Chapter 9
miRNA Sponge Technology

Abstract MiRNA Sponge technology is an innovative approach used to generate RNAs containing multiple, tandem binding sites for a miRNA seed family of interest and able to target all members of that miRNA seed family. When vectors encoding the miRNA sponges are transiently transfected into cultured cells, they depress miRNA targets as strongly as the conventional AMOs described in Chap. 6. The major advancement of this technique over the AMO technique is that it can better inhibit functional classes of miRNAs than do AMOs that are designed to block single miRNA sequences. The main principle of the miRNA Sponge technology is identical to the MT-AMO technology described in Chap. 7: targeting multiple miRNAs. The miRNA Sponge technology was established by Sharp's laboratory in 2007 [Ebert MS, Neilson JR, Sharp PA, Nat Methods 4:721–726 2007; Hammond SM Nat Methods 4:694–695, 2007]. Similar to the AMO approach, miRNA Sponge technology belongs to the "targeting-miRNA" and "miRNA-loss-of-function" strategy. The miRNA Sponge technology complies with the 'Single-Drug, Multiple-Target' [Gao H, Xiao J, Sun Q, Lin H, Bai Y, Yang L, Yang B, Wang H, Wang Z, Mol Pharmacol, 70:1621–1629, 2006] and 'miRNA Seed Family' concepts (see Sect. 2.1.4 for detail).

9.1 Introduction

As reasoned in Sect. 2.1.4 and Chap. 8, many miRNAs are members of families that share a seed sequence but may have one or more nucleotide changes in the remaining sequence (Bommer et al. 2007; Pedersen et al. 2007; Vermeulen et al. 2007). Moreover, many miRNAs are expressed from multiple genomic loci (Wang 2008). To achieve adequate miRNA-loss-of-function for elucidating a certain cellular process, the conventional AMO strategy falls short in dealing with multiple miRNAs. On the other hand, creating genetic knockouts to determine the function of miRNA families is difficult, as individual miRNAs expressed from multiple genomic loci or from multiple members of a same miRNA seed family may repress a common set

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of targets containing a complementary seed sequence. Thus, a method for inhibiting these functional classes of paralogous miRNAs in vivo is needed.

For this reason, Ebert et al. (2007) invented an innovative anti-miRNA approach termed 'miRNA sponges'. The idea behind it is to produce a single specie of RNAs containing multiple, tandem binding sites for a miRNA seed family of interest, in order to target all members of that miRNA seed family, taking advantage of the fact that the interaction between miRNA and target is nucleated by and largely dependent on base-pairing in the seed region (positions 2–8 of the miRNA). The authors constructed sponges by inserting tandemly arrayed miRNA binding sites into the 3′UTR of a reporter gene encoding destabilized GFP driven by the CMV promoter, which can yield abundant expression of the competitive inhibitor transcripts.

9.2 Protocols

The miRNA Sponge technology shares many similarities with the AMO and the MT-AMO technologies in that they are all antisense to miRNAs acting on their target miRNA by base-pairing mechanisms and producing miRNA-loss-of-function effects. There is therefore no surprise that the protocols involved in the generation and application of the miRNA inhibitors with these distinct technologies are more or less the same. The procedures described in this chapter are primarily based upon the studies reported by Ebert et al. (2007).

9.2.1 Designing miRNA Sponges

1. Select a particular miRNA seed family of your interest for study and further select a member from this seed family for designing a miRNA sponge. For example, miR-17-5p, miR-20 and miR-17–92 are within the same seed family and miR-30c, miR-30d and miR-30e are within another seed family. Pick any one member from a seed family as a template for designing a miRNA sponge;
2. Determine a binding site for the selected miRNA that is a sequence perfectly complementary to the selected member of the miRNA seed family, just like designing an AMO described in Chap. 7. But this AMO or binding site carries mismatches in the middle portion to create a bulge (4–7 A:G/G:A wobble pairs) at positions 9–12 when binding to its target miRNAs. Each of such an AMO is considered one unit that can be recognized as a binding site by multiple miRNAs that share the seed;

(The bulge is for preventing RNA interference–type cleavage and degradation of the sponge RNA through endonucleolytic cleavage by Argonaute 2. For both sponge classes, sponges with 4–7 bulged binding sites will produce stronger derepressive effects than sponges with two perfect binding sites. This difference may be due to the availability of more binding sites in the bulged sponges and/or to the greater

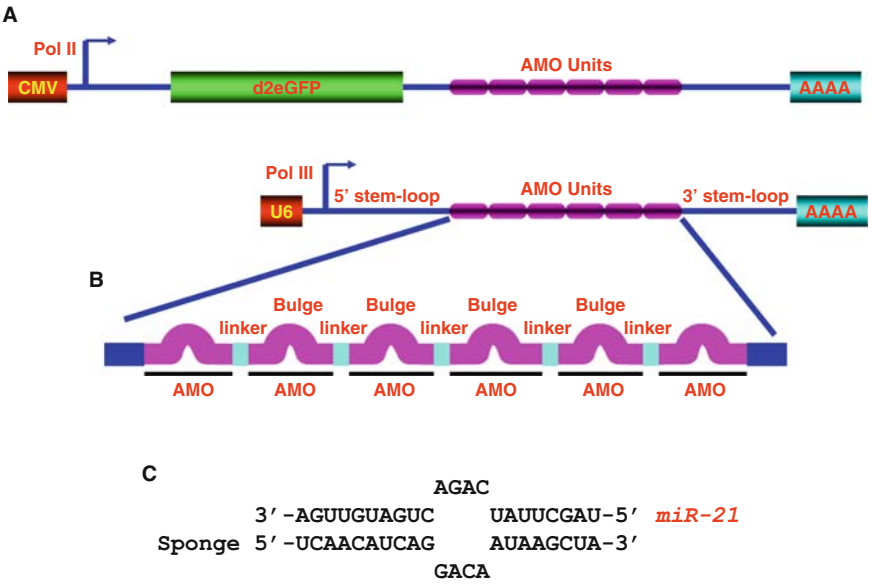

Fig. 9.1 Design of miRNA sponges according to the protocols by Ebert et al. (2007). (**a**) Construction of GFP sponges by inserting multiple identical miRNA binding sites (AMO units) into the 3′UTR of a 2-h destabilized GFP (d2eGFP) reporter gene driven by the CMV promoter. (**b**) Construction of U6 sponges by subcloning multiple identical AMO units into a vector containing a U6 snRNA promoter with 5′ and 3′ stem-loop elements. (**c**) The imperfect pairing between a miRNA and a sponge with bulged binding sites. An example is shown for miR-21. The bulge is designed to protect against endonucleolytic cleavage by Argonaute 2

stability expected of bulged sponge RNAs compared to sponge RNAs that can be cleaved by miRNA–loaded Argonaute 2. The imperfect pairing between a miRNA and a sponge with bulged binding sites is diagrammed for miR-21 in Fig. 9.1);

3. Connect the AMO units in tandem to construct multiple miRNA binding sites in a single fragment. The 5′-end AMO unit and the 3′-end AMO unit should each carry restriction enzyme recognition sequence for cloning (e.g., 5′-XhoI and 3′-ApaI in the study by Ebert et al. (2007)).
4. Ebert et al. (2007) examined 7 miR-20 binding sites in their study. Addition of more miRNA binding sites to the sponge might increase the potency of the inhibitor. These authors also tested miRNA sponges with 6, 10 and 18 sites. However, they found only a marginal increase in activity above 6 sites, with apparently saturating effect. A spacer with an irrelevant sequence might be inserted in-between binding sites to optimize the binding of miRNPs to every possible binding site. But previous results suggest that nearby sites are fully functional (Doench and Sharp 2004);
5. Design a complementary strand. Synthesize both strands. Anneal the two strands to form a double-stranded construct;

6. Construct a miRNA sponge containing RNA polymerase II promoter plasmid (pcDNA5-CMV-d2eGFP vector, Invitrogen) by inserting the fragment into the 3′UTR (at XhoI and ApaI cloning site) of a reporter gene encoding destabilized GFP driven by the CMV promoter.
7. Alternatively, one can construct a Pol III plasmid (RNA polymerase III promoter) carrying miRNA sponges to take advantage of strong Pol III promoters, which are known to drive expression of the most-abundant cellular RNAs. Subclone the fragment carrying tandemly arrayed miRNA binding sites from the GFP sponge construct described in **(3)** into a modified U6 small nuclear RNA promoter-terminator vector, which produces short (<300 nt) RNAs with stem-loop elements (Paul et al. 2003). As construct lacks an open reading frame, the U6 sponges become substrates for miRNA binding but not for translation or translational repression;
8. As a negative control, construct a sponge with repeated binding sites complementary to an artificial miRNA based on a sequence from genes but not complementary to any known miRNA;
9. Perform functional assays to validate the miRNA sponges, as outlined below.

9.2.2 Validating miRNA Sponges

1. Transfect the sponge plasmids into selected cell lines. Ideally, the cells for transfection should contain abundant endogenous target miRNAs, otherwise target synthetic miRNA (SC-miRNA; see Chap. 3) must be cotransfected. Measure the levels of targeted miRNAs using real-time qRT-PCR methods or Northern blot. The levels of miRNA members within the targeted miRNA seed family are expected to be knocked down as strongly as the conventional AMOs;
2. Construct a miRNA binding site–carrying luciferase reporter gene vector by inserting the binding site of the target miRNA seed family into the multiple cloning sites downstream the luciferase gene (3′UTR region). For example, you can insert a 22-nt fragment containing the 5′ portion 1–8 nts of the miR-17-5p seed family and an arbitrary sequence of 16 nts. Then, cotransfect a sponge plasmid and the luciferase vector at a ratio of 8:1, sponge plasmid to target plasmid, in a selected cell line. 24∼48 h after transfection, measure luciferase activity. The luciferase activity is expected to increase with application of the miRNA sponge but not with cotransfection of negative control sponges;
3. The GFP gene in the plasmid can be used for monitoring the transfection efficiency of the sponge plasmids and for tracking those cells that express high levels of the inhibitor RNA. Simply conduct quantification of GFP transcripts by real-time PCR in relation to GFP plasmid standards. It was estimated that GFP mRNAs in transiently transfected 293T cells were at least 1,000–2,000 per cell. Then, this level of expression of a miRNA sponge containing seven binding sites targeting a miRNA seed family allows inhibition of approximately

10^4 miRNAs per cell, which would be sufficient to inhibit most miRNAs in most cell types;

4. Test the ability of sponges to derepress natural miRNA target genes using Western blot and qRT-PCR methods. The target genes of the selected miRNA seed family are expected to be upregulated in their expression at the protein level. The transcript level of miRNA target genes may or may not be altered depending upon the overall complementarity between the sponge sequence and the target mRNA sequence.

9.3 Principle of Actions

miRNA sponges are transcripts expressed from strong promoters, containing multiple, tandem binding sites to a selected member of a miRNA seed family of interest. miRNA sponges act by mechanisms similar to AMO and MT-AMO; they can sequester targeted miRNAs, disrupt miRISC-mediated targeting in the cytoplasm and disrupt Drosha processing in the nucleus. But unlike AMO and MT-AMO, miRNA sponges with bulges are in theory not able to induce degradation of their targeted miRNA. Detailed comparisons among the miRNA Sponge, AMO and MT-AMO technologies are given in Table 9.1.

9.4 Applications

Like the AMO and the MT-AMO approaches, the miRNA Sponge technology is able to interfere with the function of natural, endogenous target miRNAs, the target genes of the miRNAs and therefore can be used for target validation and phenotypic analysis.

Table 9.1 Comparisons of major characteristics among the miRNA Sponge, AMO and MT-AMO technologies

	miRNA sponge	MT-AMO	AMO
Composition	mRNA containing a 3′UTR	DNA or RNA oligos	RNA or DNA oligo
Number of binding sites	Multiple (homogenous)	Multiple (homogenous or heterogeneous)	Single
Complementarity	Seed site (8 nts) complementarity	Full (22 nts) complementarity	Seed Full (22 nts) complementarity
Targeted miRNA	Targeted miRNAs intact	Targeted miRNAs degradation	Targeted miRNA degradation
Specificity	miRNA-specific & miRNA seed family-specific	miRNA-specific & miRNA seed family- specific	miRNA-specific
Outcome	Derepression of proteins	Derepression of proteins	Derepression of proteins

A potential extension of the miRNA Sponge technology would be to express sponges from stably integrated transgenes in vivo. This can be applied to studying long-term effects of miRNA-loss-of-function in cell lines, drug-inducible miRNA sponges in xenograft models to investigate miRNA contributions to tumorigenesis and to treat cancer; bone marrow reconstitution approaches to investigate miRNAs roles in immune cell development; and maybe germline transgenic sponge mice to ascertain the functions of miRNAs families at cell, tissue, organ and organism levels.

9.5 Advantages and Problems

1. miRNA sponges are at least as effective as the AMO technology in antagonizing their target miRNAs;
2. As an alternative to AMOs, miRNA sponges can be expressed in cells, as RNAs produced from transgenes; these competitive inhibitors are transcripts expressed from strong promoters, containing multiple, tandem binding sites to a miRNA of interest;
3. They specifically inhibit miRNAs with a complementary heptameric seed, such that a single sponge can be used to block an entire miRNA seed family;
4. miRNA sponges can be made stably expressed in cell lines from multicopy chromosomal insertions (by cotransfecting 293T cells with linearized GFP sponge plasmids and a puromycin selection marker) Ebert et al. (2007) reported that the stable *miR*-16 sponge–expressing cell line allowed threefold higher expression of a *miR*-16 target, relative to cells transiently transfected with sponge plasmids.

In terms of the limitation of the approach, the same concept that is applied to AMOs and MT-AMOs is also applicable to miRNA sponges; that is, the action of miRNA sponges is miRNA-specific but not specific towards a particular protein-coding gene; by knocking down a miRNA or a miRNA seed family, a miRNA sponge is deemed to affect all target genes of its targeted miRNAs. In many situations, this action generates undesirable effects. In such a case, a different approach, MiRNA-Masking Antisense Oligonucleotides (miR-Mask) technology (Chap. 10) can be employed instead.

References

Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, Zhai Y, Giordano TJ, Qin ZS, Moore BB, MacDougald OA, Cho KR, Fearon ER (2007) p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr Biol 17:1298–1307.

Doench JG, Sharp PA (2004) Specificity of microRNA target selection in translational repression. Genes Dev 18:504–511.

Ebert MS, Neilson JR, Sharp PA (2007) MicroRNA sponges: Competitive inhibitors of small RNAs in mammalian cells. Nat Methods 4:721–726.

Gao H, Xiao J, Sun Q, Lin H, Bai Y, Yang L, Yang B, Wang H, Wang Z (2006) A single decoy oligodeoxynucleotides targeting multiple oncoproteins produces strong anticancer effects. Mol Pharmacol 70:1621–1629.

Hammond SM (2007) Soaking up small RNAs. Nat Methods 4:694–695.

Paul CP, Good PD, Li SX, Kleihauer A, Rossi JJ, Engelke DR (2003) Localized expression of small RNA inhibitors in human cells. Mol Ther 7:237–247.

Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, David M (2007) Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature 449:919–922.

Vermeulen A, Robertson B, Dalby AB, Marshall WS, Karpilow J, Leake D, Khvorova A, Baskerville S (2007) Double-stranded regions are essential design components of potent inhibitors of RISC function. RNA 13:723–730.