis is a soft coral that has the ability to bioluminesce by producing a luciferase protein. This enzyme oxidizes the small molecule luciferin, which results in the emission of blue light (20.109 Transfection URL). By measuring the light emitted by different samples expressing this gene, relative expression levels may be determined, as cell extracts that emit more light express more luciferase than those that emit lower levels of light.

The purpose of this experiment was to design an siRNA with the ability to reduce the production of the Renilla luciferase protein in mouse embryonic stem cells that produce Renilla luciferase. We used a plasmid containing Renilla luciferase to transfect mouse embryonic stem cells, enabling them to bioluminesce. Mouse cells were used because they do not naturally bioluminesce, so any bioluminescence must be due to the transfected luciferase plasmid. Moreover, they do not contain mechanisms for regulating luciferase production, so any changes in bioluminescence must be due to our treatment of the cells. The transfected cells were also treated with an siRNA that was designed to bind to the Renilla luciferase mRNA transcript, resulting in its cleavage and decreased production of the luciferase protein. Luciferase expression was first quantified by measuring the amount of light emitted by the extracts of transfected cells. The cells were also visually monitored to assess if the siRNA had any off-target effects that inhibited growth. To further assess off-target effects, we ran a microarray with cDNA reverse transcribed from mRNA isolated from the samples. By designing and testing this siRNA

we were able to determine how effective it was, which indicates the effectiveness of the techniques we used to design it.

Materials and Methods

All protocols are taken from the 20.109 Fall 2007 class wiki:
[http://openwetware.org/wiki/20.109\(F07\)](http://openwetware.org/wiki/20.109(F07))

DNA and RNA

The psiCHECK2 plasmid (GenBank accession number AY535007), which contains the Renilla luciferase and Firefly luciferase genes was used to enable the mouse embryonic stem cells to express the luciferase genes. Its sequence was obtained from Promega.com to allow us to identify a target sequence to which a dsRNA could specifically bind. Ambion's siRNA Target Finder (Ambion Target Finder URL) was then used to find targets in the 469-624bp range of the Renilla Luciferase gene, using the options "ends with TT," "all G/C contents," and "avoid four or more C/Gs in a row." This program also provided the G/C content for each target sequence, and candidates with a G/C content higher than 50% or lower than 30% were not taken into further consideration. Integrated DNA Technology's Oligo Analyzer (IDT URL) was then used to obtain the melting temperature of each of the remaining candidate sequences, and OligoCalc (OligoCalc URL) was used to determine the candidate's free energy. Low T_m and free energy values are favorable, because they suggest the sequence will not have significant secondary structure that could interfere with binding. Finally, each candidate was BLASTed against the mus musculus genome to find other sequences that the siRNA might target. Only one candidate had a relatively low melting temperature (compared to the other candidates), a relatively low free energy, and only a few matching sequences in the mouse genome. Thus, this candidate was chosen for use in the siRNA experiment.

Mouse Embryonic Cell Culture and Transfection

The mouse embryonic stem cells were maintained in JI growth media, consisting of DMEM (high glucose), 10% Fetal Bovine Serum, 100U/ml Pen/Strep, 0.3mg/ml glutamine, and 50ul/L LIF. The cells were maintained in a humidified incubator at 37°C with five percent CO₂. Samples of cells were transfected with the psiCHECK2 plasmid only, a validated siRNA sequence only, both the plasmid and the validated siRNA, both the plasmid and a scrambled version of the siRNA with no homology to the mouse genome, and both the plasmid and the experimental siRNA, in order to allow them to express the luciferase genes, and to allow the siRNA to regulate the expression of those genes. The psiCHECK2 plasmid was transfected at a concentration of 10ug plasmid/100ul transfection, and the siRNA at a concentration of 5pmol RNA/100ul transfection. The carrier for the lipofection was prepared by diluting Lipofectamine 2000 3:50 in OptiMEM. It was then allowed to sit, undisturbed, at room temperature for about 15min. The DNA and RNA were prepared for transfection by diluting the plasmid 1:2500 in OptiMEM and/or diluting the siRNA 1:5x10⁶ in OptiMEM. Equal volumes of the carrier and DNA/RNA mixtures were combined and allowed to incubate for 20 min at room temperature. The cells were washed with PBS before the growth media was replaced with pre-transformation media, composed of the growth media without antibiotics. Enough of the appropriate DNA/RNA/lipofectamine mixtures were added to

each well of cells to make the media a 9.5% lipofection mixture. The media was changed to growth media after 24 hours and lysates were prepared from the cells after 48 hours to analyze luciferase activity.

Luciferase Assays

In order to measure the levels of luciferase expression in the samples, the light emitted from cell lysates was measured. Each well of cells growing in a six-well plate was washed two times with 2ml PBS. To induce lysis, 500ul of Promega's PLB reagent was added and the cells were then placed on an orbital rocker at 150rpm for 15min at room temperature. Then, 10ul of the cell lysate was added to 50ul of LARII from Promega's dual-luciferase assay kit, which contains beetle luciferin. After pipet mixing, the Firefly luciferase activity was immediately measured with the Turner Luminometer 20/20 for 10 seconds. Then, 50ul of "Stop and Glo" from Promega's dual-luciferase assay kit was added to mixture, and the Renilla luciferase activity was measured immediately after pipet mixing.

Mouse Whole Genome Microarray

Total RNA Isolation

RNA was isolated from the cells using Qiagen's RN-easy kit according to the manufacturer's instructions. This process was done to provide a template for the reverse transcription reaction that would produce the cDNA needed to hybridize to the microarray. The concentrations of the RNA samples were then determined by taking spectrophotometer readings at 260nm.

Reverse Transcription

The RNA from the cell lysates was reverse transcribed to allow it to hybridize to the microarray. The largest amount of RNA that could be taken from the most dilute sample without exceeding 10ul was 2.56ug, so this amount was reverse transcribed from each sample. The volume of the remaining sample was brought to 10ul with RNase-free water. Then, 1ul of primer/Capture Sequence I (red) was added to the sample that had been transfected with only plasmid, and 1ul of primer/Capture Sequence II (blue) was added to the sample that had been transfected with plasmid and the experimental siRNA. The primers were annealed to the RNA by heating these mixtures at 80°C for 10 minutes, and then placing them on ice for two minutes. Then, 1ul of Genisphere's Superase RNase inhibitor was added to the reactions and 8ul of Gensisphere's cDNA synthesis cocktail, containing Superscript First Strand Buffer, DTT, dNTPs, and the Superscript II enzyme was added to each sample. After gentle pipet mixing, the cDNA synthesis reaction was incubated at 42°C for 90 minutes. Finally, 3.5ul of .5M NaOH/.5M EDTA was then added to each tube to stop the reaction. The tubes were heated at 65°C for 10 minutes to degrade the RNA template and 5ul of 1M Tris at pH seven was added to each tube.

Microarray Hybridization

Agilent's Mouse Whole Genome Microarray was used to screen gene expression of the two samples. To hybridize the samples to the array, the cDNA samples were pooled and an equal volume of Agilent's 2x Hybridization Buffer was added to them. These

hybridization solutions were then heated to 80°C for 10 minutes. The microarray cover was placed in the hybridization chamber, and after the solutions cooled to room temperature, 110ul of the solution was pipetted onto the cover. The microarray was then placed face down on top of the samples, and the hybridization chamber was closed. The array was left at 60°C in a hybridization oven overnight. The next day, the unbound cDNA was washed off of the array with 0.005% Triton X-100 and 6xSSC, containing 2.4M NaCl and .24M NaCitrate, at 42° for 10 minutes. The array was washed again with 0.2X SSC/0.00016% Triton X-100 at room temperature for five minutes. The slides were then dried and rehybridized with Genisphere's Cy3 and Cy5 dendrimers for four hours at 65°C. Unbound Cy3 and Cy5 were then washed off with 2X SSC/0.0016% Triton X-100 at room temperature for five minutes and 0.2X SSC/0.00016% Triton X-100 at room temperature for 10 minutes. The slide was then dried quickly with nitrogen gas and scanned with an Agilent scanner.

Results

Table 1. Validated, scrambled, and experimental siRNA sequences

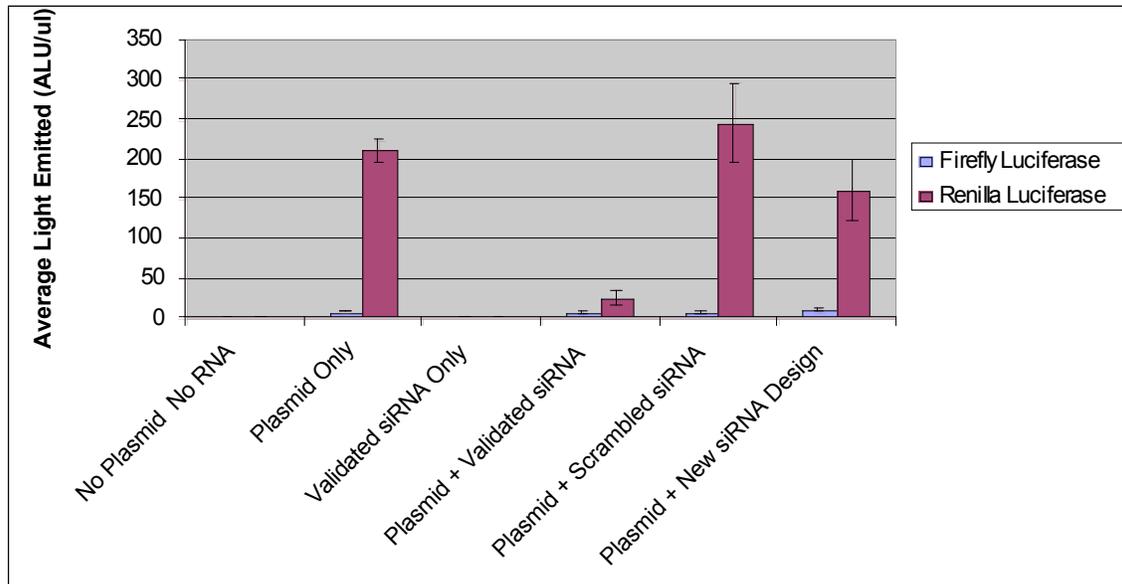
siRNA	Sequence
Validated siRNA	GGCCUUUCACUACUCCUACdTdT dTdTCCGGAAAGUGAUGAGGAUGA
Scrambled siRNA	UAAGGCUAUGAAGAGAUACdTdT dTdTAAUUCGUAUCUUCUCAUGA
Experimental siRNA 1	UCCAAUGUUCUGUCCAAAUdTdT dTdTAAAGGUUACAAGACAGGUUUA
Experimental siRNA 2	UAACUUCUUCGUCGAGACdTdT dTdTAAUUGAAGAAGCAGCUCUGG

The validated siRNA sequence is from Promega and has been demonstrated to reduce Renilla luciferase expression. The scrambled siRNA is from Dharmacon (siCONTROL Non-Targeting siRNA #2) and contains the same nucleotides as the validated siRNA sequence but has at least four mismatches to any mouse gene. The first experimental siRNA was designed by our lab group, but was not available in the laboratory. Instead, the second experimental siRNA sequence was used in the experiment.

The Renilla luciferase gene was divided into six sections, with a different group each designing an siRNA for one section in the hope that at least one of the siRNAs would be effective. Our group designed the siRNA construct labeled Experimental siRNA 1 (see Table 1), which was intended to specifically target the 469-624bp region of the mRNA transcript of the Renilla Luciferase gene for degradation by the RNAi pathway. This particular siRNA design was chosen because several of its properties suggested that it was likely to specifically bind to the luciferase mRNA. This sequence has a low melting temperature and free energy, suggesting that it is not likely to form secondary structure that could interfere with binding. Moreover, of all candidate targets, this one had the fewest homologous sequences in the mouse genome, which suggested it would bind most specifically to the target. Due to time restrictions, the staff designed and pre-ordered an experimental siRNA (Experimental siRNA 2, shown in Table 1). This siRNA was designed using the same criteria as Experimental siRNA 1, though it had a different target. Because this sequence had been pre-ordered, it was used throughout the rest of

the experiment. A validated siRNA (shown in Table 1), which is known to decrease Renilla luciferase expression, was used so that efficacy of the experimental siRNA could be compared against a functional siRNA. To ensure that differences in expression were not simply due to the presence of any siRNA in the cell, an siRNA with a scrambled version of the validated siRNA sequence that has no homology to the mouse genome was also used.

Figure 1. Experimental siRNA does not decrease light emission in cells transfected with Renilla luciferase

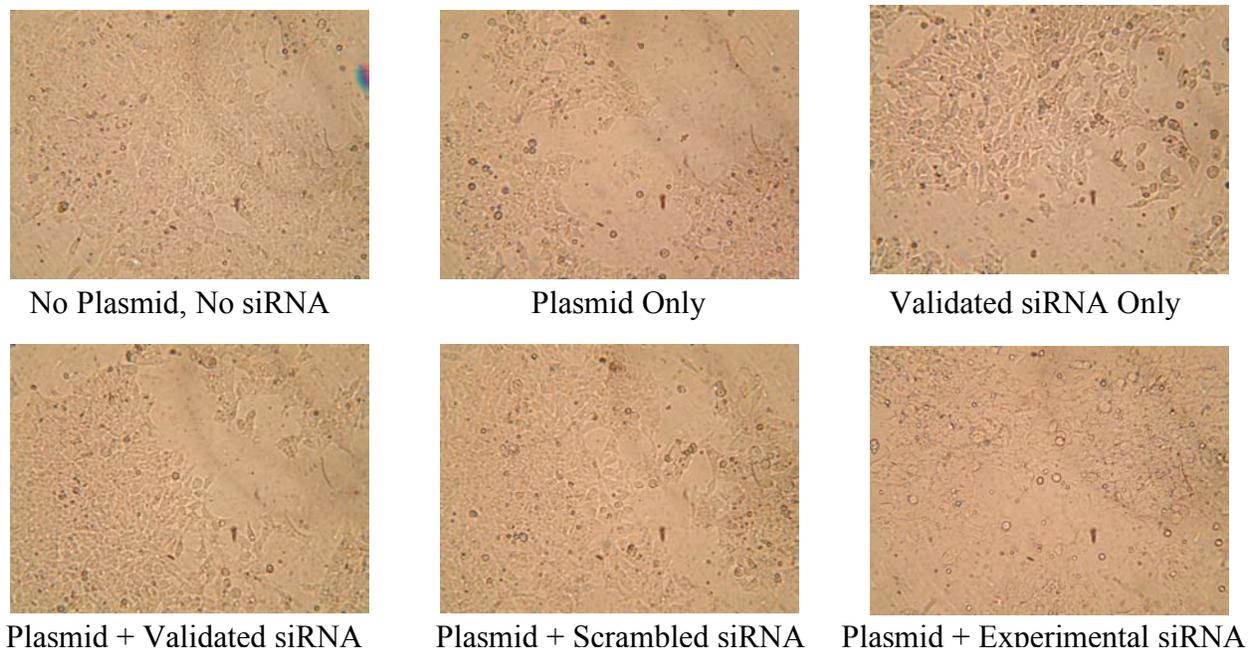


Mouse embryonic stem cells were transfected with the Renilla and firefly luciferase-containing psiCHECK2 plasmid only, the validated siRNA only, the plasmid and the validated siRNA, the plasmid and the scrambled siRNA, and the plasmid with the experimental siRNA. Two days after transfection, light emission from the cell lysates was measured. This experiment was performed with two replicates of each cell type, and the average light emitted by the two replicates is shown here. Error bars indicate 1 standard deviation above and below the mean value.

In order to determine the effect of the experimental siRNA on Renilla luciferase expression, we transfected cells with the Renilla and firefly luciferase-containing psiCHECK2 plasmid only, the validated siRNA only, the plasmid and the validated siRNA, the plasmid and the scrambled siRNA with no homology to the mouse genome, and the plasmid with the experimental siRNA. We then measured the light emitted by firefly and Renilla luciferases in the lysates from the transfected cells. Because the light emitted by firefly luciferase is approximately constant between all samples that contained a plasmid, it is reasonable to make general comparisons between the samples. The two types of cells that did not contain the psiCHECK2 plasmid that encoded the light-emitting Renilla and firefly luciferase genes – one of these cell types also contains the validated siRNA and the other contains no additional siRNA – are negative controls, and as seen in Figure 1, showed practically no light emission. The positive control cells,

which contained only the luciferase-encoding plasmid, had much higher light emission values. Likewise, the cells containing the plasmid and scrambled siRNA also had high emission values (see Figure 1). The experimental siRNA lysates emitted more light than the lysates from the cells that did not contain a plasmid, and than the lysates from the cells that contained the validated siRNA only. However, the error bars of the light emitted by the lysates from the cells that were treated with the experimental siRNA and the plasmid fall within the range of the error bars of the light emitted by the lysates from cells that were treated with the plasmid only, and within the error bars of the light emitted by the lysates from the cells that were treated with the plasmid and the scrambled siRNA, as seen in Figure 1.

Figure 2. The transfection of siRNA or psiCHECK2 plasmid into mouse embryonic stem cells leads to minor growth inhibition.



Cells transfected with either siRNA, plasmids, or both appear to exhibit less dense growth compared to cells that were not transfected with nucleic acids. These images were taken 48 hours after transfection at 10x magnification.

The transfected cells were visually monitored to determine how siRNA transfection affected growth. All samples contained cells that were healthy and growing, though the cells that were transfected appeared to be slightly less dense than those that did not contain plasmid or siRNA, as seen in Figure 2, suggesting that the transfections led to decreased cell growth.

Table 2. The experimental siRNA alters the expression of off-target genes

Observation	Possible Explanation
4,389 genes are differentially expressed 2x higher in cells containing the psiCHECK2 plasmid and the experimental siRNA 4,382 genes are differentially expressed 2x higher in cells containing the only the plasmid	It could be that experimental siRNA actually results in differential expression of the genes between the two samples, or slight differences in the cell's environment could produce these effects
35,229 genes show non-differential expression pattern	mRNA expression levels are largely the same in the samples transfected with only the psiCHECK2 plasmid, and those transfected with the plasmid and the experimental siRNA
0 genes with which the experimental siRNA has homology show differential expression patterns	The siRNA does not specifically bind to off-target mRNA transcripts
106/133 interferon genes show no differential expression pattern	It is unlikely that the interference pathway is responsible for the differential expression patterns

mRNA isolated from mouse embryonic stem cells transfected with only the luciferase-containing psiCHECK2 plasmid, and mRNA isolated from cells transfected with the plasmid and the experimental luciferase-targeting siRNA were compared on a microarray. Of the ~44,000 genes on the array, about 8,600 were differentially expressed between the two samples. No genes with which the experimental siRNA has homology were differentially expressed, and 106 of 133 interferon genes showed no differential expression between the two samples.

In order to look for off-target effects caused by the siRNA, we ran a microarray using cDNA reverse transcribed from mRNA from cell lysates that had been transfected with only the plasmid, and from cell lysates that had been treated with the plasmid and the experimental siRNA. This microarray shows that about 8,600 genes were differentially expressed between these two samples, as shown in Table 2. It is possible that these effects were due to mRNA degradation in the RNAi pathway, or that slight differences in the cells' environment led to these differential expression patterns. The siRNA was BLASTed against the mouse genome, and none of the genes to which it has the greatest homology showed differential expression between these two samples. Thus, any effects due to the siRNA were probably not due to specific binding. It is also possible that the addition of the siRNA activated the anti-viral interferon pathway, which would result in differential expression between the two samples. However, of the 133 interferon genes present in the microarray, 106 showed no differential expression (see table 2). The remaining 27 interferon genes that showed differential expression do not appear to be directly related. Thus, it appears that the experimental siRNA did result in some off-target gene regulation.

Table 3. mRNA that show the greatest differential expression between samples transfected with plasmid only and samples transfected with plasmid and experimental siRNA are unrelated

Gene Name	Systematic Name	Description	Log ₂ (adjusted plasmid only signal /adjusted plasmid & siRNA signal)
AK081157	AK081157	Unknown	-24.65
Fzd2	AK048420	Frizzled precursor	-24.50
Ptdsr	AK122317	mKIAA0585 protein	-24.34
Egfr	NM_207655	Epidermal growth factor receptor	-24.30
Pick1	AK090155	Protein that interacts with C kinase 1	-24.20
D030020J04Rik	AK142335	Unknown	-23.92
L1Md-Tf30	AK035064	Unknown	-23.82
2610028E06Rik	AK083757	Unknown	-23.64
Casp3	NM_009810	Caspase 3	-23.48
TC1658472	TC1658472	Lysin precursor	-23.39
Atp2a2	NM_009722	ATPase, Ca ⁺⁺ transporting	20.38
TC1657759	TC1657759	Ap3b1 protein	20.46
En2	NM_010134	Engrailed 2	20.48
Mrpl27	AK080324	Unknown	20.51
Tmed7	XM_128959	Emp24 protein transport domain	20.52
Ccdc117	NM_134033	Coiled-coil domain	20.92
AK035842	AK035842	Similar to histidine kinase dhkm	21.03
Slc5a2	NM_133254	Sodium/glucose cotransporter	21.04
Stambpl1	NM_029682	Stam binding protein	21.38
4932432K03Rik	NM_144535	Unknown	21.38

The 10 mRNA transcripts that showed the greatest difference in expression in the cells transfected with the plasmid only compared to the cells transfected with the plasmid and the experimental siRNA, and the 10 mRNA transcripts that showed the greatest difference in expression in the cells transfected with the plasmid and the experimental siRNA compared to those transfected with the experimental siRNA only are shown here. There does not appear to be any relationship between the proteins these mRNA transcripts produce.

In order to analyze the data collected from the microarray, we first accounted for background by subtracting the average of all median background signals over all genes from the signal measured from each gene. We chose to use the median background signal in order to avoid strong outlier signals that would skew the mean. For each gene, we then divided the signal values from the plasmid only sample by the signal values for the sample that had been treated with the plasmid and the experimental siRNA in order to obtain the fold change between their expression levels. To view these data more easily, we took the log₂ of the fold change for each gene (see table 3). Thus, genes that are expressed more than two-fold higher in the samples that were treated with the plasmid only compared to the samples treated with the plasmid and the siRNA will have positive log₂ values, and genes that are expressed more than two times higher in the samples that were treated with the plasmid and the siRNA compared to the samples treated only with the plasmid will have negative log₂ values. As can be seen in Table 3, there is no obvious

connection between the 20 genes that showed the greatest differential expression between the two samples; it does not appear that the experimental siRNA affected any specific off-target pathway. Moreover, none of these 20 genes are known to be involved in the interferon pathway, further suggesting that this pathway was not activated.

Discussion

siRNA is often used to alter gene expression at the post-translational level. In this experiment, we designed an siRNA to specifically target the Renilla luciferase gene. However, the variation of the light emitted by the lysate from cells transfected with both the experimental siRNA and the psiCHECK2 plasmid, which contains Renilla and firefly luciferase genes, was within the error bar of the light emitted by the lysates from cells transfected with the plasmid only and by the lysates from the cells transfected with the plasmid and the scrambled siRNA, suggesting the experimental siRNA is not able to reduce Renilla luciferase expression. Because of the difficulty of predicting the efficacy of siRNA designs this result seems reasonable; although we took many aspects of siRNA design into consideration, siRNA design is still not well understood, and thus there is a certain amount of randomness in the process.

Ekaterina Pak and Matthew Loper performed the same experiment and reported data showing the same luciferase expression trends (20.109 Luciferase URL); the error bars of the light emitted by the lysates of cells transfected with the experimental siRNA and the plasmid are within the error bars for the light emitted by the lysates from the cells transfected with the plasmid only and the cells transfected with the plasmid only and the scrambled siRNA. Though the two data sets follow the same trend, the Pak and Loper data show much lower emission from Renilla luciferase in all samples compared to our data, though the firefly values are similar in both data sets. This discrepancy could be due to a difference with the luciferin added to the samples to measure Renilla luciferase light emission. If the luciferin Pak and Loper used was more dilute or partially degraded, Renilla luciferase levels would be consistently decreased, as we see here.

The experimental siRNA's ineffectiveness in reducing Renilla luciferase levels could also be due to off-target effects that resulted in decreased regulation of the target mRNA. If the siRNA was used to induce RNAi of other transcripts, there would not be as much available to target the Renilla luciferase transcript. The microarray showed that about 8,600 mRNA transcripts were differentially expressed between the cells that were transfected with the plasmid only and those that were transfected with the plasmid and the experimental siRNA. However, the cells transfected with only the plasmid and those transfected with both the plasmid and the experimental siRNA showed the same level of expression for all mRNA transcripts to which the siRNA has the greatest homology. Thus, it is unlikely that the differential mRNA expression is due to specific siRNA binding. Additionally, we looked at the expression patterns of interferon genes, which the cell uses to defend against viral invasion, since the siRNA could be perceived as viral genetic material by the cell. While a few interferon genes are differentially expressed between the two samples, it is unclear that this pathway is activated. Thus, it seems likely that the siRNA affects the regulation of off-target genes.

However, it appears that the experimental siRNA's off-target effects did not significantly affect the growth of the cells into which it was transfected. After transfection, the cells were visually examined with a light microscope. Cells that had

been transfected with nucleic acids showed decreased growth compared to the cells that had been treated using the lipofection protocol, but to which no nucleic acids had been added. This decrease in growth was not more noticeable in cells transfected with the experimental siRNA than in any of the other transfected cells. Thus, it seems that the experimental siRNA itself had no effect on cell growth.

While this experiment has provided a wealth of information about the efficacy and effects of our experimental siRNA, there were also several limitations to this experiment that suggest future experiments to further elucidate the effects of the experimental siRNA. Foremost, the microarray we used contained the entire mouse genome, and thus was useful for looking at off-target effects, but it did not contain the luciferase gene that had been transfected into the mouse cells. As a result, it yielded no further information about the efficacy of the experimental siRNA. In the future, it would be valuable to include the Renilla and firefly luciferase genes in the microarray in order to obtain a second set of quantitative data describing the effect of the siRNA on its intended target. Moreover, it would be interesting to transfect cells with the plasmid and both the experimental and validated siRNAs to see if a combination of both siRNAs leads to greater Renilla luciferase depletion than either of the siRNAs by themselves.

This experiment illustrates the difficulty of using RNAi to regulate protein expression in the laboratory. Though we carefully considered the properties that are thought to be most important in designing an siRNA when we created the experimental siRNA, this siRNA cannot be considered effective and also has many off-target effects. This demonstrates that even our best understanding of siRNA design is not enough to consistently produce effective and specific siRNA. Unless significant strides are made in siRNA design, it may be more useful to implement traditional gene knockdown methods, like homologous recombination. Thus, while RNAi is a very useful technique in theory, more research must be done to increase its usefulness in practice.

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