

## SHORT COMMUNICATION

**Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3' untranslated region**S Wu<sup>1,2,4</sup>, S Huang<sup>2,4</sup>, J Ding<sup>2</sup>, Y Zhao<sup>2</sup>, L Liang<sup>2</sup>, T Liu<sup>3</sup>, R Zhan<sup>1</sup> and X He<sup>2</sup><sup>1</sup>Department of Hematology, Fujian Medical University Union Hospital, Fujian Institute of Hematology, Fuzhou, China;<sup>2</sup>State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Shanghai Jiao Tong University School of Medicine, Shanghai, China and <sup>3</sup>The International Peace Maternity and Child Health Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Cyclin-dependent kinase inhibitor 1A (CDKN1A), also known as p21Cip1/Waf1, is a master downstream effector of tumor suppressors. In this study, we experimentally demonstrate through a high-throughput luciferase reporter screen that p21Cip1/Waf1 can be directly targeted by nearly 28 microRNAs (miRNAs). The results were further confirmed by a series of mutational analyses and luciferase reporter assays. These 28 miRNAs can substantially inhibit p21Cip1/Waf1 expression, predominantly at translational level. Many of these miRNAs were upregulated in cancers and might serve as modulators of oncogenesis. Furthermore, 8 of these 28 p21-regulating miRNAs are located in the chromosome 19 miRNA cluster, the largest miRNA gene cluster in humans, and they can clearly promote cell proliferation and cell-cycle progression in choriocarcinoma cells. In conclusion, our screening strategy provides an alternative approach to uncovering miRNA modulators of an individual mRNA, and it has identified multiple miRNAs that can suppress p21Cip1/Waf1 expression by directly targeting its 3' untranslated region.

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**Introduction**

Cyclin-dependent kinase inhibitor 1A (CDKN1A), also known as p21Cip1/Waf1, is the founding member of the Cip/Kip family of cyclin kinase inhibitors (Xiong *et al.*, 1993). p21Cip1/Waf1 inhibits cell-cycle progression by targeting cyclin/cyclin-dependent kinase (CDK) complexes, which causes cells to arrest in G<sub>1</sub> phase (Harper

*et al.*, 1993). Although mutations and deletions of the p21Cip1/Waf1 gene are rare in human cancers, substantial evidence from biochemical and genetic studies indicates that p21Cip1/Waf1 acts as a master effector molecule of multiple tumor suppressor pathways. Mice lacking p21Cip1/Waf1 spontaneously develop hematopoietic, endothelial and epithelial tumors, and are more sensitive to chemical carcinogens and irradiation (Topley *et al.*, 1999; Martin-Caballero *et al.*, 2001). Similar to other cyclin kinase inhibitors, expression of p21Cip1/Waf1 is strictly regulated in a condition-specific manner. Although it is well known that p21Cip1/Waf1 can be transcriptionally regulated through p53-dependent and -independent mechanisms (Gartel and Tyner, 1999), it could also be modulated at the post-transcriptional level through mechanisms involving mRNA stability, altered subcellular localization, and/or protein stability by both ubiquitin-dependent and -independent proteasome-mediated degradation (Abbas and Dutta, 2009). Now, translational control has increasingly emerged as a prominent mechanism of p21Cip1/Waf1 regulation, especially through microRNA (miRNA) targeting.

miRNAs are a new class of small noncoding RNA molecules that have been identified as post-transcriptional regulators of gene expression. miRNAs bind to the 3' untranslated regions (UTRs) of target mRNAs, resulting in mRNA degradation or repression of mRNA translation (Ambros, 2004; Bartel, 2004). Increasing evidence shows that miRNAs function significantly in diverse biological processes. Meanwhile, deregulation of miRNAs has been observed in a wide range of human diseases, including cancer. Identification of the miRNA targets is increasingly urgent for a better understanding of miRNA functions in both physiological and pathological contexts. Computational analysis indicates that >30% of protein-coding genes may be directly modulated by miRNAs. At the same time, a single mRNA molecule can be regulated by multiple miRNA genes (Krek *et al.*, 2005; Lewis *et al.*, 2005). Previous studies showed that p21Cip1/Waf1 could be regulated at the translational level by miRNA genes such as miR-93, miR-20a/b, miR-17 and miR-106a/b, which share the same seed sequence (Ivanovska *et al.*, 2008; Petrocca *et al.*, 2008). However, the exact miRNAs that can target p21Cip1/Waf1 are largely unknown.

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In this study, we further showed that p21Cip1/Waf1 can be modulated by multiple miRNA molecules. Then, we used computational tools such as TargetScan, PicTar, miRanda and RNA22 to predict all the possible miRNA species that could target p21Cip1/Waf1. A high-throughput luciferase reporter assay was performed to screen for miRNAs that directly target the 3'UTR of p21Cip1/Waf1. Interestingly, 28 p21-targeting miRNAs were identified and further verified by mutational analysis. Unexpectedly, a set of the p21-targeting miRNAs was located on chromosome 19 in clusters, and these miRNAs were able to substantially promote cell proliferation and cell-cycle progression.

## Results and discussion

### *p21Cip1/Waf1 can be regulated by multiple miRNAs*

To determine whether p21Cip1/Waf1 was regulated by miRNAs, we globally repressed miRNA function by knocking down the expression of Dicer1, a key regulator of miRNA processing (Supplementary Figure S1A). We found that p21Cip1/Waf1 was significantly upregulated in miRNA processing-impaired cells (Supplementary Figure S1B), indicating that miRNAs downregulate the expression of p21Cip1/Waf1. To investigate whether p21Cip1/Waf1 was directly modulated by miRNAs, we introduced a luciferase reporter with the wild-type p21 3'UTR (placed immediately downstream of the luciferase gene) into the miRNA processing-impaired cells and found that the expression level of p21Cip1/Waf1 substantially increased (Supplementary Figure S1C). The 3'UTR might bind to and modulate the activities of p21-targeting miRNAs, freeing the p21Cip1/Waf1 mRNAs from being repressed by the miRNAs and possibly resulting in an increase in p21Cip1/Waf1 protein expression. These observations suggested that miRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3'UTR. Indeed, p21Cip1/Waf1 has been reported to be regulated by several miRNAs including miR-93, miR-20a/b, miR-17 and miR-106a/b, which share the same seed sequence (Ivanovska *et al.*, 2008; Petrocca *et al.*, 2008). To further examine whether p21Cip1/Waf1 was regulated by other miRNAs, a luciferase reporter with p21 that carries a mutant type of miR-93 binding sites (3'UTR-M) was introduced into the miRNA processing-impaired cells. Notably, the p21Cip1/Waf1 protein was also shown to be upregulated (Supplementary Figure S1C). These results indicated that p21Cip1/Waf1 can be regulated by miRNAs other than miR-93, miR-20a/b, miR-17 and miR-106a/b. Taken together, these findings suggest that p21Cip1/Waf1 can be modulated by multiple miRNAs.

### *Screening for miRNAs that directly target 3'UTR of p21Cip1/Waf1*

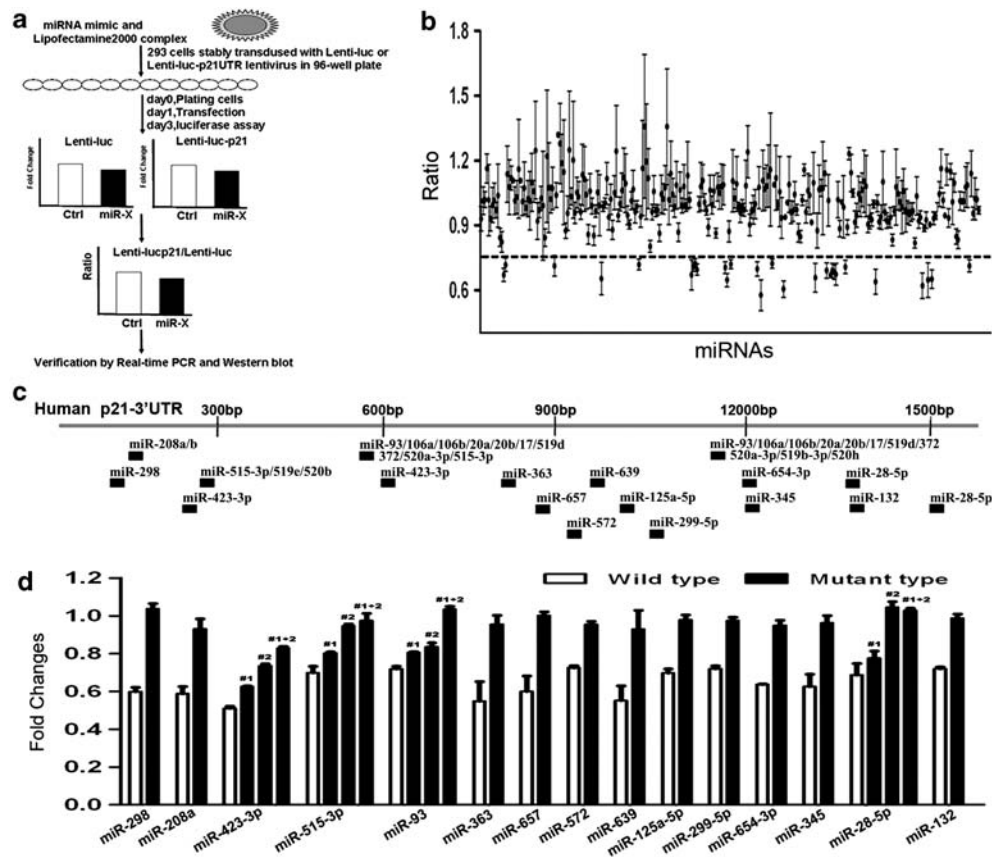
To identify miRNAs that control the expression of p21Cip1/Waf1 through its 3'UTR, we constructed a lentiviral vector containing the luciferase-coding region upstream of the 3'UTR of p21Cip1/Waf1 (Lenti-luc-p21UTR) and a control vector containing

only the luciferase construct (Lenti-luc). After lentiviral transduction of HEK 293 cells, we established two stable cell lines denoted as 293-luc-p21UTR and 293-luc. Meanwhile, four different miRNA target-prediction tools, including TargetScan, PicTar, miRanda and RNA22, were used to predict possible p21-targeting miRNAs. A pool of 266 miRNAs that could potentially target p21Cip1/Waf1 was selected (Supplementary Table 1). Then these miRNAs were individually transfected into 293-luc-p21UTR or 293-luc cells (Figure 1a). The transfection efficiency of small RNAs into HEK 293 cells was almost 100% (data not shown). Luciferase activities were assayed 36 h after transfection. Of these 266 miRNAs, 28 significantly decreased the luciferase activity of the 293-luc-p21UTR reporter compared with the control (Figure 1b and Table 1). These results were further confirmed by transient co-transfection using a dual luciferase assay (Supplementary Figure S2). It can be noted that our results appropriately included the previously identified miRNAs (miR-93, miR-106a/b, miR-17 and miR-20a/b) that could target p21Cip1/Waf1. The data indicate that this is an efficient screening strategy to identify all the possible miRNAs that can modulate the expression of a specific gene by its 3'UTR.

To clarify whether these 28 p21-targeting miRNAs directly bind to the 3'UTR of p21Cip1/Waf1, we first analyzed the location of the target sites in the 3'UTR of p21Cip1/Waf1 using target-prediction tools. As shown in Figure 1c, the target sequences distribute quite differently and some miRNAs share the same target sites. We subsequently performed serial mutations of these potential target sites, and placed the mutated p21 3'UTRs immediately downstream of a luciferase construct. The reporter assays showed that the relative luciferase activities did not drop as sharply in cells transfected with the mutated p21 3'UTRs compared with cells with their wild-type counterparts (Figure 1d), suggesting that these miRNAs can suppress p21Cip1/Waf1 expression by directly binding to the corresponding target sites.

### *p21-targeting miRNAs can inhibit the expression of p21Cip1/Waf1 mRNA and protein*

As shown above, 28 miRNAs can repress the expression of the exogenous reporter gene luciferase with a p21 3'UTR. To determine whether the endogenous p21Cip1/Waf1 can be modulated by these p21-regulating miRNAs, we introduced these miRNA mimics into HEK 293 cells and evaluated their effects on the levels of p21Cip1/Waf1 mRNA and protein. Interestingly, the level of p21Cip1/Waf1 mRNA decreased in most of the p21-targeting miRNA-expressing cells. In some cases, the level of p21Cip1/Waf1 mRNA was slightly elevated (Figure 2b). However, the level of p21Cip1/Waf1 protein was consistently and substantially downregulated (Figure 2a). Of these miRNAs, miR-298, miR-515-3p, miR-363, miR-639 and miR-132 showed the most obviously inhibitory effects (more than fourfold downregulation). These findings indicated that these miRNAs predominantly function at the translational level.



**Figure 1** Screen for candidate miRNAs that target the 3' untranslated region (3'UTR) of p21Cip1/Waf1. (a) Schematic diagram of the screening strategy. HEK 293 cells stably transduced with Lenti-luc or Lenti-luc-p21 3'UTR were plated into each well of a 96-well plate and transfected with individual miRNA mimics after 24 h. The luciferase activity was measured 36 h after transfection. The positive hits were then verified by real-time PCR and western blot. (b) Results of the luciferase screen. In all, 28 miRNAs significantly decreased the luciferase activity of luc-p21 3'UTR compared with the negative control (NC). Error bar indicates the range of triplicates. (c) A schematic diagram of the binding sites of the p21-regulating miRNAs in the wild-type p21 3'UTR. Some miRNAs shared the same target sites; thus, only 18 target sites were identified. (d) Luciferase activity assays of wild-type and mutated p21 3'UTR luciferase reporters after co-transfection with p21-regulating miRNAs. The luciferase activity of each sample was normalized to the *Renilla* luciferase activity. The normalized luciferase activity of transfected NC was set as relative luciferase activity of 1. Error bars represent s.e.m. Each data point was measured in triplicate.

### Comparisons of target-prediction tools

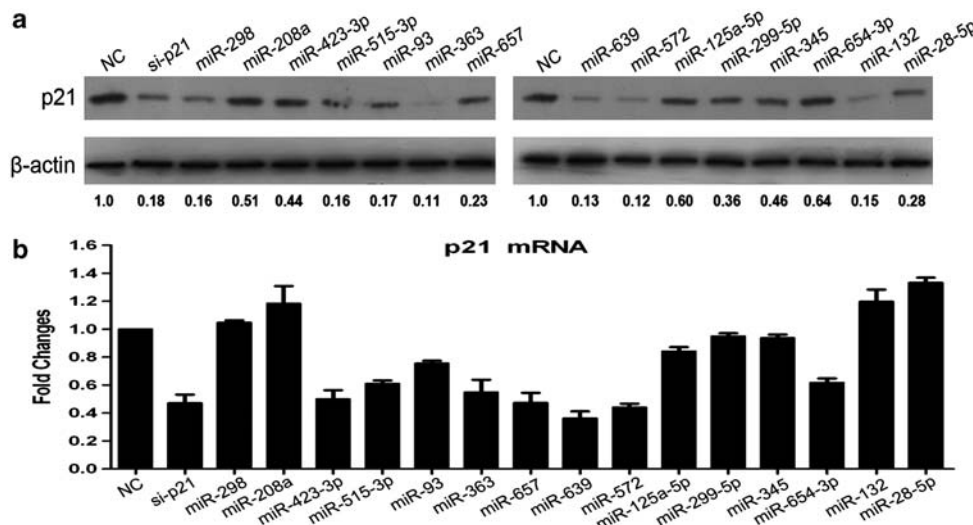
When compared with the predictions of p21-targeting miRNAs, the four target-prediction tools yielded largely nonoverlapping outputs (Figure 3a). Through our screen, we experimentally identified 28 miRNAs that can directly target and regulate p21Cip1/Waf1 expression. Considering our experimental results and the theoretical predictions, PicTar and TargetScan, which use site conservation as a prediction criterion, performed the best (7 of 8 and 8 of 24, respectively) (Figure 3b). RNA22, which does not rely on cross-species site conservation, produced the longest prediction list but had the poorest performance. Nevertheless, the fact that nonconserved target sequences still account for almost half of the target sites for the p21-targeting miRNAs should not be ignored (Figure 3c and Supplementary Table 2), because this indicates that cross-species site conservation is not the key issue for the development of computational prediction programs. In addition, current miRNA prediction methods use

perfect seed pairing as a criterion. Of the four target-prediction tools, TargetScan and PicTar require stringent seed pairing, while miRanda and RNA22 require only moderately stringent seed pairing, which allows for GU pairing. Our observations showed that GU pairing existed in half of the p21-targeting miRNAs (Figure 3d and Supplementary Table 2), suggesting that GU pairing is allowed for miRNAs to bind to its target sequences. Another difference among the prediction approaches is the scoring and ordering of the predicted targets, which is designed to help experimental biologists focus on the most probable predictions. Usually, computational biologists consider a predicted target site as a likely real target site based on how high a target ranks. However, when assessed with the results from the screen, the higher-ranked predicted target sites performed poorly, with the exception of the TargetScan rankings, which showed the most robust discrimination (10 of the top 20 predictions is authentic) (Figure 3e and Supplementary Table 3). Taken together, the interplay

**Table 1** The potential function and significance of p21-targeting miRNAs

miRNA	Chromosome	Overlap-ping transcripts	Other target gene	Related diseases
miR-28-5p	3q28	LPP	—	RCC
miR-572	4p15.33	Intergenic	—	—
miR-93/106b	7q22.1	MCM7	<i>TP53INP1, APP, PCAF, Stat3, Mapk14, E2F1</i>	ATL, AD, MM, HCC, PC, GC, OC
miR-17/20a	13q31.3	MIRHG1	<i>Stat3, Mapk14, E2F2, E2F3, PTEN, Bim, BMPR2, CCND1</i>	NB, NPC, BC, GC, CLL, LC, B-cell lymphoma, HF, cardiomyocyte hypertrophy
miR-208a/208b	14q11.2	MYH6	—	MM
miR-345	14q32.2	Intergenic	—	AML, PBC
miR-299-5p	14q32.31	Intergenic	<i>OPN</i>	—
miR-654-3p	14q32.31	Intergenic	—	TSC, RA
miR-132	17p13.3	Intergenic	<i>MeCP2, RFX4</i>	—
miR-423-3p	17q11.2	CCDC55	—	—
miR-657	17q25.3	AATK	<i>IGF2R</i>	Type II diabetes
miR-639	19p13.12	GPSN2	—	—
miR-125a-5p	19q13.41	Intergenic	<i>ERBB2, ERBB3</i>	Endometriosis, multiple myeloma
miR-372	19q13.41	Intergenic	<i>LATS2, TRPS1, KLF13, MBNL2</i>	TGCT, TSC
miR-515-3p	19q13.41	Intergenic	—	—
miR-519b-3p	19q13.41	Intergenic	<i>HuR</i>	—
miR-519d	19q13.41	Intergenic	—	—
miR-519e	19q13.41	Intergenic	—	—
miR-520a-3p	19q13.41	Intergenic	—	—
miR-520b	19q13.41	Intergenic	<i>MICA</i>	—
miR-520h	19q13.41	Intergenic	—	—
miR-298	20q13.32	Intergenic	—	—
miR-106a/20b/363	Xq26.2	Intergenic	<i>IL-10, APP, E2F1</i>	SCLC, ATL, NB, AD, GC, CRC

Abbreviations: AD, Alzheimer's disease; ATL, adult T-cell leukemia; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; GC, gastric cancer; HCC, hepatocellular carcinoma; HF, heart failure; LC, lung cancer; MM, malignant mesothelioma; miRNA, microRNA; NB, neuroblastoma; NPC, nasopharyngeal carcinoma; OC, ovarian cancer; PBC, primary biliary cirrhosis; PC, prostate cancer; RA, rheumatoid arthritis; RCC, renal cell carcinoma; SCLC, small-cell lung cancer; TGCT, testicular germ cell tumors; TSC, tongue squamous carcinoma; 3'UTR, 3' untranslated region.

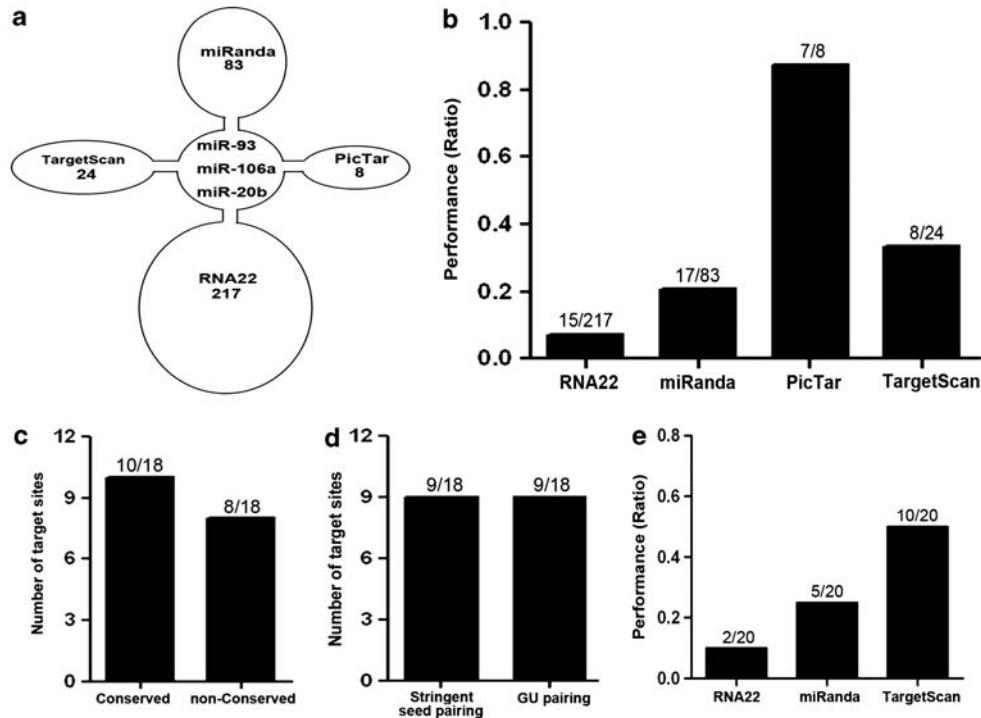


**Figure 2** p21-targeting miRNAs can inhibit the expression of p21Cip1/Waf1 mRNA and protein. (a) Western blot analysis of p21 protein levels after introduction of the p21-targeting miRNAs in HEK 293 cells. β-actin served as the internal control. The numbers below the lanes represent the relative expression levels normalized against β-actin in which NC served as 1. (b) The expression levels of p21Cip1/Waf1 mRNA were determined by quantitative real-time PCR after the introduction of p21-targeting miRNAs. β-actin was used for normalization. The relative mRNA levels were calculated using the  $2^{-\Delta\Delta C_t}$  method. Error bars represent s.e.m.

of miRNAs with their target mRNAs is very complicated, and currently available information is not sufficient for the development of the most efficient and powerful prediction tools.

*Potential function and significance of p21-targeting miRNAs*

It was surprising that so many miRNAs can significantly repress the expression of one single mRNA. We



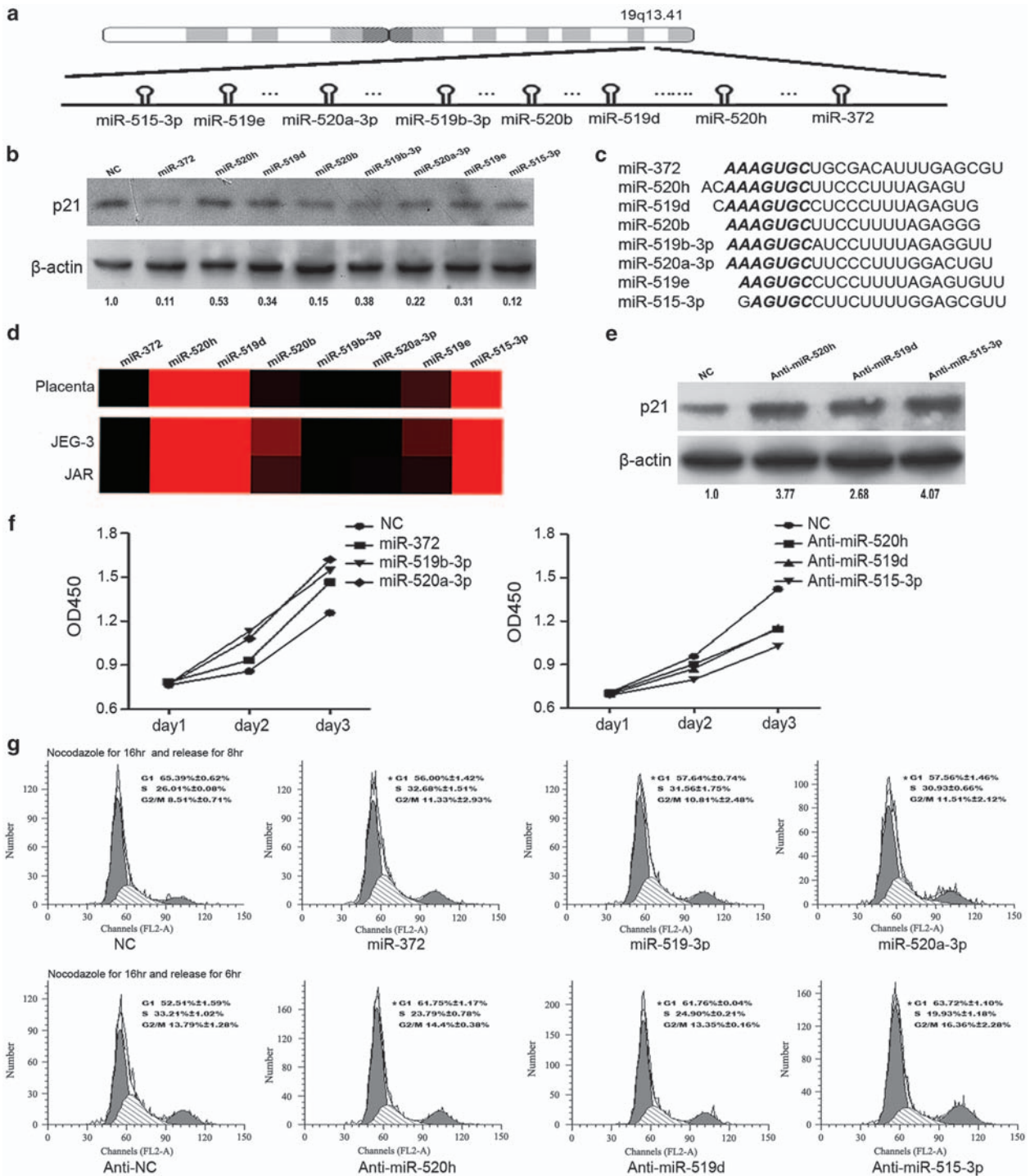
**Figure 3** Comparisons of target-prediction tools. (a) Schematic diagram of the candidate miRNAs by different prediction tools. (b) The fraction of the predicted miRNAs that targeted p21Cip1/Waf1 is calculated for all four miRNA data sets pooled, respectively. (c) The number of conserved and nonconserved target sites in the p21Cip1/Waf1 3' untranslated region (3'UTR). (d) The number of GU pairing target sites in the p21Cip1/Waf1 3'UTR. (e) The rank ordering of the performance of the three prediction approaches.

supposed that these miRNAs might have both spatial and/or temporal specificities in particular tissues or developmental stages. Indeed, the expression profiles available from the miRZ database (Hausser *et al.*, 2009) showed that the miRNAs are differently expressed in various tissues (Supplementary Figure S3). Of these miRNAs, miR-93, miR-20a/b, miR-17 and miR-106a/b have been well studied and implicated in oncogenesis in multiple types of cancer, such as T-cell leukemia, breast cancer, lung cancer, prostate cancer and gastric cancer (Table 1). Again, some of these miRNAs are reported to be upregulated in human cancers, including tongue squamous carcinoma (miR-132) (Wong *et al.*, 2008), acute myeloid leukemia (miR-299-5p) (Jongen-Lavrencic *et al.*, 2008), multiple myeloma (miR-125a-5p) (Lionetti *et al.*, 2009), testicular germ cell tumors (miR-372) (Voorhoeve *et al.*, 2006), malignant mesothelioma (miR-345) (Guled *et al.*, 2009) and renal cell carcinoma (miR-28-5p) (Gottardo *et al.*, 2007). These five miRNAs might also serve as oncogenic modulators. In addition, miR-657 and miR-208 are implicated in other diseases, such as type II diabetes (miR-657) (Lv *et al.*, 2008). In particular, miR-208, a cardiac-specific miRNA, is required for cardiomyocyte hypertrophy (van Rooij *et al.*, 2007). Intriguingly, p21Cip1/Waf1 has been shown to be downregulated and serves as an inhibitor for the development of cardiomyocyte hypertrophy. Taken together, we reasoned that p21Cip1/Waf1 might be an important functional target of miR-208 in cardiomyocyte hypertrophy. This functional linkage is noteworthy and needs to be further explored.

#### *A set of p21-targeting miRNAs located in clusters on chromosome 19 can promote cell growth and cell-cycle progression*

Unexpectedly, a number of p21-targeting miRNAs, including miR-372, miR-520h, miR-519d, miR-520b, miR-519b-3p, miR-520a-3p, miR-519e and miR-515-3p, are located on chromosome 19q13.41 (Figure 4a), a region that contains the largest human miRNA gene cluster (also referred to as the chromosome 19 miRNA cluster, C19MC) (Bentwich *et al.*, 2005). C19MC harbors 46 pre-miRNA genes that have been previously found to be mainly expressed in the placenta (Bortolin-Cavaillle *et al.*, 2009). However, the function of C19MC is largely unknown. We found that these eight C19MC p21-targeting miRNAs can significantly inhibit the expression of p21Cip1/Waf1 (Figure 4b). Remarkably, these miRNAs are highly related to each other and share a similar seed sequence (Figure 4c). We further examined the expression level of these miRNAs in the placenta and two choriocarcinoma cell lines (JEG-3 and JAR). The results showed that miR-520h, miR-519d and miR-515-3p are relatively abundant, whereas miR-372, miR-519-3p and miR-520a-3p could hardly be detected (Figure 4d). Thus, we synthesized antisense oligonucleotides of miR-520h, miR-519d and miR-515-3p, and transfected them into JAR cells, which show relatively high levels of these three miRNAs. As shown in Figure 4e, silencing of these miRNAs led to a significant increase in p21Cip1/Waf1 expression.

To further determine the biological function of the C19MC p21-targeting miRNAs, we ectopically expressed



**Figure 4** A set of p21-targeting miRNAs locate on chromosome 19 in clusters. (a) Schematic diagram of the eight p21-targeting miRNAs located on 19q13.41. Chromosome 19q13.41 contains the largest human miRNA gene cluster (also referred to as chromosome 19 miRNA cluster C19MC). (b) Western blot analysis of p21Cip1/Waf1 protein levels on introduction of the eight C19MC p21-targeting miRNAs into JAR cells. (c) Sequence alignment of the eight C19MC p21-targeting miRNAs. (d) Quantitative PCR analysis of the C19MC p21-targeting miRNAs in the placenta, JEG-3 and JAR cells. Colors indicative showed the relative expression levels (red, high expression). (e) Western blot analysis of p21Cip1/Waf1 protein after the introduction of inhibitors of miR-520h, miR-519d and miR-515-3p into JAR cells. The numbers below the lanes represent relative expression levels normalized against β-actin in which negative control (NC) was set as 1. (f) miR-372, miR-519-3p and miR-520a-3p increased JAR cell proliferation as measured by CCK-8 analysis. Inhibitors of miR-520h, miR-519d and miR-515-3p inhibited JAR cell growth as measured by CCK-8 analysis. (g) Cell-cycle distribution was analyzed by fluorescence-activated cell sorting. JAR cells were plated in a six-well plate and transfected with miRNA mimics (miR-372, miR-519-3p and miR-520a-3p) or miRNA inhibitors (miR-520h, miR-519d and miR-515-3p). Nocodazole (25 ng/ml) was added 24 h after transfection for another 16 h, then the supernatant was replaced by fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 8 h (miRNA mimics) or 6 h (miRNA inhibitors). The data were subjected to Student's *t*-test; \**P* < 0.05.

miR-372, miR-519-3p and miR-520a-3p in JAR cells. The results showed that overexpression of the three miRNAs significantly promoted cell proliferation and G<sub>1</sub>-S transition (Figures 4f and g). In contrast, silencing of miR-520h, miR-519d or miR-515-3p with transient transfection of their inhibitors in JAR cells led to a significant decrease of cell growth and restored G<sub>1</sub> accumulation (Figures 4f and g).

In conclusion, we have experimentally determined for the first time that a single mRNA can be targeted by multiple miRNAs. Our results show that about 28 miRNA species can modulate the expression of the cell CDK inhibitor, p21<sup>Cip1/Waf1</sup>, by directly binding to its 3'UTR. Some of these miRNAs are upregulated in cancers and might serve as oncogenic modulators. It is interesting that a set of these p21-targeting miRNAs is located in the chromosome 19 miRNA cluster and can promote cell proliferation and cell-cycle progression. This study provides us with abundant information to further decode the functional implications of the p21-targeting miRNA/p21<sup>Cip1/Waf1</sup> axis in the development and in human diseases, particular in carcinogenesis.

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## Materials and methods

Materials and methods are provided in the Supplementary data.

## Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)