Current updates regarding biogenesis, functions and dysregulation of microRNAs in cancer: Innovative approaches for detection using CRISPR/Cas13-based platforms (Review)

ABDULAZIZ A. ALOLIQI[1], ABDULLAH M. ALNUQAYDAN[1], AQEL ALBUTTI[1], BASMAH F. ALHARBI[1], ARSHAD HUSAIN RAHMANI[2] and AMJAD ALI KHAN[1]

[1]Department of Basic Health Sciences, College of Applied Medical Sciences, Qassim University, Buraydah, Al-Qassim 51452, Saudi Arabia; [2]Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Buraydah, Al-Qassim 51452, Saudi Arabia

Received January 15, 2025; Accepted March 4, 2025

DOI: 10.3892/ijmm.2025.5531

Abstract. MicroRNAs (miRNAs) are short non-coding RNAs, which perform a key role in cellular differentiation and development. Most human diseases, particularly cancer, are linked to miRNA functional dysregulation implicated in the expression of tumor-suppressive or oncogenic targets. Cancer hallmarks such as continued proliferative signaling, dodging growth suppressors, invasion and metastasis, triggering angiogenesis, and avoiding cell death have all been demonstrated to be affected by dysregulated miRNAs. Thus, for the treatment of different cancer types, the detection and quantification of this type of RNA is significant. The classical and current methods of RNA detection, including northern blotting, reverse transcription-quantitative PCR, rolling circle amplification and next-generation sequencing, may be effective but differ in efficiency and accuracy. Furthermore, these approaches are expensive, and require special instrumentation and expertise. Thus, researchers are constantly looking for more innovative approaches for miRNA detection, which can be advantageous in all aspects. In this regard, an RNA manipulation tool known as the CRISPR and CRISPR-associated sequence 13 (CRISPR/Cas13) system has been found to be more advantageous in miRNA detection. The Cas13-based miRNA detection approach is cost effective and requires no special instrumentation or expertise. However, more research and validation are required to confirm the growing body of CRISPR/Cas13-based research that has identified miRNAs as possible cancer biomarkers for diagnosis and prognosis, and as targets for treatment. In the present review, current updates regarding miRNA biogenesis, structural and functional aspects, and miRNA dysregulation during cancer are described. In addition, novel approaches using the CRISPR/Cas13 system as a next-generation tool for miRNA detection are discussed. Furthermore, challenges and prospects of CRISPR/Cas13-based miRNA detection approaches are described.

Correspondence to: Dr Amjad Ali Khan, Department of Basic Health Sciences, College of Applied Medical Sciences, Qassim University, Buraydah, Al-Qassim 51452, Saudi Arabia
E-mail: akhan@qu.edu.sa

Key words: microRNA, cancer, cancer detection, CRISPR/CRISPR-associated sequence 13, gene regulation

Contents

1. Introduction

MicroRNAs (miRNAs/miRs) are short non-coding RNAs, which serve a role in cell differentiation, homeostasis and organism development. miRNAs consist of ~22 nucleotides, and can induce silencing of some genes by directing argonaute (AGO) proteins to target mRNA at the 3' untranslated region (UTR) (1,2). As single-stranded short nucleic acids, miRNAs act as guides to RNA or DNA complementary sequences that are intended to be silenced (3). The translational repression and further elimination of target mRNAs occur through the miRNA-induced silencing complex (miRISC), which is formed of the miRNA, AGO proteins and other associated proteins such as Dicer (2).

At present, it has been reported that most of the human protein-coding genes (>60%) contain miRNA target sites (4), and the miRNA repository miRBase (https://www.mirbase.org) records 2,654 mature miRNAs and 1,917 precursor miRNAs (pre-miRNAs) in humans (5).

As a hallmark of cancer and other common diseases, the upregulated and downregulated expression of miRNAs serves a crucial role in disease development and progression. The upregulation of miRNAs associated with cancer promotes cancer by targeting tumor suppressor genes (6). For example, miR-21 is often upregulated in various cancer types, including breast, lung and colorectal cancer (CRC). miR-21 upregulation leads to the suppression of tumor suppressor genes such as PTEN and programmed cell death protein 4, promoting cell proliferation and survival (7). In a similar manner, miR-155 is frequently upregulated in hematological malignancies, including lymphoma and leukemias. This miRNA targets multiple genes involved in apoptosis and cell differentiation, contributing to tumorigenesis (8).

The upregulation of miRNAs such as tumor suppressor miRNAs inhibits cancer via targeting of oncogenes. For instance, miR-34a is downregulated in numerous cancer types, including pancreatic and prostate cancer. miR-34a downregulation leads to the upregulation of oncogenes such as MYC and BCL2, facilitating tumor growth and resistance to apoptosis (8). Similarly, the miR-200 family of miRNAs is often downregulated in cancer types such as ovarian and breast cancer. The loss of miR-200 expression is associated with epithelial-mesenchymal transition (EMT), a process that enhances cancer cell invasion and metastasis (9).

Cancer mortality is a threat to health worldwide. Approaches for early detection of cancer using different diagnostic procedures and cancer treatments are needed globally. A cancer-specific screening technique that is sensitive enough to identify early malignancy would be ideal. This should be specific to the cancer type and location, appropriate for the size of the tumor, affordable, user-friendly, and safe. Currently, there are numerous methods for identifying and measuring certain miRNAs, including miRNA arrays, *in situ* hybridization and immuneprecipitation to examine miRNA localization and specific miRNA activity, and total miRNA measurement (10). The accuracy, cost, effectiveness and ease of tracking miRNA dynamics vary among these methods. Furthermore, all of these approaches have some advantages and disadvantages for miRNA quantification in biochemical and medical research (11,12).

The CRISPR/CRISPR-associated sequence (Cas) system is a programmable platform, a modern world genome editing system, which has garnered trust for the management of cancer detection and treatment (13,14). The innovative approach of cancer detection using the CRISPR/Cas system is more cost effective compared with other currently known procedures (15). CRISPR/Cas systems are now a frequently used genome editing method in molecular biology labs worldwide because of their quick cycle, low cost, high efficiency, good repeatability and simple design (16,17). This system consists of a set of CRISPR-associated (Cas) genes that encode Cas proteins with endonuclease activity as well as CRISPR repeat-spacer arrays, which can be further translated into CRISPR RNA (crRNA) and trans-activating crRNA (18,19).

CRISPR/CRISPR-associated sequence 13 (Cas13) is a powerful and versatile genome-editing tool, targeting and cleaving single-stranded RNA (ssRNA) rather than DNA, and is a class 2 type VI CRISPR/Cas system (20). Cas13 belongs to the class 2 type VI CRISPR/Cas system and consists of different subtypes, including Cas13a, Cas13b, Cas13c and Cas13d. Among all these subtypes, Cas13a (also referred to as C2c2) is the most studied in terms of its structure and RNA editing approach (21). The CRISPR/Cas13 system can be effectively used for viral detection, splicing regulation, transcript tagging and RNA knockdown (22-24).

The advancements in miRNA detection with the help of the CRISPR/Cas13 system have shown some promising results. Some of the key updates include direct and accurate miRNA detection, development of amplification-free biosensors and lateral flow assays (25-27). However, several knowledge gaps remain, which need to be discussed further. The current methods of miRNA detection show high sensitivity; however, the achievement of absolute specificity in complex biological samples remains a challenging task (11). Further research is required to minimize false positives and improve the robustness of these assays. The development of cost-effective and scalable miRNA detection procedures, which are suitable for widespread clinical use, is still a hurdle. Thus, innovations that reduce the costs and simplify the detection processes are crucial. Furthermore, there is still much to learn regarding the diverse roles of miRNAs in various diseases, particularly cancer. Therefore, comprehensive studies are needed to fully understand their mechanisms and potential as therapeutic targets.

In the present review, current updates regarding the biogenesis, structural and functional aspects, and dysregulation in different cancer types of miRNAs are discussed. In addition, the significance of altered miRNA expression in tumors is discussed. Furthermore, the functional aspects of CRISPR/Cas13 as a next-generation tool in different forms for the detection of miRNAs in different cancer types are elaborated. An overview of current methods using CRISPR/Cas13-based biosensor systems is provided, emphasizing how these methods have made it possible to miniaturize electrochemical transducers and enhance their sensitivity, specificity and suitability for miRNA diagnosis. Furthermore, challenges and prospects of the use of CRISPR/Cas13 as a miRNA detection tool are discussed.

2. miRNA biogenesis

miRNA biogenesis is a multi-step process that occurs under tight temporal and spatial control by different proteins. The RNA polymerase II primarily transcribes them as primary miRNAs (pri-miRNAs), which are then converted into pre-miRNAs, which are later converted into mature miRNA duplexes (28,29) (Fig. 1). The 5p and 3p strands of the mature miRNA are derived from the 5' arm of the pre-miRNA. The A-to-I editing of pri-miRNAs is performed by adenosine deaminase proteins, which may impact subsequent biogenesis and the sequence of the mature miRNA, or mediates pri-miRNA destruction (30).

The microprocessor complex, which includes the DiGeorge syndrome critical region 8 (DGCR8) protein (31,32) and the RNase III enzyme Drosha (33), excises the pri-miRNA hairpin in the nucleus (Fig. 1). For its action, two DGCR8 proteins bind the stem and make a proper cleavage (34,35), while Drosha detects the junction between double-stranded RNA and ssRNA at the pri-miRNA hairpin base [Fig. 2A; obtained from Protein Data Bank (PDB; https://www.rcsb.org) with

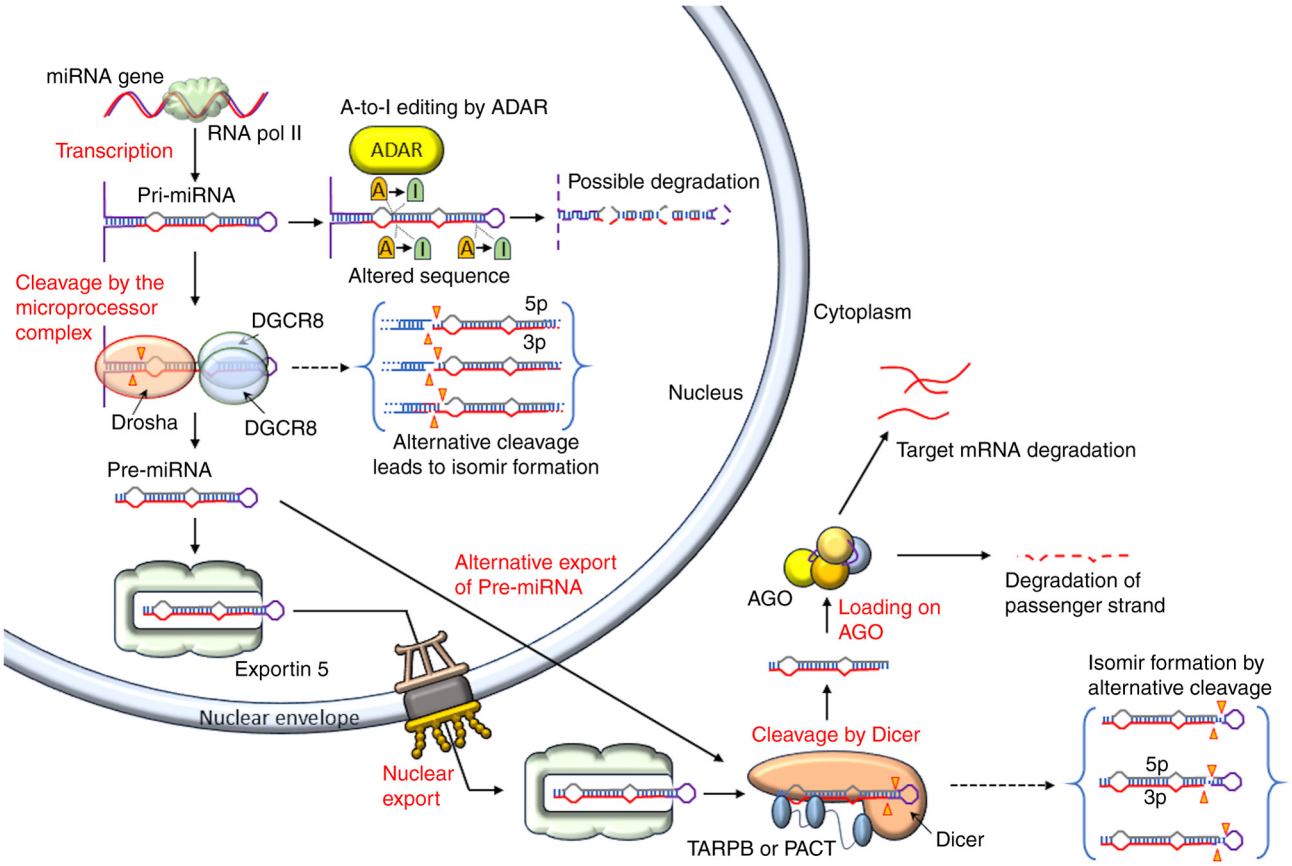

Figure 1. Overview of the different stages of miRNA biogenesis. After the transcription of pri-miRNA by RNA pol II, it is cleaved by the microprocessor unit (Drosha-DGCR8) in the nucleus. Different isomirs are formed by the microprocessor unit through alternative cleavage. The resulting pre-miRNA is exported from the nucleus by exportin 5 to the cytoplasm. Dicer cleaves it further to yield a miRNA duplex, which is later loaded into the AGO protein. At this stage, different isomirs are also formed by the alternative cleave of pre-miRNA by Dicer. Following the removal and degradation of the passenger strand, single-stranded mature miRNA is formed, which guides the AGO effector complex (now referred to as RNA-induced silencing complex) to silence complementary RNA targets. ADAR, adenosine deaminase; AGO, argonaute; DGCR8, DiGeorge syndrome critical region 8; miRNA, microRNA; PACT, protein activator of the interferon-induced protein kinase; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RNA pol II, RNA polymerase II; TARPB, trans-activation response RNA binding protein.

PDB ID 6LXD and modified with Chimera X (https://www.cgl.ucsf.edu/chimerax/; version 1.8)]. The alternative cleavage by Drosha results in the creation of isomirs (36,37). Pre-miRNAs are hairpin-shaped RNAs composed of ~70 nucleotides (38,39). The hairpin end has a 3' hydroxyl, a 5' phosphate and a 2-nucleotide overhang at the 3' end (40).

After identifying the overhang, exportin-5 (XPO-5; a transport protein) transports the pre-miRNA into the cytoplasm (41) (Figs. 1 and 2B). In a human cell line, exportin-5 (XPO-5) deletion led to decreased transportation of pre-miRNA but did not completely stop its nuclear export, indicating the existence of some other pre-miRNA nuclear export pathways (42). In the cytoplasm, Dicer (RNase III enzyme) (43,44) recognizes the 5' phosphate, 3' overhang and loop structure (45,46), and binds the pre-miRNA (Fig. 1). Dicer acts as a 'molecular ruler' that produces a mature miRNA duplex with a 2-nucleotide 3' overhang (40) after cleaving pre-miRNAs at a species-specific length (Fig. 2C). It is also possible for Dicer to produce isomirs through alternative cleavage (37).

In vertebrates, Dicer cleavage is regulated by protein activator of the interferon-induced protein kinase and trans-activating response-RNA-binding protein. The cleavage of pre-miRNA by Dicer leads to the formation of two RNA strands (guide and passenger strands). The mature miRNA 'guide' strand is loaded into AGO protein, while the 'passenger' strand is eliminated (47,48) (Fig. 1). The strand with the less securely coupled 5' end (49) is preferentially loaded on the AGO protein (Fig. 2D).

3. Functional aspects of miRNA

After being transcribed into pri-miRNA transcripts, miRNAs go through a multi-step biogenesis process that transforms them into pre-miRNAs and mature miRNAs. The expression patterns of miRNAs are tissue-specific (50) and transcriptionally regulated (51). miRNAs can originate from long non-coding RNAs or introns, and are mostly transcribed by RNA polymerase II (52,53). Pri-miRNAs can be clusters of frequently linked miRNAs or a single mature miRNA (54). Based on how similar their seed sequences are, miRNAs are categorized into different families (55). The seed region of miRNAs, which consists of nucleotides 2-8 (counting from the 5' end), is mostly responsible for targeting mRNAs (56).

Mature miRNAs act inside a single polypeptide chain AGO protein, with four distinctive domains (Fig. 3A). The four domains are the N-terminal (N), P-element induced wimpy

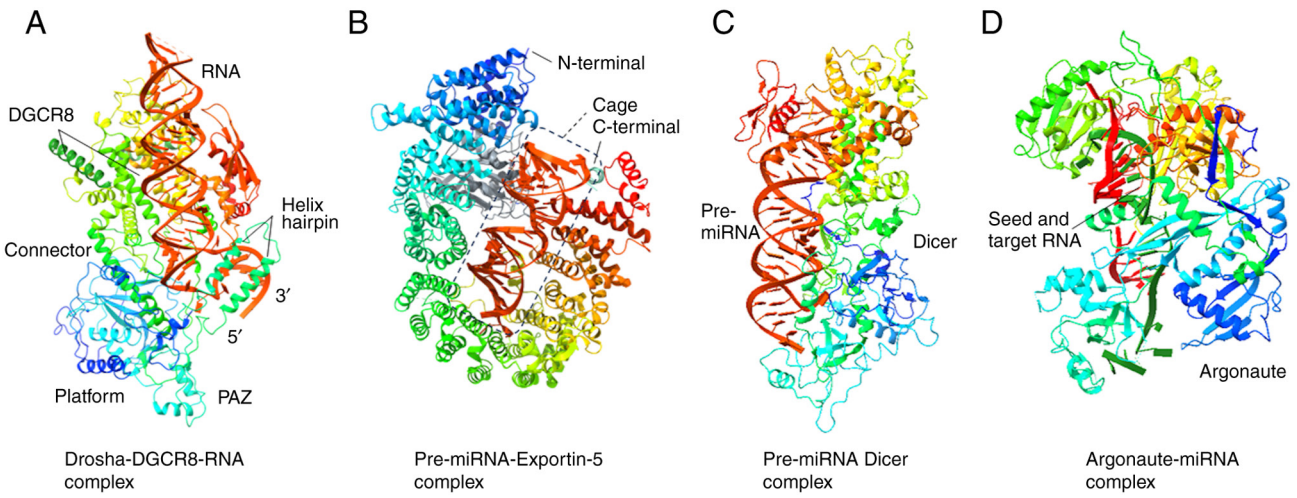

Figure 2. Overview of the 3-dimenstional structure of different proteins involved in the biogenesis of miRNA (primary miRNA to mature miRNA). The structures were obtained from PDB and edited using ChimeraX. (A) Drosha-DGCR8-RNA complex (PDB ID 6LXD). (B) Pre-miRNA-Exportin-5 complex (PDB ID 3A6P). (C) Pre-miRNA-Dicer complex (PDB ID 7XW2). (D) Argonaute-miRNA complex (PDB ID 6N4O). DGCR8, DiGeorge syndrome critical region 8; miRNA, microRNA; PAZ, Piwi-Argonaute-Zwille; PDB, Protein Data Bank; pre-miRNA, precursor miRNA.

testes (PIWI)-AGO-Zwille (PAZ), PIWI and MID domains. A linker domain (L1) is present between the N and PAZ domains, while the L2 domain is present between the PAZ and MID domains. The MID and PIWI domains make up the second lobe of AGO, while the N and PAZ domains make up the first (57,58). The PAZ domain binds the 3' nucleotides of the miRNA (59,60), whereas the MID and PIWI domains retain the 5' end of the miRNA (Fig. 3A). It has been reported that four AGO proteins (AGO1-4) are encoded by the mammalian genome. AGO2 is the most abundant and unique among the AGO proteins, with target cleaving activity when completely complementary to the guide strand of the miRNA (61,62). Numerous human miRNAs bind to all AGO proteins, although some are selectively packaged into AGO proteins (63,64). Typically found in the 3' UTR of mRNAs, miRNA target sites exhibit high complementarity to the seed region, the primary requirement for the prediction of target sites (65,66).

The strongest canonical (seed matching) target sites are those that complement miRNA nucleotides 2-8 and have an adenine opposite miRNA nucleotide 1 (referred to as 't1A'), followed by those complementing nucleotides 2-8 without a t1A and those complementing nucleotides 2-7 with t1A (67). A binding site in AGO (68,69) recognizes t1A instead of the miRNA guide strand (Fig. 3A). Target sites that complement miRNA nucleotides 2-7 or 3-8 are still considered canonical, although they have a weaker affinity (65). According to structural and single-molecule investigations, the MID and PIWI domains pre-organize the initial nucleotides 2-6 of the seed in helical conformation (59,70). This two-step mechanism is considered to be responsible for target identification (71).

The biological role of non-canonical sites has been contested because small RNA and miRNA transfection data showed no discernible suppression from these locations (65). Through translational suppression and mRNA decay, the 3' UTR binding with AGO-miRNA results in gene silencing (72) (Fig. 3B). Furthermore, glycine-tryptophan 182 (GW182; 182 kDa), a member of the glycine-tryptophan protein family, is recruited by AGO for RNA silencing (73).

By attracting the poly(A)-nuclease deadenylation complex subunit 2 (PAN2)-PAN3 and carbon catabolite repressor protein 4 (CCR4)-NOT complexes (74,75), the interaction between polyadenylate binding protein and GW182 promotes the deadenylation of mRNA. Because deadenylation encourages decapping by the mRNA-decapping enzyme subunit 1 (DCP1)-DCP2 complex (76), 5'-3' exoribonuclease 1 can quickly degrade the mRNA (77) (Fig. 3B). Through the recruitment of the likely ATP-dependent RNA helicase DEAD-box helicase 6, GW182-mediated recruitment of CCR4-NOT also results in translation suppression (78).

Eukaryotic initiation factor 4A-I (eIF4A-I) and eIF4A-II interference also inhibits translation initiation (79,80). Although the exact method of interference in initiation factors is not fully understood yet, most studies suggest that miRISC causes them to separate from target mRNAs (81,82), which prevents scanning by the ribosome and the formation of the eIF4F translation initiation unit. Translation inhibition requires the Trp-binding pockets in AGO that mediate binding with GW182, according to a study conducted in human and *Drosophila melanogaster* cells (83,84).

Complete understanding of miRISC-mediated translation inhibition remains lacking, and it has been hypothesized that the two methods of miRISC-mediated gene silencing are related (2). Ribosome profiling tests have shown that mRNA degradation typically accounts for 66-90% of silencing (72,85). The observation that translation inhibition can be restored while mRNA degradation is permanent raises the possibility that regulated pauses, or blocks, in the metabolic cascade leading to mRNA degradation could allow translation suppression without mRNA decay (86). Furthermore, multiple miRNAs can regulate the same gene (87), and it has also been reported that hundreds of genes can be muted by a single miRNA (88).

Furthermore, individual miRNAs or miRNA clusters can control whole cellular pathways (89). Cooperative suppression can be caused by nearby target site binding with miRNA on a target mRNA (90,91), which may help justify the functioning

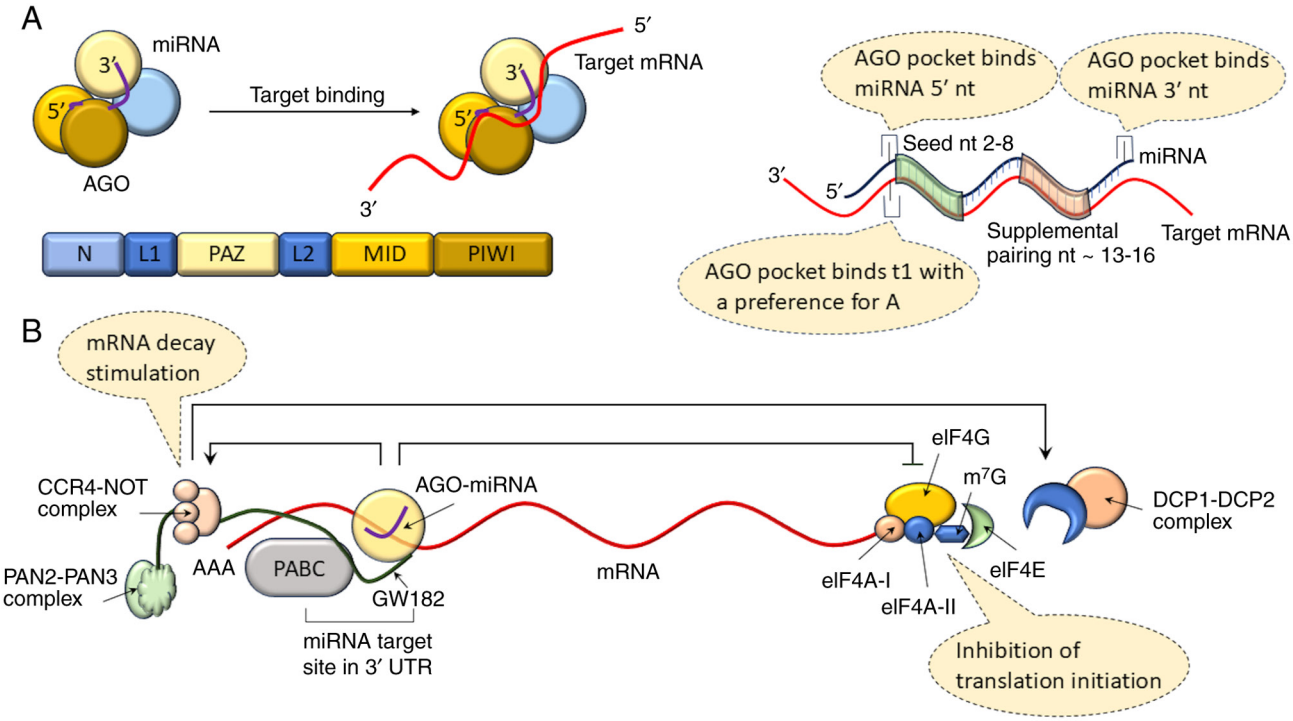

Figure 3. Overview of the regulation and function of miRNA. (A) miRNA along with the AGO protein operates as a functional unit. A total of four subdomains are present in the AGO protein, including the N, PAZ, MID and PIWI domains. There are two linker regions: L1 is present between the N and PAZ domains, while L2 is present between the PAZ and MID domains. The 5' end of miRNA is held by the MID and PIWI domains, while the 3' terminal is held by the PAZ domain. From the 5' end, nucleotides 2-8 form the seed, which is decisive for target mRNA recognition. (B) Gene expression is inhibited via silencing of translation by miRNAs at the initiation step through eIF4A-II and the decay of mRNA via GW182 protein interaction. GW182 binds PABC, the PAN2-PAN3 complex and CCR4-NOT, and the deadenylation is followed by decapping by the DCP1-DCP2 complex. AGO, argonaute; CCR4, carbon catabolite repressor protein 4; DCP, mRNA-decapping enzyme subunit; eIF4, eukaryotic initiation factor 4; GW182, glycine-tryptophan 182; MID, middle domain; m^7G, N7-methylguanosine; miRNA, microRNA; N, N-terminal; nt, nucleotide; PABC, poly(A)-binding protein-interacting C-terminal domain; PAN, poly(A)-nuclease deadenylation complex subunit; PAZ, Piwi-Argonaute-Zwille; PIWI, P-element induced wimpy testes; t1, target nucleotide 1 position; UTR, untranslated region.

of non-canonical sites relying on the occupancy of nearby canonical sites. The development of multivalent protein interactions between GW182 and AGO proteins help to explain cooperativity (84). Furthermore, miRNAs can either inhibit or regulate protein expression (92), protecting against variations (or 'noise') in gene expression levels (93).

4. miRNA dysregulation during cancer

Human cancers exhibit dysregulated miRNA expression. Cancer development through chromosome abnormalities, transcriptional regulation alterations, epigenetic modifications and flaws in the miRNA biogenesis machinery are some of the underlying mechanisms (94,95). miRNA expression is regulated at different stages of cellular activities as subsequently described.

Amplification or deletion of miRNA genes. Changes in genomic miRNA copy numbers and gene positions (amplification, deletion or translocation) are frequently related to the cause of abnormal miRNA expression in malignant cells (96). The loss of the miR-15a/16-1 cluster gene at chromosome 13q14, which is commonly seen in individuals with B-cell chronic lymphocytic leukemia, is the earliest example of a change in miRNA gene placement (97). In addition, the 5q33 region that contains miR-143 and miR-145 is frequently deleted in lung cancer, which lowers the expression levels of both miRNAs (96). On the other hand, B-cell lymphoma (98) and lung cancer (99) exhibit amplification of the miR-17-92 cluster gene, and T-cell acute lymphoblastic leukemia (100) exhibits translocation of this cluster gene, resulting in upregulation of these miRNAs in these cancer types.

High-resolution array-based comparative genomic hybridization has been used to confirm the high frequency of genomic changes in miRNA loci in 227 human cancer samples of ovarian cancer, breast cancer and melanoma (101). Numerous miRNA genes are found in genomic areas linked to cancer, according to additional genome-wide studies (102,103). These areas on chromosomes may be fragile sites, common breakpoint regions or minimal regions of amplification, which may include oncogenes, or minimal regions of loss of heterozygosity, which may carry tumor suppressor genes (102). Considering all the observations, the findings suggest that the amplification or deletion of specific genomic regions containing miRNA genes may be the cause of abnormal miRNA expression in malignant cells.

Transcriptional control of miRNAs. Since a number of transcription factors tightly regulate miRNA production, dysregulation of some important transcription factors, including p53 and c-Myc, may be linked to aberrant miRNA expression in cancer (104,105). By binding to E-box regions

in the miR-17-92 promoter, c-Myc is commonly increased through transcriptional activation, in a number of malignancies to control cell proliferation and death, and promotes the transcription of the oncogenic miR-17-92 cluster (106). In keeping with its carcinogenic function, c-Myc also inhibits the transcriptional activity of tumor-suppressive miRNAs, including the let-7, miR-26, miR-29 and mir-15a families (107).

In hepatocellular carcinoma (HCC), c-Myc and the tumor suppressor miR-122 are reciprocally regulated. By attaching itself to its promoter, c-Myc inhibits the synthesis of miR-122 and, by targeting the transcription factor Dp2 and the transcription factor E2F transcription factor 1 (E2f1), indirectly stops c-Myc transcription. Therefore, the disruption of this feedback loop between miR-122 and c-Myc is necessary for the development of HCC (108). HCC is brought on by direct binding of c-Myc to the promoters of the miR-148a-5p and miR-363-3p genes, which inhibits their synthesis and encourages the G_1 to S phase transition. miR-148a-5p, on the other hand, directly targets and suppresses the production of c-Myc, while miR-363-3p destabilizes c-Myc by targeting ubiquitin-specific protease (106,109). One of the most frequently altered genes in human cancer is TP53, which encodes the tumor suppressor p53. Another manner in which transcriptional factors control miRNA expression to exert a tumor suppressive function is the p53-miR-34 regulatory axis (110). A complex p53 network that controls cell-cycle progression and apoptosis is formed by the p53-regulated expression of several genes, including miRNA genes. Similar to p53-mediated phenotypes, the miR-34 family, comprising miR-34a, miR-34b and miR-34c, causes cell-cycle arrest, cell senescence and apoptosis in cancer (111), suggesting that p53 and miR-34 are involved in the same regulatory mechanism.

Additional research has revealed that p53 regulates the expression of several miRNAs, including miR-605 (112), miR-1246 (113) and miR-107 (114), to carry out its role. Additional transcriptional factors have been identified to control miRNA production in addition to the two most well researched transcriptional factors, c-Myc and p53. For instance, miR-223 is inhibited in a variety of cancer types, including HCC and acute myeloid leukemia (AML), and it is predominantly expressed in the hematopoietic system, which serves important roles in the formation of myeloid lineages (115).

Defects in the miRNA biogenesis pathway. As aforementioned, several enzymes and regulatory proteins, including Drosha, Dicer, DGCR8, AGO and XPO-5, intricately regulate miRNA biogenesis, enabling proper maturation of miRNA from pri-miRNA precursors. Therefore, aberrant expression of miRNAs may result from mutations or aberrant expression of any protein in the miRNA biogenesis pathway. Two important RNase III endonucleases in miRNA maturation, Drosha and Dicer, oversee the creation of pre-miRNA and the miRNA duplex (116,117). According to previous studies, both enzymes are dysregulated in some cancer types such as breast and bladder cancer (118,119).

A portion of miRNAs are controlled during the Drosha-processing stage, and this control affects miRNA expression in cancer and during embryonic development (120). It has been reported that 15% of 534 Wilms tumors had single-nucleotide substitution/deletion mutations in DGCR8 and Drosha, which resulted in reduced expression of mature Let-7a and the miR-200 family (121). In terms of Dicer dysregulation, it has been noted that CRC cells with impaired Dicer acquire a higher propensity for tumor initiation and metastasis (122). Furthermore, patients with ovarian cancer with higher Dicer and Drosha mRNA levels have a higher median survival rate (123). On the other hand, patients with lower Dicer expression have a considerably lower survival rate (124,125).

Reduced let-7 expression and poor postoperative survival have been linked to lower Dicer mRNA levels in patients with lung cancer (126). As with Dicer and Drosha, cancer also leads to deregulation of AGO. For instance, kidney tumors caused by Wilms disease frequently exhibit loss of the human eukaryotic initiation factor 2C1/human argonaute gene (127). Human AGO proteins are crucial in regulating cell-dependent gene expression. AGO2 expression levels, for example, are considerably higher in basic gastric cancer and the lymph node metastases (128), but AGO2 expression is lower in melanoma, which corresponds to a lower RNA interference efficiency, compared with in primary melanocytes (129).

A fraction of human cancers with microsatellite instability have inactivating mutations in the XPO-5 gene. The insertion of an 'A' in exon 32 in CRC cells (HCT-15 and DLD-1 cells), results in an early termination codon, which causes a frameshift mutation and the creation of a shortened protein. This shortened XPO-5 is no longer able to export pre-miRNAs. As a result, there is less miRNA processing since pre-miRNAs are confined to the nucleus. The restoration of XPO-5 activities possesses tumor-suppressor properties and restores the defective export of pre-miRNAs (130). It has also been noted that ERK phosphorylates XPO-5, preventing XPO-5 from moving pre-miRNAs from the nucleus to the cytoplasm in HCC (131).

Dysregulated epigenetic changes. A well-known characteristic of cancer is epigenetic modification, which includes the disruption of histone modification, aberrant DNA hypermethylation of tumor suppressor genes and widespread genomic DNA hypomethylation. Similar to protein coding genes, miRNAs are considered to be subjected to epigenetic modifications (132,133). For example, it has been reported that acute myeloid leukemia 1/eight twenty one, the most prevalent AML-associated fusion protein, epigenetically suppresses miR-223 expression via CpG methylation (134). A study reported that the expression levels of 17 out of 313 human miRNAs were more than three times higher in T24 bladder cancer cells after concomitant treatment with DNA methylation and histone acetylation inhibitors (134).

The downregulation of the proto-oncogene BCL6 coincides with markedly increased expression of miR-127, which is embedded in a CpG island and not expressed in cancer cells (135). These findings suggest that miRNAs may function as tumor suppressors and can be activated through DNA demethylation and histone deacetylase inhibition. In addition, it has been reported that miR-148a and the miR-34b/c cluster are susceptible to hypermethylation-associated silencing in cancer cells (136). Furthermore, *in vivo* metastasis formation was suppressed, tumor growth was decreased and cancer cell motility was inhibited when these miRNAs were restored (137). Similarly, DNA hypermethylation has been linked to lower

expression levels of miR-9-1, miR-124a and miR-145-5p in colon, lung and breast cancer, respectively (138,139). This suggests that the abnormal histone acetylation and DNA methylation of miRNA genes could be useful indicators for the diagnosis and prognosis of cancer. These findings emphasize the significance of epigenetic control in miRNA production during carcinogenesis.

5. Implications of dysregulated miRNA expression in tumors

The biological characteristics of tumor development include continued proliferative signaling, evasion of growth suppressors, replicative immortality, initiation of invasion and metastasis, induction of angiogenesis, and prevention of cell death (140). Dysregulated miRNAs may impact one or more of these cancer hallmarks for tumor initiation and development. In different situations, miRNAs act as tumor suppressors or oncogenes, depending on the genes they target. Dysregulated miRNAs affect tumorigenesis in different manners as described subsequently.

Dodging the impact of growth suppressors and retaining proliferative signals. The primary cause of tumorigenesis is aberrant cell proliferation, which is the most significant characteristic of cancer. Certain miRNAs functionally incorporate into several cell proliferation pathways, and the dysregulation of these miRNAs directs the cancer cells to continue proliferative signaling to avoid growth suppressors. miRNAs serve a role in controlling the expression of E2F proteins (cell proliferation regulators). E2F1-deficient animals develop a wide range of malignancies, and this member of the E2F family is characterized as a tumor suppressor and stimulates target gene transcription during the G_1 to S transition (141). miR-17-92, once activated by c-Myc, suppresses the translation of E2F1 (106). E2F1 protein levels may not increase markedly in response to c-Myc activation if the miR-17-92 cluster acts as a brake on this putative positive feedback loop, given that c-Myc also directly stimulates E2F1 production (142). Furthermore, the miR-17-92 cluster has been demonstrated to regulate E2F2 and E2F3 translation (143). Additionally, the expression of the miR-17-92 cluster might be triggered by the E2F transcription factors (144). Therefore, under normal conditions, the feedback loop between E2F and the miR-17-92 cluster provides a means of preserving regular cell-cycle progression. However, miR-17-92 upregulation, which is common in several cancer types such as colorectal cancer and gastric cancer, disrupts the feedback loop that encourages cell proliferation (145).

Different cyclins, Cdks and their inhibitors, extensively regulated by miRNAs, are essential for cell-cycle progression. The G_1/S transition inhibition of Drosophila germline stem cells with Dicer-1 deletion suggests that miRNAs are required for germline stem cells to pass through the normal G_1/S checkpoint (146). Additionally, Dacapo, a member of the p21/p27 family of Cdk inhibitors, is upregulated in Dicer-deficient germline stem cells, suggesting that miRNAs negatively control this protein to encourage cell-cycle progression (147). In glioblastoma cells, miR-221/222 has been found to directly target the Cdk inhibitor p27Kip1 (148), a finding that has been subsequently validated in original tumor samples and other cancer cell lines (149,150). While its inhibition causes G_1 cell-cycle arrest in malignant cells, ectopic expression of miR-221/222 enhances cell proliferation. Furthermore, several human malignancies have been reported to be associated with increased expression levels of miR-221/222, suggesting that regulation of p27Kip1 by miR-221/222 is a valid carcinogenic pathway (151).

Inducing angiogenesis. To meet the demands of food and oxygen during tumor growth and metastasis, the highly coordinated process of angiogenesis produces new blood vessels from pre-existing ones (152). Because the oxygen concentration of tumor tissues is lower than that of the surrounding normal tissues, hypoxia serves a critical role in the tumor microenvironment by facilitating the proliferation and maintenance of cancer cells. In response to hypoxia, a crucial transcription factor, hypoxia-inducible factor (HIF), affects the expression of several genes, including miRNAs (153). As a key angiogenic factor, VEGF instructs endothelial cells to form new vessels when it binds to its receptors (154). Therefore, angiogenesis is likely to be impacted by miRNAs that target the VEGF or HIF signaling pathways.

It is widely known that miRNAs intricately govern the angiogenesis process (155,156). The type of miRNA most frequently increased during hypoxia is miR-210 (157). In normoxic human umbilical vein endothelial cells, miR-210 overexpression stimulates the formation of capillary-like structures and VEGF-dependent cell migration. miR-210 blockage, on the other hand, inhibits these mechanisms (158,159). Furthermore, miR-210 stimulates angiogenesis by upregulating the expression levels of VEGF and its receptor, in addition to targeting the antiangiogenic factor receptor tyrosine kinase ligand ephrin-A3 (159).

Hypoxia causes endothelial cells to produce miR-424, which targets cullin 2, a ubiquitin ligase scaffold protein, to stimulate angiogenesis. By stabilizing HIF-1α, this step enables stabilized HIF-1α to transcriptionally trigger the expression of VEGF (160). miR-21 is another miRNA that promotes angiogenesis. To activate the downstream Akt/ERK signaling pathways and increase HIF-1α and VEGF expression, it targets PTEN (161). By inhibiting VEGF and/or HIF-1α, on the other hand, miR-20b and miR-519c negatively control angiogenesis (162,163). The downregulation of miR-107 increases tumor angiogenesis under hypoxic conditions because it not only regulates HIF-1α but also inhibits the production of HIF-1β (114).

Invasion and metastasis activation. The biology of metastasis is an intricate, multi-step and dynamic process. The loss of cell adhesion due to E-cadherin suppression and the activation of genes linked to invasion and motility are the hallmarks of the EMT, which is regarded as an initial and crucial stage of metastasis (164). Numerous miRNAs alter the expression of genes, including MMPs and their inhibitors, that control extracellular matrix (ECM) turnover and breakdown (165). Proliferation, migration, differentiation and apoptosis are among the cell phenotypic alterations that are influenced by ECM turnover or remodeling (166). Zinc-dependent endopeptidases, known as important MMPs such as MMP-1, -2, -3, -7 and -9, are crucial for cell migration, adhesion, dispersion, differentiation and ECM modeling (167). They can cause cell surface receptors to cleave

ECM proteins such as type IV collagen, interestitial collagen, elastin and casein for degradation (168). Exogenous miR-143 expression in osteosarcoma cells leads to downregulated MMP-13 protein levels and reduced cell invasion (169). In clinical samples, MMP-13 was found in cases with lung metastases and low miR-143 expression, while it was not found in patients without metastases and with high miR-143 expression (170).

It has been revealed that miR-206 decreased the amount of the cell division cycle 42, MMP-2 and MMP-9 proteins in human breast cancer (171). Due to the control of actin cytoskeleton remodeling, including filopodia formation, this protein level regulation inhibited MDA-MB-231 cell invasion and migration (171). Furthermore, downregulation of miR-340 has been associated with aggressive activity in several breast cancer cell lines (172). Because it directly targets c-Met, the stimulation of miR-340 expression suppresses the motility and invasion of breast tumor cells, thereby modulating the production of MMP-2 and MMP-9 (172).

Numerous signaling pathways, including the TGF-β pathway, are considered to govern EMT. These pathways converge on important transcription factors, including Zinc finger E-box-binding homeobox (ZEB), SNAIL and TWIST (173). TGF-β-regulated miRNAs participate in TGF-β signaling to trigger EMT and promote metastasis in advanced cancer. miR-155 is implicated in this regulation, is transcriptionally activated by TGF-β/SMAD4 signaling and is upregulated in several malignancies such as colorectal and breast cancer (174). Furthermore, miR-155 stimulates EMT by targeting RhoA GTPase, an essential modulator of cellular polarity and the formation and maintenance of tight junctions. Additionally, miR-155 suppression prevents TGF-β-induced EMT, tight junction disruption, cell migration and invasion (174). In contrast to miR-155, TGF-β inhibits miR-200 and miR-203, and it has been demonstrated that the miR-200 family influences EMT by suppressing the expression of the transcriptional repressors (ZEB1 and ZEB2) of E-cadherin (175). ZEB1 and ZEB2, in turn, also suppress the miR-200 transcript, creating a double-negative feedback loop between the miR-200 family and ZEB1/ZEB2 (176).

Cell death resistance. An important characteristic of tumor growth is evasion of apoptosis, which is considered to be controlled by miRNAs (177,178). Different strategies are developed by tumor cells to prevent or delay apoptosis. The most prevalent of these is the loss of p53 tumor suppressor activity (179). Other strategies to avoid apoptosis include the suppression of proapoptotic proteins, upregulation of anti-apoptotic regulators and blocking the death pathways (180). miRNAs either inhibit or activate the components involved in anti-apoptosis mechanisms. It has been demonstrated that numerous p53-regulated miRNAs contribute to p53 functions; some of these miRNAs can feedback-modify p53 activity (181). For example, in multiple myeloma, p53 transcriptionally activates three miRNAs (miR-192, miR-194 and miR-215) to bind directly with the mRNA of mouse double minute 2 homolog (Mdm2) and prevent p53 from being destroyed, thus inhibiting Mdm2 expression (182). The development of multiple myeloma is influenced by the downregulation of these miRNAs, which are positive regulators of p53 (183).

Another negative feedback regulation takes place between p53 and miR-122 by targeting cyclin G1 (184) and cytoplasmic polyadenylation element-binding protein (185). The creation of a chemotherapy and miRNA-based therapeutic combination for HCC is made possible by the stimulation of p53 activity by miR-122, which also increases cell sensitivity to doxorubicin (186). In addition, cancer cells are resistant to death due to other dysregulated p53-regulated miRNAs. For example, the miR-17-92 cluster represents a novel target for p53-mediated transcriptional repression during hypoxia. While its upregulation prevents apoptosis, its downregulation makes cells more vulnerable to hypoxia-induced apoptosis. Consequently, tumor cells that express more miR-17-92 may be able to evade the apoptosis caused by hypoxia (187). All these aforementioned findings demonstrate that, in healthy circumstances, p53 and miRNAs regulate a network that intricately determines cell destiny. However, dysregulation of p53 or its target miRNAs may allow cancer cells to evade cell death.

Some miRNAs that serve a role in cell death may target proapoptotic factors (Bax, Bim and Puma) and anti-apoptotic regulators (Bcl-2 and Bcl-xL). In chronic lymphocytic leukemia, miR-15a and miR-16-1 are downregulated, and their expression is inversely associated with that of Bcl-2 (97). Additional research revealed that these two miRNAs caused apoptosis and suppressed Bcl-2 expression (188). Other miRNAs, including miR-204 (189), miR-148a (113) and miR-365 (190), also control Bcl-2 expression. miR-491-5p effectively causes ovarian cancer cells to undergo apoptosis by causing Bim accumulation and directly suppressing Bcl-xL expression (191).

The role of some miRNAs and their relationship with tumor occurrence, development, metastasis and survival is shown in Table I.

6. Current miRNA detection and quantification methods

Different approaches are currently available for the identification and quantification of miRNAs, which have the capacity to measure either total miRNAs or specific miRNAs, and their localization within cells. For the quantification of total miRNA, these methods have been devised based on hybridization, amplification, sequencing and enzyme-based approaches (10). The hybridization-based methods include northern blotting, microarrays and bead array-based profiling. The amplification-based methods of miRNA quantification include reverse transcription-quantitative PCR (RT-qPCR), rolling circle amplification (RCA) and next-generation sequencing. The enzyme-based methods of miRNA detection include invader assays, luciferase-based assays, assay using miRNA *in vivo* activity reporters and the molecular beacon imaging method. All these detection and quantification assays vary in efficiency, cost, accuracy and monitoring convenience (10). The currently used miRNA detection approaches using different detections tools, and their strengths and limitations are summarized in Table II.

7. Structural and functional aspects of the CRISPR/Cas13 system

CRISPR/Cas systems have been widely used for biotechnological and clinical research following the identification of

Table I. Association of different miRNAs with tumor occurrence, development, metastasis and survival.

First author/s, year	miRNA	Tumor occurrence	Tumor development	Metastasis	Survival rate	(Refs.)
Sheedy and Medarova, 2018	miR-10b	Upregulated in metastatic cancers	Promotes invasion and metastasis	Directly involved in metastasis	Poor prognosis, associated with high metastatic potential	(250)
Kolenda *et al*, 2020	miR-18a	Upregulated in multiple cancers	Promotes tumor growth	Promotes metastasis	Decreased survival rate	(251)
Feng and Tsao, 2016	miR-21	Upregulated in various cancers, acts as an oncogene	Promotes cell proliferation and inhibits apoptosis	Enhances migration and invasion	Poor prognosis, associated with lower survival rates	(252)
Zhang *et al*, 2019	miR-34a	Tumor suppressor, downregulated in cancers	Induces cell cycle arrest and apoptosis	Inhibits EMT and metastasis	Improved prognosis, associated with higher survival rates	(253)
Mahesh and Biswas, 2019	miR-155	Upregulated in several cancers, acts as an oncogene	Promotes tumor growth and inflammation	Facilitates metastasis by targeting tumor suppressor genes	Poor prognosis, linked to aggressive cancer types	(254)
Lam *et al*, 2018	miR-143/145	Downregulated in colorectal and bladder cancers	Inhibits tumor growth	Inhibits metastasis	Improved survival rate	(255)
Cavallari *et al*, 2021	miR-200 family	Downregulated in metastatic cancers	Inhibits EMT and promotes the epithelial phenotype	Suppresses metastasis	Improved prognosis, associated with reduced metastasis	(256)
Zaccagnini *et al*, 2017	miR-210	Upregulated in breast, lung and pancreatic cancer	Promotes tumor growth	Promotes angiogenesis	Decreased survival rate	(257)
Torres-Berrío *et al*, 2020	miR-218	Downregulated in cervical and colorectal cancer	Inhibits tumor growth	Inhibits metastasis	Improved survival rate	(258)
Abak *et al*, 2018	miR-221	Upregulated in liver and breast cancer	Promotes cell proliferation	Promotes invasion	Decreased survival rate	(259)

EMT, epithelial-mesenchymal transition; miR/miRNA, microRNA.

Table II. Current miRNA detection approaches using different tools with strengths and limitations.

A, Hybridization-based methods

First author/s, year	Detection tools	Strengths	Limitations	(Refs.)
Ahmad *et al*, 2021	Northern blot	Simplicity and cost-effectiveness, distinguishes between precursor and mature miRNAs, specificity, and visualization	Low sensitivity, labor-intensive and time-consuming, requires large quantities of RNA, and not quantitative	(260)
Russo *et al*, 2003	Microarrays	Large-scale screening, comparative analysis, high throughput and established technology	Requires miRNA annotation, not quantitative, potential cross-hybridization and data analysis complexity	(234)
Gaarz *et al*, 2010	Bead-array-based Profiling	High throughput, quantitative, cost-effective, speed and flexibility	Complexity, sensitivity, equipment requirement and data interpretation	(261)

B, Amplification-based methods

First author/s, year	Detection tools	Strengths	Limitations	(Refs.)
Mehta, 2022	RT-qPCR	High sensitivity, quantitative, specificity, wide dynamic range and speed	Complexity, cost, normalization, potential for bias and equipment requirement	(262)
Tan *et al*, 2022	Amplification assays	High sensitivity, specificity, versatility, quantitative and compatibility with various platforms	Complexity, cost, potential for bias, normalization challenges and equipment requirement	(263)
Pervez *et al*, 2022	Next-generation sequencing	High sensitivity and specificity, comprehensive profiling, identification of novel miRNAs, quantitative data, and high throughput	Cost, complexity, data analysis, turnaround time and equipment requirement	(264)

C, Enzyme-based assays

First author/s, year	Detection tools	Strengths	Limitations	(Refs.)
Li *et al*, 2022	Invader assay	High sensitivity, specificity, quantitative, isothermal process and high throughput	Complexity, cost, equipment requirement and data interpretation	(265)

D, Luciferase and GFP-based miRNA reporters

Jin *et al*, 2013	miRNA transcription and target site reporters	Direct measurement, quantitative, high specificity, versatility and high throughput	Complexity, cost, potential for artifacts, normalization challenges and data interpretation	(266)

Table II. Continued.

D, Luciferase and GFP-based miRNA reporters				
First author/s, year	Detection tools	Strengths	Limitations	(Refs.)
Oh and Lee, 2013	*In vivo* miRNA activity reporters	Real-time monitoring, spatial and temporal resolution, physiological relevance, comprehensive analysis, and versatility	Complexity, cost, time-consuming, potential for artifacts and data interpretation	(267)
Hwang *et al*, 2018	Molecular beacon *in vivo* imaging system	High sensitivity and specificity, real-time monitoring, non-invasive, quantitative, and spatial and temporal resolution	Complexity, cost, potential for artifacts, data interpretation and delivery challenges	(268)

GFP, green fluorescent protein; miRNA, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

RNA-programmable nucleases from the prokaryotic adaptive immune system (192,193). Among different types of CRISPR/Cas systems, the type VI system exclusively targets ssRNA (194,195). In this system, different subtypes have been identified as type VIA (having Cas13a)-VID (having Cas13d), Cas13X and Cas13Y (196,197), among which Cas13a is the best known so far. Innovative diagnostic methods for the identification of certain miRNAs have been developed using this platform.

Cas13 protein. The Cas13 protein of *Leptotrichia buccalis* consists of a single effector molecule (~1,159 aa residues) and a 64-66 nucleotide crRNA, which make up the CRISPR/Cas13 system (198,199) (Fig. 4). The Cas13a type protein crystal structure along with the crRNA secondary structure has been studied in more detail compared with that of other Cas13 (b-d, x and y) types (200,201). The Cas13a protein, isolated from the Gram-negative bacterium *Leptotrichia shahii*, has been identified to catalyze two reactions: One that matures crRNA and the other that cleaves the target RNA (202). In this single effector protein, two domains, the nuclease (NUC) domain and the crRNA recognition (REC) lobe, make up the entire molecule (Fig. 4A). The N-terminal domain (NTD) and a domain known as helix-1 are both present in the REC lobe (202). The non-conserved area of Cas13a known as the NTD is made up of two subdomains: A smaller subdomain with three α-helices, a β-hairpin and a β-sheet, and a bigger subdomain with an ordered fragment made up of seven α-helices and a disordered fragment (203) (Fig. 4).

The V-shaped structure of the helix-1 domain is formed by seven α-helices. The crRNA binding channel is formed by the positively charged surface of the helix-1 domain facing the NTD domain (204). The two conserved higher eukaryotic and prokaryotic nucleotide-binding (HEPN) domains (HEPN-1 and HEPN-2), a linker that joins the two HEPN domains, and a helix-2 domain are all found in the NUC lobe (202). The helix-1 and HEPN domains are responsible for the two enzymatic functions of Cas13a. The helix-2 domain further sub-divides the HEPN-1 domain into two subdomains. Three α-helices make up the HEPN 1-II subdomain, whereas four α-helices and a brief β-hairpin make up the HEPN-1 I subdomain (Fig. 4B). Seven α-helices plus a double-stranded β-sheet directly make up the HEPN2 domain structure (202). Between the two HEPN-1 subdomains lies the helix-2 domain, which is made up of eight bean-shaped α-helices (204). The Cas13a/crRNA complex targets the matching RNA under the direction of crRNA, triggering the protection of prokaryotes against RNA viruses (205,206) (Fig. 4F and G).

crRNA structure. The structure of the crRNA is composed of a spacer sequence [guide RNA (gRNA)] that mediates target recognition by RNA-RNA hybridization and a repeat stem-loop region (referred to as the 5'-handle) that prevents this region from being cleaved during the cleavage of the target RNA by Cas13a (207) (Fig. 4C). The stem-loop is composed of two single-stranded bases at the base, a nine-base loop, neighboring motifs at both ends and a stem made up of five base pairings. The repeat region of crRNA can twist slightly to generate a helical shape in addition to a stem-loop secondary structure (202). This structure is primarily sustained by the

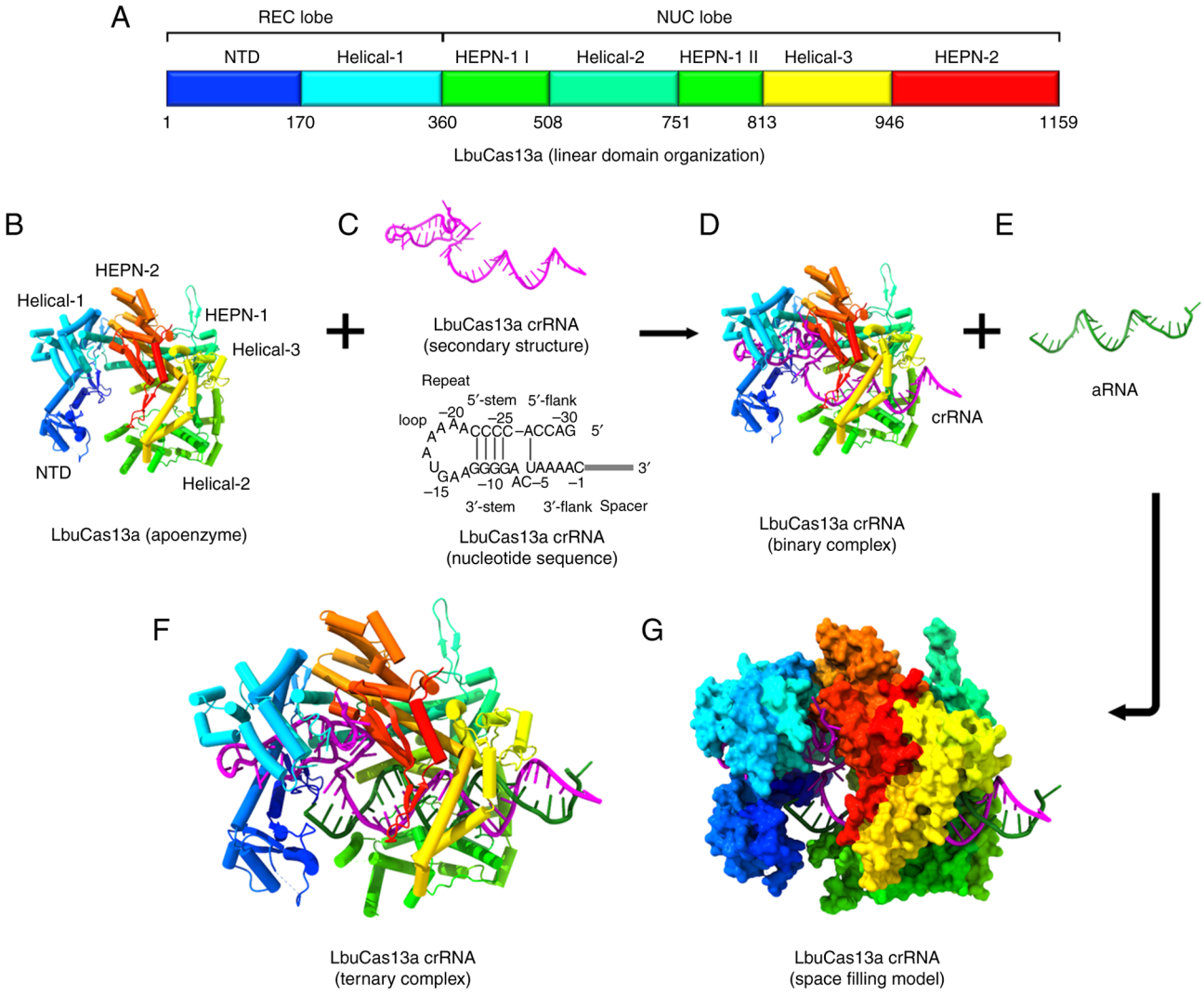

Figure 4. Overview of the structural features of CRISPR/Cas effectors of type VI-A. (A) Linear domain organization of LbuCas13a, presenting different domains, and the REC and NUC lobes, annotated with corresponding amino acid residues. (B) Cartoon representation of LbuCas13a apoenzyme. (C) Secondary structure of LbuCas13a crRNA and its nucleotide sequence. (D) Binary structure of the LbuCas13a-crRNA complex. (E) Secondary structure of the aRNA. (F) LbuCas13a-crRNA-aRNA ternary complex (cylinders and stubs form). (G) Space filling model of LbuCas13a ternary complex. The crystal structure of the LbuCas13a-crRNA-target RNA ternary complex was downloaded from PDB (https://www.rcsb.org/; PDB ID 5XWP) and was edited using University of California, San Francisco ChimeraX software. aRNA, activator RNA; Cas, CRISPR-associated sequence; crRNA, CRISPR RNA; HEPN, higher eukaryotic and prokaryotic nucleotide-binding; LbuCas13a, *Leptotrichia buccalis* Cas13a; NTD, N-terminal domain; NUC, nuclease; PDB, Protein Data Bank; REC, crRNA recognition.

creation of strong hydrogen bonds between the HEPN2 domain and the backbone of the crRNA (204). It is possible to cause the Cas13a protein to recognize the stem-loop structure of the crRNA in a sequence-specific way by altering stem nucleotides, which is necessary for its nuclease cleavage activity (204) (Fig. 4D).

Within the crRNA guide region, one to four nucleotides oversee the 5'-end being attached to the void created by the HEPN-1 and helix-2 domains, making a U-turn. The linker and the groove created by the HEPN-2 domain bind the final three or four nucleotides (16). To identify the target RNA, the Cas13 protein buries a spacer of eight or nine nucleotides at the 5'-end, while the middle region and 3'-end are exposed to the peripheral solvent (204). To determine the ideal gRNA, researchers have created a computational model, and identified that the properties of crRNA and the environment of the target RNA are important constraints on the cleavage effectiveness of Cas protein by assessing the activities of 24,460 gRNAs and looking for mismatches between gRNAs and the target sequence (208).

Cleavage mechanism of RNA by CRISPR/Cas13. Cas13 action does not require crRNA maturation; in fact, pre-crRNA can detect target RNAs (also referred to as activator RNA; Fig. 4E) (209). The ribonucleoprotein complex (RNP) undergoes a conformational change when crRNA binds to a target RNA (Fig. 4F). A catalytic site is formed in part by the close interaction of two HEPN domains (201). The binding of crRNA is anticipated to result in the cleavage of the target RNA as well as other ssRNAs surrounding the RNP complex, possibly including host RNA, because of the distance between the catalytic site and the crRNA-RNA duplex. This activity of the CRISPR/Cas13 system, known as collateral damage or collateral cleavage, refers to the off-target cleavage of endogenous RNA (205,210).

According to one study, sequence-specific activation of non-specific RNA cleavage may help to protect the nearby cells by causing cell dormancy or death, or it may improve the prevention of phage replication by removing all RNA from a cell in bulk (195). This ability has been widely used in RNA knockout strategies, disease treatment, interference with viral infection, screening of loss of function mutants, molecular detection of biological agents and CRISPR-based antimicrobials (211-213). Therefore, the CRISPR/Cas13 system can simultaneously cleave target RNA and non-target RNA (206,214). Furthermore, in Cas13, the mutation of arginine in the HEPN domain, which is responsible for RNA cleavage, results in the formation of catalytically inactive Cas13, known as dead Cas13 (dCas13). This form of Cas13 has been used to increase the application of Cas13 in RNA research. Through the binding of fluorescent proteins or enzymes, mutant dCas13 persistently attaches to target RNA and offers a platform for detecting transcripts in living cells (20,215).

8. Novel approaches for cancer detection using CRISPR/Cas13

miRNA expression profiling is technically difficult as mature miRNAs are small in size, highly similar and not too abundant in bodily fluids (216). Different platforms have been engineered that link CRISPR-based methods for miRNA detection with the advantages of enzyme-assisted signal amplification and enzyme-free amplification biosensing technologies (217). Electrochemical detection using biosensors is a novel low-cost and sensitive diagnostic strategy for the identification of nucleic acids. This approach offers excellent sensitivity, accuracy and fewer diagnostic constraints as this strategy is inexpensive, relying on effective sensors and a straightforward, miniature readout (218).

Colorimetric miRNA detection

Visual miRNA detection. RCA is a common isothermal enzymatic DNA replication technique for creating DNA, RNA and protein sensors (219,220). In an RCA reaction, a short DNA or RNA strand is amplified to produce a long, single-stranded DNA (ssDNA) or RNA using a circular DNA template by a specialized isothermal strand displacement DNA or RNA polymerases (219,220). The RCA process results in a concatemer that has $>10^9$ tandem DNA repeats that are complementary to the circular DNA or RNA template (221). This visual detection platform utilizing a CRISPR/Cas13 system, known as the visual Cas (vCas) system, has been used for the specific and sensitive detection of miRNA (222) (Fig. 5A).

Theoretically, when Cas13a/crRNA recognizes the target miRNA, it collaterally cleaves the pre-primer that contains uracil ribonucleotide (rU). DNA polymerase-mediated RCA can be started using the leftover 5'-DNA fragment of the pre-primer. Following its 3'-end, a T4 polynucleotide kinase restores the aforementioned process to produce a lengthy G-rich repeat sequence that can form a tandem G-quadruplex to function as a DNAzyme that mimics HRP and T4 polynucleotide kinase catalyzes the oxidation of the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (223) (Fig. 5A). Consequently, within 10 min, the colorless mixture turns green, allowing the target miRNA to be observed (223). The results using this approach show that vCas can be used to evaluate miRNAs in serum and cell extracts (222), and can offer a limit of detection (LOD) of 1 fM for miR-10b, a type of miRNA highly expressed in breast cancer cells (224,225). However, compared with one-step Cas13a detection platforms, the LOD of this approach is four orders of magnitude lower (222).

Naked-eye gene detection platform. Colorimetric assays typically examine the color shift within the test solution. Without the need for complex equipment, miRNA detection can be performed quickly and easily with the use of a colorimeter or the human eye (226). Once Cas12a/crRNA, Cas13a/crRNA or Cas14/crRNA identify their target DNA or RNA, they exhibit a transition to the active state, where their collateral cleavage activity cleaves the ssRNA or ssDNA substrates (227,228). With the aid of CRISPR/Cas recognition for miRNA sensing, researchers have created a DNA probes and gold nanoparticles (AuNPs)-based platform. Cas12a/crRNA's programmable recognition of DNA and Cas13a/crRNA's programmable recognition of RNA initiates ssRNA or trans-ssDNA cleavage of their corresponding complementary target (229). For the intended AuNPs-DNA probe pair, target-induced trans-ssDNA or -ssRNA cleavage results in a change in aggregation behavior, which allows direct visual observation in an hour of various samples, including miRNAs (229). For naked-eye gene detection platforms, a universal linker ssRNA or ssDNA acts as a trans-cleavage substrate for the CRISPR/Cas13a or CRISPR/Cas12a systems. Furthermore, two universal AuNPs DNA probes have been created to hybridize with either ssRNA or linker ssDNA (229).

Since trans-cleavage is not triggered in the absence of a target (such as miRNA sequences), the linker ssDNA or ssRNA stays intact during the reaction. An aggregated state is created by hybridization-induced cross-linking of the AuNPs-DNA probe pair (229). After the trigger of trans-cleavage activity, the linker nucleotides are broken down once the CRISPR/Cas systems identify their target nucleotides. By identifying cross-linked and scattered AuNPs-DNA probes (229), visual detection can be approximated (Fig. 5B). The sensitivity of miRNA detection is as low as 500 fM, which is on par with or better than that of techniques based on dual-labeled fluorescent ssRNA reporters (229). When comparing 1.0 nM target miRNA-17 with miRNA-10b, miRNA-21 and miRNA-155 by colorimetry at the same concentration, this approach demonstrated a strong level of specificity (229). Furthermore, family miRNA members with highly related sequences, such as miRNA-17, miRNA-20a, miRNA-20b and miRNA-106a, can be distinguished by single- or double-nucleotide variations at a concentration of 1.0 nM using CRISPR/Cas-based colorimetric assays (229).

Fluorescence-based miRNA detection

Cascade CRISPR/Cas13 (casCRISPR) system. A specific and sensitive biosensor, known as casCRISPR has been developed for quick and precise miRNA detection, even in cell extracts and serum samples, without the need for a target amplification procedure such as PCR or recombinase polymerase amplification (RPA) (230). By using this method, the trans-cleavage activity of Cas13a/crRNA is triggered following miRNA recognition. Such trans-ribonuclease activity has been

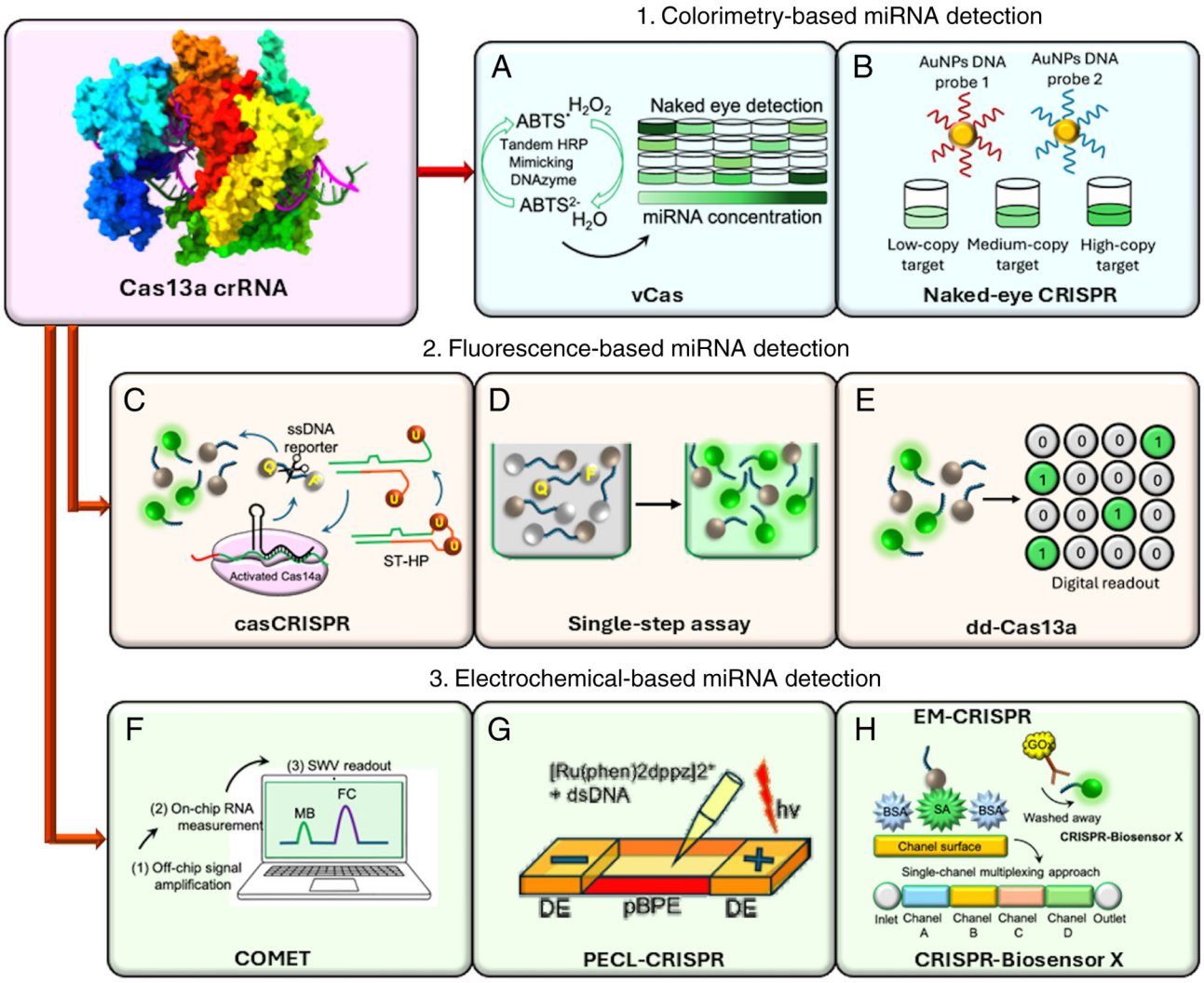

Figure 5. Overview of the different Cas13-based miRNA detection platforms. (1) miRNA detection by colorimetric methods. (A) vCas is a naked eye visual detection platform and the results can also be interpreted on a microplate reader by checking the absorbance at 405 nm. (B) In the naked eye CRISPR/Cas13-mediated colorimetric assay, single-stranded RNA cleavage initiation (by Cas13a-crRNA) or trans ssDNA cleavage (by Cas12a-crRNA) changes the behavior of the designed AuNPs-DNA probe pair. The AuNPs-DNA probe pairs lose the hybridization linkers and become dispersed, enabling miRNA detection by the naked eye. (2) Fluorescence-based miRNA detection. (C) miRNA detection by the Cas13a-Cas14a cascade (CasCRISPR). Cas14a-single guide RNA (ST-HP) has a locked trigger functioning as a bridge between Cas14a and Cas13a, acting as a substrate of Cas13a trans-cleavage triggering the Cas14a-mediated trans-cleavage for miRNA detection. (D) In single-step miRNA detection using the Cas13a-crRNA complex, a poly-U RNA reporter labelled by a quencher, black hole quencher 1, and a fluorophore, carboxyfluorescein, is cleaved by the collateral activity of Cas13a. This leads to the elimination of the fluorescence resonance energy transfer effect between the two fluorophores and releases the fluorescence signals. (E) dd-Cas13a assay for miRNA quantification. Oil is used to emulsify with miRNA and Cas13 into thousands of picolitre-sized droplets, each containing 0-1 miRNA target molecules. The target miRNA induces the cleavage of 10^4 fluorescent reporters, once recognized by a crRNA. This leads to the production of a fluorescence-positive droplet. (3) Electrochemical-based miRNA detection. (F) The approach of the COMET assay for miRNA detection includes off-chip signal amplification, on-chip RNA measurement and square wave voltmetry (SWV) readouts. The two-stage signal is amplified in the off-chip stage as Cas13-based collateral cleavage of the trigger molecule and the enzyme-free allosteric catalysis of the catalytic hairpin DNA circuit are performed in one pot. To perform the baseline drift correction, a dual-reporter approach is used for the on-chip electrochemical quantification. (G) Quantitative miRNA detection on a PECL-CRISPR chip. At the anode of the BPE, the $[Ru(phen)2dppz]2^+$-DNA complex oxidation, indicated by electrochemiluminescence is proportional to the target miRNA concentration. (H) On-chip miRNA detection using the EM-CRISPR assay. The amperometric signal obtained from the enzymatic readout reflects the immobilized Gox-conjugate. In the sample, this immobilized complex is inversely proportional to the target concentration. Non-specific biomolecule adsorption is prevented by BSA. Furthermore, the previous electrochemical microfluidic biosensor has been advanced as CRISPR-Biosensor X, which was implemented by dividing the channel into subsections for simultaneous quantification and amplification-free detection of up to eight miRNAs. This strategy led to the creation of four novel chip designs. ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; $ABTS^{\cdot}$, ABTS free radical; $ABTS^{2-}$, ABTS radical anion; AuNP, gold nanoparticle; CRISPR, clustered regularly interspaced short palindromic repeats; Cas13, CRISPR-associated sequence 13; CasCRISPR, cascade CRISPR/Cas13; COMET, Cas-catalytic hairpin DNA circuit-powered electrochemical RNA sensing technology; crRNA, CRISPR RNA; dd-Cas13a, droplet-digital Cas13a assay; DE, differential expression; dsDNA, double-stranded DNA; EM, electromicrofluidic; FC, ferricyanide; GOx, glucose oxidase; hv, energy; MB, methylene blue; miRNA, microRNA; pBPE, photonic barcoded particle encoding; PECL-CRISPR, CRISPR/Cas13a powered portable electrochemiluminescence chip; SA, streptavidin; ssDNA, single-stranded DNA; ST-HP, stem-loop hairpin probe; SWV, square wave voltammetry; vCas, visual Cas.

investigated using a hairpin-structured DNA oligonucleotide (stem loop-hairpin or 'locked trigger') with an unpaired rU in the loop. Accordingly, the phosphodiester bond adjacent to the rU of the Cas14a/single guide RNA (sgRNA) 'locked-trigger' is broken (Fig. 5C). Through strand displacement, the latter causes the 'locked-trigger' to change from a stable structure to

a 5'-toehold duplex structure that can start the interaction with Cas14a/sgRNA (230). The quencher, black hole quencher 1 (BHQ1) and fluorophore [6-carboxyfluorescein (6-FAM)] present on the ssDNA reporter are efficiently separated once the 'locked-trigger' is cleaved, resulting in Cas14a-based trans-cleavage and the production of fluorescence signals (230) (Fig. 5C). In casCRISPR, the trans-cleavage activity of Cas13a and Cas14a enables the detection of miR-17 with a LOD of 1.33 fM, even by a difference of one nucleotide (single base specificity), which is ~1,000 times lower than that of direct Cas13a-based miRNA detection (199). As a promising tool for miRNA diagnostics, casCRISPR may offer greater miRNA detection specificity without amplification of the target as required for RT-qPCR because of the Cas13a/crRNA high-fidelity recognition capacity (230). Researchers have engineered casCRISPR v2, a second version, by adding Cas13a and Cas12a coupled with casCRISPR, and have compared this with ordinary casCRISPR (version 1). This platform exhibited an advanced performance, because Cas12a also functions as a ssDNA-activated DNase (230,231). The fluorescence intensity curves showed that casCRISPR v1 could detect miR-17 at as low as 6.25 fM, whereas casCRISPR v2 could detect miR-17 at as low as 100 fM. However, Cas13a-Cas14a (casCRISPR v1) could reach a lower detection limit and a greater signal to background ratio than Cas13a-Cas12a, which may be explained by the fact that Cas14a has an improved cleavage efficiency compared with Cas12a when ssDNA is utilized as the activator (230).

Single-step Cas13a-triggered miR-17 signal amplification method. The *Leptotrichia buccalis* Cas13a (LbuCas13a) protein has been utilized to directly detect miRNAs with great specificity and simplicity compared with stem-loop RT-PCR, which requires three oligonucleotides, a cDNA synthesis step and different temperature cycles to replicate amplicons (232). To achieve an enhanced fluorescence signal, a crRNA and moderate isothermal conditions are used. According to the fluorescence spectroscopy results, the poly-U RNA probe (fluorescent quencher 5' U) labeled with fluorophore (6-FAM) and quencher (BHQ1) is successfully cleaved when introduced into the miRNA detection test, resulting in fluorescent signals (233) (Fig. 5D). Based on the trans-ribonuclease activity of Cas13a and base pairing between crRNA and miR-17 (selected as the model target), researchers have able to detect the miR-17 at as low as 4.5 aM concentration within 30 min (233). Furthermore, rationally programmed crRNA is able to direct the LbuCas13a/crRNA complex to distinguish single-nucleotide changes, even at the end of target miRNA (234). The relative measurement of miR-17 in four different cell line samples, including three human breast cancer cell lines, validated the uniqueness and validity of the approach (233). The high sensitivity of identifying miRNAs in total small RNA preparations from serum samples also suggests a useful application of the one-step Cas13a/crRNA-based detection approach for miRNA-related disease diagnostics (233).

Direct single-molecule miRNA quantitation using a droplet-digital Cas13a assay (dd-Cas13a). A novel approach known as dd-Cas13a increases the concentration of local molecules for an effective reaction or detection (234). Researchers have adopted a droplet microfluidic technology to increase the local concentration of the RNA reporter and target sequence for the Cas13a system in cell-sized volumes (235,236). Thousands of picolitre-sized droplet reactors are filled with an oil-emulsified combination of target RNAs and Cas13a. Such droplets can be illuminated by the cumulative fluorescence signal from the collateral cleavage of a single RNA target-activated Cas13a (235,236) (Fig. 5E). Under these circumstances, a single-target RNA can cause the cleavage of >100 quenched fluorescent RNA reporters following recognition by a crRNA, producing a fluorescence-positive droplet. Therefore, dd-Cas13a eliminates the necessity for reverse transcription and amplification, and enables absolute digital quantification of single unlabeled RNA molecules (235,236). The utility of the dd-Cas13 assay was assessed by measuring miRNA-17 expression in several cell lines, such as human glioma cells, normal human breast cells and adenocarcinoma cells, and 100% results that corresponded with RT-qPCR data were obtained (235).

Electrochemical miRNA detection

Cas-catalytic hairpin DNA circuit (CHDC)-powered electrochemical RNA sensing technology (COMET). The CRISPR/Cas13a platform and a CHDC can be integrated on an electrochemical biosensor, known as COMET. This approach consists of a two-stage signal amplification system for high-sensitivity estimation of six RNAs related to non-small cell lung cancer (NSCLC), including miR-17, miR-19b, thyroid transcription factor-1 RNA, miR-155, EGFR mRNA and miR-210. All components are carefully combined to enable dual signal amplification for the CRISPR/Cas13a platform and catalyzed hairpin DNA circuit (Cas-CHDC)-powered RNA detection (237).

In a measuring volume of 10 μl, COMET allows for RNA detection with a dynamic range between 50 aM and 5 nM, with a readout time of 6 min and an overall time of 36 min. To differentiate early-stage patients (n=20) with NSCLC from healthy participants (n=30) and patients with benign lung disease (n=12), the biosensor could sensitively and selectively detect minimal expression RNA targets. The latter shows that the electrochemical CRISPR/CHDC system can be a quick and accurate way to diagnose cancer at an early stage (237).

To achieve quick RNA detection on a small point-of-care (POC) testing device, scientists have incorporated chip-based Cas-CHDC amplification into an electrochemical biosensing technology (237) (Fig. 5F). The COMET chip could differentiate between miR-20a and miR-20b (two-base mismatches) and between miR-17 and miR-106a (1-base mismatch) (237). Furthermore, a 1:1,000 M mixture of miR-17 with miR-155 and thyroid transcription factor-1 mRNA has been used to assess the capacity of the COMET chip to identify lower expression target RNAs in special samples such as benign lung disease and NSCLC samples (237).

Because non-target RNAs are highly expressed in the circulation of patients, scientists have identified that the COMET chip could detect miR-17 at zeptomolar (10^{-21} M) concentrations and as low as 0.1% of background RNA mixture. This is crucial for future clinical use, and the chip and reagents can be purchased for as little as USD 0.27 per test (237).

CRISPR/Cas13a powered portable electrochemiluminescence (ECL) chip (PECL-CRISPR) for miRNA detection. Based on the ECL approach and employing the trans-cleavage

activity of CRISPR/Cas13a, scientists have engineered a sophisticated platform known as PECL-CRISPR, which mediates the exponential amplification of the ultrasensitive detection of miRNAs that differ even by a single-base (232).

Quantitative miRNA detection is made possible by the ECL signal on a bipolar electrode (BPE), which directly reflects the oxidation reaction of the ruthenium (II) polypyridyl complex [Ru(phen)2dppz]2^{+} and DNA at the BPE anode, and is directly related to the miRNA concentration (232) (Fig. 5G). By identifying miR-17 from various human cancer cells, the application of PECL-CRISPR has been examined, proving that the platform has great potential for miRNA diagnosis (232).

By logically designing the crRNA, the platform exhibits the ability to differentiate between highly homologous members of the miR-17 and let-7 families by introducing a mismatch at the precise location of the crRNA, independent of the single distinct base locations at the 5' or 3' end of the target (233). The LOD of this platform for miR-17 was $1.0x10^{-15}$ M, which is approximately three orders of magnitude lower than that of the direct detection of miR-17 (LOD, $1.0x10^{-12}$ M) using LbuCas13a (233).

Detection of specific miRNAs using electromicrofluidic (EM)-CRISPR/Cas13a. For on-site detection of miRNAs, a CRISPR/Cas13-based biosensor has been created as a user-friendly and cheaper electrochemical microfluidic platform (EM-CRISPR/Cas13a), which substitutes a Cas13a-driven signal amplification for synthetic nucleic acid amplification steps (238). In this assay, a streptavidin is applied to the chip inlet to functionalize the immobilized surface area of the biosensor for the CRISPR/Cas13a-powered miRNA detection. All unbound biomolecules are eliminated by vacuuming the channel inlet (238). To activate Cas13a and perform the collateral cleavage of the reporter RNA (reRNA), the samples (biotin and 6-FAM-tagged reRNA), and the other sample (containing the target miRNA and the Cas13a effector with its target-specific crRNA) are mixed separately (238) (Fig. 5H).

An enzymatic readout of the assay is made possible by the addition of anti-fluorescein antibodies bonded with glucose oxidase (GOx), which bind only to the uncleaved reRNAs (Fig. 5H). Since GOx catalyzes its substrate to produce H_2O_2, which is amperometrically measured in the electrochemical cell, a glucose solution is used in this assay for the readout (238). Without performing nucleic acid amplification, researchers have used this new combination to detect the miRNA levels of the putative brain tumor markers miR-19b and miR-20a in serum samples. With a setup time of <4 h and a readout time of 9 min, the EM-CRISPR/Cas13a biosensor achieved a detection limit of 10 pM with a measuring volume of <0.6 liters. Additionally, this biosensor platform was able to identify miR-19b in serum samples of children with brain cancer, proving that this electrochemical CRISPR-powered system is a low-cost, readily scalable and target amplification-free molecular approach for miRNA-based diagnostics (238).

Simultaneous quantification of miRNA using CRISPR-Biosensor X. The development of diagnostic tools and techniques for multiplexing approaches is based on analyzing the abundance of a number of specific components in individual samples from a patient. However, the variety of Cas protein types and the potential for cross-reactions between distinct Cas13a proteins may restrict the use of Cas13a (239). This might be resolved using a multichannel microfluidic chip technique.

Researchers have developed various multiform versions of electrochemical microfluidic biosensors by segmenting the channels into subsections, using the same principle as the EM-CRISPR/Cas13a system (Fig. 5H). This resulted in four unique chip designs for the simultaneous quantification of a maximum of eight miRNAs using the innovative system called CRISPR-Biosensor X. Without altering the sensor or measurement configuration, this system can work on a single clinical sample with a single effector protein (239).

Electrochemical CRISPR/CHDC assay. Because of their high sensitivity, selectivity and low instrument costs, electrochemical biosensors are emerging as a potent detection technique platform (240). Using the CRISPR/Cas13a platform in conjunction with the catalytic hairpin assembly (CHA) process, an ultrasensitive electrochemical biosensing platform has been designed for miRNA-21 detection (241). CHA is a high-efficiency, isothermal, enzyme-free amplification technique that may be used in a variety of analytical formats, such as electrophoretic (242), colorimetric (243), fluorescence (231), surface plasmon resonance (244) and electrochemical approaches (245).

This electrochemical biosensor exhibited a strong biosensing activity from 10 fM to 1.0 nM with an LOD of 2.6 fM using the CRISPR/Cas13a-mediated cascade signal amplification approach (241). The use of the biosensing platform for miRNA-21 detection was assessed. A clinical sample (human serum; diluted ten times), was used to test the analytical reliability before being tested for various concentrations of miRNA-21 (10 fM to 1.0 nM). The electrochemical recoveries were ~98.2%, demonstrating that the CRISPR/CHDC assay can be used to identify miRNA-21 in biological materials (241).

Other RNA sensors utilizing the CRISPR/Cas13 machinery. The off-target consequences of the CRISPR/Cas13a system are minimal. One to two mismatches in the core of a gRNA diminish or eliminate its activity, enabling a mismatch control for each targeting gRNA (20,246). In this context, the specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) detection system, employs the CRISPR/Cas13a system as a quick DNA or RNA detection technique with aM (10^{-18} M) sensitivity and single-base mismatch specificity (247). This approach uses isothermal RPA, transcription and detection by Cas13 platforms (247). The signal can be recorded on a colorimetric lateral-flow strip or can be observed by fluorescence signal readout to facilitate the rapid identification of most types of suitable Cas13 RNA target. SHERLOCK enables single base-pair mismatch specificity, quick (setup time, <15 min) and accurate nucleic acid detection at concentrations as low as ~2 aM (248).

A summary of CRISPR/Cas13 systems used in miRNA detection platforms is shown in Table III.

9. Challenges and future directions

The use of innovative methods for miRNA detection is revealing novel information regarding the processing and function of miRNAs, as well as providing a basic understanding of how

Table III. Summary of CRISPR/Cas13-based biosensor platforms for miRNA detection highlighting the readout techniques and the LOD.

First author/s, year	Protein effector	miRNA target	Platform	Amplification step	Readout techniques	LOD	(Refs.)
Sha *et al*, 2021	LbuCas13a	miR-17	casCRISPR	-	Fluorescence	1.33 fM	(230)
Shan *et al*, 2019		miR-I0b, miR-17, miR-21, miR-155	Single-step assay	-		4.5 aM	(233)
Tian *et al*, 2020		miR-17	dd-Cas13a	-		3.0 aM	(235)
Zhou *et al*, 2020		miR-17	PECL-CRISPR	EXPAR	Electrochemiluminescence	1.0 fM	(232)
Zhou *et al*, 2021		miR-10b, miR-17, let-7a, let-7b, let-7c	vCas	RCA	Colorimetry	1.0 fM	(222)
Bruch *et al*, 2019	LwaCas13a	miR-19b, miR-20a	EM-CRISPR	-	Electrochemical microfluidic assay	10 pM	(238)
Bruch *et al*, 2019		miRNA-19b, miRNA-20a	CRISPR-Biosensor x	-	Amperometric readout (μA cm^{-2})	10 pM	(238)
Sheng *et al*, 2021	Cas13a	miR-17, miR-155, miR-19b, miR-210	COMET	-	Electrochemical	50 aM	(237)
Cui *et al*, 2021		miR-21	CRISPR/CHDC assay	CHA		2.6 fM	(241)
Yuan *et al*, 2020	AsCas12a/LbuCas13a	miR-17	Naked-eye CRISPR	RPA/PCR	Colorimetry	500 fM	(229)

aM, attomolar (10^{-18} M); As, Acidaminococcus sp.; Cas13, CRISPR-associated sequence 13; casCRISPR, cascade CRISPR/Cas13; CHA, catalytic hairpin assembly; CHDC, catalytic hairpin DNA circuit; COMET, Cas-CHDC-powered electrochemical RNA sensing technology; dd-Cas13a, droplet-digital Cas13a assay; EM, electromicrofluidic; EXPAR, exponential amplification reaction; fM, femtomolar (10^{-15} M); Lbu, *Leptotrichia buccalis*; LOD, limit of detection; Lwa, *Leptotrichia wadei*; miR/miRNA, microRNA; PECL-CRISPR, CRISPR/Cas13a powered portable electrochemiluminescence chip; pM, picomolar (10^{-12} M); RCA, rolling circle amplification; RPA, recombinase polymerase amplification; vCas, visual Cas.

important miRNA factors and substrates function. There are next steps to follow for most of the experimental procedures that have been described. For instance, structural investigations of miRNA biogenesis factors have only evaluated a small number of miRNAs. Since the biogenesis efficiency of endogenous miRNAs varies greatly, it will be instructive for both microprocessor and Dicer substrates to include a variety of miRNAs in future investigations. This could provide information regarding microprocessor cofactors that serve a role in the synthesis of clustered and/or inefficient miRNAs.

Direct viewing of dynamic miRNA processing and regulatory complexes is made possible only by single-molecule imaging. *In vivo* experiments have largely focused on AGO and not enough research has been performed on other miRNA biogenesis factors. *In vitro* investigations of microprocessors have been lacking. The subcellular environments of microprocessors and Dicers are functionally significant and require additional investigation in the future.

Furthermore, the implications of miRNA biology, such as whether organismal phenotypes are actually mediated by the regulation of specific miRNA targets, must not be overlooked. Most of the research on miRNA-target biology is still correlative, and just because numerous derepressed targets are found when a miRNA is lost, this does not always suggest that they all serve a functional role in miRNA phenotypes.

CRISPR mutagenesis has demonstrated the potent phenotypic influence of target mRNAs and validated the growing number of target-directed miRNA degradation target sites that regulate miRNA abundance. To completely comprehend the role of miRNA in development and disease, it is necessary to establish clear links between target site-mediated gene regulation and *in vivo* phenotypes.

Prior to the full clinical use of CRISPR/Cas13 technologies, a few issues need to be resolved, including: i) As the fidelity of the CRISPR/Cas13 platform is directly linked to the tolerance of RNA mismatches, a wider range of mismatch tolerance by Cas13 effectors may encourage off-target activity (22); ii) it may be challenging to meet the specific protospacer flanking sequence requirement for Cas13 effectors; iii) RNA recognition of small RNA target sequences (<22 nt) because of the requirement that crRNAs be long enough for binding (205); and iv) RNA segments derived from effective ssRNA cleavage mediated by Cas13 may be toxic in eukaryotic cells (206,249).

10. Conclusion

miRNAs serve a role in the regulation of gene expression. It is now well documented that dysregulated miRNA expression is a fundamental cause of different diseases. including cancer. The dysregulation of miRNA expression can occur via the amplification or deletion of their genes, aberrant transcriptional control, dysregulated epigenetic modifications and flaws in their biogenesis machinery. Aberrant miRNA expression leads a cell to achieve the ability to maintain the proliferative signals, avoid growth suppressors, initiate invasion and metastasis, trigger angiogenesis, and withstand cell death, and all these changes lead to the transformation to cancerous cells. The exploration of programmable RNA-targeting regulators makes it easier to detect endogenous RNA and track various mRNA species in real time within cells. It is important to develop methods that could identify and target miRNA sequences in a broad manner, especially those with low expression levels. CRISPR/Cas13-based miRNA detection strategies have proven to be a versatile platform, as this approach of miRNA-detection is beneficial in all aspects compared with other currently used methods, which are expensive, and require special expertise and instruments. CRISPR/Cas13-based miRNA detection systems hold promise for the future with the potential to revolutionize diagnostic and personalized medicine. CRISPR/Cas13-based miRNA detection with high sensitivity and specificity can enable early and accurate detection of diseases such as cancer, even when miRNA levels are low. Future developments may allow for precise differentiation between miRNA isoforms. This can provide insights into disease mechanisms and guide targeted therapies. CRISPR/Cas13-based assays can be designed for rapid, POC diagnostics, eliminating the need for complex laboratory setups. Overall, the future of CRISPR/Cas13-based miRNA detection is bright. With continued research and development, this technology has the potential to transform the field of diagnostics, enabling early disease detection, personalized medicine and potentially even miRNA-based therapies.

Acknowledgements

The researchers would like to thank the Deanship of Graduate Studies and Scientific Research at Qassim University for financial support (QU-APC-2025).

Funding

The present study was supported by the Deanship of Graduate Studies and Scientific Research at Qassim University (QU-APC-2025).

Availability of data and materials

Not applicable.

Authors' contributions

AAA and AAK conceived the study. AAA, AMA and AAK wrote the original draft. AAA, AMA, AA, BFA, AHR and AAK reviewed and edited the manuscript. Data authentication is not applicable. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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