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Review

Cancer Research

Emerging Role of CRISPR/Cas9 Technology for MicroRNAs Editing in Cancer Research

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Abstract

MicroRNAs (miRNA) are small, noncoding RNA molecules with a master role in the regulation of important tasks in different critical processes of cancer pathogenesis. Because there are different miRNAs implicated in all the stages of cancer, for example, functioning as oncogenes, this makes these small molecules suitable targets for cancer diagnosis and therapy. RNA-mediated interference has been one major approach for sequence-specific regulation of gene expression in eukaryotic organisms. Recently, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system, first identified in bacteria and archaea as an adaptive immune response to invading genetic material, has been explored as a sequence-specific molecular tool for editing genomic sequences for basic research in life sciences and for therapeutic purposes. There is growing evidence that small noncoding RNAs, including miRNAs, can be targeted by the CRISPR/Cas9 system despite their lacking an open reading frame to evaluate functional loss. Thus, CRISPR/Cas9 technology represents a novel gene-editing strategy with compelling robustness, specificity, and stability for the modification of miRNA expression. Here, I summarize key features of current knowledge of genomic editing by CRISPR/Cas9 technology as a feasible strategy for globally interrogating miRNA gene function and miRNA-based therapeutic intervention. Alternative emerging strategies for nonviral delivery of CRISPR/Cas9 core components into human cells in a clinical context are also analyzed critically. *Cancer Res; 77(24); 6812–7. ©2017 AACR.*

Introduction

MicroRNAs (miRNA) include a set of short, noncoding protein, and small RNA molecules with a length of 20–24 nt, which are generated by the RNase-III-type enzyme Dicer from an endogenous transcript that contains a local hairpin structure (1). The key signature of miRNAs is their master role in repressing the expression of multiple protein-coding genes at the transcriptional, post-transcriptional, and/or translational levels by binding to the promoter regions, 3′ untranslated region (3′-UTR), 5′-UTR, and the coding regions of target sequences (2).

Growing evidence suggests that miRNAs might play an unanticipated role in pathogenesis of all types of human cancers (2). In this regard, miRNAs can inhibit the expression of tumor suppressors and/or carry out inadequate repression of protein-coding oncogenes. For example, miR-29a/b/c may function as anticancer treatment by targeting several oncogenic pathways including induced myelogenous leukemia cell differentiation protein (Mcl-1) and cell division protein kinase 6 (CDK6; refs. 3, 4). Conversely, miR-29b may promote tumor cell migration, invasion, and apoptotic resistance through direct targeting of the tumor suppressor phosphatase and tensin homolog (PTEN; ref. 5). Because miRNAs play a role in nearly all aspects of cancer biology, including proliferation, apoptosis, invasion/metastasis, and angiogenesis (6, 7), several miRNAs are expected to be identified as oncogenes or as tumor suppressors. Therefore, miRNAs can be readily used as potential diagnostic and prognostic tools in cancer. In this regard, for molecular oncologists, it is desirable to experimentally recreate a mutation identified in human tumors and interrogating its effects in a cell line or within the context of an entire organism to recapitulate a wide array of oncogenic events, such as inactivation of a tumor suppressor or point mutation of a proto-oncogene (8). However, methods to manipulate miRNAs *in vivo* for therapeutic purposes should become more precise.

Genetic modification is an essential approach to study gene function in normal development and disease. The rapid advancement of gene-editing technologies such as TALENs (transcription activator-like effector nucleases), ZFNs (zinc finger nucleases), and the CRISPR (clustered regularly-interspaced, short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system have greatly accelerated the development of human genome manipulation at the molecular level (9).

Three types (I–III) of CRISPR systems have been identified in bacteria and archaea as an immune defense system conferring resistance on foreign nucleic acids of viruses (e.g., phages) and other genetic elements (10, 11). The *Streptococcus pyogenes* type II CRISPR locus includes the Cas9 endonuclease and a Cas9-associated single guide RNA (sgRNA). Cas9 requires 17–20 nt of complementarity between sgRNA and a target site (protospacer) adjacent to a 5′-NGG protospacer adjacent motif (PAM) via Watson-Crick base pairing (Fig. 1A; refs. 12, 13). In principle, any DNA sequence that is followed by a PAM can be recognized and cleaved by the sgRNA:Cas9 ribonucleoprotein (sgRNP) complex to achieve a desired editing outcome (Fig. 1A; refs. 12, 13). The easy design, high targeting efficiency, and low off-target mutation frequency of the CRISPR/Cas9 system have rapidly made it that is most commonly used for high-throughput and

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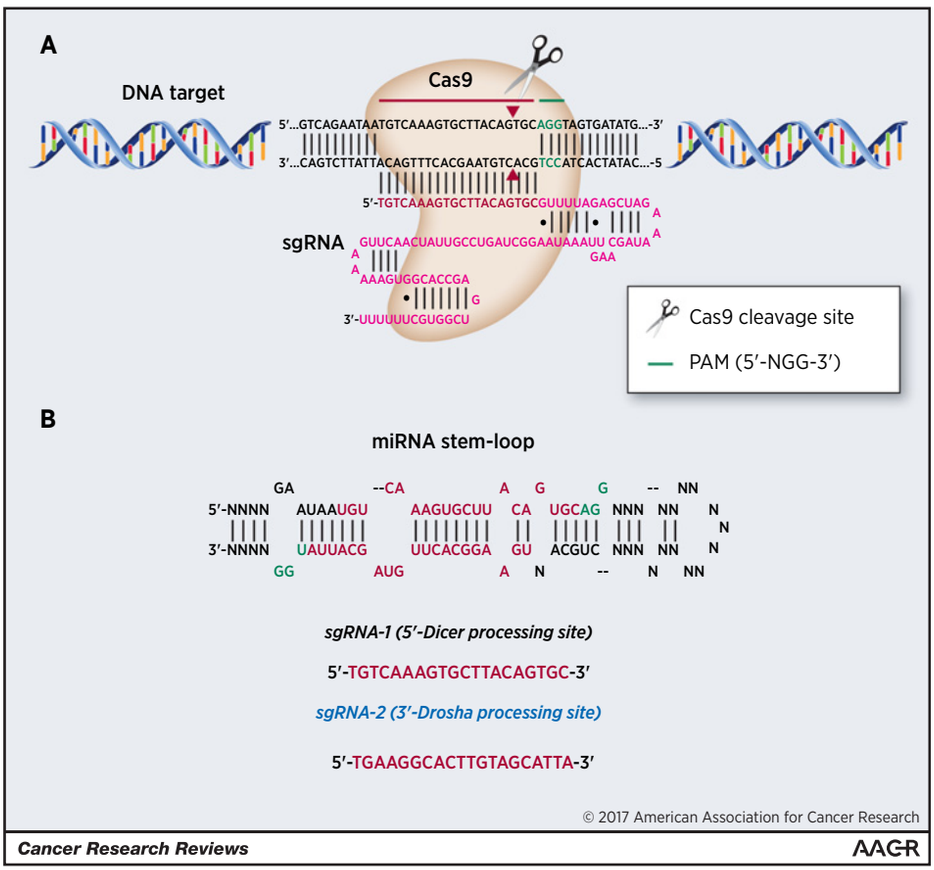

Figure 1.

Schematic diagram of the mechanism of CRISPR/Cas9-mediated miRNA editing. **A,** The Cas9 nuclease from *S. pyogenes* is targeted to genomic DNA (e.g., the *hsa-miR-17* gene) by an sgRNA consisting of a 20-nt guide sequence (red) and a scaffold (magenta). The guide sequence pairs with the DNA target (red line on top strand) directly upstream of a required 5′-NGG adjacent motif (PAM; green). The Cas9 nuclease introduces a DSB in targeted genomic DNA (e.g., Drosha or Dicer processing sites) approximately 3 bp upstream of the PAM (red triangles). The DSB generated by Cas9 is repaired by host-mediated DNA repair mechanisms. nt, nucleotide; bp, base pairs. **B,** CRISPR/cas9 can alter the biogenesis process of miRNA. SgRNAs targeting the sequences within/adjacent to Drosha- and Dicer-processing sites, respectively, in the secondary stem-loop structure of primary miRNA sequences can lead to the reduction of mature miRNAs in cells. Both arms of the mature miR-17 sequence in the stem-loop of the hairpin are depicted in red. PAM sequences are highlighted in green. The mechanism of editing of *hsa-miR-17* mediated by sgRNA-1 is depicted in **A**.

multiplexed gene-editing approaches (14, 15). Thus, taking advantage of the endogenous DNA repair machinery of cell, the CRISPR/Cas9 system has been broadly applied as a genetic engineering tool in a wide range of organisms, and *in vivo* and *in vitro* studies of human diseases (16, 17).

Here, I critically examine the current status and recent progress of miRNA-targeted therapeutics through the CRISPR/Cas9 system, as well as alternative emerging strategies to specifically combine different delivery platforms and cell-fate engineering for the elucidation of miRNA function and miRNA-based therapeutic intervention.

CRISPR/Cas9-Based Genome Editing: A Feasible Strategy for Globally Interrogating *miRNA* Gene Function

The ability to manipulate any genomic sequence by gene-editing tools has created diverse opportunities for treating many different diseases and disorders (18). As a consequence, investigating the cause and function of aberrantly expressed miRNAs is important to elucidate the clinical relevance of miRNA-mediated oncogenic pathways in tumorigenesis. However, knockout of protein-coding genes is relatively straightforward, because a small deletion or insertion can disrupt the open reading frame so that no functional gene product is synthesized. In the case of primary miRNA (pri-miRNA)—the direct transcript of *miRNA* gene and then processed by Drosha and Dicer to form short mature miRNA—a small deletion or insertion may cause functional knockout. In this regard, it has been demonstrated that both the flanking and internal structure of pri-miRNA dictate the efficiency of RNase-III-type Drosha processing and, in turn, the biogenesis of miRNA (19, 20). Therefore, by using CRISPR/cas9 technology, it was possible to repress miRNA expression by targeting the terminal loop or 5′ region of pre-miRNA (21, 22). Furthermore, CRISPR/cas9 technology has been employed for targeting the sequences

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either in or adjacent to Drosha and Dicer processing sites in the secondary stem-loop structure of primary miRNAs (Fig. 1B), which are reported to be critical for processing miRNA biogenesis (23). Recently, by using two human colon cancer lines, HCT116 and HT-29, Chang and colleagues (23) transfected CRISPR/cas9 vectors containing the individual sgRNAs specifically targeting the biogenesis processing sites of *miR-17*, *miR-200c*, and *miR-141* genes, respectively, and examined the alteration of these miRNA expressions up to 30 days posttransfection. Interestingly, the authors demonstrated the long-term stability of miRNA knockdown in nude mice, after subcutaneous administration of the HCT116 and HT-29 cells with the miR-17 knockdown phenotype generated by CRISPR/cas9 (23). This study provided strong evidence that mutations generated by CRISPR/cas9 on miRNA Drosha- and Dicer-processing sites leads to downregulation of mature miRNA by interrupting the process of biogenesis, and this study also supports the relatively high specificity of the CRISPR/cas9 system for editing miRNA sequences, avoiding crossing off-target effects among miRNA members of the same family or those with highly conserved sequences (23).

Application of the CRISPR/Cas9 System in Cancer Research to Modulate the miRNA Expression

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults and it represents the second leading cause of cancer-related deaths in the world (24). One of the major risk factors for HCC development is persistent hepatitis B virus (HBV) infection, which accounts for more than 50% of HCC worldwide (25).

In a very recent study, the role of miR-3188 in HCC and the underlying mechanism of miR-3188 regulation and cancer-related signaling pathways were investigated (26). For this purpose, Zhou and colleagues (26) manipulated miR-3188 expression in human HCC cell lines by means of CRISPR/Cas9 genome-editing technology, targeting the sequences adjacent to Drosha processing sites in the secondary stem-loop structure of miR-3188. Through this strategy, the authors demonstrated that miR-3188 knockout suppressed malignant features in HCC cells and inhibited xenografts tumor growth in nude mice. This previously noted study is a representative example of the manner in which CRISPR/cas9 technology can be applied for miRNA loss-of-function studies and provides evidence that miR-3188 may be a potential therapeutic target in HBV-related HCC.

Renal cell carcinoma

Previous studies have revealed the involvement of miRNA in the pathogenesis and progression of various human malignancies, including cell clear renal cell carcinoma (ccRCC; ref. 27), the most prevalent histological subtype, accounting for 70% to 80% of all RCC cases (28). For example, miRNAs have been implicated in different cellular processes in ccRCC, including proliferation, migration, invasion, and apoptosis (29, 30). Recently, Yoshino and colleagues (31) targeted the two versions of miR-210, miR-210-3p and miR210-5p, employing the CRISPR/Cas9 system in three renal cell carcinoma (RCC) cell lines (786-o, A498 and Caki2). This report suggested that knockdown efficiency in terms of sgRNA targeting miR-210-3p was more than 98%, whereas the knockdown efficiency for miR-210-5p was between 81 and 90%. Upon deleting miR-210-3p, the authors found that its downregulation resulted in increased tumorigenesis, both *in vitro* and *in vivo* (31). Furthermore, the authors also found an enhanced expression of oncogenic TWIST1 when miR-210-3p expression was downregulated, suggesting that RCC progression was promoted by TWIST1 upregulation. Therefore, the applicability of the CRISPR/Cas9 system to study specific miRNAs for this particular carcinoma was demonstrated (31).

Pancreatic cancer

Several key oncogenic miRNAs have been identified in pancreatic cancer—for example, miR-483-3p (32) or miR-371-5p (33) to name a couple. To identify novel transcription factors involved in pancreatic ductal adenocarcinoma (PDAC) pathogenesis, Vorvis and colleagues (34) performed miRNA-profiling analyses and found that forkhead box protein A2 (FOXA2) is a transcription factor that was significantly downregulated in PDAC relative to control pancreatic tissues. In addition, the authors found that FOXA2 expression is directly regulated by miR-199a, whereas suppression of FOXA2 expression through the CRISPR/Cas9 technology increased tumor growth in pancreatic tumor xenografts. These findings revealed the high efficiency of the CRISPR/Cas9 system to permanently knockout FOXA2 in a pancreatic cancer cell line for studying the function of FOXA2 *in vivo*, demonstrating its tumor-suppressive role in pancreatic cancer via miR-199a regulation.

Ovarian cancer

Several miRNAs have been reported to be associated with drug resistance (35). Thus, some miRNAs could alter the sensitivity of cancer cells to anticancer treatments through targeting genes associated with apoptosis and drug resistance. In epithelial ovarian cancer, the X-linked inhibitor of apoptosis protein (XIAP) is reported to be upregulated, and this upregulation may be partially responsible for the generation and development of ovarian cancer (36). Very recently, Li and colleagues (37) investigated the molecular mechanism of XIAP dysregulation in ovarian cancer. For this purpose, the authors disrupted miR-137 expression via CRISPR/Cas9 genome editing. Cas9 nuclease and miR-137-specific sgRNA were delivered into A2780 ovarian cancer cells after transduction with lentiviral particles. Li and colleagues (37) found that, upon miR-137 genome editing, apoptosis was inhibited and there was an upregulation of XIAP expression in A2780 cells. Furthermore, the ovarian cancer cells were sensitized to cisplatin-induced apoptosis, providing new insight into overcoming drug resistance in ovarian cancer cells through the miR-137 knockdown phenotype.

Another representative study was reported by Huo and colleagues (38), who constructed two lentiviral CRISPR/Cas9 sgRNA vectors that target different regions of the precursor miR-21 sequence. The evidence shown in this study suggests that disruption of pre-miR-21 sequences leads to reduced cell proliferation, migration, and invasion in ovarian cancer SKOV3 and OVCAR3 cell lines. Also, CRISPR/Cas9-mediated miR-21 gene editing sensitizes both SKOV3 and OVCAR3 cells to chemotherapeutic drug treatment and, as well, the loss of miR-21 leads to the inhibition of epithelial-to-mesenchymal transition, which it has

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been associated with tumor metastasis and chemoresistance (39). These results indicate that lentiviral CRISPR/Cas9-mediated *miRNA* gene editing is an effective approach to address miRNA function in ovarian cancer cells (38).

Chronic myeloid leukemia

There has been an interest in analyzing the role of miRNAs in hematopoiesis and related disease states (40). In a recent study, it was reported that myeloid differentiation in chronic myeloid leukemia cells (K562) is mediated by the loss of miRNA182-5p expression (41). In this study, Arya and colleagues used the CRISPR/Cas gene-editing approach to targeting miRNA182 locus and abrogate miRNA182-5p expression. After deletion of the *MIR182* locus, myeloid differentiation of K562 cells was observed, which in turn contributed to tyrosine kinase inhibitor (TKI) resistance (41). This study suggested the role of miRNA182-5p in a conserved lineage program of leukemic cells and proposes the use of miRNA182-5p for therapeutic intervention combined with the conventional treatments (e.g., TKI therapy) for this hematological cancer (41).

Application of CRISPR/Cas9 Technology for miRNA Therapeutics: The Delivery-Related Concerns

Gain- and loss-of-function approaches have been utilized to study the roles of miRNAs *in vitro* and *in vivo*. Because of the potential of CRISPR/Cas9-mediated genome editing, the roles of miRNAs in therapeutic approaches have become an area of intense research in recent years.

However, for therapeutic genome editing, it is desirable to limit the expression of CRISPR components in specific tissues to avoid potential off-target activities in nontarget tissues. As a result, it is possible to restrict the expression of Cas proteins in a particular tissue using tissue-specific promoters. Nevertheless, the natural function of Cas9 is to act as a nuclease by creating DNA double-strand breaks (DSB) via RuvC and HNH endonuclease domains, each of which cleaves one strand of the target DNA (42). An alternative to further limit off-target activities of the CRISPR/Cas9 technology is the use of modified versions of the Cas9 enzyme that possess only one active catalytic domain (designated "Cas9-nickase" or Cas9n) for binding DNA based on sgRNA specificity, but are only capable of cutting one of the DNA strands, resulting in a "nick", or single-stranded break, instead of a DSB (43, 44). Thus, "dual nickase" or "double nick" CRISPR system is a method that could be intended to decrease off-target cleavage frequency at genomic locations that resemble the intended target for miRNAs editing by using a single Cas9 nickase and two different sgRNAs, which can bind in close proximity on opposite strands of the noncoding DNA, to create a DSB. In addition, to minimizing off-target effects, sgRNA length has also been optimized. For example, it has been reported that truncated sgRNAs with <20 base homology display less off-target activity (45). Furthermore, two new Cas9 variants have been generated to display low off-target activity at nearly undetectable levels due to rationally designed mutations: (i) enhanced *S. pyogenes* Cas9 (eSpCas9) with helicase activity reduced without altering on-target editing efficiency (46) and (ii) high-fidelity variant of SpCas9 (SpCas9-HF1), which contains engineered mutations in the SpCas9 residues that normally form hydrogen bonds with DNA (47). Any of these variants could be used for CRISPR/Cas9-mediated miRNAs editing.

On the other hand, targeting a single miRNA region in the genome is not sufficient to achieve a full therapeutic effect because some diseases, including cancer, have a multigenic source, and it would require targeting more than one member of the intricate network of miRNAs to disrupt the pathological state of the cell associated with these. Thus, different approaches would involve the use of multiple sgRNAs able to target multiple genomic loci, for designing multiplex CRISPR/Cas9-based genome-engineering tools (48). Previously, Kabadi and colleagues (49) designed and constructed a single lentiviral system for expressing and delivering a Cas9 nuclease and up to four sgRNAs expressed from independent and different RNA polymerase III promoters, all of these configured into the same vector. Interestingly, each sgRNA was efficiently expressed and it was possible to promote multiplex gene editing and sustained transcriptional activation in HEK-293T and primary human dermal fibroblasts cells (49), providing a useful methodology that could also be applicable for miRNA-based biomedical research. Notwithstanding this, employing different promoters or performing several treatments may increase the risk of adverse side effects occurring through off-target effects (50). To overcome these limitations partially, multiple sgRNAs could be simultaneously expressed from a single promoter and each sgRNA can be flanked by two self-cleaving ribozymes and/or self-cleaving transfer RNA (tRNA) to perform trimming activities for the release of individual and functional sgRNAs (51) against different miRNA sequences. This represents an attractive strategy to avoid co-delivery of multiple sgRNAs-encoding constructs and reduce the amount of CRISPR/Cas9 components and the potential adverse side effects.

However, beyond possible off-target effects, a major challenge to achieve the therapeutic potential of the CRISPR/Cas system is the lack of a safe and effective *in vivo* delivery method. Thus, CRISPR/Cas9 technology will require the adoption of appropriate delivery systems in practical applications.

Currently, nucleic acid-based aptamers—small, single-stranded DNA or RNA oligonucleotides capable of recognizing, with high specificity and affinity, a wide variety of molecules—have been successfully adapted for the targeted delivery of active therapeutics *in vitro* and *in vivo* via specific cell surface receptors (52). Recently, Zhen and colleagues (53) developed an aptamer-liposome-CRISPR/Cas9 chimera incorporating an RNA aptamer that specifically binds prostate cancer cells that express the prostate-specific membrane antigen as a ligand. Subsequently, therapeutic CRISPR/Cas9 delivered by cationic liposomes targeted the survival gene, polo-like kinase 1, expressed in tumor cells. The authors demonstrated that this aptamer-liposome-CRISPR/Cas9 chimera-based approach possessed significant cell-type specificity in binding and a significant gene-silencing effect *in vitro*, but also an important tumor regression *in vivo*, while demonstrating a lower immune response (53). To my knowledge, this is the only applicability of the aptamer-cationic liposome approach to achieve cell type–specific CRISPR/Cas9 delivery, which represents an attractive strategy for the development of CRISPR/Cas9-based therapeutics for miRNA editing in a wide variety of tumor tissues. Nevertheless, although targeting tumors using aptamers comprises an attractive application of CRISPR/Cas9 system; this strategy implies a huge challenge because there are few RNA aptamers adapted for the targeted

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delivery via specific cell surface receptors. In fact, the isolation of high-affinity aptamer ligands involves a difficult task *per se*. The latter indicates the need to implement other plasmid DNA-free CRISPR approaches for CRISPR/Cas9 delivery.

Recently, Mout and colleagues (54) reported a highly efficient editing strategy based on the co-delivery of Cas9 protein complexed with an sgRNA (Cas9–RNPs complex), through the co-engineering of Cas9 protein and carrier nanoparticles for efficient delivery into the cytoplasm, with concomitant transport to the nucleus. Interestingly, in this study, it was suggested that the nano-assembly mediated Cas9-RNP delivery occurred preferably through a cholesterol-dependent membrane fusion-like process, but not via cellular endocytosis, avoiding the endosomal accumulation of both Cas9 and sgRNA (54). By means of this DNA-free CRISPR approach, the authors achieved up to approximately 90% delivery efficiency in a range of cell types, with subsequent gene-editing efficiency up to 30% (54).

However, the therapeutic applicability of CRISPR/Cas9 for *in vivo* miRNA editing represents other concerns for translational potential. For example, for miRNAs with an essential role in cellular physiology regulating many different mRNA targets, partial loss-of-function studies are important for our understanding of their function. In this regard, development and modification of Cas9 variants has led to a single-platform technology with capabilities far beyond DNA mutagenesis (55). Currently, technologies have been reported that are based on nuclease-deactivated Cas9, termed dCas9, which can be directed near the promoter sequence of a target gene to exert RNA-guided transcription regulation without permanently modifying the genome. Hence, by repression through CRISPR interference (CRISPRi), or by activation through CRISPR activation (CRISPRa), transient or stable control of miRNA expression without altering the genomic sequence would be intended. For example, miRNA-transcription regulation can be modulated through sgRNA-guided DNA-binding proteins, which can be fused with an epigenetic modifier (e.g., methylase or acetylase), for the activation (CRISPRa) or repression (CRISPRi; ref. 56) of miRNA expression. Furthermore, several approaches have been intended to design inducible CRISPR/Cas9 systems that use minimal genetic elements to achieve conditional expression of Cas9, for example, placing Cas9 or sgRNAs under the control of a hybrid tetracycline-responsive promoter (57), as well as fusing a hormone-binding domain of the estrogen receptor with Cas9 (58). Furthermore, by using an miRNA-complementary sequence in the 5′-UTR of mRNA encoding *S. pyogenes* Cas9, its genome-editing activity can be modulated through endogenous miRNA signatures in mammalian cells (59). By means of this strategy, Hirosawa and colleagues (59) developed an miRNA-responsive CRISPR-Cas9 system called miR-21-Cas9 or miR-302-Cas9 switches, which efficiently responded to miR-21-5p in HeLa cells or miR-302a-5p in human induced pluripotent stem cells, and posttranscriptionally attenuated Cas9 activity only in the presence of each miRNA in these target cells.

Concluding Remarks

Despite the ethical controversies regarding to nonresearch applications of CRISPR/Cas technology, it is clear that the CRISPR/Cas nuclease system represents a cutting-edge tool suitable for basic research and future therapeutic modalities. Given the flexibility in the design of sgRNA, it is now possible to target a wide range of DNA sequences containing PAM within the stem-loop structure of pri/pre-miRNA transcripts (Fig. 1B). In addition, developments in nanotechnology and materials for nonviral delivery systems, including liposomes, polymers, nanoparticles, and cell-penetrating peptides have increased the alternatives available for the delivery of Cas proteins and sgRNAs (60). Therefore, we must encourage research efforts to investigate the potential application of CRISPR technologies as a traditional miRNA-editing strategy for the elucidation of miRNA function and miRNA-based therapeutic intervention, with emphasis on combining one or more delivery strategies that might increase the success rate of delivery of CRISPR/Cas components in cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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