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# Construction and detection of expression vectors of microRNA-9a in BmN cells*

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**Abstract:** MicroRNAs (miRNAs) are small endogenous RNAs molecules, approximately 21–23 nucleotides in length, which regulate gene expression by base-pairing with 3′ untranslated regions (UTRs) of target mRNAs. However, the functions of only a few miRNAs in organisms are known. Recently, the expression vector of artificial miRNA has become a promising tool for gene function studies. Here, a method for easy and rapid construction of eukaryotic miRNA expression vector was described. The cytoplasmic actin 3 (A3) promoter and flanked sequences of miRNA-9a (miR-9a) precursor were amplified from genomic DNA of the silkworm (*Bombyx mori*) and was inserted into pCDNA3.0 vector to construct a recombinant plasmid. The enhanced green fluorescent protein (EGFP) gene was used as reporter gene. The *Bombyx mori* N (BmN) cells were transfected with recombinant miR-9a expression plasmid and were harvested 48 h post transfection. Total RNAs of BmN cells transfected with recombinant vectors were extracted and the expression of miR-9a was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot. Tests showed that the recombinant miR-9a vector was successfully constructed and the expression of miR-9a with EGFP was detected.

**Key words:** miRNA-9a (miR-9a), EGFP gene, *Bombyx mori* N (BmN) Cells, Expression vector
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## 1 Introduction

MicroRNAs (miRNAs) are endogenous small RNA molecules (~22 nt) that post-transcriptionally regulate expressions of target genes (Bartel, 2004). miRNA genes are transcribed by RNA polymerase II in the nucleus to give primary miRNA transcripts, which are capped at 5′ end and polyadenylated at 3′ end (Bushati and Cohen, 2007). These primary miRNA transcripts are processed by the RNase III enzyme Drosha into ~70-nt precursor miRNAs, which form an imperfect stem-loop structure (Filipowicz *et al*., 2008). Precursor miRNAs are then transported into the cytoplasm by the transporter exportin-5, where they are cleaved by the cytoplasmic RNase III enzyme Dicer to yield ~22-nt miRNA duplexes. This duplex is then loaded into the multiprotein RNA-induced silencing complex (miRISC) which regulates the stability and translation of mRNAs that are partially or fully complementary to specific miRNA (Pillai *et al*., 2007; Tang *et al*., 2008).

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miRNAs play a crucial role in regulating development, cell differentiation, proliferation, species evolution, and diseases pathogenesis (Liu, 2008; Bartel, 2009). Some studies confirmed that artificial miRNA can be processed in a similar biogenesis pathway of natural miRNA and generate functional mature miRNA, as long as the basis of precursor remains unchanged or very few alterations within an miRNA sequence occur (Vaucheret *et al*., 2004; Trujillo *et al*., 2010). This result makes it possible to construct artificial miRNA expression vector targeting any gene of interest for further functional study (Parizotto *et al*., 2004; Niu *et al*., 2006; Park *et al*., 2009).

The silkworm, *Bombyx mori*, is used as a model organism to represent lepidopteran insects that have been used for study of insect physiology, genetics and molecular biology. At present, by using computational and experimental methods, a total of 487 silkworm miRNAs have been identified on the miRBase database (http://www.mirbase.org/) (Tong *et al*., 2006; Cao *et al*., 2008; He *et al*., 2008; Yu *et al*., 2008; 2009; Huang *et al*., 2009; Zhang *et al*., 2009; Cai *et al*., 2010; Jagadeeswaran *et al*., 2010; Liu *et al*., 2010). However, their functions in the silkworm are largely unknown. The miRNA-9a (miR-9a) is highly conservative and spreads in animals from insects to human. Ambros (2003) showed that the high conservation of miRNA is involved in physiological functions. Previous studies indicated that the miR-9a is involved in the developmental regulation of the wings and nervous system differentiation in *Drosophila* (Li *et al*., 2006; Bejarano *et al*., 2009; Biryukova *et al*., 2009). Liu *et al*. (2009) found that miR-9a was highly expressed in the silkworm embryos, implying that miR-9a may play an important functional role during embryogenesis. Up until recently, few functional studies were performed in the silkworm miRNAs, leaving function of miR-9a unknown. We were interested in constructing a eukaryotic expression vector system containing the silkworm miR-9a gene. The miR-9a expression vector contains the silkworm actin 3 (A3) promoter and an enhanced green fluorescent protein (EGFP) reporter gene. In this study, an miR-9a expression vector was successfully constructed based on the flanked sequence of miR-9a precursor, expressed in *Bombyx mori* N (BmN) cells and confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot. These results will make this expression vector to facilitate further functional study of miR-9a in the silkworm.

## 2 Materials and methods

### 2.1 Materials

The silkworm strain used in this study was p50 and was reared in the Sericulture Research Institute, Chinese Academy of Agriculture Sciences. The plasmids of pCDNA3.0 (Invitrogen) and pEGFP-N3 (Clontech) were preserved in our laboratory. The silkworm ovary cells line BmN was gifted by the Institute of Life Sciences, Jiangsu University. Restriction enzymes, T4 DNA ligase and PCR reagents were obtained from TaKaRa. Primer was purchased from Sangon. Fetal bovine serum (FBS), the cell culture medium TC-100 and transfection reagent were from Invitrogen.

### 2.2 PCR amplifications of A3 promoter, EGFP and pre-miR-9a fragments

A3 promoter fragment was amplified from silkworm genomic DNA with the primer (up) 5′-AGATCTCCGCTACGATATCATTATCATA-3′ and (down) 5′-AAGCTTCTTGAATTAGTATAGTATTATTAAATAAGT-3′, which contained *Bgl*II and *Hin*dIII sites, respectively. The EGFP open reading frame (ORF) from pEGFP-N3 plasmids was amplified with the primer (up) 5′-AAGCTTGCGTGAGCAAGGGCGAGGA-3′ and (down) 5′-GGATCCTTACTTGTACAGCTCGTCCATGC-3′, which contained *Hin*dIII and *Bam*HI sites, respectively. The 290-bp DNA fragment containing miR-9a precursor was amplified with the primer (up) 5′-GGATCCAATTGAATAGAAAAAAAATATAACTTATC-3′ and (down) 5′-GAATTCGACAGATATAGGGAAAGAGAA-3′, which contained *Bam*HI and *Eco*RI sites, respectively. The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 40 s, and final extension at 72 °C for 5 min.

### 2.3 Generation of plasmids

The cytomegalovirus (CMV) promoter was replaced by *Bombyx mori* A3 promoter in pCDNA3.0 vector because CMV did not have the biological activity in insect cells. The A3 promoter fragment was

treated with *Bgl*II and *Hin*dIII and then ligated into the pCDNA3.0 vector, which was treated using same restriction enzyme to form pBmA3 vector. The EGFP ORF was obtained from the pEGFP-N3 plasmid by PCR, and it was subcloned into the downstream of the A3 promoter at *Hin*dIII and *Bam*HI sites of pBmA3 vector to generate pBmA3-EGFP vector. Then, the expression vector pBmA3-EGFP-miR-9a was generated by inserting the miR-9a gene fragment into the *Bam*HI/*Eco*RI sites of pBmA3-EGFP. The EGFP was used as a reporter gene in this vector. The construction process is shown in Fig. 1. These constructs were identified by sequencing performed by a commercial service provider (Sangon Biotech).

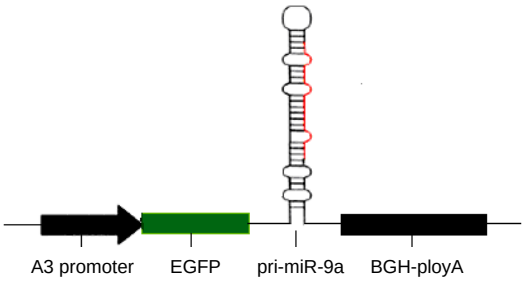


**Fig. 1 Schematic map of pBmA3-EGFP-miR-9a vector construction**

### 2.4 Cell transfection and detection of the EGFP fluorescence

The BmN cells were grown in a TC-100 medium supplemented with 10% (v/v) FBS, 200 μg/ml penicillin, and 100 μg/ml streptomycin at 27 °C with 5% $CO_2$. Transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions. Cell images with fluorescence were taken using Nikon E600 microscope equipped with Olympus camera and were processed by OpenLAB software.

### 2.5 RT-PCR assay

The BmN cell small RNA samples were isolated using mirVana™ miRNA isolation kit (Ambion) according to the manufacturer's instructions. The cDNAs were synthesized from small RNAs by using miRNA specific stem-loop primers according to the criteria as described previously (Chen *et al*., 2005; Feng *et al*., 2009). The stem-loop RT-primer was 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATAC-3′; the primer was 5′-GCGGCTCTTTGGTTATCTAG-3′ (up); and the primer was 5′-GTGCAGGGTCCGAGGT-3′ (down). The cDNAs synthesis was performed using 1 μl of diluted first-strand cDNA (1/10, v/v). PCRs were performed in a 20-μl mixture containing 1 μl cDNA, 0.5 μmol/L up and down primers, 10× PCR buffer, 0.25 μmol/L each of deoxyribonucleoside triphosphate (dNTPs) and 2 U Taq polymerase, under the following conditions: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

### 2.6 Northern blotting

The expression of miR-9a was detected by Northern blotting analysis at 48 h after transfection. Total RNA was extracted from BmN cells using a TRIzol reagent and quantified by GeneQuant spectrophotometer (Bio-Rad) before loading sample. For each sample, 20 μg of total RNAs were run on 12% (v/v) polyacrylamide gel (SequaGel, National Diagnostics) and transferred to a Nytran SuPerCharge Signal membrane (Schleicher & Schuell). The miR-9a was detected with a purified 3′ P-end-labelled oligo probe, 5′-TCATACAGCTAGATAACCAAAGA-3′, which is complementary to the mature miR-9a sequence.

## 3 Results

### 3.1 Identification of recombinant plasmid

The miR-9a expression vector was successfully constructed based on the flanked sequence of the miR-9a precursor, and this vector contained the silkworm A3 promoter and an EGFP reporter gene (Fig. 1). The miR-9a precursor was amplified by the use of recombinant plasmid template. The PCR products were detected in a 0.01 g/ml agarose gel by electrophoresis containing ethidium bromide, and photographed under ultraviolet (UV) light. A band at approximately 290 bp was separated, which was consistent with the expected molecular mass (Fig. 2a). The miR-9a precursor fragment could also be isolated from the pBmA3-EGFP-miR-9a vector after the recombinant plasmid was digested with *Bam*HI and *Eco*RI (Fig. 2b). These results implied that the recombinant plasmid pBmA3-EGFP-miR-9a was successfully constructed.

### 3.2 EGFP reporter gene expression

EGFP was observed under a fluorescent

microscope. At 48 h post transfection with the recombinant miR-9a expression plasmid, a fluorescence signal was detected in the most of the cells (Fig. 3).

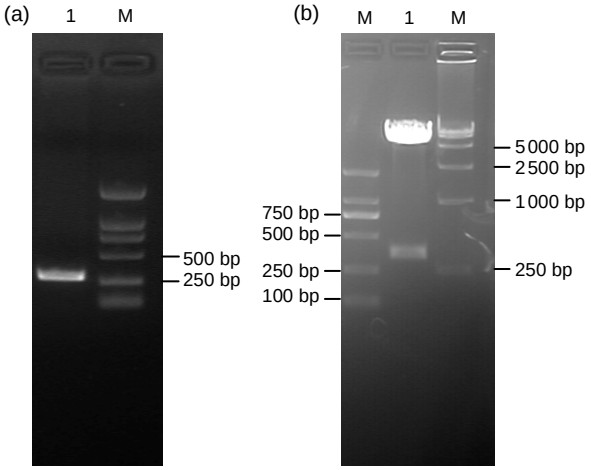


**Fig. 2 Amplification of miR-9a precursor and double enzymatic digestion**

(a) PCR amplification of the miR-9a precursor DNA (M: DL2000 maker; Lane 1: PCR products); (b) Characterization of the recombinant plasmids after digestion with restriction enzymes (M: DNA maker; Lane 1: pBmA3-EGFP-miR-9a digested by *Bam*HI and *Eco*RI)

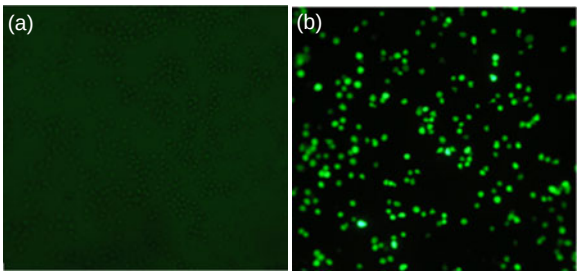


**Fig. 3 BmN cells transfected with pBmA3-EGFP-miR-9a plasmid-bright light (a) and pBmA3-EGFP-miR-9a plasmid-fluorescence (b)**

### 3.3 Detection of expression by RT-PCR

The BmN cells transfected with the recombinant miR-9a expression plasmid were harvested 48 h post transfection. The RT-PCR results showed that a ~60-bp fragment was amplified, suggesting that the recombinant plasmid of miR-9a was successfully transfected into BmN cells and miR-9a precursor can be cut into mature miR-9a (Fig. 4). The results implied that the maturity of approximately 20 bp miR-9a was successfully expressed in BmN cells.

### 3.4 Northern blot assay

To accurately assess whether the recombinant plasmid was appropriately processed into the mature miRNA, the pBmA3-EGFP-miR-9a vector was transfected into BmN cells and the total RNA was isolated. Northern blot analysis showed that miR-9a was expressed most obviously in the transfected BmN cells (Fig. 5), but miR-9a was not detected in untransfected BmN cells or BmN cells transfected with pBmA3-EGFP plasmid, suggesting that endogenous miR-9a was not expressed in this cell line. This suggests that miR-9a expression vector is recognized by Drosha and Dicer as a miRNA precursor and processed into mature miRNA-9a in BmN cells. These results show that the plasmid can express miR-9a gene effectively in BmN cells.

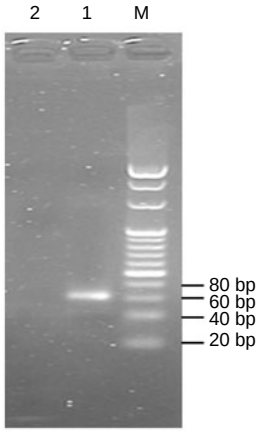


**Fig. 4 RT-PCR amplification of mature miR-9a**

M: 20-bp ladder maker; Lane 1: PCR products amplified with recombinant plasmid in transfected BmN cells; Lane 2: no PCR products amplified in untransfected BmN cells

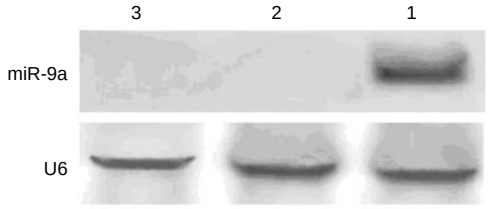


**Fig. 5 Northern blot detection of miR-9a expression in BmN cells**

Lane 1: BmN cells transfected with pBmA3-EGFP-miR-9a plasmid; Lane 2: BmN cells transfected with pBmA3-EGFP plasmid; Lane 3: normal BmN cells. U6 served as an internal control

## 4 Discussion

Research evidence indicates that miRNAs participate in a variety of biological activities, including cell differentiation, biological development, genesis of disease, tumorigenesis, and host-viral interactions (Bartel, 2004; Stefani and Slack, 2008). In the past few years, the important role played by miRNAs in gene regulation has been recognized (Filipowicz *et al*., 2008). It is estimated that miRNAs comprise about 1%–3% of genes in animal and may regulate 10%–30% protein-coding genes (Friedman *et al*., 2009; Hong *et al*., 2009). Although the role played by miRNAs in target regulation has been recognized, very little is known about their functions.

Currently, there are primarily three approaches for the functional research of miRNAs: construction of eukaryotic expression vectors, in vitro transcription (Hu *et al*., 2010; Schmollinger *et al*., 2010), and direct chemical synthesis (Shibata *et al*., 2007; Hu *et al*., 2009; Shan *et al*., 2009; 2010). Compared with the chemical synthesis or in vitro transcription, construction of the eukaryotic expression vector should be more economical, and applied in long-term research of its biological functions. Endogenous primary miRNA transcripts share some similar structural characteristics that comprise a stem, a terminal loop, long flanking sequences, and some internal loops or bulges, which likely contribute to efficient processing in miRNA pathways (Ritchie *et al*., 2007; Scherr and Eder, 2007; Qiu *et al*., 2008). Previous experiments confirmed that the flanking sequences at 5′ and 3′ ends of miRNA precursors are necessary for miRNA expression (Chen *et al*., 2004; Zhou *et al*., 2005; Chang *et al*., 2006; Fukuda *et al*., 2006). Zeng *et al*. (2002) first successfully utilized miR-30 precursor to express functional mature miRNA. The sequence flanking miRNA precursor has also proven critical for primary miRNA transcripts to be processed into the miRNA precursor by Drosha complex (Zeng and Cullen, 2005; Rumi *et al*., 2006). Sun *et al*. (2006) also used an extended miR-30 hairpin of 118 nt as a backbone for artificial miRNA expression. Based on these previous results, various studies have been carried out successfully with regards to the construction of miRNA expression vectors (Han *et al*., 2006; Molnar *et al*., 2009; Xu *et al*., 2009; Sun *et al*., 2010). So, we reasoned that the flanked sequence of the extended miR-9a hairpin of 100 nt on each side of miR-9a is essential and sufficient for the expression of miR-9a. The eukaryotic expression method is considered fairly inexpensive and can generate functional miRNAs to suppress target genes in cells (Silva *et al*., 2005; Gou *et al*., 2007). In addition, there are also commercial kits for miRNA expression vector construction, but these expression vectors can express miRNAs only in mammalian cells and are not suitable for miRNA expression in insect cells.

Exogenous genes can be faithfully and efficiently transcribed in BmN cells derived from the ovarian tissue of the silkworm (Sakurai *et al*., 1990). Previous studies have confirmed that EGFP ORF was placed at the 5′ end of miRNA gene, and EGFP and mature miRNAs were expressed (Cai *et al*., 2004; Du *et al*., 2006). In our study, based on knowledge on the primary miRNAs driven by polymerase II and natural miRNA biogenesis, a method for the generation of miRNA expression vectors was developed, which can efficiently expressed miRNAs in silkworm BmN cells. This study demonstrated that the construction of miRNA expression vectors is a better approach for further function study of miRNAs in vitro or in the silkworm.

## References

Ambros, V., 2003. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell*, **113**(6): 673-676. [doi:10.1016/S0092-8674(03)00428-8]

Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**(2):281-297. [doi:10.1016/S0092-8674(04)00045-5]

Bartel, D.P., 2009. MicroRNAs: target recognition and regulatory functions. *Cell*, **136**(2):215-233. [doi:10.1016/j.cell.2009.01.002]

Bejarano, F., Smibert, P., Lai, E.C., 2009. miR-9a prevents apoptosis during wing development by repressing *Drosophila LIM-only*. *Dev. Biol*., **338**(1):63-73. [doi:10.1016/j.ydbio.2009.11.025]

Biryukova, I., Asmar, J., Abdesselem, H., Heitzler, P., 2009. *Drosophila mir-9a* regulates wing development via fine-tuning expression of the LIM only factor, dLMO. *Dev. Biol*., **327**(2):487-496. [doi:10.1016/j.ydbio.2008.12.036]

Bushati, N., Cohen, S.M., 2007. microRNA functions. *Annu. Rev. Cell Dev. Biol*., **23**(1):175-205. [doi:10.1146/annurev.cellbio.23.090506.123406]

Cai, X., Hagedorn, C.H., Cullen, B.R., 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA*, **10**(12): 1957-1966. [doi:10.1261/rna.7135204]

Cai, Y., Yu, X., Zhou, Q., Yu, C., Hu, H., Liu, J., Lin, H., Yang, J., Zhang, B., Cui, P., *et al*., 2010. Novel microRNAs in silkworm (*Bombyx mori*). *Funct. Integr. Genomics*, **10**(3):405-415. [doi:10.1007/s10142-010-0162-7]

Cao, J., Tong, C., Wu, X., Lv, J., Yang, Z., Jin, Y., 2008. Identification of conserved microRNAs in *Bombyx mori* (silkworm) and regulation of fibroin L chain production by microRNAs in heterologous system. *Insect Biochem. Mol. Biol*., **38**(12):1066-1071. [doi:10.1016/j.ibmb.2008.09.008]

Chang, K., Elledge, S.J., Hannon, G.J., 2006. Lessons from Nature: microRNA-based shRNA libraries. *Nat. Methods*, **3**(9):707-714. [doi:10.1038/nmeth923]

Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R., *et al*., 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic. Acids Res*., **33**(20):e179. [doi:10.1093/nar/gni178]

Chen, C.Z., Li, L., Lodish, H.F., Bartel, D.P., 2004. MicroRNAs modulate hematopoietic lineage differentiation. *Science*, **303**(5654):83-86. [doi:10.1126/science.1091903]

Du, G., Yonekubo, J., Zeng, Y., Osisami, M., Frohman, M.A., 2006. Design of expression vectors for RNA interference based on miRNAs and RNA splicing. *FEBS J*., **273**(23):5421-5427. [doi:10.1111/j.1742-4658.2006.05534.x]

Feng, J., Wang, K., Liu, X., Chen, S., Chen, J., 2009. The quantification of tomato microRNAs response to viral infection by stem-loop real-time RT-PCR. *Gene*, **437**(1-2):14-21. [doi:10.1016/j.gene.2009.01.017]

Filipowicz, W., Bhattacharyya, S.N., Sonenberg, N., 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet*., **9**:102-114. [doi:10.1038/nrg2290]

Friedman, R.C., Farh, K.K., Burge, C.B., Bartel, D.P., 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*., **19**(1):92-105. [doi:10.1101/gr.082701.108]

Fukuda, Y., Kawasaki, H., Taira, K., 2006. Construction of microRNA-containing vectors for expression in mammalian cells. *Methods Mol. Biol*., **338**:167-173. [doi:10.1385/1-59745-097-9:167]

Gou, D., Zhang, H., Baviskar, P.S., Liu, L., 2007. Primer extension-based method for the generation of a siRNA/miRNA expression vector. *Physiol. Genomics*, **31**(3):554-562. [doi:10.1152/physiolgenomics.00005.2007]

Han, J., Lee, Y., Yeom, K.H., Nam, J.W., Heo, I., Rhee, J.K., Sohn, S.Y., Cho, Y., Zhang, B.T., Kim, V.N., 2006. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell*, **125**(5):887-901. [doi:10.1016/j.cell.2006.03.043]

He, P.A., Nie, Z., Chen, J., Lv, Z., Sheng, Q., Zhou, S., Gao, X., Kong, L., Wu, X., Jin, Y., *et al*., 2008. Identification and characteristics of microRNAs from *Bombyx mori*. *BMC Genomics*, **9**(1):248. [doi:10.1186/1471-2164-9-248]

Hong, X., Hammell, M., Ambros, V., Cohen, S.M., 2009. Immunopurification of Ago1 miRNPs selects for a distinct class of microRNA targets. *PNAS*, **106**(35):15085-15090. [doi:10.1073/pnas.0908149106]

Hu, T., Fu, Q., Chen, P., Ma, L., Sin, O., Guo, D., 2009. Construction of an artificial microRNA expression vector for simultaneous inhibition of multiple genes in mammalian cells. *Int. J. Mol. Sci*., **10**(5):2158-2168. [doi:10.3390/ijms10052158]

Hu, T., Chen, P., Fu, Q., Liu, Y., Ishaq, M., Li, J., Ma, L., Guo, D., 2010. Comparative studies of various artificial microRNA expression vectors for RNAi in mammalian cells. *Mol. Biotechnol*., **46**(1):34-40. [doi:10.1007/s12033-010-9264-7]

Huang, Y., Zou, Q., Tang, S.M., Wang, L.G., Shen, X.J., 2009. Computational identification and characteristics of novel microRNAs from the silkworm (*Bombyx mori* L.). *Mol. Biol. Rep*., **37**(7):3171-3176. [doi:10.1007/s11033-009-9897-4]

Jagadeeswaran, G., Zheng, Y., Sumathipala, N., Jiang, H., Arrese, E.L., Soulages, J.L., Zhang, W., Sunkar, R., 2010. Deep sequencing of small RNA libraries reveals dynamic regulation of conserved and novel microRNAs and microRNA-stars during silkworm development. *BMC Genomics*, **11**(1):52. [doi:10.1186/1471-2164-11-52]

Li, Y., Wang, F., Lee, J.A., Gao, F.B., 2006. *MicroRNA-9a* ensures the precise specification of sensory organ precursors in *Drosophila*. *Genes. Dev*., **20**(20):2793-2805. [doi:10.1101/gad.1466306]

Liu, J., 2008. Control of protein synthesis and mRNA degradation by microRNAs. *Curr. Opin. Cell Biol*., **20**(2):214-221. [doi:10.1016/j.ceb.2008.01.006]

Liu, S., Zhang, L., Li, Q., Zhao, P., Duan, J., Cheng, D., Xiang, Z., Xia, Q., 2009. MicroRNA expression profiling during the life cycle of the silkworm (*Bombyx mori*). *BMC Genomics*, **10**(1):455. [doi:10.1186/1471-2164-10-455]

Liu, S., Li, D., Li, Q., Zhao, P., Xiang, Z., Xia, Q., 2010. MicroRNAs of *Bombyx mori* identified by Solexa sequencing. *BMC Genomics*, **11**(1):148. [doi:10.1186/1471-2164-11-148]

Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., Baulcombe, D., 2009. Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *Plant J*., **58**(1):165-174. [doi:10.1111/j.1365-313X.2008.03767.x]

Niu, Q.W., Lin, S.S., Reyes, J.L., Chen, K.C., Wu, H.W., Yeh, S.D., Chua, N.H., 2006. Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat. Biotechnol*., **24**(11):1420-1428. [doi:10.1038/nbt1255]

Parizotto, E.A., Dunoyer, P., Rahm, N., Himber, C., Voinnet, O., 2004. In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. *Genes Dev*., **18**(18):2237-2242. [doi:10.1101/gad.307804]

Park, W., Zhai, J., Lee, J.Y., 2009. Highly efficient gene silencing using perfect complementary artificial miRNA targeting *AP1* or heteromeric artificial miRNA targeting *AP1* and *CAL* genes. *Plant Cell Rep*., **28**(3):469-480. [doi:10.1007/s00299-008-0651-5]

Pillai, R.S., Bhattacharyya, S.N., Filipowicz, W., 2007. Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol*., **17**(3):118-126. [doi:10.1016/j.tcb.2006.12.007]

Qiu, L., Wang, H., Xia, X., Zhou, H., Xu, Z., 2008. A construct with fluorescent indicators for conditional expression of miRNA. *BMC Biotechnol*., **8**(1):77. [doi:10.1186/1472-6750-8-77]

Ritchie, W., Legendre, M., Gautheret, D., 2007. RNA stem-loops: to be or not to be cleaved by RNAse III. *RNA*, **13**(4):457-462. [doi:10.1261/rna.366507]

Rumi, M., Ishihara, S., Aziz, M., Kazumori, H., Ishimura, N., Yuki, T., Kadota, C., Kadowaki, Y., Kinoshita, Y., 2006. RNA polymerase II mediated transcription from the polymerase III promoters in short hairpin RNA expression vector. *Biochem. Biophys. Res. Commun*., **339**(2):540-547. [doi:10.1016/j.bbrc.2005.11.037]

Sakurai, H., Izumi, S., Tomino, S., 1990. In vitro transcription of the plasma protein genes of *Bombyx mori*. *Biochim. Biophys. Acta*, **1087**(1):18-24. [doi:10.1016/0167-4781(90)90115-I]

Scherr, M., Eder, M., 2007. Gene silencing by small regulatory RNAs in mammalian cells. *Cell Cycle*, **6**(4):444-449. [doi:10.4161/cc.6.4.3807]

Schmollinger, S., Strenkert, D., Schroda, M., 2010. An inducible artificial microRNA system for *Chlamydomonas reinhardtii* confirms a key role for heat shock factor 1 in regulating thermotolerance. *Curr. Genet*., **56**(4):383-389. [doi:10.1007/s00294-010-0304-4]

Shan, Z., Lin, Q., Deng, C., Li, X., Huang, W., Tan, H., Fu, Y., Yang, M., Yu, X., 2009. An efficient method to enhance gene silencing by using precursor microRNA designed small hairpin RNAs. *Mol. Biol. Rep*., **36**(6):1483-1489. [doi:10.1007/s11033-008-9339-8]

Shan, Z., Lin, Q., Deng, C., Zhou, Z., Tan, H., Fu, Y., Li, X., Zhu, J., Mai, L., Kuang, S., *et al*., 2010. Comparison of approaches for efficient gene silencing induced by microRNA-based short hairpin RNA and indicator gene expression. *Mol. Biol. Rep*., **37**(4):1831-1839. [doi:10.1007/s11033-009-9618-z]

Shibata, A., Iwaki, A., Fukumaki, Y., 2007. A novel expression system for artificial miRNAs containing no endogenous miRNA precursor sequences. *J. RNAi Gene Silencing*, **3**(1):237-247.

Silva, J.M., Li, M.Z., Chang, K., Ge, W., Golding, M.C., Rickles, R.J., Siolas, D., Hu, G., Paddison, P.J., Schlabach, M.R., *et al.*, 2005. Second-generation shRNA libraries covering the mouse and human genomes. *Nat. Genet*., **37**:1281-1288. [doi:10.1038/ng1650]

Stefani, G., Slack, F.J., 2008. Small non-coding RNAs in animal development. *Nat. Rev. Mol. Cell Biol*., **9**(3):219-230. [doi:10.1038/nrm2347]

Sun, D., Melegari, M., Sridhar, S., Rogler, C.E., Zhu, L., 2006. Multi-miRNA hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. *Biotechniques*, **41**(1):59-63. [doi:10.2144/000112203]

Sun, H., Li, Q.W., Lv, X.Y., Ai, J.Z., Yang, Q.T., Duan, J.J., Bian, G.H., Xiao, Y., Wang, Y.D., Zhang, Z., *et al*., 2010. MicroRNA-17 post-transcriptionally regulates polycystic kidney disease-2 gene and promotes cell proliferation. *Mol. Biol. Rep*., **37**(6):2951-2958. [doi:10.1007/s11033-009-9861-3]

Tang, G., Tang, X., Mendu, V., Jia, X., Chen, Q.J., He, L., 2008. The art of microRNA: various strategies leading to gene silencing via an ancient pathway. *Biochim. Biophys. Acta*, **1779**(11):655-662. [doi:10.1016/j.bbagrm.2008.06.006]

Tong, C.Z., Jin, Y.F., Zhang, Y.Z., 2006. Computational prediction of microRNA genes in silkworm genome. *J. Zhejiang Univ.-Sci. B*, **7**(10):806-816. [doi:10.1631/jzus.2006.B0806]

Trujillo, R.D., Yue, S.B., Tang, Y., O′Gorman, W.E., Chen, C.Z., 2010. The potential functions of primary microRNAs in target recognition and repression. *EMBO J*., **29**(19):3272-3285. [doi:10.1038/emboj.2010.208]

Vaucheret, H., Vazquez, F., Crete, P., Bartel, D.P., 2004. The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev*., **18**(10):1187-1197. [doi:10.1101/gad.1201404]

Xu, J., Zeng, J.Q., Wan, G., Hu, G.B., Yan, H., Ma, L.X., 2009. Construction of siRNA/miRNA expression vectors based on a one-step PCR process. *BMC Biotechnol.,* **9**(1):53. [doi:10.1186/1472-6750-9-53]

Yu, X., Zhou, Q., Li, S.C., Luo, Q., Cai, Y., Lin, W.C., Chen, H., Yang, Y., Hu, S., Yu, J., 2008. The silkworm (*Bombyx mori*) microRNAs and their expressions in multiple developmental stages. *PLoS One*, **3**(8):e2997. [doi:10.1371/journal.pone.0002997]

Yu, X., Zhou, Q., Cai, Y., Luo, Q., Lin, H., Hu, S., Yu, J., 2009. A discovery of novel microRNAs in the silkworm (*Bombyx mori*) genome. *Genomics*, **94**(6):438-444. [doi:10.1016/j.ygeno.2009.08.007]

Zeng, Y., Cullen, B.R., 2005. Efficient processing of primary microRNA hairpins by Drosha requires flanking nonstructured RNA sequences. *J. Biol. Chem*., **280**(30):27595-27603. [doi:10.1074/jbc.M504714200]

Zeng, Y., Wagner, E.J., Cullen, B.R., 2002. Both natural and designed microRNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol. Cell*, **9**(6):1327-1333. [doi:10.1016/S1097-2765(02)00541-5]

Zhang, Y., Zhou, X., Ge, X., Jiang, J., Li, M., Jia, S., Yang, X., Kan, Y., Miao, X., Zhao, G., *et al.*, 2009. Insect-specific microRNA involved in the development of the silkworm *Bombyx mori*. *PLoS One*, **4**(3):e4677. [doi:10.1371/journal.pone.0004677]

Zhou, H., Xia, X.G., Xu, Z., 2005. An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. *Nucleic Acids Res*., **33**(6):e62. [doi:10.1093/nar/gni061]