

**IDENTIFICATION AND CHARACTERIZATION OF BOVINE POL III  
PROMOTERS TO EXPRESS A SHORT-HAIRPIN RNA**

A Thesis

by

MICHAEL DAVID PEOPLES

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Genetics

**IDENTIFICATION AND CHARACTERIZATION OF BOVINE POL III  
PROMOTERS TO EXPRESS A SHORT-HAIRPIN RNA**

A Thesis

by

MICHAEL DAVID PEOPLES

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of  
MASTER OF SCIENCE

Approved by:

Chair of Committee,	Mark Westhusin
Committee Members,	Charles Long
	James Womack
Intercollegiate Faculty Chair,	Craig Coates

December 2010

Major Subject: Genetics

## ABSTRACT

Identification and Characterization of Bovine Pol III Promoters to Express a Short-Hairpin RNA. (December 2010)

Michael David Peoples, B.S.; B.S., Oregon State University

Chair of Advisory Committee: Dr. Mark Westhusin

The use of molecular biology as a means to advance agriculture has proven beneficial in many fields. However, the development lentiviral vectors that utilize a livestock promoter to express short hairpin RNA (shRNA) has been limited to date. The goal of this research project was to develop and characterize lentiviral bovine Pol III mir30 shRNA expression vectors for future use in livestock research. The bovine Pol III promoter (7sk, U6-2, or H1) was inserted directly upstream of a mir30 shRNA expression sequence in the lentiviral vector pNef-GT. A transient luciferase knockdown assay in human embryonic kidney (HEK) 293T cells was used to compare the functionality of these vectors. The bPol III mir30 shRNA expression vector was co-transfected with the pGL3 luciferase expression vector and the renilla expression vector pLB at a ratio of 5:10:1 respectively. The vectors were allowed 48 hrs to produce their respective products before luciferase activity was measured with the Stop-n-Glo Assay (Promega). Each bPol III promoter was able to express a functional shRNA resulting in a reduction of luciferase activity greater than 68%. The bH1 and bU6-2 Luc shRNA vectors were the most effective vectors when transfected with >76% (p-value <0.05)

reduced luciferase activity. To confirm that these promoters were functional after integration into a bovine genome, recombinant lentivirus was made from these vectors. These particles were then used to transduce a bovine kidney (MDBK) cell line that expressed luciferase. After transduction, transgenic cells were selected by the addition of the antibiotic, Geneticin to the culture media until a population of 100% bPol III expression cells were observed for two passages and luciferase activity was measured. The 7sk promoter was the most effective bPol III promoter that reduced luciferase activity in these cells by 72% (p-value <0.05), while the bU6-2 and bH1 were moderately effective at reducing luciferase levels (37%, 46% respectively). These experiments were the first to quantify the bovine Pol III promoter function after integration into a bovine genome. While variability was observed, for livestock based research, the b7sk lentiviral vector appears to be the best choice to express a shRNA from the genome of a bovine genome.

## TABLE OF CONTENTS

	Page
ABSTRACT .....	iii
TABLE OF CONTENTS .....	v
LIST OF FIGURES.....	viii
LIST OF TABLES .....	ix
 CHAPTER	
I INTRODUCTION AND LITERATURE REVIEW .....	1
Introduction .....	1
RNA Interference .....	5
The RNAi Pathway .....	6
Triggers of the RNAi Pathway.....	11
Promoters to Express RNAi Effector Molecules .....	16
Livestock Pol III Promoters .....	21
Applications of RNAi.....	24
Retroviral Transgenesis.....	25
Lentivirus .....	26
Process of Lentivirus Infection .....	27
Utilization of Multiple Vectors to Construct Recombinant Lentivirus.....	29
rLentiviral Vector Utilized in These Experiments .....	32
Different Promoter's Potential for shRNA Expression Following a rLentivirus Transduction.....	33
Enhancing Livestock Production Characteristics.....	34
Assisted Reproductive Technologies .....	35
Transgenic Livestock .....	36
Methods to Produce Transgenic Livestock .....	40
Research Objective.....	44
II MATERIALS AND METHODS .....	45
Identification of Bovine Pol III Promoters.....	45
Developing Bovine Pol III Driven mir-30 shRNA Lentiviral Expression Vector .....	46

CHAPTER	Page
Nef-GT mir-30 shRNA Expression Vector.....	46
PCR Amplification of bPol III Promoter .....	47
PCR Mediated Insertion of Restriction Enzyme Sites to Bovine Pol III Promoter and Cloning into Nef-GT.....	48
Functional Analysis of Nef-GT bPol III Vector.....	51
Short Hairpin RNA Design, Amplification, Ligation into Nef-GT bPol III.....	51
Transient Luciferase Assay to Confirm Function of Nef-GT bPol III Vector.....	52
Statistical Analysis of Relative Luciferase Activity .....	53
Functionality of Nef-GT bPol III shRNA Transgene after Integration into Bovine Genome .....	54
Development of Luciferase Expressing MDBK Cells .....	54
Construction of rLentivirus .....	55
Transduction of MDBK+Luc Cells with bPol III mir-30 shRNA Recombinant Lentivirus .....	56
 III RESULTS .....	 58
Identification and PCR Amplification of Bovine Pol III Promoters .....	58
Development of Lentiviral Bovine Pol III mir-30 Based shRNA Expression Vectors.....	58
Confirmation of Nef-GT bPol III Vector Function with Transient Luciferase Assay .....	59
Confirmation of bPol III Promoter Function After Integration of Transgene in the Bovine Genome .....	62
 IV DISCUSSION AND CONCLUSION .....	 65
Use of bPol III Promoters to Express a mir-30 Based shRNA .....	65
Conclusion.....	72

	Page
REFERENCES.....	73
APPENDIX A .....	90
APPENDIX B .....	110
VITA .....	112

## LIST OF FIGURES

FIGURE	Page
1 RNAi Pathway.....	10
2 Examples of RNAi Triggers.....	17
3 Illustration of a Retrovirus (Lentivirus) .....	28
4 Nef-GT mir-30 Based shRNA Lentiviral Expression Vector .....	33
5 Nef-GT bPol III mir-30 Based shRNA Lentiviral Expression Vectors .....	50
6 rLentiviral Vector PEIZ Luc .....	55
7 Production of Recombinant Lentivirus .....	57
8 Transient Luciferase Knockdown Using Nef-GT bPol III shRNA Expression Vector .....	61
9 Transduced Nef-GT bPol III shRNA Mediated Luciferase Knockdown Assay in MDBK+Luc Cells .....	64



**LIST OF TABLES**

TABLE		Page
1	Relative Luciferase Activity in a Transient Luciferase Knockdown Assay Mediated by bPol III shRNA Lentiviral Expression Vectors.....	60
2	Relative Luciferase Activity of MDBK+Luc Cells after Integration of a Bovine Pol III shRNA Expression Transgene.....	63
3	Primers Utilized for PCR Reactions.....	110
4	shRNA Sequences .....	111

## CHAPTER I

### INTRODUCTION AND LITERATURE REVIEW

#### Introduction

RNA interference (RNAi) is an evolutionary conserved gene silencing mechanism found in plants, insects, and mammalian cells. The primary function of this pathway is to mediate the targeted reduction of gene products by translational inhibition, or mRNA degradation. In the laboratory, the RNAi pathway has proven to be a powerful tool for manipulating protein expression and studying gene function [1]. In addition, it has been used to develop model animals with increased muscle mass, and increased resistance to viral infections [2, 3]. Harnessing the potential of RNAi could be particularly beneficial in the animal agricultural field where creating gene knock-out specimens is more difficult than the common rodent model. Utilizing the RNAi pathway has contributed to the knowledge of physiological pathways in animal agriculture [4, 5]. In addition, it has been used to develop animals with the potential to be resistant to Transmissible Spongiform Encephalopathy [6], commonly known as Mad Cow Disease in cattle. Also RNAi has been used to decrease expression of porcine endogenous retroviruses [7], which are a key concern for the use of swine as organ donors in xenotransplantation experiments. Overall, the potential of utilizing RNAi in livestock research and production seems limitless.

---

This thesis follows the style of *BMC Biotechnology*.

Another potentially powerful tool in transgenic animal agriculture research is the utilization of lentiviruses to permanently integrate DNA sequences of interest into the genome of cells or zygotes. Lentiviruses are a subfamily of retroviruses that efficiently introduce their genome into infected cells. Through the use of multiple plasmids providing essential proteins and RNA sequences, these highly infective viruses have been manipulated to be relatively safe for laboratory research. They have been manipulated to be replication incompetent, infect dividing and non-dividing cells and integrate a transgene that can be designed in the laboratory [8, 9]. The use of these recombinant lentiviruses results in the stable integration of a transgene of interest, which can be subsequently passed on to daughter cells and germ cells when used to develop transgenic animals. As a result of these qualities, the use of recombinant lentiviruses to transduce zygotes is a promising efficient method to introduce a gene of interest to create transgenic livestock [10].

A major goal of our laboratory is to use lentiviral transgenesis and the RNAi pathway to develop transgenic livestock with enhanced production characteristics. Since the domestication of animals, humans have been trying to enhance production from different desired traits. These traits include: meat production, milk production, wool or fur, and resistance to diseases. Selective breeding and artificial reproductive technologies are most commonly used to enhance these traits. However, the increase in production is minimal from generation to generation when using standard breeding practices. In

addition, selective breeding can lead to the reduction of genetic variation and loss of potentially valuable traits from the population. Researchers have tried to directly increase these production traits, as well as introduce others by inserting genes into livestock genomes to create transgenic animals [11-15]. These animals were produced with hopes of increasing muscle mass, enhancing milk and wool production, as well as, producing pharmaceuticals. Recently, the potential of creating transgenic animals which utilize the RNAi pathway to obtain the desired phenotype has been explored to enhance livestock production traits. The enhanced production characteristics our laboratory is interested in include increased muscle development and enhanced resistance to agricultural viruses.

There are three basic questions that require further research in the production of animals that utilize exogenous RNAi precursors to modulate gene products, particularly in animals other than rodents. First, which RNAi precursor is the most effective at decreasing only the targeted gene product or products and does not affect other physiological pathways. Some of these concerns are off-target effects of the siRNA and triggering innate immune responses [16, 17]. Another question to address, are which expression cassettes for RNAi precursors are best suited for the desired goals of the research. The majority of the promoters utilized are responsible for natural transcription of different housekeeping genes, unfortunately some of these promoters vary in function between cell types and stages of animal development [18]. A promoter can be too strong

at driving expression of the RNAi precursor which overloads the endogenous RNAi pathway and decreases the ability of RISC to perform its normal cellular functions [19]. In addition, if a RNAi precursor in a specific tissue is desired, specific promoters must be identified and tested. Overall, the ability of the promoter to express sufficient amounts of the RNAi precursor that results in a biological effect in all desired tissues is the primary goal. The third question is how to efficiently introduce a expression cassette into the genome that is functional in a transgenic animal. Currently, the primary methods to produce transgenic animals use pronuclear injection of a gene construct, or somatic cell nuclear transfer (SCNT) of genetic material from a manipulated cell line, or recombinant retroviruses to deliver a transgene that contains the expression cassette. Each of these methods has pro's and con's, but the primary limiting factor is the efficiency of producing a non-rodent transgenic animal. While transgenic livestock expressing RNAi precursors have been developed, further research is required to determine methods that result in a greater efficiency of producing transgenic livestock that have the desired levels of gene product knock-down.

The goal of this research was to focus on the expression of an RNAi precursor and identify efficient promoters to express the RNAi effector molecule both transiently and after integrated in the genome. The purpose of this research project was to identify and analyze the ability of three bovine Pol III promoters to express an RNAi effector molecule (short hairpin RNA) in human and bovine cells.

## RNA Interference

The first observations of RNA having an inhibitory role were noted in the early 1980's. When introduced into the cell, antisense RNA resulted in decreased gene and protein expression in animal and plant models [20-23]. Later, Napoli *et. al.* in 1990 attempted to alter flower color in a petunias with over-expression of a transgene for Chalcone synthase (CHS), the protein responsible for pink or violet coloring. Surprisingly, they noticed a significant decrease in flower color that was correlated to a block in the anthocyanin biosynthesis or decrease of CHS. At the time, it was believed that the antisense RNA would hybridize with the coding mRNA resulting in double-stranded RNA (dsRNA) which either inhibited translation or was targeted for destruction by cellular ribonucleases. It wasn't until 1998 when Fire and Mello set out to study the requirements for structure and delivery of interfering RNA that they discovered dsRNA was significantly more effective than targeting single stranded RNA[24]. For these experiments, they injected 500-1,000 bp sense, antisense, or mixed (sense+antisense) RNA sequences corresponding to mRNA for four different proteins in *Caenorhabditis elegans* that result in observable phenotypes when the protein is not present. To their surprise, the sense or antisense injections only had a marginal effect. However, when the two strands were co-injected creating dsRNA, the greatest phenotypic traits were observed and some of the characteristics proceeded through multiple generations. Their data was the first publication proving that dsRNA is responsible for gene product knock-down. This achievement was rewarded in 2006 as Craig Mello and Andrew Fire were awarded the Nobel Prize in Physiology or Medicine. In addition, this research provided

the foundation for further research into how dsRNA interferes with protein production and thus, the RNA-mediated interference (RNAi) field was born.

### *The RNAi Pathway*

Following the discovery of potent dsRNA mediated interference; the next goal of researchers was to determine the effector molecule. Researchers hypothesized that the antisense RNA sequence acts as the guide strand for specific post-transcriptional gene silencing (PTGS). However in the early studies, the full length antisense RNA was never detected in hybridization assays. As a result, Hamilton and Baulcombe set out to determine the size of the antisense RNA (effector molecule) that resulted in the gene specific knockdown [25]. They transformed tomato plants and analyzed them for PTGS of the target protein 1-aminocyclopropane-1-carboxylate oxidase (ACO). In the selected plants that exhibited reduced levels of ACO, they purified low molecular weight nucleic acids and used a labeled ACO sense RNA probe to detect the anti-sense RNA. In their findings a high level of antisense RNA approximately 25 nucleotides long was observed. To confirm these findings, an antisense 25 nucleotide RNA sequence targeting green fluorescent protein (GFP) was developed and transformed into GFP positive leaves. Reduced levels of GFP as well as the 25 nt targeting RNA were observed in only the transformed leaves. As a result of this research, they determined the RNAi effector molecule is approximately 25 nt. Supporting these findings, Hammond *et. al*, used *Drosophila melanogaster* cell extracts and also confirmed the presence of an RNA

effector product of ~25 nt [26]. To further confirm the size of the effector molecule, Zamore *et. al*, determined that radiolabeled dsRNA is cleaved into 21-23 nt fragments [27]. As a result of these studies, the effector RNA molecule resulting in specific PTGS has been determined to be 21-23 nt long.

In addition, to determining the size of the effector molecule, these early studies also confirmed that the sequence specific nuclease activity was protein and ATP dependent. Hammond *et. al*, named the enzyme that co-precipitated with the targeting RNA the effector protein or later RNA-induced silencing complex (RISC) [26]. The next logical question was to determine if this complex was responsible for both the processing of the dsRNA into small interfering RNA (siRNA) and subsequently PTGS [28]. They used ultra-centrifugation as a means to fractionate the drosophila cell lysates. The PTGS activity could be removed during this process within the pellet. However, the protein responsible for the processing of the dsRNA into 22 nt products was in the supernatant. As a result, they analyzed candidate RNase III proteins based upon the knowledge that the processing of the dsRNA trigger into the 22 nt siRNA required ribonuclease activity and specificity to bind dsRNA. A drosophila protein (CG4792) was found to consistently process dsRNA into 22 nt products and was subsequently named Dicer.. These initial findings of the RNAi pathway determined that Dicer, was responsible for the cleavage of the trigger dsRNA into an siRNA approximately 21-23 nt in length and the effector protein, RISC, was required for siRNA mediated mRNA degradation.

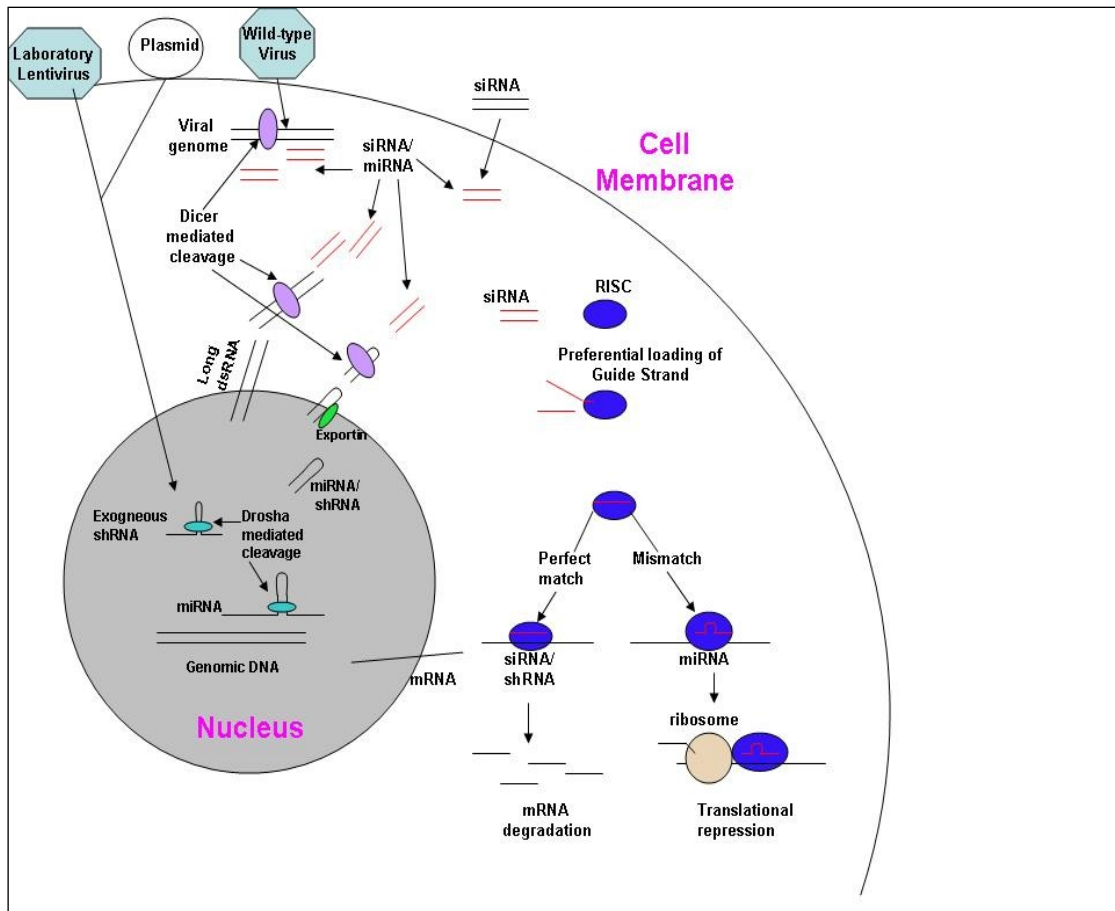


As with many important pathways, understanding of the multi-protein complexes became a priority. One particular interest was determining the protein responsible for the cleavage of the mRNA after the binding of the RISC. The RISC complex was immunoprecipitated to study the components of the complex. As a result of these studies two proteins were identified Argonaute 1 and Argonaute 2 (Ago 1 and Ago 2). Eventually, it was proven that Ago 2 exclusively retained the cleavage ability observed during PTGS. Thus, Ago 2 is the catalytic enzyme responsible for mRNA cleavage by RISC in eukaryotic cells.

With a better understanding of the downstream processing of dsRNA and hundreds of naturally occurring endogenous small RNA products subsequently discovered, termed micro RNA (miRNA). The question of the nuclear pre-processing was still unanswered. Previously, it was found that miRNAs are transcribed as primary transcripts (pri-miRNAs) and later processed into a stem-loop pre-miRNA structure approximately 70 bp in length. Lee *et. al*, used the miRNA30 as a candidate pre-miRNA[29]. They determined that it had a 3' overhang of 2 nt, suggesting RNase III activity. As a result of these findings, human protein databases were analyzed and three RNase III enzymes were found. One of these proteins, Drosha was known to be localized in the nucleus. Further analysis of this protein revealed that it was responsible for creating dsRNA products approximately 70 nt in length. Subsequently, it has been determined that Drosha is the nuclear initiator protein of the RNAi pathway.

Both miRNA and siRNA result in a reduction of gene products by similar means in the RNAi pathway, but vary in origin and action. A miRNA is generated within the cell, and the sequence is highly conserved. It is rarely perfectly complementary with mRNA sequences, but it can affect protein translation and mRNA decay by binding to its imperfectly matched target sites of a mRNA. A siRNA is usually generated from the cleavage synthetic or exogenous dsRNA by Dicer into ~21-nt in size. Then the targeting strand is preferentially loaded into the RISC complex with Ago2. This complex then cleaves its target mRNA if it is perfectly complementary. A siRNA can act like a miRNA if the loaded RISC binds an imperfect complementary mRNA sequence and results in translational inhibition. In addition, if the targeting strand from a miRNA in the RISC complex binds a perfectly complementary sequence, then it can act as a siRNA and result in the degradation of the mRNA. While a siRNA and miRNA may both be similar and result in PTGS, they are different.

The RNAi pathway has been found to be highly conserved among many eukaryote cell types (Fig. 1).[30]. Initially, the primary miRNA is transcribed in the nucleus. Then Drosha recognizes a specific binding sequence and structural elements to direct the nuclease activity of the enzyme resulting in a 2 nt 3' overhang. This 70 nt stem-loop structure is exported to the cytoplasm where the loop is removed by Dicer. Thus, creating a miRNA approximately 22 nt in length. The anti-sense or guide strand is preferentially loaded into the RNA-induced silencing complex (RISC), while the



**Fig. 1.** RNAi Pathway. There are various dsRNA structures that can enter the RNAi pathway for processing and resulting in decreased gene expression. Within the nucleus, dsRNA triggers can be expressed from transfected plasmids, or from the genome as naturally occurring pre-miRNA or as shRNA from transgene introduced by retroviral transgenesis. Typically these triggers form a stem-loop structure as part of a mRNA sequence. The stem-loop structure is cleaved from the mRNA by the protein Drosha then shuttled into the cytoplasm by the protein Exportin V. Once in the cytoplasm, the dsRNA RNA is recognized by Dicer, which cleaves the 22 bp dsRNA from the stem-loop structure, turning it into an siRNA. Also within the cytoplasm, other RNAi triggers are recognized as dsRNA Fig.and processed by Dicer into small 21-23 nt siRNAs. In addition, synthetic siRNA can be transfected into the cells. Then the guide (mRNA complimentary) strand of the miRNA/siRNA is preferentially loaded into RISC where it seeks out complimentary mRNA sequences. If the exact sequence is recognized, the RISC protein directs mRNA cleavage. However, if a near complimentary sequence is recognized (i.e. miRNA or off-target siRNA) RISC mediates translational inhibition.

passenger strand is degraded. The RISC is responsible for recognizing the near homologous regions of the mRNA for sequence specific gene silencing. A miRNA loaded into the RISC recognize near perfect homologous sequences and result in translational repression [31]. Both siRNA's and miRNA's are effective tools to inhibit protein expression. However as research continues, the functions of si/miRNA's continue to diversify and have roles in enhancing transcription, increasing gene expression, and even altering chromatin and centromeric structure [32, 33]. While more new and surprising roles of these small RNA's are discovered every day, their robust ability to decrease gene expression still remains their primary use in the laboratory.

#### *Triggers of the RNAi Pathway*

In the laboratory, dsRNA or triggers of the RNAi pathway can be introduced as many forms into cells. Initially, long targeting sequences (>30 nt) of dsRNA corresponding to the target gene of interest were used to mediate the PTGS [34]. These proved to be very effective in many models and various gene products. The long dsRNA homologous to the target mRNA is cleaved by Dicer into various siRNAs of 21-23 nt in length. As a result of this random processing, some of the siRNAs would be effective at reducing the target gene of interest. However in mammalian cells, long dsRNA sequences have been shown to induce an interferon response [35, 36]. Another negative use of long dsRNA is the possibility of off target effects [37]. Many gene sequences share homology, especially in gene families with similar conserved actions. Consequently, due to the

random siRNA sequences created by Dicer, some of these siRNAs could target the conserved regions resulting in undesired protein knockdown. As a result of these findings and advancements in RNAi research the use of long dsRNA has been substituted with the use of synthetic siRNAs.

Since the discovery of the RNAi pathway, research has evolved to effectively utilize synthetic siRNAs as tools for rapid gene product knockdown. However, the effectiveness of a siRNA does not only depend upon homologous sequence binding with a target mRNA. Multiple siRNAs targeting different regions of the same mRNA can have various levels of knockdown. As a result, researchers have compiled extensive data sets that included nucleotide composition, length, secondary structure, sugar backbone, and sequence specificity of functional si/miRNA. Computer analysis of the characteristics of the guide strand and the mRNA sequence are used to determine optimal RNAi target sites [38]. Some general rules have been employed to enhance a siRNA's effectiveness. One is that the antisense sequence should be 19 nt in length. Another is the 3' end of guide strand should contain two uridine residues. In addition, to increase the efficiency, the siRNA should have a 5'-phosphate group as well as a 3'-hydroxyl group [39]. Unfortunately even with these rules and the extensive data analyzed, the algorithms are not perfect at providing a single optimum siRNA. As a result, the suggested siRNAs should be used as a sampling of target sites within mRNA

sequence, and it is up to the researcher to determine the most effective for their experiments.

A siRNA is a desirable tool for a knockdown experiment to better understand the function or eliminate the presence of a gene product. Because of their small size, they can effectively enter a cell by various transfection methods. Time is another benefit of using synthetic siRNAs, within hours of the transfection the concentration of siRNAs are to a point to elicit an effect. Once inside the cell, the siRNA can be directly loaded into RISC. However, a synthetic siRNA based experiment is also limited by time because eventually the transfected population of siRNAs reduces to a critical level where their effect diminishes. This is typically 3-5 days depending upon the gene product [40]. As a result, most experiments can be concluded in two days and the knockdown of the gene product can be analyzed. However, if a protein is highly stable, the biological effect can outlast the mRNA reduction. As a result, researchers must be aware of the products they are analyzing and modify the experiment time to compensate. This experimental period is shorter than the weeks, months, or even years required when using gene-knockout technology to analyze protein function. In addition, if the siRNA target region is conserved, the same siRNA can be used to analyze protein knockdown in different species. A drawback to a siRNA experiment is cost. Even with advancements in oligonucleotide synthesis the costs of synthetic siRNA are still relatively high for

extensive testing and utilization. However, siRNAs are ideal for many research studies where quick analysis of a gene/protein knockdown is desired.

While the use of a synthetic siRNA has many benefits, they are still costly for many preliminary experiments and some experiments require a longer inhibition of gene products. As a result of these limitations, alternative means for expressing siRNAs from plasmids were explored. Plasmids actively reproduce their siRNA precursor in the cells for weeks or even months. This continuous production results in an effective population of siRNA for a longer period than synthetic siRNAs. This quality allows an observed knockdown for a longer period of time when compared to synthetic siRNA. When comparing costs, DNA oligo sequences corresponding to the siRNA product can be ordered and cloned into the expression plasmid for a fraction of the cost when compared to synthetic siRNAs. In addition, once the expression plasmid is created it can be readily replicated in the lab for a relatively low cost. Because of the costs and longer expression, the use of plasmids to express a precursor of the siRNA has become a powerful tool in RNAi research.

With a better understanding of the RNAi pathway various dsRNA triggers have been developed to be expressed from a plasmid. There are four main types of siRNA precursors that have been used (Fig. 2). One trigger developed directly mimics the siRNA and does not require the early processing as other triggers (Fig. 2.A). Two Pol

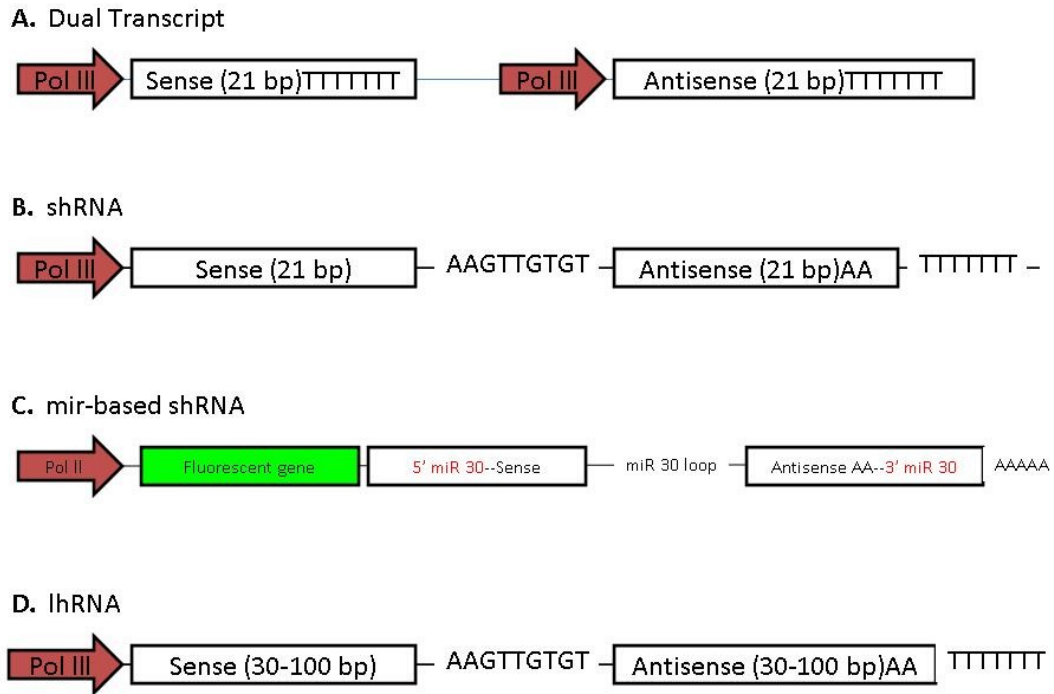
III promoters separately transcribe either the sense or antisense RNA strand, and the RNA products hybridize to form the siRNA [41]. Another trigger developed was the short hairpin RNA (shRNA) (Fig. 2.B)[42]. The shRNA is transcribed as a single sense-loop-antisense RNA sequence that forms a tight hairpin structure with a 3' TT overhang. The targeting sequence (anti-sense) is only 20-23 bp resulting in a siRNA after removal of the loop structure by dicer. The loop sequence does not appear to be important in the efficiency of shRNA processing. Sequences have been successfully used that are between 4-9 bp in length. However, the most commonly used sequence is 5'-UUCAAGAGA-3' [42]. Another variation of the shRNA trigger is to mimic an endogenous miRNA structure, where the 22 bp sense and antisense sequence is replaced with the desired siRNA sequence while the other bumps and loop of the miRNA are maintained (Fig. 2.C) [43, 44]. Research has shown that triggers that enter the RNAi pathway through a more natural route, like the miRNA based shRNA, result in a more effective knockdown. Another effective trigger expressed from a plasmid is a long hairpin RNA (lhRNA) (Fig. 2.D). The lhRNA structure comprises of a long sense and targeting antisense sequence (30-90 bp) with a loop structure between [45]. In the cytoplasm, this long dsRNA structure is randomly processed by dicer resulting in multiple different siRNA effector molecules. A lhRNA can be designed to target multiple mRNA targets or have all of the random siRNAs targeting a single mRNA. However, because of the random processing of dicer, off target affects are harder to predict [37]. As well, in mammalian cells, lhRNA have a greater chance of inducing an



immune response. For many research projects utilizing either a basic shRNA or a mir-based shRNA are ideal.

### *Promoters to Express RNAi Effector Molecules*

As the popularity of using plasmids for RNAi based research increased, promoters were evaluated for their ability to express functional siRNA triggers. There are two classes of promoters used for constitutive expression of RNAi triggers based upon the RNA polymerase they recruit. One class recruits the Pol II promoter. These promoters have typically been used to express a mir-based shRNA, or tissue specific, or inducible shRNA expression. The other type of promoter used, recruits a Pol III polymerase. These promoters have been typically used to express very precise sequences like those required in the sense-antisense hybridization siRNA expression plasmid or a basic shRNA. While both types of promoters are commercially available, neither a Pol III nor Pol II promoter has been found to be superior. It is primarily up to the researcher to determine if a Pol III or Pol II of promoter will work best for their research studies.



**Fig. 2.** Examples of RNAi Triggers. **A.** The sense and antisense strands corresponding to the siRNA complex are driven by individual Pol III or T7 promoter. **B.** A single Pol III promoter drives the transcription of a DNA sequence corresponding to the sense strand, a 4-9 bp loop (non-complimentary) sequence, and antisense strand. Immediately following this DNA sequence are 5-7 thymines that result in the termination of transcription. **C.** A single Pol II promoter drives the transcription of a long transcript that includes a fluorescent marker, as well as, an endogenous miRNA sequence where the effective siRNA sequence is replaced by a design siRNA sequence. **D.** A single Pol III promoter drives the transcription of a stem loop structure. However the sense strand is longer than 22 bp, which results in multiple siRNA products when the dsRNA is cleaved by Dicer.

Many different promoters have been effectively used to knockdown gene products. Pol II promoters were initially utilized to mimic endogenous miRNA expression [46]. Pol II promoters are responsible for the majority of mRNA transcription, miRNAs, and naturally transcribe long sequences. In shRNA expression plasmids, the Pol II polymerase was used to transcribe a long mRNA sequence that included the gene for a fluorescent marker as well as the mir-based shRNA in the same transcript [47]. This combination of expression is desirable because the fluorescence can be directly correlated to shRNA transcription. Early studies used the human cytomegalovirus immediate-early (CMV) promoter to express a mir-based shRNA [48, 49]. However, some experiments suggested that the promoter is too effective and elicits an interferon response in some cells. Another Pol II promoter that also has been frequently used for plasmid based shRNA expression is the human ubiquitin C promoter [50]. More recently, the elongation factor 1- $\alpha$  (EF1- $\alpha$ ) promoter has been utilized in commercial mir-based shRNA expression vectors. The EF1- $\alpha$  promoter expresses the alpha subunit of the EF-1 complex. This housekeeping complex is responsible for the enzymatic delivery of tRNAs to the ribosome [51]. The EF1- $\alpha$  promoter has been very effective for *in vitro* based RNAi experiments [18]. The majority of the shRNA expression plasmids in our laboratory use the EF1- $\alpha$  promoter to express a fluorescent protein and a mir-based shRNA as a single transcript. Another Pol II promoter that has been increasingly used is the CAG promoter. This promoter is unique in that it was developed by combining the CMV early enhancer element, with the chicken beta-actin promoter, and the first intron of chicken beta actin [52]. The combination of these elements has

resulted in a very strong promoter that is active in many cell types. Many Pol II promoters are successfully utilized in the commercially available mir-based shRNA expression vectors and further research is required to determine which will work best for the desired experiments.

However, some experiments are interested in tissue specific or inducible shRNA expression. As a result, many Pol II tissue specific promoters have been explored to locally express a shRNA [53, 54]. Other experiments might require expression of a shRNA at a certain time or stage of development. Because of this, inducible promoters also have been developed to express a shRNA from a plasmid [50, 55, 56]. Two types of inducible elements are commonly used with a Pol II promoter in shRNA expression vectors. One depends on a Cre-Lox recombination reaction to induce expression of a shRNA. Where in the presence of the recombinase Cre, an inhibitory sequence is removed that then allows the expression of the shRNA. Another type of inducible shRNA expression vector has inducible elements in the promoter that are tetracycline dependent for activation/suppression of the promoter to initiate or suppress expression of a shRNA. Research is continuing to advance, improve, and diversify expression of a shRNA from Pol II based shRNA expression plasmids.

While Pol II promoters were explored to mimic the miRNA pathway and produce long transcripts, Pol III promoters have also been used to express precise shRNA sequences.

These are effective because they are used to transcribe small nuclear RNAs that are required for normal cellular function. As a result, their activity should be consistent among cell types. Also, Pol III promoters have a well defined transcriptional start site and termination sequence of 5-7 consecutive thymines. Because a Pol III promoter has the ability to transcribe a precise transcript constitutively among many cell types they have been extensively used in shRNA expression vectors.

Three Pol III promoters have been widely used for the expression of a shRNA from a plasmid. The first Pol III promoter used to express a shRNA was the human U6 small nuclear RNA (snRNA) promoter. The snRNA is the noncoding component of the U6 small nuclear ribonucleoprotein. This housekeeping ribonucleoprotein is required for pre-mRNA processing. When the U6 promoter was used to express a shRNA, it resulted in the effective knockdown of firefly luciferase and neuron specific  $\beta$ -tubulin [57]. The U6 promoter has become one of the most popular Pol III promoters on commercially available shRNA expression vectors. Another frequently used promoter is the H1 RNA promoter [58]. The H1 protein is a subunit of the Ribonuclease P (RNase P) complex. RNase P is a ribozyme responsible for the modifications of tRNA molecules, and plays a role in Pol III transcription. A unique advantage of the H1 promoter is the short effective promoter region, only ~100 nt are required to recruit a Pol III polymerase, as compared to 350 nt for the U6 promoter region. A third promoter used for shRNA expression in many plasmids is the 7SK RNA promoter [59-61]. 7SK RNA has been

found to be an inhibitory protein that binds elongation factor P-TEFb. P-TEFb is responsible for activating the Pol II polymerase for transcription. While other Pol III promoters have been explored the U6, H1, and 7SK are the most frequently used in shRNA expression plasmids.

#### *Livestock Pol III Promoters*

RNAi technologies in animal agriculture based research could be a valuable tool to better understand basic physiology and molecular pathways. It is particularly beneficial because creating knockout livestock models to study a molecular pathway is impractical and very difficult when compared to rodent models. However, the majority of shRNA expression vectors to date has used either human or mouse promoters. While the mouse and human promoters are functional in livestock species; the use of species specific promoters to express a shRNA has been explored. Thus far, bovine, porcine, and chicken Pol III promoters have been identified and characterized [59, 61-65]. When these promoters have been compared to the mouse or human counterparts sequence differences among key promoter elements have been reported. In addition, some livestock promoters have been more effective than the human or mouse promoter in knockdown assays *in vitro*. These previous experiments have shown that Pol III promoters from other species might be better suited for RNAi based research projects.

RNAi applications in animal agriculture research could have many benefits. Cattle are important in agriculture research as early embryo models, and for milk and meat production. To determine if homologous Pol III promoters were advantageous in shRNA expression, bovine Pol III promoters were identified. These promoters were transiently tested for their ability to express an shRNA resulting in the knockdown of EGFP in various cell lines. The bovine Pol III promoters analyzed to date include: 7SK, U6-1, U6-2, U6-3 [59, 66]. The U6 promoters are unique because there are numerous copies of the U6 snRNA in the genome. It is estimated within the human genome that there could be over 200 U6 genes, and to date 9 promoter regions have been identified. As a result, it can be assumed that these (U6-1,-2,-3) are probably not the only three U6 promoter regions in the bovine genome-. When the three U6 promoters were analyzed, two (U6-2, -3) resulted in a similar knockdown of EGFP and U6-1 was slightly less effective. In addition, the bovine U6-2 and U6-3 shRNA expression plasmid were more effective at reducing fluorescent levels than the commercially available mouse U6 expression plasmid pSilencer 1.0-U6 [66]. However, the bovine 7SK expression plasmid resulted in the greatest knockdown almost 2-5 times better than the pSilencer 1.0-U6 in all of the cell lines utilized [59]. These cell lines included: Vero (monkey), L929 (mouse), DF-1 (chicken), MDBK (bovine), and Hela (human). As a result of this research, bovine Pol III promoters U6-2, U6-3, and 7SK were more efficient at expressing a shRNA in bovine cells than the commercially available pSilencer 1.0-U6. In conclusion, it appears that bovine Pol III shRNA expression vectors are a better choice for transient experiments using bovine cells.

Another livestock species where RNAi research could assist with a better understanding of physiology and enhance production is swine. Three functional porcine promoters have been identified to date pY1, pY3, and pU6 [63]. The Y RNAs are small cytoplasmic RNAs that are components of the Ro ribonucleoprotein complex (RNP). To determine the promoter strength shRNA expression vectors were made using the above mentioned promoters to drive expression of a luciferase targeting shRNA and reduction of luciferase activity was analyzed. The most effective promoter was the U6 promoter. Its activity resulted in only about 5% luciferase activity (or 95% luciferase knockdown) when compared to the negative controls. In addition, the cells using the Y1 promoter to express the luciferase targeting shRNA were observed to reduce luciferase activity by >85%. While not as effective with a range of 70-82% the Y3 promoter was still efficient at producing a functional shRNA. In this study the promoters were not compared to mouse or human promoters, however, they are very effective and are a viable option for porcine based research.

The chicken is a widely used livestock model for studies of vertebrate development and genome function. RNAi could prove to be very beneficial in these fields and many chicken Pol III promoters have been analyzed. Currently, the chicken 7sk promoter and 4 U6 promoters (annotated U6-1, -2, -3, -4) have been identified and analyzed for their ability to express an shRNA [62, 64, 65]. Previously, it was reported in other species that the species specific 7sk promoter is more efficient at expressing a shRNA than



currently available shRNA expression plasmids [59]. However, when the chicken 7SK was analyzed in chicken DF-1 cells, the pSilencer 1.0 U6 resulted in a slightly greater knockdown [62]. The chicken 7SK shRNA expression vector was able to achieve a >50% knockdown, and was concluded by the author to be a viable option for future RNAi research. Two independent laboratories tested the chicken U6 promoters' ability to express shRNAs targeting either the fluorescent protein EGFP or the Luciferase [64, 65]. When targeting luciferase in chicken ovary cells (COV1), the positive control (a human U6 promoter) resulted in a 41% knockdown, while the 4 chicken U6 promoters resulted in a knockdown range of (47-26%). The chicken U6-2 and U6-3 were the most effective at reducing luciferase activity. When these promoters were used to target EGFP in DF-1 cells, U6-3 and U6-4 were the most efficient of the chicken Pol III promoters with a reduction of fluorescence approximately 55-60%. In addition, these knockdown levels were comparable to the positive pSilencer 1.0-U6, 62%. Based upon these studies, the chicken promoters are comparable to commercially available promoters and can be effectively used in RNAi studies of chicken cells.

### *Applications of RNAi*

RNAi has become a powerful tool utilized for research, pharmaceuticals, and agricultural advancements. In the research field, the RNAi pathway is an effective quick alternative to creating loss of function mutants to study protein function, complex protein pathways, and functional genomics in both *in vitro* and *in vivo* studies. In our

laboratory, we are utilizing the RNAi pathway to study the roles of DNA methyl transferases and histone modifiers in early embryo development. Also, the first transgenic animal that our laboratory produced which expressed a shRNA was a prion knock-down goat fetus [6]. The laboratory is continuing research in transgenic animal development by targeting the endogenous protein myostatin, which is a muscle growth inhibitor protein that should result in transgenic animals with greater muscle mass. In addition, we are also utilizing the RNAi pathway by developing animals or *in vitro* models that express shRNAs targeting livestock viral genomes (FMDV and Equine Infectious Anemia Virus). The use of dsRNA as short-lived small-interfering RNA's (siRNA) or constitutively expressed short-hairpin RNA's (shRNA) are being explored as medicines to combat viruses like HIV and hepatitis, or utilized against macular degeneration and liver failure [45, 67]. In agriculture, RNA interference is being explored in crops as possible means for additional disease and pest resistance [68]. RNAi has become a vital component to many different fields; however, effective dsRNA delivery systems have not been optimized for specialized uses.

### **Retroviral Transgenesis**

Just as the utilization of the natural RNAi pathway has proven to be a powerful tool in research; the manipulation of retroviruses to introduce foreign DNA into target genomes has proven invaluable. The use of recombinant retroviruses to shuttle DNA into the genome of early embryos was first used to make transgenic mice before being used to

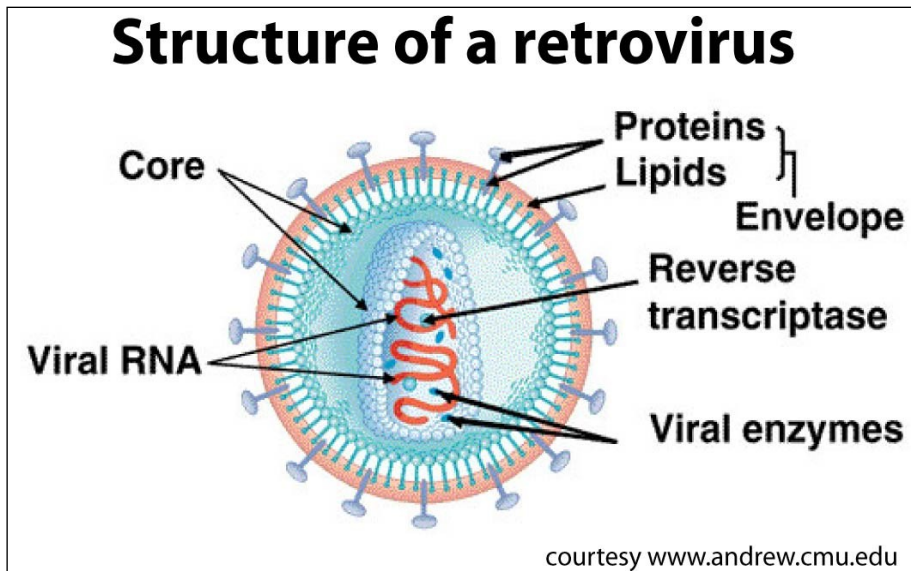
develop transgenic livestock models. Transgenic mice were made using the Moloney leukemia virus (MLV) to introduce its genome into all organs of the mouse by transducing 4-8 cell preimplantation embryos [69]. This was the first time that a gammaretrovirus infected a tissue it was believed to not be able to infect and was able to pass the transgene to subsequent offspring. However, other research studies using similar early generation vectors found that after integration, majority of the transgenes were silenced during the embryo's development [70]. Another disadvantage to the use of these early gammaretroviruses as a gene delivery system is that they can only transduce actively dividing cells; thus limiting their potential for *in vivo* gene delivery in nonproliferating cells [71]. To overcome this, Naldini et. al; 1996, developed a similar gene transfer system using components of the lentivirus HIV-1.

### *Lentivirus*

Natural lentiviruses are a genus of the Retroviridae family. Some examples of these viruses include: Human immunodeficiency virus (HIV), Simian immunodeficiency viruses (SIV), and Feline immunodeficiency virus (FIV). They are unique in that they can infect dividing and non-dividing cells. The lentivirus actively integrates large amounts (~10 kb) of DNA into the infected cell's genome; thus, allowing long term expression of RNA. It is because of this unique trait that lentiviruses have been modified for gene therapies for *in vitro* and *in vivo* experiments [72, 73].

### *Process of Lentivirus Infection*

Lentiviruses are positive strand RNA viruses encased in an envelope. They are approximately 80-100 nm in diameter. The envelope contains glycoproteins that target a specific extracellular protein, i.e. HIV recognizes the glycoprotein CD4 on helper T cells, macrophages, monocytes, and dendritic cells. Within the envelope is a cone-like protein based nucleocapsid that contains two RNA genomes, and proteins required for replication and integration. Some of these proteins include reverse transcriptase, protease, and integrase (Fig. 3). Once the envelope glycoproteins bind onto the cell receptors, they mechanically pull the virus to the cell membrane. After fusion, a pore is created and the contents of the virus are extruded into the cytoplasm of the cell; while the envelope and capsid are degraded. The RNA is first transcribed into a complimentary strand of DNA. The single stranded DNA is reverse transcribed resulting in double-stranded DNA. A sequence of the dsDNA is recognized by the integrase protein that shuttles the DNA through a nuclear pore. Once inside the nucleus, the integrase makes a random nick in the cell's DNA and ligates the viral RNA into the genome. Unique to the viral genome that facilitate the integration and replication of the viral from the cells genome are long terminal repeats (LTR). Each LTR contain a promoter recognition sequence (5'/3' UTR). During the reverse transcription, the 3' LTR of the viral RNA becomes the 5' LTR and is responsible for driving expression of the viral RNA after integration into the host genome. Once integrated, the virus then uses the cell's machinery to make more positive-strand mRNA and production of virus.



**Fig. 3.** Illustration of a Retrovirus (Lentivirus). The retrovirus is protected by an envelope protein case that has glycoproteins that bind specific cell receptors on target cells. Within the envelope, a cone-like capsid contains two positive-strand RNA genomes, as well as the viral proteins: reverse transcriptase, integrase, and protease.

### *Utilization of Multiple Vectors to Construct Recombinant Lentivirus*

Because of the highly infective nature of these lentiviruses, key components were removed from the viral genome to make them safe for use as gene delivery tools. The essential components required to construct a recombinant lentivirus include the viral proteins: integrase, reverse transcriptase, transactivators, internal capsid proteins, as well as the glycoenvelope proteins. In addition the RNA to be packaged and integrated as DNA into the genome must be present. Multiple vectors were developed to deliver the key components in trans after being transfected in a packaging cell line. The goal of these manipulations was to create a virus that would only go through the initial stages of infection, genome integration, and not replicate to produce additional infective viral particles.[8, 73].

One of the vectors is commonly called a packaging vector provides all the key viral proteins required for packaging and integrating the viral RNA. A packaging vector (pCMV $\Delta$ R9) was created that used the CMV promoter drive expression of the viral genome. However, key deletions were made to the HIV genome in the 5' LTR packaging sequence and in the *env* gene. In addition, the 3' LTR of the genome was replaced in this vector with a polyadenylation sequence. This modification added another safe-guard to decrease the chance that RNA encoding the viral proteins essential for packaging, reverse transcription, and integration from being included in the recombinant virus. Over generations of developing this packaging vector, researcher's

have systematically deleted genes of this modified HIV genome to determine the proteins essential for viral production and integration. As a result, they determined the viral products Vif, Vpr, Vpu, and Nef were not required for effective packaging and integration transgene RNA and subsequently deleted from packaging vector (pCMVΔR8.9) [74]. The essential genes in the packaging vector are *gag*, *pol*, *tat*, and *rev*. The *gag* gene encodes viral products essential to form the capsid of the recombinant lentivirus. The other essential viral products, reverse transcriptase and integrase are encoded in the *pol* gene. The two Tat proteins are transcriptional transactivators required to activate transcription from the 5'LTR. The Rev protein binds the RRE sequence of the RNA and assists in the shuttling of RNA through the nucleus to the cytoplasm. By minimizing the genes expressed from this packaging vector and deleting key components, only the proteins are expressed during the production of recombinant lentiviruses and the gene sequences should not be transferred after transduction.

Another requirement for an effective gene delivery rlentivirus is an envelope that can readily bind many cell types. To accomplish this, the vesicular stomatitis glycoprotein envelope (VSV-G) is utilized [75]. The envelope protein was also provided on a supplemental vector (pMDg) to limit the chances of replication competent escape viruses. The advantage of using the VSV-G envelope protein is that its corresponding extracellular receptor is ubiquitously expressed in animal cells, including those typically

used for *in vitro* experiments [76]. Another advantage of the envelope is that it is durable and protects the virus from adverse conditions. This quality is advantageous to maintain a high percent of infective recombinant viral particles when purifying by ultracentrifugation or other methods, and also enhances long term storage of the virus. The utilization of the VSV-G envelope has enhanced the capabilities of recombinant lentiviruses for gene transfer.

The final component required to produce replication incompetent lentivirus is the vector that provides the packaged RNA. Within this RNA sequence are the specialized genes of interest that the researcher wants to integrate into the target cells genome. These can include but are not limited to: fluorescent markers, antibiotic resistance, RNAi expression cassettes, exogenous proteins, inducible promoters, or other genes or DNA sequences of interest. As a result, many varieties of vectors are available that can be packaged into a recombinant HIV-based lentivirus. The majority of them share key features that facilitate transcription, packaging, and integration. Flanking the specialized sequences are 5' and 3' LTRs. The 3' LTR has had a region deleted that is required for promoter recruitment and initiation of transcription [9]. This is important, because after infection the viral RNA is reverse transcribed into dsDNA and the sequence corresponding to 3' LTR is now the 5' LTR. As a result of this deletion, once integrated into the genome the LTR can not initiate transcription of the entire DNA sequence, thus further decreasing the chances of replication competent escape viruses. In addition to



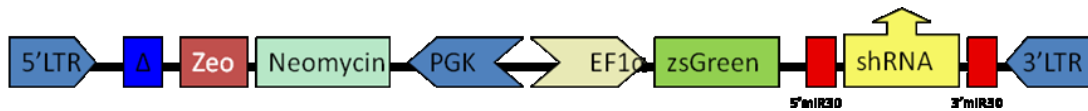
the LTRs, the vector has to have a packaging sequence specific to the lentivirus genome being used in the packaging vector. Most vectors also have a central poly-purine tract sequence (cppt) near the 5' viral promoter region that enhances transduction efficiency [77]. Another cis-acting DNA element in the lentiviral vector is the woodchuck post-regulatory element (WPRE). It is cloned 3' to the transgene near the polyA signal of the 3' LTR and enhances integration of the transgene [78]. These viral elements provided on the vector facilitate and enhance the integration of genes of interest to the researcher.

Researchers have continued to refine and diversify the viral requirements in this multi-vector system. The goal of this system was to provide the necessary proteins in trans that are required to create a recombinant lentivirus that integrates the packaged RNA but is unable to replicate after infection. As a result of these manipulations, a very effective laboratory safe method of using viral mediated transgenesis of cells was developed.

#### *rLentiviral Vector Utilized in These Experiments*

In our laboratory, HIV-based lentiviral vectors are utilized as tools for long term expression of shRNAs targeting endogenous proteins and exogenous viral genomes. The lentiviral vector that was used for this research was called Nef-GT (Fig. 4). The integrated transgene contains the EF1 $\alpha$  promoter for the expression of an mRNA sequence that encodes the fluorescent marker zsGreen and a mir30 based shRNA. The

zsGreen is important to visualize transduction rates. In addition, a human phosphoglycerate kinase (PGK) promoter drives the expression of an antibiotic resistance gene, neomycin, for selection of transgenic cells. Also, Nef-GT contains all of the key viral components that allow it to be packaged and integrated into the genome.



**Fig. 4.** Nef-GT mir-30 Based shRNA Lentiviral Expression Vector. Transgene has dual opposing Pol II promoters. Elongation Factor 1  $\alpha$  promoter is responsible for driving the fluorescent marker zsGreen and the mir30 based shRNA sequence in the same transcript. The Phosphoglycerate Kinase (PGK) promoter is responsible for the expression of the antibiotic resistance, G418 selection gene, and zeocin resistance as well.

#### *Different Promoter's Potential for shRNA Expression Following a rLentivirus*

##### *Transduction*

Recombinant lentiviruses have been the delivery system of choice for long-term, low cost dsRNA expression where protein knockdown is desired by the RNAi pathway. During the search for optimal promoters in vector mediated shRNA delivery systems, Pol III promoters were initially explored. They are ideal for expression of precise short RNA sequences because of their specific transcriptional start and termination sequences. One of the first vectors developed used the human U6 small nuclear RNA promoter to express shRNAs targeting the firefly luciferase gene [79]. Since then, Pol III promoters

have been the primary choices for commercial companies for shRNA production. The most widely used promoters for shRNA expression include the human U6 and human H1. In addition, the human 7sk promoter has shown to express high levels of shRNAs in various cell types [80]. While these promoters have proven successful in various experiments, Lambeth et. al, 2006 reported that bovine U6-2 driven shRNA expression vector resulted in a greater knockdown of the target protein EGFP than its human U6 counterpart in various cell types. In addition, the bovine 7SK promoter based shRNA expression vector achieved the greatest knockdown in his experiments. Due to these previous studies we decided to pursue species specific (bovine) Pol III promoters to express shRNAs in our lentiviral vector Nef-GT. These unique bovine Pol III based shRNA expression vectors were used to create rlentivirus and introduce a shRNA targeting genes of interest into the bovine genome that could theoretically enhance production of the transgenic animal.

### **Enhancing Livestock Production Characteristics**

Since the domestication of animals over 11,000 years ago, humans have had tried to increase the production of desirable traits. Originally, this was done by selecting superior animals that exhibited the desired trait or traits, thus the development of selective breeding. Some of these traits included: wool or coat production, increased meat production, increased rate of growth, better meat quality, increased milk production, better mothering instincts, and many others. Unfortunately, while selecting for specific enhanced characteristics other important physiological and metabolism

pathways have been lost. Over generations of livestock production, selective breeding has been effective at increasing the quality of the traits.

### *Assisted Reproductive Technologies*

A limitation of selective breeding in animal agriculture has been the availability of superior genetics to a producer. Before the mid-late 1900's the producer was limited to either the best of the current herd or was required to purchase an animal to introduce new genetics. As a result of the constrained distribution of genetics, the increased production of selected traits within breeds was limited. Reproductive research and private industry have increased the availability of superior genetics by developing methods for safely collecting, storing, and transferring male and female gametes resulting in pregnancies [81, 82]. These assisted reproductive technologies (ART) facilitated the international distribution of superior genetics to producers for a cost far less than purchasing the animal. In addition, ART allowed the producers to distribute a superior animal's genetics and produce more offspring from this animal than traditional breeding practices. However, even with all of these technology advancements the genetic gain of the desired traits has been reported to be 1-3% per generation. But over the past 20 years with the common use of ART in animal agriculture a significant increase in important production characteristics has been observed.

### *Transgenic Livestock*

Alternative biotechnology tools have been explored to enhance a desired trait, or to introduce a new trait by creating transgenic livestock. In 1982 transgenic mice were produced by microinjection of a gene construct into the pronuclei of a zygote. This gene construct used the heavy metal inducible mouse promoter Metallothionein-I (MT) to express either rat or human growth hormone (hGH) [13]. The transgene was developed with the ultimate goal of significantly increasing growth and muscle development.

These mice not only exhibited an increase in hGH mRNA, but also a significant increase in growth, up to 4 times greater than controls, and muscle mass. In addition, these genes were passed through the germ line to subsequent generations. Based upon this research a similar transgene was used to develop the first transgenic livestock (rabbits, sheep, and pigs) by microinjection into the pronuclei of a zygote [83]. These were the first reports proving that researchers could introduce foreign genes into the genomes of livestock.

Further research efforts were pursued to increase the muscle mass by creating transgenic pigs. The following data was nicely reviewed by Pursel V. G., and Pinkert C. A., *et al.* 1989. Researchers used microinjection to develop transgenic pigs expressing transgene containing either hGH, bovine growth hormone (bGH), recombinant growth factor (rGF), or human insulin like growth factor-1 (hIGF-1). Only about 8% of the 7000 microinjected eggs resulted live births and of those only 7% were transgenic (40 animals). The economic importance of increasing the efficiency of weight gain,

decreased body fat, and overall better body composition provided incentive for this research. These transgenic animals did not respond to the increased levels of growth hormone like the mice but a significant increase in average daily weight gain of 11-15% was observed. In addition, when the bGH was used a 16-18% increase in feed efficiency was also observed. These enhanced production characteristics were significant enough to have an economical impact in commercial herds, but the efficiency of producing these animals was not practical for commercial applications.

In addition, numerous negative side effects were observed in these founder animals. They had a higher incidence of pericarditis, early onset of arthritis leaving some animals unable to walk, and peptic ulcers. Also, many of the founder animals had impaired fertility, making the development of transgenic lines very difficult. Also, the expression of an exogenous hormone for human consumption has risen many concerns not only among the public and the FDA[84]. As a result of these negative characteristics and the low success rates, the enthusiasm of utilizing transgenic livestock as everyday meat animals diminished.

Transgenic livestock have been particularly beneficial for pharmaceutical production and human medicine. The mammary gland naturally produces various proteins in high quantities for the nutritional support and development of young. Researchers have harnessed these qualities by using mammary specific promoters to express recombinant

proteins and turned dairy animals into efficient pharmaceutical bioreactors. Like many technologies that end up in livestock laboratories, this was first accomplished in a mouse model where transgenic mice were created that had produced the human tissue plasminogen activator protein driven by the murine whey acid promoter [85]. Shortly after this research was published, the potential of expressing recombinant human proteins in milk exploded in the academic and pharmaceutical research fields [86, 87]. The use of a transgenic livestock herd as bioreactors to produce pharmaceutical recombinant proteins has the potential to replace large facilities for tissue culture based bioreactors that are currently used to produce similar quantities of the recombinant protein. The first transgenic livestock were sheep that produced 63 g/l of  $\alpha$ -1-antitrypsin in their milk [88]. In the following years, other mammary gland specific promoters were characterized for their ability to express a variety of recombinant human proteins important to the pharmaceutical industry. Currently, some transgenic goats express a recombinant form of the human anti-clotting protein antithrombin, as well as the coagulation factors Factor VIIa, Factor IX, and Factor VIII[89]. These clotting proteins are important for the treatment of Type A hemophilia and are administered to patients to avoid episodes of excessive bleeding. The low overhead once the transgenic animals are developed and the value of the products they produce have resulted in an important niche in the pharmaceutical field.

Another area of human medicine where the utilization of transgenic livestock has been explored is xenotransplantation. Xenotransplantation is defined as; transplanting cells, tissues, or organs from one species into another. Currently in the United States there are over 103,000 people waiting for transplants and 60% of the patients will die before they can be matched to a donor. Transgenic pigs someday might be able to provide a viable option for some of these donors. One of the first experiments testing the potential of pig organs for transplant was conducted in 1988. A pig heart was transferred into a non-human primate. Unfortunately in this early xenotransplantation experiment the organ only lasted hours. Other early xenotransplant organs were able to survive up to 5 days before being recognized as being foreign and rejected by the host's immune system[90].

Researchers have persisted and made significant strides in the development of transgenic pigs (1,3 galactosyl transferase gene knockouts or expressing the  $\alpha(1,2)$  fucosyltransferase gene) that have delayed the rejection in some cases up to 6 months for a heart when combined with novel immunosuppressant agents[91-93]. Besides the rejection, another concern that has been raised is the possibility of porcine endogenous retrovirus (PERV) developing into a competent virus that infects organ recipients. Researchers have developed transgenic pigs expressing shRNAs targeting highly conserved sequences of these PERV[94]. *In vitro* studies showed a 73% reduction of the reporter plasmids expressing PERV proteins, in addition when the transgenic pigs were tested for the presence of PERV, none were observed by western blots or



immunofluorescent staining. While there are still questions about the true potential of xenotransplantation as a means of extending a transplant patients life until a suitable donor can be found, it appears that specific transgenic knockouts coupled with anti-rejection drugs and RNAi are all major advancements in research that might facilitate an important need for transgenic pigs.

#### *Methods to Produce Transgenic Livestock*

The use of transgenic livestock to enhance or introduce new production characteristics has been beneficial in many fields. To continue these advancements the efficiency of producing animals must improve. Initially, transgenic livestock were created by microinjection. Microinjection involves injecting a chosen gene construct directly into the pronucleus of a fertilized ovum. In these original studies microinjection resulted in very few live transgenic offspring. About 2,000 pig and rabbit ovum were injected and transferred into recipients resulting in about 200 viable fetuses being developed per species and a range of 10.4% (pig) to 12.8%(rabbit) of them having the transgene incorporated into the genome. However, the experiments involving the sheep only had 1 transgenic fetus from 1,032 ova injected and 73 fetuses collected. Later research studies, reported similar low efficiencies ~2% of injected ova resulting in the production of transgenic livestock [83, 95, 96]. The efficiency of microinjection is too low for the practical applications of large scale transgenic animal production.

In the early '90's another invaluable technology was developed that revolutionized transgenic livestock research, cloning. The first offspring was born from nuclear transfer of cultured cells [97]. Previously, nuclear transfer has been successful in the multiplication of elite embryos either from the 16-cell, embryonic discs, or cultured embryonic cells [98, 99]. However, this was the first study where cultured adult cells successfully resulted in a live offspring. As most pioneering studies the success rate was extremely low. Out of 277 fused couplets only 29 morulas developed and 1 live offspring for an initial success rate of less than 0.4%. Since then, advancements in the procedure have significantly increased the success rate of producing live cloned offspring in many different livestock species [100]. The increase success rate of cloning has been instrumental to the development of many transgenic livestock and animal research models which include but not limited to: sheep, goats, pigs, cattle, dogs, and cats [101]. Researchers can introduce the genes of interest into the genomes of an *in vitro* cell population and create single cell clonal populations. These populations can then be tested for productive integration sites and subsequently used to create cloned transgenic livestock. The advantage of this is that researcher knows that every embryo created is transgenic and has the gene of interest inserted. Cloning also allows for selecting genetically uniform same sexed offspring. While the success rate of creating transgenic clones is as low as 2%; it is still advantageous to use this method instead of pronuclear injection to create live offspring.

Recombinant lentivirus has been utilized to produce a variety of transgenic animals. Two primary methods that have been explored to create transgenic livestock are zona-free embryos cultured in lentiviral particles, or injecting the particles into the perivitelline (PV) space of all embryos or ova followed by IVF. The culturing of zona-free or 2-cell embryos or blastocyst, in lentiviral particles and transferring them into recipient animals has successfully resulted in transgenic animals. It was also observed that when blastocysts were cultured after hatching or without the zona pellucida that placenta specific expression was observed [102]. This localized transduction could be a powerful tool in helping better understand the maternal-fetal interface for reproductive physiology and human medicine. The most common method to create transgenic animals using lentiviral transgenesis is to inject the lentiviral particles into the PV space of early embryos or ova. This method has shown far superior abilities to maintain embryo viability after manipulation (>70%) when compared to pronuclear injection (<18%) [10, 103]. In addition, the pronuclear injection success rate of resulting in a live transgenic pig was 0.3-1.8%. In comparison, the preliminary studies using lentiviral transduction of livestock species was near 80% efficiency for producing transgenic embryos and 50% of those embryos resulted in a live birth [10]. Overall, the use of lentiviral transgenesis has been a promising tool to introduce transgenes into germ or embryonic cells for various experiments and development of transgenic livestock.

When using rlentiviruses as gene delivery tools, one must be aware of the potential hazards and adverse effects. The VSV-G glycoproteins of the rlentivirus envelope recognize an ubiquitously expressed mammalian cell receptor. While this is great for lentiviral transgenesis *in vitro*, it presents a potential danger if not handled properly because it can readily infect majority of cells, including cells found within skin. After the rlentivirus binds the cell, it can mediate the integration and subsequent expression of the transgene within the cell. In addition, the envelope protein provides a strong protective surface for the virus and can survive for an extended period of time and still remain infective. As a result, researchers must be very mindful when handling any equipment or fluids that could have been in contact with rlentivirus. Besides the biosafety hazards associated with the use of rlentivirus as gene delivery tools, they have concerns the recombinant viral RNA enters the cell. Even though many manipulations have been conducted to make them replication incompetent, and replication competent lentivirus have not been observed when using these later generation vectors [9, 104]. It is theoretically possible a replication competent lentivirus could develop [105]. There are also, concerns because of the unpredictable integration of the transgene. Two main concerns associated with integration include gene disruption or trans-activation of oncogenes [106, 107]. rLentiviruses are excellent gene delivery tools, however when they are used, knowledge of the potential risks and concerns is required.

## **Research Objective**

The long term goal of the Reproductive Sciences Laboratory at Texas A&M University is to use molecular biology tools and technologies to advance the field of animal agriculture. Currently, the laboratory is interested in enhancing production characteristics as well as developing disease resistance in livestock via transgenic technologies. The laboratory routinely utilizes recombinant lentiviruses to deliver a transgene that can express one or multiple shRNAs depending on the target. Preliminary research must be conducted to determine effective promoters to drive the expression of these shRNA in livestock species. Previous research suggests that livestock Pol III promoters are equally or more efficient at expressing a shRNA when compared to their human or mouse counterparts [59]. The goal of this research study was to identify the bovine Pol III promoters 7SK, U6-2, and H1 and clone them into the lentiviral vector Nef-GT. Then functionality and strength of the promoter to express a shRNA was analyzed both transiently and after integration into the bovine genome. These studies are essential before the vectors can potentially be used to create transgenic livestock.

## CHAPTER II

### MATERIALS AND METHODS

#### **Identification of Bovine Pol III Promoters**

Bovine Pol III promoters have been reported to be efficient at expressing simple shRNA complexes when transfected. However, they have not been analyzed for their ability to express a mir-based shRNA, nor their functionality after integration into a bovine genome. The goal of this research project was to determine the efficiency of bovine Pol III promoters to express a miRNA-30 based shRNA from the lentiviral vector Nef-GT. Three bovine Pol III promoters were selected for this research project, bU6-2, b7sk, bH1. The bU6-2 promoter region was previously identified by Lambeth (2006) and the sequence (432 bp) is publically available on the National Center for Biotechnology Information (NCBI) website, accession number DQ150531. In addition, Lambeth (2006) also identified and characterized a b7sk (695 bp) promoter region. The third promoter that we selected, bH1, was identified with the assistance of Dr. Kevin Wells, University of Missouri. A 204 bp region directly upstream of the H1 ribonucleoprotein coding region on chromosome 10 was selected for our research study.

## **Developing Bovine Pol III Driven mir-30 shRNA Lentiviral Expression Vector**

### *Nef-GT mir-30 shRNA Expression Vector*

The lentiviral vector Nef-GT (Fig. 4) was a gift from Dr. Michael Golding at Cold Springs Harbor, NY. The Nef-GT vector contains many key elements that were desired in our experiments. The integrated transgene utilizes the Pol II EF1 $\alpha$  promoter to express the fluorescent marker zsGreen to visualize transduction rates, as well as a Mir-30 based shRNA expression sequence. The shRNA expression sequence contains the cloning sites EcoR1 and Xho1 to insert a designed shRNA. Between the zsGreen protein and the mir-30 shRNA expression sequence is the restriction site Not1. This is the target site to insert the bovine Pol III promoter sequence. In addition, the transgene also contains a neomycin resistance gene to for selection of transgenic cells. Another key feature of this original vector is that between the shRNA insertion restriction sites there are only 6 nt. This small byproduct is then easily removed during purification steps after the restriction digest, decreasing the chance of background ligations. In addition, after the ligation of the PCR amplified shRNA into the Nef-GT, a restriction digest can be used to determine if the shRNA was inserted. As a result, fewer products have to be sequenced to confirm that the correct shRNA sequence is present in the vector. Because of these qualities, the Nef-GT vector was manipulated to utilize the bovine Pol III promoter (7SK, U6-2, or H1) to express a mir-30 based shRNA.

### *PCR Amplification of bPol III Promoter*

The bovine Pol III promoters were PCR amplified from the bovine genome before they were inserted directly upstream of the mir-30 shRNA expression cassette of Nef-GT. Primers were designed specifically to amplify the promoter regions from the bovine genome. The 5' (forward) primer was designed based either on the upstream region of previously published sequences; or for the bH1, a 21 bp region that would work well for primer annealing within the desired promoter range. The 3' (reverse) primer started at -1 transcription site and included 19 bp upstream. A single genomic DNA preparation obtained from a mix breed Bos Taurus Bos Indicus animal from Dr. James Womack's lab, Texas A&M University was used as the template for the bovine Pol III promoters. The promoter sequence was initially amplified with the respective forward and reverse primer: bU6-2 A1, A2; b7sk A3, A4; bH1 A5, A6 (Appendix B. Table 3). A high fidelity polymerase was used in the PCR master mix, which included 7% DMSO for genomic amplification. Detailed protocols can be found in Appendix A.1. The PCR products were subcloned into the pGEM Easy-T vector from Promega and grown in *Stbl3 Escherichia coli* (*E. coli*; Appendix A. 2, A. 3). Plasmid DNA was collected for restriction enzyme test digestion, sequence confirmation, and long term product storage (Appendix A. 4). Sequencing was performed by the Gene Technologies Laboratory at Texas A&M University using the internal T7 FWD primer sequence to confirm the products creating pGEM-bU6-2, pGEM-b7sk, pGEM-bH1.



*PCR Mediated Insertion of Restriction Enzyme Sites to Bovine Pol III Promoter and Cloning into Nef-GT*

The pGEM-bPol III vectors were used as DNA templates in a PCR reaction that added restriction enzyme sites Not 1 on the 5' end of the promoter and PsP0M1 on the 3' end (Appendix A.1-4). The following primers w/restriction sites (forward, reverse respectively) were used for bU6-2 B1, B2; b7sk B3, B4; bH1 B5, B6 (Appendix B. Table 3). Two restriction sites were used because when digested with these restriction enzymes, the cut sites resulted in compatible cohesive ends and allowed the promoter to be inserted in the Not1 site of Nef-GT, which is directly upstream of the mir-30 based shRNA expression cassette. Unique to this procedure, the Not1 site of the Nef-GT bPol III vector is maintained on the 5' end of the promoter while the sequence on the 3' end is not recognized by the Not1 restriction enzyme. This technique allows future digestions of the Not1 site without disrupting the orientation of the inserted promoter. Because the Not1 restriction enzyme is not very effective when the recognition sequence is near the edges of linear DNA product, the PCR products were inserted into the pGEM-Easy T vector creating pGEM-bU6-2+Not1/PsP0M1, pGEM-b7sk+Not1/PsP0M1, pGEM-bH1+Not1/PsP0M1 (Appendix A.2-4). Plasmid DNA was collected and digested sequentially to maintain 100% restriction enzyme efficiency and purified by ethanol precipitation between the two digestions. The digested samples were run on a 1% agarose gel to separate the digested bovine Pol III promoter regions and gel purified (Appendix A. 5-7). In addition, 3.2 ng of the Nef-GT vector was also purified via ethanol precipitation following Not1 digestion to maintain the sticky ends and quantity

of the DNA. The DNA pellet was resuspended in ddH<sub>2</sub>O for a concentration near 100 ng/  $\mu$ l.

The purified DNA products were electrophoresed on a 1.2% agarose gel and compared to the 2-log 1 kb Ladder (New England Biolabs) to confirm the quality and quantity of the DNA. Based upon these results the ligation mix using Quick Ligase (New England Biolabs) was modified to have a 6:1 molar ratio of bovine Pol III promoter:Nef-GT (Appendix A. 8). For the ligations, 150 ng of Nef-GT was used in the ligation reaction and the ng quantities of the respective bovine Pol III promoters (bU6-2 36 ng, b7sk 63 ng, bH1 18 ng) were determined using the Biomath calculator available from the Promega website. The ligations were transformed into Stb13 cells (Appendix A. 3, 4.1, 4.2, 8, 4.4, 4.5, 9, 10) and the Nef-GT bPol III plasmids were sequenced resulting in Nef-GT bU6-2, Nef-GT b7sk, Nef-GT bH1 (Fig. 5).

A.



B.



C.



**Fig. 5.** Nef-GT bPol III mir-30 Based shRNA Lentiviral Expression Vectors. Transgene has dual opposing Pol II promoters. Elongation Factor 1  $\alpha$  promoter is responsible for driving the fluorescent marker zsGreen. The Phosphoglycerate Kinase (PGK) promoter is responsible for the expression of the antibiotic resistance, G418 selection gene, and zeocin resistance as well. In addition each vector has a bovine Pol III promoter sequence directly upstream of the mir30 shRNA expression region, making unique bPol III shRNA expression cassettes. **A.** Is a representation of Nef-GT b7sk, **B.** Nef-GT bH1, **C.** Nef-GT bU6-2.

## **Functional Analysis of Nef-GT bPol III Vector**

### *Short Hairpin RNA Design, Amplification, Ligation into Nef-GT bPol III*

Once the novel lentiviral bPol III mir-30 shRNA expression vectors were developed, a functional assay was performed. To determine that the promoters could express functional a mir-30 shRNA a quantitative luciferase knockdown assay was utilized. Thus, two shRNAs were designed specific for this project, one targeting the luciferase mRNA and the other being a non-targeting shRNA control. To assist with the shRNA design, the publically available web tool, RNAi Central website, hosted by Cold Spring Harbor was utilized. The website used a computer algorithm to analyze the mRNA for the best shRNA target regions. The program outputs the shRNA as part of a mir-30 based shRNA stem-loop complex. This stem-loop complex is unique in that it includes a Drosha recognition sequence and the loop sequence is based on the endogenous miRNA confirmation. The luciferase mRNA sequence was entered into the computer algorithm to design shRNA's. The algorithm returned three possible targeting or positive control shRNA sequences. The first option was selected that targeted 1308 bp to 1328 bp of the luciferase mRNA. In addition, we used a previously designed shRNA sequence targeting the Foot and Mouth Virus as the non-targeting control (Appendix B. Table 4).

The 97 bp oligo sequence corresponding to the stem loop structure containing the unique shRNA sequence was ordered from Integrated DNA Technologies. The DNA was rehydrated to a 100 mM concentration with ddH<sub>2</sub>O and PCR amplified (primers S1, S2;

Appendix B. Table 3) to insert restriction enzyme sites for Xho1 and EcoR1 respectively on the 5' and 3' ends. (Appendix A.11) The PCR products were ethanol precipitated and digested with EcoR1 and Xho1 restriction enzymes as well as the Nef-GT bPol III vector. The digested products were ethanol precipitated and ligated into Nef-GT as described in Appendix A.12, 4.3, 5.2, 8) resulting in the vectors: Nef-GT bU6-2 Luc, Nef-GT bU6-2 NT; Nef-GT b7sk Luc, Nef-GT b7sk NT; Nef-GT bH1 Luc, Nef-GT bH1 NT.

*Transient Luciferase Assay to Confirm Function of Nef-GT bPol III Vector*

The ability of the promoters to express a functional mir-30 based shRNA was initially tested using a transient transfection luciferase knockdown assay. For this experiment, the vector's ability to knock down the luciferase protein was analyzed. The relative luciferase protein levels were quantified using the Stop n Glo Assay (Promega) and a luminometer. The pGL3 (Promega) vector was used as the luciferase expression vector, and as a transfection control, the pRL vector (Promega) was used to express the Renilla luminescent protein. In addition, for this *in vitro* preliminary experiment, human embryonic kidney cells (HEK) 293-T cells were utilized. The cell culture media utilized in this experiment and subsequent cell culturing was DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1x concentration of Penicillin/Streptomycin (Invitrogen). The experimental vectors that were transfected were: Nef-GT bU6-2 Luc, Nef-GT bU6-2 NT; Nef-GT b7sk Luc, Nef-GT b7sk NT; Nef-

GT bH1 Luc, Nef-GT bH1 NT The ratio for the transient transfection was 1:10:5 of Renilla: Luciferase: Experimental Vector or 40 ng pRL: 400 ng pGL3: 200 ng Nef-GT bPol III Luc or Nef-GT bPol III NT. To deliver the vectors into the HEK 293-T cells calcium phosphate mediated transfection was utilized (Appendix A.13). The plasmids were allowed 48 hrs to produce their respective products before luciferase and renilla activities were measured with the Stop and Glo Assay (Appendix A. 14). The measurements were made with a luminometer and then exported to an Excel file.

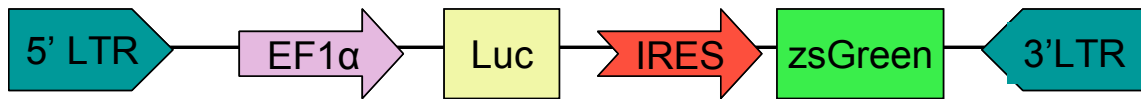
#### *Statistical Analysis of Relative Luciferase Activity*

The relative percent of luciferase activity was calculated by dividing the relative luciferase activity of the Luc targeting controls by the Null controls for that vector. The relative percent of luciferase activity was then arranged to be exported into the statistical program JMP. ANOVA was used to determine statistical differences among the group of samples. In addition, the means were compared pairwise by a Student's t-test. This test determined if there was a statistical difference between the targeting Luc samples and to their respective Null control, as well as differences among promoter function. Finally, the data was reported as percent luciferase knockdown when compared to its non-targeting control.

## **Functionality of Nef-GT bPol III shRNA Transgene after Integration into Bovine Genome**

### *Development of Luciferase Expressing MDBK Cells*

The purpose of this experiment was to determine if the Nef-GT bPol III shRNA expression cassette was able to produce a functional shRNA after it was integrated into the cell's genome. For this experiment, the luciferase protein being targeted was expressed from the genome of the immortalized Madin-Darby bovine kidney (MDBK) cells. The luciferase gene was inserted into the MDBK genome using the PEIZ+Luc recombinant lentivirus (Fig. 6; Appendix A.15 - 19, 21). The integrated transgene has an EF1 $\alpha$  promoter expressing the luciferase coding mRNA sequence followed by an internal ribosome entry site to express the zsGreen fluorescent protein. Following this transduction, the MDBK cell line constitutively expressed the luciferase protein and low levels of zsGreen. As a result of these cells (MDBK+Luc) not having a selectable marker, the transgenic cells had to be sorted. For the cell sorting, a flow cytometer was used with stringent high accuracy parameters. Following the sorting, a population of >99% zsGreen positive MDBK+Luc cells were grown and maintained. In addition, the luciferase activity for  $8 \times 10^8$  cells was measured in triplicate to calculate a background measurement.



**Fig. 6.** rLentiviral Vector PEIZ Luc. This vector utilizes the Elongation Factor 1  $\alpha$  promoter to express an mRNA sequence whose coding sequence is inserted into the multiple cloning site (MCS). In addition the vector has a fluorescent green marker, zsGreen, that is expressed by an internal ribosomal entry site (IRES).

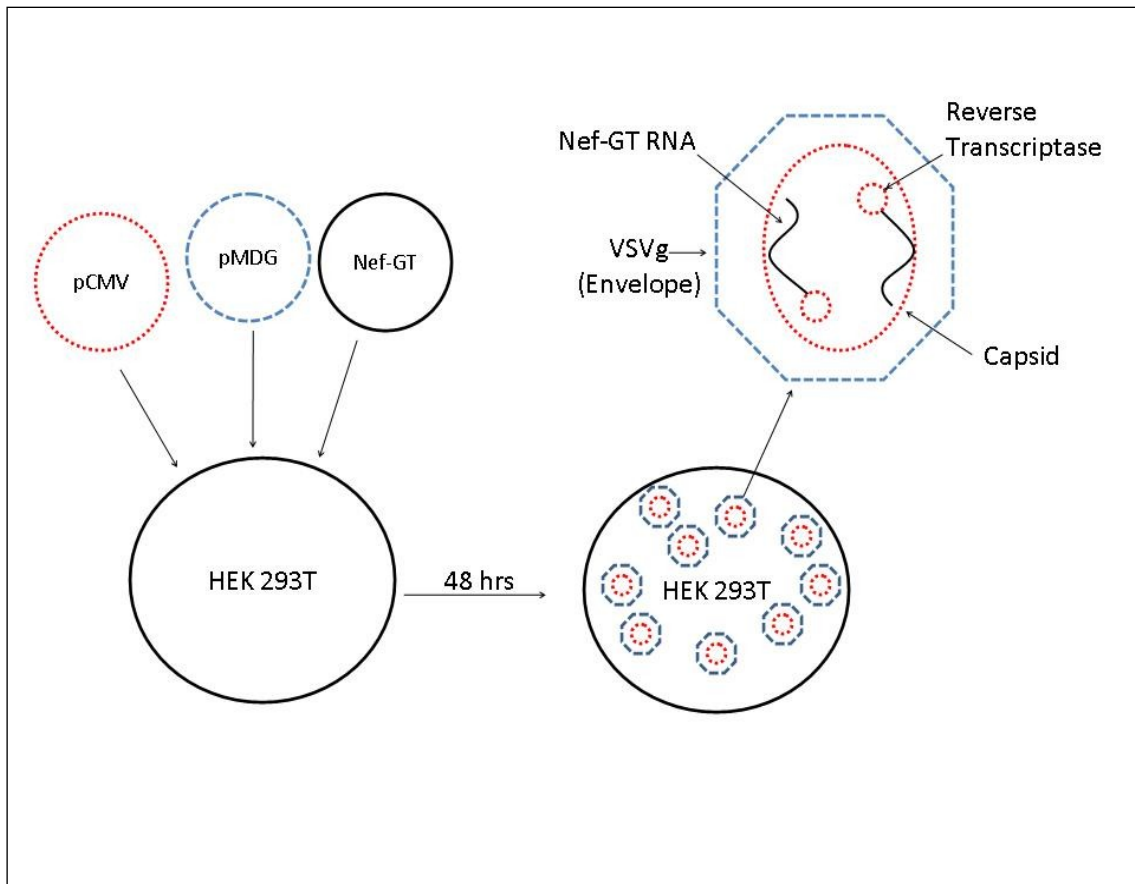
### *Construction of rLentivirus*

To construct recombinant lentivirus in our laboratory, two other vectors are co-transfected with the lentiviral vector Nef-GT (Fig. 7). The packaging vector that we use in our system is pCMV $\Delta$ R8.91. The third vector we use is the VSV-G expressing plasmid pMDG. These vectors are transfected by CaPO<sub>4</sub> into human embryonic kidney 293T cells at an approximate ratio of 3: 2: 1 (Nef-GT: pCMV $\Delta$ R8.91:pMDG). Twelve to fifteen hours later the media is replaced with fresh media. The cells are then given 48 hrs to produce recombinant lentiviruses that are present in the media. The media is then collected and filtered through a .45  $\mu$ m filter to remove cells and cellular debris. This media containing the virus can either be used for transduction, frozen for future use, or the virus can be purified to make a high-titer viral preparation.



*Transduction of MDBK+Luc Cells with bPol III mir-30 shRNA Recombinant Lentivirus*

To test bPol III promoter function after integration, the MDBK+Luc cells were transduced in a 6 well plate at 60% confluency with the bPol III based recombinant lentivirus (Nef-GT bU6-2 Luc, Nef-GT bU6-2 NT; Nef-GT b7sk Luc, Nef-GT b7sk NT; Nef-GT bH1 Luc, Nef-GT bH1 NT) (Appendix A.19). Following transduction, the cell population was cultured and expanded until a fluorescent population of cells was able to be visualized. The transgenic MDBK+Luc+Nef-GT bPol III cells were selected using 250 µg/ml of G418 (Sigma) until a 100% Nef-GTbPol III population was observed and then they were maintained under selection. The selected MDBK+Luc cell samples: Nef-GT bU6-2 Luc, Nef-GT bU6-2 NT; Nef-GT b7sk Luc, Nef-GT b7sk NT; Nef-GT bH1 Luc, Nef-GT bH1 NT, were measured for luciferase activity by using the Luciferase Assay kit (Promega; Appendix A.20, 16) for eight observations per sample. To help normalize the data,  $8 \times 10^8$  cells were collected per observation. Luciferase activity was measured and statistically analyzed as previously described.



**Fig. 7.** Production of Recombinant Lentivirus. A multi-vector system is used to construct recombinant lentiviral particles in our laboratory. The pCMV $\Delta$ R8.91 vector provides all of the essential viral proteins important for packaging and integration to the system in trans. The pMDG encodes the VSV-G envelope protein. Nef-GT provides the RNA to be packaged and ultimately reversed transcribed into a dsDNA transgene that is inserted into the target cells genome. These vectors are transfected in an approximate ratio of 3:2:1 of Nef-GT:pCMV:pMDG. Our current method of transfection is CaPO<sub>4</sub>. The media containing the transfection components is replaced after 12 hrs. 48 hrs later media is collected that contains the recombinant lentiviral particles.

## CHAPTER III

### RESULTS

#### **Identification and PCR Amplification of Bovine Pol III Promoters**

Two previously described bovine Pol III promoters, bU6-2, b7sk, and the novel bH1 promoter were identified for this project as promoters to drive the expression of a mir-based shRNA. The same primer sequences were used to successfully amplify the b7sk and bU6-2 promoters from a bos taurus/bos indicus mix breed genomic preparation [59]. A bioinformatics approach was used to determine the location of the bovine H1 snRNA coding sequence and primers were designed to successfully amplify a 204 bp promoter sequence. All of the promoters were cloned into the pGEM Easy TA-vector and sequencing confirmed successful amplification.

#### **Development of Lentiviral Bovine Pol III mir-30 Based shRNA Expression Vectors**

Bovine Pol III promoters were PCR amplified to introduce the restriction enzyme sites Not1 (5'-end) and PspOM1 (3'-end) onto the promoters. After restriction enzyme digestion and ligation, all three promoters were successfully introduced into the base vector Nef-GT; creating the empty lentiviral vectors Nef-GT bU6-2, Nef-GT b7sk, and Nef-GT bH1.

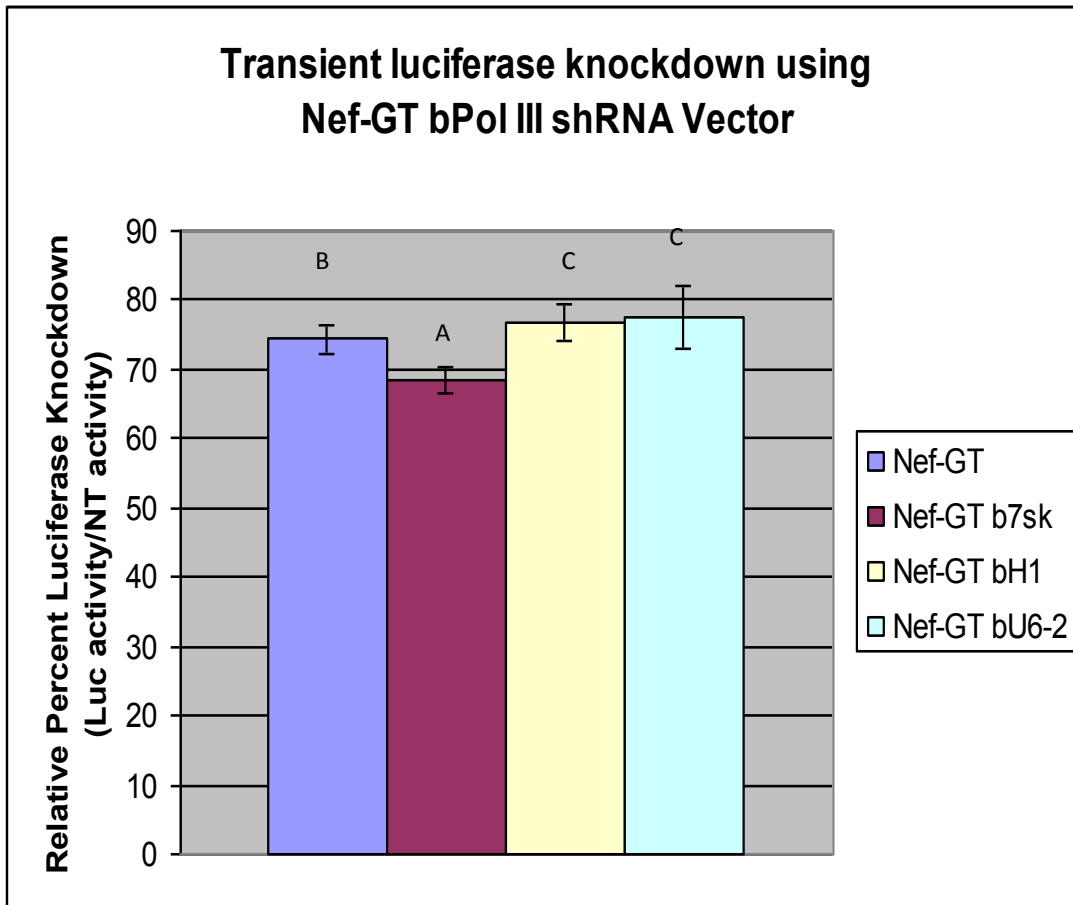
### **Confirmation of Nef-GT bPol III Vector Function with Transient Luciferase Assay**

To test the functionality of the bPol III shRNA expression vectors, a transient luciferase knockdown assay was used. Two shRNA sequences (a targeting Luc control, and a null (NT) control) were developed and individually inserted into the control Nef-GT vector as well as each bovine Pol III vector; creating the following vectors: Nef-GT bU6-2 Luc, Nef-GT bU6-2 NT; Nef-GT b7sk Luc, Nef-GT b7sk NT; Nef-GT bH1 Luc, Nef-GT bH1 NT. When the means were compared, each promoter Nef-GT, Nef-GT bU6-2, Nef-GT b7sk, Nef-GT bH1 that expressed the targeting Luc shRNA resulted in a range of percent luciferase activity between 31% and 22% (Table 1) or a 69-78% (Fig.8) reduction of luciferase activity ( $P < 0.0001$ ) when compared to the null control. In addition, there was a significant difference of percent luciferase activity among the promoters expressing the Luc shRNA. The Nef-GT b7sk promoter for this experiment was the least affective with a reduction of percent luciferase activity at 69% ( $P < 0.0001$ ). Furthermore, it was also the least effective in this transfection mediated luciferase assay when compared to all other vectors expressing the targeting Luc shRNA (p-value,  $< 0.0001$ ). The Nef-GT vector (Pol II based EF1 $\alpha$  promoter) was moderately effective at 74% reduction of luciferase activity when compared to the other vectors, but was significantly lower than both the Nef-GT bH1 ( $P = 0.0111$ ) and Nef-GT bU6-2 ( $P = 0.0005$ ). When compared to all of the vectors, there was no difference observed between the two most effective promoters, Nef-GT bH1 and the Nef-GT bU6-2, with a knockdown of luciferase activity of 77% and 78% respectively.

**Table 1**

Relative Luciferase Activity in a Transient Luciferase Knockdown Assay Mediated by bPol III shRNA Lentiviral Expression Vectors. The relative luciferase activity was calculated by dividing the promoter sample luciferase activity (Luc) by the null (NT) control activity per promoter set . P <0.0001 signified by superscript letters.

<b>Vector</b>	<b>Observations</b>	<b>Relative Luciferase Activity</b>	<b>Standard Error</b>
Nef-GT	12	0.2568 <sup>B</sup>	0.0206
Nef-GT b7sk	12	0.3146 <sup>A</sup>	0.0186
Nef-GT bH1	12	0.2328 <sup>C</sup>	0.0273
Nef-GT bU6-2	12	0.2248 <sup>C</sup>	0.0474



**Fig. 8.** Transient Luciferase Knockdown Using Nef-GT bPol III shRNA Expression Vector. Relative percent knockdown of transient luciferase activity via transfected Nef-GT bPol III vectors. The luciferase knockdown was calculated using the relative luciferase activity.  $P < 0.0001$  signified by superscript letters.

## **Confirmation of bPol III Promoter Function after Integration of Transgene in the Bovine Genome**

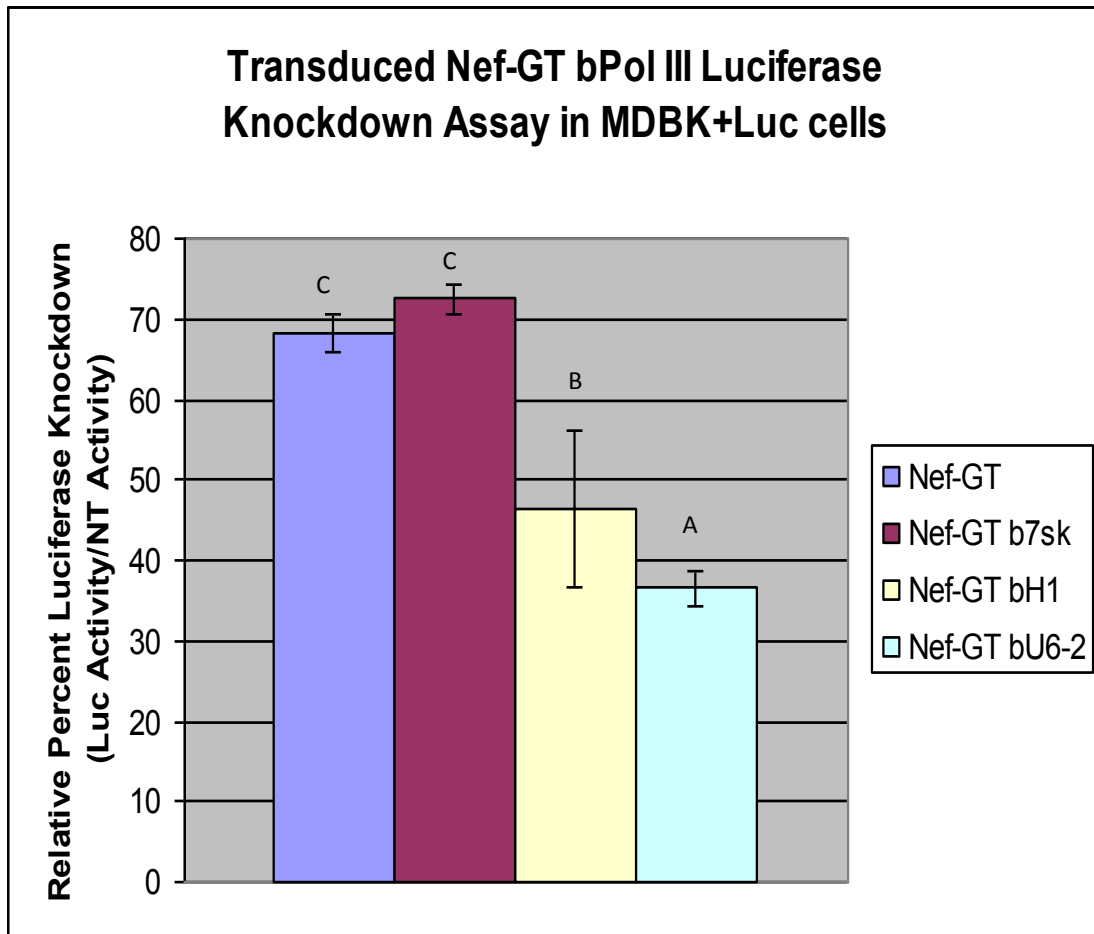
Once the bPol III based lentiviral vectors were proven effective at expressing a functional shRNA with the transient transfection. The next experiment was to prove that the bPol III expression cassettes are functional after being integrated into the bovine genome. To test their activity a MDBK cell line that expressed the luciferase protein was utilized and luciferase knockdown was analyzed. The cells were transduced with a recombinant lentivirus containing transgenes for the following shRNA expression vectors: Nef-GT Luc, Nef-GT NT, Nef-GT bU6-2 Luc, Nef-GT bU6-2 NT; Nef-GT b7sk Luc, Nef-GT b7sk NT; Nef-GT bH1 Luc, Nef-GT bH1 NT. Following Geneticin selection, specific to the shRNA expression cassette, the cells were lysed and luciferase measurements were recorded. All of the samples expressing the targeting Luc shRNA resulted in a reduced level of activity (Table 2). The range of knockdown for these transduced experiments were 37% to 73% ( $P < 0.0001$ ) (Fig. 9). In addition, there were significant differences observed between all of the bovine Pol III promoters. In this study, the bU6-2 was least effective, with a reduction of relative luciferase activity by 37% ( $p$ -value  $< 0.001$ ). The bH1 promoter was moderately effective at 46% ( $P < 0.0001$ ). The b7sk promoter had the greatest observed reduction of the bovine Pol III promoters with a knockdown of the luciferae activity by 72% ( $P < 0.001$ ). The control Nef-GT Luc targeting vector had similar knockdown at 68% reduced relative luciferase activity. Surprisingly, in this experiment the integrated promoters had vast differences of activity but all were effective to some degree.

**Table 2**

Relative Luciferase Activity of MDBK+Luc Cells after Integration of a Bovine Pol III shRNA Expression Transgene. The relative luciferase activity was calculated by dividing the promoter sample luciferase activity (NT or Luc) by the non-targeting (NT) control activity per promoter set. P <0.001 signified by superscript letters.

<b>Vector</b>	<b>Observations</b>	<b>Relative Luciferase Activity</b>	<b>Standard Error</b>
Nef-GT	8	0.3171 <sup>C</sup>	0.0239
Nef-GT b7sk	8	0.2755 <sup>C</sup>	0.0199
Nef-GT bH1	8	0.5352 <sup>B</sup>	0.0987
Nef-GT bU6-2	8	0.6343 <sup>A</sup>	0.0216





**Fig. 9.** Transduced Nef-GT bPol III shRNA Mediated Luciferase Knockdown Assay in MDBK+Luc Cells. Relative knockdown of luciferase activity in MDBK cells expressing luciferase following transduction of the control vector Nef-GT or Nef-GT bPol III, either expressing an shRNA targeting luciferase (Luc) or non-targeting shRNA (NT). The relative luciferase knockdown was calculated dividing the Luc sample luciferase activity (NT or Luc) by the (NT) control activity of the respective promoter and converted to percent knockdown.  $P < 0.001$  signified by superscript letters.

## CHAPTER IV

### DISCUSSION AND CONCLUSIONS

#### **Use of bPol III Promoters to Express a mir-30 Based shRNA**

The most common commercially available Pol III promoters to express a shRNA are of mouse or human origin. These promoter regions are highly conserved and are effective at expressing a shRNA in many cell types.. However, species divergence could have a role in the efficiency of the promoter in livestock models[64-66]. Differences in the efficiency of the b7sk and mU6 promoter to express a shRNA have been observed between species and cell types [59]. As a result of these observations, promoter optimization in agricultural animal cells is required.

Previously, livestock based Pol III promoters have been identified and characterized in cattle, chicken, and swine for their ability to drive expression of a shRNA. The first livestock Pol III promoters to be characterized were the bovine and chicken U6 and 7sk [59, 62, 64-66]. Swine based Pol III promoters were also shown to be effective at expressing a shRNA[63-66]. When these Pol III promoter sequences were analyzed, differences in spacing and sequences of key enhancer and core elements were reported. Variation in spacing and sequence specificity of key Pol III promoter elements have been reported to change the efficiency of transcription from the genome or after transient transfection [108]. In these initial studies of characterizing the function of livestock Pol

III promoters to express a shRNA, the promoter strength was determined by transient transfection. All of the reported livestock based Pol III promoters were functional to varying degrees at expressing a shRNA that resulted in a decrease expression of the fluorescent protein, eGFP.

It is difficult to extrapolate how these promoters would function in a transgenic animal when initiating shRNA expression from the genome. When the promoter is driving expression of a shRNA from a plasmid, the promoter strength can be skewed because a greater number of active shRNA expression cassettes are present. In addition, the conformation of the promoter in a plasmid is more accessible to the Pol III polymerase. As a result, a transfected cell might be producing a far greater number of shRNAs than the threshold to observe a physiological affect. Where as a transgenic animal will have far fewer active shRNA expression cassettes. Also, since these expression cassettes are integrated into the genome, the chromatin structure of the promoter region, as well as possible DNA methylation or other cellular silencing mechanisms can result in varying quantities of shRNA transcripts being produced [10, 109]. As a result, further research is required to quantify the strength of livestock Pol III promoters to drive shRNA expression from a genome.

The goal of this project was to evaluate different bovine based Pol III promoters in our lentiviral shRNA expression system for future research experiments. Three different

bovine Pol III promoters (bU6-2, b7sk, bH1) were utilized to express a mir-30 based shRNA after being integrated in the bovine genome. The study described in this work was the first to characterize and use the bovine H1 promoter to express a shRNA. The bU6-2, b7sk, bH1 promoters were effectively incorporated into the lentiviral vector Nef-GT resulting in the first bovine based mir-30 shRNA expression vectors. These vectors were initially tested by a transient transfection based luciferase knockdown assay. All of the promoters were highly effective. When compared to the bU6-2 and bH1 promoters, the b7sk promoter was the weakest promoter to drive expression of a shRNA. This observation was in contrast to previous results that reported the b7sk to be the most active promoter in multiple cell lines. Although, this initial experiment to confirm functionality of the promoters was conducted in human kidney cells (293T). Previously, when the b7sk promoter was used to express an eGFP targeting shRNA in a Vero cell line (monkey kidney epithelial) its ability to reduce fluorescence was significantly decreased when compared to other cell types [59]. Also, the b7sk promoter had variable knockdown of fluorescence in multiple cell types from 70% in Vero cells to >90% in bovine kidney cells. These previous observations support a theory that the arrangement and sequence of the enhancer and the core elements of pol III promoters are important for the efficiency of the promoter in different cell types and species. The variability of the individual promoter function observed in these experiments suggests that different promoters may be more effective depending upon cell or species of origin. In our experiment, significant differences were observed between promoter efficiency and all bPol III mir-30 based shRNA expression vectors resulted in desired knockdown levels in

human 293T cells. The Nef-GT bPol III vectors exhibited that they can be effectively used transiently for *in vitro* studies.

Further testing is required to determine if these bovine based rlentiviral vectors would express a functional shRNA in a transgenic animal. The first step to address this long term goal was to determine the individual bPol III promoter's ability to drive expression of a shRNA after being integrated into the bovine genome. We were the first to quantify the function of these promoters after integration into a homologous cell line. Significant differences were observed between promoter function after integration into a bovine genome. In these experiments, the b7sk was the most effective of the three bPol III promoters we used. Similar observations were reported when comparing the human 7sk and U6-1 promoters *in vivo* [110]. This preliminary experiment proved that the bPol III based rlentiviral vectors could produce functional rlentivirus and integrate its transgene. The transgene was also functional after random integration into the bovine genome. In addition, the Nef-GT b7sk is the most efficient at reducing a target mRNA when expressing an shRNA from the genome of MDBK cells *in vitro*.

It is unlikely that the reduced promoter activity observed in the MDBK+Luc cell populations produced from the transduction of Nef-GT bU6-2 Luc or Nef-GT bH1 Luc is a result of differences in integration of the transgene. The lentiviral mediated introduction of transgenes results in random integration and within the cell population

there is assumed to be hundreds to thousands of different integration sites. Initially, when fluorescence was observed 3-4 days post-transduction, a similar transduction rate was observed. This observation suggests similar number of integrations per cell in each population. After selection, the cell populations appeared to have similar levels of zsGreen expression, further supporting that a similar number of integrations were observed between the different Nef-GT bPol III transduced cell populations. In addition, the random populations of MDBK cells were maintained and individual cells were not selected to make clonal populations. As a result, the chances of integration number or location resulting in the significant differences observed among the bPol III promoters strength to express a shRNA resulting in decreased luciferase activity is highly unlikely.

The orientation of the different components of the Nef-GT bPol III construct could have an influence on the varied promoter strengths we are reporting. The expression of the zsGreen fluorescent protein is directly upstream of the bPol III expression cassettes. The EF1 $\alpha$  promoter recruits a Pol II polymerase to transcribe the zsGreen coding sequence and may potentially continue down the DNA sequence into bPol III promoter regions. Thus, the Pol II promoter might be interfering with the recruitment of the Pol III promoter that is responsible for the expression of the shRNA. The b7sk promoter sequence was the longest at 695 bp, where as the bU6-2 and bH1 were 432 bp and 204 bp respectively. The efficiency of the U6 promoter is dependent upon the upstream enhancer elements octamer element (OCT) and SPH to stabilize the Pol III promoter for

transcription initiation [108] In both the b7sk and bU6-2, these elements are approximately 250 bp upstream of the transcriptional start site[59]. The extra sequence between the end of the coding region for zsGreen and these elements could be providing a buffer for the 7sk promoter. While the bH1 promoter sequence is much shorter than the bU6-2, its unique modification of a compact promoter sequence could have an influence on its strength in this integrated transgene [111]. Further research is required differentiate between actual promoter strength or if the orientation of these components within the Nef-GT bPol III are influencing the efficiencies of these promoters after integration.

As a result of these observations two conclusions can be made as a consequence of the post-integration activity of the bU6-2 and bH1. First, further research in different experimental conditions or targets are required before their observed reduced activity can be confirmed in livestock models. The other is that if this reduced activity is confirmed, they can still be useful to express shRNAs in experiments that analyze an animal's or cell's response to varying levels of reduced protein expression. While the observations from this post-integration experiment were unexpected, we are able to conclude that b7sk promoter is highly effective after integration into the MDBK genome and coupled with previously reported data in other cell lines, this promoter is the best candidate to produce a transgenic animal expressing a single shRNA.

The potential of using RNAi to alter an animal's gene expression is highly dependent upon the promoter expressing the RNAi effector molecule, as well as the other components of the integrated transgene and location of integration. Previous research has shown that both Pol II and Pol III promoters are highly variable depending upon the cell type and species [18, 59]. In addition, the composition of the transgene can result in variable expression. Some exogenous gene sequences have been shown to be more readily silenced after integration into a genome [112-114]. The location of the integrated transgene in the genome can influence the active recruitment of the polymerase[109]. Therefore, one integration site might be expressing a greater number of shRNAs than another. In addition, the integrated transgene can be influenced by chromatin remodeling if it integrates in a region that is variable between tissues[114, 115]. Consequently, the effectiveness of a promoter to express a mir-based shRNA could be highly variable from animal to animal because of these potential variations of expression and low integration numbers associated with lentiviral transgenesis to create transgenic animals, As a result, further research in producing live transgenic cattle is required to determine optimal Pol III promoters and transgene constructs for expression of a shRNA. Based upon our results and the average of hundreds to thousands of integrations into a bovine genome we conclude that the b7sk Pol III is a viable option for expressing a mir-based shRNA in a transgenic bovid.



## **Conclusion**

The bU6-2, b7sk, and bH1 promoters were successfully incorporated into the lentiviral vector Nef-GT creating the first functional bovine based mir-30 shRNA expression vectors. When these vectors were transfected, all of the bPol III promoters were very effective at reducing target protein levels by expressing a mir-30 based shRNA. The strong promoter activity of Nef-GT and Nef-GT b7sk was maintained after integration into the bovine genome. With regards to the bU6-2 and newly characterized bH1 promoters, further research is required to fully characterize their activity after integration in bovine genomes. In conclusion, the b7sk promoter was the strongest promoter to express a mir-30 based shRNA after being integrated into the genome of bovine cells. We further conclude that the Nef-GT b7sk is the best option to continue research in further characterizing shRNA expression in transgenic cattle.

## REFERENCES

1. Lopez-Fraga M, Martinez T, Jimenez A: **RNA interference technologies and therapeutics: from basic research to products.** *BioDrugs* 2009, **23**:305-332.
2. Kinouchi N, Ohsawa Y, Ishimaru N, Ohuchi H, Sunada Y, Hayashi Y, Tanimoto Y, Moriyama K, Noji S: **Atelocollagen-mediated local and systemic applications of myostatin-targeting siRNA increase skeletal muscle mass.** *Gene Ther* 2008, **15**:1126-1130.
3. Joyappa DH, Sasi S, Ashok KC, Reddy GR, Suryanarayana VV: **The plasmid constructs producing shRNA corresponding to the conserved 3D polymerase of Foot and Mouth Disease virus protects guinea pigs against challenge virus.** *Vet Res Commun* 2009, **33**:263-271.
4. Nganvongpanit K, Muller H, Rings F, Hoelker M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Schellander K, Tesfaye D: **Selective degradation of maternal and embryonic transcripts in in vitro produced bovine oocytes and embryos using sequence specific double-stranded RNA.** *Reproduction* 2006, **131**:861-874.
5. Furbass R, Tomek W, Vanselow J: **Upstream stimulating factors 1 and 2 enhance transcription from the placenta-specific promoter 1.1 of the bovine cyp19 gene.** *BMC Mol Biol*, **11**:5.

6. Golding MC, Long CR, Carmell MA, Hannon GJ, Westhusin ME: **Suppression of prion protein in livestock by RNA interference.** *Proc Natl Acad Sci U S A* 2006, **103**:5285-5290.
7. Ramsoondar J, Vaught T, Ball S, Mendicino M, Monahan J, Jobst P, Vance A, Duncan J, Wells K, Ayares D: **Production of transgenic pigs that express porcine endogenous retrovirus small interfering RNAs.** *Xenotransplantation* 2009, **16**:164-180.
8. Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D: **In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector.** *Science* 1996, **272**:263-267.
9. Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM: **Development of a self-inactivating lentivirus vector.** *J Virol* 1998, **72**:8150-8157.
10. Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pfeifer A: **Generation of transgenic cattle by lentiviral gene transfer into oocytes.** *Biol Reprod* 2004, **71**:405-409.
11. Clark AJ, Ali S, Archibald AL, Bessos H, Brown P, Harris S, McClenaghan M, Prowse C, Simons JP, Whitelaw CB, et al.: **The molecular manipulation of milk composition.** *Genome* 1989, **31**:950-955.
12. Murray JD, Nancarrow CD, Marshall JT, Hazelton IG, Ward KA: **Production of transgenic merino sheep by microinjection of ovine metallothionein-ovine growth hormone fusion genes.** *Reprod Fertil Dev* 1989, **1**:147-155.

13. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM: **Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes.** *Nature* 1982, **300**:611-615.
14. Pursel VG, Pinkert CA, Miller KF, Bolt DJ, Campbell RG, Palmiter RD, Brinster RL, Hammer RE: **Genetic engineering of livestock.** *Science* 1989, **244**:1281-1288.
15. Su HY, Jay NP, Gourley TS, Kay GW, Damak S: **Wool production in transgenic sheep: results from first-generation adults and second-generation lambs.** *Anim Biotechnol* 1998, **9**:135-147.
16. Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS: **Expression profiling reveals off-target gene regulation by RNAi.** *Nat Biotechnol* 2003, **21**:635-637.
17. Judge A, MacLachlan I: **Overcoming the innate immune response to small interfering RNA.** *Hum Gene Ther* 2008, **19**:111-124.
18. Fleury S, Simeoni E, Zuppinger C, Deglon N, von Segesser LK, Kappenberger L, Vassalli G: **Multiply attenuated, self-inactivating lentiviral vectors efficiently deliver and express genes for extended periods of time in adult rat cardiomyocytes in vivo.** *Circulation* 2003, **107**:2375-2382.
19. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA: **Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways.** *Nature* 2006, **441**:537-541.

20. Ecker JR, Davis RW: **Inhibition of gene expression in plant cells by expression of antisense RNA.** *Proc Natl Acad Sci U S A* 1986, **83**:5372-5376.
21. Izant JG, Weintraub H: **Inhibition of thymidine kinase gene expression by anti-sense RNA: a molecular approach to genetic analysis.** *Cell* 1984, **36**:1007-1015.
22. Melton DA: **Injected anti-sense RNAs specifically block messenger RNA translation in vivo.** *Proc Natl Acad Sci U S A* 1985, **82**:144-148.
23. Simons RW, Kleckner N: **Translational control of IS10 transposition.** *Cell* 1983, **34**:683-691.
24. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: **Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*.** *Nature* 1998, **391**:806-811.
25. Hamilton AJ, Baulcombe DC: **A species of small antisense RNA in posttranscriptional gene silencing in plants.** *Science* 1999, **286**:950-952.
26. Hammond SM, Bernstein E, Beach D, Hannon GJ: **An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells.** *Nature* 2000, **404**:293-296.
27. Zamore PD, Tuschl T, Sharp PA, Bartel DP: **RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals.** *Cell* 2000, **101**:25-33.

28. Bernstein E, Caudy AA, Hammond SM, Hannon GJ: **Role for a bidentate ribonuclease in the initiation step of RNA interference.** *Nature* 2001, **409**:363-366.
29. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN: **The nuclear RNase III Droscha initiates microRNA processing.** *Nature* 2003, **425**:415-419.
30. Cerutti H, Casas-Mollano JA: **On the origin and functions of RNA-mediated silencing: from protists to man.** *Curr Genet* 2006, **50**:81-99.
31. Ambros V: **microRNAs: tiny regulators with great potential.** *Cell* 2001, **107**:823-826.
32. Allshire R: **Molecular biology. RNAi and heterochromatin--a hushed-up affair.** *Science* 2002, **297**:1818-1819.
33. Check E: **RNA interference: hitting the on switch.** *Nature* 2007, **448**:855-858.
34. Diallo M, Arenz C, Schmitz K, Sandhoff K, Schepers U: **Long endogenous dsRNAs can induce complete gene silencing in mammalian cells and primary cultures.** *Oligonucleotides* 2003, **13**:381-392.
35. Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R: **Induction of an interferon response by RNAi vectors in mammalian cells.** *Nat Genet* 2003, **34**:263-264.
36. Lee SB, Rodriguez D, Rodriguez JR, Esteban M: **The apoptosis pathway triggered by the interferon-induced protein kinase PKR requires the third**

**basic domain, initiates upstream of Bcl-2, and involves ICE-like proteases.**

*Virology* 1997, **231**:81-88.

37. Kulkarni MM, Booker M, Silver SJ, Friedman A, Hong P, Perrimon N, Mathey-Prevot B: **Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays.** *Nat Methods* 2006, **3**:833-838.
38. Li W, Cha L: **Predicting siRNA efficiency.** *Cell Mol Life Sci* 2007, **64**:1785-1792.
39. Agrawal N, Dasaradhi PV, Mohammed A, Malhotra P, Bhatnagar RK, Mukherjee SK: **RNA interference: biology, mechanism, and applications.** *Microbiol Mol Biol Rev* 2003, **67**:657-685.
40. Holen T, Amarzguioui M, Wiiger MT, Babaie E, Prydz H: **Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor.** *Nucleic Acids Res* 2002, **30**:1757-1766.
41. Donze O, Picard D: **RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase.** *Nucleic Acids Res* 2002, **30**:e46.
42. Brummelkamp TR, Bernards R, Agami R: **A system for stable expression of short interfering RNAs in mammalian cells.** *Science* 2002, **296**:550-553.
43. Chang K, Elledge SJ, Hannon GJ: **Lessons from Nature: microRNA-based shRNA libraries.** *Nat Methods* 2006, **3**:707-714.
44. Ling X, Li F: **Silencing of antiapoptotic survivin gene by multiple approaches of RNA interference technology.** *Biotechniques* 2004, **36**:450-454, 456-460.

45. Sano M, Li H, Nakanishi M, Rossi JJ: **Expression of long anti-HIV-1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations.** *Mol Ther* 2008, **16**:170-177.
46. Zeng Y, Wagner EJ, Cullen BR: **Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells.** *Mol Cell* 2002, **9**:1327-1333.
47. Qiu L, Wang H, Xia X, Zhou H, Xu Z: **A construct with fluorescent indicators for conditional expression of miRNA.** *BMC Biotechnol* 2008, **8**:77.
48. Xia H, Mao Q, Paulson HL, Davidson BL: **siRNA-mediated gene silencing in vitro and in vivo.** *Nat Biotechnol* 2002, **20**:1006-1010.
49. Zeng Y, Cullen BR: **Sequence requirements for micro RNA processing and function in human cells.** *RNA* 2003, **9**:112-123.
50. Xia XG, Zhou H, Xu Z: **Multiple shRNAs expressed by an inducible pol II promoter can knock down the expression of multiple target genes.** *Biotechniques* 2006, **41**:64-68.
51. Negrutskii BS, El'skaya AV: **Eukaryotic translation elongation factor 1 alpha: structure, expression, functions, and possible role in aminoacyl-tRNA channeling.** *Prog Nucleic Acid Res Mol Biol* 1998, **60**:47-78.
52. Niwa H, Yamamura K, Miyazaki J: **Efficient selection for high-expression transfectants with a novel eukaryotic vector.** *Gene* 1991, **108**:193-199.



53. Gou D, Narasaraaju T, Chintagari NR, Jin N, Wang P, Liu L: **Gene silencing in alveolar type II cells using cell-specific promoter in vitro and in vivo.** *Nucleic Acids Res* 2004, **32**:e134.
54. Song J, Pang S, Lu Y, Yokoyama KK, Zheng JY, Chiu R: **Gene silencing in androgen-responsive prostate cancer cells from the tissue-specific prostate-specific antigen promoter.** *Cancer Res* 2004, **64**:7661-7663.
55. Ventura A, Meissner A, Dillon CP, McManus M, Sharp PA, Van Parijs L, Jaenisch R, Jacks T: **Cre-lox-regulated conditional RNA interference from transgenes.** *Proc Natl Acad Sci U S A* 2004, **101**:10380-10385.
56. Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ: **A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells.** *Proc Natl Acad Sci U S A* 2005, **102**:13212-13217.
57. Yu JY, DeRuiter SL, Turner DL: **RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells.** *Proc Natl Acad Sci U S A* 2002, **99**:6047-6052.
58. Abbas-Terki T, Blanco-Bose W, Deglon N, Pralong W, Aebischer P: **Lentiviral-mediated RNA interference.** *Hum Gene Ther* 2002, **13**:2197-2201.
59. Lambeth LS, Wise TG, Moore RJ, Muralitharan MS, Doran TJ: **Comparison of bovine RNA polymerase III promoters for short hairpin RNA expression.** *Anim Genet* 2006, **37**:369-372.

60. Grimm D, Pandey K, Kay MA: **Adeno-associated virus vectors for short hairpin RNA expression.** *Methods Enzymol* 2005, **392**:381-405.
61. Cummins D, Doran TJ, Tyack S, Purcell D, Hammond J: **Identification and characterisation of the porcine 7SK RNA polymerase III promoter for short hairpin RNA expression.** *J RNAi Gene Silencing* 2008, **4**:289-294.
62. Bannister SC, Wise TG, Cahill DM, Doran TJ: **Comparison of chicken 7SK and U6 RNA polymerase III promoters for short hairpin RNA expression.** *BMC Biotechnol* 2007, **7**:79.
63. Chuang CK, Lee KH, Fan CT, Su YS: **Porcine type III RNA polymerase III promoters for short hairpin RNA expression.** *Anim Biotechnol* 2009, **20**:34-39.
64. Kudo T, Sutou S: **Usage of putative chicken U6 promoters for vector-based RNA interference.** *J Reprod Dev* 2005, **51**:411-417.
65. Wise TG, Schafer DJ, Lambeth LS, Tyack SG, Bruce MP, Moore RJ, Doran TJ: **Characterization and comparison of chicken U6 promoters for the expression of short hairpin RNAs.** *Anim Biotechnol* 2007, **18**:153-162.
66. Lambeth LS, Moore RJ, Muralitharan M, Dalrymple BP, McWilliam S, Doran TJ: **Characterisation and application of a bovine U6 promoter for expression of short hairpin RNAs.** *BMC Biotechnol* 2005, **5**:13.
67. Shrey K, Suchit A, Nishant M, Vibha R: **RNA interference: emerging diagnostics and therapeutics tool.** *Biochem Biophys Res Commun* 2009, **386**:273-277.

68. Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, Johnson S, Plaetinck G, Munyikwa T, Pleau M, et al: **Control of coleopteran insect pests through RNA interference.** *Nat Biotechnol* 2007, **25**:1322-1326.
69. Jaenisch R: **Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus.** *Proc Natl Acad Sci U S A* 1976, **73**:1260-1264.
70. Pannell D, Ellis J: **Silencing of gene expression: implications for design of retrovirus vectors.** *Rev Med Virol* 2001, **11**:205-217.
71. Verma IM: **Gene therapy: hopes, hypes, and hurdles.** *Mol Med* 1994, **1**:2-3.
72. Carbonaro DA, Jin X, Petersen D, Wang X, Dorey F, Kil KS, Aldrich M, Blackburn MR, Kellems RE, Kohn DB: **In vivo transduction by intravenous injection of a lentiviral vector expressing human ADA into neonatal ADA gene knockout mice: a novel form of enzyme replacement therapy for ADA deficiency.** *Mol Ther* 2006, **13**:1110-1120.
73. Naldini L, Blomer U, Gage FH, Trono D, Verma IM: **Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector.** *Proc Natl Acad Sci U S A* 1996, **93**:11382-11388.
74. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D: **Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo.** *Nat Biotechnol* 1997, **15**:871-875.

75. Hopkins N: **High titers of retrovirus (vesicular stomatitis virus) pseudotypes, at last.** *Proc Natl Acad Sci U S A* 1993, **90**:8759-8760.
76. Coil DA, Miller AD: **Phosphatidylserine is not the cell surface receptor for vesicular stomatitis virus.** *J Virol* 2004, **78**:10920-10926.
77. Park F, Kay MA: **Modified HIV-1 based lentiviral vectors have an effect on viral transduction efficiency and gene expression in vitro and in vivo.** *Mol Ther* 2001, **4**:164-173.
78. Zufferey R, Donello JE, Trono D, Hope TJ: **Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors.** *J Virol* 1999, **73**:2886-2892.
79. Ma X, Lu X, Peng J, Tan S, Yuan Z, Yin F, Wang H, Wu X, Hou Y: **[Inhibition of Luciferase expression in mammalian cells by AAV vector plasmid mediated Luciferase shRNA].** *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* 2002, **16**:253-255.
80. Czauderna F, Santel A, Hinz M, Fechtner M, Durieux B, Fisch G, Leenders F, Arnold W, Giese K, Klippel A, Kaufmann J: **Inducible shRNA expression for application in a prostate cancer mouse model.** *Nucleic Acids Res* 2003, **31**:e127.
81. Betteridge KJ, Rieger D: **Embryo transfer and related techniques in domestic animals, and their implications for human medicine.** *Hum Reprod* 1993, **8**:147-167.

82. Mapletoft RJ, Hasler JF: **Assisted reproductive technologies in cattle: a review.** *Rev Sci Tech* 2005, **24**:393-403.
83. Hammer RE, Pursel VG, Rexroad CE, Jr., Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL: **Production of transgenic rabbits, sheep and pigs by microinjection.** *Nature* 1985, **315**:680-683.
84. Gandhi RS, S. : **Consumer Concerns About Hormones in Food.** In *Cornell University Program on Breast Cancer and Environmental Risk Factors in New York State*, vol. June Fact Sheet #37; 2000.
85. Pittius CW, Hennighausen L, Lee E, Westphal H, Nicols E, Vitale J, Gordon K: **A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice.** *Proc Natl Acad Sci U S A* 1988, **85**:5874-5878.
86. Simons JP, Wilmut I, Clark AJ, Archibald AL, Bishop JO, Lathe R: **Gene transfer into sheep.** *Nat Biotech* 1988, **6**:179-183.
87. Ebert KM, Selgrath JP, DiTullio P, Denman J, Smith TE, Memon MA, Schindler JE, Monastersky GM, Vitale JA, Gordon K: **Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression.** *Biotechnology (N Y)* 1991, **9**:835-838.
88. Clark AJ, Bessos H, Bishop JO, Brown P, Harris S, Lathe R, McClenaghan M, Prowse C, Simons JP, Whitelaw CBA, Wilmut I: **Expression of human anti-**

- hemophilic factor IX in the milk of transgenic sheep.** *Nat Biotech* 1989, 7:487-492.
89. Edmunds T, Van Patten SM, Pollock J, Hanson E, Bernasconi R, Higgins E, Manavalan P, Ziomek C, Meade H, McPherson JM, Cole ES: **Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin.** *Blood* 1998, **91**:4561-4571.
90. Hammer C: **Xenotransplantation and its future.** *Forensic Sci Int* 1994, **69**:259-268.
91. Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, et al: **Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning.** *Science* 2002, **295**:1089-1092.
92. Ramsoondar JJ, Machaty Z, Costa C, Williams BL, Fodor WL, Bondioli KR: **Production of alpha 1,3-galactosyltransferase-knockout cloned pigs expressing human alpha 1,2-fucosyltransferase.** *Biol Reprod* 2003, **69**:437-445.
93. Cowan PJ, Aminian A, Barlow H, Brown AA, Chen CG, Fiscaro N, Francis DM, Goodman DJ, Han W, Kurek M, et al: **Renal xenografts from triple-transgenic pigs are not hyperacutely rejected but cause coagulopathy in non-immunosuppressed baboons.** *Transplantation* 2000, **69**:2504-2515.

94. Karlas A, Kurth R, Denner J: **Inhibition of porcine endogenous retroviruses by RNA interference: increasing the safety of xenotransplantation.** *Virology* 2004, **325**:18-23.
95. Saeki K, Matsumoto K, Kinoshita M, Suzuki I, Tasaka Y, Kano K, Taguchi Y, Mikami K, Hirabayashi M, Kashiwazaki N, et al: **Functional expression of a Delta12 fatty acid desaturase gene from spinach in transgenic pigs.** *Proc Natl Acad Sci U S A* 2004, **101**:6361-6366.
96. Reh WA, Maga EA, Collette NM, Moyer A, Conrad-Brink JS, Taylor SJ, DePeters EJ, Oppenheim S, Rowe JD, BonDurant RH, et al: **Hot topic: using a stearoyl-CoA desaturase transgene to alter milk fatty acid composition.** *J Dairy Sci* 2004, **87**:3510-3514.
97. Campbell KH, McWhir J, Ritchie WA, Wilmut I: **Sheep cloned by nuclear transfer from a cultured cell line.** *Nature* 1996, **380**:64-66.
98. Westhusin ME, Pryor JH, Bondioli KR: **Nuclear transfer in the bovine embryo: a comparison of 5-day, 6-day, frozen-thawed, and nuclear transfer donor embryos.** *Mol Reprod Dev* 1991, **28**:119-123.
99. Campbell KH, Loi P, Cappai P, Wilmut I: **Improved development to blastocyst of ovine nuclear transfer embryos reconstructed during the presumptive S-phase of enucleated activated oocytes.** *Biol Reprod* 1994, **50**:1385-1393.
100. Mastromonaco GF, King WA: **Cloning in companion animal, non-domestic and endangered species: can the technology become a practical reality?** *Reprod Fertil Dev* 2007, **19**:748-761.

101. Smith LC, Yoo JG: **Animal cloning by somatic cell nuclear transfer.** *Methods Mol Biol* 2009, **550**:267-279.
102. Okada Y, Ueshin Y, Isotani A, Saito-Fujita T, Nakashima H, Kimura K, Mizoguchi A, Oh-hora M, Mori Y, Ogata M, et al: **Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer.** *Nat Biotech* 2007, **25**:233-237.
103. Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A: **Efficient transgenesis in farm animals by lentiviral vectors.** *EMBO Rep* 2003, **4**:1054-1060.
104. Levine BL, Humeau LM, Boyer J, MacGregor RR, Rebello T, Lu X, Binder GK, Slepushkin V, Lemiale F, Mascola JR, et al: **Gene transfer in humans using a conditionally replicating lentiviral vector.** *Proc Natl Acad Sci U S A* 2006, **103**:17372-17377.
105. Quinonez R, Sutton RE: **Lentiviral vectors for gene delivery into cells.** *DNA Cell Biol* 2002, **21**:937-951.
106. Wu X, Li Y, Crise B, Burgess SM: **Transcription start regions in the human genome are favored targets for MLV integration.** *Science* 2003, **300**:1749-1751.
107. Leib-Mosch C, Brack-Werner R, Salmons B, Schmidt J, Strauss PG, Hehlmann R, Erfle V: **The significance of retroviruses in oncology.** *Onkologie* 1990, **13**:405-414.



108. Domitrovich AM, Kunkel GR: **Multiple, dispersed human U6 small nuclear RNA genes with varied transcriptional efficiencies.** *Nucleic Acids Res* 2003, **31**:2344-2352.
109. Weis J, Fine SM, David C, Savarirayan S, Sanes JR: **Integration site-dependent expression of a transgene reveals specialized features of cells associated with neuromuscular junctions.** *J Cell Biol* 1991, **113**:1385-1397.
110. Koper-Emde D, Herrmann L, Sandrock B, Benecke BJ: **RNA interference by small hairpin RNAs synthesised under control of the human 7S K RNA promoter.** *Biol Chem* 2004, **385**:791-794.
111. Myslinski E, Ame JC, Krol A, Carbon P: **An unusually compact external promoter for RNA polymerase III transcription of the human H1RNA gene.** *Nucleic Acids Res* 2001, **29**:2502-2509.
112. Teschendorf C, Warrington KH, Jr., Siemann DW, Muzyczka N: **Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumor cell lines using recombinant adeno-associated virus.** *Anticancer Res* 2002, **22**:3325-3330.
113. Ellis J: **Silencing and variegation of gammaretrovirus and lentivirus vectors.** *Hum Gene Ther* 2005, **16**:1241-1246.
114. Hong S, Hwang DY, Yoon S, Isacson O, Ramezani A, Hawley RG, Kim KS: **Functional analysis of various promoters in lentiviral vectors at different stages of in vitro differentiation of mouse embryonic stem cells.** *Mol Ther* 2007, **15**:1630-1639.

115. **Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project.** *Nature* 2007, **447**:799-816.

## APPENDIX A

### A.1 PCR Amplification of bPol III promoter from Genomic DNA

PCR Master Mix (50 ul rxn)

Genomic Amplification	Volume ( $\mu$ l)	Vector Amplification	Volume ( $\mu$ l)
10X buffer with MgCl <sub>2</sub>	5	10X buffer with MgCl <sub>2</sub>	5
10 mM dNTP mix	1	10mm dNTP mix	1
10 mM forward primer	1	10 mM forward primer	1
10 mM reverse primer	1	10 mM reverse primer	1
Dimethyl Sulfoxide (DMSO)	3.5	Vector DNA (100 ng)	1
Genomic DNA (100 ng)	1	Polymerase	1
Polymerase	1	ddH <sub>2</sub> O	40
ddH <sub>2</sub> O	36.5		

PCR Program:

1. 94<sup>0</sup>C Denaturing step 5 minutes
2. 94<sup>0</sup>C Denaturing step 30 seconds
3. 58<sup>0</sup>C Annealing step 30 seconds
4. 68<sup>0</sup>C Extension step 30 seconds
5. Repeat steps 2-4 29 times
6. 68<sup>0</sup>C Final Extension step 7 min

**A.2 TA-Cloning PCR products into pGEM Easy**

1. 1  $\mu$ l of Bovine Pol III Promoter PCR Product
2. 1  $\mu$ l of Vector (50 ng)
3. 3  $\mu$ l of H<sub>2</sub>O
4. Mix Steps 1-3 Together
5. Add 5  $\mu$ l of 2x Ligation Buffer
6. Add 1  $\mu$ l of Ligase
7. Briefly Mix by Pipeting
8. Incubate at room temperature for 1 hr or overnight at 4<sup>0</sup>C

**A.3 Transformation of plasmid DNA into Stbl3 Cells**

1. The Stbl3 cells were removed from -80<sup>0</sup>C storage and thawed on ice
2. 50  $\mu$ l aliquots of the Stbl3 cells were placed in chilled and labeled 500  $\mu$ l tubes
3. Add 7  $\mu$ l of ligated plasmid DNA (pGEM-Easy, Nef-GT bPol III, Nef-GT)
4. Keep on ice for 20 min to allow plasmids to enter cells
5. Heat Shock the Stbl3 Cells for 50 Sec. to trap the plasmid inside
6. Chill on ice for 1 minute
7. Add 300  $\mu$ l of SOC media
8. Incubate for 45 minutes at 37<sup>0</sup>C
9. Plate on 1% low-salt LB agarose plates supplemented with Ampicilian (50  $\mu$ g/ml) selection
10. Culture 37<sup>0</sup>C overnight no longer than 18 hrs

11. Check for colony formation

#### **A.4 Confirmation of Bovine Pol III PCR Product Insert in pGEM Easy-TA Vector**

##### *A.4.1 Growth of Colonies for mini prep plasmid DNA extraction*

Following colony formation, they must be grown and the plasmid DNA removed to confirm the insert of the PCR product and used for subsequent sequencing. Eight colonies were plucked from the plates and placed in 6 ml of low salt LB broth for incubation at 37<sup>0</sup>C overnight or again no longer than 18 hrs. Five ml of the cultured cells were collected and plasmid DNA was collected with the Qiagen plasmid. One ml of cultured cells were reserved for long term storage of plasmid at -80<sup>0</sup>C after correct sequence was confirmed by sequencing.

##### *A.4.2 Plasmid Purification (mini-prep)*

1. 5 ml of cultered cells were centrifuged at ~4,000 g for 10 minutes
2. Supernatant was removed and pellet was dried by inverting collection tube
3. 250 µl of P1 was used to resuspend the cell pellet and transferred to 1.5 ml microfuge tube
4. 250 µl of P2 lysis buffer was mixed with the P1/cells
5. 300 µl of N3 neutralizing buffer was added and tubes inverted 5-8 times to mix
6. The tubes were centrifuged for 10 min at 13,000 g

7. Supernatant was placed in purification column and spun 1 min at 13,000 g
8. Flow through was discarded
9. 700  $\mu$ l of PE was placed in the purification column and spun 1 min at 13,000g
10. Flow through was discarded
11. Purification column was spun 1.5 min at 13,000g to remove the alcohol and dry column
12. 75  $\mu$ l of ddH<sub>2</sub>O was added to the purification column and allowed to rehydrate column for 10 min
13. The DNA was eluted from the column in the 75  $\mu$ l of ddH<sub>2</sub>O by spinning for 1.5 minutes at 13,000 g
14. DNA was stored at 4<sup>0</sup>C if it was being used in the near future or -20<sup>0</sup>C for long term storage

#### *A.4.3 Double digestion of vector DNA*

1. 3  $\mu$ l of 10x Buffer 3
2. 1  $\mu$ l of 5' restriction enzyme
3. 1  $\mu$ l of 3' restriction enzyme
4. 5  $\mu$ l of eluted DNA mini prep sample
5. 20  $\mu$ l of ddH<sub>2</sub>O

6. Incubate at 37<sup>0</sup>C for 1 hr

Following restriction enzyme digestion, 20 µl of the reaction was run on a 1.2% agarose gel at 150 constant volts for approximately 20 minutes and visualized with an ultraviolet light.

#### *A.4.4 Sequencing of vector at gene technologies laboratory Texas A&M University*

We provided the vector and sequencing primer to the facility to let the experts run the amplifying and sequencing reactions.

#### *A.4.5 Long term storage of Stbl3 cells*

1. 850 µl of cultured cells were placed into labeled 1.5 ml microfuge tubes
2. 150 µl of glycerol
3. Briefly vortex to mix the two solutions
4. Store at -80<sup>0</sup>C until seeding of culture media for plasmid purification

### **A.5 Two step double digestion of donor vector**

#### *A.5.1 Initial single restriction digest*

1. 3 µl of 10x Buffer 4
2. 1 µl of restriction enzyme

3. 5  $\mu$ l of eluted DNA mini prep sample
4. 21  $\mu$ l of ddH<sub>2</sub>O
5. Incubate at 37<sup>0</sup>C for 1 hr

#### *A.5.2 Ethanol precipitation of DNA*

1. Add 20  $\mu$ l of ddH<sub>2</sub>O (up to 50  $\mu$ l)
2. Add 5  $\mu$ l of 3M sodium acetate
3. Add 150  $\mu$ l of cold 200 proof ethanol
4. Chill in liquid nitrogen for 30-45 seconds
5. Spin at 16,000 g for 35 min
6. Remove supernatant
7. Rinse DNA pellet with 150  $\mu$ l ice cold 80% ethanol
8. Remove all ethanol and air dry for 10 minutes
9. Resuspend DNA pellet in 26  $\mu$ l of 65<sup>0</sup>C ddH<sub>2</sub>O\*
10. Heat at 65<sup>0</sup>C for 10 minutes to fully dissolve DNA pellet

\* dependent upon future use of product: 26  $\mu$ l for single restriction enzyme digestion, 25  $\mu$ l for double restriction enzyme digestion, 30  $\mu$ l for ligation



### *A.5.3 Single Restriction Enzyme Digestion Following Ethanol Precipitation*

1. Add 3  $\mu\text{l}$  of 10x Buffer 3
2. 1  $\mu\text{l}$  Restriction Enzyme NotI
3. Mix and incubate at 37<sup>0</sup>C for 1 hr

### **A.6 Single restriction enzyme digestion of receiving vector prior to ligation**

1. 3  $\mu\text{l}$  of 10x Buffer 4
2. 1  $\mu\text{l}$  of restriction enzyme
3. 3.2  $\mu\text{g}$  of eluted Nef-GT (1  $\mu\text{g}/\mu\text{l}$ )
4. 23  $\mu\text{l}$  of ddH<sub>2</sub>O
5. Incubate at 37<sup>0</sup>C for 1 hr

### **A.7 Gel Purification of DNA from an agarose gel**

1. While visualizing the product over the UV light a scalpel blade was used to cut the section of agarose containing the respective band and placed in a labeled 1.5 ml microcentrifuge tube
2. 600  $\mu\text{l}$  of Buffer QG was added to the tube and incubated at 55<sup>0</sup>C for 10 minutes to dissolve the agarose and suspend the DNA in the solution

3. The Buffer QG was then placed in a purification column and spun at 13,000 g for 1 minute
4. The flow through was discarded
5. 750  $\mu$ l of PE buffer was added and spun at 13,000 g for 1 min
6. The flow through was discarded
7. The empty purification column was spun 1.5 min at 13,000g to remove the alcohol and dry column
8. 30  $\mu$ l of ddH<sub>2</sub>O was added to the purification column and allowed to rehydrate column for 10 min
9. The DNA was eluted from the column in the 30  $\mu$ l of ddH<sub>2</sub>O by spinning for 1.5 minutes at 13,000 g

#### **A.8 Ligation Protocol for PCR product insert into receiving vector**

##### **(6:1 molar ratio of donor vector: receiving vector)**

1. 1.5  $\mu$ l (150 ng) of Nef-GT
2. 2-3.5  $\mu$ l of purified Pol III Promoter DNA
3. ddH<sub>2</sub>O for total volume 5  $\mu$ l
4. 5  $\mu$ l 2x ligation buffer

5. 1  $\mu$ l Quick Ligase
6. Incubate on bench top for 8 minutes
7. Chill on ice at least 1 minute before transformation

#### **A.9 Test Digestion of Bovine Pol III Promoter Ligation into Not1 site of Nef-GT**

1. 3  $\mu$ l of 10x buffer 3
2. 1  $\mu$ l of restriction enzyme Not1
3. 1  $\mu$ l of restriction enzyme Ecor1
4. 7  $\mu$ l of mini-prep DNA (Nef-GT+bPol III promoter)
5. 18  $\mu$ l of ddH<sub>2</sub>O
6. Mix and incubate at 37°C for 1.5 hours
7. Following restriction enzyme digestion, 20  $\mu$ l of the reaction was run on a 1.2% agarose gel at 150 constant volts for approximately 20 minutes and visualized with an ultraviolet light.

### **A.10 Plasmid Purification using Qiafilter Plasmid Purification Midi kit**

1. Fresh or -80<sup>0</sup>C stored Stbl3 cells were used to seed 150 ml of low-salt LB media
2. Bacterial cells were pelleted by 6,000 g for 15 min at 4<sup>0</sup>C
3. Supernatant was removed and pellet was dried by inverting the collection container
4. The bacteria pellet was fully resuspended in 4 ml of Buffer P1
5. The cells were then lysed by that addition of 4 ml of Buffer P2 and mixed by inversion and incubated for 4-5 minutes
6. The lysis reaction was neutralized and proteins and genomic DNA were precipitated out by the addition 4 ml of ice cold P3 buffer.
7. The neutralized lysate was immediately transferred to the barrel of the QIAfilter Cartridge and incubated for 10 min on the bench top.
8. During the incubation period the QIAGEN-tip 100 was equilibrated with 4 ml of Buffer QBT
9. Following the equilibration, the precipitate was removed from the lysate by forced filtration and collected in the QIAGEN-tip 100
10. Allow the cleared lysate to enter the resin and bind the DNA by gravity flow through

11. The resin filter was washed 2 times with 10 ml of Buffer QC by gravity flow through
12. The DNA was eluted in 5 ml of warm 65<sup>0</sup>C Buffer QF
13. To precipitate the DNA for a greater concentration, 3.5 ml of isoproponal was added
14. The precipitated DNA was pelleted by centrification at 15,000 g for 30 minutes in 4<sup>0</sup>C
15. The supernatant was removed and the DNA pellet was washed with 70% ethanol then centrifuged at 15,000 g for 30 minutes in 4<sup>0</sup>C.
16. The supernatant was removed and the DNA pellet was allowed to air dry for 10 minutes
17. The dried DNA pellet was resuspended in 500 µl of warm 65<sup>0</sup>C ddH<sub>2</sub>O
18. Then the DNA was quantified using the Nanodrop-1000

**A.11 PCR Master Mix for Amplifying shRNA (50 ul rxn)**

1. 10X buffer with MgCl<sub>2</sub> ..... 5 µl
2. 10mm dNTP mix ..... 1 µl
3. 10 mM forward primer ..... 1 µl
4. 10 mM reverse primer ..... 1 µl
5. Oligo DNA (150 ng) ..... 1 µl
6. Polymerase ..... 1 µl
7. ddH<sub>2</sub>O ..... 40 µl

**PCR Program:**

1. 94<sup>0</sup>C Denaturing step 5 minutes
2. 94<sup>0</sup>C Denaturing step 30 seconds
3. 57<sup>0</sup>C Annealing step 30 seconds
4. 68<sup>0</sup>C Extension step 30 seconds
5. Repeat steps 2-4 29 times
6. 68<sup>0</sup>C Final Extension step 7 minutes

**A.12 Digestion of PCR amplified shRNA**

1. 3  $\mu$ l of 10 x Buffer 2
2. 1  $\mu$ l of Ecor1 restriction enzyme
3. 1  $\mu$ l of Xho1 restriction enzyme
4. 25  $\mu$ l of rehydrated ethynol precipitated PCR product
5. Digest at 37<sup>0</sup>C for 1.5 hours

**A.13 Transient Transfection of HEK 293-T for Stop n Glo Assay**

1. HEK 293-T cells were subpassed into a 6-well tissue culture plate and allowed to plate and grow to 70-80% confluence the morning before transfecting.
2. For the transfection the following vectors and solutions were combined in a 1.5 ml microfuge tube:
  - a. 40 ng of Renilla
  - b. 400 ng pGL3
  - c. 200 ng Experimental Vector
  - d. 12.6  $\mu$ l of CaCl<sub>2</sub>
  - e. ddH<sub>2</sub>O to 100  $\mu$ l

- f. Briefly vortexed to mix
3. Drop wise 100  $\mu$ l 2x Hank's Buffered Saline Solution was added while mixing.
4. A final 10 second vortex was used to mix and allowed to incubate at room temperature for 15 minutes
5. The calcium phosphate solution with the vectors was applied to the individual wells of the HEK 293-T and dispersed randomly through the media by a 4 point cross rotation
6. The cells were then washed the following morning (16 hours) and 2 ml of culture media was added

#### **A.14 Stop-n-Glo luciferase assay**

1. 100  $\mu$ l of Lar II media was loaded into alternating wells of a 96 well reader plate
2. 10  $\mu$ l x 2 of each sample of cellular lysate was added to those wells
3. The plate was mixed and incubated for 5 minutes
4. The reading Gain and PMT were adjusted per plate to maximize the non-targeting readings without maxing out
5. The plate was read two times with a 2 min wait period between readings
6. Then 100  $\mu$ l of Stop n Glo media was added to each well to stop the luciferase activity and activate the Renilla luminescent protein
7. The plate was mixed and incubated for 5 min



8. The plate was read two times with the same settings and a 2 minute wait period between readings
9. The files were saved and exported into an excel file to be later statistically analyzed.

#### **A.15 Calcium phosphate transfection of single vector, PEIZ + Luc**

1. HEK 293-T cells were subpassed into a 6-well cell culture plate (\_\_\_\_\_) and allowed to plate and grow to 70-80% confluence the morning before transfecting.
2. For the transfection the following vectors were combined in a 1.5 ml microfuge tube:
  - a. 400 ng PEIZ+Luc
  - b. 12.6  $\mu$ l of  $\text{CaCl}_2$
  - c. ddH<sub>2</sub>O to 100  $\mu$ l
  - d. Briefly vortexed to mix
3. Drop wise 100  $\mu$ l 2x Hank's Buffered Saline Solution was added while mixing.
4. A final 10 second vortex was used to mix and allowed to incubate at room temperature for 15 minutes
5. The calcium phosphate solution with the vectors was applied to the individual wells of the HEK 293-T

6. The cells were then washed the following morning (16 hours) to remove the transfection media and 2 ml of culture media was added

#### **A.16 Luciferase assay**

1. 100  $\mu$ l of Lar II media was loaded into alternating wells of a 96 well luminometer reader plate
2. 10  $\mu$ l x 2 of each sample of cellular lysate was added to those wells
3. The plate was mixed and incubated for 5 minutes
4. The reading Gain and PMT were adjusted per plate to maximize readings without maxing out
5. The plate was read two times with a 2 min wait period between readings
6. The files were saved and exported into an excel file to be later statistically analyzed.

#### **A.18 Production of recombinant lentivirus**

1. HEK 293-T cells were plated the morning of the transfection to be 80-85% confluent in a 100 mm plate by the afternoon.
2. For the transfection the following vectors were combined in a 1.5 ml microfuge tube:
  - a. 6  $\mu$ g of VSVg expression vector pMDG

- b. 12  $\mu\text{g}$  of Delta
  - c. 18  $\mu\text{g}$  of PEIZ+Luc
  - d. 36  $\mu\text{l}$  of  $\text{CaCl}_2$
  - e. ddH<sub>2</sub>O to 300  $\mu\text{l}$
  - f. Briefly vortexed to mix
3. Drop wise 300  $\mu\text{l}$  2x Hank's Buffered Saline Solution was added while mixing.
  4. A final 10 second vortex was used to mix and allowed to incubate at room temperature for 15 minutes
  5. The calcium phosphate solution with the vectors was applied to the HEK 293-T plates
  6. The cells were then washed the following morning (16 hours) and 6 ml of culture media was added
  7. Following the wash, the cells were allowed to produce the recombinant lentivirus for 48 hrs
  8. The media containing the lentiviral particles was collected and centrifuged at low speed 2,000 g for 5 minutes to pellet cellular debris
  9. The supernatant was then filtered through .45 micron filter and stored in FBS coated polypropylene tubes at 4<sup>0</sup>C

**A.19 Lentiviral transduction of MDBK**

1. The MDBK cells were subpassed in the morning into a 6 well plate \_\_\_\_\_ to achieve a 60% confluency that afternoon in 2 ml of culture media
2. 1 ml of media was removed and replaced with 2 ml of media containing PEIZ+Luc recombinant lentivirus
3. 10 mM of Polybrene was added to the media to assist in the transformation
4. The virus was given 24 hours to infect the MDBK cells before being washed and replaced with 2 ml of culture media

**A.20. Preparation of cells for luciferase assay of MDBK+Luc/+Nef-GT bPol III shRNA**

1. The culture media was removed and the cells were washed with 1x DPBS
2. The cells were trypsinized and suspended in DPBS
3. The cells were counted using a hemocytometer and the volume of  $8 \times 10^8$  cells was determined.
4. The cells were pelleted by briefly centrifuged at 800 G for 3 min
5. The supernatant was removed and the cells were lysed with 100  $\mu$ l of Lysis Buffer for 10 min

6. The lysed cell solution was collected in a 1.5 ml centrifuge tube
7. The cellular debris was pelleted by a centrifugation of 8,000 g for 1 min

#### **A.21 Cell sorting of MDBK+Luc cells**

1. Population of 90% confluent transduced MDBK+Luc cells in a T75 flask were washed and trypsinized
2. The cells were resuspended in growth media to neutralize the trypsin
3. 3/5<sup>th</sup> of the cells were centrifuged to pellet the cells
4. The supernatant was removed and the cells were resuspended as single cells in 3 ml of 1X PBS solution
5. To guarantee single cells were processed in the flow-cytometer, the suspended cells were filtered through a 40 micron filter.
6. The single transgenic cells were then sorted in the flow cytometer using stringent high accuracy parameters
7. The transgenic cells were sorted into 1x PBS
8. The cells were collected by centrifugation and resuspended in growth media
9. The cells were then plated in a T25 tissue culture flask and grown to populate a T75 tissue culture flask and maintained at 60-90% confluence

10. A population of the cells were frozen in 50% FBS and 5% DMSO for long-term storage and future use

## APPENDIX B

**Table 3.** Primers Utilized for PCR Reactions

<b>Primer Name</b>	<b>Description</b>	<b>Sequence</b>
A1	5' bU6-2 amplifying	CAGTAGTTGCGGGGATCAC
A2	3' bU6-2 amplifying	GCAGTTTATAGTATATGTGCTGCC
A3	5' b7sk amplifying	GGATGAGGAAGGAGCGTGAG
A4	3' b7sk amplifying	TCAACCCTGGCGATCAATGG
A5	5' bH1 amplifying	CGAACGCTGACGTCATCAGTC
A6	3' bH1 amplifying	CCGCACGCAAATAGCACTT
A7	5' zsGreen amplifying	GCA ATGGCCCAGTCCAAGCACGG
A8	3' zsGreen amplifying	GCAGATGCGATCCTTAGGGCAAGG
A9	5' Luciferase amplifying	ATGGAAGACGCCAAAAACATAAAG
A10	3' Luciferase amplifying	TTTTACCACATTTGTAGAGGTTTTA
B1	5' bU6-2 + NotI RE	GATGCGGCCGCTAAGTAGTTGCGGGGATCAC
B2	3' bU6-2 + PsP0M1 RE	CAGGGCCCTTTTAGTATATGTGCTGCC
B3	5' b7sk + NotI RE	GATGCGGCCGCGGATGAGGAAGGAGCGTGAG
B4	3' b7sk + PsP0M1 RE	CAGGGCCCTCAACCCTGGCGATCAATGG
B5	5' bH1 + NotI RE	GATGCGGCCGCAACGCTGACGTCATCAGTC
B6	3' bH1 + PsP0M1 RE	CAGGGCCCCACGCAAATAGCACTT
B7	5' zsGreen + BamHI	GCAGGATCCATGGCCCAGTCCAAGCACGG
B8	3' zsGreen + NotI	GCATGCGGCCGCGATGCGATCCTTAGGGCAAGG
B9	5' Luciferase + EcoRI	CGGAATTCCCATGGAAGACGCCAAAAACATAAAGAAA
B10	3' Luciferase + XbaI	GCTCTAGAATTACACGGCGATCTTTCCGCCCTT
S1	5' shRNA oligo amplifying (5' mir30 XhoI primer)	CAGAAGGCTCGAGAAGGTATATTGCTGTGACAGTGAGCG
S2	3' shRNA oligo amplifying (3' mir30 EcoRI primer)	CTAAAGTAGCCCCTTGAATTCCGAGGCA GTAGGCA

**Table 4.** shRNA Sequences

Name	Target	Sequence
Luc, Targeting	Luciferase	TGCTGTTGACAGTGAGCG <b>CCCGCCTGAAGTCTCTGATT</b> AATAGTGAAGCCACAGATGTATTAATCAGAGACTTCAGGC GGTTGCCTACTGCCTCGGA <sup>a</sup>
NT, Non- targeting	Foot and Mouth Virus	TGCTGTTGACAGTGAGCG <b>CCCTGTCGCTTTGAAAGTGA</b> AATAGTGAAGCCACAGATGTATTTCACTTTCAAAGCGACA GGTTGCCTACTGCCTCGGA <sup>a</sup>

<sup>a</sup>mRNA targeting siRNA 22 bp sequence in **bold**, and complimentary sequence is in *italics*.



**VITA**

NAME: Michael David Peoples

ADDRESS: Veterinary Physiology and Pharmacology  
College of Veterinary Medicine  
TAMU MS 4466  
College Station, Tx 77843

EMAIL ADDRESS: [mpeoples@cvm.tamu.edu](mailto:mpeoples@cvm.tamu.edu)

EDUCATION: B.S. Animal Science. Oregon State University. 2003  
B.S. Bioresource Research. Oregon State University. 2003