# MiRNA–RBP Binding Functions

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MicroRNAs (miRNAs), short-chain RNAs of 18–22 nt chain length, are expressed in all vertebrates and control tissue development, differentiation, cell growth, and apoptosis in non-cancer and cancer cells. Additionally, miRNAs target mRNAs on the basis of their sequence and decrease protein production by inhibiting mRNA translation or destroying the mRNAs, thereby controlling cellular homeostasis. miRNA expression is controlled through DNA modification, such as by methylation and transcriptional factors through the signal-transduction pathway. miRNAs are transcribed as pri-mature from DNA and are then processed by the Drosha complex, thus generating pre-miRNAs. These pre-miRNAs are transported by exportin 5 from the nucleus to the cytoplasm and further processed by Dicer to form the double-stranded miRNA RNA-induced silencing complex (RISC). RISCs involving single-strand miRNAs, comprising Ago, Dicer, and trans-activation-responsive RNA-binding protein (TRBP) 2, are directed to the mRNA targets, thus regulating protein expressions. To date, the bioinformatics databases TargetScan (http://www.targetscan.org/vert_72/, 26 September 2021) and miRTarBase (http://mirtarbase.cuhk.edu.cn/php/index.php, 26 September 2021) have been used to predict the interaction between miRNAs and target mRNAs.

Keywords: microRNA ; RNA-binding protein

## 1. RNA-Binding Proteins

In most cases, functional protein regulation by miRNAs without translational regulations through binding with mRNAs was mediated by a complex of miRNA–RNA-binding proteins (RBPs). RBPs are molecular groups with RNA-binding domains associated with RNA regulation, including the processing, transportation, splicing, translation, and stabilization of mRNAs (**Figure 1**A), thereby maintaining cellular functions such as differentiation, development, apoptosis, and inflammation [1][2][3]. For instance, we demonstrated the heterogeneous ribonucleoprotein (hnRNP) A1 to stabilize the trefoil factor 2, which regulates apoptosis, enhances epithelial restoration, and attenuates intestinal injury in T cell-activated enteritis model [4]. Human 424, mouse 413, fly 257, and worm 244 RBPs were registered in RBPDB (http://rbpdb.ccbr.utoronto.ca/, 28 October 2021). Additionally, 16 RNA-binding domains (RNA recognition motif [RRM], K homology [KH], CCCH zinc finger, like Sm domain, cold-shock domain [CSD], PUA domain, ribosomal protein S1-like [S1], Surp module/SWAP [SURP], Lupus La RNA-binding domain [La], PWI domain, YTH domain, THUMP domain, Pumilio-like repeat [PUM], sterile alpha motif [SAM], $C_2H_2$ zinc finger, and TROVE module), which specifically recognize the sequences, structures, or both of target RNAs, have been identified; the interaction of miRNA with RBPs are mediated by these domains. Additionally, the recognition sequences of the RNA-binding domain of each RBPs were registered in RBPDB.

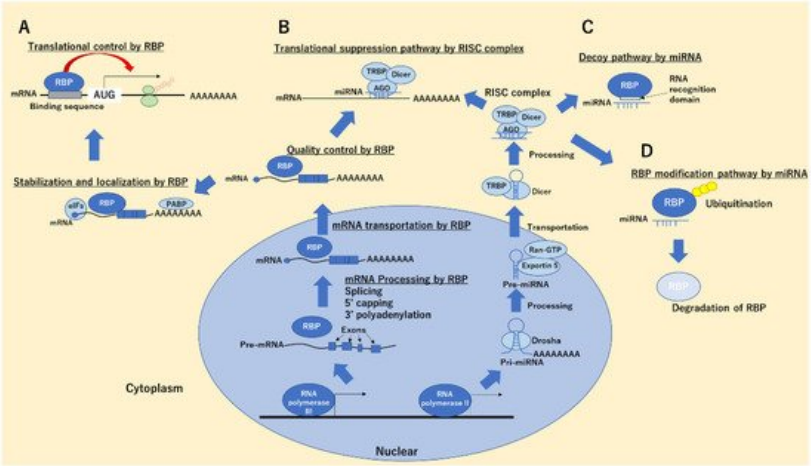


**Figure 1.** Working system of microRNA (miRNA) targeting RNA-binding protein (RBP). RBPs support the regulation of translation by binding to messenger RNAs (mRNAs) (**A**). Conversely, microRNA (miRNA)-containing RNA-induced silencing complexes (RISCs) suppress the translation through the sequence-dependent binding to mRNAs (**B**). Some

miRNAs, which have similar sequences with RNA-binding domains of RBP, can be decoy mRNA and interfere with the translation mediating the RBP–mRNA binding (**C**) and induce RBP ubiquitination mediating the direct binding (**D**).

Previous investigations suggested that some RBPs are directly associated with the maturation of miRNAs and transportation in cell–cell communication. hnRNP A1 directly binds to the pri-miR-18a through a specific sequence recognized by RRM of hnRNP A1 and promotes the processing and maturation of miR-18a [5]. Lin28 also directly binds to pri-let7 via CSD and CCHC zinc knuckle domain and inhibits its processing by Dicer, thereby regulating pluripotency and tissue development and differentiation [6]. Interestingly, RBPs, including AGO2, HuR, hnRNP A2/B1, YBX1, and SYNCRIP, recognize the RNA sequence motifs and/or secondary conformation and control the packaging of RNA into extracellular vesicles [7]. Therefore, this database has a high data availability and better ability to screen miRNA–RBP interactions when RBPs were not post-transcriptionally modified.

## 2. RBPs in Cancer Progression and Suppression

Previous investigations have suggested that RBPs are associated with the progression and suppression of various types of cancer [8]. Changes in the expression of RBPs such as hnRNP AB [9] and hnRNP K [10] have been reported in gastrointestinal cancer cells and are correlated with prognosis. RBPs exhibit cancer-promoting and suppressive functions that mediate RNA stabilization, transportation, and degradation. For instance, we showed that hnRNP H1, which is highly expressed in colorectal cancer tissues, directly binds with and stabilizes 54 apoptosis-related mRNAs, including sphingosine-1-phosphate lyase 1 mRNA, thereby promoting the growth of colorectal cancer cells [11]. Conversely, Quaking, which is downregulated in colorectal cancer, accelerates the translation of p27 and β-catenin mRNA, thereby suppressing proliferation in colorectal cancer [12]. Tristetraprolin (TTP), which is also downregulated in colorectal cancer, destabilizes VEGF mRNA and suppresses tumorigenesis in human colon cancer [13].

## 3. miRNA–RBP Binding Functions in Cancer Cells

Although 2654 miRNAs (*Homo sapiens*) have been registered in the database (http://miRbase.org/, 28 October 2021), only a few miRNAs that bind to target proteins and change the cellular functions without a direct binding between miRNA and mRNA have been identified (**Table 1**). We searched the PubMed database using the keywords “(miRNA) AND (decoy) NOT (circRNA) NOT (lncRNA) AND (cancer)” and found 53 articles. Of these, we selected original studies on miRNA-mediated RBP functional regulation in cancer progression or suppression.

**Table 1.** Protein-targeted miRNAs and regulation systems.

| miRNA | Type of Pathway | Target | Type of Cancer | Function | Reference |
|---|---|---|---|---|---|
| miR-328 | Decoy | hnRNP E2-CEBPα mRNA | Leukemic blasts | Differentiation | Eiring AM, Cell 2010 |
| | Canonical | PIM1 mRNA | | Decreased survival | |
| miR-29 | Decoy | HuR-A20 mRNA | Sarcoma | Differentiation | Balkhi MY, Sci signal, 2013 |
| miR-26a, -584 | Decoy | hnRNP A1-CDK6 mRNA | Colorectal cancer | Cell growth suppression | Konishi H, Biochem Biophys Res Commun. 2015 |
| miR-574-3p | Decoy | hnRNP L-VEGFA mRNA | Myeloid cells | Inhibition of cell proliferation | Yao P, Nucleic Acids Research, 2017 |
| | Canonical | EP300 mRNA | | | |
| miR-574-5p | Decoy | CUGBP1-mPGES-1 mRNA | Lung tumor | Cell growth promotion | Saul MJ, FASEB J, 2019 |
| miR-18a | Degradation | hnRNP A1 | Colorectal cancer | Apoptosis induction | Fujiya M, Oncogene, 2014 |

The most-investigated function of direct binding between miRNA and RBPs is the “decoy” system (**Figure 1**B,C). miRNA directly binds to target RBPs through its RNA-binding domain (RBD) on the basis of their sequences and cancels out the functions of RBP, including the inhibition/promotion of mRNA translations. This system was first reported by Eiring et al. in leukemic blast cells. They showed that miRNA-328 bound to hnRNP E2, releasing CCAAT/enhancer-binding protein alpha (C/EBPα) mRNA from hnRNP E2, thereby restricting the translation of C/EBPα mRNA and supporting the differentiation of progenitor cells in leukemic blast cells (**Figure 2**A) [14]. Interestingly, miRNA-328 interacts with hnRNP E2 without RISC-

associated proteins, such as Ago, suggesting that this system works independently of the mRNA-associated gene silencing mechanism of miRNA. In contrast, Balkhi et al. revealed that miR-29, which has a complementary sequence of 3′ UTR of tumor-suppressive TNFAIP3 mRNA, was decreased in patients with sarcoma, and it directly bound to the RBPs HuR, inhibited the recruitment of RISC to the 3′ UTR of TNFAIP3 mRNA, and negatively regulated NFkB signaling, thereby suppressing tumorigenesis in sarcoma cells [15]. We also globally assessed hnRNP A1-binding RNAs through microarray and whole transcriptome analyses combined with RNA immunoprecipitation. The results demonstrated that miR-26a, miR-584, and CDK6 mRNA had a high affinity for the RBD of hnRNP A1. The induction of miR-26a or miR-584 inhibited the binding between hnRNP A1 and CDK6 mRNA, which is recognized and stabilized by hnRNP A1 mediating RBD, and decreased CDK6 expression, resulting in apoptosis induction in colorectal cancer cells (**Figure 2**B) [16]. Yao et al. revealed that miR-574-3p works as a decoy for hnRNP L, which supports the translation of VEGFA mRNA by interacting 3′ UTR-localized CA-rich elements and inhibiting the translation of VEGF, resulting in tumor suppression in lymphoma cells [17]. Saul et al. revealed that miR-574-5p, which contains a GU-rich sequence, is highly induced in patients with non-small cell lung cancer and acts as an RNA decoy to CUG RNA-binding protein 1 (CUGBP1), which suppress the translation of microsomal prostaglandin E synthase-1 (mPGES-1) by directly binding mediating CU-rich element 1 and 2 in 3′ UTR of mPGES-1 mRNA, and antagonizes the function of CUGBPs, thereby supporting tumorigenesis in non-small cell lung cancer [18].

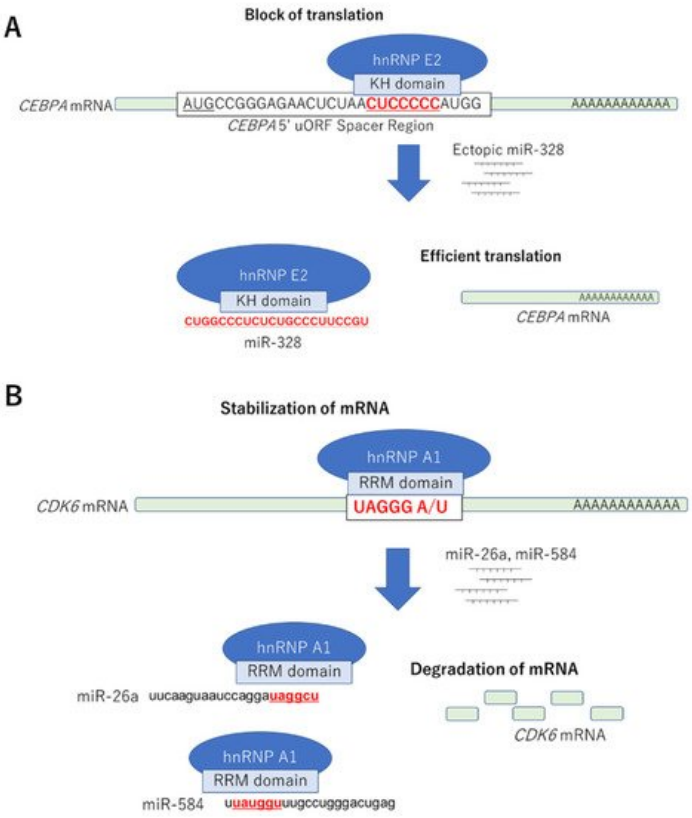


**Figure 2.** Decoy system mediating miRNA. miR-328 directly binds to the KH domain of hnRNP E2 and inhibits the binding of hnRNP E2 and CEBPA mRNA, thereby accelerating the translation of CEBPA mRNA (**A**). miR-26a and 584 directly bind to the RRM domain of hnRNP A1 and inhibit the binding of hnRNP A1 and CDK6 mRNA, thereby inducing the destabilization of CDK6 mRNA (**B**).

## 4. Strategies for Identifying Interactions between miRNAs and RBPs

Most reports have analyzed and identified specific miRNA and RBP decoy systems using expressional analysis targeting miRNAs and complementary sequences recognized by the RBD of RBPs. For instance, Eiring et al. compared the miRNA expressions in patient-derived chronic myelocytic leukemia (CML)-blast crisis (BC) CD34+ versus CML-chronic phase (CP) CD34+ bone marrow progenitors using microarray analysis and found that various miRNAs, including miRNA-328, were downregulated in CML-BC. They focused on miR-328 because its mature form harbors a C-rich sequence that resembles the negative regulatory hnRNP E2-binding site included in the CEBPA intercistronic mRNA region [14]. However, previous studies, including our study, have shown that the binding affinities of RBPs and RNAs are influenced by the posttranslational modification of RBPs. Therefore, the direct binding must be confirmed through other molecular biological methods, such as pulldown assays combined with RT-PCR and electrophoretic mobility shift assays. Notably, specific RBP–miRNA interactions based on the RBD of RBP cannot rank the affinity of each miRNA–RBP binding in cellular physiological conditions.

A useful strategy for identifying novel miRNA–RBP interactions is RNA immunoprecipitation (RNA-IP) combined with microarray analysis [16] (**Figure 3**). In this study, the cancer-associated RBP hnRNP A1 was subjected to a pulldown assay using immunoprecipitation in colorectal cancer cells. RNAs were eluted from the precipitant via phenol–chloroform extraction, and microarray analysis was conducted to detect the miRNAs interacting with RBPs. Notably, this method determines the physical binding of miRNAs and RBPs in cancerous cells; therefore, the tumor therapeutic binding of miRNA and RBP is enhanced. As listed in **Table 2**, numerous miRNAs are directly bound to hnRNP A1. Furthermore, RBPs have a high affinity for a specific RNA motif recognized by the RBD. To confirm the RBP–miRNA binding, RNA competencies of miRNAs, which are chemically synthesized artificial short RNA similar to the RNA-binding motif sequence, were developed. We demonstrated that RNA competence could inhibit the specific binding of miR-26a, miR-584, and hnRNP A1, thus preventing miRNA-induced apoptosis in colorectal cancer cells [16].

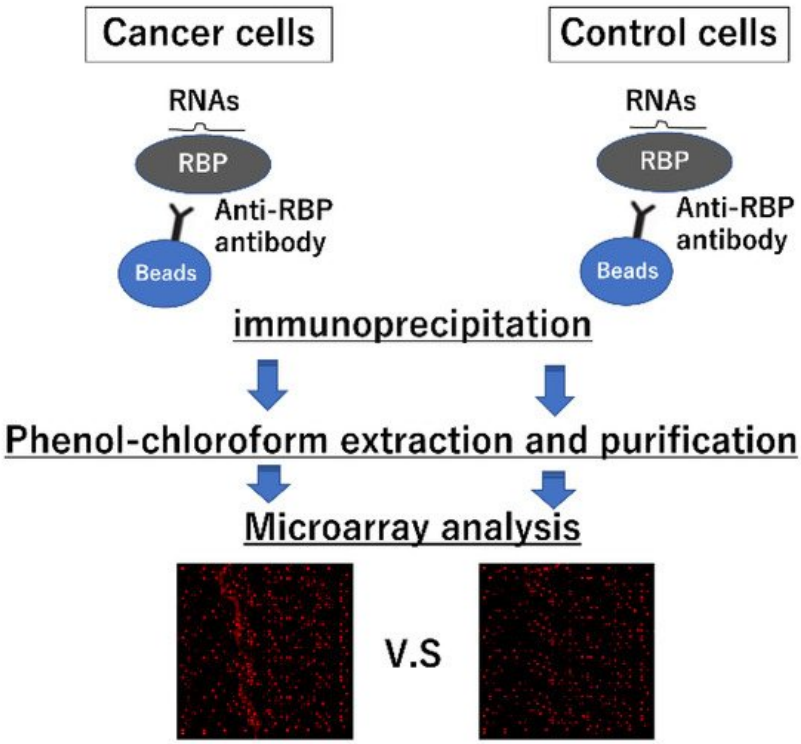


**Figure 3.** Methodology for the identification of RNA-binding protein (RBP)-binding micro RNAs (miRNAs) and messenger RNAs (mRNAs). RNA-IP with microarray analysis is a powerful strategy to exhaustively identify unknown PBP-miRNA or messenger RNA (mRNA) interactions. miRNAs and mRNAs, which interact with a specific RNA-binding protein (RBP) in cancer cells, are subjected to pulldown assays by immunoprecipitation using anti-RBP antibodies extracted through the phenol–chloroform extraction method and identified via microarray analysis.

**Table 2.** hnRNP A1 interacting miRNAs identified by RNA-IP in combination with microarray analysis.

| **miRs with Greater than 4-Fold Expression Compared to the Isotype Control IgG** | | | |
|---|---|---|---|
| **Name** | **ID** | **Ratio (hnRNP A1/IgG)** | **LOG2ratio** |
| **hsa-miR-29a-3p** | **MIMAT0000086** | **11.49** | **3.52** |
| **hsa-miR-26a-5p** | **MIMAT0000082** | **11.37** | **3.51** |
| **hsa-miR-584-5p** | **MIMAT0003249** | **9.93** | **3.31** |
| **hsa-miR-107** | **MIMAT0000104** | **9.73** | **3.28** |
| **hsa-miR-106b-5p** | **MIMAT0000680** | **8.99** | **3.17** |
| **hsa-miR-1229-5p** | **MIMAT0022942** | **8.88** | **3.15** |
| **hsa-miR-29b-3p** | **MIMAT0000100** | **8.07** | **3.01** |
| **hsa-miR-194-5p** | **MIMAT0000460** | **8.07** | **3.01** |
| **hsa-miR-142-3p** | **MIMAT0000434** | **7.97** | **2.99** |
| **hsa-miR-18a-5p** | **MIMAT0000072** | **7.93** | **2.99** |

| miRs with Greater than 4-Fold Expression Compared to the Isotype Control IgG | | | |
|---|---|---|---|
| **Name** | **ID** | **Ratio (hnRNP A1/IgG)** | **LOG2ratio** |
| **hsa-let-7c-5p** | **MIMAT0000064** | **7.31** | **2.87** |
| **hsa-miR-16-5p** | **MIMAT0000069** | **7.22** | **2.85** |
| **hsa-miR-500a-3p** | **MIMAT0002871** | **6.96** | **2.8** |
| **hsa-miR-200b-3p** | **MIMAT0000318** | **6.89** | **2.78** |
| **hsa-miR-19a-3p** | **MIMAT0000073** | **6.55** | **2.71** |
| **hsa-miR-222-3p** | **MIMAT0000279** | **6.4** | **2.68** |
| **hsa-let-7b-5p** | **MIMAT0000063** | **5.98** | **2.58** |
| **hsa-miR-23a-3p** | **MIMAT0000078** | **5.97** | **2.58** |
| **hsa-let-7d-5p** | **MIMAT0000065** | **5.85** | **2.55** |
| **hsa-miR-431-3p** | **MIMAT0004757** | **5.7** | **2.51** |
| **hsa-miR-200c-3p** | **MIMAT0000617** | **5.62** | **2.49** |
| **hsa-miR-23b-3p** | **MIMAT0000418** | **5.27** | **2.4** |
| **hsa-miR-27b-3p** | **MIMAT0000419** | **5.23** | **2.39** |
| **hsa-miR-19b-3p** | **MIMAT0000074** | **5.05** | **2.34** |
| **hsa-miR-103a-3p** | **MIMAT0000101** | **4.95** | **2.31** |
| **hsa-miR-1246** | **MIMAT0005898** | **4.73** | **2.24** |
| **hsa-let-7a-5p** | **MIMAT0000062** | **4.66** | **2.22** |
| **hsa-miR-20a-5p** | **MIMAT0000075** | **4.5** | **2.17** |
| **hsa-miR-27a-3p** | **MIMAT0000084** | **4.49** | **2.17** |
| **hsa-miR-141-3p** | **MIMAT0000432** | **4.32** | **2.11** |
| **hsa-miR-21-5p** | **MIMAT0000076** | **4.25** | **2.09** |
| **hsa-miR-17-5p** | **MIMAT0000070** | **4.24** | **2.08** |
| **hsa-miR-106a-5p** | **MIMAT0000103** | **4.15** | **2.05** |
| **hsa-miR-20b-5p** | **MIMAT0001413** | **4.11** | **2.04** |

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