Down-regulation of the cyclin E1 oncogene expression by microRNA-16-1 induces cell cycle arrest in human cancer cells

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INTRODUCTION

Progression of a cell through or its arrest in a specific cell cycle phase requires the integration, processing and initiation of a variety of signal transduction pathways. Critical for the function of the cell cycle machinery are the activities of cyclin dependent kinases (Cdkks) and their activating subunits, the cyclins. Cyclin E1 (CCNE1), an essential cyclin activating Cdk2, regulates the G1-S phase transition of the mammalian cell division cycle (1). Its timing expression plays a direct role in the initiation of DNA replication (2), the control of histone biosynthesis (3), and the centrosome cycle (4).

CCNE1 expression is largely restricted to the G1-S phase transition in normal dividing cells. Previous studies have revealed that such a periodical expression of CCNE1 is controlled by transcriptional regulation and ubiquitin-dependent proteolysis (5). However, in different types of human cancers, CCNE1 expression is uncoupled from cell cycle progression (6, 7). A growing studies suggest that CCNE1 is expressed significantly higher than physiological levels in many human tumors, in particular breast cancer, and also non-small cell lung cancer, leukemia and others (8). And the genomic locus at which the CCNE1 gene is located (19q12-q13) is frequently amplified in human cancers (9, 10). Transgenic mouse models where CCNE1 is constitutively expressed develop malignant diseases (11), which further supporting the notion of CCNE1 as a dominant oncogene. Clearly, uncoupling CCNE1 expression from the cell cycle control is a critical factor in human cancer development, while the known regulatory mechanisms cannot fully explain such a dysregulation.

MicroRNAs (miRNAs) have recently come into focus as a novel class of post-transcriptional regulatory elements. They are abundant endogenous, 22-nucleotides (nt) RNAs and function by mainly binding to complementary sites on 3' untranslated region (3' UTR) of multiple target mRNAs to repress the target translation or induce the target degradation (12). A number of miRNAs have been found to be involved in a large variety of cellular processes, including cell proliferation, differentiation, cell cycle and apoptosis (13), and their aberrant expression has been linked to disease. Emerging studies have uncovered both the tumor suppressive and oncogenic potential of a number of miRNAs (14, 15), underscoring their importance in human cancers.

The findings of the generally altered miRNA expression in different tumors led us to question if CCNE1 mRNA is normally under the regulation of microRNAs. We initially conducted a survey of the potential microRNAs that can bind to CCNE1 mRNA using bioinformatics databases. MiR-16-1 was identified as a strong candidate due to its crucial function in cell cycle control (16-18). We undertook studies to confirm the predicted role of miR-16-1 in the regulation of CCNE1. Additionally, we showed that miR-16-1 induced a significant G0/G1 cell cycle arrest, which at least in part through down-regulation of CCNE1.

RESULTS

Two evolutionary conserved target sequences for miR-16-1 are found in the 3' UTR of CCNE1

Several prediction programs have been developed to identify potential miRNA targets (19-21). In initial studies, we adopted the bioinformatic approach to identify miRNAs with putative
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Fig. 1. Two conserved miR-16-1 target regions in the 3’ UTR of CCNE1. (a) Diagram showing the predicted results from database miRGator. (b) Schematic representation of the two putative miR-16-1 binding sites in the 3’ UTR of CCNE1. (c) Comparison of nucleotides between the miR-16-1 seed sequence and its two target sites in five species.

binding sites in CCNE1 3’ UTR. By the on-line search of miRGator (22), an integrated database of three most used software algorithms (TargetScan, PicTar, and MiRanda), we found that 3 candidate miRNAs were in the intersection of three programs (Fig. 1a). Of the 3 miRNAs, 2 miRNAs (miR-16-1, and miR-195) share the identical seed region and belong to the miR-16 family. The other microRNA is miR-138, whose function is poorly understood. As noted, miR-16-1 is considered to be an important regulator of cell cycle (16-18) and has been found downregulated in many cancers (23, 24). Therefore, in this study, we have focused on studying the role of miR-16-1 in regulating CCNE1 expression.

As shown in Fig. 1b, there are two potential target sites in 3’ UTR of CCNE1 for miR-16-1, and the calculated minimum free energies for its hybridization with the predicted target sites are ∼24 kcal/mol and ∼24.8 kcal/mol, determined by RNA hybrid analysis (25), which is consistent with the authentic miRNA targeting (26). Comparing the human sequence for interspecies homology, we found that the miR-16-1 target sequences at nt229-254 and nt459-492 of the CCNE1 3’ UTR are highly conserved among five species (Fig. 1c).

miR-16-1 downregulates CCNE1 expression
To determine whether miR-16-1 downregulate the endogenous CCNE1 expression, we first generated a construct that can express precursor forms of miR-16-1 and GFP under the control of a CMV promoter. The GFP gene was interrupted by the genomic fragment encoding the endogenous miR-16-1 locus and its correct splicing from GFP gene resulted in expression of miR-16-1. The advantage of this construct is that we can select miR-16-1 expression cells by sorting GFP-positive cells. Then human breast cancer MCF-7 cells, in which CCNE1 is overexpressed, were transfected with this miR-16-1 overexpression plasmid or a control plasmid. After sorting GFP-positive cells by flow cytometry, western blot analysis were performed. The results showed that enforced expression of miR-16-1 led to a significant decrease in endogenous CCNE1 proteins (Fig. 2a), suggesting that the endogenous expression of CCNE1 is downregulated by miR-16-1.

We further investigated whether miR-16-1 also targeted CCNE1 mRNA for degradation by real-time PCR analysis of RNAs isolated from MCF-7 cells transfected with the miR-16-1 overexpression plasmid or control plasmid. As shown in Fig. 2b, CCNE1 mRNA levels in cells transfected with the miR-16-1 overexpressed plasmid were correspondingly decreased about 60% compared to that in cells transfected with the control plasmid. The mRNA levels of the CCNE1 was shown and normalized against that of GAPDH. The ctrl value was set to 1. Error bars are means of three separated experiments.

CCNE1 is a direct target of miR-16-1
The predicted interaction between miR-16-1 and its target sites in CCNE1 3’ UTR is illustrated in Fig. 3a. To determine whether the negative regulatory effects of miR-16-1 on CCNE1...
expression were indeed mediated through binding to the predicted target sites at the 3' UTR of target miRNAs, we cloned the full length 3' UTR of CCNE1 immediately downstream of the renilla luciferase open reading frame (ORF) in the plasmid pRL-TK. Transient transfection of HEK293 cells with synthetic miR-16-1 duplex and the pRL-CCNE1-3' UTR reporter construct, led to a significant decrease of the luciferase reporter activity as compared with the control miRNA duplex (Fig. 3b).

To further investigate which putative target site was regulated by miR-16-1, we introduced point mutation to the corresponding seed sequences at pRL-CCNE1-3' UTR to eliminate the predicted binding by miR-16-1 (Fig. 3a). As shown in Fig. 3b, suppression of the reporter activity by miR-16-1 was partially relieved by mutation of the single conserved seed complementary site, and mutations of both seed complementary sites almost fully rescued the repression for CCNE1, denoting that both of the two matching sites identified strongly contribute to the miRNA:mRNA interaction that mediates the post-transcriptional inhibition of the CCNE1 expression.

Reciprocal results were observed in experiments carried out in HeLa cells using 2'O-Methylated anti-miR-16-1 (Fig. 3c), which binds to endogenous miR-16-1 and thereby antagonizes its activity. Taken together, these data suggest that miR-16-1 directly binds to the 3' UTR of CCNE1 and downregulates its expression.

miR-16-1 induces G0/G1 cell cycle arrest partially through down-regulation of CCNE1

Since CCNE1 has a prominent role in cell cycle, particularly drives cells progressing from G1 to S phase, we then examined if miR-16-1 regulation of CCNE1 expression correlates with cell cycle regulation. A549 human lung carcinoma cells were transfected with synthetic miR-16-1 duplex or siRNAs against CCNE1, then the cell cycle distribution of the transfected cells treated with microtubule depolymerizing drug nocodazole 24 hours post-transfection were analyzed by flow cytometry. Compared with cells transfected with negative control (NC) duplex, cells transfected with siRNA against CCNE1 triggered an accumulation of cells at the G0/G1 stage, whereas the numbers of cells in S-phase and G2/M-phase accordingly decreased (Fig. 4a), which consistent with the role of CCNE1 in cell cycle progression from G1 to S phase. As predicted for downregulation of CCNE1 expression by miR-16-1, transfection with miR-16-1 yielded a phenocopy of the phenotype generated by siRNA against CCNE1, and the effects were more profound, featured by a greater G0/G1-cell accumulation and a greater decrease in cell population in S-phase and G2/M-phase (Fig. 4a). Comparable results were also observed in human breast cancer MCF-7 cells (Supplementary Fig. 1).

One explanation for the more profound cell cycle arrest at G0/G1 phase elicited by miR-16-1 than siRNA against CCNE1 is that multiple cell cycle genes coordinating cell cycle progression are targeted by miR-16-1. CCNE1 is only one of such targets regulated by miR-16-1 in modulating cell cycle progression.

To further establish a functional connection between miR-16-1 and CCNE1, we addressed whether high level of CCNE1 is account for the cell cycle phenotype caused by the reduced level of miR-16-1. Anti-miR-16-1 was used to reduce the miR-16-1 activity in A549 human lung carcinoma cells. As shown in Fig. 4b, compared to control-treated cells, transfection with anti-miR-16-1 resulted in a reduced level of G0/G1-cells and a correspondingly accumulation of cells in S phase. If the anti-miR-16-1-induced phenotype depends on increased levels of CCNE1, then reducing the level of CCNE1 should abrogate the...
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Fig. 4. Ectopic miR-16-1 triggers G0/G1 arrest in A549 cells, and si-CCNE1 rescues the anti-miR-16-1-induced phenotype. (a) A549 cells were transfected with synthetic miR-16-1 duplex, siRNA against CCNE1 (si-CCNE1) or negative control (NC) duplex, 24 hr post-transfection, cells were then treated with nocodazole for 16∼20 hr. Cell cycle distribution were analyzed by flow cytometry. (b) A549 cells were transfected anti-miR-16-1 and negative control (NC), anti-miR-16-1 and si-CCNE1, or NC and scramble anti-miR control (SC). The cells were collected 48 hr after transfection and subjected to flow cytometry analysis. In each instance, flow cytometry was performed three times, the shown data represent three independent experiments.

effect. To test this hypothesis, anti-miR-16-1 was cotransfected with siRNA against CCNE1. The result showed that si-CCNE1 led to substantial, although not complete, rescue of anti-miR-16-1-induced cellular phenotype (Fig. 4b). These data suggest that CCNE1 is required for the anti-miR-16-1 phenotype and indicate for further that CCNE1 is only one of the targets modulated by miR-16-1 in cell cycle regulation. Overall, these results show that miR-16-1 contributes to induction of G0/G1 arrest in A549 cells and MCF-7 cells, which is partially through down-regulation of CCNE1.

DISCUSSION

MicroRNAs are of ever increasing importance as post-transcriptional regulators of gene expression following transcription. They have been demonstrated to play a significant role in carcinogenesis by altering expression of oncogenes and tumor suppressor genes (15). In this study, we have confirmed by multiple methods that miR-16-1 controls the expression of a positive cell cycle regulator CCNE1 oncogene by directly targeting the 3' UTR of its mRNA, which reveals a mechanism of post-transcriptional regulation of CCNE1 expression and connects the function of miR-16-1 with a cell cycle gene.

Our bioinformatic analysis has suggested that in addition to miR-16-1, miR-195 in miR-16 family and some other miRNAs may also regulate the expression of CCNE1 at post-transcriptional level. Meanwhile, it is likely that CCNE1 is only one of the multiple targets coordinately regulated by miR-16-1 in controlling cell cycle progression. This can be reflected by the findings that siRNA against CCNE1 only partially phenocopied the cellular phenotype of miR-16-1 and incompletely rescued the anti-miR-16-1-induced phenotype (Fig. 4). An emerging common theme is that microRNAs modulate cell cycle progression by a coordinated repression of multiple genes. In other words, multiple targets regulated by a single miRNA can act in concert, rather than individually, to regulate the same biological process. Coordinated regulation of many targets by a single miRNA may allow for a prompt cellular response to the progression of the cell cycle and also for rapid reversal of the microRNA-induced cell cycle regulation upon changes in microRNA synthesis, stability or localization (27).

We found that miR-16-1 also triggered G0/G1 arrest in MCF-7 breast cancer cells, but led to a less G0/G1 accumulation phenotype as compared to that in A549 lung carcinoma cells (supplementary Fig. 1). One possible explanation for this finding is that the genetic context in individual cell line is distinct from the other, for example, the expression profile of the targeted genes of miR-16-1 differs in these two cell lines.

The present study helps to further our understanding of the molecular pathways involved in the development and progression of lung carcinoma and breast cancer, which may implicate new therapeutic strategies in the treatment of these diseases. MiR-16-1 could negatively regulate CCNE1 oncogene overexpressed in the development of a subset of human cancers, and thus has a strong rationale for cancer therapy in the future.
MATERIALS AND METHODS

Cell lines and transfection
HEK293 and HeLa cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (Gibco) at 37°C in a humified atmosphere of 5% CO2. MCF-7 and A549 cells were grown at the same condition except that the fetal bovine serum (FBS) was substituted for the 10% new-born calf serum (Gibco). Transfections were performed by Lipofectamine 2000 reagent (Invitrogen) for plasmids or Oligofectamine reagent (Invitrogen) for RNA oligos according to the manufacturer’s protocol.

Plasmids and RNA oligos
A miRNA construct expressing miR-16-1 was designed by our laboratory. In brief, a genomic fragment spanning the miR-16-1 locus from human chromosome 13 was cloned into the Xho I/Sac II restriction site of a mammalian expression vector (Promega). Mutant 3' UTRs were generated by overlap-extension PCR method by the following primers: CCNE-MUT1 FW 5'-CGA TAC CAT GGA AGG TGC TCC A-3' and CCNE1-UTR RV 5'-CTG GGG GCC CTG TCT CAA AAA CAG TAT TAT TT-3' and inserted at the XbaI and Apa I site, immediately downstream of the luciferase gene in the modified pRL vector (Promega). Mutant 3' UTRs were generated by overlap-extension PCR method by the following primers: CCNE1-UTR FW 5'-CCA CAC CTG ACA AAG AAG ATG ATG GCC ATC AC-3', RV, 5'-TCC ACC ACC CTG TTG CTG TA-3'. The full length 3' UTR of CCNE1 was amplified from HeLa cell genomic DNA by PCR using the following primers: CCNE1-UTR FW 5'-CTA GTC TAG ACC ACC CCA TTC TTC-3' and CCNE1-UTR RV 5'-CTT ACA AAA CAG TAT TTC TT-3' and inserted into the XbaI and Apa I site, immediately downstream of the luciferase gene in the modified pRL vector (Promega). Mutant 3' UTRs were generated by overlap-extension PCR method by the following primers: CCNE-MUT1 FW 5'-CGA TAC CAT GGA AGG TGC TCC A-3' and CCNE-MUT1 RV 5'-CGC CAA UAU UUA CGU GCU G-3', anti-sense: 5'-CGC CAA UAU UUA CGU GCU G-3'), scramble anti-miR control (5'-UUG GCU GCA GAA GAG GGU G dTdT-3').

RNA extraction and qRT-PCR
Total RNA was extracted from the cultured cells using Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. qRT-PCR was used to confirm the expression level of mRNAs. cDNA was produced with random primers and reverse transcription was performed according to the protocol of M-MLV Reverse Transcriptase (Promega), and qPCR was performed as described in the method of SYBR Green Realtime PCR Master Mix (ToYoBo, Japan) with Rotor-Gene 3000 Multi-filter Real-time Cycler detection system (Corbett Research, Sydney, Australia) supplied with analytical software. The PCR reaction was conducted at 95°C for 5 min followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. GAPDH mRNA levels were used for normalization. The oligonucleotides used as PCR primers were: GAPDH FW, 5'-ACC ACA GTC CAT GCC ATC ACC TA-3'; RV, 5'-TCC ACC ACC CTG TGG TCA TAA-3'. CCNE1 FW, 5'-CCA CAC CTG ACA AAG AAG ATC-3'; RV, 5'-GAG CCT CTG GAT GGT GCA ATA AT-3'.

Western blot
Cells were lysed then the lysates were bold for 5 min and centrifuged at 12,000 r/min at 4°C for 10 min. The whole cell lysate of 40 μg was loaded per lane and separated using 12% SDS-acrylamide gels, and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk in TBS, the membrane was probed with primary monoclonal antibody specific to CCNE1 (1: 1,000, Epitomics, CA) or β-actin (1: 10,000, Sigma), which was used as an internal control for protein loading. The membrane was further probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1: 10,000, Sigma) and the protein bands were visualized using enhanced chemiluminescence detection reagents (Pierce).

Luciferase activity assay
For luciferase analysis, HEK293 or HeLa cells were transiently transfected with 200 ng of each renilla luciferase reporter plasmid plus 40 ng pGL3-promoter (Promega), in combination with 50 nM of synthetic miR-16-1 duplex or 2-O-Methylated anti-miR-16-1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Luciferase activity was measured 24 hr after transfection using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Three independent experiments were performed in triplicate.

Flow cytometry
Flow cytometry analysis was done as described previously (28, 29) but with some modifications. Briefly, one day before transfection, equal numbers of A549 cells or MCF-7 cells (2.0 × 10⁴) were seeded into 12-well tissue culture plates without antibiotics so they will be about 30% confluent at the time of transfection. Next day cells were transiently transfected with negative control siRNA or miR-16-1 duplex or siRNA against CCNE1 at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, nocodazole (100 ng/ml; Sigma-Aldrich) was added and cells were further incubated for 16 to 20 h before harvesting. The cells were collected by centrifugation, fixed with ice-cold 70% ethanol at -20°C, washed with phosphate-buffered saline (PBS), and resuspended in 0.5 ml of PBS containing propidium iodide (50 μg/ml) and RNase A (1 mg/ml). After a final incubation at 37°C for 30 min, cells were analyzed by the EPICS ALTRA II flow cytometer (Beckman Coulter). A total of 10,000 events were counted for each sample. Data were analyzed using Multicycle AV software (Beckman Coulter).

Statistical analysis
Results were expressed as means ± SD unless indicated other-
wise. Differences between groups were assessed by unpaired, two-tailed Student’s t-test, p < 0.05 was considered significant. All data were plotted using the GraphPad Prism 4.0 program (www.graphpad.com).

Acknowledgements
We would like to gratefully thank Yan Wang and Weihuang Liu (Medical college, Wuhan University, China) for the flow cytometry technical assistance and data analysis. This work is supported by National High-tech R&D Program (863 Program) of China through grant 2007AA02Z100 awarded to Y. Zhang and by the National Basic Research Program (973) of China through grant 2005CB724604 awarded to Y. Zhang and X.-D. Fu.

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