MQP-BIO-DSA-9196

Silencing the HSL Gene in Human 293 Cells

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

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January 10, 2007

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ABSTRACT

Hormone-sensitive lipase (HSL) is thought to be the major enzyme responsible for the release of free fatty acids from adipose tissues. HSL hydrolyses triacylglycerides to diacylglyceride, releasing free fatty acids from fats (lipolysis). Using RNAi to silence the gene would be advantageous in the study of obesity, so three highly effective 19-nucleotide short hairpin RNAs (shRNAs 1-3) were designed against the HSL gene. Their ability to reduce HSL protein levels was verified in human 293 cells using immunoblots. shRNA-2 sequence was inserted into an adenovirus genome, and a high titer viral solution was created which could be used in the future to silence the HSL gene *in vivo*.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Keith Luo for allowing me to work in his research laboratory at Welgen, Inc (Worcester, Massachusetts) and for his help and instruction throughout the entire project. I would also like to thank Michelle Paksosa for her assistance in the laboratory, with managing the data, and with investigating laboratory procedures. Finally, I would also like to acknowledge Dave Adams for focusing this project and for his assistance on the MQP report.

2. BACKGROUND

2.1. Post-Transcriptional Gene Silencing (PTGS)

2.1.1. History and Discovery of Gene Silencing

Two forms of gene silencing have been discovered: transcriptional gene silencing (TGS), and post-transcriptional gene silencing (PTGS). TGS involves the gene-specific methylation which helps prevent the synthesis of unwanted RNA in the cell. PTGS is a complex mechanism involving RNA inhibition or interference (RNAi), small inhibitor RNAs (siRNAs) or microRNAs (miRNAs), and double-stranded RNA nucleases, and this process affects RNA synthesis and degradation. For the purpose of this MQP, the focus will be upon post-transcriptional gene silencing. The PGTS process was first noticed in plants such as petunias (Baulcombe, 1996), and was considered for a long time to be a rare phenomenon. Research has since found that PTGS has many applications in scientific research, and the discovery of more organisms that use this mechanism has lead to many uses of gene silencing technology in the laboratory.

The original discovery of gene silencing began with Rich Gorgenson and his study of petunias (Napoli, *et al.*, 1990). When he and his colleagues introduced a pigment-producing gene into petunias under the control of a previously powerful promoter, it was expected that the flowers would produce a richer purple color. Instead, the flowers were pale or even white (Napoli, *et al.*, 1990; Ambion, 2002). At that time, this process was called "cosuppression" due to the fact that both the petunia's natural color genes as well as the introduced gene were suppressed (Ambion 2002).

Cosuppression was later found to exist in a number of organisms, particularly fungi such as *Neurosporra crassa*, flies such as *Drosophila melanogaster,* nematodes such as *C. elegans*, and many forms of bacteria (Mechler, 2002). Through the study of mutations in gene silencing, several proteins have been found to be involved in the PTGS process including PPD, Dicer, Helicases, and RNA dependent RNA polymerase (RdRP). Mutations in the genes for these proteins inhibit some, but not all, of PTGS activity

(Mechler, 2002). Gene silencing can be induced by in the introduction of transgenes or certain viruses into cells, and from recent experiments is has been determined that double stranded RNA (dsRNA) can initiate the gene silencing within plants, nematodes, and flies (Ambion, 2002). In fungi, the process of PTGS through the introduction of a transgene is known as quelling. The injection of dsRNA was shown to completely silence homologous gene expression in nematodes (Fire, *et al.*, 1998). The injection of dsRNA into cells to induce PTGS is now known as RNA interference (RNAi).

2.1.2. PTGS: The Mechanism of Gene Silencing and RNAi

The effects of gene silencing are noticed in the cytoplasm, rather than in the genes themselves. Transcripts of genes are produced, but they are rapidly degraded in the cytoplasm and thus do not accumulate or translate into proteins. Importantly, the specific genes which are supressed have 3' sequences that are identical to, or very similar to, the dsRNA which is introduced into the cell (Watson, 2004).

The RNaseIII-like enzyme known as Dicer (Figure-1) recognizes and digests the long dsRNA into smaller fragments. These fragments, which are typically about 23 nucleotides long, are often called small interfering RNAs (siRNAs) and are responsible for the inhibition of the homologous mRNA. SiRNAs can also be manufactured outside of the cell and introduced in the laboratory. These siRNAs trigger the destruction of the mRNA from the homologous gene; they inhibit the translation of that mRNA, and at times they induce modifications in the chromatin within the promoter of the homologous gene to silence the gene (Watson, 2004).

Several processes take place which lead to this inhibition within the cell, and most of them use the same cellular machinery. Proteins from the Argonaut family interact with the siRNA to form a complex known as RISC (RNA-induced silencing complex) (Figure-1, center of the diagram) (RNAi Web, 2004). The siRNA is denatured in an ATP-dependent manner (Watson, 2004) and the RISC complex binds to a strand mRNA transcribed from the homologous gene that is complementary to that of the siRNA. Depending upon the match between the siRNA and the target mRNA, the mRNA is either silenced or degraded by a nuclease property of RISC (Figure 1, bottom of the diagram) (Watson, 2004).

RISC can also enter the nucleus and associate with complementary stands of homologous DNA to the siRNA in order to silence the gene. The complex binds to the complementary region and recruits other proteins which modify the chromatin around the gene, which silences transcription.

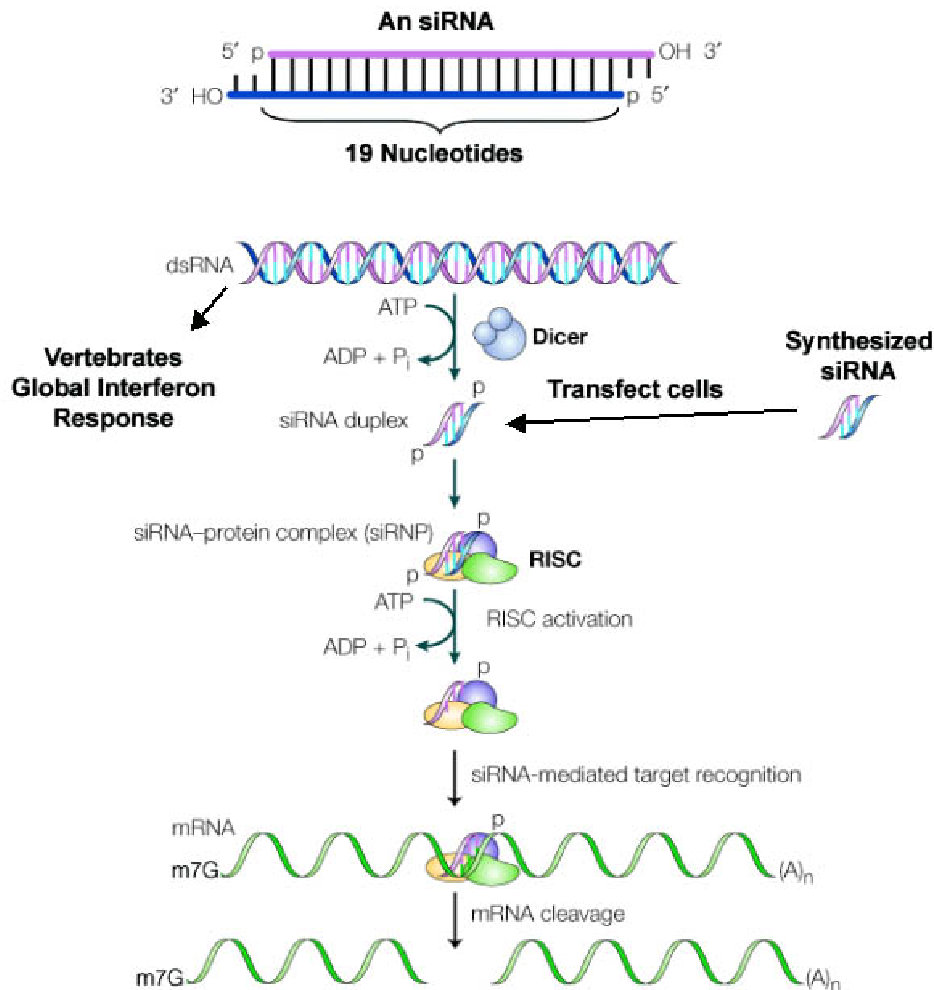

Figure 1: RNAi as induced by siRNA (Shapiro, 2003)

The process of RNAi is expremely efficient; only small amounts of dsRNA need be introduced into a cell in order to incite gene silencing because the siRNAs are recycled. The strenth of this reaction is further increased by RNA-dependent RNA polymerase, which is required in many cases of RNAi, and amplifies the siRNA after it is recruited to the mRNA by the original siRNA (Watson, 2004).

2.1.3. Applications of Gene Silencing in the Laboratory and Research

Potential uses for gene silencing as a functional genomics tool are the focus of much research. RNAi can be used to ascertain the fuction of specific genes; a gene in a cell may be selectively silenced and the resulting effects upon the cell can then be determined. Transfection of siRNAs is beginning to be widely used in mammalian cell systems (Ambion, 2002). Originally, siRNAs were produced synthetically chemically, but now methods that allow for *in vitro* transcription of siRNAs from plasmids or viruses are sometimes used. These vectors can be constructed to express small, tight double-stranded hairpin loops termed short hairpin RNAs (shRNAs) that get recognized like siRNA and carry out gene specific silencing. A number of companies also produce custom siRNAs as a service industry to the biotech industry, and microinjection of these RNAs is also a common method for introducing siRNAs into the cell.

2.1.4. pQuiet Plasmid as a Vector for Inducing RNAi

The pQuiet plasmid (Figure-2) is one such vector that expresses small hairpin loops of RNA for gene silencing. It was developed and is commercially available through Welgen, Inc. (Worcester. MA). This system has been widely used in different academic and industrial settings. Several authors silencing important genes have reported success with this system (Sheng *et al,* 2006; Miyoshi *et al*., 2006). pQuiet-U6 uses the human U6 snRNA promoter (yellow in the diagram) to direct the expression of the desired siRNA sequence for RNAi (Luo and Wang, 2004). A short cDNA containing the siRNA of interest can be ligated into the plasmid adjacent to the promoter (red in the diagram). Several common cut sites present in the shRNA domain include an Spe I site, an Mlu I site, and a Cla I site. The plasmid can be transfected into bacterial cells plated on tetracycline plates to validate a successful DNA

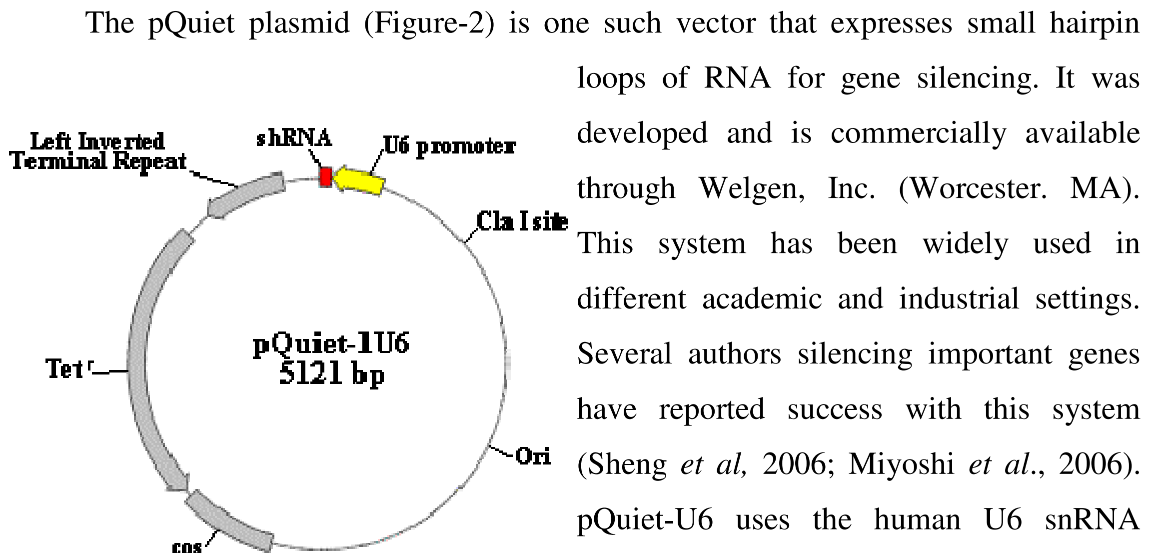

Figure 2: pQuiet-U6 Plasmid Vector (Luo and Wang, 2004).

insertion. This plasmid can also be easily converted into an adenovirus vector using the SpeAd procedure (Luo and Wang, 2004).

2.2. Adenovirus Packaging

Once a vector has been constructed and validated using standard genetic engineering techniques to contain the correct gene of interest, the pQuiet plasmid can then be integrated into an adenovirus to be used to infect mammalian cells to deliver the siRNA sequence. Adenoviruses are double-stranded DNA viruses that are classified as nonenveloped icosahedral viruses (Figure 3). The adenovirus genome is linear dsRNA about 35 Kbp of which about 30 Kbp can be replaced with foreign DNA (Wikipedia, 2006). The virus is able to replicate in the host cell's nucleus using the cell's own replication machinery, although it does encode for it's own DNA polymerase and packaging proteins (Hunt, 2005).

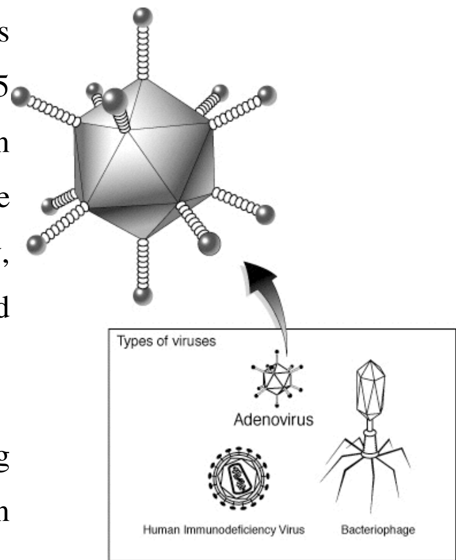

Figure 3: Adenovirus (Wikipedia, 2006)

Adenoviral infection falls under the following categories: productive, abortive, or latent (Figure-4). In productive infections, the viral genome is transcribed in the nucleus, allowing mRNA from the virus to be translated in the cytoplasm. Virions also self-assemble within the nucleus, and virus-host recombinants can also be found). In latent infections, and in transformed and tumor cells, viral DNA is integrated into the host genome (Doerfler, 2005).

Type of Interaction	Functional Definition	Biologic System
Productive infection	Complete replication of infectious virions	Cultured human cells
Abortive infection	Synthesis of viral gene products without production of infectious virions	Cultured hamster or monkey cells
Semipermissive infection	Complete replication with low yields of infectious virions	Cultured rat cells
Malignant transformation	Associated with integration of viral DNA and differential viral and cellular gene expression	Cultured rodent cells
Tumor induction	Associated with integration of viral DNA and differential viral and cellular gene expression	Newborn hamsters (mice)
Viral latency	Persistence of viral genome	Human tonsils

Figure-4: Various Means of Adenovirus Infection (Hunt, 2005).

Productive infection (Figure-5) is one desired route for laboratory use; the virus enters the host cell through viropexis or by directly penetrating the membrane (right side of the figure). Adenovirus often prefers epithelial cells. Once inside the host cell, the viral DNA is uncoated in steps, and transported to the nucleus (left side of diagram). Once the viral DNA resides within the nucleus, it can be transcribed and replicate. Adenoviral mRNA undergoes processing in the nucleus before traveling to the cytoplasm to be translated into viral proteins using polysomes. Early proteins produced include those for transcription, and adenovirus construction, and those that alter the expression of host cell genes (Hunt, 2005). The newly synthesized adenovirus proteins then enter the nucleus where the replicated viral DNA can self-assemble into virions (Doefler, 2005).

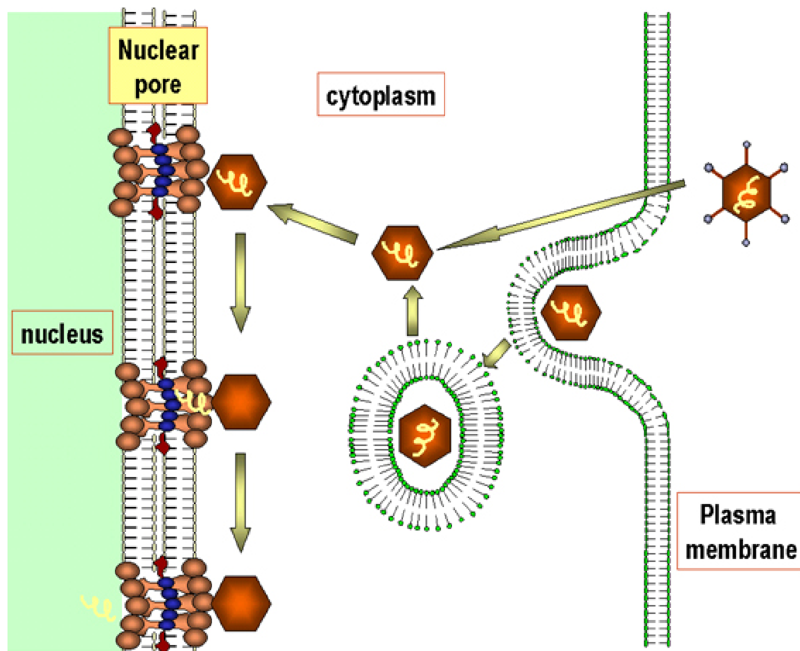

Figure 5: Adenovirus and Host Cell Interactions (Doerfler, 2005)

The cell then lyses releasing large amounts of the virion into the culture supernatant, from which it can be collected and purified. Adenoviral vectors are very efficient at transducing target cells *in vitro* and *in vivo*, and can be produced at high titres ($>10^{11}$/ml) (Doefler, 2005).

2.3. Mouse Hormone-Sensitive Lipase (HSL)

Hormone-sensitive lipase (HSL), also known as triacylgyceride lipase (TAG lipase), is thought to be the major enzyme responsible for the metabolism of free fatty acids (FFA) from adipose tissues (Mulder, *et al*., 1999). HSL hydrolyses triacylglycerides to diacylglyceride, releasing free fatty acids from fats (lipolysis). Studies have shown that it is the rate-limiting enzyme for cholesterol ester and diacylglycerol hydrolysis in adipose tissue (Kraemer and Shen, 2006). HSL is essential for complete hormone stimulated lypolysis. HSL is a neutral intracellular lipase with a broad specificity catalyzing the hydrolysis of triacylglycerol, diacylglycerol, monoacylglycerol, and cholesteryl esters, as well as retinyl esters; however, it possesses no phospholipase activity (Kraemer and Shen, 2006).

2.4.1 Free Fatty Acids and Metabolism

Free fatty acids (FFAs) are an important source of energy for many tissues in mammals (Kraemer and Shen, 2006). FFAs circulating in the plasma are thought to be derived from tissue lypolysis of stored adipose tissue rather than from the primary digestion of food. Adipose tissue is the main storage repository for triacylglycerol and is therefore integral in the study of obesity. Hormones and blood sugar levels regulate the lypolysis of adipose tissue. Increases in glucagon (associated with low blood glucose) and epinephrine (linked with increased metabolic demands) withing the body signal that energy is needed, and the oxidation of fatty acids is increased to meet that need. Glucagon, norepinephrine, and epinephrine bind to a G-protein-coupled receptor that activates adenylate cyclase to produce cyclic AMP; cAMP next activates protein kinase A, which phosphorylates (and thus activates) HSL. It has been shown that HSL knockout mice are resistant to obesity, although further analysis of lypolysis and adipose tissue metabolism must be completed before HSL can be considered for therapeutic treatment (Berg *et al*., 2002).

2.3.2. HSL Gene Mapping

The mouse HSL gene sequence is known, so it can be used to design siRNAs complementary to it in RNAi strategies. The mouse HSL gene is located on chromosome 7, and occupies base pairs 25088287-25104747 (Figure 6). The genes for digestive activity, as well as adipose tissue related genes, are represented (Figure 6, yellow colored transcripts) in the mouse mRNAs that are transcribed from this region of the chromosome (The Jackson Laboratory, 2006). For more information on the HSL gene, also known as the Lipe gene, see Appendices 1-3.

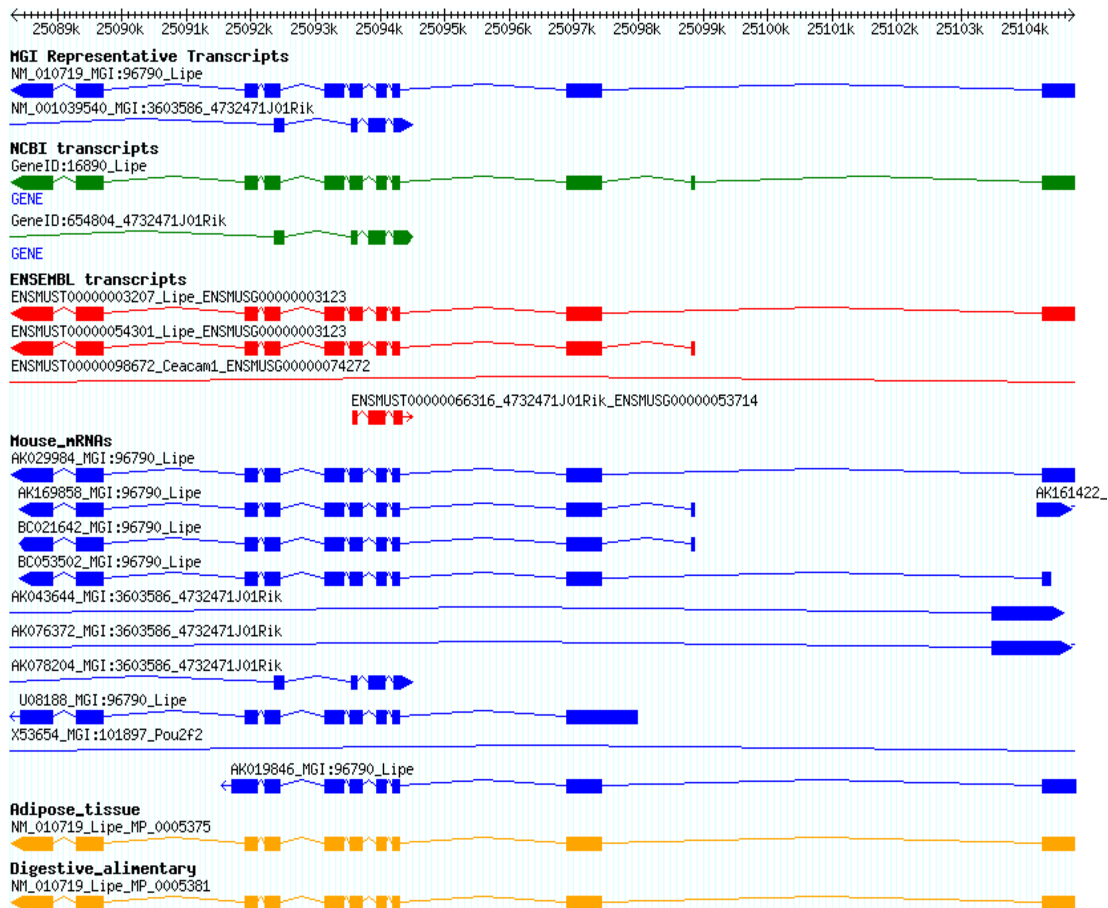

Figure 6: Gene Map of Mouse Chromosome 7, 25088287-25104747: HSL gene (MGI, 2006).

PROJECT PURPOSE

Because of the key role of hormone-sensitive lipase (HSL) in the release of circulatory system-damaging fatty acids from stored adipose tissue, HSL might be a good target for gene silencing in obese patients via RNAi therapy. A siRNA for HSL gene silencing could also have potential in the study of obesity and free fatty acid metabolism. The purpose of this MQP was to select and validate various mouse HSL siRNAs. Three short hairpin RNAs (shRNAs), each 19 nucleotides in length (Table-1 in Methods), will be cloned into the Spe I/ Mlu I site of the pQuiet-U6 RNA expression plasmid, and the inserts will be verified through enzymatic digestion and selective screening. After the three plasmids (titled pQmHSL-shRNA1, pQmHSL-shRNA2, and pQmHSL3) are verified for the presence of insert, they will be transformed into *E. coli* cells for amplification, and then purified. Purified plasmid will then be transfected into 293 cells to test siRNA expression and HSL protein knockdown by immunoblots. The most successful plasmid will be used to create an adenoviral vector with a titer of at least 1 x 10^{12} virus particles per milliliter for future *in vivo* delivery.

4. METHODS

4.1. Construction of pQHSL-shRNA

Three short hairpin RNA (shRNA) sequences shRNA-1, -2, and -3 (Table-1) were chosen for cloning into RNA expression vector pQuiet-U6 based on the mouse hormone-sensitive lipase (HSL) gene sequence (Background Figure-6).

Table 1: siRNAs for Insertion into pQuiet-U6 Plasmid for Testing HSL RNAi in 293 Cells.

siRNA designation	Sequence	Base Pair Location
MHSL-shRNA 1	GCTGGTGACACTCGCAGAA	(94-116)
MHSL-shRNA 2	GCAAGAGTATGTCACGCTA	(526-545)
MHSL-shRNA 3	GCTGAAAGTGGTAGAAGAT	(2212-2231)

All three shRNAs were cloned into the SpeI/ MluI site of the pQuiet-U6 plasmid (located immediately downstream from the U6 RNA promoter) using the pQuiet-U6 vector cloning kit provided by Welgen. Inc. The DNA sequences were synthesized as individual complementary oligomers (whose sense sequence is shown in Table 1) which were then hybridized to each other to make double stranded DNAs flanked by SpeI and MluI restriction sites. The vector pQuiet-U6 was cut with MluI and SpeI, then ligated to the annealed oligos, to generate plasmids pQmHSL-shRNA-1, -2, and -3. Ligated plasmids were transformed into competent *E. coli*, and the cells were plated onto LB tetracycline plates. Tetracycline-resistant colonies were grown overnight in liquid cultures, and plasmid DNA was prepared from 1 ml of culture.

For screening, plasmid DNA was cut with EcoR5 and XbaI to release shRNA insert, then analyzed on 1 % agarose gels. The positive clones were grown in 5 ml LB overnight and plasmid DNA was purified with the Qiagen mini-plasmid purification kit.

The shRNA sequences were further verified by DNA sequencing through an outside source (data not available).

4.2. Silencing HSL in 293 Cells

4.2.1 Co-transfection experiments

The target gene expression plasmid pCMV-mHSL (encoding murine hormone-sensitive lipase) and the three HSL RNAi plasmid vectors were transfected into 293 cells. The amount of DNA used for each transfection experiment is listed in Table 2. A control shRNA vector containing a non-specific whRnA sequence, known as scramble shRNA, was used as a negative control.

Table 2: Components of Each Co-Transfection Reaction, for Each Well of a 6-Well Plate.

	Scramble shRNA	**mHSL**	**shRNA 1**	**shRNA 2**	**shRNA 3**
pCMV-mHSL cDNA (target)	-	1.0	1.0	1.0	1.0
pQ-HSL-shRNA	-	-	2.0	2.0	2.0
pLep3 vector DNA	3.0	2.0	-	-	-
Total DNA	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**

Human 293T cells were grown in a 6-well microtiter plate to a density of about 5 x 10^5 cells per ml and then transfected with as indicated in Table 2 using the Lipofection 2000 reagent. At 48 hours post-transfection, the 293T cells were lysed in SDS-PAGE buffer and then subjected to electrophoresis on 10% SDS-PAGE gels.

4.2.1 Western Blotting

Western blot analysis was used to verify HSL protein knockdown via the siRNA expression. The Western blot was performed using an HSL antibody, provided by a

proprietary customer, at 1:1000 dilution, followed by an HRP conjugated secondary antibody. HRP was detected through chemiluminescence using ECL reagents.

4.3. Construction of Adenovirus AdQmHSL

An effective shRNA was chosen for conversion to adenovirus using the SpeAd system provided at Welgen, Inc. The pQmHSL-shRNA-2 vector was digested with Pi-PsPI, then ligated into an equivalently linearized pLep plasmid that contains the remailing adenovirus genome (Adenovirus type-2). The ligation product was packaged into a cosmid using Epicentre's lambda phage packaging kit. The packaging products were used to infect *E. coli* cells. After incubation overnight, the positive clones were selected, and cosmid DNA purified. The purified cosmid DNA (2 μg) was digested with I-Ceu I, and then transfected into 293 cells with Lipofectamine 2000 according to manufacturer's instructions. The 293 cells were grown at 37º C with 5% CO_2. The adenovirus plaques were seen 7 days after transfection. A low titer of virus (approximately 10^9 virus particles per milliliter (vp/ml) was obtained from a single well of a 6-well plate.

4.4 Amplification and Purification of Adenovirus

The low titer of adenovirus was further amplified to 10^{12} vp/ml using DMEM seeded plates. The amplified adenovirus was purified on two sequential cesium chloride gradients, and then dialyzed against 1X PBS buffer containing 10% glycerol, pH 7.4, to reduce the salt concentration. The purified virus was measured for its titer using a spectrophotometer; in a cuvette 10 μl of viral sample was added into 990 μl 0.1% SDS buffer and incubated at room temperature for 15 minutes, the OD was then measured at 260 nm. The adenovirus titer was calculated using the following formula, where D is the dilution factor:

$$\textit{adenovirus titer in vp/ml} = \textit{OD value} \times D \times 1.1 \times 10e^{12}$$

RESULTS

Construction of Plasmids pQmHSL-shRNA-1, -2, and -3

The first phase of this project involved ligating three different short hairpin RNAs (shRNA-1, -2, and -3) whose sequences were designed to be complementary to the 3' UTR of hormone-sensitive lipase (HSL) into the pQuiet RNA expression vector. The ligation products were transformed into competent *E. coli*, and plated onto tetracycline medium plates. Plasmid DNA was prepared from randomly selected tet-resistant colonies, then screened by cutting with EcoRV and XbaI to release the 0.5 KB shRNA insert. Positive shRNA-1 colonies (Figure-7) are seen in the first 3 sample lanes (lanes 2-4). The upper band is the plasmid vector DNA and the lower band, if present, is the successful insert. One colony from the plasmid of lane 1 was chosen for further analysis.

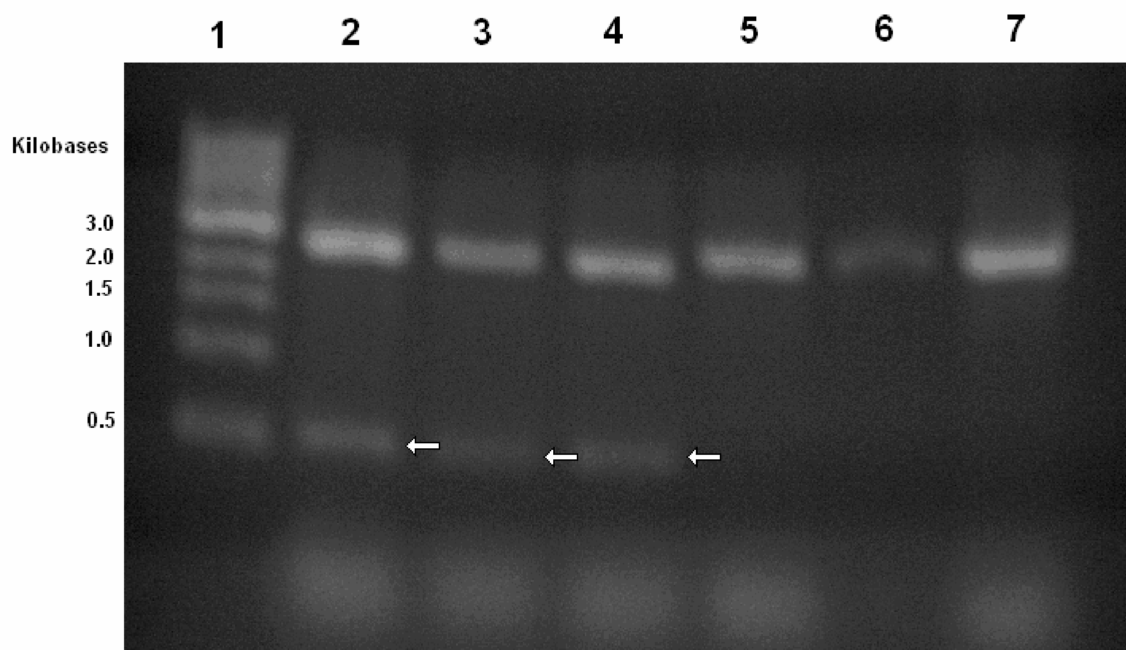

Figure 7: Restriction digestion screening of plasmid pQmHSL-shRNA-1. Samples were digested with EcoRV and XbaI to release a 0.5 kb insert. Positives (indicated by white arrows) are seen in lanes 2-4.

The screening for pQMHSL-shRNA-2 (Figure-8) shows positives in lanes 2, 3, 4, 8, and 11. The sample shown in lane-2 was chosen for further analysis.

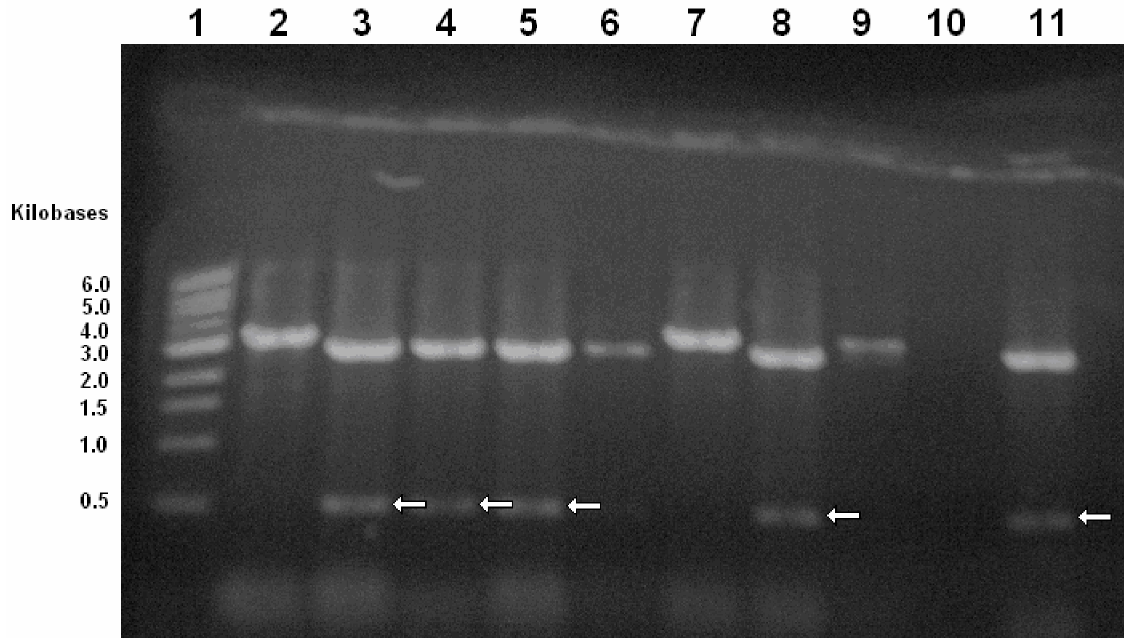

Figure 8: Restriction digestion screening of plasmid pQmHSL-shRNA-2. Samples were digested with EcoRV and XbaI to release a 0.5 kb insert. Positives (denoted by white arrows) are seen in lanes 3-5, 8, 11.

The screening of plasmid pQmHSL-shRNA-3 (Figure 9) shows positives in lanes 1 and 2. One colony, represented by lane 1, was chosen for further analysis.

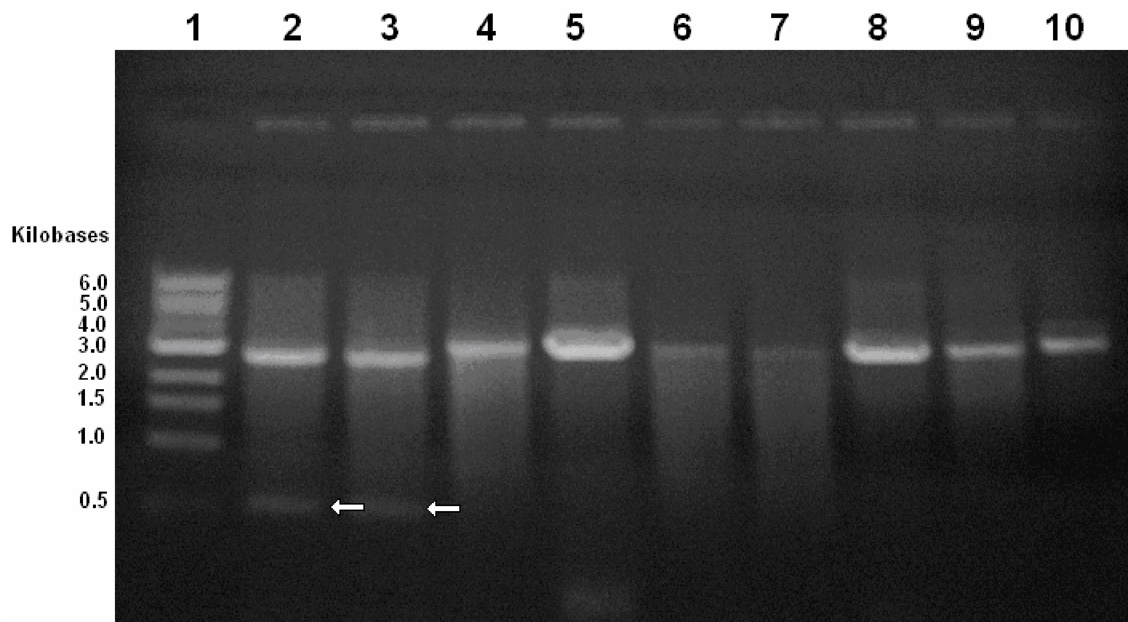

Figure 9: Restriction digestion screening of plasmid pQmHSL-shRNA-3. Samples were digested with EcoRV and XbaI to release a 0.5 KB insert. Positives (denoted by white arrows) are seen in lanes 1-2.

Verification of HSL Protein Knockdown by siRNA

Co-transfection experiments were performed with human 293T cells to verify a successful knock-down of the HSL gene by the three shRNAs. 293T cells were co-transfected with plasmid DNAs for each of the 3 shRNAs plus plasmid pCMV-mHSL (encoding human HSL) as the target vector. Lysates were prepared from the plasmid transfected cells, and analyzed for total cellular levels of HSL protein using an immunoblot (Figure-10). Negative controls were transfected with a vector that does not produce HSL. A scrambled sequence "control shRNA" was included in the experiments to ensure the shRNA specificity. Figure 10 shows that each the three shRNAs reduced the amount of HSL expression relative to the scrambled shRNA, but shRNA-2 showed the best silencing efficiency, removing almost 95% of HSL proteins.

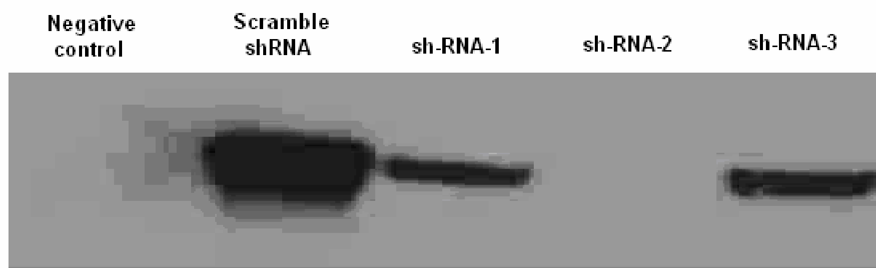

Figure 10: HSL Immunoblot to test for HSL knock-down in cells co-transfected with HSL reporter plasmid and the three shRNAs designed in this MQP. shRNA-2 (second from the right) produced the best HSL knockdown.

DISCUSSION

The selection and validation of three murine HSL siRNAs for construction into Adenovirus type 2 was successful. Three short hairpin RNAs (shRNAs), each 19 nucleotides in length, were cloned into the Spe I/ Mlu I site of the pQuiet-U6 plasmid and the inserts were verified through restriction digestion screening and plasmid sequencing. One positive colony for each type of shRNA plasmid was chosen for testing in co-transfection experiments with human 293T cells treated with pCMV-mHSL target plasmid and a plasmid encoding shRNAs-1, -2 or -3. Each shRNA reduced the expression of HSL protein levels in the 293T cells relative to a scrambled shRNA, and shRNA-2 worked best knocking down HSL to undetectable levels. While all three showed a reduction in HSL, shRNA-2 showed almost 95% RNA silencing for the HSL gene. The conclusion of this MQP is that shRNA-2 should be the best choice for research using RNAi to study hormone sensitive lipase metabolism in mice. The final viral titer of AdQmHSL for shRNA-2 was 4.5×10^{12} virus particles per ml for use in such *in vivo* experiments.

An example of a future experiment that could be performed with the HSL knockdown adenovirus produced in this project with respect to lipid metabolism would be one that studies the effects upon adipose tissue when HSL is silenced in that tissue. In 2004, a study by the Institute of Molecular Biosciences, University of Graz in Austria, determined that a non-obese phenotype of HSL knock-out (HSL-KO) mice retained an accumulation of diglycerides in their adipose tissue, which suggests that HSL may be responsible for releasing those diglycerides from that tissue, and there may be one or

more additional lipases in adipose tissue that preferentially hydrolyzes the first ester bond of the triglyceride molecule. They discovered that another enzyme, adipose triglyceride lipase (ATGL), catalyzes the initial step in triglyceride hydrolysis (Zimmermann, *et al.*, 2004). The HSL enzyme, which has 10 times higher affinity for diglycerides over triglycerides, then continues with hydrolysis (Zimmermann, *et al.*, 2004). A later study by this group also showed that mice could survive with few complications if they were HSL-defective, but ATGL-suppressed mice showed fat cell hypertrophy and mild obesity (Haemmerle, 2006). The inability to mobilize these fat stores by ATGL leads to energy starvation, resulting in reduced energy expenditure, a decline in body temperature, and premature death when *Atgl*(–/–) animals are stressed by cold exposure or food deprivation (Haemmerle, 2006). This was an important discovery considering that prior knowledge had lead scientists to believe that HSL was the sole enzyme responsible for adipose tissue metabolism. Using this information, a future experiment in which both ATGL and HSL are silenced would confirm the fact that ATGL and HSL are the only enzymes involved in lipid metabolism in adipose tissue. Another experiment using the adenovirus construct designed in this project to silence HSL in cells might be in the study of HSL silencing as a weight-loss tool in diabetic or obese mice. Using some form of silencing as a therapy, transient HSL reduction could be induced, as compared to the permanent knock-down of the gene in HSL-KO mice to ascertain whether a transient reduction in HSL might be more clinically manageable.

While transgenic mouse knockouts are available for the HSL gene, the creation of the adenovirus construct for this MQP appears to be the first case in which mouse HSL has been silenced using RNAi.

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Appendix 1: FASTA genomic sequence for Lipe (HSL) 16890 bp long (NCBI 2006)

Chromosome 7: 25088286-25104747

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Appendix 2: Transcribed sequence for Lipe (HSL) from NCBI refSeq NM_010719 (NCBI 2006)

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 241 ttaaaggcgg agctccgcga ggagcctgcc caggattgga tggtttgggg gcctggcagt
 301 ggtgtgtaac taggattgac tctaagagag gaacttgatc gctggaattg ggccccgcgc
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 421 tccaagcagg gcaaagaagg atcgaagaac cgcagtcgca ggcggtggcg aaaaggcaag
 481 atcaaagcct cagcgttctc acacagcatg gatttacgca cgatgacaca gtcgctggtg
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Appendix 3: NM_010719 Translation (NCBI 2006)

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