Ago HITS-CLIP expands microRNA-mRNA interactions in nucleus and cytoplasm of gastric cancer cells

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Abstract

Background: Intensive investigations have identified a collection of microRNAs (miRNAs) and their functional machineries in cytoplasm. However, a comprehensive view of miRNAs and mRNAs in cytoplasm and nucleus has not been explored. This study aims to reveal the mechanisms of miRNA-RNA interactions in nucleus and cytoplasm.

Methods: In this study, the miRNAs and their target mRNAs in the Argonaute2 (Ago2) complex of nucleus and cytoplasm of gastric cancer cells were characterized using high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP). Then, the selected miRNAs were verified by Northern blot. The target mRNAs in the Argonaute2 (Ago2) complex of nucleus and cytoplasm of gastric cancer cells were analyzed through Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis.

Results: The results revealed that there were 243 miRNAs and 265 miRNAs in the Ago2 complexes of nucleus and cytoplasm, respectively. The majority of mature miRNAs existed in cytoplasm. The analysis of miRNA targetome from the Ago2 complexes indicated that a lot of mRNAs with high expression level existed in nucleus. The target genes of miRNAs in the Ago2 complexes of nucleus and cytoplasm played important roles in cell proliferation, cell differentiation, innate immune response and tumorigenesis.

Conclusions: microRNA-mRNA interactions occur in nucleus and cytoplasm of gastric cancer cells. Therefore, our study demonstrated that miRNA-mRNA interactions not only took place in cytoplasm but also in nucleus.

Keywords: Ago HITS-CLIP, miRNA-RNA interaction, Nucleus and cytoplasm, Gastric cancer cell
combination of microarray analysis and small RNA deep sequencing [10]. Some reports have pointed out the existence of active RISC in nucleus because of the findings of human Ago proteins and other RNAi factors (DICER1, TARBP2 and GW182) in nucleus of cells [11–14]. However, the miRNAs in nucleus have not been intensively explored.

Generally, the interaction between a miRNA and its target requires base pairing with only 6–8 nucleotides. Therefore, it is a major challenge to predict target miRNAs. Based on evolutionarily conserved seed sequence and free binding energy, computational predictions have greatly improved the potentials to find bona fide miRNA target sites [15]. Nonetheless, different prediction algorithms produce a large number of divergent results for one given miRNA [16, 17]. In addition, computer programs usually predict targets on a genome-wide scale irrespective of mRNA and miRNA that are specific to tissues or cell lines. Thus, it has become increasingly important to develop biochemical tools based on physical interactions between miRNAs and their specific target RNAs. As miRNA target sites are parts of RISC that contain, except for miRNAs, Argonaute (Ago) protein and guiding miRNAs, co-immunoprecipitation (Co-IP) of Ago protein together with associated target miRNAs is an effective tool for the identification or validation of miRNA target candidates [18, 19].

In order to explore the miRNA-mRNA interaction in nucleus and cytoplasm, the miRNAs and mRNAs in the Ago complexes of nucleus and cytoplasm of gastric cancer cells (MGC-803) were characterized using high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (MGC-803) were characterized using high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP) in this study. The results revealed that there existed 243 miRNAs and 265 miRNAs in Ago2 complexes of nucleus and cytoplasm, respectively. The targetome analysis indicated that these miRNAs could function in nucleus or cytoplasm.

**Methods**

**Cell culture**

Human gastric cancer cells (MGC-803) were cultured in RPMI-1640 Medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) at 37 °C. The cell line was purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China in the year of 2014.

**Isolation of nucleus and cytoplasm**

MGC-803 cells were cross-linked at 254 nm and then washed twice with ice-cold phosphate-buffered saline (PBS). Subsequently, the cells were warmed at room for 45 s and lysed by TM-2 buffer [10 mM Tris-HCl, 2 mM MgCl2, phenylmethanesulfonyl fluoride (PMSF), pH 7.4] at room temperature for 1 min. To isolate nuclei and cytoplasm of cells, the lysate was centrifuged at 1000×g at 4 °C for 6 min.

**Western blot analysis**

8% SDS–PAGE was used to separate the protein samples. Then the protein samples were transferred to a nitrocellulose membrane (Bio-Rad, USA) at 80 V for 1.5 h. The membrane was blocked with 3% milk at room temperature for 2 h. Subsequently, the membrane was incubated with a primary antibody (anti-tubulin IgG, anti-Lamin A/C IgG or anti-Ago2 IgG) at 4 °C overnight. Tubulin and Lamin A/C antibodies were purchased from Abcam (USA). The anti-Ago2 IgG was prepared in our laboratory. After washes with TBST, the membrane was immersed in 2% milk containing AP-conjugated goat antimouse IgG (Sigma, USA) for 1 h. At last, NBT and BCIP solutions (BBI, Canada) were used to detect the protein.

**Co-immunoprecipitation (co-IP) of Ago2 complex**

The lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, pH 7.5) was prepared to lyse the isolated nuclei and cytoplasm. After treatment with RNAsin (Promega, USA), the lysate was incubated with RQ1 DNAase (Promega) for 5 min at 37°. Subsequently the Ago2 complex was immunoprecipitated using the polyclonal antibody against Ago2 for 3 h at 4 °C and then incubated with protein A-Sepharose (Bio-Rad) for 3 h at 4 °C. After washes with lysis buffer, the Ago2 complex was collected.

**Sequencing and sequence analysis of RNAs of Ago2 complex**

RNA was extracted from immunoprecipitated Ago2 complex and subjected to sequencing using GA-I genome analyzer (Illumina, San Diego, CA). To analyze small RNAs, the ACGT V3.1 program developed by LC Sciences (Houston, USA) was employed. Then the high-quality sequences were obtained by removing adaptor sequences, mRNA, rRNA, tRNA, snRNA, snoRNA and other noncoding RNA sequences available in Rfam (http://www.sanger.ac.uk/software/Rfam). The high quality sequences were then blasted with known human miRNAs in miRBase 19.0 to obtain miRNAs in this study. To analyze the longer RNAs, the ACGT V3.1 program developed by LC Sciences (Houston, USA) were employed. Then the high-quality reads were aligned to human mRNA database (ftp://ftp.ensembl.org/pub/release-70/fasta/homo_sapiens/).

**Northern blot**

Total RNAs were extracted from the Ago2 complex of nucleus or cytoplasm using a mirVanaPTMP miRNA isolation kit according to the manufacturer’s protocol (Ambion, USA) and then separated on a denaturing 15%
polyacrylamide gel containing 8 M urea. After transferred to a Hybond-N+ membrane (Amersham Biosciences, Buckinghamshire, UK), the RNAs were cross-linked under ultraviolet. The membrane was pre-incubated with DIG Easy Hyb granules buffer (Roche, Basel, Switzerland) for 1 h at 42 °C and then the miRNAs were probed against with complementary DIG-labeled miRNA probe at 42 °C overnight. The RNAs were detected using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, USA). NBT and BCIP solutions (BBI, Canada) were used to detect the RNA.

**Prediction of genes targeted by miRNAs**
The transcriptome sequences of the Ago2 complexes of nucleus and cytoplasm served as a database for miRNA target prediction. MiRNA target genes were predicted by employing TargetScan 5.1 (http://www.targetscan.org) and miRanda (http://www.microrna.org/) as described in previous study [18].

**Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis**
The coding sequences of transcripts obtained from the Ago2 complexes were aligned with the protein sequences in the GO database with the blast E value <1e−5 [20]. The best hit GO identities were described the properties of these genes and gene products. The coding sequences of transcripts were analyzed by KEGG Pathway (239 KEGG) to analyze the relationship between genes and enzyme annotations in KEGG, and the information mapped to pathway.

**Results**

**Acquirement of Ago2 complexes from cytoplasm and nucleus of gastric cancer cells**
To identify miRNAs and mRNAs targeted by miRNAs in Ago2 complex of cytoplasm and nucleus, co-immunoprecipitation (Co-IP) assays with Ago2-specific antibody were performed using gastric cancer cells (MGC-803). The nuclear and cytoplasmic fractions of gastric cancer cells were extracted. Western blot analysis indicated that β-tubulin, a cytoplasmic marker, was detected in cell lysate and cytoplasm but not nucleus (Fig. 1a). At the same time, Lamin A/C, a nuclear marker, was found in cell lysate and nucleus (Fig. 1a). These data showed that cytoplasm and nucleus were successfully isolated.

The isolated cytoplasm and nucleus were subjected to Co-IP assays using Ago2-specific antibody. The results revealed that the Ago2 complexes of cytoplasm and nucleus were obtained (Fig. 1b).

**Characterization of miRNAs in Ago2 complexes of cytoplasm and nucleus of gastric cancer cells**
To characterize the miRNAs in Ago2 complexes of cytoplasm and nucleus, RNAs were extracted from Ago2 complexes and then subjected to deep sequencing. After removal of mRNA, rRNA, tRNA, snRNA and snoRNA sequences, high-throughput small RNA sequencing yielded an average of 7,813,944 high-quality reads. The results showed that most of small RNA reads were 20–25 nucleotides (nt) in length, which is typical for products generated by the enzyme Dicer (Fig. 2a). Of high-quality small RNA reads, 4,697,199 reads in nucleus and 4,545,958 in cytoplasm were mapped to known miRNAs in miRBase 19.0. In total, 243 miRNAs in the Ago2 complex of nucleus and 265 miRNAs in the Ago2 complex of cytoplasm were identified (Fig. 2b, Table 1), indicating that there was no difference of miRNA number between nucleus and cytoplasm of gastric cancer cells. The sequencing analysis revealed that there was a strong preference of U at the first nucleotide of small RNAs’ 5’ ends from the Ago2 complexes (Fig. 2c), showing that these small RNAs were in line with the characteristics of mature miRNA.

The results indicated that the expression profile of miRNAs in cytoplasm was different from that in nucleus (Fig. 2d). A lot of mature miRNAs existed in cytoplasm (Fig. 2d).
To confirm the existence of mature miRNAs in the Ago2 complexes of cytoplasm and nucleus, 5 miRNAs were randomly selected for Northern blot analysis. The results of Northern blots were consistent with those of miRNA sequencing (Fig. 2e).

Identification of miRNA targetome in Ago2 complexes of nucleus and cytoplasm of gastric cancer cells
To obtain an overview of gene expression profiles of miRNA targetome in cytoplasm and nucleus, cDNA libraries of target mRNA tags of miRNAs were prepared from the Ago2 complexes of cytoplasm and nucleus. After removal of repetitive and low-quality reads, an average of 12.2 million high-quality reads were aligned to human mRNA database. A total of 191,134 unigenes in nucleus and 191,134 unigenes in cytoplasm were obtained. The results showed that a large proportion of the target mRNA reads, 64.7% in nucleus and 55% in cytoplasm, were mapped to human mRNA database. Among the top 100 mRNAs with higher expression level, 80 genes were downregulated in cytoplasm and 20 upregulated (Fig. 3a), indicating that the main proportion of mRNAs existed in nucleus.

The analysis of cellular component by gene ontology (GO) revealed that the differentially expressed genes could be categorized into molecular function, cellular component and biological process (Fig. 3b). In the molecular function category, the most common functional annotations were the zinc ion binding, nucleotide binding, ATP binding and metal ion binding (Fig. 3b). A lot of differentially expressed genes could function as cellular components of cytoplasm and nucleus (Fig. 3b). In terms of biological process, most of genes were implicated in signal transduction, multicellular organismal development and translational elongation (Fig. 3b). The results of KEGG analysis indicated that the differentially expressed genes were classified into 239 KEGG pathways. Many genes were involved in the MAPK signaling pathway, the Wnt signaling pathway, focal adhesion, the TGF-beta signaling pathway and pathways in cancer (Fig. 3c). These data showed that the target genes of miRNAs in the Ago2 complexes of nucleus and cytoplasm played important roles in cell proliferation, cell differentiation, innate immune response and tumorigenesis.
| miRNAs in Ago2 complex of nucleus | miRNAs in Ago2 complex of cytoplasm |
|----------------------------------|-------------------------------------|
| let-7a-3p                        | miR-18a-5p                          | let-7a-3p                        | miR-191-5p                          | miR-330-5p                          | miR-769-3p                          |
| let-7a-5p                        | miR-1908                            | let-7a-5p                        | miR-192-5p                          | miR-331-5p                          | miR-769-5p                          |
| let-7b-3p                        | miR-191-5p                          | let-7b-3p                        | miR-193a-3p                         | miR-331-5p                          | miR-873-5p                          |
| let-7b-5p                        | miR-192-5p                          | let-7b-5p                        | miR-193a-5p                         | miR-335-3p                          | miR-876-5p                          |
| let-7c                           | miR-193b-3p                         | let-7c                           | miR-193b-3p                         | miR-335-5p                          | miR-877-5p                          |
| let-7d-3p                        | miR-194-5p                          | let-7d-3p                        | miR-193b-5p                         | miR-338-3p                          | miR-92a-5p                          |
| let-7d-5p                        | miR-196a-5p                         | let-7d-5p                        | miR-194-5p                          | miR-338-5p                          | miR-92a-3p                          |
| let-7e-5p                        | miR-196b-5p                         | let-7e-5p                        | miR-196a-5p                         | miR-339-3p                          | miR-92b-3p                          |
| let-7f-1-3p                      | miR-197-3p                          | let-7f-3p                        | miR-196b-3p                         | miR-339-5p                          | miR-92b-5p                          |
| let-7f-5p                        | miR-199a-5p                         | let-7f-5p                        | miR-196b-5p                         | miR-33a-5p                          | miR-93-5p                           |
| let-7g-5p                        | miR-199b-3p                         | let-7g-5p                        | miR-197-3p                          | miR-340-3p                          | miR-941                             |
| let-7i-5p                        | miR-19a-3p                          | let-7i-5p                        | miR-199a-5p                         | miR-340-5p                          | miR-95                              |
| miR-100-3p                       | miR-19b-3p                          | miR-100-3p                       | miR-199b-3p                         | miR-342-3p                          | miR-96-5p                           |
| miR-100-5p                       | miR-200c-3p                         | miR-100-5p                       | miR-199b-5p                         | miR-342-5p                          | miR-98-3p                           |
| miR-101-3p                       | miR-203a                            | miR-101-3p                       | miR-199b-5p                         | miR-345-5p                          | miR-98-5p                           |
| miR-103a-3p                      | miR-204a                            | miR-101-5p                       | miR-199b-5p                         | miR-34a-3p                          | miR-99a-3p                           |
| miR-105-5p                       | miR-210                             | miR-103a-3p                      | miR-200c-3p                         | miR-34a-5p                          | miR-99a-5p                           |
| miR-106b-3p                      | miR-2110                            | miR-106b-5p                      | miR-203a                            | miR-361-3p                          | miR-99b-3p                           |
| miR-106b-5p                      | miR-212-5p                          | miR-107                          | miR-203a                            | miR-361-5p                          | miR-99b-5p                           |
| miR-107                          | miR-21-3p                           | miR-4454                         | miR-10a-3p                          | miR-210                             | miR-362-5p                           |
| miR-10a-3p                       | miR-215                             | miR-450a-5p                      | miR-10a-5p                          | miR-2110                            | miR-365b-3p                          |
| miR-10a-5p                       | miR-21-5p                           | miR-450b-5p                      | miR-10b-5p                          | miR-212-5p                          | miR-3687                             |
| miR-10b-5p                       | miR-221-3p                          | miR-452-5p                       | miR-1180                            | miR-21-3p                           | miR-374a-3p                           |
| miR-1180                         | miR-221-5p                          | miR-454-3p                       | miR-1247-3p                         | miR-214-3p                          | miR-374a-5p                           |
| miR-1247-5p                      | miR-222-3p                          | miR-454-5p                       | miR-1255a                           | miR-21-5p                           | miR-374b-3p                           |
| miR-1254                         | miR-222-3p                          | miR-455-3p                       | miR-125a-3p                         | miR-219-5p                           | miR-374b-5p                           |
| miR-1255a                        | miR-224-3p                          | miR-4677-3p                      | miR-125a-5p                         | miR-221-3p                           | miR-378a-3p                           |
| miR-125a-3p                      | miR-224-5p                          | miR-484                          | miR-125b-3p                         | miR-221-5p                           | miR-421                             |
| miR-125a-5p                      | miR-23a-3p                          | miR-500a-3p                      | miR-125b-5p                         | miR-222-3p                           | miR-423-3p                           |
| miR-125b-2-3p                    | miR-23b-3p                          | miR-501-3p                       | miR-126-3p                          | miR-22-3p                            | miR-423-5p                           |
| miR-125b-5p                      | miR-24-2-5p                         | miR-502-3p                       | miR-126-5p                          | miR-224-3p                           | miR-424-5p                           |
| miR-126-3p                       | miR-24-3p                           | miR-503-3p                       | miR-126-5p                          | miR-224-5p                           | miR-424-5p                           |
| miR-126-5p                       | miR-25-3p                           | miR-505-3p                       | miR-1277-3p                         | miR-22-5p                            | miR-425-3p                           |
| miR-1277-3p                      | miR-25-5p                           | miR-532-3p                       | miR-1277-5p                         | miR-225-3p                           | miR-425-5p                           |
| miR-128                          | miR-26a-5p                          | miR-532-5p                       | miR-128                             | miR-23a-3p                           | miR-450b-5p                           |
| miR-1285-3p                      | miR-26b-3p                          | miR-545-3p                       | miR-1285-3p                         | miR-23b-3p                           | miR-452-5p                           |
| miR-1285-5p                      | miR-26b-5p                          | miR-548a-3p                      | miR-1301                            | miR-24-5p                            | miR-454-3p                           |
| miR-1291                         | miR-27a-3p                          | miR-548d-5p                      | miR-1303                            | miR-24-3p                            | miR-454-5p                           |
| miR-1292-5p                      | miR-27b-3p                          | miR-548e                         | miR-1304-3p                         | miR-25-3p                            | miR-455-3p                           |
| miR-1301                         | miR-27b-5p                          | miR-550a-5p                      | miR-1307-3p                         | miR-25-5p                            | miR-4677-3p                           |
| miR-1303                         | miR-28-3p                           | miR-570-3p                       | miR-130a-3p                         | miR-26a-5p                            | miR-484                             |
| miR-1304-3p                      | miR-28-5p                           | miR-574-3p                       | miR-130b-3p                         | miR-26b-3p                            | miR-491-5p                           |
| miR-1307-3p                      | miR-296-3p                          | miR-574-5p                       | miR-130b-5p                         | miR-26b-5p                            | miR-500a-3p                           |
| miR-130a-3p                      | miR-29a-3p                          | miR-576-3p                       | miR-132-3p                          | miR-27a-3p                            | miR-501-3p                           |
Table 1  MiRNAs in Ago2 complex of nucleus and cytoplasm of gastric cancer cells (Continued)

| miRNAs in Ago2 complex of nucleus | miRNAs in Ago2 complex of cytoplasm |
|----------------------------------|-------------------------------------|
| miR-130b-3p                      | miR-29b-3p                           |
| miR-130b-5p                      | miR-582-3p                           |
| miR-132-3p                       | miR-132-5p                           |
| miR-132-5p                       | miR-27a-5p                           |
| miR-134                          | miR-301a-3p                          |
| miR-135b-3p                      | miR-305-5p                           |
| miR-135b-5p                      | miR-3074-5p                          |
| miR-140-3p                       | miR-641                              |
| miR-143                          | miR-30a-3p                           |
| miR-146a-5p                      | miR-652                              |
| miR-146b-5p                      | miR-671                              |
| miR-148a-5p                      | miR-7-1-3p                           |
| miR-148b-3p                      | miR-744                              |
| miR-148b-5p                      | miR-30d-3p                           |
| miR-149-5p                       | miR-766                              |
| miR-151a-3p                      | miR-767                              |
| miR-151b                         | miR-155                              |
| miR-152                          | miR-15a                              |
| miR-155-5p                       | miR-15b-3p                           |
| miR-156                          | miR-320a                             |
| miR-15b-5p                       | miR-92a-1-5p                         |
| miR-15c-3p                       | miR-15b-3p                           |
| miR-16-2-3p                      | miR-92b-3p                           |
| miR-16-5p                        | miR-181a-3p                          |
| miR-17-3p                        | miR-93-3p                            |
| miR-17-5p                        | miR-181a-5p                          |
| miR-181a-2-3p                    | miR-941                              |
| miR-181a-5p                      | miR-95                               |
| miR-181b-5p                      | miR-96-5p                            |
| miR-181c-3p                      | miR-181b-3p                          |
| miR-181c-5p                      | miR-98-3p                            |
| miR-181d                         | miR-181c-3p                          |
| miR-182                          | miR-181c-5p                          |
| miR-183                          | miR-183-3p                           |
| miR-183-5p                       | miR-183-5p                           |
| miR-185                          | miR-330-3p                           |
| miR-186                          | miR-330-3p                           |

Pathways mediated by miRNAs with higher expression level

In order to characterize the potential interactions between miRNAs and their targets in cytoplasm and nucleus, 10 miRNAs with higher expression level in nucleus and cytoplasm (miR-125b-5p, miR-92b-3p, miR-17-5p, miR-30b-5p, miR-20a-5p, miR-21-3p, let-7d-3p, miR-98-5p, miR-424-5p and miR-155-5p) and their predicted targets were subjected to GO and KEGG analyses. The results of GO analysis demonstrated that most of the miRNA targets were involved in single-organism process, positive regulation of biological activity and cellular component including...
cytoplasm, intracellular organelle and organelle (Fig. 4a). The data of KEGG analysis revealed that most target genes participated in axon guidance, natural killer cell mediated cytotoxicity and T cell receptor signaling pathway.

**Discussion**

Over a decade of years, investigations have identified a collection of miRNA functional machineries. Recently, more and more investigations focus on miRNA functions in nucleus. It is found that miR-9 can result in degradation of MALAT-1 mRNA by targeting the metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1) in nucleus [21]. MiR-671 could regulate the expression level of the circular non-coding (ncRNA) natural antisense transcript in nucleus [22]. These studies demonstrate that miRNAs in nucleus can also regulate the expressions of genes. Consistent with previous studies, our study characterized 243 miRNAs and 265 miRNAs in the Ago2 complexes of nucleus and cytoplasm of gastric cancer cells in this study. Report has shown that miRNA could translocate from cytoplasm to nucleus, which has been demonstrated by using superquencher molecular probes complementary to the mature miR-122 [23]. And RISC-associated proteins are involved in transport from the cytoplasm to the nucleus in human cells [24, 25]. For instance, Exportin-1 and importin-8 play positive roles in the nuclear import of miRNAs [24, 25]. However, miRNAs in nucleus and cytoplasm have not been extensively compared. In the present study, the results revealed that the expression pattern of miRNAs in cytoplasm of gastric cancer cells were obviously different from that in nucleus. Therefore, our study contributed a comprehensive view of miRNAs and their targets in nucleus and cytoplasm.

MiRNAs, loaded into the miRISC, are thought to target multiple mRNAs, affecting the translation or stability of several target mRNAs [2, 4, 6, 26]. In this study, the
miRNA-mRNA map in nucleus and cytoplasm was investigated using high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP). The targetome of Ago2 complexes in nucleus and cytoplasm was obtained. The results showed that the main proportion of mRNAs existed in nucleus, while miRNAs function predominantly in cytoplasm. The existence of miRNAs in the Ago2 complexes of nucleus and cytoplasm revealed that miRNAs could function in nucleus and cytoplasm. Decades of studies have elucidated the function and mechanism of miRNAs in cytoplasm [1, 2, 4, 5]. In recent years, more and more investigations focus on the function and mechanism of miRNAs in nucleus. In C. elegans, it is reported that the intronic regions of pre-mRNA could be silenced by dsRNAs [27]. In human HeLa cells, the U6 small nuclear RNA transcripts are also susceptible to RNAi-mediated post-transcriptional silencing [28]. These findings support our miRNA-mRNA-Ago interactions in nucleus of gastric cancer cells. In nucleus, miR-320 can trigger the heterochromatinization and transcriptional gene silencing of POLR3D by binding the promoter locus of POLR3D [29]. In addition, some splicing factors, such as U2 and U5 snRNP core subunits, SRSF1, SRSF3, SRSF7, SRSF10, PTBP1, PTBP2, KHDRBS1 and snRNAs, could be detected in immunoprecipitated Ago complex, indicating a cross-talk between splicing factors and nuclear RISC [30]. However, the regulatory mechanism of nuclear miRNAs needs to be further investigated.

Acknowledgements
We are grateful for the funding of National Natural Science Foundation of China and National Program on the Key Basic Research Project.

Funding
This work was supported by National Natural Science Foundation of China (31430089) and National Program on the Key Basic Research Project (2015CB755903). No specific funding was received for this study.

Availability of data and materials
The data of this article are available in this manuscript.

Authors’ contributions
YLC and BS designed the experiment. YLC, BS and XYZ analyzed the data. YLC and XYZ wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This study did not involve human subjects or human material. The cell lines in this study did not require ethics approval for their use.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 16 April 2018 Accepted: 26 December 2018
Published online: 08 January 2019

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