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Mini Review

The Use of CRISPR/Cas9 in MicroRNA Function Studies

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Abstract

miRNAs are an important class of gene regulators. Different studies have shown that the deregulation of these miRNAs is linked to diseases and reproductive problems in humans and animals. Thus, characterizing the function of these regulators is of strategic importance in understanding the development of clinical therapies and features of economic interest. CRISPR/Cas9 is a technique that has been used as a strategy for analyzing miRNA function due to its high efficiency and specificity in *in vivo* study. The use of CRISPR/Cas9 in studies of miRNA function is growing and promising results have been presented for use in aquaculture for addressing issues relevant to production, such as muscle growth and reproduction. However, studies of loss of function using CRISPR/Cas9 are still limited.

Keywords: MicroRNA; CRISPR-Cas9; Zebrafish

CRISPR/Cas9: Principle of Technique

CRISPR is a genomic editing technique that has been popular in different research areas due to its precision, low cost and usability [1,2]. The Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR, as it is popularly known, is a region of the bacterial genome characterized by the presence of short and repeated DNA sequences [3,4].

CRISPR-Cas9 was adapted from a naturally occurring genome editing system in bacteria, in which pieces of DNA from invading viruses are inserted between the replicates [5]. This system works like a memory in a future infection, where the bacteria produce enzymes (Cas9 is the best known) to excise the virus that was inserted in its DNA. Based on this bacterial defense mechanism, the scientists discovered how to guide Cas9 enzyme in the sequence to be edited in the genome. The target sequence is recognized using an RNA sequence called a guide RNA (sgRNA) constructed according to the sequence of interest of the genome. In this way the sgRNA leads the Cas9 protein to the target genome sequence to cut the double strand of DNA (Figure 1). Through the DNA repair mechanisms present in the cell, activated every time the DNA is damaged, the CRISPR technique can join the two ends of the DNA fragment that has been cut, allowing the inactivation of genes; or it can insert an exogenous DNA sequence into the organism during the repair, allowing the insertion of genes [6]. Thus, the understanding of how to use the mechanisms of breakdown and repair of regions of interest of the genome allowed developing different CRISPR systems capable of editing the genome of any living organism.

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Genome editing is of great interest in the prevention and treatment of human diseases. Many advances in understanding the development of complex diseases, such as cancer [7,8], heart disease [9] and HIV [10], have been achieved using this technique.

MicroRNAs

MicroRNAs (miRNA) constitute a class of non-coding RNAs that play key roles in the regulation of gene expression [11]. MiRNA are highly conserved sequences of RNA (20 to 24 nucleotides) that regulate post-transcriptional gene expression by binding to the 3' untranslated (3' UTR) region of specific mRNAs (Figure 2). They target the mRNA 3′-UTR, causing the mRNA down regulation either through its destabilization or protein translation inhibition [12]. The

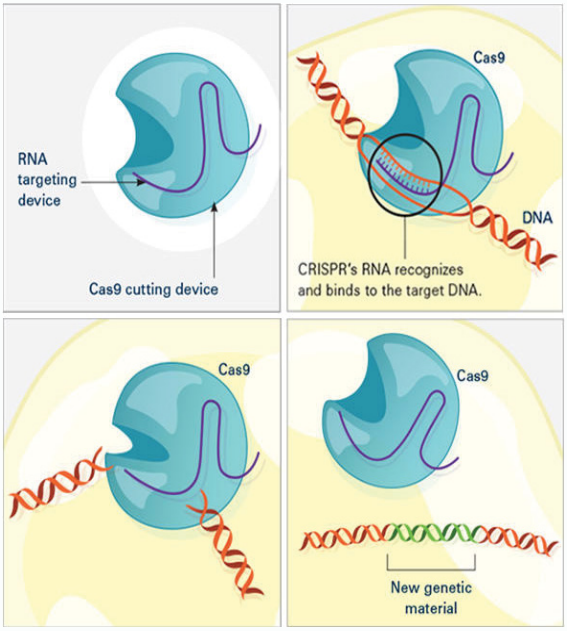

Figure 1: Principle of CRISPR/Cas9 technique; the guide RNA (RNA-targeting device, purple) associated to the Cas9 enzyme (blue) matches up the target DNA (orange), then Cas9 cuts the DNA. A new segment of DNA (green) can then be added or not. Credit: National Institute of General Medical Sciences, National Institutes of Health.

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miRNAs are expressed in the nucleus in a larger primary miRNA form (pri-miRNA) and, after cleavage, results in precursor microRNA (pre-miRNA), which one is transported into the cytoplasm. There, the pre-miRNA sequence will pass through other cleavages to reach its mature form, the miRNA, capable of regulating the expression of target genes.

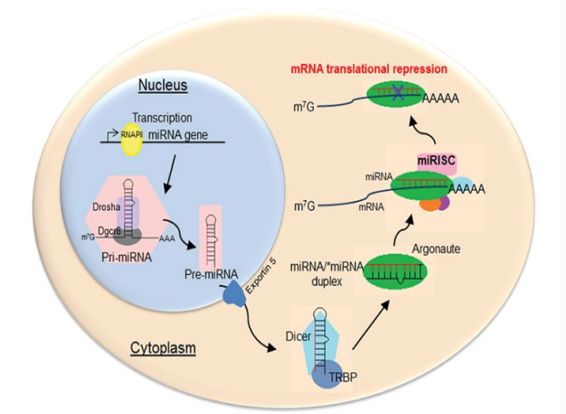

Figure 2: Principle of CRISPR/Cas9 technique; the guide RNA (RNA-targeting device, purple) associated to the Cas9 enzyme (blue) matches up the target DNA (orange), then Cas9 cuts the DNA. A new segment of DNA (green) can then be added or not. Credit: National Institute of General Medical Sciences, National Institutes of Health.

MiRNAs regulate diverse physiological aspects and understanding their biological role is proving increasingly important. Analysis of miRNA function can provide valuable information because the deregulation of its function can lead to human diseases such as cancer, cardiovascular and muscle disease [13,14]. Some muscle-specific miRNAs, such as miR-1 and miR-133 for example, have an essential role in the regulation of muscle energy and protein metabolism [15,16]. The miR-29b plays an important role in the pathogenesis of muscle atrophy in diverse models [14,17]. Over expression of miR-29b promotes muscle atrophy, while reducing miR-29b attenuates muscle atrophy. Consequently, disrupting the expression of miR-29b would be a therapeutic approach for muscle atrophy in several diverse models.

For aquaculture species as Nile Tilapia the miR-29 and miR-129 are over expressed in ovary and they are involved in oogenesis and ovary maturation, respectively [18]. In medaka, miR-202 was associated with reproductive characteristics, such as the number of spawning and the number of eggs per spawning [19].

Identifying Mirnas Involved in a Physiological Process

Gene expression analysis in a given tissue is a common way to characterize the function of a known miRNA. Many studies have revealed this strategy to be effective in associating miRNAs dysregulation with diseases or physiological processes [19-21]. However, to use the study of gene expression as a strategy it is necessary to have miRNA candidates, that is, involved with the function studied at some level.

MicroRNA sequencing (miRNA-seq) is a type of RNA-Seq that use of next-generation sequencing to sequence miRNAs. miRNA-seq allows to identify tissue-specific expression patterns, disease associations, and isoforms of miRNAs, and to discover previously uncharacterized miRNAs [22-24]. Despite presenting advantages, the technique still has a high cost, requires infrastructure and potential artifacts. After detecting the miRNA present in a specific sample or some specific situation, it is possible to identify which genes the miRNAs have homology to using in silico analysis. Thus, it is possible to start organizing the miRNAs sequenced in clusters and, thus, start screening the miRNAs according to the research interest. Different strategies can be used to confirm miRNA function *in vivo*, such as CRISPR, morpholinos, mimics and antisense inhibitors.

Establishing a knockout population using CRISPR/Cas9

CRISPR/Cas9 can be used to perform a miRNA function study, because through gene editing, we can silence the expression of the miRNA and analyze the results directly in the studied organism [25]. Therefore, it is necessary to establish a line in which the gene sequence of the miRNA of interest is absent, and analyze the different genotypes (+/+, +/-, -/-).

Before performing CRISPR/Cas9 it is essential to check whether there is any polymorphism in the individuals. Thus, depending on the region to be edited, genotyping of individuals is necessary in order to verify whether the targets used will be able to hybridize to the genome and allow the deletion to be carried out [26,27].

The creation of a mutant strain is based on design of RNA guides that will be used in CRISPR to signalize to Cas9 the cutting site [27]. To synthesize the guides, a sequence of techniques is needed, which include amplification of the gene region, sequencing, purification, cloning and after in vitro transcription to mRNA, these guides will be microinjected into embryos of up to 2 cells. After the animals grow, they must be genotyped and sequenced to the target region of CRISPR / Cas9 in order to identify whether the mutation is generated.

Table 1: Summary of studies on miRNAs of model and aquaculture species.

Species	miRNA	Associated function	Reference
Oryza latipes (medaka)	miR-202; miR-430c and miR-26a	Oogenesis, folliculogenesis and spermatogenesis; gonad-specific	[19,29]
Danio rerio (zebrafish)	13-miRNA,miR-202-5p,miR-144-5p; miR122a-3p	follicle activation; ovaries	[24,34]
Oreochromis niloticus (nile tilapia)	miR-1 and miR-206; miR-29; miR-129; miR-4585	Myoblast differentiation; myoblast proliferation; Oogenesis; ovary maturation; sex determination	[18,33,35]
Colossoma macropomum (tambaqui)	miR-122; miR-1 and miR-206	Cholesterol metabolism regulation; myoblast differentiation	[36]
Oncorhynchus mykiss (rainbow-trout)	miR-21, miR-30d, miR-90a, miR-200 and miR-26	Cell differentiation and embryo development	[37]
Piaractusmesopotamicus (pacu)	miR-133a/b	Myoblast proliferation	[38]
Ctenopharyngodonidella (grass carp)	miR-1a, miR-181a, miR-133a/b, miR-214, miR-206, miR-146 and miR-26a	Fast-twitch skeletal muscle growth	[39]

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Animals containing the same indel of mutation will be screened for breeding, aiming to obtain animals -/- in F2. Through crosses between homozygous individuals for the desired mutation with each new generation the lineage will be established and analyzes can be performed.

miRNA loss-of-function studies using CRISPR/Cas9

Loss-of-function analysis of miRNA genes has been traditionally challenging due to lack of appropriate knockout tools. The CRISPR/Cas9 technique made studies feasible and allowed advances to be made in large steps in some areas. Studies using the CRISPR/Cas9 technique to study miRNA function are increasing in different species, such as human, zebrafish and medaka, and has been allowing to evaluate phenotypes in the miRNA absence in more accurate way through the establishment of knockout animals [19,24,28]. For example, miR-202-5p was initially identified as gonad-specific present throughout spermatogenesis and was only detected at the early stages of oogenesis, this sex biased expression pattern suggested that miR-202-5p might be a crucial candidate in male differentiation and development [29,30]. However, studies of loss-of-function *in vivo* using knockout animals by CRISPR/Cas9 have shown that miR-202 is crucial for fertility in medaka females [19].

Studies using model species are essential to provide data for advancing the use of CRISPR/Cas9 for aquaculture species. Despite limited, there is an increase in the number of studies identifying miRNAs linked to productive traits, such as muscle growth and game to genesis [19,31-33] (Table 1). In recent years, the number of miRNAs identified is increasing in model and aquaculture species, but there are still few studies of loss-of-function. Establishing knockout populations takes time, varies according to the species used, because it is necessary to analyze the phenotype that individuals present, in addition to genotyping, for each generation. It means that at each new generation all animals must be genotyped and screened to identify the carriers of the same mutation indel, after growth and sexual maturation the animals must have the phenotype evaluated. Genetic knockout of miRNA is the most reliable technique on study of loss-of-function of miRNA with high efficiency and specificity for *in vivo* analysis; however, the manipulation procedure is complicated and the process is time consuming.

Conclusion

Genome editing technologies, such as CRISPR/Cas9, have significant potential to accelerate advances in studies of miRNA function. Establishing a knockout population using CRISPR/Cas9 takes time, due to the screening and growth time of the animals each generation; besides requiring a sequence of laboratory techniques for its execution. However, is the most reliable technique on study of loss-of-function of miRNA with high efficiency and specificity for *in vivo* analysis.

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