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REVIEW

IsomiRs have functional importance

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Abstract

Since the inception of deep sequencing, isomiRs are consistently observed to be produced by most miRNA genes in a variety of cell types. IsomiRs appear as a variation in length from the canonical sequence annotated in miRBase, due to an addition or deletion of one or more nucleotides at the 5' or 3' ends or both. As the seed sequence is located at the 5' end of the microRNA, the target mRNA will be theoretically different. Therefore, 5' isomiRs might potentially target a new set mRNA compared to their canonical counterpart. This article gives an overview of investigations that explored the functional potential of isomiRs such as their ability to incorporate into Argonaute protein, the differential expression of isomiRs in various tissue types and cell lines, and the differences of mRNA targets between isomiR and its canonical microRNA. In addition, this article provides a brief introduction of RNA sponges as a potential way to inhibit isomiRs.

Keywords: IsomiRs, microRNA, messenger RNA, molecular, target prediction

INTRODUCTION

MicroRNAs were first discovered in C. *elegans* by Lee *et al*., (1993)[1] as two overlapping transcripts of lin-4 gene, which are about 22 and 61 nucleotides in length. These transcripts inhibited lin-14 mRNA through complementarity to the 3' untranslated region (UTR). Subsequently, lin-4 and let-7 were found to regulate the timing of C. *elegans* development. MicroRNAs were also identified in humans, fruit flies, chickens, frogs, zebrafish, molluscs, sea urchins and mouse.[2-4]

MicroRNAs are about 19-25 nucleotides in length and belong to one of the classes of non-coding RNAs which are functional RNAs that do not translate into protein. Non-coding RNAs consist of transfer RNA (tRNA), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA), microRNA (miRNA), small interfering RNA (siRNAs), small nuclear RNA (snRNA), piwi-interacting RNA (piRNA) and long ncRNA.[5] So far, based on miRBase database, approximately 1881 pre-miRNAs and 2588 mature human miRNAs have been identified (miRBase, June 2014).[6,7] MicroRNAs are found to have important post-transcriptional roles in almost every cellular process in eukaryotes, which include the regulation of embryonic development, signalling pathways, apoptosis, metabolism, organogenesis and involvement in pathological conditions like viral diseases, genetic disorders and cancer.[8-10] In addition, embryonic stem cell specific microRNA, i.e. miR-302 cluster, has been used to reprogram or facilitate the reprograming of somatic cells to induced pluripotent stem cells.[11-12]

Biogenesis of miRNA

MicroRNA genes can be located in various parts of the human genome, i.e., between genes as well as within the intron or exon regions of other genes (Figure 1). The miRNA genes are mostly transcribed into primary miRNA (pri-miRNA) by RNA polymerase II.[13,14] The pri-miRNAs are hundreds to thousands of nucleotides in length and can encode multiple precursor miRNAs, for example the miR-302 cluster (Figure 1).[15] The pri-miRNA undergoes processing by Drosha, an RNase III endonuclease.[16] Drosha forms a microprocessor complex with DGCR8 (DiGeorge syndrome critical region gene 8), which is called Pasha in Drosophila and PASH-1 in C. *elegans*.[15,17,18] This complex binds to stem loops within pri-miRNA and can excise and release

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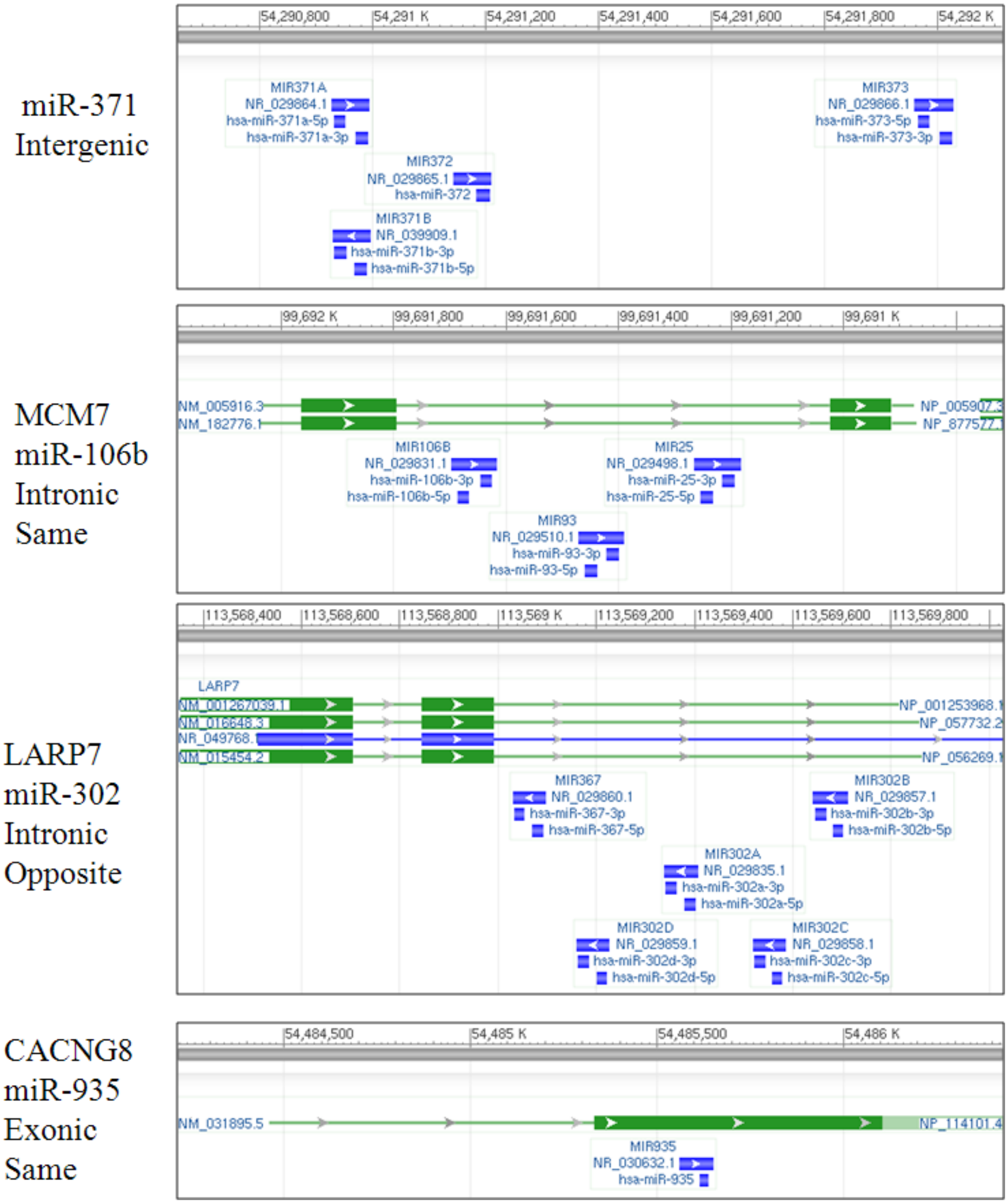

FIG. 1: Examples of location of microRNA genes in human genome. (Reproduced from thesis of Tan GC. Imperial College London 2013)

precursor miRNA (pre-miRNA).[16,19] DGRC8 assists Drosha to cleave approximately 11 bp away from the ssRNA-dsRNA junction.[20] The hairpin of pre-miRNA is about 70 nt in length. Some miRNAs are not dependent upon Drosha-mediated processing, these include miRNAs called mirtrons that are processed by splicing.[21,22] Subsequently, the pre-miRNA is transported into the cytoplasm by Exportin-5,[23,24] where it is being processed by Dicer (a RNase III endonuclease) resulting in the generation of an approximately 22 nt miRNA-miRNA* duplex.[25,26]

Mechanism of target selection

Mature miRNA is incorporated into the RNA-induced silencing complex (RISC) that guides the miRNA towards target mRNAs that lead to reduce protein production, via mechanisms that are still under investigation. Bartel's group found that mRNA destabilization explains most miRNA mediated repression.[27]

MiRNAs pair with target mRNAs at sites complementary to the miRNA 5' region. Most effective sites map to 3' untranslated regions

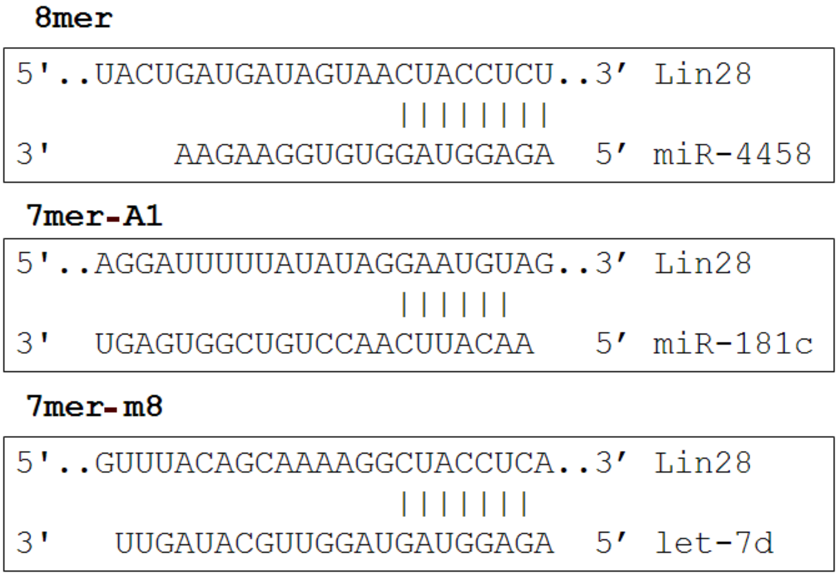

FIG. 2: Examples of the canonical type of miRNA–mRNA target interaction. Vertical line represents Watson-Crick base pairing.

(3’ UTRs) and pair perfectly with the miRNA seed (nucleotides 2–7), with an additional pair at nucleotide 8 and/or an A across from nucleotide 1.[27,28] The canonical site of target recognition is known as the “seed region” which is located at nucleotides 2 to 7 or 2 to 8 at the 5’ end of the miRNA and often has perfect complementarity pairing to the target mRNA[28] (Figure 2). In addition, central pairing (nucleotides 4 to 15) has been shown to lead to Argonaute protein 2 (Ago2) mediated target cleavage.[29]

IsomiRs

The usage of high-throughput deep sequencing has led to the detection of large numbers of miRNAs.[5] Intriguingly, miRNAs encoded by the same gene frequently displayed variation in length from the canonical sequence annotated in miRBase, due to an addition or deletion of one or more nucleotides at the 5’ or 3’ ends or both. These variants are termed as isomiRs.[30] They are categorised into 5’ isomiRs, 3’ isomiRs and mixed (Figure 3). 3’ isomiRs are

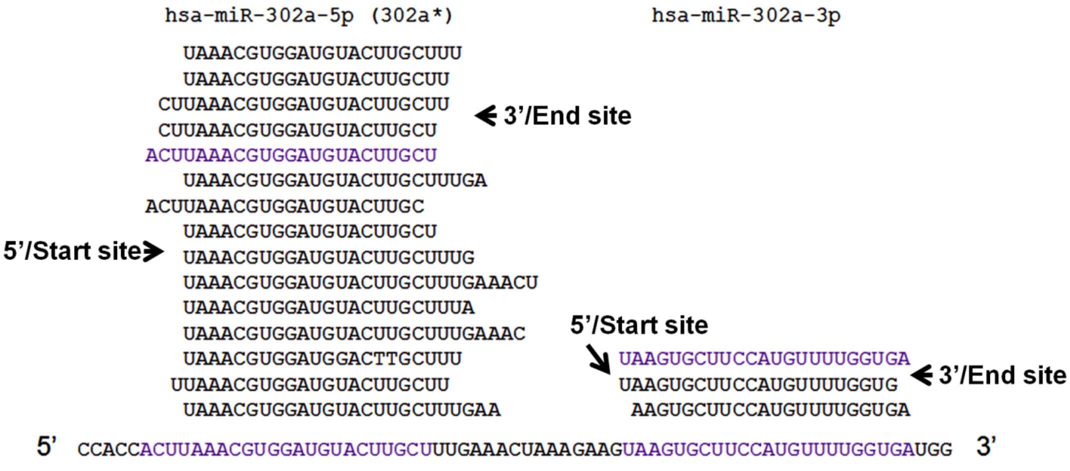

FIG. 3: Different species of isomiRs, using miR-302a as an example (those that are highlighted in purple represent the canonical microRNA)

more frequently observed than 5' isomiRs.[31-35] This heterogeneity in length is thought to arise in part from imprecise cleavage by Drosha or Dicer.[30] The presence of 3' isomiRs are thought to have resulted from trimming, adenylation or uridylation.[31,36-38] Interestingly, Liu *et al.*, (2011)[37] showed that knockdown of *Nibbler* (a 3' to 5' exoribonuclease) was accompanied by loss of some 3' isomiRs.

Some studies suggested that the 3' ends of miRNA extend from the PAZ domain of the Argonaute protein and are therefore available to exonucleolytic attack,[39,40] whereas the 5' ends of miRNAs are buried within the MID domain, hence might be protected.[30] This suggests that 5' end of microRNA might be more important and protected from unwanted alteration. On the other hand, Wu *et al.*, (2009)[41] showed that alternative processing of primary miRNA by Drosha and DGCR8 can generate precursor miRNA with or without 5' end variation. Eventually, these precursor miRNAs may undergo 3' end modification which produces mature miRNAs having 5', 3' or mix variations.[41] In principle, 5' isomiRs have different seed regions to their canonical miRNA and therefore could have a different subset of target genes. Although miRBase (June 2014) has included isomiRs in their database, miRNAs are still annotated as a single mature miRNA sequence.

IsomiRs have been detected in a variety of cell lines, tissues and cancers such as hESCs, endothelial cells, 293T cells, various mouse tissues, prostate cancer, gastric cancer, breast cancer and leukemic cells.[5,35,42-48] There are a number of isomiR databases in the web such as miRBase,[6] YM500,[49] Hood lab (Institute of System Biology 2012 - http://hood.systemsbiology.net/), miRGator v3.0[50] and SeqBuster[51] (Table 1).

Functional significance of isomiRs

There have been some concerns that isomiRs are simply sequencing artefacts. However, "spike in" synthetic RNA oligonucleotide experiments indicate that isomiR identification far exceeds error rates.[31] In addition, bioinformatics analysis using target prediction program predicted conserved target sites for isomiRs, northern blot study showed isomiRs are differentially expressed in tissue and cell lines, immunoprecipitation study showed isomiRs are capable of incorporation into argonaute proteins and luciferase assay showed isomiRs are functionally different from their canonical microRNA.[35]

Target prediction programs

Target prediction programs have been created to attempt to generate predictions of miRNA targets

TABLE 1: List of isomiR databases

No	Name of the database	Web-link	Reference
1	miRBase	http://www.mirbase.org/	Griffith-Jones *et al.*, 2004[6]
2	miRGator v3.0	http://mirgator.kobic.re.kr/	Cho *et al.*, 2013[50]
3	SeqBuster	http://code.google.com/p/seqbuster/ (need to download software)	Pantano *et al.*, 2010[51]
4	Hood lab	http://hood.systemsbiology.net/cgi-bin/isomir/find.pl	Institute of systems biology (ISB) 2012
5	YM500	http://ngs.ym.edu.tw/ym500/	Cheng *et al.*, 2013[49]

based on genome wide computational search for microRNA and mRNA UTR complementary sites. The most significant contribution to target recognition was the identification of Watson-Crick microRNA-mRNA perfect complementarity of 6 to 8 bp at the 5' end of microRNA and 3' UTR of mRNA.[52,53] As a result, the initial method of target prediction was based on complementarity of the miRNA to the target site and the predicted free energy of the microRNA-mRNA duplex.[53,54] Subsequently, a new generation of miRNA target prediction programs emerged in 2005 that are based on more extensive bioinformatics analysis using cross-species comparison, called TargetScan.[55]

In TargetScan, miRNA targets are predicted by searching for Watson-Crick base pairing matches between the seed region and 3' UTRs that are conserved via whole genome alignment. Based on a prediction study, more than 5300 human genes were predicted targets of miRNA, which represented about 20 to 30% of the human gene set.[55] Figure 4 illustrates the conserved predicted microRNA target sites in the 3' UTR of NCAM2 (Figure 4A) (long red arrows). Intriguingly, there are a few other conserved sites (short yellow

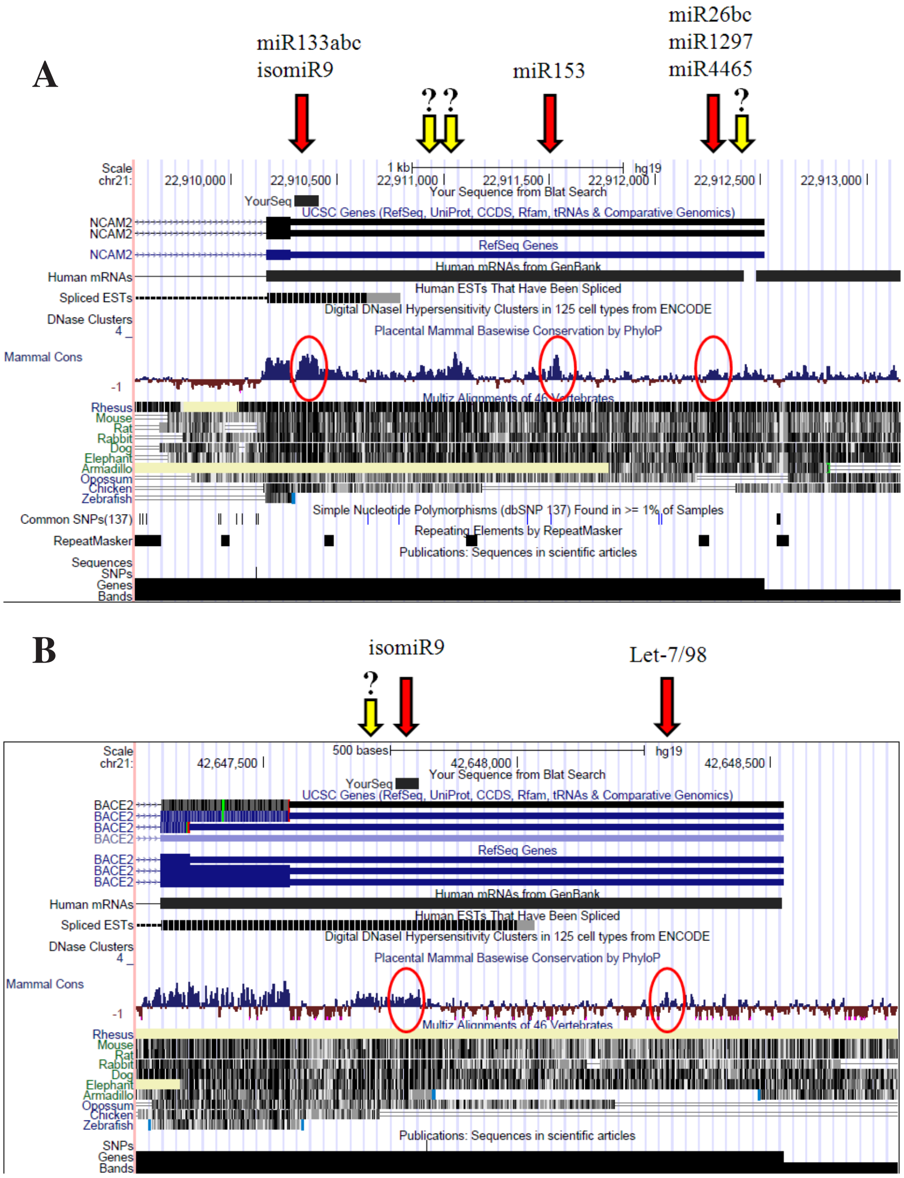

FIG. 4: **Conserved miRNA target sites in the 3' UTR of NCAM2 and BACE2.** Long red arrows represent known miRNA target sites. Short yellow arrows denote conserved sites that are not known to be a target of any canonical/ annotated miRNA. Reproduced and modified from USCS genome browser.

arrows) that are not predicted target sites of any canonical/ annotated microRNA. These sites could be undiscovered target sites of isomiRs or perhaps targets of RNA binding proteins. Another related example is the BACE2 (Figure 4B), where there are 3 highly conserved sites and one of these is a predicted target site of let-7. Notably, one of the remaining two conserved sites is a target site of isomiR-9 (unpublished data).

There are some miRNA target prediction programs available on the web. These programs differ in their selection criteria like the stringency of seed complementarity and measurement of base pairing stability and selection of different UTR sequence.[28,56] Different prediction databases may predict different sets of target genes. The differences in prediction might result from the use of different 3' UTR sequence in the prediction programs.[28] So far, only a small proportion of these predictions have been experimentally validated.[57-59] Bioinformatics analysis of the microRNAs and isomiRs using Targetscan Human and Targetscan Custom predicts that there are many specific targets of isomiRs and that the percentage of common targets is surprisingly low with an average value of about 22%.[35]

Differential expression of isomiRs in tissue and cell lines

Based upon sequencing data, a number of groups have reported that isomiR expression patterns differ between cell lines or tissue types and in some cases the changes are as much as ten-fold.[33,60] IsomiRs were readily detected in a variety of human cell lines and mouse tissue types by northern blotting. Interestingly, the relative ratio of isomiRs encoded by the same microRNA gene varied between cell types.[35] This suggests that isomiRs might play different roles or are required to function selectively in different cell types.

Functional analysis of isomiRs

MicroRNAs execute their function by firstly incorporating into Argonaute protein which belongs to a component of RNA-induced silencing complex (RISC) or microRNA ribonucleoprotein complex (miRNP). Subsequently RISC/miRNP will guide the microRNA to the target mRNA, preventing the translation process. By using co-immunoprecipitation testing, it was shown that isomiRs were able to associate with argonaute (Ago) proteins (Ago1 or Ago2).[35] Similarly, a few other studies have also showed the association of isomiRs with Ago.[5,32,61]

Fukunaga and colleagues described an *in-vivo* study where Dicer partner proteins may bind to Dicer and generate different isomiRs of a miRNA. Loquacious-PA generates a 21-mer miR-307a and loquacious-PB generates a 23-mer miR-307a. Thus altering the Dicer partner proteins changes the choice of the cleavage site, producing isomiRs with different target specificities. Furthermore, the authors found glycerol kinase and taranis were targets of 23-mer miR-307a but not 21-mer miR-307a.[62] This finding complements our *in-vitro* study that isomiRs have a different set of mRNA targets (see below). Humphrey *et al*., (2012)[63] has also presented preliminary evidence to indicate that miR-133a and its isomiR have different target specificities in murine cardiomyoctyes.

In our study, we found that miR-367 and its isomiR-367 (3' isomiR) were able to repress PTEN, a predicted target common to both miR-367 (canonical) and isomiR-367. Subsequently, bioinformatics was used to focus on 5' isomiRs that have different targets to their canonical microRNA. Interestingly, bioinformatics show one of the predicted targets of miR-9 (canonical) is CDH1, while its most abundant isomiR-9 targets DNMT3B and NCAM2 instead. Using *in-vitro* luciferase assay, isomiR-9 indeed targets DNMT3B and NCAM2 and has lost its ability to repress CDH1. Seed sequence mutation studies confirmed that the predicted seed target sites were crucial for the recognition of both miRs and isomiRs.[35]

MicroRNA sponges were first described by Ebert *et al*., (2007)[64] and Franco-Zorrilla *et al*., (2007).[65] These sponges are decoy mRNAs that compete with endogenous mRNA for base pairing with miRNAs. The effectiveness of sponges in general is likely to be dependent upon the relative concentration of endogenous miRNA and sponge expression level (Figure 5). In order to reassure that our finding is valid, we constructed two RNA sponge expression vectors that have specific binding site for miR-9 and isomiR-9, respectively. Our RNA sponges contain six repeated binding sites for either miR-9 or isomiR-9. These binding sites have the same sequence as the target sites within the 3'UTRs of CDH1 or DNMT3B. The "isomiR-9 sponge" could specifically sequester isomiR-9 at a better efficiency than the canonical miR-9, which has just one base difference at the 5' end, and *vice-versa*. This observation reassured

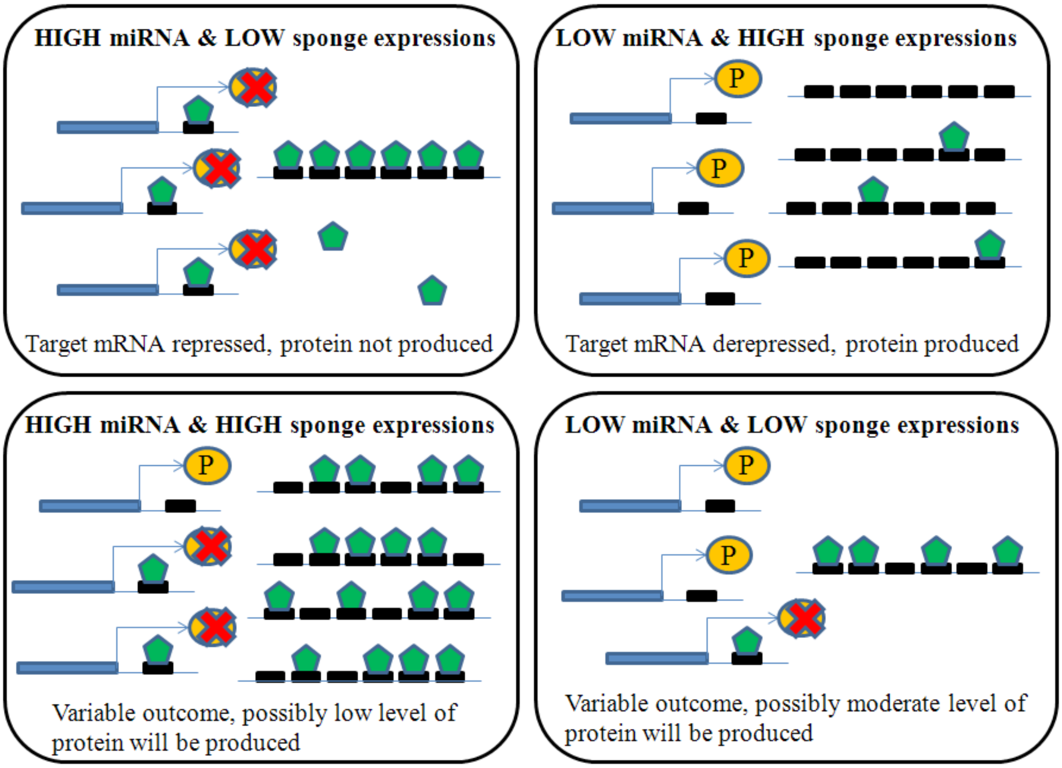

FIG. 5: **Sponges compete with target mRNA for binding with miRNA and the various outcomes as a result of the concentration differences between the miRNA and sponge.** Long blue bar – target mRNA; short black bar – miRNA binding site; orange oval – protein; green pentagon – endogenous miRNA; red cross – protein not produced; arrow – protein translation. In an environment where there is high level of miRNA concentration but low sponge expression, the most likely outcome is protein will not be produced. Conversely, if there is high sponge expression coupled with a low miRNA concentration, most invariably protein will be produced. The situation becomes unpredictable when there is either high level of both miRNA and sponge or low level of both miRNA and sponge**.**

that isomiRs can recognise different targets to canonical/ annotated microRNAs.[35]

Ma and colleagues showed MiR-9 was upregulated in breast cancer cells and repressed CDH1, which promotes cancer cells motility and invasiveness. MiR-9 mediated downregulation of CDH1 is also associated with the activation of vascular endothelial growth factor through the upregulation of beta catenin signaling, which increases tumour angiogenesis. Inhibition of miR-9 by miRNA sponge reduces metastasis formation.[59] Therefore, miRNA sponge might be a useful research tool for future studies as well as a potential inhibitor of oncomiR.

In conclusion, these findings indicate that a microRNA may not be composed of a single strand of nucleotides, instead it is a group of microRNAs that are very similar but differ only in their length with one or more nucleotides at either or both ends. Thus, the function of microRNAs is very complex.

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