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TRANSGENIC RNAi IN MOUSE OOCYTES

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Prague
2009

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Declaration

I declared this thesis is my own work elaborated under the supervision of thesis leader with the use of mentioned literature.

Prague, 2.9.2009

Acknowledgement

I would like to thank to Dr. Petr Svoboda for patience and wiliness during leading of my diploma thesis. My thanks belongs to my colleges from Laboratory of epigenetic regulations, especially to Dr. Radek Malík, who help me to solve occurring problems and made me understand principles and improvements that could be useful to my future work and life.

Furthermore, I would like to thank to Mgr. Radek Blatný for help with statistical data evaluation.

The last but not least, I would like to thank to my family, especially to my parents Olga Sarnová and Josef Sarna, and to my friends for broad support and patience that help me to overcome difficult points of my life and study.

Abstract

RNA interference (RNAi) is double-stranded RNA (dsRNA)-mediated mRNA degradation. RNAi has been widely used to investigate gene functions. Many methods to induce transient or stable RNAi have been developed. Transient RNAi can be induced by delivering of a long dsRNA or short interfering RNAs (siRNAs). Stable RNAi may be induced by introducing plasmids expressing a long or a short hairpin RNA. Both small and long RNAs have been used to induce transient RNAi in mouse oocytes. Nevertheless, only long hairpin-expressing system has been used to trigger stable RNAi in oocytes. Although, this system appeared to be highly efficient and specific, it has several disadvantages as complicated long inverted repeat cloning or limited possibility to test these vectors in the cell culture.

Here, we constructed a short hairpin-expressing vector suitable for transgenic RNAi induction in mouse oocytes. The new vector, pTMP_ZP3_sh, was derived from a lentiviral short hairpin vector selected based on comparative study of different short hairpin-expressing plasmids.

The pTMP_ZP3_sh vector was tested by targeting Moloney sarcoma oncogene (*Mos*) mRNA, which is a common model for RNAi in mouse oocytes. We designed several candidate short hairpin sequences and tested their efficacy. Subsequently, the most efficient one was selected and transgenic mice were prepared.

Unfortunately, analysis of transgenic mice revealed that the short hairpin-expressing transgene is silenced. The exact reasons of its silencing will be further investigated. Our results suggest that short hairpin-expressing system is not superior to currently available RNAi-inducing systems, because short hairpin selection is as time-consuming as long inverted repeat cloning. Long hairpin-expressing vectors remain the most suitable tool for RNA interference-based functional studies in oocytes.

1 Abbreviations

ANOVA	analysis of variance
AGO	Argonaute protein
bp	base pair
BSA	bovine serum albumin
CMV	cytomegalovirus
CP	crossing point
DMEM	Dulbecco’s modified Eagle medium
dsRNA	double-stranded RNA
EGFP	enhanced green fluorescent protein
GV	germinal vesicle
HCG	human choriogonadotropine
IFN	interferon
LTR	long terminal repeat
MAP kinase	mitogen activated protein kinase
MAZ	Myc-associated zinc finger protein
MII	metaphase II
miRNA	microRNA
Mos	Moloney sarcoma oncogene
MPF	maturation promoting factor
mRNA	messenger RNA
nt	nucleotide
PBS	potassium phosphate buffer
PCR	polymerase chain reaction
PEG	polyethylenglycol
PenStrep	Penicillin and Streptomycin
PGK	phosphoglycerate kinase
PNK	polynucleotide kinase
PKR	protein kinase R
PMSG	pregnant mare’s serum gonadotropine
PNI	pronuclear injection
pol II	RNA polymerase II
pol III	RNA polymerase III

qPCR	quantitative polymerase chain reaction
RDRC	RNA-Directed RNA Polymerase Complex
RIG-I	retinoic acid/inducible gene-I
RISC	RNA-induced silencing complex
RITS	RNA-Induced Transcriptional Silencing complex
RNAi	RNA interference
RT	reverse transcription
SAP	Shrimp alkaline phosphatase
SEM	standard error of the mean
shRNA	short hairpin RNA
SIBR	synthetic inhibitory BIC-derived RNA
siRNA	short interfering RNA
SPF	specified pathogen free
TGN	transgenic
UTR	untranslated region
ZP3	zona pellucida glycoprotein 3

2 Introduction

2.1 RNA silencing

RNA silencing is a set of eukaryotic mechanisms, in which short RNAs participate in regulation of gene expression. Short RNAs can regulate gene expression at different levels: transcriptional, posttranscriptional or translational. RNA silencing plays an important role during the development and as a defense mechanism against parasitic sequences.

Sequence-specific mRNA degradation mediated by dsRNA, known as RNA interference (RNAi), was one of the discovered posttranscriptional RNA silencing mechanisms. RNAi was first observed in *Caenorhabditis elegans* (Fire, et al., 1998). Initially, scientists supposed that mRNA translational repression could be mediated by interaction with complementary RNA. Fire et al. revealed that not the antisense RNA but dsRNA caused gene silencing by degradation of mRNAs complementary to dsRNA. Since the time, the RNAi phenomenon was observed in many diverse organisms as insects (Kennerdell and Carthew, 1998), amphibians (Oelgeschlager, et al., 2000) and mammals (Svoboda, et al., 2000; Wianny and Zernicka-Goetz, 2000).

RNAi (Fig. 1) takes place in the cytoplasm where a long dsRNA is cleaved by RNase III Dicer to 21 – 23 bp long dsRNAs called short interfering RNAs (siRNAs). siRNAs have two-nucleotide overhangs at 3´ends originating from Dicer cleavage. Overhangs are recognized by the PAZ domain of Argonaute proteins (AGO), which are main components of the RNA-induced silencing complex (RISC) (Lingel, et al., 2004). RISC is a ribonucleoprotein complex, which unwinds siRNA and binds one of its strands while the second strand is degraded. Strand selection depends on the thermodynamic stability of the siRNA ends. The RISC is preferentially loaded by the strand with less stable 5´end that contains more A-U pairs (Khvorova, et al., 2003). The loaded RISC finds and binds an mRNA complementary to the siRNA strand and cleaves the target mRNA in the middle of the complementary region.

In parallel, another RNA silencing pathway, the microRNA pathway, was discovered. In this pathway, short RNA precursors are coded in the genome (see

the chapter 2.2 for details). While RNAi serves mainly as defensive mechanism against parasitic sequences, miRNAs are concerned in the control of development.

Small RNAs and RNAi machinery also participate in silencing at the transcriptional level. Regulation of heterochromatin assembly involving RNAi components was described in yeast and plants (Martienssen, et al., 2005; Wassenegger, 2005). In *Schizosaccharomyces pombe* two complexes essential for heterochromatin formation in the centromeric region were described: RNA-Directed RNA Polymerase Complex (RDRC) and RNA-Induced Transcriptional Silencing complex (RITS) (Motamedi, et al., 2004; Verdel and Moazed, 2005). In addition, several mutations in RNAi components in *Drosophila melanogaster* and *Caenorhabditis elegans* led to the disruption of heterochromatic silencing (Pal-Bhadra, et al., 2004; Sijen and Plasterk, 2003). Although siRNA-induced transcriptional silencing has been reported in mammalian cells (Janowski, et al., 2006; Kim, et al., 2006), the actual underlying silencing mechanism is still a matter of discussion.

2.2 miRNA pathway

The miRNA pathway (Fig. 1) is closely related to RNAi (Grishok, et al., 2001). miRNAs are transcribed from the genome in the form of a hairpin-containing precursor RNA (pri-miRNA) by RNA polymerase II (Lee, et al., 2004a). Nuclear RNase III Drosha releases the hairpin (pre-miRNA) from the pri-miRNA. Like Dicer, Drosha produces two-nucleotide overhangs at the 3´end. Pre-miRNAs translocate to the cytoplasm via Exportin 5 (Lund, et al., 2004). A double-stranded stem structure longer than 16 bp and a short 3´overhang are required for successful transport (Zeng and Cullen, 2004). In the cytoplasm, pre-miRNAs are cleaved by Dicer to release mature miRNAs. Mature miRNAs have the same length and similar structure as siRNAs, except that a miRNA duplex typically contains bulges. One miRNA strand is loaded into RISC complex, which mediates mRNA translational repression and/or mRNA degradation. Endogenous miRNAs are usually not perfectly complementary to target mRNAs, bind to 3´UTRs and induce translational repression. The whole mRNA-RISC complex relocates to the cytoplasmic structures called P-bodies, where target mRNAs are stored and ultimately degraded.

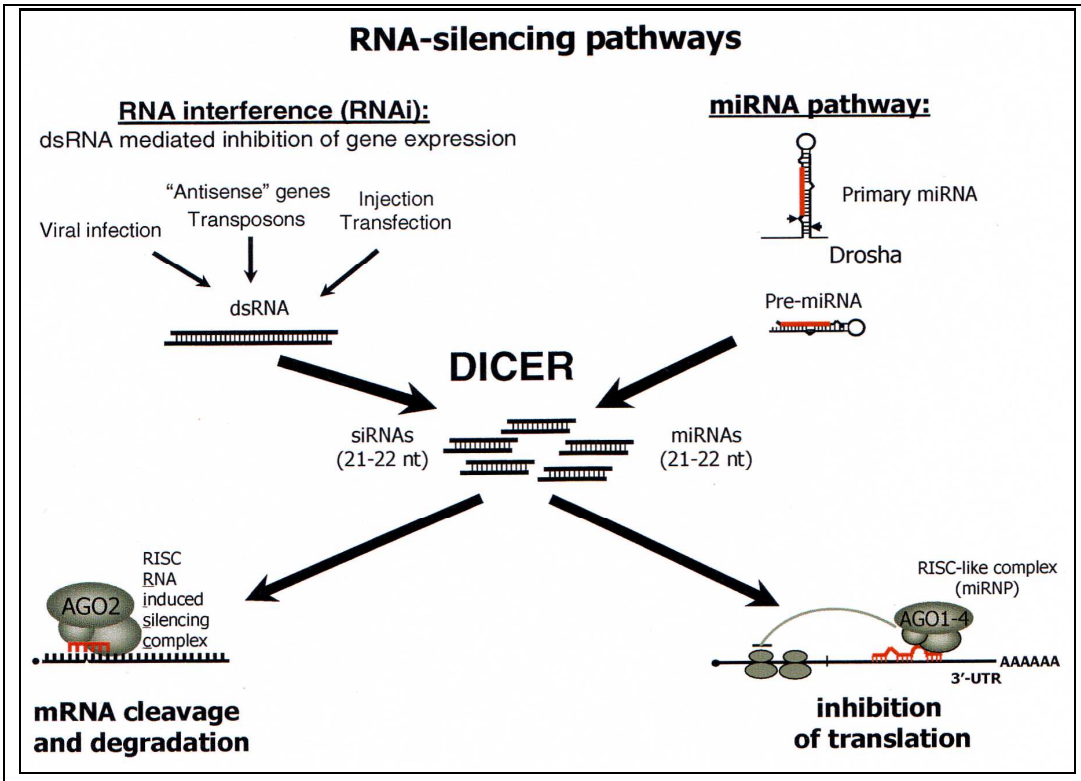


Figure 1: RNA silencing pathways in mammals

In both RNAi and miRNA pathways, short dsRNAs with 3´ overhangs arise after cleavage by Dicer. Whether these short RNAs cause mRNA degradation or translation repression depends on the level of short RNA-mRNA complementarity but not on the short RNA origin. However, endogenous miRNA are not usually perfectly complementary to mRNA and thus inhibit translation, while siRNAs usually mediate mRNA cleavage.

2.3 RNAi in mammalian cells

When RNAi was described in *C. elegans*, it was not expected RNAi could work in mammals, because even a small amount of dsRNA longer than 30 bp triggers a series of reactions generally called the interferon (IFN) response in mammalian somatic cells (Manche, et al., 1992). The activation of IFN pathway leads to translational repression, indiscriminate mRNA degradation and, eventually, apoptosis. The IFN response involves activation of a number of proteins, including protein kinase R (PKR), retinoic acid/inducible gene-I (RIG-I) and/or family of 2'-5'-oligoadenylate synthetases. dsRNA binding to domains at the amino acid terminus of PKR leads to conformation changes and to PKR activation by autophosphorylation. Activated PKR phosphorylates and inhibits translation initiation factor eIF2a, causing general repression of protein synthesis. dsRNA also activates 2´, 5´-oligoadenylate synthetase. Synthesized oligoadenylates induce dimerization and activation of the latent endonuclease

RNaseL. Active RNaseL indiscriminately degrades RNA in the cell. Oligoadenylate/RNaseL system can also induce apoptosis by degrading mRNAs coding anti-apoptotic or cell survival factors (reviewed in Barber, 2001). The IFN response is a defensive mechanism against viruses, which frequently produce dsRNA during their life cycle. Interestingly, oocytes and early embryos lack sequence-independent response to the long dsRNA (Stein, et al., 2005) and long dsRNA can thus induce specific RNAi in these cells (Svoboda, et al., 2000; Wianny and Zernicka-Goetz, 2000). It is unknown why oocytes and early embryos lack the IFN pathway. Interestingly, these are the only cells, where endogenous siRNAs have been detected so far (Watanabe, et al., 2008).

In contrast to long dsRNA-induced RNAi, miRNAs and siRNAs are functional in all mammalian cells. The same enzymes and complexes involved in long dsRNA-triggered RNAi pathway mediate the action of both types of small RNAs. Mammals have only one Dicer and four AGO proteins (AGO1-4). The presence of a specific AGO protein in the RISC complex determines whether target mRNA is cleaved or not (Meister, et al., 2004). mRNA cleavage occurs only when the RISC complex contains AGO2 but not other AGO proteins (Meister, et al., 2004). Next to the AGO type, complementarity between miRNA/siRNA and its target plays an important role. Cleavage requires the perfect complementarity (Hutvagner and Zamore, 2002) while the presence of mismatches prevents cleavage but may result in translation repression.

In some organisms, like *Drosophila melanogaster*, RNAi and miRNA pathways genetically diverged. Dicer-1 and AGO1 are involved in the miRNA pathway while Dicer-2 and AGO2 are specific for the RNAi pathway (Lee, et al., 2004b; Okamura, et al., 2004).

2.4 RNAi as an experimental tool

Since its discovery, RNAi has become a powerful technique to inhibit gene function. Two ways to experimentally induce RNAi exist. The first one is based on delivering of exogenous siRNAs or long dsRNAs. However, resulting gene down-regulation is only transient. The second way is to use dsRNA-expressing system, which allows for transient or stable inhibition of the target gene. Particular RNAi-mediating systems are described later.

Before RNAi discovery, homologous recombination was the method of choice for gene silencing. In this method, a selection marker flanked by sequences homologous to the target gene is transfected into cells where it replaces the target gene during a homologous recombination, which takes place during mitosis (reviewed in Sedivy and Dutriaux, 1999). Such recombination inactivates the target gene and simultaneously enables selection of affected cells. However, Founder animals carry only one mutated allele, because the frequency of homologous recombination is very low. Therefore, gene targeting by homologous recombination requires inbreeding to obtain homozygous mutants, which makes the procedure even more time-consuming.

In comparison to homologous recombination, RNAi provides several advantages. The main advantages of RNAi are speed and a favorable cost. RNAi may induce transient or stable silencing. Transient RNAi is relatively fast method suitable for using in cell cultures. In addition, preparation of animals with stable silencing provided by transgenic RNAi is also less time-consuming process compared to homologous recombination. Transgenic RNAi does not require breeding to obtain homozygotes in transgene, because it mediates down-regulation of transcripts from both corresponding chromosomes simultaneously. Moreover, while gene targeting by homologous recombination leads to complete gene silencing, RNAi mediates only partial silencing and remaining weak expression of target gene may prevent eventual lethality.

Alternatively, conditional RNAi can be used to analyze genes whose knock-out is lethal at specific developmental stage. In this approach, the transgene is expressed from a tetracyclin-inducible promoter and its activity can be regulated by doxycyclin. Doxycyclin-mediated regulation is reversible and can be induced repeatedly. However, tetracycline-inducible regulation may not always be 100% reliable.

2.4.1 RNAi in mouse oocytes

Maternal mRNAs are transcribed during oocyte maturation and are stored in mature oocytes where no new RNA synthesis occurs. It is difficult to make a classic gene knock-out specifically in oocyte. RNAi is suitable for studying maternal mRNA function. Stored mRNAs are translated only upon resumption of

meiosis. So theoretically, there is long time for RNAi to totally degrade target maternal mRNA during the period when no fresh proteins are synthesized, whose prolonged stability might affect the overall RNAi efficacy in oocyte.

Moloney sarcoma oncogene (*Mos*) is well investigated maternal mRNA. Its well-characterized phenotype makes this gene a suitable target for development of RNAi-based methods. MOS is a protein kinase participating in meiosis regulation. MOS is active only in oocytes and is degraded upon fertilization. A negative regulatory sequence in the promoter of the *Mos* gene inhibits its expression in somatic cells. During oocyte development, MOS indirectly activates maturation promoting factor (MPF) via activation of mitogen activated protein (MAP) kinase. MOS inhibits proteolytic degradation of cyclin-B, thus leads to the accumulation of cyclin-B between meiosis I and II and MPF activity remains high during metaphase arrest (reviewed in Heikinheimo and Gibbons, 1998). *Mos* knock-out expression leads to the disruption of the meiotic block in metaphase II and parthenogenetic activation of oocytes (Colledge, et al., 1994; Hashimoto, et al., 1994) leading to the extrusion of the second polar body without fertilization by the sperm, which provides a readily detectable phenotype.

2.4.2 Nonspecific effects in RNAi experiments

Although RNAi successfully down-regulates target gene expression in a sequence-specific manner, it can also cause unspecific changes in the expression of unrelated genes. siRNAs may function like endogenous miRNAs and bind 3´UTR regions of transcripts with partial homology resulting in the repression of their translation. This undesirable effect is called off-targeting (Jackson, et al., 2003; Persengiev, et al., 2004). Off-target effect can be reduced when the amount of an introduced siRNA is as low as possible and/or when a pool of siRNAs targeting the same gene but differing in their target sequences is used. Using a pool of siRNAs with the same total siRNA amount delivered, concentration of each individual siRNA from the pool is lower compared to single siRNA delivery. Lower siRNA concentration has reduced probability of off-targeting effects. The fact that a pool of siRNAs arising from a long dsRNA in oocytes does not evoke off-targeting supports this explanation (Stein, et al., 2005).

Introducing the smallest possible amount of siRNAs (miRNAs) also prevents the saturation of the endogenous miRNA pathway, which may be detrimental for the cell. This problem becomes apparent when miRNAs-mimicking hairpins (described later) are used to induce RNAi (Grimm, et al., 2006). The critical point is the miRNA transport to the cytoplasm. Overexpressed recombinant pre-miRNAs seem to saturate Exportin 5 pathway and constrain the transport of endogenous pre-miRNAs into the cytoplasm and their further processing by Dicer (Grimm, et al., 2006).

In addition, some specific nucleotide stretches, which stimulate immune response if contained within siRNA sequence, have been described (Hornung, et al., 2005; Judge, et al., 2005). Eventually, blunt-ended short dsRNAs can be recognized by RIG-I helicase and trigger immune response via activating the IFN pathway (reviewed in Marques and Williams, 2005). However, majority of short RNAs do not trigger IFN response in mammalian cells.

2.4.3 Transient RNAi

As mentioned above, transient RNAi can be triggered by delivering siRNAs or long dsRNA. Three delivery methods are commonly used in different model systems: transfection, electroporation and microinjection. Transfection and electroporation are typically used for experiments using cultured cells. Contrary to cell cultures, chemical transfection is difficult to use in mouse oocytes. Commercial transfection reagents are usually highly toxic for mouse oocytes (Svoboda, 2004). Lazer et al. described successful transfection of rat oocytes (Lazar, et al., 2004). Electroporation is a suitable method when more oocytes are needed to be targeted simultaneously (Grabarek, et al., 2002).

Microinjection is the most common method of delivering nucleic acids into oocytes Pilot experiments using transient RNAi in oocytes were performed by microinjection of long dsRNA complementary to *Mos* mRNA. Subsequent real-time PCR analysis confirmed sequence-specific *Mos* mRNA down-regulation (Svoboda, et al., 2000) confirming that dsRNA microinjection led to the strong and specific silencing in mouse oocytes. Since that time, many other genes were successfully down-regulated by delivering dsRNA or siRNAs into oocytes by

microinjection (Anger, et al., 2005; De La Fuente, et al., 2004; Gui and Joyce, 2005; Svoboda, et al., 2004).

2.4.4 Transgenic RNAi

Many studies require stable or continuous inhibition of gene expression. This initiated a development of RNAi-mediating expression systems, which could be stably integrated in the genome. Most systems express transcripts with a hairpin structure (McManus, et al., 2002; Shinagawa and Ishii, 2003; Svoboda, et al., 2001). Some others express sense and antisense RNA strands separately, but these systems do not seem to mediate RNAi efficiently (Miyagishi and Taira, 2002). Developed vectors can be divided according to the type of expressed hairpin into three categories: 1) long hairpin, 2) short hairpin (shRNA) and 3) miRNA-like hairpin (Fig. 2).

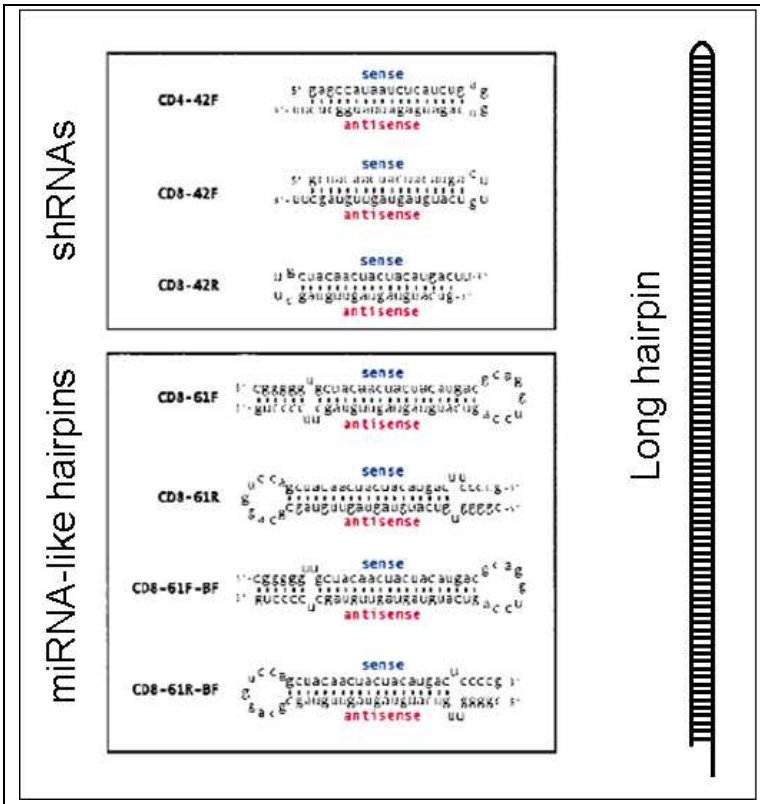


Figure 2: Types of hairpins used for RNAi induction.

Short hairpin (shRNA) and long hairpin arise from inverted repeat and make a perfect dsRNA. miRNA-like hairpins have the similar structure as endogenous miRNAs and contain bulges and mismatches in double-strand region. The figure is based on (McManus, et al., 2002).

Hairpins differ in stem length, loop length and in the presence of mismatches in the stem structure (described later). All three types can induce transgenic RNAi in oocytes. RNA polymerase III as well as RNA polymerase II promoters may be used to control hairpin expression. Choice of hairpin and promoter type depends on a particular situation. Properties of individual hairpins and promoters are discussed later.

2.4.4.1 Long hairpin expressing systems

Long dsRNA hairpin arises by transcription of a large inverted repeat. The hairpin is usually at least 500 bp long and mimics a long dsRNA. The expression systems producing long hairpin are applicable only to oocytes, early embryos and stem cells in mammals where the IFN pathway in not induced (Svoboda, et al., 2001; Yu, et al., 2004). However, long hairpins are commonly used in RNAi experiments in invertebrates (Kennerdell and Carthew, 2000; Tavernarakis, et al., 2000) and plants (Wesley, et al., 2001).

Long hairpin expression elicits the same inhibitory effect as microinjected dsRNA in oocytes (Stein, et al., 2003). This system is highly efficient and unspecific effects were not found (Stein, et al., 2005). So far, only long hairpins have been used to mediate transgenic RNAi in mouse oocytes. Several long hairpin-expressing systems have been constructed. The simplest system contains a promoter followed by an inverted repeat (Svoboda, et al., 2001). More advanced systems contain also reporter genes such as enhanced green florescent protein (EGFP) (Svoboda, et al., 2001). Although long 3´overhangs block RNAi *in vitro* (Elbashir, et al., 2001b), long 3´or 5´ overhangs do not apparently limit RNAi *in vivo*, so one promoter can simultaneously drive hairpin and marker expression (Svoboda, et al., 2001). However, mutual position of hairpin and marker coding sequences influences marker expression (Svoboda, et al., 2001). The hairpin expression was not influenced by the position of EGFP coding sequence, but EGFP expression was detectable only when localized upstream of the hairpin. Introduction of an intron sequence next to the EGFP increased its expression, but an intron localized next to the hairpin blocked RNAi. A system containing an intron and EGFP followed by an inverted repeat appeared to be the most effective (Svoboda, et al., 2001) and it was chosen for transgenic RNAi (Stein, et al., 2003).

Shinagawa and Ishii presented an alternative system generating long hairpin, which should not trigger IFN response in mammalian somatic cells (Shinagawa and Ishii, 2003). Long dsRNA triggers IFN response after its transport to the cytoplasm (Stark, et al., 1998). Polyadenylation of the 3´ end and 5´ cap are required for successful transport of RNA to the cytoplasm. If these structures are excised, transcript is retained in the nucleus. In their system, Shinagawa and Ishii flanked long hairpin-coding inverted repeat with a ribozyme sequence at the 5´end and with Myc-associated zinc finger protein (MAZ) sequence at the 3´end. The ribozyme cut off 5´cap and the presented MAZ sequence terminated transcription prematurely. Produced long hairpin RNA was probably digested by Dicer present in the nucleus and siRNAs were transported to the cytoplasm. This system was used for successful down-regulation of Ski gene expression in mouse embryos without triggering the immune response (Shinagawa and Ishii, 2003).

2.4.4.2 Short hairpin expressing systems

Short hairpin-expressing vectors have been used since 2002 (Brummelkamp, et al., 2002; McManus, et al., 2002). Short expressing systems are able to mediate RNAi in all mammalian cells contrary to the long hairpins, which function only in oocytes and early embryos. Other advantage of short hairpins is their easier cloning compared to long ones.

A variety of short hairpin-expressing vectors have been described since then (Coumoul, et al., 2005; Gupta, et al., 2004; Chung, et al., 2006; Stern, et al., 2008; Ventura, et al., 2004; Xia, et al., 2006). Short hairpin-expressing systems can be divided into class I and II (McManus, et al., 2002).

Class I hairpins, usually called short hairpin RNAs (shRNA), contain perfectly complementary 19-21 bp long stem structure and 4 – 9 nt long loop. If the loop is placed between the 3´end of the strand complementary to target mRNA (antisense strand) and the 5´end of the passenger strand, the hairpin triggers RNAi as efficiently as corresponding synthetic siRNA. In the opposite orientation, the hairpin RNAi efficiency is impaired (McManus, et al., 2002). Class I hairpin expression is usually driven by RNA polymerase III-dependent promoters (for details see the chapter 2.4.4.2.1).

Class II hairpins mimic endogenous miRNAs and they are often called miRNA-like hairpins (Dickins, et al., 2005). These hairpins are typically transcribed by RNA polymerase II. Stem structure of these hairpins also contains bulges and mismatches. RNases Drosha and Dicer are involved in class II hairpin maturation (for details see the chapter 2.4.4.2.2).

Other small RNA-expressing systems were described. The system developed by Miyagishi and Taira (Miyagishi and Taira, 2002) expresses siRNA strands separately from two independent promoters. However, this system is not commonly used.

2.4.4.2.1 RNA polymerase III (Pol III) expression systems

Pol III systems are predominantly used to express class I shRNAs, but may be used for the expression of miRNA-like hairpins as well. Hairpin expression is typically driven by U6 or H1 promoters. The main advantage of pol III is the precisely defined transcription termination. A stretch of thymidines directly following the hairpin sequence provides the transcription stop signal and creates TT 3´overhang.

The major disadvantage of pol III systems is the difficulty to achieve tissue-specific hairpin expression, since there are no tissue-specific pol III promoters. Pol III-mediated tissue-specific expression may be achieved indirectly by Cre-loxP systems. In Cre-activated systems, the loxP-flanked sequence of a marker (e.g. EGFP gene) is placed between the promoter and shRNA coding sequence. Thus, shRNA is expressed only after Cre-mediated deletion of the loxP-flanked marker sequence (Coumoul, et al., 2005; Ventura, et al., 2004).

In Cre-inactivated systems, loxP sites flank shRNA coding sequence, whose deletion upon recombination leads to the lost of silencing (Ventura, et al., 2004). Breeding of transgenic mice containing loxP-flanked shRNA cassette with a mice expressing Cre recombinase in selected tissue allows for tissue-specific shRNA expression *in vivo*. The disadvantage of these Cre-loxP regulated systems is that achieving tissue-specific shRNA expression requires extensive breeding and that Cre-mediated recombination changes are irreversible.

2.4.4.2.2 RNA polymerase II (Pol II) expression systems

Class II miRNA-like hairpins are expressed by RNA polymerase II similarly to endogenous miRNAs (Lee, et al., 2004a). In comparison to pol III systems, higher expression levels of miRNA-like hairpins are achieved from pol II promoters (Dickins, et al., 2005) and a wide range of tissue-specific pol II promoters are available. Most pol II systems are based on human miR-30 sequence, which was analyzed in detail in Bryan Cullen's laboratory (Zeng and Cullen, 2003). The miR-30 precursor creates an approximately 80 nt long hairpin structure, which contains many bulges and mismatches. Irregular structure probably avoids triggering of IFN response (Bridge, et al., 2003). In pol II systems, sequence corresponding to mature miR- 30 is replaced by target gene sequence, while flanking sequences are preserved. These sequences contain regions recognized by nuclear RNase Drosha (Chen, et al., 2004). miRNA-like hairpins with the most similar secondary structure to the original miR-30 work more efficiently then structurally distorted hairpins (Zhou, et al., 2005). Some endogenous miRNAs reside in introns of protein coding genes. Thus, pol II systems allowing simultaneous expression of a hairpin and a marker gene from a single promoter were constructed (Chung, et al., 2006; Xia, et al., 2006).

Chung et al. developed a vector using the mouse non-coding BIC sequence. The third BIC exon contains miR-155 precursor, which has been used for the construction of the synthetic inhibitory BIC-derived RNA (SIBR) cassette expressing miRNA-like hairpin. SIBR cassette can be embedded within an exon as well as an intron, but its expression from intron is stronger (Chung, et al., 2006). Multiple SIBR cassettes expressing different shRNAs targeting one or more gene(s) can be expressed from a single promoter. Such arrangement was proved to lower off-targeting and enhance RNAi effect (Chung, et al., 2006).

2.4.4.3 Aims of diploma thesis

The major aim of this study was to develop an shRNA-expressing system mediating transgenic RNAi in mouse oocytes. The issue was approached through the following steps (Fig. 3):

- selection the most appropriate shRNA expression system
- design and selection of functional shRNA hairpin(s) targeting the mouse *Mos* gene
- construction of a vector expressing *Mos*-targeting shRNA specifically in oocytes
- production of transgenic mouse line(s)
- phenotype analysis of transgenic animals

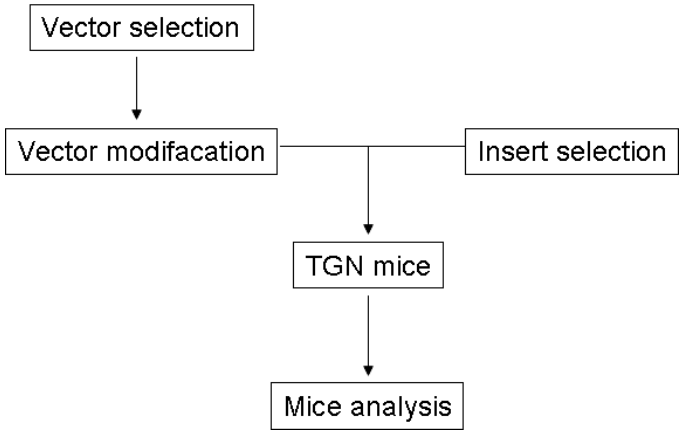


Figure 3: Thesis project outline

The thesis aim was approached by several indicated stepwise goals. We selected suitable shRNA-expressing vector and performed changes to make oocyte-specific expression system. We designed functional *Mos*-targeting shRNA hairpin(s) and inserted into prepared vector. Finally, transgenic mouse lines were established and analyzed.

3 Materials and methods

3.1 Instruments

Centrifuge 5424 (Eppendorf)

Centrifuge 5415 R (Eppendorf)

Centrifuge BR4i (Jouan)

Electrophoresis HE 33 Mini Submarine Unit (Amersham Biosciences)

Electrophoresis D3-14 Wide Gel System (Thermo Scientific)

Vortex Mixer VX-100 (Labnet)

Electroporator Gene Pulser (BioRad)

Spectrophotometer NanoDrop ND-1000

Thermocycler XP (Bioer)

Thermocycler iQ5 (BioRad)

Shaker 311DS (Labnet)

Incubator shaker Innova 4000 (New Brunswick Scientific)

Biological thermostat BT 120M (Laboratorní přístroje Praha)

Laminar flow box Bio-II-A (Telstar)

Incubator Model 321 (Thermo Electron Corporation)

Microscope SZX16 (Olympus)

3.2 Primers and oligonucleotides

5´loxP.fwd	GAT CGC GGC CGC ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA TA
5´loxP.rev	GAT CTA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT GCG GCC GC
3´loxP.fwd	TCG ACA ATA AAA TAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT GCG GCC GCG
3´loxP.rev	TCG ACG CGG CCG CAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATT TTA TTG
LMP_MunI.fwd	CCA ACA GAA GGC TCG AGC AAC CAC AAT TGA AGG GGC TAC TTT AGG AGC AAT TAT CTT GTT TAC
LMP_MunI.rev	GTA AAC AAG ATA ATT GCT CCT AAA GTA GCC CCT TCA ATT GTG GTT GCT CGA GCC TTC TGT TGG
ZP3_BglII_Fwd	GAC AGA TCT CTG GGA GTT CAA GGC CAG
ZP3_BglII_Rev	GAG AGA TCT TAA TGA GAG GCT GAC ACC ACT G

LMP_oligo.fwd	CAG AAG GCT CGA GAA GGT ATA TTG CTG TTG ACA GTG AGC G
LMP_oligo.rev	CTA AAG TAG CCC CTT GAA TTC CGA GGC AGT AGG CA
FL_1	TGC TGT TGA CAG TGA GCG ACG TAC GCG GAA TAC TTC GAA ATA GTG AAG CCA CAG ATG TAT TTC GAA GTA TTC CGC CTA CGG TGC CTA CTG CCT CGG A
FL_2	GAT CCC CCG TAC GCG GAA TAC TTC GAT TCA AGA GAT CGA AGT ATT CCG CGT ACG TTT TTA
FL_3	AGC TTA AAA ACG TAC GCG GAA TAC TTC GAT CTC TTG AAT CGA AGT ATT CCG CGT ACG GGG
FL_4	GCT GAT TTC GAA GTA TTC CGC GTA CGT TTT GGC CTC TGA CTG ACG TAC GGG AAA CTT CGA AAT C
FL_5	TCC TGA TTT CGA AGT TTC CCG TAC GTC AGT CAG AGG CCA AAA CGT ACG CGG AAT ACT TCG AAA T
LMP_Rev_Seq	CTC CAG ACT GCC TTG GGA AAA GC
LMP_Fwd_Seq	CCT CAT CAC CCA GGT TAA GAT
ZP3_end.fwd	TAA GAA CAG TGG TGT CAG CCT C
Mos_1	TGC TGT TGA CAG TGA GCG AAC GAC AAC ATA GTT CGG GTT GTA GTG AAG CCA CAG ATG TAC AAC CCG AAC TAT GTT GTC GTG TGC CTA CTG CCT CGG A
Mos_2	TGC TGT TGA CAG TGA GCG ACC GAA GAC TCC AAC AGC CTA GTA GTG AAG CCA CAG ATG TAC TAG GCT GTT GGA GTC TTC GGG TGC CTA CTG CCT CGG A
Mos_A	TGC TGT TGA CAG TGA GCG AGG AGA TCC TGA AAG GAG AGA TTA GTG AAG CCA CAG ATG TAA TCT CTC CTT TCA GGA TCT CCG TGC CTA CTG CCT CGG A
Mos_B	TGC TGT TGA CAG TGA GCG CCC ATA GAC TGG GAA CAG GTA TTA GTG AAG CCA CAG ATG TAA TAC CTG TTC CCA GTC TAT GGA TGC CTA CTG CCT CGG A
Mos_C	TGC TGT TGA CAG TGA GCG AAG GGT TTG GCT CGG TGT ATA ATA GTG AAG CCA CAG ATG TAT TAT ACA CCG AGC CAA ACC CTC TGC CTA CTG CCT CGG A
Mos_D	TGC TGT TGA CAG TGA GCG CCC GGA GAT CCT GAA AGG AGA GTA GTG AAG CCA CAG ATG TAC TCT CCT TTC AGG ATC TCC GGA TGC CTA CTG CCT CGG A
Mos_E	TGC TGT TGA CAG TGA GCG ATC GGT GTA TAA AGC CAC TTA CTA GTG AAG CCA CAG ATG TAG TAA GTG GCT TTA TAC ACC GAG TGC CTA CTG CCT CGG A

Mos_F	TGC TGT TGA CAG TGA GCG CGG GTT TGG CTC GGT GTA TAA ATA GTG AAG CCA CAG ATG TAT TTA TAC ACC GAG CCA AAC CCT TGC CTA CTG CCT CGG A
OcAGlobin55.Fwd	GCA GCC ACG GTG GCG AGT AT
OcAGlobin312.Rev	GTG GGA VAG GAG VTT GAA AT
Mos2.Fwd	GGG AAC AGG TAT GTC TGA TGCA
Mos2.Rev	CAC CGT GGT AAG TGG CTT TAT ACA

3.3 Plasmid cloning

3.3.1 Bacterial strains

DH5α –electrocompetent cells (Invitrogen)

MDS42 RecA Blue cells (Scarab Genomics)

XL1-Blue supercompetent cells (Stratagene)

XL10-GOLD competent cells (Stratagene)

3.3.2 Commercial kits

Zyppy™ Plasmid Miniprep Kit (Zymo Research)

QIAprep Spin Miniprep Kit (Qiagen)

GeneJET™ Plasmid Miniprep Kit (Fermentas)

NucleoBond Xtra Midi (Machery - Nagel)

HiSpeed Plasmid Midi Kit (Qiagen)

QIAquick Gel Extraction Kit (Qiagen)

Zymoclean™ Gel DNA Recovery Kit (Zymo Research)

DNA Clean & Concentrator™ (Zymo Research)

Quick Change II XL Site-Direct Mutagenesis Kit (Stratagene)

3.3.3 Chemical transformation

A mixture of 1- 5 µl of a plasmid diluted in water and 50 µl of competent bacteria was placed in a 1.5 ml microtube and incubated on ice for 30 minutes, then at 42 ºC for 30 seconds. After 2 minutes on ice 450 µl of LB media (20 g/l) was added and the mixture was shaken at 37 ºC and 600 rpm for 1 hour in the mixing block. The culture was plated on a LB agar (15 g/l) containing Ampicilin

(100 µg/ml). Plates were incubated at 37 ºC overnight. Single colonies were picked up and inoculated into LB media (3-4 ml for Miniprep or 50-80 ml for Midiprep) with Ampicilin (100 µg/ml) and incubated at 37 ºC and 250 rpm overnight. Plasmids were isolated using one of the commercial Mini- or Midiprep kits specified above according to the manufacturer's instructions.

3.3.4 Electroporation

A mixture of 1 µl plasmid and 50 µl bacteria was placed to the electroporation cuvette (Bio-Rad) and electroporated (pulse: 12.5 kV/cm, time constant: 4.4 – 5.0 msec) on the Gene Pulser (Bio-Rad). Immediately after the pulse, 950 µl of LB media was added and culture was incubated in 14 ml tube at 37 ºC and 250 rpm for 1 hour. The culture was plated on a LB agar containing Ampicilin. Plates were treated as described above.

3.3.5 Glycerol stocks

Important bacterial cultures were stored as glycerol stocks at -80 °C. 0.25 ml of 60% glycerol was mixed with 0.75 ml bacteria culture in cryotubes and placed in a -80 °C freezer immediately after glycerol addition.

3.3.6 Restriction cleavage and dephosphorylation

For restriction digests, 1 – 2 µg of a plasmid was mixed with 0.5 – 1 µl of a restriction endonuclease (usually 10 U/µl, Fermentas or New England Biolabs) and corresponding buffer. Water was added up to the total volume of 20 µl. The mixture was incubated at 37 ºC for 1 hour. If dephosphorylation was required, 8 µl of water, 1 µl of the restriction buffer used and 1 µl of the Shrimp Alkaline Phosphatase (SAP, Fermentas) were added. The mixture was incubated at 37 ºC for additional 30 minutes and then at 65 ºC for 15 minutes to inactivate SAP.

3.3.7 Ligation

A cleaved vector (~ 50 ng) and an insert were mixed with T4 ligase buffer (1x), 2 µl polyethylenglycol (PEG) 4000, 1 µl T4 ligase (Fermentas) and water to total volume 20 µl. Concentration of insert ends was at least three-fold higher than concentration of vector ends. The mixture was incubated overnight in water bath at 16 ºC or for 1 hour at room temperature.

3.3.8 Oligonucleotides annealing

Oligonucleotides (2 µg each) were added into 100 µl of water. Oligonucleotides were annealed in a gradually cooling water bath (1 liter) after boiling for 5 minutes. Alternatively, oligonucleotide mixture was incubated in a thermocycler for 5 minutes at 95 ºC and then the thermocycler was turned-off to allow for gradual cooling.

If phosphorylation was required, the following reaction was set up prior to annealing:

Oligonucleotide in water	15 µl (2 µg)
T4 ligase Buffer	2 µl
ATP (100mM)	2 µl
T4 Polynucleotide Kinase (PNK)	1 µl

The mixture was incubated at 37 ºC for 20 minutes. Then, PNK was inactivated at 75 ºC for 10 minutes. Phosphorylated oligonucleotides were annealed as described above.

3.3.9 Mutagenesis

Quick Change II XL Site-Direct Mutagenesis Kit (Stratagene) was used to introduce single nucleotide mutations. Primers containing mutations were designed according to the manufacturer's instructions. PCR reaction for incorporating a mutation was following:

Plasmid DNA in water (50 ng)	30 µl
Buffer (10x)	5 µl
Primer.fwd (125 ng)	5 µl
Primer.rev (125 ng)	5 µl
Quick Solution	3 µl
dNTP mix	1 µl
Pfu Ultra polymerase	1 µl

PCR parameters: 95 ºC for 4 minutes; 18 cycles of 95 ºC for 30 seconds, 58 ºC for 1 minute, 68 ºC for 12 minutes; 68 ºC for 7 minutes and 4 ºC until samples were removed from the thermocycler.

The PCR product was cleaved by DpnI restriction enzyme to degrade non-mutated plasmids. 1 µl of DpnI was added to 10 µl of PCR reaction. Mixture was incubated at 37 ºC for 2 hours. Subsequently, XL10-GOLD competent cells (30 µl) provided with Mutagenesis kit were chemically transformed with mutated plasmids.

All introduced mutations were verified by sequencing (Laboratory of DNA Sequencing, Faculty of Science, Charles University in Prague).

3.4 Electrophoresis

Instruments:

HE 33 Mini Submarine Unit (Amersham Biosciences)

D3-14 Wide Gel System (Thermo Scientific)

Chemicals:

UltraPure Agarose (Invitrogen)

SeaKem LS Agarose (Lonza)

TBE buffer:

89 mM Tris

89 mM boric acid

2 mM EDTA

LB buffer:

5mM lithium borate

1% or 1.5% agarose in TBE or LB buffer with ethidium bromide (250 µg/l) was used for size-separation of DNA fragments. Commercial 1 kbp or 100 bp DNA ladder (Fermentas or NEB) served to determine fragments size. When some fragment was required for further processing, it was excised from the gel and DNA was isolated using one of the gel extraction kits according to the manufacturer's instructions.

3.5 Polymerase chain reaction (PCR)

PCR reactions were prepared as follows:

DNA in water (200 ng)	33 µl
Primer.fwd (10 µM)	3 µl
Primer.rev (10 µM)	3 µl
Pfu buffer without Mg (10x)	5 µl
$MgSO_4$ (25mM)	4 µl
dNTP´s (12.5 mM)	1 µl
Pfu polymerase	1 µl

All reagents were obtained from Fermentas.

PCR reaction conditions were following: 94 ºC for 4 minutes; 12 cycles of 94 ºC for 30 seconds, 57 ºC for 1 minutes, 68 ºC for 1 minute per 1 kb sequence; 68 ºC for 3 minutes and 18 ºC until samples were removed from the thermocycler.

3.5.1 Quantitative real-time RT-PCR (qPCR)

qPCR was used for the analysis of mRNA expression in oocytes. Individual oocytes were collected separately in 5 µl of water. 1 µg of stuffer rRNA (16S + 23S, Roche) and 15 pg of external standard rabbit β-globin mRNA (Sigma) were added to each sample. All samples were snap-frozen and stored at -80 °C until further processing. Before qPCR, samples were incubated at 85 °C for 5

minutes to lyse oocytes and then were placed on ice. 1 µl of Oligo(dT) primer (50 µM) and water up to 13 µl were added to all samples. Mixtures were incubated for 5 minutes at 65 °C and placed directly on ice for at least 1 minute. Reverse transcriptase master mix (7 µl) was added into each sample:

5X Reaction buffer (Fermentas)	4 µl
dNTPs (10mM)	1 µl
DTT 0.1 M	1 µl
RiboLock RNase Inhibitor (40U/µl) (Fermentas)	0.5 µl
RevertAid M-MuLV Reverse transcriptase (200 U/µl) (Fermentas)	0.5 µl

Reverse transcriptase was omitted in control (-RT) samples.

For cDNA synthesis, samples were incubated for 60 minutes at 42 °C, followed by 10 minutes incubation at 50 °C. Resulting cDNA was diluted 3:2 with water. A 3 µl aliquot of prepared cDNA was used as a template for qPCR. qPCR was performed on the Bio-Rad iQ5 machine using Maxima SYBR Green qPCR Master Mix (Fermentas). Specific primers for mouse *Mos* (Mos2.Fwd, Mos2.Rev) and rabbit β-globin mRNAs (OcAGlobin55.Fwd, OcAGlobin312.Rev) were used (for sequences see the chapter 3.2). Final PCR reaction mix was as follows:

Maxima SYBR Green qPCR Master Mix (2X)	5 µl
Primers mixture (Fwd and Rev; 2 µM each)	2 µl
cDNA	3 µl

qPCR reactions were cycled 50 times at 95 °C for 15 seconds, 60 °C for 20 seconds, 72 °C for 30 seconds; final extension 10 minutes at 72 °C was performed. qPCR data were analyzed by the iQ5 software (Bio-Rad) and values of crossing points (CPs) were evaluated for each reaction. PCR efficiency was calculated for each individual reaction using the exponential regression model (Tichopad, et al., 2003). CPs values were corrected according to particular reaction efficiency. Statistical significance of changes of *Mos* mRNA levels normalized to the external β-globin standard were analyzed by the pair-wise fixed reallocation randomization test using the REST 2008 software (Pfaffl, et al., 2002).

3.6 Tissue culture

3.6.1 Cell lines

HeLa - human epithelial cells from a cervical carcinoma transformed by human papillomavirus 18 (HPV18)

HEK293 - human embryonic kidney cell line

NIH3T3 - mouse fibroblast cell line established from Swiss mouse embryo tissue

3.6.2 Solutions

Dulbecco's Modified Eagle medium (DMEM, 2x):

Component	g/l	Component	g/l
NaCl	12.8	L serine	0.084
KCl	0.8	L threonine	0.19
$CaCl_2.2H_2O$	0.53	L tryptophan	0.032
$MgSO_4.7\ H_2O$	0.4	L tyrosine 2Na H_2O	0.2076
$Fe(NO_3)_3.9\ H_2O$	0.0002	L valine	0.188
NaH_2PO_4	0.2156	Folic acid	0.008
L arginine HCl	0.168	D-Ca pantothenate	0.008
L cysteine 2HCl	0.1252	choline chloride	0.008
Glycine	0.06	Myo-inositol	0.0144
L histidine HCl H_2O	0.084	Niacinamide	0.008
L isoleucine	0.21	Pyridoxal HCl	0.008
L leucine	0.21	Ribofavin	0.0008
L lysine HCl	0.292	Thiamine	0.008
L methionine	0.06	Glucose	9
L phenylalanine	0.132	Phenol red	0.032

Phosphate-buffered saline (PBS; 1x):

Component	g/l
NaCl	8
KCl	0.2
KH_2PO_4	0.2
Na_2HPO_4	1.15

3.6.3 Cell culture

Cells were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS), Penicillin 100 U/ml and Streptomycin 100 μg/ml. All reagents were obtained from Media service of Institute of Molecular Genetics, Academy of Science of the Czech Republic.

3.6.3.1 Transfection

Cells were seeded in 24-well plates at the initial density 30000 (HEK293 and NIH3T3) or 60000 (HeLa) cells per well in 0.5 ml of DMEM. 24 hours later, cells were transfected with 0.5 μg of plasmid DNA per well. TurboFect (Fermentas) was used as the transfection reagent. TurboFect to DNA ratio was cell-line specific; 1 μl and 2 μl of TurboFect per 1 μg DNA were used in HEK293/NIH3T3 and HeLa cells, respectively.

Corresponding amount of plasmid DNA and TurboFect were mixed in 0.1 ml DMEM without FBS. The mixture was incubated 30 minutes at room temperature and then added dropwise directly to the cells. 1 ml of fresh DMEM culture media was added 6 hours post-transfection. Each transfection was performed at least in duplicates. Cells were collected 48 hours post-transfection and used for analysis.

3.6.4 Dual Luciferase assay

Knock-down effect produced by different vectors was measured by dual luciferase assay. Cells were transfected with a tested short hairpin-expressing plasmid, a plasmid expressing *Renilla* luciferase and a plasmid expressing firefly luciferase. Expression of one of the luciferases was specifically down-regulated with hairpin expressed from tested vector. The expression of the second luciferase was unaffected and served for normalization purposes.

Analysis of variance (ANOVA) was used for statistical analysis of results (www.blatny.com/code/import.csv.files.and.run.single.factor.multiple.comparison.anova.R). All ANOVA results (p-values) have been adjusted for multiple comparison.

Cells were typically transfected with 50 ng of a firefly luciferase coding plasmid, 1 ng of a *Renilla* luciferase coding plasmid, 50 ng of a tested hairpin-coding vector, and pBluescript up to the total DNA amount 500 ng per well. In some cases, different concentrations of a tested plasmid (20 – 450 ng) were transfected. Control transfection did not include an shRNA-expressing vector.

3.6.4.1 Plasmids used in this study

pGL2 Control Vector (Promega)

pGL4.10 (Promega, see appendix for map) with added promoter:

- pGL4.10_SV40: SV40 promoter was amplified from pRL-SV40 (Promega) and cloned between BglII and HindIII restriction sites

pRL_SV40 (Promega, see appendix for map) and its modified variants:

- pRL_SV40_mMos: mouse *Mos* cognate sequence was cloned into the XbaI site in the 3'UTR region of *Renilla* luciferase gene
- pRL_SV40_ inverted_mMos: mouse *Mos* cognate sequence was cloned into the XbaI site in an inverted orientation

pBluescript II KS (Stratagene, see appendix for plasmid map)

3.6.4.2 Luciferases activity measurement

Dual-Luciferase® Reporter Assay System (Promega) was used to measure luciferase activity. Cells were harvested 48 hours post-transfection. Cells were washed twice with 0.5 ml PBS. PBS was removed and 150 µl of Passive Lysis Buffer was added into each well. Plates were gently shaken for 20 minutes at room temperature. Lysed cells were collected into 1.5 ml microtubes and centrifuged for 1 minute and 14 000 rpm using Centrifuge 5424 (Eppendorf). A 10 µl aliquot of each lysate was pipetted to a 96-well plate. Luciferases activity was measured using a luminometer Modulus Microplate (Turner BioSystems), which injected 50 µl of each luciferase substrate to each sample during measurement. All reagents used were prepared according to the manufacturer's instructions (Promega).

3.6.5 Bradford protein assay

In some experiments, total protein amount in the lysate was used to normalize results from luciferase assay. Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) was used for protein measurement. Standards (Tab. 1) or 2 µl of cell lysate were pipetted into 96-well plate. 200 µl of dye reagent (0.2x) was added to each well. After incubation for at least 5 minutes (no more than 1 hour), the absorbance was measured at 595 nm.

Protein amount (µg) corresponding to 1 µl of sample									
4	*3*	*2*	*1.5*	*1*	*0.75*	*0.5*	*0.375*	*0.25*	*0.125*

Table 1: Standards used for Bradford protein assay

Range of standard protein (IgG) concentrations used to construct the calibration curve.

3.7 Transgenic mice

3.7.1 Vector preparation for transgenesis

To release the transgene from the TMP_ZP3_sh (see appendix for map) vector carrying the mouse *Mos*-targeting hairpin, the plasmid was cleaved with NotI and NdeI restriction enzymes. Fragments were separated by agarose gel electrophoresis. The functional cassette (~ 4.5 kbp long fragment) was excised and extracted from the gel by Gel Extraction Kit (Qiagen) and eluted to 500 µl H_2O. The cassette was cleaned twice using DNA Clean & Concentrator kit (Zymo Research). The cassette purity was verified by agarose gel electrophoresis and given to the transgenic facility of Institute of Molecular Genetics Academy of Science of the Czech Republic.

3.7.2 Oocyte microinjection and embryo transfer

Fertilized donor oocytes were obtained from super-ovulated 3-4 weeks old C57Bl/6N females (Charles Rivers Laboratories). Hormonal stimulation was carried out as follows: 5U of Pregnant Mare's Serum Gonadotropine (PMSG/Folligon; Intervet) was injected into peritoneum. Forty-five hours later, 5U of human Choriogonadotropine (HCG, Sigma) was injected into peritoneum and mice were mated with C57Bl/6N males. One day later, one-cell stage embryos were isolated from plugged females.

Pronuclear injection (PNI) of transgene DNA into male pronucleus was performed. Embryo transfer was performed either at one-cell stage directly after PNI or at the two-cell stage after an overnight culture. This was dependent on the amount of foster mice available on a specific day.

Pseudopregnant CD1 females were used as foster mothers. Females were paired with vasectomized CD1 males (for optimal stimulation of the female) a night before the transfer. Embryos were transferred into oviduct (15-25 embryos per recipient, into one or both oviducts). The embryo transfer was done under sterile conditions on laminar bench in SPF (specified pathogen free) area of animal house. CD1 mice were obtained from in-house breeding.

3.7.3 Genotyping

The biopsies were obtained from 3-4 weeks old mice. GFP expression was analyzed by fluorescent stereomicroscope SZX16 (Olympus). Subsequently, tails were dissolved in one of the following lysis buffers.

Buffer I:
100 mM TRIS-HCl, pH 8.5
5 mM EDTA
0.2% SDS
200 mM NaCl

Buffer II:
50 mM KCl
10 mM TRIS-HCl, pH 8.3
0.1 mg/ml gelatin
0.45% NP 40
0.45% Tween 20

0.2 ml of lysis buffer supplemented with proteinase K (50:1) was added to each tail. Tails were shaken (700 rpm) in the mixing block MB-102 (Bioer) at 55 °C until completely dissolved. Proteinase K was inactivated by incubation at 95 ºC for 10 minutes. When buffer I was used for the lysis, DNA was purified by the phenol-chloroform extraction (see 3.7.3.1 for details). Tails lysed in buffer II were used directly for PCR.

PCR amplification of Mos hairpin sequence using ZP3_end.fwd and LMP_Rev_Seq primers and Taq DNA polymerase was performed. PCR

conditions were as follows: 94 ºC for 5 minutes; 40 cycles of 94 ºC for 30 seconds, 57 ºC for 30 seconds, 72 ºC for 1 minute; 72 ºC for 10 minutes. PCR products were analyzed by electrophoresis using 1.5 % agarose gel.

3.7.3.1 Phenol-chloroform extraction

DNA from tails digested in lysis buffer I was subsequently purified by phenol-chloroform extraction. Samples were mixed with one sample volume of phenol solution equilibrated with 10 mM Tris HCl to pH 8.0 (Sigma Aldrich), mixed and centrifuged for 15 minutes at 12 000 rpm in centrifuge 5415R (Eppendorf) to separate the lower phenol and the upper water phase. The upper phase containing DNA was transferred and supplied with one volume of phenol and chloroform mixture (1:1) and mixed thoroughly. After centrifugation (15 minutes, 12000 rpm), the upper phase was transferred and mixed with one volume of chloroform and centrifuged as previously. DNA from the upper phase was precipitated by adding 0.1 volume 3 M sodium acetate and 3 volumes ice-cold 96% ethanol. The mixture was incubated at -20 °C for 10 minutes and then centrifuged as previously in a cooled centrifuge 5415R (Eppendorf). Supernatant was removed carefully and the pellet was washed by 1 ml of 70% ethanol. After centrifugation, the supernatant was removed and the pellet was dried and resuspended in 0.2 ml of water.

3.7.4 Primary tail fibroblasts culture

A small piece of mouse tail (about 0.5 cm) was minced by scalpel and resuspended in 10 ml of 1:1 mixture DMEM with PenStrep and collagenase (1 mg/ml in PBS). The suspension was shaken for 1 hour at 37 ºC. The supernatant (after tissue sedimentation) was transferred to a clean tube. The fresh mixture of DMEM and collagenase was added to the rest of the sample and incubated as previously. The supernatant was collected and mixed with the previously collected one. The rest of the sample was stirred in 6 ml DMEM supplemented with FBS and PenStrep and cultured in 6 cm Petri dish at 37 ºC and 5% CO_2. Collected supernatants were centrifuged for 5 minutes at 6000 rpm in the BR4i centrifuge (Jouan). The supernatant was discarded and the pellet was resuspended in 6 ml

DMEM supplemented with FBS and PenStrep and cultured in 6 cm Petri dish in 37 ºC and 5% CO_2.

3.7.4.1 Puromycine resistance testing

Primary tail fibroblasts from transgenic and wild type mice were cultured for at least five days. Then, medium was changed and puromycine was added to the final concentration 2.5 μg/ml. Cells were cultured at 37 ºC until the control cells (from wild-type mice) died.

3.7.5 Oocyte isolation

Eight weeks old mice were superovulated by intraperitoneal injection of 0.1 ml (5 units) of PMSG (Folligon; Intervet). Forty-four hours after the injection, ovaries were isolated and placed in 4 ml of M2 medium supplemented with 4 μg of isobutylmethylxanthine (IBMX, 200mM). They were mechanically disintegrated to release cumulus-enclosed oocytes. Cumulus cells were removed with a thin glass capillary. Oocytes with germinal vesicle (GV) were washed three-times in M2 medium (approximately 0.2 ml) and twice in PBS. Oocytes were used for RT-PCR analysis or for maturation to the MII phase (in this case, IBMX was omitted).

M2 medium (per 1L):

Component	g/l
NaCl	5.53193
KCl	0.35635
$CaCl_2.2H_2O$	0.25137
KH_2PO_4	0.162
$MgSO_4$	0.1649
$NaHCO_3$	0.35
HEPES	5.42726
Sodium lactate	3.31985
Sodium pyruvate	0.0363
D-Glucose	1
Albumin, Bovine Fraction V	4
Phenol Red	0.0106

3.7.5.1 Maturation to MII phase

A few drops (60 – 80 µl) of CZB medium supplemented with glutamine (5 µl of 3% glutamine per 1 ml CZB) were pipetted to a Petri dish. Drops were covered by paraffin oil to prevent evaporation. The dish was incubated at 37 ºC under 5% CO_2 overnight. Next day, 5 – 8 oocytes (GV) were put into each drop and incubated at 37 ºC and 5% CO_2 for 18 hours to mature to MII phase.

CZB medium:

Component	per 1l
bovine serum albumin (BSA)	5 g
$CaCl_2.2H_2O$	0.251 g
D-Glucose	1 g
KCl	0.36 g
KH_2PO_4	0.16 g
L-glutamine	0.146 g
$MgSO_4.7H_2O$	0.29 g
NaCl	4.77 g
$NaHCO_3$	2.11 g
sodium lactate	5.848 ml
sodium pyruvate	0.029 g
Penicillin G	100,000 U
phenol red	0.01 g
streptomycin sulfate	0.1 g

4 Results

The results are presented in four sections:

1. In the first section, I performed comparative study of various available systems mediating RNAi via short hairpin (shRNA) expression to confirm that vectors selected for our system construction (pTMP and pLMP) have a potential to down-regulate target gene efficiently.
2. In the second section, I made few changes in selected pTMP and pLMP vectors to make them compatible with oocyte-specific expression.
3. I the third section, I designed and selected the most efficient shRNA sequences targeting *Mos* gene. The best anti-*Mos* shRNA was cloned into prepared vector and used to make transgenic mice.
4. In the last section, obtained transgenic mice were bred and their phenotype was analyzed.

4.1 Vector selection

Various short hairpin RNA-expressing systems mediating RNAi have been described. We decided to base our system on pLMP and pTMP vectors.

pTMP and pLMP vectors express miRNA-like hairpin derived from endogenous miR-30 driven by tetracycline-regulated cytomegalovirus (CMV) promoter and viral long terminal repeat (LTR) promoter, respectively. Both vectors also express EGFP and puromycine resistance genes driven by PGK promoter.

We compared the functionality of these plasmids to other systems to assure the selected vectors are optimal for down-regulation target genes. Altogether, we compared six expression systems (Tab. 2), four of them expressing miRNA-like hairpins and two of them expressing class I hairpins. We also compared the efficacy of modified pLMP and pTMP vectors (containing loxP and NotI sites; described later in the chapter 4.2) with other systems.

Short hairpins targeting firefly luciferase were cloned into tested vectors. siRNA sequence was adopted from the literature (Elbashir, et al., 2001a).

Vector name	Hairpin type	Reference
pLMP	miRNA-like (class II)	Open Biosystems
pTMP	miRNA-like	Open Biosystems
pTRIPZ	miRNA-like	Open Biosystems
pUI2-GFP-SIBR	miRNA-like	(Chung, et al., 2006)
pSuper.retro.puro	shRNA (class I)	OligoEngine
pSuper2xTRE	shRNA	unpublished plasmid, a gift from J. Jaworski

Table 2: Vectors used for the comparative study

A list of available vectors used for shRNA expression, whose functionality was tested in our assays. See appendix for maps of these vectors.

For hairpin cloning to pTRIPZ, pLMP/pTMP vectors and their loxP variants we designed 97 nt long oligonucleotide FL_1 (for sequences see the chapter 3.2) containing miRNA-like hairpin sequence. Insert preparation and cloning procedure was performed as described in the manufacturer's manual (Open Biosystems, http://www.openbiosystems.com/collateral/rnai/pi/LMP-TMP%20vector%20product%20insert_Glycerol.pdf). Briefly, hairpin inserts were amplified by PCR using LMP_oligo.fwd and LMP_oligo.rev primers. PCR products and vectors were cleaved by EcoRI and XhoI restriction enzymes and ligated together.

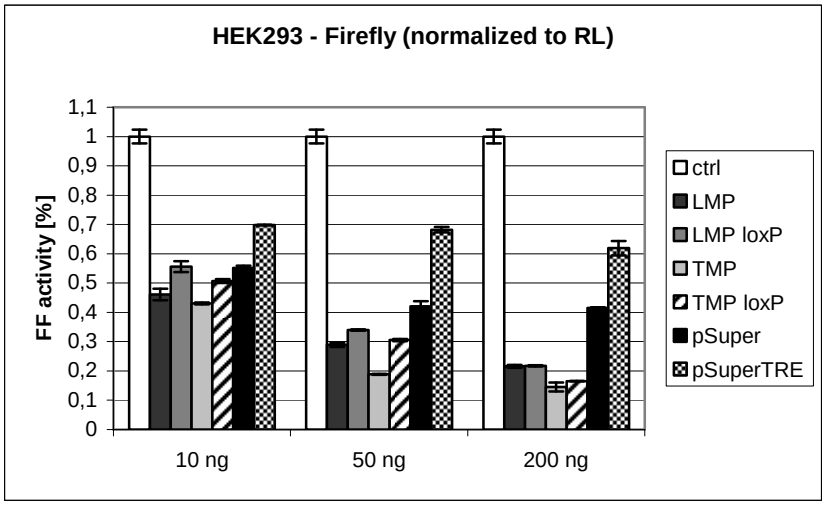
The insert for cloning into pSuper and pSuper2xTRE vectors was prepared by annealing oligonucleotides FL_2 and FL_3 (for sequences see the chapter 3.2). Annealed oligonucleotides were cloned into BglII and HindIII sites of target vector.

Hairpin cloning to the UI2-GFP-SIBR vector was performed similarly to the cloning into the pSuper vector. Annealed oligonucleotides FL_4 and FL_5 (for sequences see the chapter 3.2) were ligated with a BpiI-cleaved vector.

We compared the performance of pTMP, pLMP vectors and their loxP variants with pSuper.retro.puro and pSuper 2xTRE vectors using dual luciferase assay. The experiment was performed in HEK293 and HeLa cells. HeLa cells were transfected by 100 ng pGL2, 2 ng pRL_SV40, 10 or 50 or 200 ng tested plasmid. HEK293 cells were transfected by 100 ng pGL2, 1 ng pRL_SV40, 10 or 50 or 200 ng tested plasmid. pBluescript was added to all transfections to make total DNA amount up to 500 ng per well (if not otherwise noted, pBluescript was

used as a stuffer plasmid in all experiments). All transfections were performed in duplicates.

A)



B)

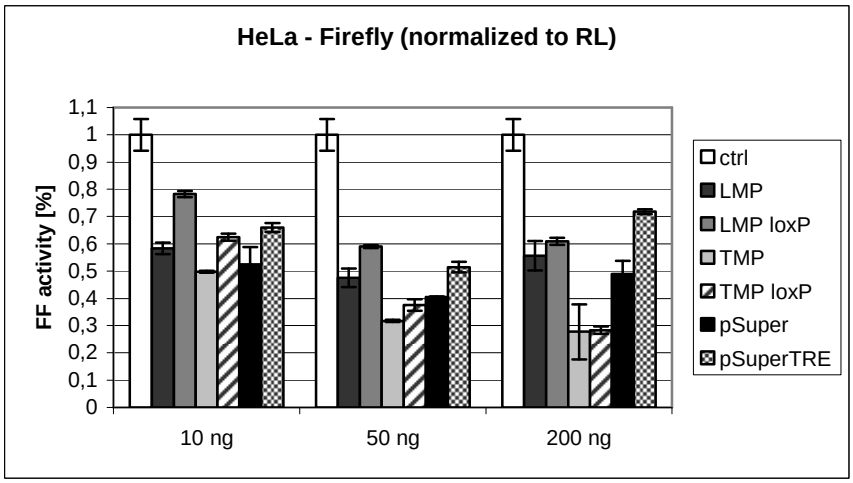


Figure 3: Plasmids efficacy test.

Different concentrations of tested shRNA-expressing vectors were co-transfected with reporter plasmids (pGL2) in (A) HEK293 and (B) HeLa cells. Luciferase activity was measured by dual luciferase assay. Measured values were normalized to protein content measured by Bradford method. Firefly luciferase activity was normalized to control *Renilla* luciferase (pRL_SV40 plasmid). Firefly luciferase activity in control sample (omitting shRNA-expressing plasmid) was set to 100%. Data are expressed as an average +/- SEM (standard error of the mean). All tested samples were significantly down-regulated compared to the control (ANOVA; $p < 0.001$).

In HEK293 cells, all tested vectors significantly down-regulated reporter activity; pTMP and pLMP vectors performed better compared to pSuper.retro.puro

and pSuper2xTRE plasmids. The effect of all plasmids in HEK293 cells was concentration-dependent (Fig. 3 A). The loxP variants performed similarly as original pTMP/pLMP vectors (but variants of both vectors - 10 ng and pTMP variants - 50 ng and significantly differed, ANOVA, $p < 0.01$). In HeLa cells, the effect of all plasmids was similar but less pronounced compared to HEK293 cells. because However, the highest concentration of shRNA-expressing plasmid did not show the highest level of down-regulation suggesting that saturation effect may exist at concentrations above 50 ng in HeLa cells (Fig. 3 B).

After cloning appropriate UI2_GFP_SIBR and pTRIPZ derivates, we repeated the experiment with all vectors (Fig. 4). In contrast to the previous experiment, only single concentration of shRNA-expressing plasmids was used. HEK293 and HeLa cells were transfected by 100 ng of pGL2, 1 ng of pRL_SV40 and 50 ng of tested vectors. Transfections were performed in quadruplicates. Variants of tested vectors expressing shRNA targeting an irrelevant gene were used as negative controls to verify the specificity of firefly luciferase down-regulation (Fig. 4 B, D).

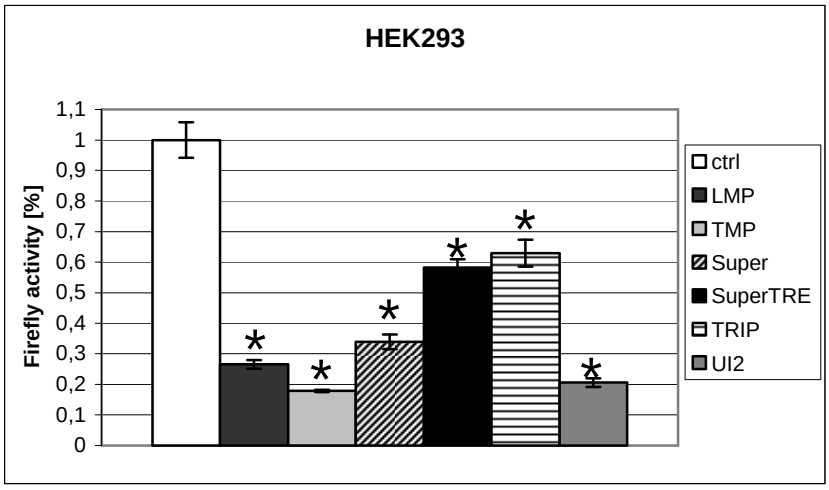
In HEK293 cells, all tested vectors specifically down-regulated luciferase reporter activity (Fig. 4 A). UI2_GFP_SIBR plasmid was similarly potent as pTMP/pLMP vectors, down-regulating the activity by ~ 80%. pTRIPZ and pSuper2xTRE plasmids performed worse, down-regulating the activity only by ~ 40%. (Fig. 4 A). All tested plasmids, except the pSuper2xTRE, significantly down-regulated luciferase activity in absence of anti-firefly hairpin that reflects non-specific effects (Fig. 4 B). The extent of non-specific action of pTRIPZ vector was similar to its “specific” down-regulation, suggesting its malfunction. The non-specific effects of other plasmids were acceptable compared to the level of their specific down-regulation (Fig.4 A, B). In HeLa cells, the effect of all plasmids was similar but less pronounced compared to HEK293 cells. pTRIPZ vector did not down-regulate reporter activity (Fig. 4 C). No plasmids showed any non-specific effect in HeLa cells (Fig. 4 D).

We observed cell line-dependent effects of shRNA-expressing plasmids. The level of down-regulation was higher in HEK293 compared to HeLa cells. Less efficient transfection of HeLa cells (assayed by flow cytometry after EGFP transfection; unpublished results) could explain observed differences in down-regulation efficiency. However, absolute values of control *Renilla* luciferase

activity were higher in HeLa cells (data not shown) suggesting that promoter activity is cell-line dependent and may influence the outcome of luciferase assay. The cell line-differences may be influenced by amount of shRNA expressed in individual cell lines. However, the amount of expressed shRNAs was not directly evaluated.

Together, loxP variants of pTMP/pLMP vectors selected for generation of our new system worked satisfyingly and are suitable for anti-*Mos* shRNA cloning and transgenesis.

A)



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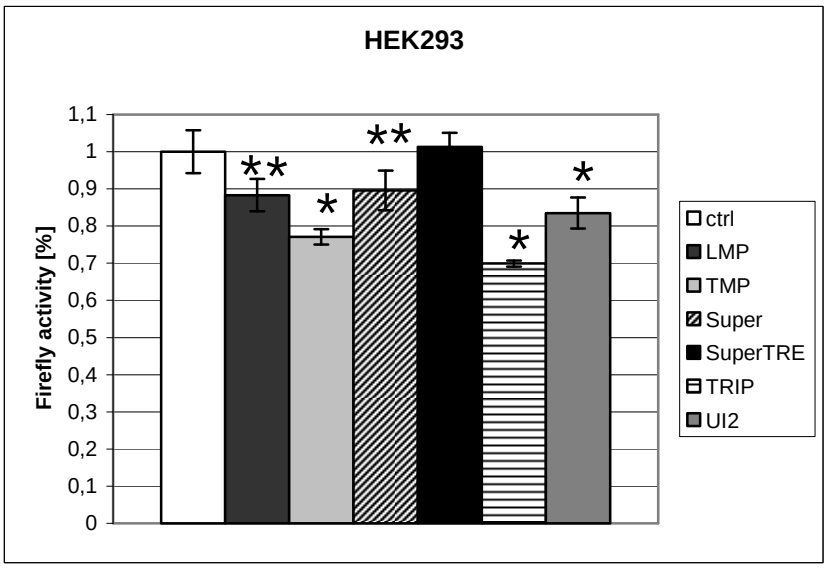
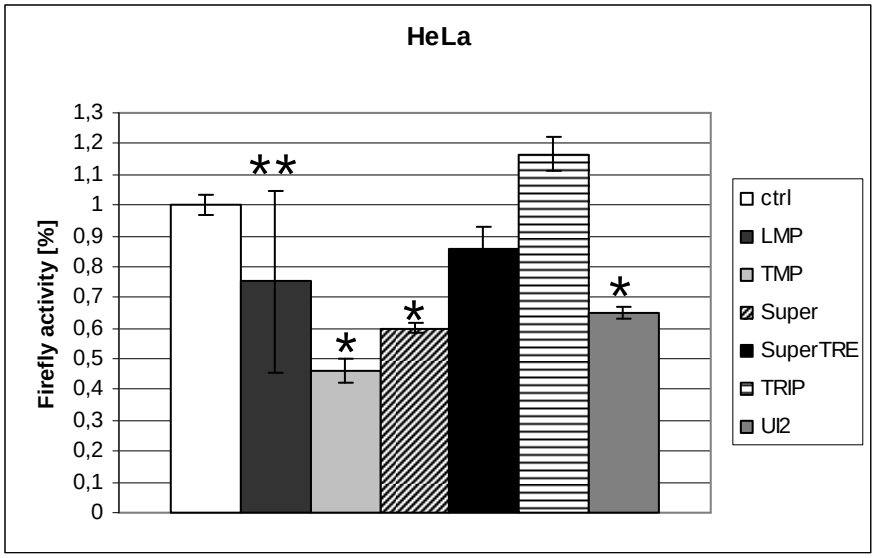


C)



D)

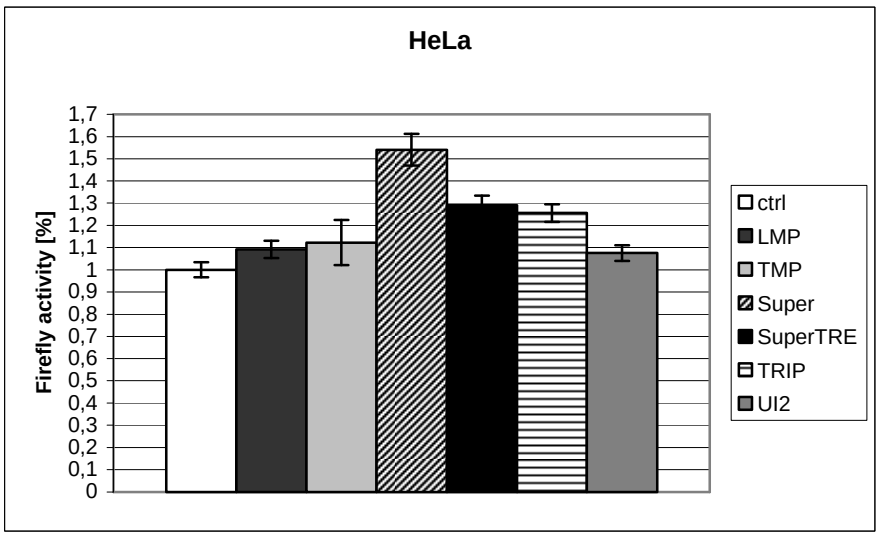


Figure 4: Luciferase assay comparing functionality of short hairpin-coding plasmids

50 ng of tested anti-firefly shRNA-expressing vectors was co-transfected with reporter plasmid (pGL2) in (A) HEK293 and (C) HeLa cells to test vectors efficacy. The same amount of tested vectors expressing an irrelevant shRNA was co-transfected with reporter plasmid (pGL2) in (B) HEK293 and (D) HeLa cells to test vectors specificity. Luciferase activity was measured by dual luciferase assay. Measured values were normalized to protein content in particular samples measured by Bradford method. Firefly luciferase activity was normalized to control *Renilla* luciferase (pRL_SV40 plasmid). Firefly luciferase activity in control sample (omitting shRNA-expressing plasmid) was set to 100%. Data are expressed as an average +/- SEM (standard error of mean). * $p < 0.001$;** $p<0.05$ (ANOVA)

4.2 Construction and testing of miRNA-like hairpin expressing vector

Retroviral vectors pTMP and pLMP (Open Biosystems; see appendix for plasmid maps) were used for construction of our oocyte-specific shRNA-expressing system.

We made the following changes in pLMP and pTMP vectors (Fig. 5). We inserted sequences containing NotI restriction site and loxP site behind the 5´UTR and behind EGFP gene. All four oligonucleotides used for the construction (5´loxP.fwd, 5´loxP.rev, 3´loxP.fwd and 3´loxP.rev; for sequences see the chapter 3.2) were phosphorylated and annealed. The 5´loxP insert was cloned into BglII site behind the 5´UTR and the 3´loxP insert was cloned into SalI site behind the EGFP gene. Only one BglII site downstream the 5´loxP insert was kept. This site was subsequently used for ZP3 promoter insertion.

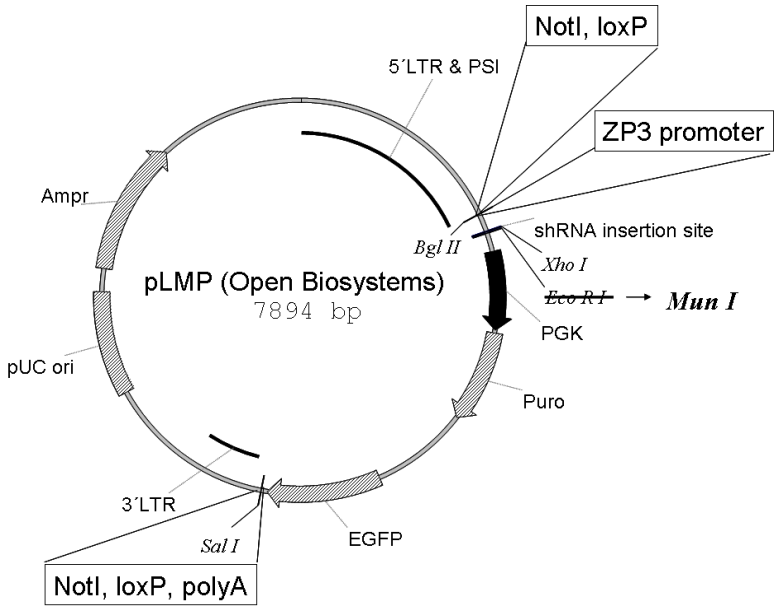


Figure 5: Construction of pLMP_loxP vector

Schematic representation of changes made in the parental pLMP plasmid. NotI and loxP sites were inserted behind the 5´LTR and before the 3´LTR to flank functional cassette expressing shRNA and marker genes. EcoRI restriction site present within shRNA insertion site in the original plasmid was mutated to MunI site. Identical changes were performed in pTMP plasmid simultaneously (new system was constructed from functional transgenic cassette from pLMP and retroviral sequences from pTMP, which is self-inactivated vector. Oocyte-specific ZP3 promoter was inserted between loxP and MunI sites in pLMP to get pLMP_ZP3_sh. The NotI-flanked cassette was transferred to pTMP_loxP to get pTMP_ZP3_sh.

NotI sites allow for releasing the functional transgenic cassette for microinjection in a simple digest. LoxP sites allow for site-specific Cre-mediated recombination. Cre-loxP recombination provides an alternative way of transgenesis, such as recombination into loxP sites integrated in the genome of specific embryonic stem lines. Cre-loxP recombination may be also used for the phenotype rescue to reveal unspecific phenotypes. If phenotype remains unchanged after recombination (only loxP sites retained in the genome after recombination), an observed phenotype likely arises from the disruption of a gene, in which the transgene has been inserted, not by the transgene itself.

The functionality of loxP sites was tested in bacteria expressing the Cre recombinase. A plasmid part flanked by loxP sites was efficiently excised during Cre-mediated recombination and bacteria would produce shorter plasmid. Both plasmids pLMP_loxP and pTMP_loxP were chemically transformed into the Cre recombinase-expressing *E. coli* strain (obtained from Sylvia Arber, FMI, Basel).

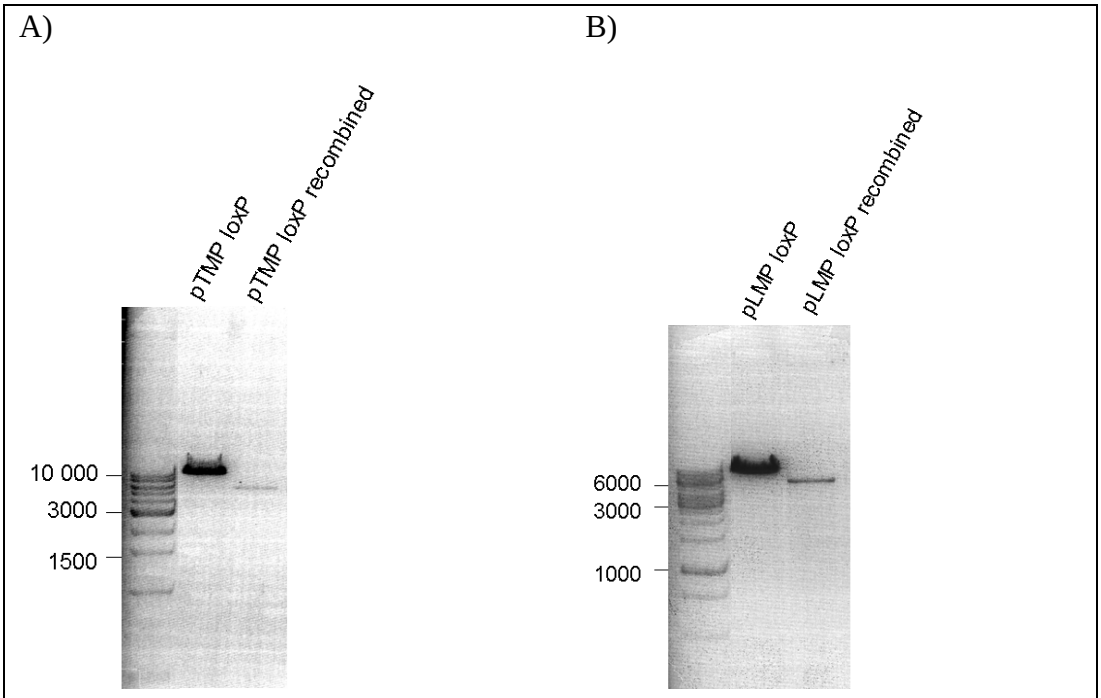


Figure 6: Test of LoxP sites functionality

pTMP_loxP (panel A) and pLMP_loxP (panel B) plasmids were transfected to Cre recombinase-expressing bacterial strain. Plasmids were isolated and linearized by restriction digestion. Sizes of linearized original and recombined plasmids were compared using agarose gel electrophoresis.

Recombined plasmids were linearized by HindIII cleavage and original plasmids were linearized by EcoRI cleavage. The size of original and recombined plasmids was compared on agarose gel electrophoresis. pLMP and pTMP recombined plasmids were about 3 kbp and 3.5 kbp in length shorter compared to parental plasmids, respectively. Plasmid size reduction confirmed the functionality of inserted loxP sites (Fig. 6).

To achieve oocyte-specific expression of shRNA, ZP3 promoter was inserted into the pLMP_loxP vector. The ZP3 promoter could guarantee robust oocyte-specific shRNA expression, which may be sufficient to effectively down-regulate *Mos* mRNA. Unfortunately, ZP3 promoter contains several EcoRI sites, which are used for shRNA insertion in the original pTMP and pLMP vectors. As the shRNA insertion site has to be unique, we replaced EcoRI site with MunI, which generates overhangs compatible with EcoRI and it allows for cloning the same oligonucleotides designed for original pLMP/pTMP vectors. Primers LMP_MunI.fwd and LMP_MunI.rev (for sequences see the chapter 3.2) were used for mutagenesis.

We inserted ZP3 promoter into pLMP_loxP_MunI to get pLMP_ZP3_sh. Sequence of ZP3 promoter was amplified from a ZP3-containing plasmid used in a previous transgenic RNAi experiment (Stein, et al., 2003) by PCR using primers ZP3_BglII_Fwd and ZP3_BglII_Rev (for sequences see the chapter 3.2). The PCR product and the pLMP_loxP_MunI vector were cleaved by BglII and the plasmid was dephosphorylated. Plasmid and amplified ZP3 promoter were ligated together. Insertion was verified by restriction analysis using BamHI and XhoI. To avoid mutations in the ZP3 promoter, pLMP_ZP3_sh clones were sequenced using LMP_Rev_Seq and LMP_Fwd_Seq primers (for sequences see the chapter 3.2).

Direct insertion of ZP3 promoter into pTMP_loxP_MunI instead of TRE-CMV promoter would be complicated due to absence of unique restriction site immediately behind the TRE-CMV promoter. Thus, the functional ZP3 cassette from pLMP_ZP3_sh was transferred into pTMP_loxP_MunI using NotI digestion to get pTMP_ZP3_sh. pTMP_ZP3_sh plasmid was constructed for the case of using retroviral transduction for transgene delivery. In contrast to pTMP, pLMP is not self-inactivating (SIN) retroviral vector and strong promoter present in the 5´LTR region could influence shRNA expression. Construction of pTMP_ZP3_sh plasmid will guarantee that only ZP3 promoter will drive the hairpin expression.

The final pTMP_ZP3_sh construct was used for cloning functional *Mos*-targeting shRNA and transgenesis.

4.3 Mos siRNA sequence selection

Mos gene was successfully down-regulated by transgenic RNAi mediated by long RNA hairpin. *Mos* down-regulation resulted in parthenogenetic activation of oocytes (Stein, et al., 2003). The absence of MOS leads to disruption of the second meiotic block and parthenogenetic activation of oocyte. The second polar body is extruded and mouse is infertile. We decided to test our pTMP_ZP3_sh system on the *Mos* gene, because the characteristic phenotype observed long hairpin application allows comparing the effects of long and short hairpin-mediated RNAi in oocytes.

Eight various miRNA-like shRNA sequences targeting mouse *Mos* (NM_020021.2) were designed (Tab. 3). Mos_1 and Mos_2 hairpin sequences originally designed for pTER plasmid (J. Filkowski, unpublished results) were extended to miRNA-like shRNAs using RNAi Oligo Retriever (http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA). Other six Mos hairpin sequences were designed using RNAi Codex database (Olson, et al., 2006), BIOPREDsi (Huesken, et al., 2005), RNAxs (Tafer, et al., 2008) and RNAi Oligo Retriever prediction algorithms. Different algorithms predict different siRNA sequences. There is higher probability that a siRNA will be efficient when predicted by more algorithms. In any case, reliability of prediction algorithms is not absolute and all selected siRNAs have to be experimentally tested to reveal the most efficient one.

Hairpin	Sequence
Mos 1	CA**CGACAACATAGTTCGGGTT**G
Mos 2	CC**CGAAGACTCCAACAGCCTA**G
Mos A	CGGAGATCCTGAAAGGAGAGAT
Mos B	TCCATAGACTGGGAACAGGTAT
Mos C	GAGGGTTTGGCTCGGTGTATAA
Mos D	TCCGGAGATCCTGAAAGGAGAG
Mos E	CTCGGTGTATAAAGCCACTTAC
Mos F	AGGGTTTGGCTCGGTGTATAAA

Table 3: Selected shRNA targeting mouse *Mos* mRNA
Mos target sequences of selected short hairpins are shown. Bold letters indicate originally designed 19 bp long sequences (J. Filkowski, unpublished results).

We used corresponding siRNA sequences to design 97 nt long oligonucleotides Mos_1, Mos_2, Mos_A-F as is described in pLMP/pTMP manual (Open Biosystems, http://www.openbiosystems.com/collateral/rnai/pi/LMP-TMP%20vector%20product%20insert_Glycerol.pdf). Inserts were prepared by PCR using LMP_oligo.fwd and LMP_oligo.rev primers (for sequences see the chapter 3.2) adopted from the manufacturer's manual. PCR product was cleaned using DNA Clean & Concentrator kit (Zymo Research), cleaved by EcoRI and XhoI restriction enzymes, and cloned to pLMP_loxP and pTMP_loxP plasmids. Hairpins effects were tested in HeLa, HEK293 and NIH3T3 cells. The final version of developed vector, pTMP_ZP3_sh, was not used for the test, because the oocyte-specific ZP3 promoter is not active in somatic cell lines. The efficacy of each Mos shRNA was measured using dual luciferase assay.

In the first experiment, the efficacy of Mos_1 and Mos_2 hairpins was tested. HEK293 cells were transfected with 100 ng of pGL4.10_SV40, 50 ng of pRL_SV40_mMos and 20, 50 or 150 ng of hairpin-expressing pLMP_loxP or pTMP_loxP plasmids. In contrast to other experiments, Nanofectin reagent (PPA) was used for transfections (150 μl Nanofectin solution per sample). Transfection was performed in triplicates.

Only pTMP_LoxP_Mos_1 and pLMP_LoxP_Mos_2 at the concentration 20 ng per well significantly down-regulated reporter activity by ~ 50 - 60 % (Fig. 7). Remaining vectors did not work at any concentration tested. Higher plasmid concentration should result in increased reporter activity down-regulation. In this experiment, increasing plasmid concentration showed no tendency to enhance down-regulation of reporter activity suggesting that tested hairpins were non-functional. These results were rather surprising since originally constructed pTER_Mos_1/2 plasmids had been functional in previous experiments (J. Filkowski, unpublished results).

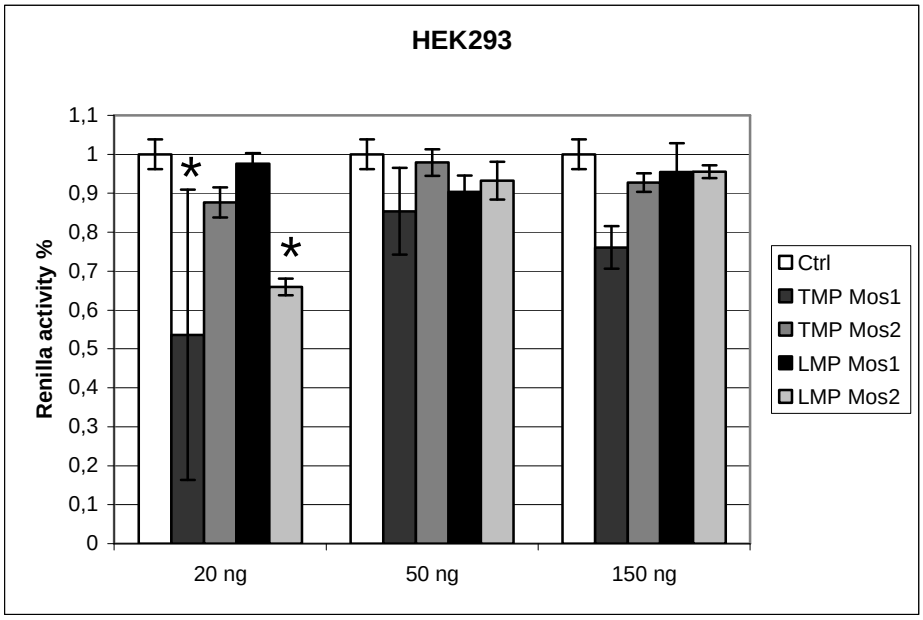


Figure 7: Test of Mos_1 and Mos_2 shRNA hairpins

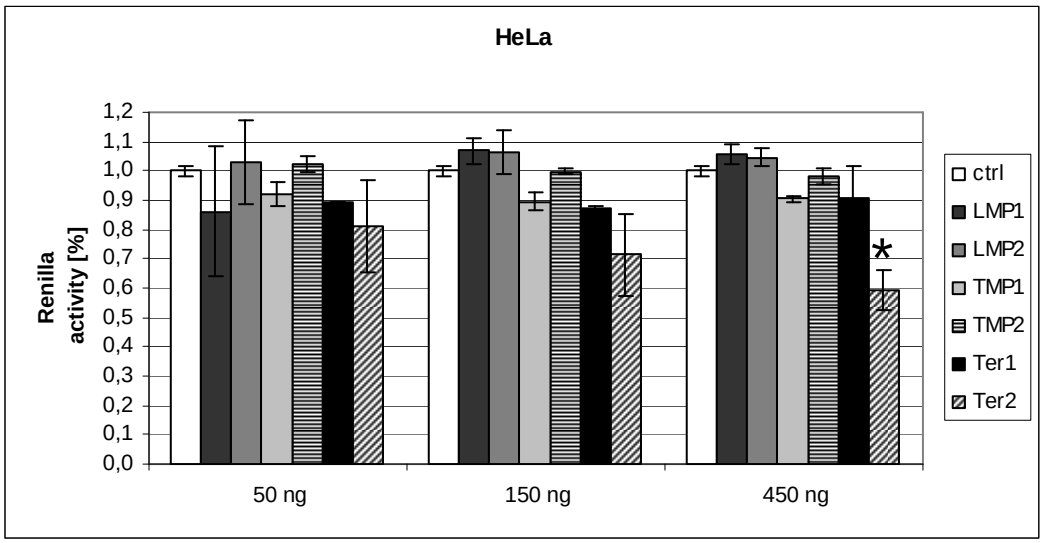
Different concentrations of tested shRNA-expressing vectors were co-transfected with reporter plasmid (pRL_SV40_mMos) in HEK293 cells. Luciferase activity was measured by dual luciferase assay. *Renilla* luciferase activity was normalized to control firefly luciferase (pGL4.10_SV40 plasmid). *Renilla* luciferase activity in control sample (omitting shRNA-expressing plasmid) was set to 100%. Data are expressed as an average +/- SEM (standard error of mean). * $p < 0.05$ (ANOVA).

Subsequently, we compared pLMP/pTMP_loxP_Mos_1/2 plasmids direct with originally constructed pTER_Mos_1/2 plasmids in a single experiment. This test was performed in HEK293 and HeLa cells. Cells were transfected with 150 ng of pGL4.10_SV40, 20 ng of pRL_SV40_mMos and 50, 150 or 450 ng of Mos_1/2-expressing pLMP/pTMP_loxP or pTER plasmids. Transfections were performed in duplicates.

Results from HeLa cells suggested that only pTER_Mos_2 down-regulated *Renilla* luciferase activity by ~ 20 – 40 % in a concentration-dependent manner. However, the reduction of reporter activity was statistically significant only at the highest pTer_Mos_2 concentration. Remaining vectors did not work at any concentration tested (Fig. 8 A).

Similar results were obtained from HEK293 cells (Fig. 8 B). The apparent down-regulation (by ~ 40-50%) present in all samples is probably caused by an erroneous measurement of luciferase activity in control samples (Fig.8 B; note high SEM values). In HEK293 cells, only pTer_Mos_2 significantly down-regulated reporter activity at all concentrations tested (ANOVA; $p < 0.05$).

A)



B)

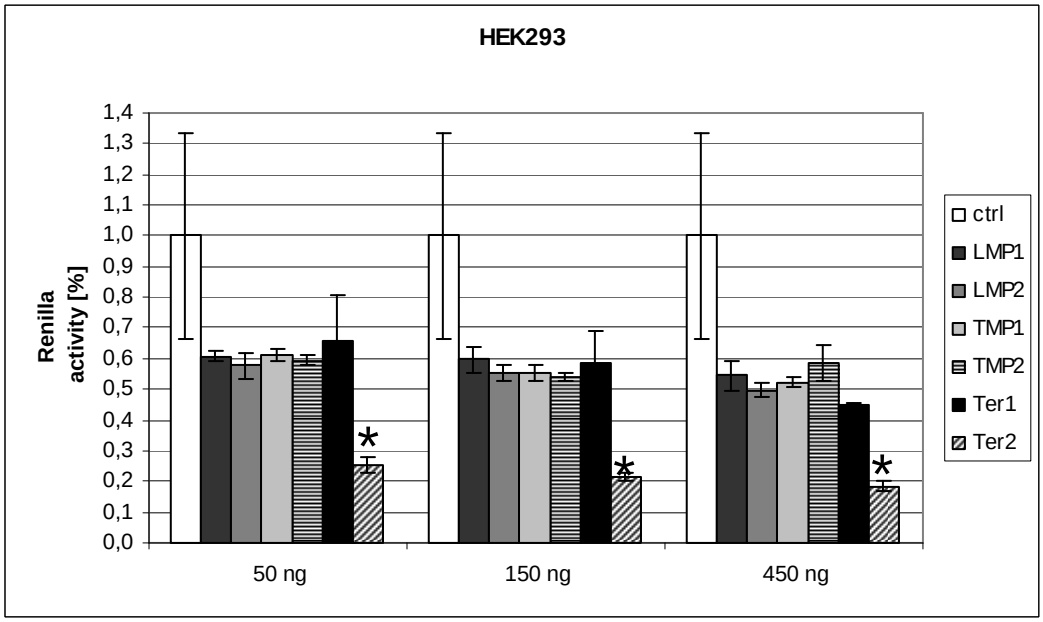


Figure 8: Comparison of Mos_1 and Mos_2 hairpins expressed from pLMP/pTMP_loxP and pTER plasmids.

HeLa (A) and HEK293 (B) cells were co-transfected with different concentrations of tested shRNA-expressing vectors and reporter plasmids (pRL_SV40_mMos, pGL4.10_SV40). Luciferase activity was measured by dual luciferase assay. *Renilla* luciferase activity was normalized to control firefly luciferase. Normalized *Renilla* luciferase activity in control sample (without shRNA-expressing plasmid) was set to 100%. Data are expressed as an average +/- SEM. * $p < 0.05$ (ANOVA).

Based on these results, we decided not to use Mos_1 and Mos_2 shRNA hairpins for transgenesis and decided to design and test additional hairpins, Mos_A–F.

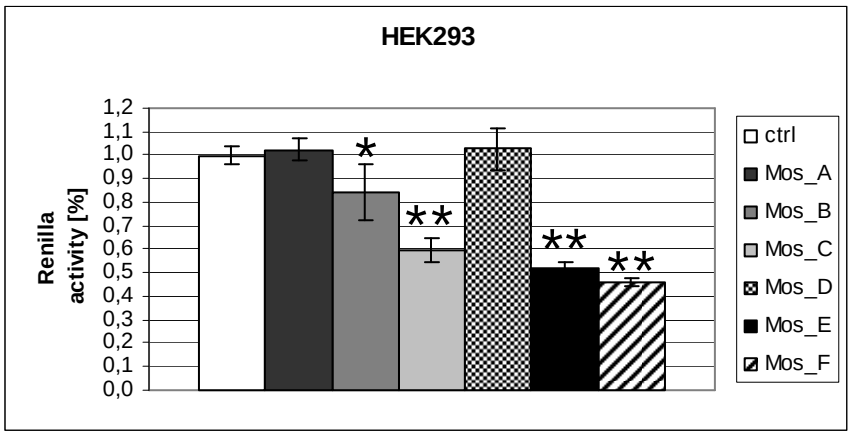
Cells were transfected with pRL_SV40_mMos to elicit hairpin efficacy as in the previous experiment. Moreover, we also performed sense strand loading test using plasmid expressing *Renilla* luciferase tagged with an inverted cognate *Mos* mRNA sequence (pRL_SV40_inverted_mMos). The RISC complex should be preferentially loaded by the anti-sense strand of shRNA depending on the shRNA thermostability. shRNA sequences are designed to be less stable at their 5´end of anti-sense strand to support its preferential loading. Potentially loaded sense strand, which has no specific target in the cell, could bind to many partially homologous mRNAs and down-regulate their expression eventually leading to an unspecific phenotype. Control pRL_SV40_inverted_mMos plasmid should detect loading of sense strand on the RISC complex.

Cells were transfected with 50 ng LMP loxP expressing hairpins Mos_A–F, 50 ng pGL4.10_SV40 and 1 ng pRL_SV40_mMos (or pRL_SV40_inverted_mMos). Transfection was performed in quadruplicates. Efficacy test was performed using HEK293, HeLa and NIH3T3 cells. Sense strand loading test was performed using HEK293 and NIH3T3 cells.

Hairpins Mos_A and D did not down-regulate reporter activity in any cell lines used (Fig. 9). Hairpins Mos_C, E and F significantly down-regulated *Renilla* expression by ~ 40-60 % in HEK293 and NIH3T3 cells (Fig. 9 A, C). In HeLa cells, only Mos_F hairpin down-regulated *Renilla* activity by more than 40 % (Fig. 9 B). Mos_B hairpin significantly down-regulated reporter expression in all cell lines but only by approximately 20 % (Fig. 9).

No hairpin affected reporter *Renilla* activity in the sense strand loading test (Fig. 10) suggesting that all selected hairpins targeted specifically Mos mRNA and shRNA sense strand was not loaded on the RISC complex.

A)



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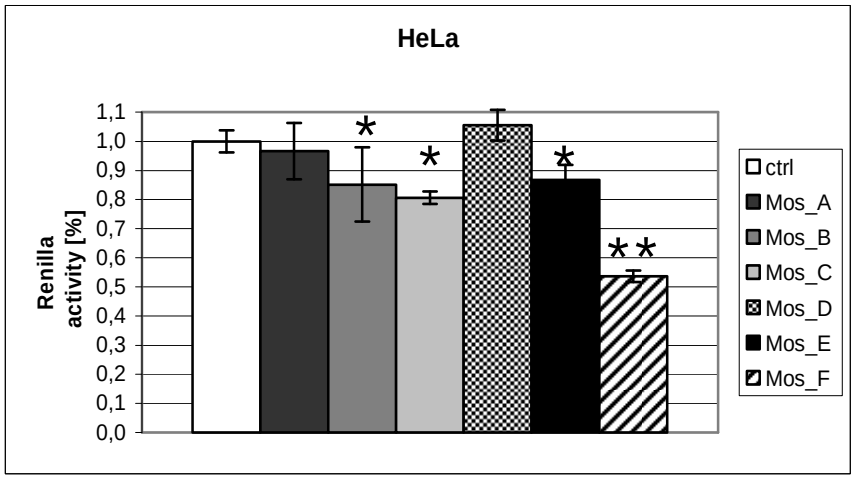


C)

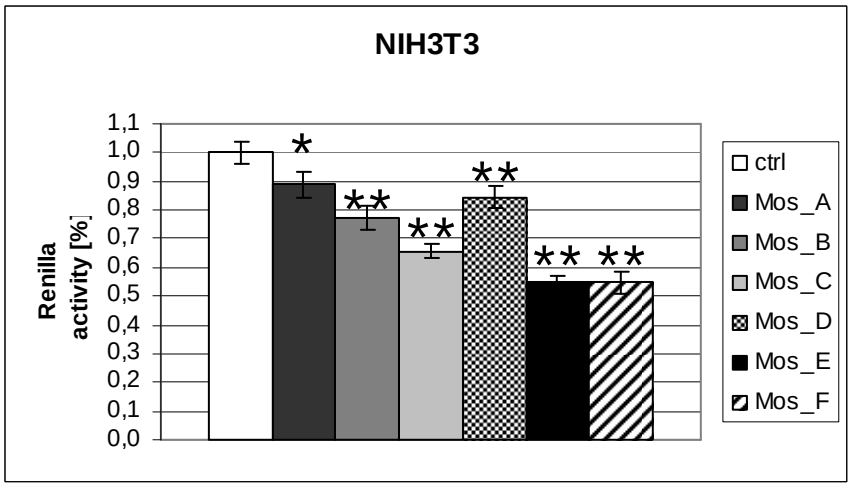
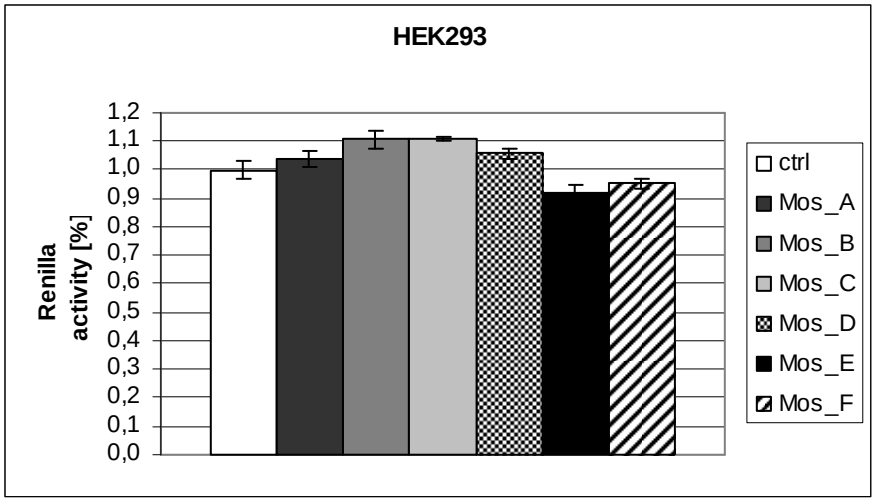


Figure 9: Mos hairpins efficacy test.

HEK293 (A), HeLa (B) and NIH3T3 (C) cells were transfected with Mos hairpin-expressing plasmid, firefly luciferase coding plasmid and Mos-tagged *Renilla* luciferase plasmid. Activity of both luciferases was measured by dual luciferase assay. *Renilla* luciferase activity was normalized to control firefly luciferase. Normalized *Renilla* luciferase activity in control sample (without shRNA-expressing plasmid) was set to 100%. Data are expressed as an average +/- SEM. * $p<0.001$; ** $p<0.05$ (ANOVA)

A)



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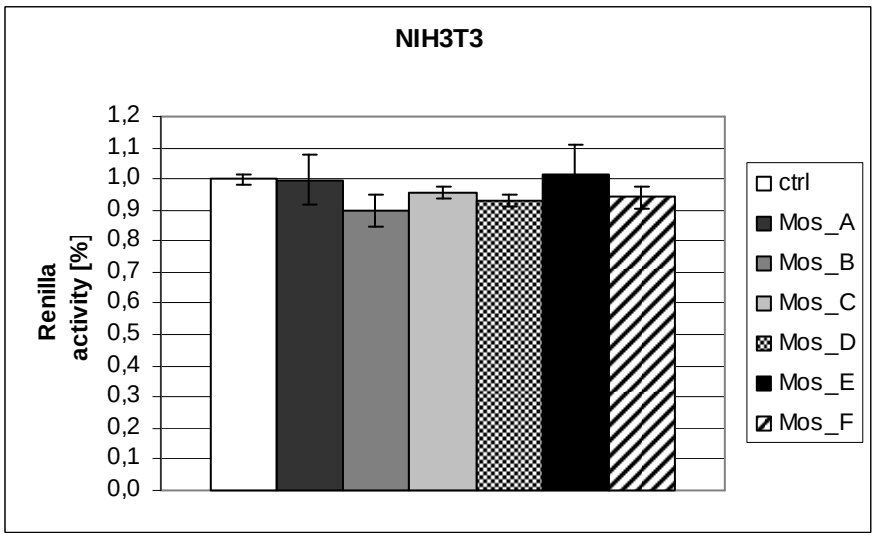

Figure 10: Mos hairpins sense strand loading test

Plasmids coding shRNA, firefly luciferase and *Renilla* luciferase tagged with inverted Mos sequence were co-transfected into HEK293 (A) and NIH3T3 (B) cells. Activity of luciferases was measured by dual luciferase assay. *Renilla* luciferase activity was normalized to control firefly luciferase. Normalized *Renilla* luciferase activity in control sample (without shRNA-expressing plasmid) was set to 100%. Data are expressed as an average +/- SEM.

Finally, we tested the concentration dependency of shRNA-expressing vector. NIH3T3 mouse fibroblasts were transfected with 50 ng, 150 ng or 450 ng LMP_loxP_Mos_A, B, C, E or F (non-functional Mos_D was omitted), 50 ng pGL4.10_SV40 and 1 ng pRL_SV40_mMos. Transfection was performed in triplicates.

Observed shRNA-mediated down-regulation of reporter *Renilla* plasmid was slightly concentration-dependent up to 150 ng of shRNA-expressing plasmids

(Fig. 11). However, significant non-specific down-regulation of control firefly luciferase activity was observed at higher concentrations used (data not shown). Our unpublished data suggested that global transfection efficacy decreases when growing amount of a large plasmid (such as pLMP_loxP) is used in transfections. Nevertheless, the data confirmed that the Mos_F and Mos_C were the most functional hairpins.

We decided to use the hairpin Mos_F (it appeared to be more functional in previous experiment - Fig. 9) for further cloning into the pTMP_ZP3_sh vector and making transgenic animals.

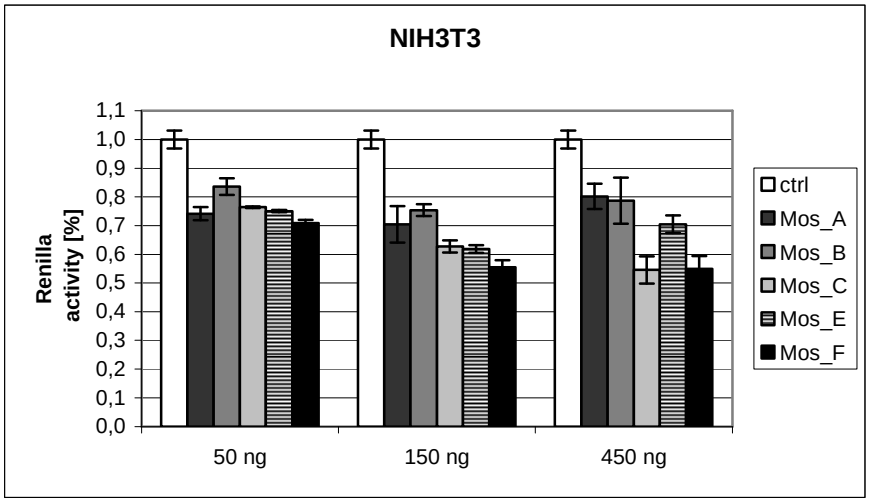

Figure 11: Concentration dependency test of Mos-targeting shRNAs

NIH3T3 fibroblasts were transfected with three concentrations of shRNA-expressing plasmid and stable amount of luciferase reporter plasmids. Activity of *Renilla* and firefly luciferase was measured by dual luciferase assay. *Renilla* luciferase activity was normalized to control firefly luciferase. Normalized *Renilla* luciferase activity in control sample (without shRNA-expressing plasmid) was set to 100%. Data are expressed as an average +/- SEM. All values were significantly lower compared to the control (ANOVA; $p<0.05$).

4.4 Transgenic mice analysis

Mos_F hairpin was cloned into the pTMP_ZP3_sh plasmid. Functional shRNA-expressing cassette, released by NotI digestion, was given to the transgenic facility, where it was microinjected into male pronucleus. One- or two-cell stage embryos were transferred to the oviducts of stimulated foster mothers.

4.4.1 Genotyping

Overall, we obtained 56 mice. EGFP expression in mouse tails was screened under the microscope. EGFP expression should be apparent in all tissues of transgenic mice, because EGFP expression is under the control of PGK promoter in contrast to shRNA expression, which is driven by oocyte-specific ZP3 promoter. However, no mouse had EGFP-positive tail.

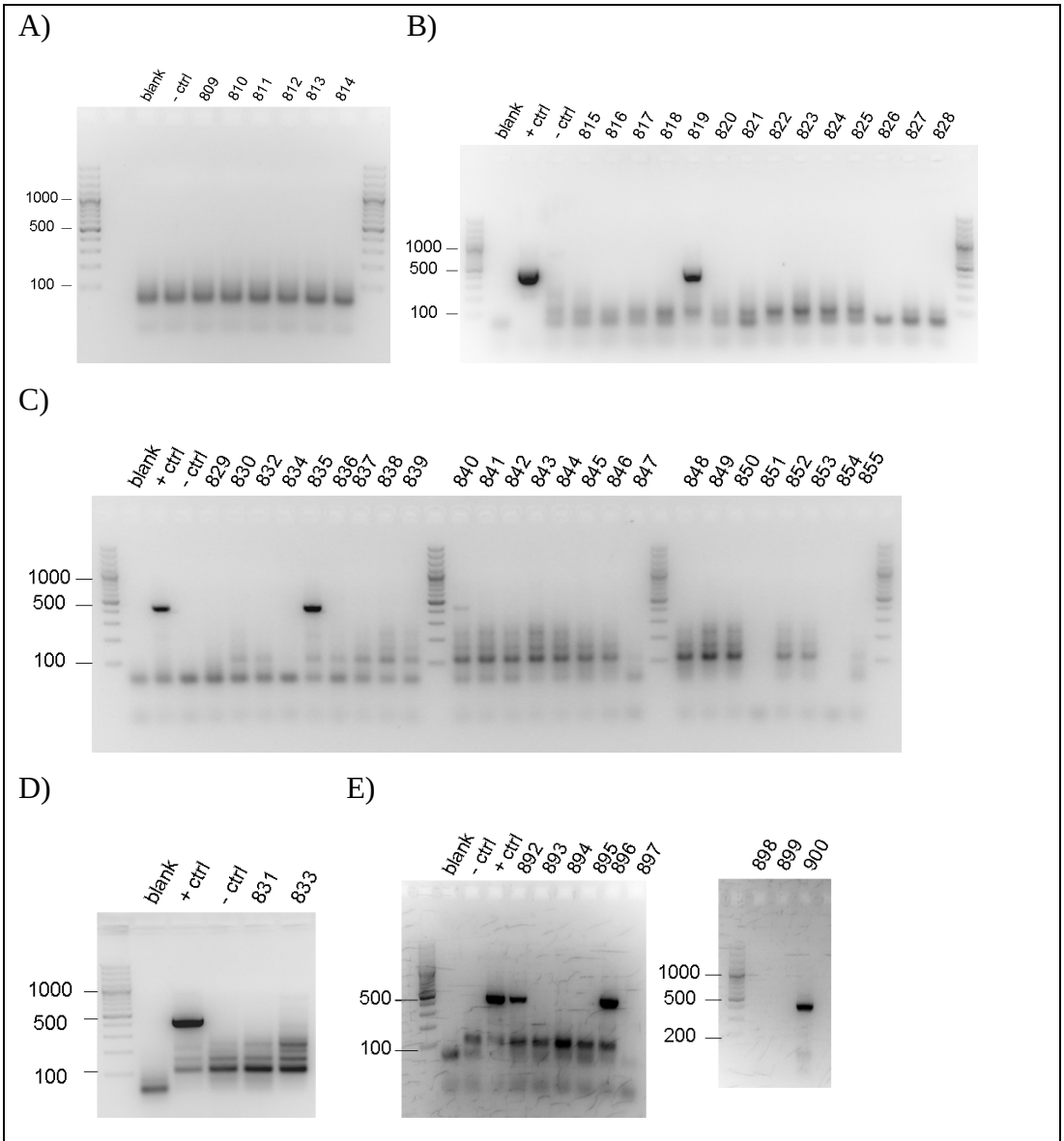

Figure 12: Mouse tail genotyping

Mice were genotyped by PCR using primers specific for inserted DNA. Five rounds of genotyping were performed (A-E) as tail biopsies were obtained from the mouse facility. Specific product is ~ 500 nt long. GeneRuler 100bp DNA Ladder (Fermentas) was used as a standard for size determination.

Then, tails were lysed and genotyping by PCR was performed using transgene-specific LMP_Rev_Seq and ZP3_end.fwd primers (for sequences see the chapter 3.2). Six transgenic mice were detected (Fig. 12). Four males and one female were strongly PCR-positive, the second female was weakly positive.

4.4.2 Analysis of transgenic markers

There are several possible reasons, why tails of transgenic mice were EGFP-negative. A transgene may be inserted in a transcriptionaly inactive region or silenced as a parasitic sequence. Other reason may be an altered EGFP expression. EGFP is expressed as the second gene together with puromycin resistance in a bi-cistronic transcription unit driven by the PGK promoter. If IRES or PGK promoter were functionally altered, EGFP would not be expressed. Hairpin expression is driven by an independent ZP3 promoter so the EGFP absence does not automatically mean that the shRNA hairpin is not expressed.

To confirm silencing of transgene, primary tail fibroblasts were isolated from transgenic mice and treated with puromycine. Simultaneously, primary cultures were prepared from wild type mice as controls. No puromycine-resistant fibroblasts were obtained neither from transgenic nor from wild-type animals. These results suggest that at least the expression of marker genes is disrupted. However, the expression status of shRNA should be determined directly only in oocytes, because shRNA is not expressed in any other tissues.

4.4.3 Mice breeding

Transgenic mice were interbred with wild type C57Bl/6NCrl mice. We expected transgenic females to be infertile (in the case of complete *Mos* down-regulation) or have decreased fertility (in the case of partial *Mos* disruption). Moreover, both transgenic females we obtained gave birth as frequently as wild type females. Number of pups (about 8) in litters was also comparable to wild type females.

4.4.4 Oocyte analysis

Oocytes were isolated from one transgene-positive and one transgene-negative female offspring of transgenic male No. 819. The *Mos* phenotype and *Mos* mRNA expression levels were analyzed in isolated oocytes.

Analysis of offspring of other transgenic mice is in progress.

4.4.4.1 Oocyte maturation

Several isolated oocytes (in GV phase) were moved to CZB medium supplemented with glutamine and let mature to elicit whether fraction of transgenic oocytes undertake parthenogenetic activation. Moreover, oocytes from transgenic as well as wild-type animals matured only to MII phase suggesting that the second meiotic block was maintained and the *Mos* gene is functional in transgenic animals.

4.4.4.2 qPCR

Hypothesis that *Mos* is partially down-regulated but not enough to cause the null phenotype was tested by qPCR. *Mos* mRNA expression levels were analyzed in individual oocytes isolated from transgenic and control mice. Four transgenic and seven wild-type oocytes were used for single cell RT-PCR. Reverse transcriptase was omitted in one sample from each group. This “minus RT” (-RT) control allowed to distinguish PCR fragment amplified from cDNA and genomic DNA. In the case of *Mos* analysis, the (-RT) control is necessary, because *Mos* is an intron-less gene and genomic DNA is amplified together with cDNA. Rabbit β-globin mRNA was added into each sample and amplified as an external standard.

Results showed non-significant difference in *Mos* mRNA expression from transgenic oocytes compared to control ones (relative expression = 0.59; $p = 0.305$) suggesting that the integrated transgene was non-functional, probably due to complete silencing of its expression.

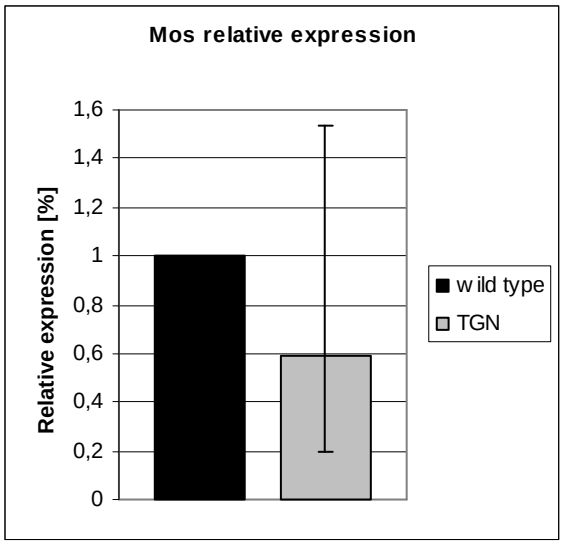

Figure 13: *Mos* relative expression in transgenic oocytes

Mos mRNA amount was analyzed by single-cell qPCR. Rabbit β-globin was used as an external standard. Data indicate relative *Mos* expression in transgenic oocytes compared to wild-type oocytes. Results were analyzed using the REST 2008 software.

5 Discussion

Up to now, only systems expressing long hairpin RNAs have been used for transgenic RNAi experiments in mouse oocytes (Coumoul, et al., 2005; Stein, et al., 2003; Yu, et al., 2004). Time-consuming preparation of long hairpin RNA-expressing vectors is the main disadvantage of this system. We have prepared pTMP_ZP3_sh vector mediating transgenic RNAi in oocytes via the expression of miRNA-like short hairpin RNAs that should overcome the disadvantages of long hairpin-expressing system.

pTMP_ZP3_sh vector is derived from commercial pTMP and pLMP vectors. Vector efficacy may be influenced by several factors as hairpin type and its sequence, promoter type or target cell type used. Our comparative study of different shRNA-expressing systems suggests that majority of plasmids expressing the class II hairpins (miRNA-like hairpins) work better compared to the class I hairpin-expressing plasmids. Original pLMP, pTMP vectors and their loxP variants constructed during this thesis significantly down-regulated target gene expression in transient transfection assays. These results strongly supported the usage of miRNA-like hairpin for development of our oocytes-specific expression system.

The final version of our expression vector, pTMP_ZP3_sh, could not be directly tested in cell culture because of strong oocyte-specific activity of ZP3 promoter. However, ZP3 promoter was successfully used in several studies for RNAi in oocytes and guarantees sufficient gene expression in transgenic animals (Stein, et al., 2003; Yu, et al., 2004).

The advantage of pTMP_ZP3_sh vector is that two markers, EGFP and puromycine resistance, are expressed in all tissues providing easy recognition of transgenic mouse (EGFP positivity), while the hairpin is expressed only in oocytes. In addition, this vector can be delivered into oocytes or other cells by three different approaches: (a) via microinjection of linearized cassette into the zygote or oocytes, (b) via Cre-loxP recombination into stem cells or (c) via retroviral transduction. LoxP sites contained in pTMP_ZP3_sh vector provide an option to perform a rescue experiment, which could reveal potential false positive phenotype arising from a disruption of any gene caused by transgene insertion.

We tested pTMP_ZP3_sh vector by targeting *Mos* dormant maternal mRNA, which was commonly studied using RNAi. We selected and tested several short hairpins targeting *Mos* and the best one, Mos_F, was used to establish transgenic mouse line. We obtained fifty-six mice but only six of them carried integrated transgene. Unfortunately, none of them expressed EGFP in somatic cells. Moreover, primary fibroblasts isolated from transgenic mouse tail were not resistant to puromycine suggesting that the whole transgene was silenced. While marker genes expression is driven by the PGK promoter, hairpin expression in driven by the strong oocyte-specific ZP3 promoter, which is not active in somatic tissue. Thus, the possible functional expression of shRNA in oocytes cannot be excluded solely based on these results.

Previous experiments revealed that mouse females with down-regulated *Mos* were infertile (Stein, et al., 2003). Both transgenic females we obtained gave birth to pups indicating that the transgene is non-functional. It was still possible that the hairpin is expressed in oocytes, but the expression level is insufficient to cause *Mos* null phenotype. However, there was no significant difference in *Mos* mRNA amount in transgenic and wild type oocytes. Together, these results suggested that the transgene was completely silenced. Although the transgene is under the control of a strong promoter, the local environment at the integration site can significantly influence the transgene expression (reviewed in Matthaei, 2007). However, it is unlikely that all six animals we have obtained would have suffered from such inactivation.

Linear pieces of DNA, similar as pTMP_ZP3_sh fragment used in our study, used for transgenesis integrate into genome randomly and preferentially in tandems, not as a single copy (reviewed in Matthaei, 2007). This possible unfavorable effect would be eliminated in transgenesis using viral transduction, which provides a single copy integration. The vector used in our study is of retroviral origin and thus lacks any introns. On the other hand, mammalian genome is rich in intronic sequences and intron-less regions are frequently recognized as parasitic sequence, which may be silenced by epigenetic mechanisms (reviewed in Matzke, et al., 2000). Up to now, viral transduction of our vector has not been performed and is a possibility for future studies.

In any case, all performed experiments suggest that our new expression system is not mediating efficient transgenic RNAi in mouse oocytes. Whether

vector sequence itself or other events (such as mouse strain used for transgenesis) are a reason of transgene failure will be further analyzed.

Our work also suggests that the construction of short hairpin-expressing vector is not advantageous compared to the preparation of long hairpin plasmids. Short shRNA cloning is easier, but the proper selection of efficient shRNAs is time-consuming and costly. Not all designed short hairpins are really functional *in vitro* and *in vivo* and it is necessary to design more short hairpins and to test their efficiency throughoutly. On the other hand, the long hairpin is usually functional regardless of the sequence (Coumoul, et al., 2005; Stein, et al., 2003; Yu, et al., 2004). Moreover, even the most rigorously selected short hairpin RNA shows off-targeting effects while long hairpin is practically free of non-specific action in oocytes (Stein, et al., 2005).

We conclude that long hairpin-expressing vectors remain the most suitable tool for RNA interference-based functional studies in oocytes. Short hairpin-expressing systems are alternative approaches when the long-hairpin expression cannot be used.

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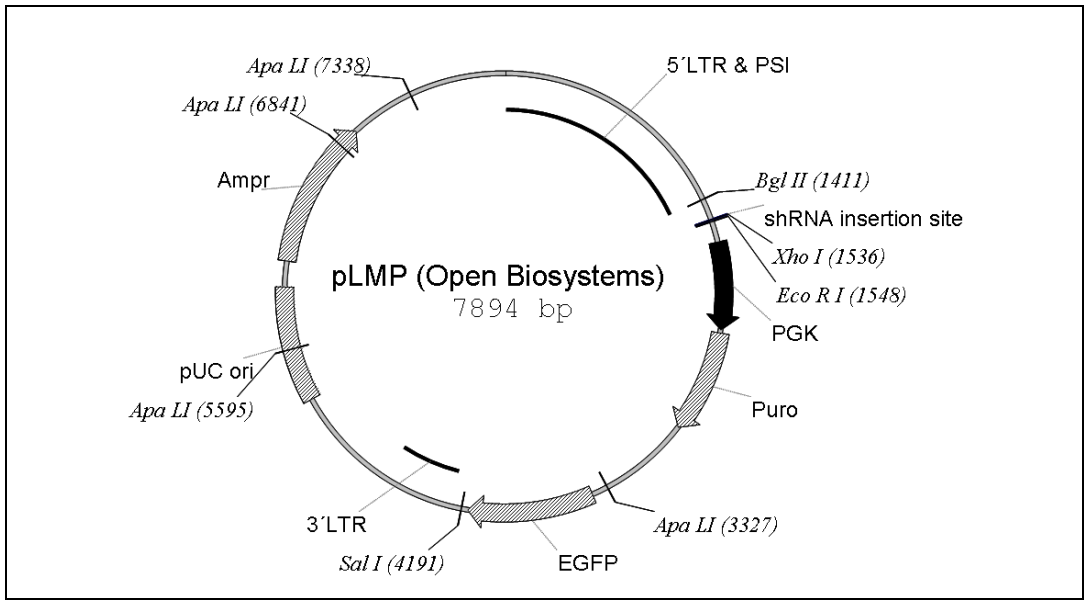
7 Appendix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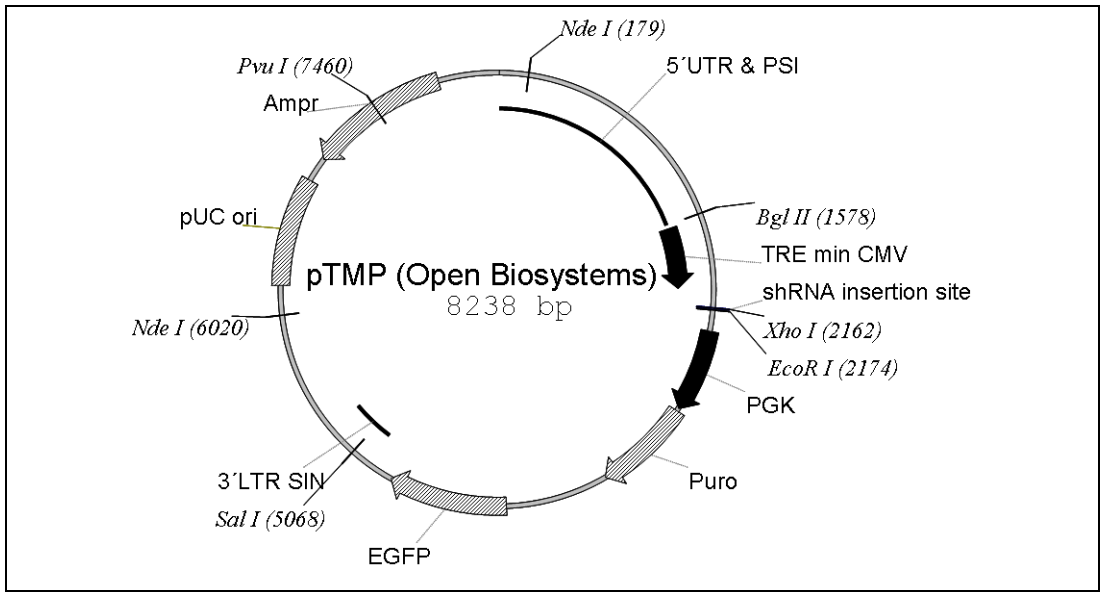
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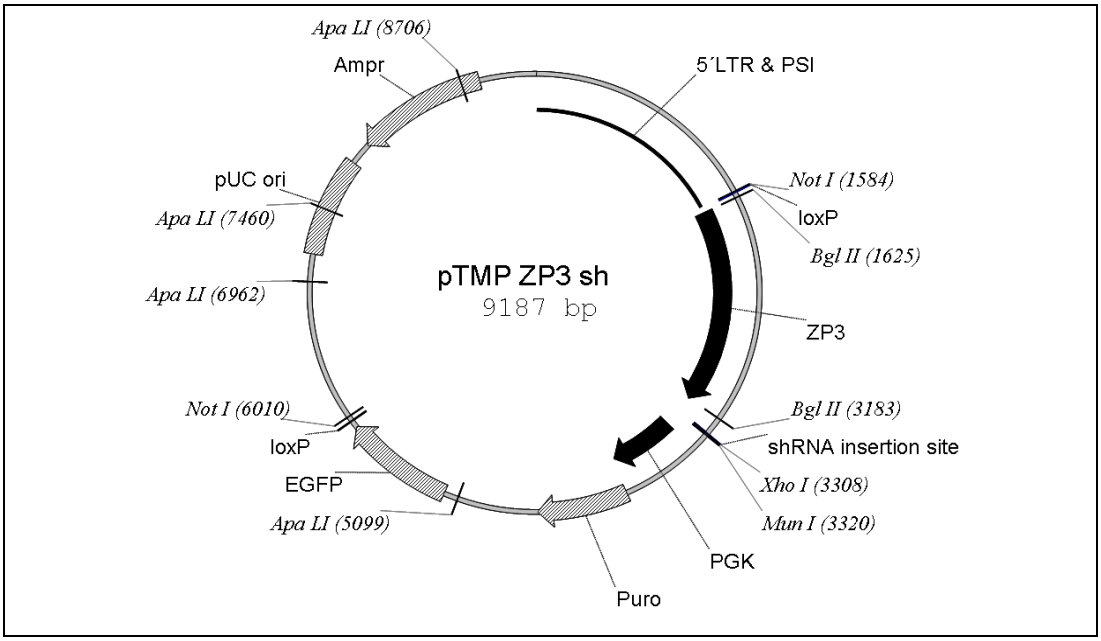
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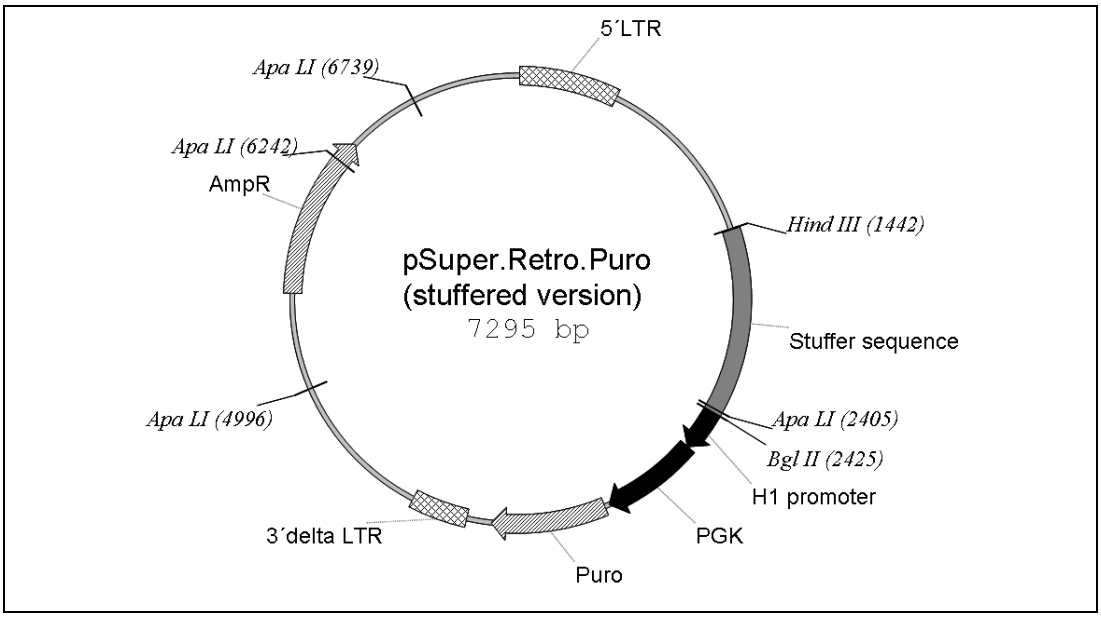
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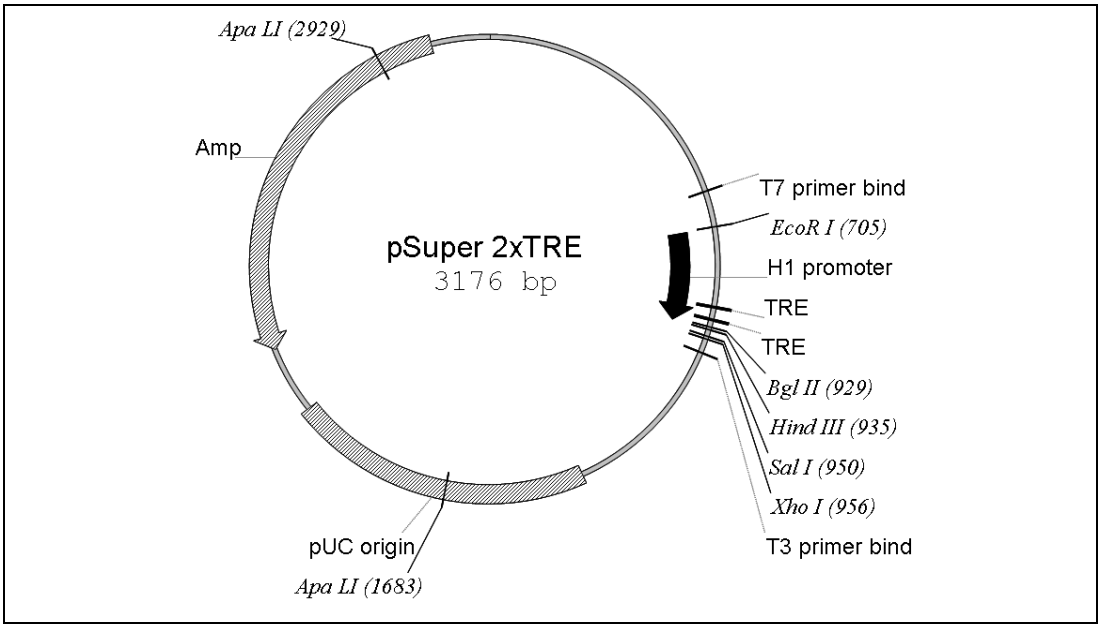
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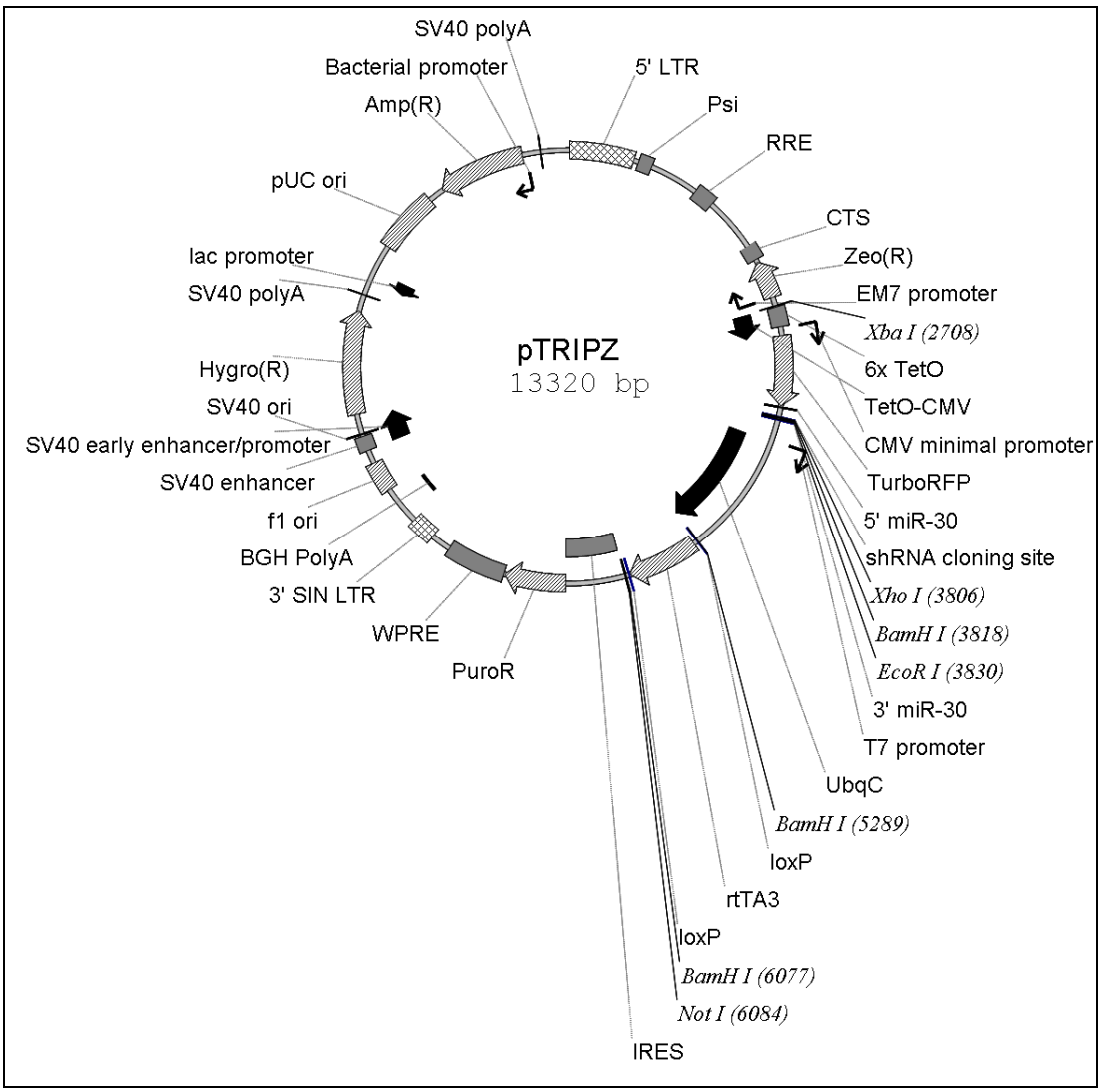
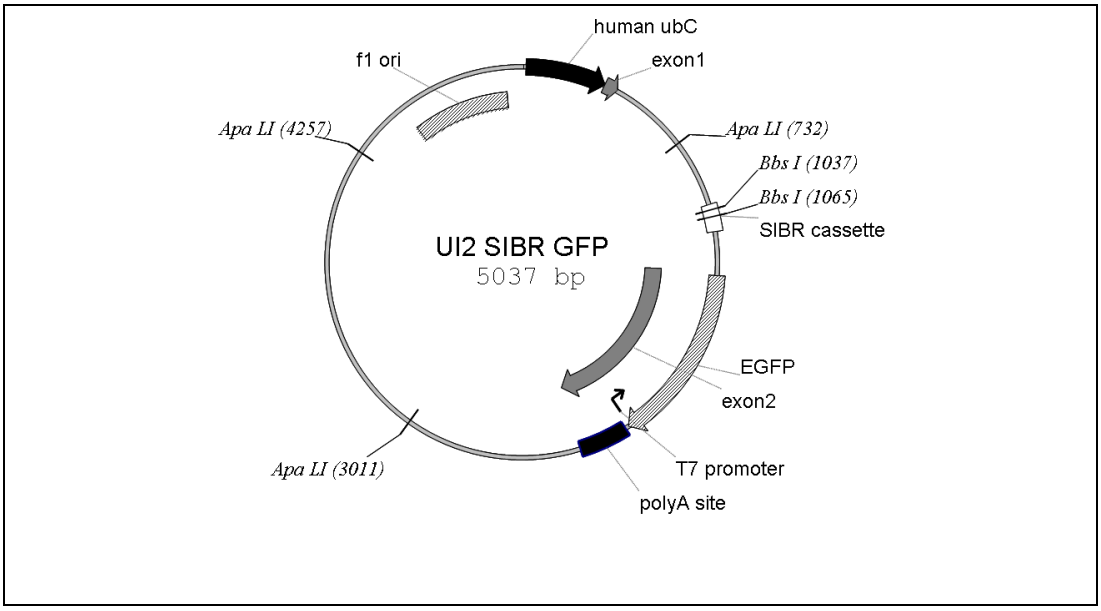
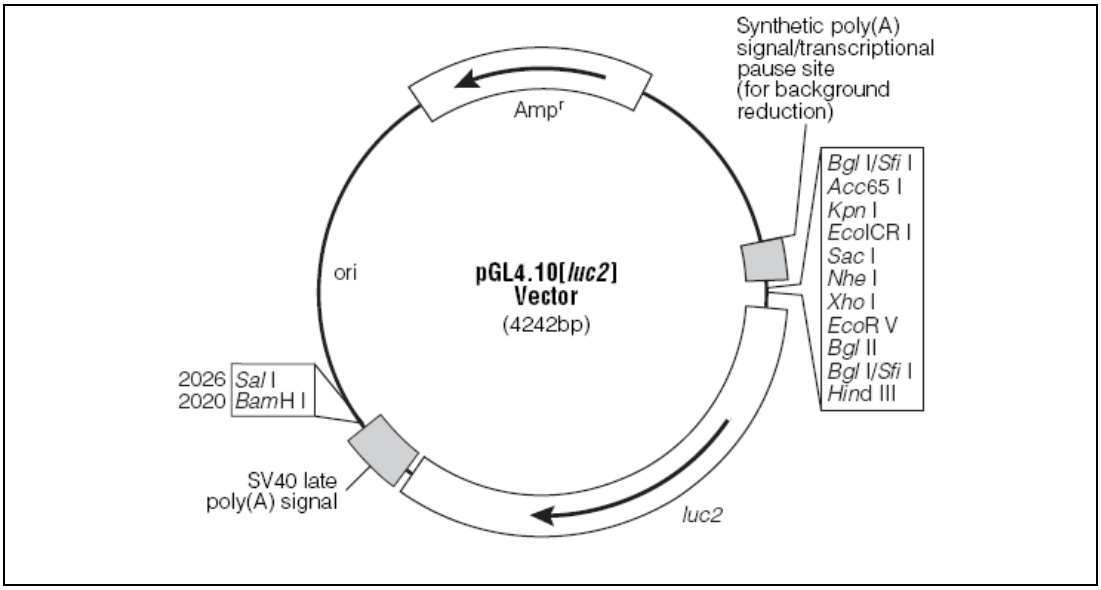
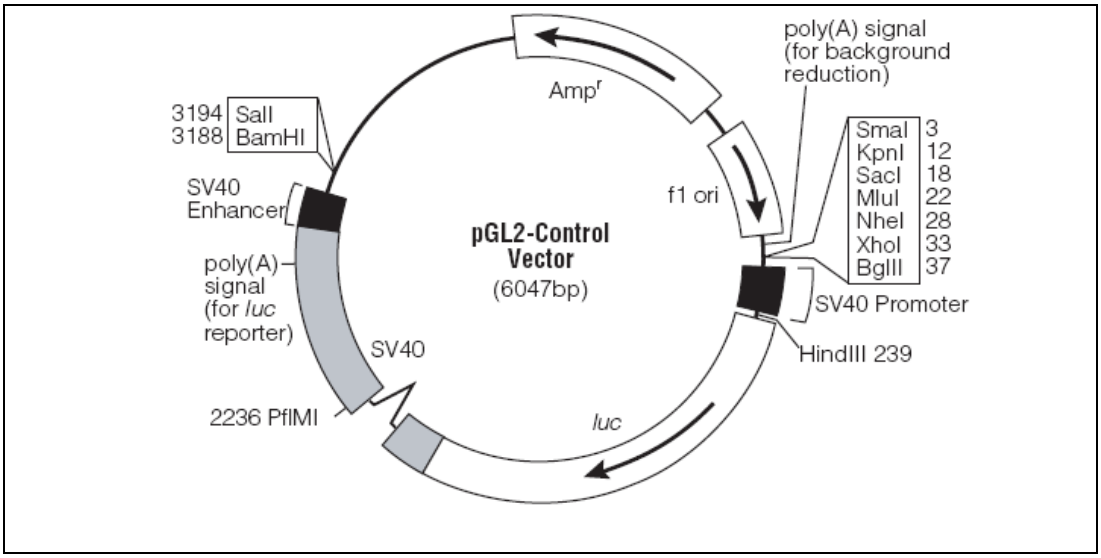
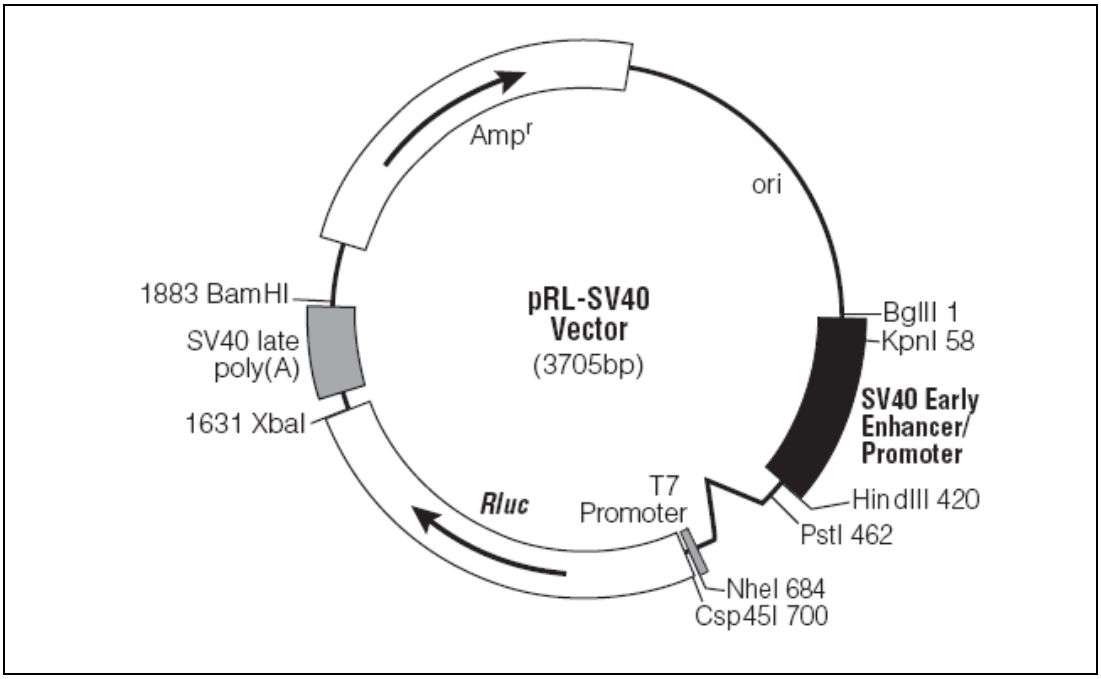
pUC origin
Apa LI (1683)

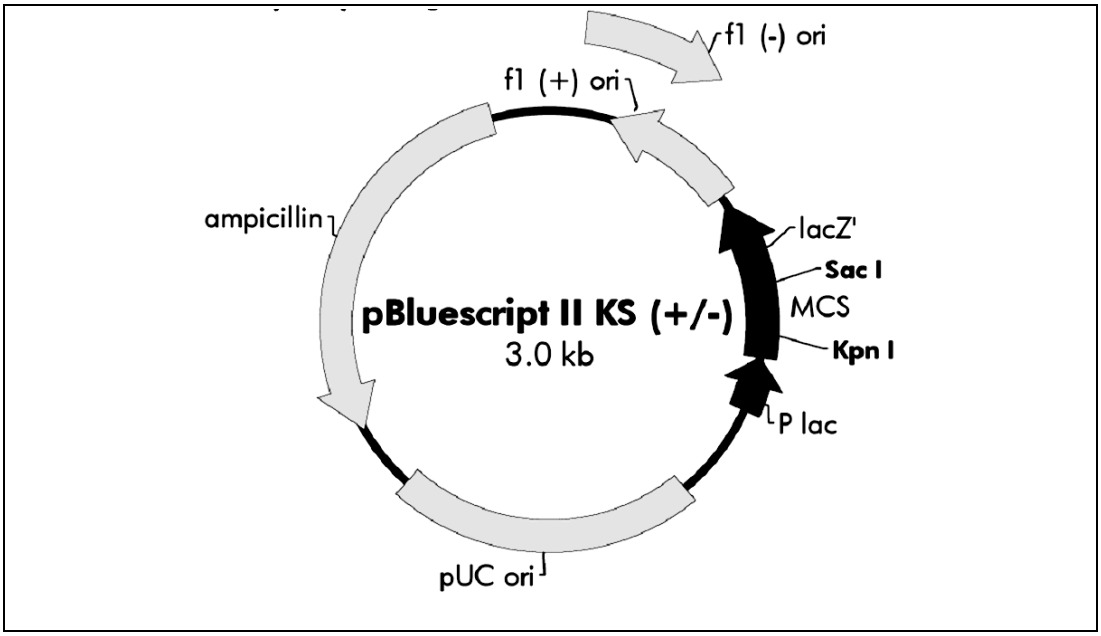

SV40 polyA
Bacterial promoter
5' LTR
Amp(R)
Psi
RRE
pUC ori
CTS
Zeo(R)
lac promoter
SV40 polyA
EM7 promoter
Xba I (2708)
pTRIPZ
13320 bp
Hygro(R)
6x TetO
SV40 ori
TetO-CMV
SV40 early enhancer/promoter
CMV minimal promoter
SV40 enhancer
TurboRFP
f1 ori
5' miR-30
BGH PolyA
shRNA cloning site
3' SIN LTR
Xho I (3806)
WPRE
BamH I (3818)
PuroR
EcoR I (3830)
3' miR-30
T7 promoter
UbqC
BamH I (5289)
loxP
rtTA3
loxP
BamH I (6077)
Not I (6084)
IRES


human ubC
exon1
f1 ori
Apa LI (4257)
Apa LI (732)
Bbs I (1037)
Bbs I (1065)
SIBR cassette
UI2 SIBR GFP
5037 bp
EGFP
exon2
Apa LI (3011)
T7 promoter
polyA site


Synthetic poly(A) signal/transcriptional pause site (for background reduction)
Amp^r
Bgl II/Sfi I
Acc65 I
Kpn I
EcoICR I
Sac I
Nhe I
Xho I
EcoR V
Bgl II
Bgl II/Sfi I
Hind III
ori
pGL4.10[luc2] Vector
(4242bp)
2026 Sal I
2020 BamH I
SV40 late poly(A) signal
luc2


poly(A) signal
(for background
reduction)
Amp^r
3194 SalI
3188 BamHI
SV40
Enhancer
f1 ori
SmaI 3
KpnI 12
SacI 18
MluI 22
NheI 28
XhoI 33
BglII 37
pGL2-Control
Vector
(6047bp)
poly(A)
signal
(for luc
reporter)
SV40 Promoter
HindIII 239
SV40
2236 PflMI
luc


Amp^r
ori
1883 BamHI
pRL-SV40
Vector
(3705bp)
BglII 1
KpnI 58
SV40 late
poly(A)
SV40 Early
Enhancer/
Promoter
1631 XbaI
Rluc
T7
Promoter
HindIII 420
PstI 462
NheI 684
Csp45I 700


f1 (-) ori
f1 (+) ori
ampicillin
lacZ'
Sac I
MCS
Kpn I
pBluescript II KS (+/-)
3.0 kb
P lac
pUC ori