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MicroRNA "SPONGE": PROOF OF CONCEPT FOR A NOVEL MicroRNA TARGET IDENTIFICATION TECHNIQUE

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ABSTRACT

This project provides proof of concept for a microRNA "sponge", a novel microRNA sequestration technique which will aid characterization of the function of different microRNAs by showing the effects of the depletion of specific cellular microRNAs. To prove that microRNA "sponges" will effectively bind and hold native microRNA *in vitro*, plasmids were constructed encoding luciferase reporter mRNAs whose 3' UTRs contain four experimental sponges to bind either microRNA Let7 or miR122, or to contain two mutant sequences that do not bind miRNAs. The plasmids were transfected into Huh7 and Hela cell lines, and the levels of luciferase activity were quantified by luminescence. The results showed a decreased luciferase activity for the sponge containing plasmids, but not the mutant plasmids, indicating they successfully bound their miRNAs to block reporter mRNA translation.

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BACKGROUND

MicroRNAs

Overview

MicroRNAs (miRNAs) are small RNA molecules found in the cytoplasm and nuclei of plants and animals. miRNAs are approximately 21 nucleotides long, and usually function by binding the 3'-untranslated regions (3'-UTR) of a target mRNA to block further translation of the mRNA, thus regulating the production of specific proteins (Lu et al., 2008). It is estimated that 1-4% of genes in the human genome encode miRNAs, and that any one miRNA could regulate up to 200 genes (Lim et al., 2003; Lu et al., 2008).

miRNA Functions

Each miRNA is thought to regulate multiple genes. Given the prediction that 200 to 500 unique miRNAs may be present in eukaryotes, the potential range of regulatory functions for miRNAs is enormous (Lim et al., 2003). It is expected that over one third of gene expression is regulated by miRNAs. miRNAs have been implicated in the regulation of a diverse array of functions, including early development (Reinhart et al., 2000), cell proliferation, cell death (Brennecke et al., 2003), apoptosis, fat metabolism (Xu et al., 2003), and cell differentiation (Dostie et al., 2003). Recent studies have revealed possible links between miRNA and cancer (Michael et al, 2003; Calin et al, 2004) , viral disease (Pfeffer et al., 2004), and developmental diseases (Krichevsky et al., 2003).

miRNA Mechanisms

The vast majority of miRNAs studied in animals act to regulate protein levels of a targeted gene or genes, apparently without affecting the levels of the corresponding mRNAs. This situation contrasts with the function of small interfering RNAs (siRNAs) where the target mRNAs are usually cleaved and degraded (Chu and Rana, 2006). Studies on miRNAs from *C. elegans* show that miRNA does not affect mRNA polyA tail length, transport by exportin-5 into the cytoplasm, nor entry into the appropriate polysomes for translation. Thus, steps downstream from translation are likely influenced by miRNA function (Olsen and Ambros, 1999).

The RNA-induced silencing complex (RISC) is understood to be a major factor in blocking translation of mRNA, and is already known to be an important part of how siRNAs function to regulate protein levels. RISC is the primary molecule employed by cells to regulate the induced silencing of mRNA translation. The miRNA must associate with RISC in order to accomplish this regulation of translation. Immunoprecipitation with antibodies against RISC yields endogenous miRNAs, providing further evidence that RISC most likely associates with miRNAs *in vivo*. miRNAs in animals bind imperfectly to its binding site, presenting a non-complementary bulge in the middle of the site. This results in the RISC/miRNA complex binding and holding the target mRNA and blocking translation.

The situation with miRNAs contrasts with the function of siRNAs. siRNAs also complex with RISC to regulate translation, but the resulting mechanism for mRNA silencing is different (**Figure-1**). siRNA binds perfectly with the target site (diagram right side), and this perfect complementarity leads to cleavage of the mRNA at the binding site (Chu and Rana, 2006). This process of targeted cleavage by RISC is known as RNA interference or RNAi (Filipowicz, 2005). In plants, miRNAs are more often perfectly complementary to their binding sites, and thus also

degrade target mRNAs through the RNAi pathway, although it is likely that plants also possess imperfectly binding miRNAs. In addition to these processes, there is further evidence that the RISC/miRNA complex also acts to block the transcription of some genes in plants, yeast, and possibly in animals, by guiding the methylation of chromatin in the nucleus (Rhoades et al., 2002).

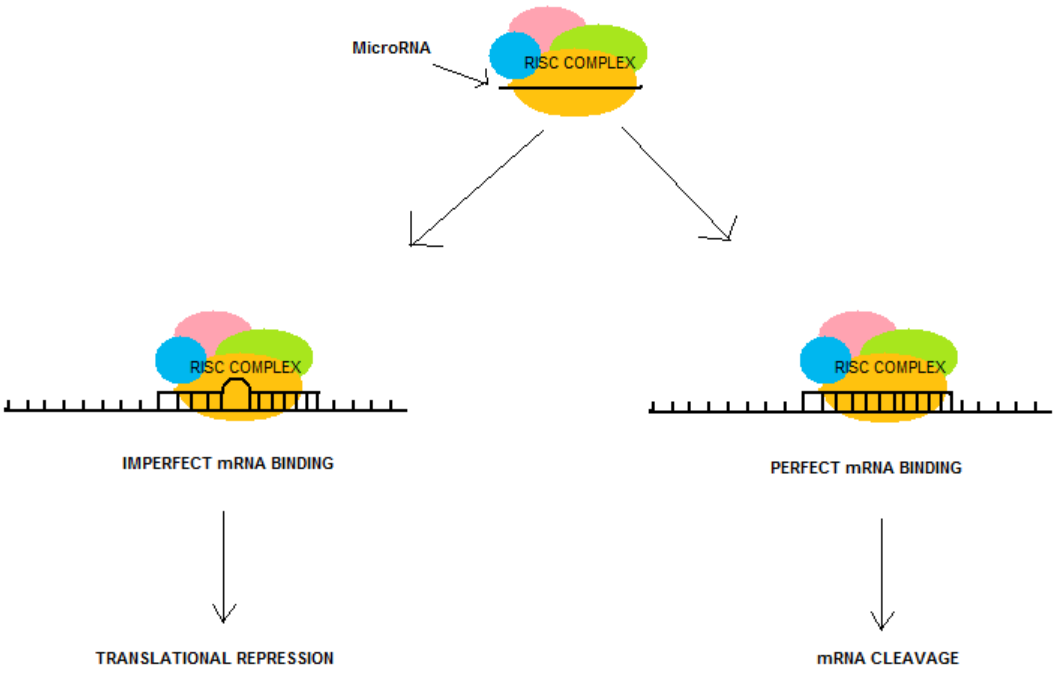

Figure 1: RISC/miRNA-Induced mRNA Repression Versus Cleavage. The diagram on the left side shows translational repression resulting from imperfect base-paired hybrids between a miRNA and its mRNA target sequence. The right side shows mRNA degradation resulting from miRNA forming a perfect hybrid with its mRNA target. Diagram drawn by MQP author.

It has also been shown that mRNAs containing multiple miRNA binding sites are more highly affected by miRNA translation regulation than those only possessing a single binding site. It has been shown that for mRNAs containing 2, 4, or 6 miRNA binding sites, translation decreases more with additional binding sites. This evidence suggests that, at least in animals, gene expression can be fine-tuned by varying the concentrations and types of miRNAs in the cell

(Zeng and Cullen, 2003). Such an miRNA network would help explain why many miRNAs appear to have such a wide array of functions, and why the expression of certain genes seems to be so complex. The ability of some miRNAs to regulate mRNA translation but not degradation could help explain why gene expression based upon mRNA analysis alone does not always correlate specifically with protein expression (Kern et al., 2003).

miRNA Production

The production of miRNAs is still not entirely understood. Mounting evidence seems to show that miRNAs are originally transcribed as part of a primary or "Pri-miRNA" (**Figure-2**, see within the nucleus), a long single-stranded RNA molecule which forms "hairpin loop" double-stranded structures (Lee and Ambros, 2001; Winter et al., 2009). These hairpin loops are processed into 70 to 100 nucleotide double-stranded "Pre-miRNA" molecules (Figure-2, nucleus) by the ribonuclease "Drosha" (Lee and Ambros, 2001). These molecules are then transported into the cytoplasm by the export protein exportin-5 (diagram, purple), where they are further processed by another ribonuclease called "Dicer" (diagram, blue) (Yi et al., 2003). The resulting molecule is double-stranded, with one of the strands being the mature miRNA. The mature miRNA strand then dissociates from the double-stranded molecule (diagram, lower) and associates with the protein complex RISC. This final complex of RISC and the mature miRNA strand is the mechanism used in mRNA regulation (Schwarz et al., 2003).

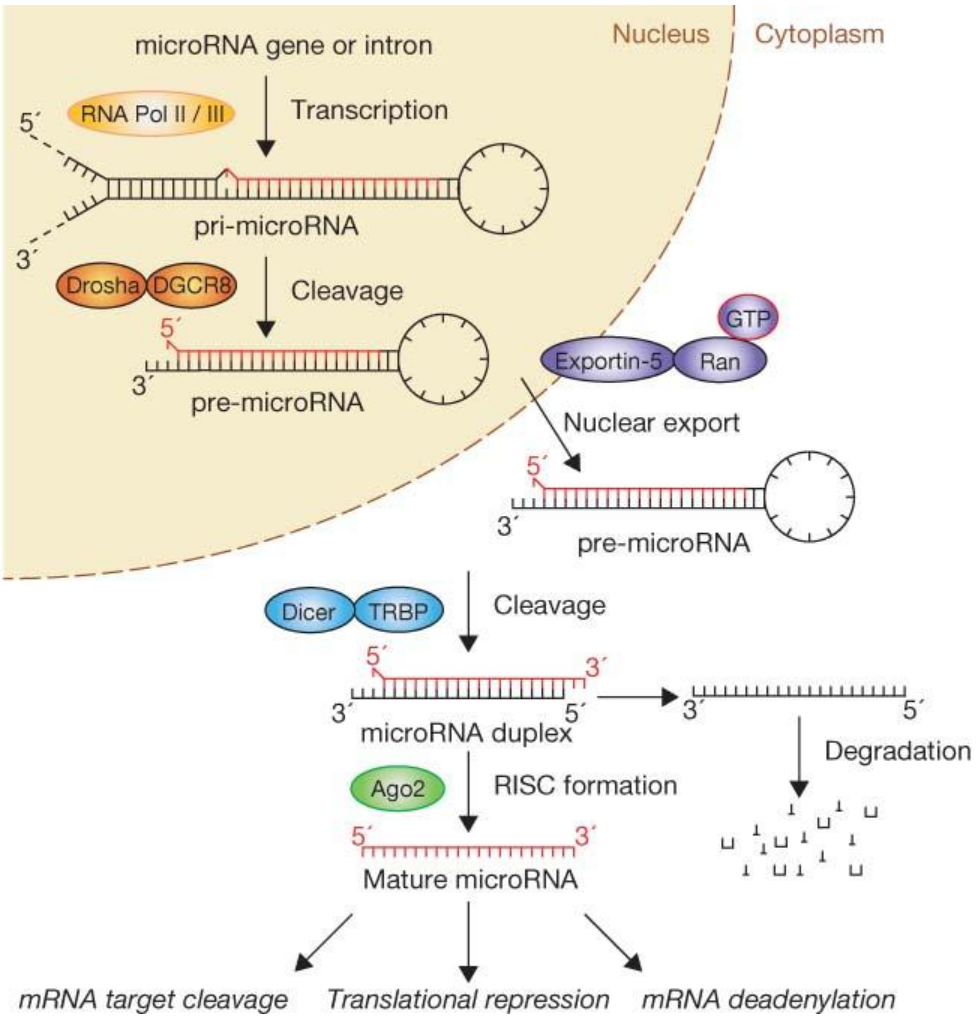

Figure 2: MicroRNA Production. Diagram shows the formation of Pri-miRNAs, Pre-miRNAs, douoble-stranded miRNAs, and mature single-stranded miRNAs. (Winter et al., 2009)

Role of miRNAs in Disease

Given the wide array of genes expected to be regulated in some way by miRNAs in animals, it is expected that miRNAs and their mutagenesis likely plays a role in various diseases. It is expected that a mutation in an miRNA gene may have just as great an pathogenic role in genetic diseases as a mutation in a protein encoding gene. Three different types of mutations should cause a malfunction in the miRNA regulation of a gene: (1) a mutation in the sequence of the miRNA itself, (2) a mutation in the miRNA target-binding site on an mRNA, and (3) a

mutation in the proteins which process and export the miRNAs to their final mature form (Meola et al., 2009).

Interestingly, it was initially argued that miRNA mutations could not play a significant role in genetic diseases for two main reasons. First, a mutation which would result in a complete failure to regulate the target mRNA(s) of a miRNA would seemingly be incompatible with the cell surviving at all. And second, given the large amount of redundancy in most cells' use of miRNAs, a mutation in a single miRNA may not cause even a measurable change in cell function. However, it has since been shown that miRNAs do indeed play a strong pathogenic role in the development of several genetic diseases (Meola et al., 2009).

One study showed that a knockdown of miRNAs expressed in cerebellar regions of mice results in a gradual neurodegeneration (Schaefer et al., 2007). This suggests that a mutation in miRNAs could easily be a cause of neurodegenerative diseases. Another study showed that a knockdown of miRNA expressed in T-cells severely affects cell functionality; mice with a knockout of the ribonuclease “dicer”, critical in miRNA development, lose their ability to properly form functioning T-cells, and develop a severe auto-immune disease within weeks of birth (Liston et al., 2008). This dicer knockout technique has been used to downregulate the expression of miRNAs in many other tissues, and the result is most often a significant dysfunction. These results demonstrate the role that miRNA plays in cell development and function in animals, and their potential roles in diseases (Meola et al., 2009).

Role of miRNAs in Cancer

MicroRNAs have also been implicated in the development of cancer in animals. It is likely that various miRNAs have multiple roles in controlling cell growth and death, so any

mutation in a miRNA involved in regulating growth or death pathways could lead to cancerous growth *in vivo*. A miRNA involved in the development of cancer is called an *oncomir* (Meola et al., 2009).

For example, studies have shown that miRNAs derived from the miR-12-92 polycistron may play a role in the development of B-cell lymphomas. MicroRNAs from this locus are substantially elevated in tissue samples from these cancers in comparison to normal B-cell samples (He et al., 2005). There is further evidence that miRNAs miR-15a and miR-16-1 are deleted in cases of chronic lymphocytic leukemia. The downregulation of these miRNAs results in a lack of regulation of downstream translation of the proto-oncogenes MCL-1 and TCL-1, which results in the development of leukemia in humans (Mraz et al., 2009). These are only two examples of studies which have begun to show the role of miRNA in cancer, but mounting evidence indicates that malfunctioning miRNAs may have a role in most human cancers (Meola et al., 2009).

MicroRNA Target Identification

The bioinformatic development of a sequence library of miRNA target sites and target mRNAs would be a major step toward furthering miRNA research by identifying potential miRNA/mRNA interactions which could be subsequently proven by mutagenesis. Unfortunately, in animals this is particularly challenging because of the imperfect complementarity of the miRNA/mRNA interaction. Moreover, only a few predicted mRNA targets of miRNAs have been experimentally confirmed (Wang and Wang, 2006). Thus, a technique must be developed to rapidly determine which mRNAs are targeted by a specific miRNA.

Several different techniques have been studied for their effectiveness in miRNA target identification. Some groups have created bioinformatic algorithms which theoretically determine the predicted target of a miRNA based on various elements of the miRNA target recognition process such as sequence complementarity, binding free energy, and the level of cross-species conservation of the sequence (Wang and Wang, 2006). In another approach, a gene encoding a specific miRNA is transfected into a desired cell line, and *over-expressed* to observe changes in cell function as quantified by microarrays, which can potentially reveal which mRNAs are targeted by the enhanced miRNA (Wang and Wang, 2006). Another approach is to *knock down* the expression of a specific miRNA, then observe alterations in gene expression to predict mRNA targets. Although plasmids encoding siRNAs are usually used to knock down specific gene expression, this RNAi process cannot be used for this purpose as the target for knock down is an miRNA, not a mRNA. Thus, a different technique must be developed to knock down the miRNA. Although *total* miRNA production can be knocked down through the genetic knockout of "Drosha" or "Dicer", this does not lower production of a *specific* miRNA. One possible approach for this purpose is to create a microRNA "sponge".

MicroRNA "Sponges"

A miRNA "sponge" is a novel technique consisting of a single-stranded mRNA with no translational start codon, but whose sequence contains repeated copies of a specific miRNA binding site. Each sponge would be able to bind and hold up to 7 miRNA copies. Expressing the sponge gene in a cell could result in a decrease in the cellular levels of the target miRNA, thus causing the deregulation of the mRNA regulated by the miRNA. This deregulation might produce a quantifiable change in the biochemistry or microarray profile of the cell, which could

then be measured. Building a library of the specific targets of each miRNA would allow for a much greater application of miRNA research to various diseases.

PROJECT PURPOSE

The purpose of this MQP project is to demonstrate proof of concept in the development of a functioning miRNA sponge as a novel technique for decreasing the cellular levels of specific miRNAs to help identify their mRNA targets. Although technically, a sponge mRNA would contain several miRNA-binding sequences and would not be translated (i.e. would not contain any translational start codon), for purposes of this project the "sponge" will consist of a functional firefly (FF) luciferase reporter mRNA containing multiple miRNA-binding sequences within its 3' UTR to allow assay of alterations in FF luciferase activity as a measure of miRNA binding. Binding sequences for microRNAs Let7 and miR122 will be used to bind their respective miRNAs. When bound, we expect to see a reduced FF luciferase translation and activity. A repeat sequence containing 7 copies of the Let7 or miR122 miRNA binding sites, or their mutated binding sites, will be inserted into plasmid pGL3, which already contains the FF luciferase gene, to create four experimental sponges: Let7, mut-Let7, miR122, and mut-miR122. After the sponge sequences have been cloned into pGL3, the Let7 sponges will be transfected into Huh-7 and Hela cell lines, alongside a Renilla luciferase transfection control plasmid. Once in the cell, the FF luciferase mRNA will be expressed containing the sponge repeat sequence within its 3' UTR. The effective binding of endogenous miRNA to the sponge will result in a knockdown of expression of the FF luciferase gene by blocking its translation, as quantified by a Promega Dual Luciferase Assay.

In the future, RT-PCR or Northern blots will be used to verify an unchanged level of FF luciferase mRNA (sequestration not degradation) under experimental conditions where the FF luciferase *activity* decreases. In addition, miRNA Northern blots will be used to determine

whether the cellular levels of specific miRNAs changes detectably in the presence of the sponge. And *in situ* hybridization assays will be used to determine whether the sponge treatments alter mRNA localization.

METHODS

Sponge Plasmid Production

Sponge inserts containing 7X miRNA binding sites were synthesized commercially as single stranded DNAs, then were annealed to make double stranded inserts containing XbaI and ApaI overhangs (Guanping Gao, personal communication). The sequences of the binding sites for each sponge are shown in **Table 1**.

Target miRNA	7X miRNA-Binding Repeat Sequence
Let7	AACTATACAAAACCTACCTCA
mutLet7	AACTATACAAAACCTA**AAGA**A
MiR122	CAAACACCATACAACACTCCA
mutMiR122	CAAACACCATACAACA**AGAA**A

Table 1: miRNA-Binding Sequences for Each Experimental MicroRNA Sponge. Let 7 and miR122 binding sequences are shown. Mutant nucleotides are shown in red.

Firefly luciferase reporter plasmid pGL3 was doubled-digested by XbaI and ApaI to prepare its multiple cloning site for receiving a sponge insert. First an overnight digestion by ApaI was performed at 25^{o}C in NEB Enzyme buffer 4 with BSA supplement. The following morning, XbaI was added to the digestion mix, and the temperature was raised to 37^{o}C for 3 hours to complete the digestion.

Each sponge insert was ligated into the XbaI-ApaI cut pGL3 using a T4 DNA Ligase procedure in a 20 µl total reaction volume. 2 uL of cut pGL3 at a concentration of 50 ng/µl were combined with 3 µl of sponge insert at a concentration of 150 ng/µl. To the mix was added 1 µl NEB T4 DNA Ligase and 10 µL of 10X T4 Ligase buffer. 4 µl of water were added to bring the reaction tube to a total of 20 µL. The ligation reaction was incubated at 16^{o}C in a thermocycler

overnight. The ligation product was then transformed into DH5α cells, and plated to ampicillin plates to select for transformed cells. Colonies were picked and grown in 3 mL of LB broth. Qiagen mini-prep kit procedure was used to isolate plasmid DNA for sequencing. Suspended cells were spun down then resuspended for solution 1, 2, 3 lysis. DNA was precipitated, and vacuum filtration columns were used to isolate plasmid DNA.

Transfection of Cell Lines

Once each experimental sponge insert had been successfully cloned into pGL3, the Let7 and mutant Let7 experimental plasmids were transfected into Huh-7 and Hela cell lines, along with a Renilla luciferase control plasmid pGL4. Each experimental plasmid and control was mixed with Invitrogen Lipofectamine^{TM} and added to 500 μl of cell culture. Each transfection was allowed to incubate at 37^{o}C in a CO_2 incubator overnight to complete transfection. After transfection, the cultures were allowed to incubate for a further 72 hours to achieve expression. A separate cell line was transfected with pGL3 without the sponge insert as a control, and one culture each of Hela and Huh-7 cells were left untransfected (UTC) as negative controls.

Dual Luciferase Assay

In order to quantify the resulting firefly and Renilla luciferase activities in the cell lines, a Promega Dual-Luciferase® Reporter Assay System was employed. Each cell culture, in multi-well plates, were mixed with Promega Passive Lysis Buffer (PBL) and shaken for 15 minutes. The resulting lysates were stored at 4^{o}C for assay with the Dual-Luciferase Reporter Assay Kit. Per the instructions of the kit, the lysates were not cleared by centrifugation because it is not necessary.

20 µl of each test sample was added to a luminometer tube containing 100 µl of Luciferase Assay Reagent II (LAR II), and placed into the luminometer without vortexing. This reagent induces luminescence from the firefly luciferase expressed from pGL3. After recording the luminescence, 100 µl of Stop & Glo® Reagent was added to the reaction, vortexed quickly, and the tube was returned to the luminometer. This reagent quenches the luminescence of firefly luminescence and simultaneously induces the reaction of the Renilla luciferase for control luminescence (as a transfection marker). After both readings had been taken, the sample was discarded and the next sample was assayed.

RESULTS

Cloning Sponge Sequences Into Expression Plasmid pGL3

The four synthetic sponge sequence inserts (Let7, mut-Let7, miR122, and mut-miR122), were assembled from synthetic oligos (data not shown) to make double-stranded DNA duplexes for restriction digestion and cloning. Each sponge was 121 bp long, and contained a 5' XbaI site and a 3' ApaI restriction site. This restriction site orientation allows the sponge DNAs to be cloned into a matching site in firefly (FF) luciferase expression plasmid pGL3 immediately downstream (3') of the Luciferase reporter to affect its mRNA's expression. To add an ApaI a restriction site to pGL3 downstream of the Luciferase reporter, a synthetic linker ~30 basepairs long was inserted at the XbaI restriction site (**Figure-3**). With the XbaI and ApaI enzymes in place in pGL3, each annealed sponge assembly could then be cloned into this site with the 5' end oriented upstream at the XbaI site, and the 3' end downstream at the ApaI site. The ApaI-modified pGL3 was double-digested with XbaI and ApaI (data not shown), and the sponge was ligated into the site (**Figure-3**).

After the ligation of sponge inserts into pGL3, each ligation reaction was transformed into competent *E. coli* DH5α cells, and allowed to incubate overnight on ampicillin plates to select for cells containing plasmids. After overnight culture incubation, several randomly chosen ampicillin-resistant colonies were picked, and grown in 3mL cultures for a Qiagen© miniprep. Following plasmid DNA extraction, each test sample was then assayed for the presence of the 121 bp sponge insert by double restriction digestion. The screening used a BamHI site farther downstream than the ApaI site, so positives should produce a fragment approximately 600 bp long, while negatives produce a fragment approximately 500 bp long. The plasmids were double

digested with XbaI/BamHI, and analyzed by gel electrophoresis alongside digested and undigested parental pGL3 for comparison (data not shown).

Positive plasmids containing sponge inserts were confirmed by sending the samples for sequence analysis by Operon Inc. and their SimpleSeq^{TM} service. The sequencing results (data not shown) indicated that all positives contained the expected four sponge sequences. Selected positives were then used to grow 2 liter cultures for large scale plasmid isolations and CsCl gradients to produce high quality plasmid DNA for performing mammalian cell transfections.

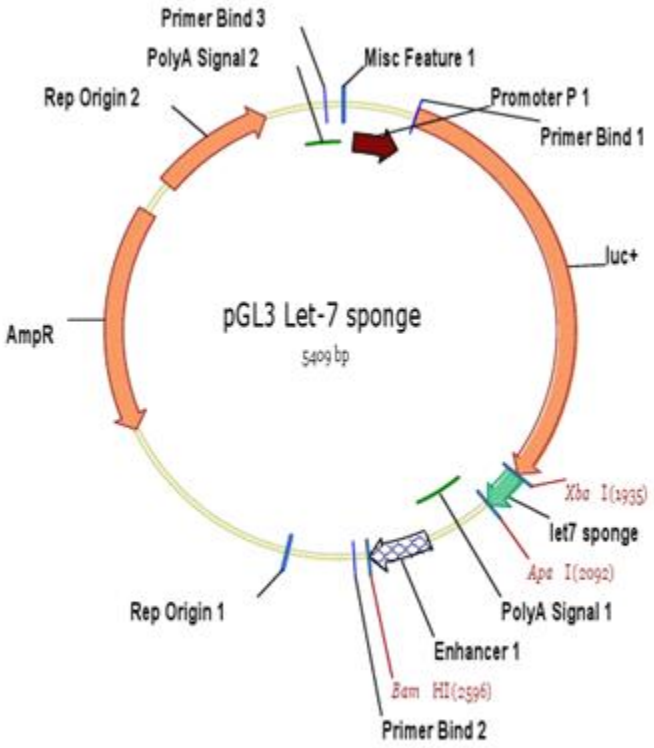

Figure 3: Diagram of Expression Plasmid pGL3 Containing a Let7 Sponge Downstream from Luciferase. The diagram shows the pGL3 parental plasmid containing a luciferase reporter gene (orange, diagram right) with a Let7 sponge sequence (green arrow) cloned into the XbaI/ApaI site immediately downstream of the reporter. The sponge sequence lies upstream of the poly(A) transcription termination signal (green line), so it will be present within the 3' UTR of the luciferase mRNA where it will bind endogenous cellular Let7 miRNA to induce blockage of luciferase mRNA translation.

Cell Transfections and Luciferase Assays

CsCl-purified plasmid DNAs were transfected into two types of mammalian cell lines, Huh7 and Hela cells. These two cell lines were chosen because they each contain the target miRNA Let7, whereas cell lines such as HEK293 do not. The cells were also double transfected with a Renilla luciferase reporter plasmid to monitor transfection efficiency. Following 72 hrs of incubation to ensure expression of the Luciferase reporter (mRNA and protein), cell lysates were prepared and assayed for each type of Luciferase (firefly luciferase from the engineered pGL3 containing the miRNA sponges, and Renilla luciferase) using a Dual-Luciferase Assay (Promega®). The average luminescence for each experimental sample was measured in triplicate, and the mean is shown in **Figure-4**.

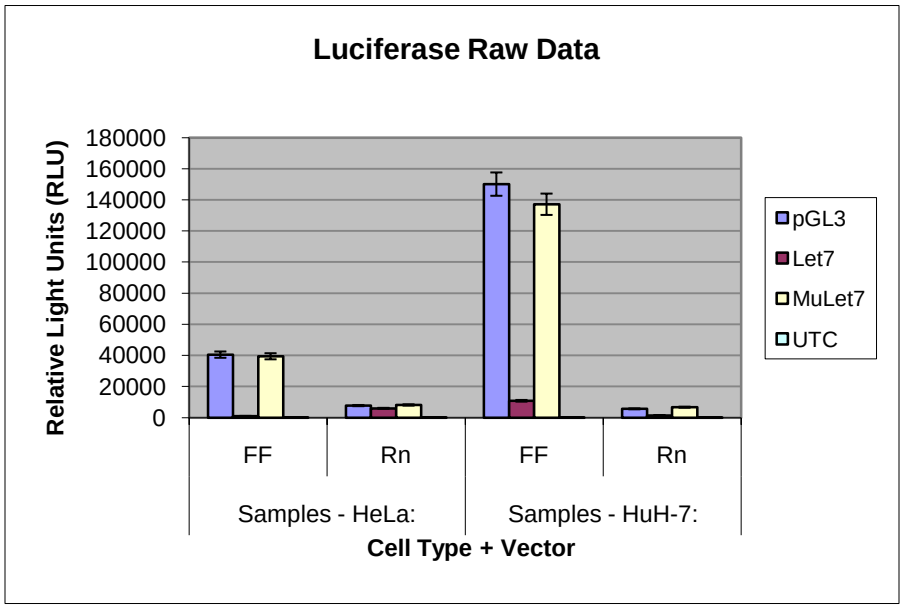

Figure 4: Average Luminescence of Each Transfected Cell Line. A dual luciferase assay was performed for firefly luciferase (FF) (expressed from the engineered pGL3 containing the sponge sequences), and for renilla luciferase (RN) (expressed from the transfection control plasmid). Each histobar represents the mean of three observations. Error bars denote standard error. FF luciferase activity was found to be high in parental pGL3 and Mut-Let7 plasmids (not binding miRNA), and low in Let7 plasmid or untransfected cells (UTC). The Renilla control luciferase is similar in each cell line.

For each cell line, Firefly (FF) luciferase activity (expressed from pGL3) is high in parental pGL3 and in Mut-Let7 plasmids, neither of which contains a Let7 miRNA binding site. And FF luciferase activity is low in the Let7 plasmid and in untransfected cells (UTC). The activity of the Renilla control luciferase is similar in each cell line. The low level of FF luciferase observed for the Let7 plasmid likely results from luciferase mRNA sequesteration induced by endogenous cellular miRNA Let7 binding its sponge sequence at the 3' UTR of the mRNA. Thus, the data indicates that the Let7 sponge was highly effective in binding endogenous Let7 in each cell line, and the binding was sequence specific.

Due to time constraints, the miR122 sponge cloned as a part of this project was not tested, nor were the cellular levels of luciferase mRNA as assayed by RT-PCR.

DISCUSSION

The data shown in this project demonstrate that the microRNA sponge technique applied to miRNA Let7 was effective in binding and holding endogenous miRNA *in vitro,* as evidenced by the decreased activity of Firefly (FF) luciferase expressed from the mRNA containing seven Let7 binding sequences in its 3' UTR. A clear decreased activity of FF luciferase was observed in each human cell line transfected with the Let7 plasmid, but not for cells transfected with the mutant Let7 plasmid, indicating base-pairing interactions are required for the activity decrease. Let7 binding sites perfectly complementary to Let7 miRNA should be more effective in the decrease than mutated binding sites that bind miRNA less efficiently.

This data shows that the miRNA sponge concept is effective and has potential for future applications *in vivo* for both miRNA research and therapeutics. The ability of this technique to decrease cellular levels of specific miRNAs should help determine the effects of the depletion on genes affected by specific miRNAs. A bioinformatics approach for theoretically identifying potential miRNA binding sites in the UTRs of mRNAs has a somwhat limited potential (Wang and Wang, 2006). The sponge technique should allow for faster and easier identification of target sites to aid our understanding and use of miRNAs, instead of creating genetic knockouts or generating complex bioinformatic algorithms (Wang and Wang, 2006).

The work performed in this project encountered some difficulties. The Let7, mut-Let7, and mut-miR122 sponge sequences were all cloned into plasmid pGL3 within the first month of the project, however the miR122 sequence remained stubborn. Repeated ligations, transformations, and screenings yielded many plasmid DNA samples containing a ~121 bp

insert, but whose sequence did not match that of the purchased synthetic DNA oligos. This problem continued for several months until a second set of miR122 sponge inserts was annealed and cloned into the plasmid successfully.

This cloning problem led to this project ceasing at the proof of concept stage. Not tested in the luciferase assays were either of the two cloned miR22 plasmids. Nor was RT-PCR used to assay the cellular levels of luciferase mRNA to determine whether the levels remained unchanged (sequestered) as translation decreased. Also not tested was the RT-PCR of any mRNA known to be affected by cellular levels of Let7 or miR122 to determine whether a lowering of specific miRNAs had indeed occurred.

Allowed more time, this project could also have moved into *in vivo* studies with mice to determine whether infection with viruses engineered with sponge sequences affect gene expression patterns in mice. So the next step in the development of this technique involves the incorporation of sponge sequences into an Adeno-Associated Virus (AAV) gene therapy vector. The AAV vector is a new developing technique for gene therapy which takes advantage of the inability of AAV to recombine with host DNA to reconstitute its pathogenicity. AAV also has an extremely low risk of insertion near any known oncogene, making it very promising as a gene therapy vector. The use of AAV vectors to deliver miRNA sponges *in vivo* will allow the effects of miRNA lowering on complex organ systems. The next target for research *in vivo* is a family of miRNAs known to be involved in the development of familial hypercholesterolemia. The sponge will be used to knockdown overexpressed levels of an miRNA which helps cause the disease. This potential therapeutic use would be a major development in our understanding of the role of miRNA in disease, and potential treatments for diseases.

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