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Application of Genome Editing Technology to MicroRNA Research in Mammalians

Lei Yu, Jennifer Batara and Biao Lu

Additional information is available at the end of the chapter

Abstract

Targeted nucleases have recently emerged as a powerful genome editing tool. The ability of introducing targeted, desired changes into mammalian genome makes them an invaluable tool to unravel functions of miRNAs in biology and disease. In combination with homologous donor vector, targeted nucleases can achieve high efficiency and precision, enabling bi-allelic ablation of miRNA in cultured somatic cells. Here we review the structure and function of miRNA as well as the unique implementation of genome editing technology in modifying miRNA sequences in mammalians. This chapter discusses the four mainstay genome editing technologies: meganuclease, zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeat-associated nuclease Cas9 (CRISPR-Cas9), focusing on TALEN.

Keywords: Genome editing, miroRNA, TALEN, ZFN, CRISPR-Cas9, Meganuclease, Non-homologous end joining, Homologous recombination

1. Introduction

Recent achievements in genome editing have resulted in progress beyond any imagination decades ago. This new technology provides tools for fast and precise alterations into genome with unprecedented efficiency and specificity. For instance, a group of targeted nucleases have been successfully used to modify genomic sequences in a wide variety of cells and organisms, including mammalians.[1-4] This modification is realized by the combination of conventional gene knockout technology with genome editing, empowering the researchers to make any desired changes in a given gene or its regulatory elements to establish casual linkage between genetic variations and biological phenotypes. This new methodology completes a framework for studying gene function and regulation *in vitro* and *in vivo*.

1.1. Conventional gene knockout technology

Mammalian genomes contain billions of DNA bases and are difficulty to manipulate.[5-7] Conventional genome editing is inefficient and must undergo a number of complicated and time-consuming procedures to obtain double knockouts. Because this technology had been built upon homologous recombination (HR), the targeted gene modifications occur at an extremely low frequency (1 in $10^6 \sim 10^9$ cells).[8] This is the reason why this conventional approach is mainly used for producing knockout mice, for which a large amount of high quality embryonic stem cells is available. Although knockout mice have provided great insights into the fundamentals of developmental biology and physiology; the pathological roles of genes have yet to be addressed in relevant disease models or human samples.

1.2. Novel genome editing technology: Targeted nucleases

To overcome limitations of conventional gene knockout technology, a number of nuclease-based methods have been developed in the past decade.[9-12] These novel technologies exploit the ability of endonucleases to induce double-stranded DNA breaks (DSBs) and stimulate subsequent damage repair mechanisms in mammalian cells.[11, 13-17] Remarkably, this nuclease-induced DSBs not only make sequence changes at the break points but also enhance HR rate to an astonish frequency of 1-40%.[7, 18] This approach provides a simple and effective way to streamline the genome editing with the potential to generate double knockout models in both cell lines and animals. Because of this technical breakthrough, nuclease-based methods quickly gain popularity for gene editing in variety of cell types and species. To date, four major classes of targeted nucleases are prominent: meganucleases derived from microbial mobile elements, zinc finger nuclease (ZFN) based on eukaryotic transcription factor DNA binding motif, transcription activator-like effector nuclease (TALEN) derived from a plan-invasive bacterial protein and most recently the bacterial type II clustered, regularly interspaced, short palindromic repeat-associated nuclease Cas (CRISPRE-Cas).[19] These technologies are collectively termed as targeted nucleases because they all have more or less a programmable DNA binding domain for specific genome targeting. The first three nucleases all recognize specific DNA sequences through protein-DNA interaction, whereas the last CRISPRE-Cas is targeted by a short guide RNA that recognizes the unwind DNA via Watson-Crick base pairing. The purpose of the nuclease is to make localized DSBs. These DSBs will invoke powerful non-homologous end-joining as well as homologous-direct recombination repair pathway for the versatile and precise modification of complicated mammalian genome.[14, 18, 20-32]

2. miRNA

Large fraction of the genome is transcribed without protein coding potential. [33, 34] These non-coding transcripts can be broadly categorized into short and long non-coding RNAs, in which the arbitrary size delineation is at ~ 200 bases in length. Short non-coding RNAs are less 200 bases including microRNA, tRNAs and snoRNAs. An abundant class of small regulatory

RNAs are termed microRNAs (miRNAs) that have been identified to play major roles in gene regulation.[35-38] Like coding mRNAs, miRNAs are transcribed by polymerase II and processed into ~ 22 nucleotide non-coding transcripts, which repress translation by binding to target sites within the 3' untranslated region of mRNA.[39-41] Recent genomic studies have discovered over 1000 miRNAs in human and mouse genome.[34] It is estimated that up to 80% of human genes are regulated by miRNAs.[36, 42] Some mRNAs are clustered in polycistronic transcripts and allow coordinated regulation, while others are expressed in a tissue-specific and developmental stage-specific manner.[35, 40] The roles of miRNAs have been extensively studied at molecular and cellular levels in a number of species including mammals.[43, 44] Furthermore, their roles in physiology and development in animals have been established by conditional knockins and knockouts.[45-47] In fact, miRNAs are expressed across genome, and many of them show spatial and temporal expression. Similar to transcription factors, miRNAs may function as master regulators of embryonic pluripotency, differentiation, and tissue/organ formation.[37] Recently, increasing evidence suggests that miRNAs are implicated in numerous disease states as well. For instance, miR-21 was found to be overexpressed in virtually all types of human cancers and thus has emerged as an important therapeutic target in cancer treatment.[45, 48, 49] Additionally, miR-21 and other miRNAs have been shown to play crucial regulatory roles in basic cell functions such as cell growth, proliferation and apoptosis.[48, 49] Their pathological roles in multiple human diseases such as autoimmune, cardiovascular and neurological disorders and obesity are emerging.[50, 51]

2.1. Genome organization and biogenesis

MicroRNA genes have a great diversity in mammalian genomes. They are located in either introns of annotated protein-coding genes or outside the context of an annotated genes.[34] Gemone analysis studies reveal that up to 42% of human miRNAs are in clusters of two or more with pairwise chromosome distance of at most 3000 nucleotides. This pattern of clustering allows similar levels of expression and coordinated regulation. Examples of the two most famous clusters are miR-17~92 and miR-302~367. While the deletion of miR-17~92 cluster causes skeletal and growth defects, overexpression of miR-302-367 cluster enhances somatic reprogramming to pluripotent status. These clustered miRNAs appear to function together; therefore alteration of a specific member may not result in expected changes in physiology.

Although the exact mechanism of the regulation of each miRNA remains to be determined, the biogenesis of miRNA is becoming more apparent. From their gene loci, miRANs are initially transcribed by RNA polymerase II as long primary transcripts, which are processed into approximately 70-nucleotide precursors by the RNAse III enzyme Drosha in the nucleus. [52] These highly structured precursors are termed pre-miRNAs and subsequently transported from the nucleus to the cytoplasm by an Exportin-5 protein shuttler.[40] In cytoplasm, these pre-miRNAs are further cleaved by another RNase III enzyme, Dicer, resulting in imperfect miRNA:miRNA duplexes of about 18 ~ 24 bp in length.[53, 54] Although either strand of the duplex may potentially mature as a functional miRNA, only one strand is usually chosen and subsequently incorporated into the RNA-induced silence complex (RISC) where the miRNA and its mRNA target interact.[55, 56]

2.2. Molecular tools for miRNA study

Despite their fundamental importance, the exact role of miRNAs in the context of human development and disease processes remain largely unknown. This is partly due to lack of effective methods for completely abolishing their expression in human cells and disease-relevant models. Although miRNA knockdown by short-interfering RNAs (siRNAs) provides a rapid and inexpensive tool to study most protein-coding genes, it cannot be used to reduce mature miRNAs in a sensible way at the cellular level. Other alternatives include small molecule inhibitors, antisense oligo-nucleotides, anti-miR vectors and miRNA sponges.[57, 58] However, the major limitations of these methods are transient nature of their effects and a high risk of off-target effects as well as toxicity. It is no surprise to see reports on discrepancies between the effects of miRNA inhibitors and genetic knockouts. With the completion of the genome sequencing, genome-wide gene targeting knockout of miRNA have been taken.[59] As a result, resources for the conditional ablation of miRNAs in mouse have been attempted. [60] In one study, generation of 162 miRNA targeting vectors and 46 germline-transmitted miRNA knockout mice was reported.[60] However, this homologous donor based knockout technology is expected to have tremendous hurdles to apply to other mammalian species such as rat, pig and monkeys. These larger animals are more close to humans; however, they all lack large scale culture of high quality embryonic cells to make use of this conventional technology. Nevertheless, the work in mice will provide an important basis for elucidating the physiological roles of certain miRNAs in at least one animal species.

3. Targeted nucleases

Programmable DNA-binding proteins have emerged as an exciting platform for engineering targeted nucleases for precise genome editing.[61] The key component of these engineered nucleases is the DNA recognition domain that is capable of directing the nucleases to the specific genome loci therefore generating DNA double-strand breaks (DSBs) near or at the target sites.[62] In mammalian cells, these DSBs are repaired by one of two mechanisms, non-homologous end-joining (NHEJ) or homologous recombination (HR).[18] Repair by NHEJ is error-prone and often results in small insertions or deletion mutations, termed indels, at the break point. At the same time, DSBs can also greatly stimulate high-fidelity HDR repair mechanism in fast dividing cells. In mammalian cells, NHEJ is dominant over HR, but the frequency of latter can be substantially increased when large amount of exogenous homologous donors are co-delivered into cells.[25]

With these strategies, various methods, including meganucleases, ZFNs, TALENs and CRISPRE-Cas have been reported for genome editing in a wide variety of mammalian species. [63-70] For these methods, a DNA endonuclease enzyme for generating DSBs is brought in place by a guide molecule. In the first three scenarios, the guider is a protein, whereas the last (CRESPRE-Cas) is a short stretch of RNA. Figure 1 illustrates the action of targeted nucleases and the binding mode of guide molecules. In general, the protein guider is more specific than the RNA guider, where degeneration is governed by hybridization mechanism. The pros and cons as well as their features of four main technologies are discussed below.
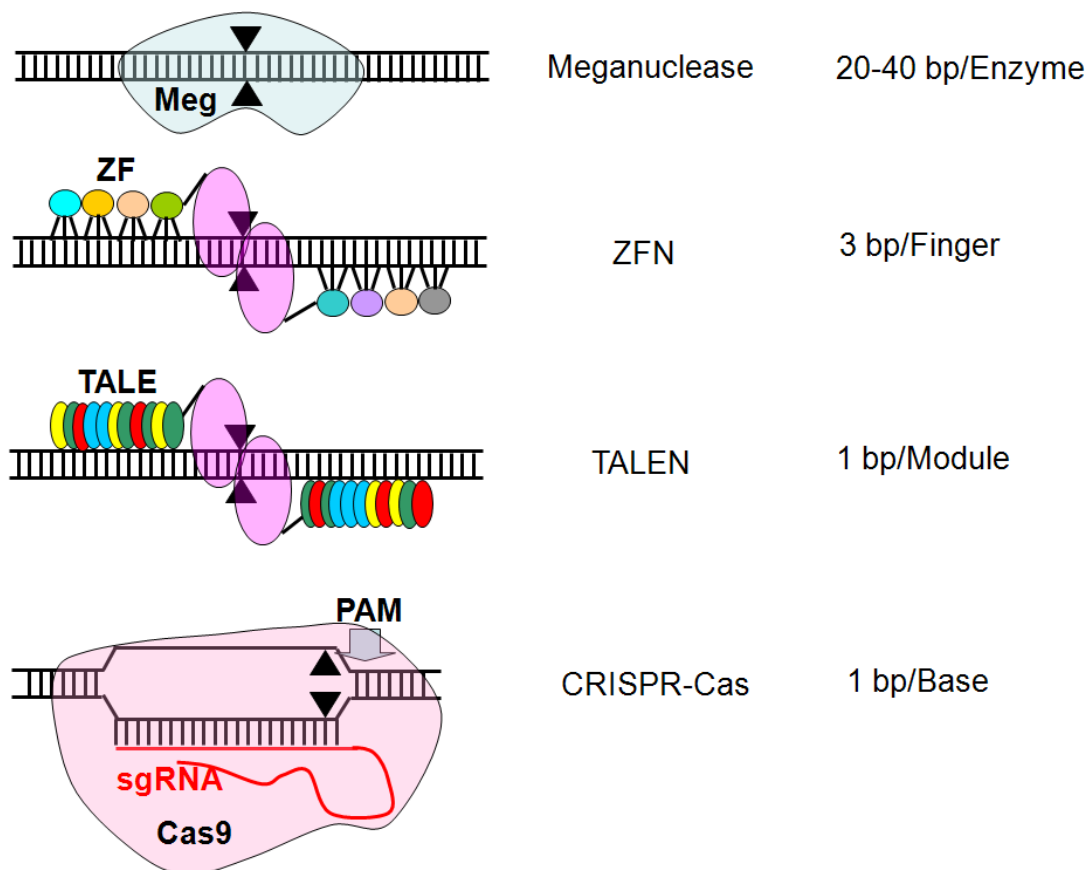
Figure 1. Action of the Targeted Nucleases and Their DNA Binding Mode. Genomic DNA is shown horizontally in black and double stranded, with the site of DNA cleavage indicated by arrowheads. For meganuclease, the holoenzyme binds and cleaves the target DNA. For ZFN and TALENs, they function as a pair; with one zinc finger DNA binding domain (ZF) binding to the upper strand while the other ZF binding to the lower strand. Once this fused FokI enzyme (purple oval) is oriented to form a homodimer, it is activated to cut DNA. For CRISPR-Cas, the Cas9 holoenzyme (orange oval) is directed to the target site by the guide RNA and cleave the DNA at the position close to the PAM motif (grey arrow).

3.1. Meganuclease

Meganucleases are endonucleases with a large DNA recognition site of 12 ~ 45 bp in length.[71] As a result, this site may occur only once in most mammalian genome.[6] Although Meganuclease possesses high degree precision and low toxicity, its target range is limited. Moreover, the intertwined DNA-binding domain and nuclease domain restrict its capacity to reprogram for other targets, and the probability of finding a meganuclease for cutting a desired locus is extremely slim.[72] For example, the phiC31 integrase mediates recombination between a donor DNA of two 34 bp sequences, termed as attachment sites (att) and the other in mammalian genome. In the introduction of phage integrase, a phiC31 integrase can insert a plasmid donor DNA of any size and requires no additional co-factors. Other advantages of using phiC31 integrase include non-viral delivery, sustainable transgene expression, and functions in species like bacteria, yeast, plants, frogs, chickens, mice, rats, pigs, cows, and humans.[2, 73] However, potential sites of phiC31-based genome editing are limited. In human genome,

of the 106 mapped integration sites of phiC31, ~ 39% are within coding genes and ~ 61% are in the intergenic regions.[74]

3.2. Zinc finger nuclease

First described in 1996, ZFN is a chimeric protein that is composed of two distinctive domains, a programmable DNA binding domain and an endonuclease, FokI.[75] Because of its programmability, ZFN have been successfully employed to modify almost all genome types including bacteria, plants, and animals.[1, 76, 77] In fact, ZFNs have been used for the correction of a number of hereditary diseases such as hemophilia B, sickle cell anaemia, a-1 antitrypsin deficiency and gene therapies for viral infections.[77-80] ZFN-based HIV gene therapy is already under clinical trials, because of its specificity and safety profile.[81, 82] However, the use of ZFNs has been partly hindered by the complex and time-consuming strategies to generate highly specific zinc-finger arrays with sufficient affinity and specificity. It is worthy to note that ZFNs also suffer some constrains in targeting range with about one potential target site every 500 bp.[83] It is conceivable that one may find it difficult to design ZFNs to precisely targeting a smaller gene in genome, such as miRNAs.

3.3. Transcription activator-like effector nuclease

Similar to ZFNs, TALENs are also fusion proteins that comprise of a programmable DNA binding domain with an endonuclease, FokI. The DNA-binding domain of TALEN consists of ~34 amino acid repeats, followed by a single half repeat of 20 amino acids.[84] Interestingly, the tandem repeats are nearly identical, except for two amino acid codons at positions 12 and 13, referred to as "repeat variable di-residue" (RVD). [85]Each of the four most common RVDs specifies the binding to one of the four nucleotide bases (Table 1). The natural RVD for G is NN with asparagine at positions 12 and 13. NN binds G with high affinity, but also recognizes and binds A with relative low affinity.[86] Although artificial NH or NK provides good specificity for G, the binding affinity to G is relative low as compared to NN.[86] It is worth to note that TALE proteins bind DNA sequences with an invariable base T in the first position of the target. The corresponding module is not the repeat but the cryptic sequences flanking the repeats. Because the first binding base is invariable, the DAN-binding domain can theatrically be programmed to bind any sequences starting with a "T". Taking advantage of the simplicity of the coding principle, the DNA-binding domain can be easily designed to allow binding of almost any sequences within genomes. Owing to the repetitive nature of the DNA binding domain, the assembly of the custom TALENs by direct synthesis or traditional cloning is expensive and technically challenging.[87] Realizing the potential of TALEN technology, a number of approaches for TALEN assembly have been devised to allow low ~ medium throughput, or high-throughput with automation. Fortunately, a number of Biotech companies provide either assembly kit and/or service due to its technical difficulty (Table 2). Like ZFN, this genome editing technology has been shown to function in a wide variety of cells and organisms, including bacteria, yeast, plant, insect, zebrafish and mammal.[21, 88-92] Furthermore, unlike meganuclease or ZFN that limit the choice of targets, TALEN can virtually bind any loci in the genome with new design that removes the 5' first "T" base constrain.

34 amino-acid repeat	RVD	Deoxynucleotide
LTPEQVVAIASNIGGKQALETVQRLLPVLCQAHG	NI	A
LTPEQVVAIASNGGGKQALETVQRLLPVLCQAHG	NG	T
LTPEQVVAIASNNGGKQALETVQRLLPVLCQAHG	NN(NH*/NK*)	G or A
LTPEQVVAIASHDGGKQALETVQRLLPVLCQAHG	**HD**	**A**

*Note: NH and NK favor specificity rather than activity. They bind to G more specifically but with less affinity as compared to NN.

Table 1. TALEN DNA binding repeat and its simple code scheme

Company	Web Address	Tool Kit and Service
Addgene (Cambridge, Massachusetts)	www.addgene.org	Collections of TALEN assembly kits for multiple organisms
Cellectis (Paris, France)	www.cellectis.com	TALEN or meganuclease-based services
GenCopoea (Rockville, Maryland)	www.genecopoeia.com	TALEN design and assembly; HR donor design and cloning; Indel detection kits
GenScript (Piscataway, New Jersey)	www.genscript.com	TALEN gene synthesis
Life Technologies (Carlsbad, California)	www.lifetechnologies.com	TALEN design and assembly services for genome editing; Targeted gene activator/ silencer cloning services

Table 2. Selected companies with TALEN tool kit and service

3.4. CISPRE-Cas9

Unlike ZFNs or TALENs, CRISPR-Cas needs a short RNA for target site recognition, which is mediated by Watson-Crick DNA base pairing. To form a triplex with genomic DNA, CRISPR-Cas9 also requires a NGG protospacer adjacent motif (PAM) immediately downstream of the hybrid region.

The bacterial CRISPRE-Cas9 system can be reconstituted in mammalian cells using three components: a programmable, specificity-determining CRISPR RNA (crRNA), an auxiliary trans-activating (tracrRNA), and a CRISPRE-associated endonuclease Cas9 (Cas9).[93, 94] In current applications, crRNA and tracrRAN duplexes are fused to generate a chimeric sgRAN that mimics the natural crRNA-tracrRNA hybrid. The single sgRNA has been shown to interact with the holoenzyme Cas9 to generate efficient cleavage.[95] The adaptation of the CRISPR-Cas9 by the research community have been phenomenal, and the system has been used to modify genes in most model species, including drosophila, silkworm, plant, C elegan, zebrafish, Xenopus, rat, mouse, pig, and human.[96-106] The success is mainly due to some significant advantages. First, a single protein Cas9 is required and remains the same. This means no time-consuming protein engineering is required, in contrast to that of meganuclease,

ZFN or TALEN. Second, genome targeting depends on an oligonucleotides (20 ~ 30 in length), which are very easy and cheap to produce. Third, among the established programmable DNA-binding domains, CRISPR-Cas9 is most easily to facilitate genome-scale perturbations owing to its one-binding to one target. Last, this is an open system, where most of them are established and can be purchased from the non-profit distributor Addgene in Cambridge Massachusetts. However, the sequence of individual guide RNA makes difference in terms of efficacy and specificity, not all guide RNA provides the same high levels of genome editing activities.[107] Bad guide RAN can elicit off-target effects, causing cyto-toxicity to cells.[108] This can be very problematic for therapeutic applications.

The technologies are still quickly evolving, and tools to better assess both efficiency and specificity will be essential to improve the system. Choosing certain method shall be carefully balanced to serve the need of research. For example, ZFNs, TALENs and CRISPR-Cas9 are reasonable options to target most coding genes. ZFNs and TALENs shall be preferred when specificity and low-toxicity are required. For genome-wide screen or in dealing with multiple targets, CRISCR-Cas9 is more suitable because of its robustness and one molecule for one target. For small gene manipulations, TALENs may be of choice since they have broad rang access in the genome and edit precisely the target with high specificity with much less toxicity and off-target effects as compared to CRISCR-Cas9.

## 4. Design strategy and experimental approaches

4.1. Design strategies for miRNA targeting

One of the challenges in knocking out a miRNA is that the mature and fully functional miRNA is only ~ 22 nucleotides in length. Therefore, sequence alterations outside this 22-nucleotide region may have little or no effects on function of miRNA. To design useful targeted nucleases, sites have to be carefully chosen so that targeted nucleases are directed to the critical region of miRNA gene loci. To this end, the relative small but highly structured pre-miRNA (~70 bp) appears appropriate since it contains sequences vital for miRNA biogenesis and function.[35, 43] Within pre-miRNA, the seed regions of 5p and 3p as well as the dicer processing sites are of choice (Figure 2). Indeed, a number of reports have used this strategy for miRNA knockouts; studies have shown that small indels targeting these sites are effective to abolish miRNA expression in mammalian cells.[45, 46, 59]

Here, we use miR-21 as an example to illustrate the valid target sites that were successfully used in knockout studies. We choose miR-21 for a number of reasons. First, the human miRNA-21 was one of the first mammalian genes identified. MicroRNA-21 is located on plus strand of chromosome within a protein coding gene TMEM49. It is independently transcribed as a ~3433 nuclotides long primary transcript, where the pre-miR-21 (72 nucleotides) has a typical stem-loop (hairpin) structure similar to other miRNAs.[109] Second, the mature miRNA sequence is typically processed from the 5'-arm of the miR-21 precursor.[110] Last, miR-21 is strongly conserved and its role in physiology and pathology has been extensively

explored and firmly established.[45, 48, 49, 109] Together, miR-21 provides general design strategies for miRNA gene editing.

As illustrated in Figure 2, the region corresponding to 1-8 nucleotides of the mature miRNA is the most preferred site of cleavage. This is because a small indel in this seed region is expected to abolish miRNA activity.[111] The second choice would be the adjacent Dicer processing site of the seed region. In principle, the chosen of process sites shall be preferred to the one that close to the seed region, indels involves both process site and seed sequences would most likely results in complete knockout. According to above consideration, the 5′ miRNA seed region and the adjacent Dicer processing site are usually chosen for miR-21 knockout. Similarly, the 3′-arm of the miRNA precursor shall be preferred when the mature miRNA is of 3p miRNA.
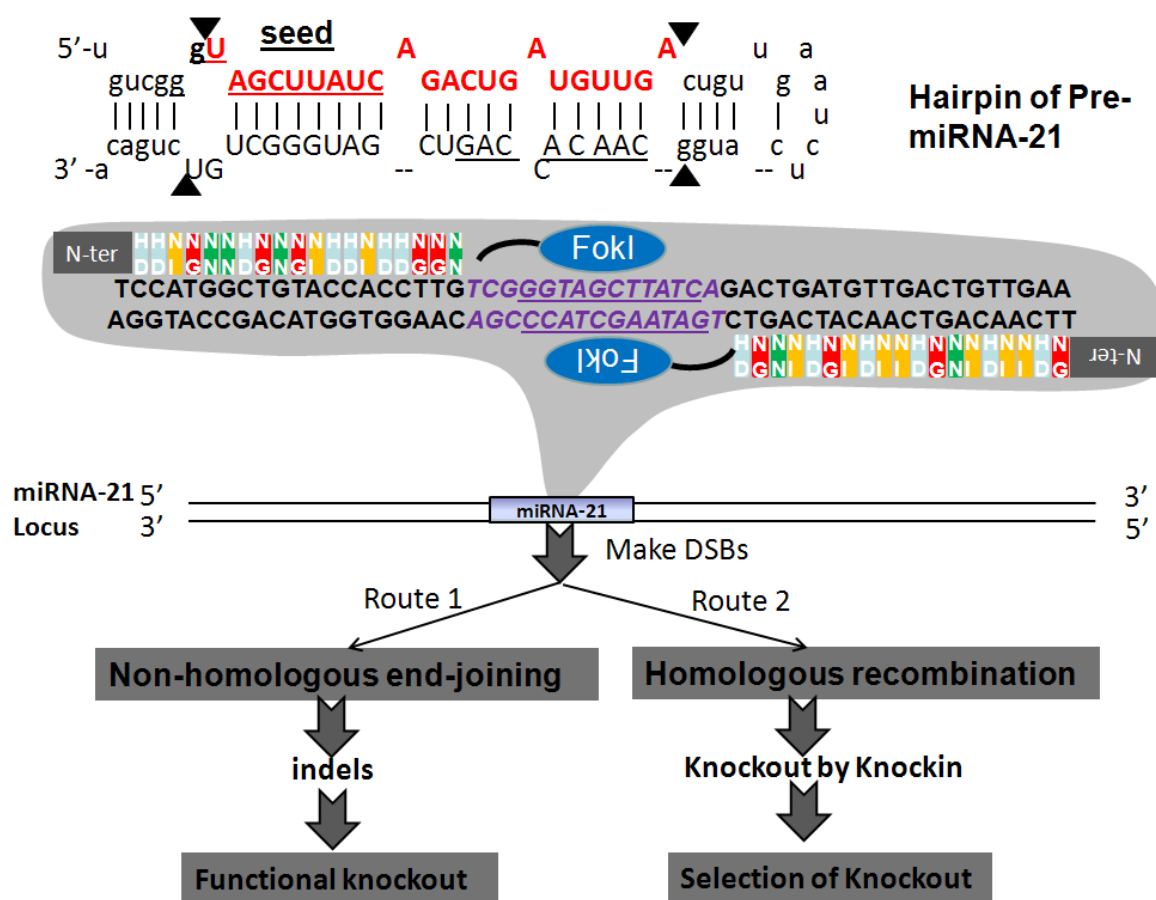
Figure 2. MicroRNA knockout strategy and the preferred cleavage sites. The upper panel shows the stem-loop structure of miR-21, with mature miR-21 shown in red and seed region underlined. The middle panel shows the TALEN pair and their binding sequences, separated by a 15-bp spacer. The cleavage site normally falls in the middle of the spacer, where the FokI dimerizes and makes double-strand breaks (DSBs) at the seed and/or Dicer process site. These DSBs can lead to two potential consequences as illustrated in the low panel. The predominant route 1 leads to indels via non-homologous end joining mechanism, where the alternative route 2 may lead to a precise genome editing via homologous recombination. Repair by homologous recombination can be used to bring in any desired changes at the targeted site. For knocking out miRNA-21, replacement of pre-miRNA with selection markers can facilitate the enrichment and selection of edited cells.

Here we introduce the potential sites as a general strategy for miRNA knockout utilizing the dominant NHEJ mechanisms. In fact ZFNs, TALENs and CRISPR-Cas have been used to functionally knockout a number of miRNAs in mammalian cells.[45, 59, 112-116] Among the three types of programmable nucleases, TALENs are suitable for small loci with narrow targetable regions, whereas ZFNs and CRISPR-Cas are limited by availability of binding modules and the requirement of PAM motif respectively. It is important to keep in mind; the binding sites of targeted nucleases may be different from the cleavage sites in terms of design. For ZFNs and TALENs, the cleavage site is situated in the middle of their binding sites of ZFNs or TALENs. For CRSPR-Cas, the cleavage site is within the guider RNA and close to the PAM motif (Figure 2).

4.2. TALEN design and assembly

Free online tools such as TALEN Targeter and SAPTA are available to design DNA binding domain of TALEN that is specific and has a low risk of of-target effects.[117, 118] Similar to ZFNs, TALENS s requires the design of protein pairs, which bind two optimal anchoring positions on opposite strands, usually spaced 15 ~ 25 bp apart to allow for FokI dimerization and cutting.[119] Length of DNA binding sequences may vary, typically ranging from 14 ~ 20 bp. In humans, 20 bp may offer high specificity, considering the genome size. It is worthy to note that longer domains (18 ~ 20) may decrease cell toxicity by reducing the risk of off-target effects.[120]

A number of approaches have been developed for rapid assembly of custom TALENs. With these advances, TALEN pairs can be generated easily and economically in a matter of days. [121] Most of the methods rely on the ability of type IIS restriction enzymes to assemble premade repeats into fully functional TALEN scaffold (Figure 3). A TALEN scaffold is comprised of a number of domains from the N-terminus to the C-terminus, including a nuclear localization signal, part of the N-terminal sequences for the first "T" recognition, flowed by the last half-repeat and part of C-terminal sequences fused with a nuclease FokI. Because the last binding nucleotide can be any one of the four, the TALEN scaffolds normally have four different flavours. To make the TALEN fully functional, an eukaryotic gene promoter is normally placed at 5' of TALEN coding region and a poly A signal sequences at the 3' end of TALEN.

Most assembly protocol is based on the Golden Gate method, which relies on the ability of type IIS restriction enzymes to cut outside their recognition site. Type IIS recognition sites arranged in inverse orientation at the 5' and 3' end of DNA fragment will be removed upon cleavage, slowing simultaneous restriction and ligation. The continuous re-digestion of unwanted ligation products increases the formation of the desired construct.[122, 123] As type IIS fusion sites can be designed to have different sequences, Golden Gate cloning enables directional and seamless assembly of multiple DNA fragments. Based on this principle, one-step or two-step assembly protocol or kits are developed and commercially available to allow do-it-yourself assembly of TALEN in any molecular biology laboratories.[121]
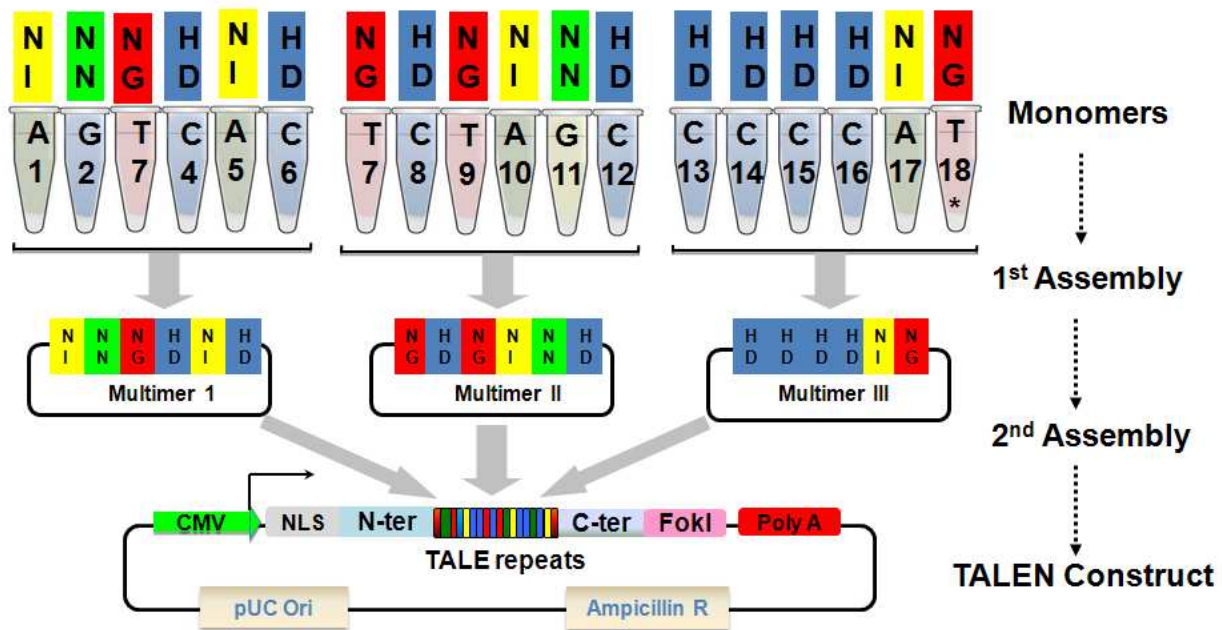
Figure 3. Golden Gate assembly of TALENs and molecular structure. Two-step assembly strategy can increase the correct joining of monomers into multimers and subsequently into the TALEN scaffold. A TALEN, from its N- to C-terminus, is composed of a nuclear localization signal peptide (NLS), modified N- and C-termini flanking the assembled RVDs, and fused nuclease FokI at C-terminus. CMV (cytomegaloviral) promoter situates at the 5′ of the TALEN and drives its expression in mammalian cells. The polyadenylation signal (Poly A) is to add a poly(A) tail to the TALEN mRNA.

4.3. HR donor design and construction

While it is possible to disrupt genes in mammalian genome with TALENs alone, the frequency of gene editing is typically 2 ~ 40%, averaging ~16% for mono-allelic disruptions.[87] Cells carrying bi-allelic disruption are rare and require time-consuming signal cell-derivation and subsequent screening.[5] One strategy is to combine TALENs targeting to the miRNA seed region with a homologous recombination of donor vector carrying a selectable marker.[46, 59, 114] This approach enables convenient positive selection, and the combination of NHEJ with stem-loop deletion results in efficient bi-allelic miRNA gene ablation, which can be as high as >90%.[46]Additionally, by using HR donors, endogenous loci can be potentially modified with custom sequences such as IRES-florescent proteins to allow functional assessment of endogenous miRNA expression and regulation.[124]

4.4. Generation of knockout cell clones

All targeted nucleases can be used in mammalians to create miRNA knockouts.[45, 46, 59, 113-116] Success of miRNA gene editing depends largely on the ability to deliver all the

reagents efficiently to the cells. For transgenics, direct injection of DNA vector or sometime *in vitro* transcribed nuclease mRNAs into embryos is effective.[88, 125] For cultured cells, the options include plasmid DNA transfection, viral deliver, and transfection with synthetic mRNA or proteins.[126-130] Normally, donor DNAs can also supplied via plasmid format and co-delivered with nucleases using the same methodology. To deal with difficult cell types, viral transduction appears to be effective. Although lentiviral system appears to work well for both ZFNs and CRISPR-Cas, it is not suitable for TALEN delivery due to its incompatiblility with the tandem repeats of TALEN.

Following the delivery, small-scale sequence changes are introduced at the break by NHEJ. [18] These indels are typically assayed by polymerase chain reaction (PCR) amplification of the region, followed by DNA sequencing or by a gel electrophoresis assay using T7E1 endonuclease, or alternatively by high-resolution melting analysis.[131] In addition to detecting changes at genomic level, a reverse transcripts-PCR on the expression can also be performed to confirm the reduced/ablated expression of miRNA at transcript level. PCR-based genotyping can be used to make distinctions between HR and NHEJ events when donor DNA is used.[131]

5. Applications of targeted nucleases for miRNA research

Targeted nucleases are powerful genome editing tools for uncovering gene functions. Though relatively new, they have been successfully employed in a broad variety of systems and produced exciting results for miRNA research.

5.1. Knockout of the miR-200 family

To understand the biological and pathological significance of miRNAs, a talen-based knockout library for 274 highly conserved human miRNAs has been established.[59] To demonstrate the genome editing activities of the TALEN library, 66 TALEN pairs against 33 miRNA loci are selected. All TALEN pairs tested induce mutations as assessed by a mismatch-senstivie T7EI assay with a frequency above 0.5%.[132] To gain some insight of functional role miRNAs, the authors conduct detail analysis on members of the miR-200 family.

There are at least two members in the highly conserved miR-200 family, miR-141 and miR-200c. [59] Interestingly, miR-141 and miR-200c have largely indistinguishable activity and differ only in the seed region by one nucleotide. This imposes a great difficulty to use either over-expression or complementary inhibitor-based knockdown to investigate the potential functional divergence without complications. Using TALEN technology, however, the authors can target the seed region of the 5p strand for miR-141 but choose the Drosha processing site in the 3p strand for miR-200c, hence avoid cross-targeting.[52, 110] With this design, both single and double knockout clones were obtained and their corresponding expression of mature miRNAs was confirmed by RT-PCR.[59] Using these cell models, the authors found that miR-141 represses the expression of mRNAs that have miR-141 motif at the 3'-untranslated region. Similarly, miR-200c represses expression of mRNAs that have miR-200c motif. These

data indicate the two closely related miRNAs do not cross-react notably and may control largely nonoverlapping group of genes. Together, TALEN-based method may provide unprecedented tools for miRNA research with great precision and specificity.

5.2. miR-21 ablation and cancer research

MicroRNA-21 gene knockout in the cultured human cells was achieved independently by two research groups.[45, 131, 133] The first group used a combination of TALEN pair with a HR donor. TALENs were designed to position the miR-21 seed region in the central portion of the spacer, directing the cleavage to the functionally essential miRNA motif. The HR donor construct was created corresponding to the cleavage location of the TALEN pair and carried 509-bp (5' arm) and 600-bp (3' arm) regions of homology to the miR-21 genome sequences. TALEN pair and HR donor were delivered together using transfection proto-col. In the case of HR events, the donor replaces the entire miR-21 precursor with two selectable marker genes (red florescent protein and puromycin resistant gene). Clonal population of cells in which an HR event occurred can be easily selected by puromycin treatment. Because NJEJ is the predominant repair mechanism induced by DSBs, selecting for HR events would most likely produce clones that harbour bi-allelic modifications, with the second allele carrying an indel in the seed region. In fact, this approach demonstrated bi-allelic miR-21 gene disruption at very high frequency of 87% in cultured HEK293 cells. [131] Analysis of three independent clones showed a total loss of miR-21expression. Phenotypical examination revealed an increase in miR-21 target gene expression, reduced cell proliferation, and alteration of global miRNA expression profiles, which is in agree-ment with the role of miR-21 in cancer biology.[45, 48]

5.3. miRNA knockout for transgenics

To explore the gene specific-function *in vivo*, generation of gene-targeting animal is powerful strategy. To this end, targeted nucleases have demonstrated that all nucleases are applicable for generating knockout animal and have several advantages over the conventional embryonic stem cells or nuclear transfer technologies. Recently, ZFNs, TALEN and CRISPRE-Cas were able to generate knockout animals in several model species in addition to the established spices of mice.[19] One of the strong merit is that they are free of constrain from using embryonic stem cells. The genome of the fertilized zygotes can be directly modified by injecting the targeted nuclease. Additionally, these new tools are robust enough to allow a simultaneous knockout of multiple genes, thus greatly accelerating the experimental speed and answering complicated biological questions.

Targeted knockout of miRNAs in mice by TALEN has also been reported.[113] In this study, microinjection of synthesized mRNA of TALEN was carried out in one cell stage of embryo. Embryos were allowed to develop to two-cell stage and subsequently transferred into pseudopregnant female mice. With optimized protocol, these mice were able to produce 29.6% mono-allelic offspring. Further cross-mating of the heterozygous founder mice with the wild type strain was able to produced heterozygote offspring, suggesting transmittable of the mutated miRNA.

6. Prospects and challenges

Targeted nuclease technology has become one the most powerful and versatile platforms for engineering biology. The new technology is enabling systematic interrogation of genome, including miRNAs in mammals with high precision and efficacy. It is superb over other technologies by creating null mutations that lead to complete suppression of gene expression. Furthermore, bi-allelic ablation of miRNAs in cultured somatic cells opens new avenue to study the pathological role of miRNAs in relevant disease samples. Knowledge gained from these studies is more likely lead to discovery of novel drug targets, accelerating new therapeutics towards clinic application. In deed, a number of animal studies as well as clinical trials using targeted nucleases already provide encouraging results. In addition to repairing mutations underlying inherited diseases, targeted nucleases can be used to create productive mutations in tissues to combat viral infections or complex diseases such as familial hypercholesterolemia and hypertension.

Targeted nucleases can be tweaked to carry out other functions, such as modifying DNA associated histones, activating or inhibiting gene transcriptions and monitoring chromatin dynamics in living cells. It becomes increasing apparent that targeted nuclease is a versatile and common platform for elucidating gene function and epigenetic regulation.

However, many challenges still lie ahead. The most prominent issue may be the off-target effect. In this aspect, ZFNs and TALENs appear less problematic owing to the intimate interaction with their binding sites and the requirement of correct binding of two molecules. In contrast, CRISPRE-Cas tends to have higher off-target effects, because it binds DNA via base pairing. One or more mispairings may be tolerated, which may cause detrimental unintended effects.[134] Attempts to solve this issue include improvement of guide RNA scaffold, use of computational algorithms to predict off-targets, and development of high throughput method to assess unintended cuts. The challenge, though, is difficult to detect the off-target cleavage. Hence, one shall interpret the data with caution, and is aware of the potential off-target effects and its related toxicity. Secondly, although nuclease molecule may have high level of activity within cells, one may find that some tissue types are still difficult to deliver into. In this case, multiple delivery methods shall be tried, suitable one shall be optimized. Finally, for further development toward clinical application, it will be essential to thoroughly the safety and toxicity profile using a variety of mammalian models.

7. Conclusion

Advance in genome engineering technology based on the targeted nucleases are enabling the systematic interrogation of mammalian gene function. Using this system, miRNA gene sequences within mammalian genome can be easily edited with high efficacy and precision. Targeted miRNA editing will empower researcher to reveal the complex regulatory circuits governed by miRNAs and to realize, in the long-term, their full diagnostic and therapeutic potential.

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