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Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest

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Abstract **miRNAs regulate gene expression by inhibiting translation or by targeting messenger RNA (mRNA) for degradation in a post-transcriptional fashion. In the present study, we show that ectopic expression of miR-34a reduces both mRNA and protein levels of cyclin D1 (CCND1) and cyclin-dependent kinase 6 (CDK6). We also demonstrate that miR-34a targets the 3′-untranslated mRNA region of CCND1 as well as CDK6, which in turn interferes with phosphorylation of retinoblastoma. In addition, we show that overexpression of miR-34a induces a significant G1 cell-cycle arrest in the A549 cell line. Taken together, our data suggest that the effects of miR-34a on G1 cell cycle arrest are through the down-regulation of CCND1 and CDK6, which is associated with other targets of miR-34a either additively or synergistically.**

Keywords: MicroRNA; miR-34a; CCND1; CDK6; Cell cycle

1. Introduction

Neoplasia is considered to be the result of dysregulation of the cell cycle machinery. Studies on cell cycle regulation and cancer genetics have revealed that multiple cell cycle regulatory proteins play key roles in oncogenesis. In the G1/S phases of the cell cycle, cyclin-dependent kinase 4/6 (CDK4/6) in complex with cyclin D1 phosphorylate the Rb family [1–4].

Phosphorylated pRb then loses the repressive activity for the E2F transcription factor. The activated E2F complexes, as transcriptional activators, work on the target genes whose products regulate both the G1/S transition and DNA replication [5,6]. Loss of regulation at the G1/S transition appears to be a common event among virtually all types of human tumor. According to recent in-depth investigation on microRNA (miRNA) functions, proteins involved in cell cycle progression and cell survival increasingly represent good candidate targets of miRNAs.

miRNAs are a non-coding family of genes involved in post-transcriptional gene regulation [7,8]. In general, miRNAs negatively regulate the stability and translation of target messenger RNAs (mRNAs) at the 3′-untranslated region (3′-UTR) and are involved in diverse processes, such as cell-cycle control, apoptosis and carcinogenesis [9,10]. Emerging evidence suggests that several miRNAs target genes that are involved in cell cycle progression and cellular proliferation [11]. For example, miR-221 and miR-222 repress the expression of the cell cycle regulator p27Kip1 in glioblastoma cells [12]. More recent studies suggest that miR-16 family directly regulates cell cycle progression by controlling the G1 checkpoint. As a result, over-expression of miR-16 family leads to G1 arrest in cultured human tumor cells [13].

Recently, some reports have revealed that abnormal miR-34a is involved in different types of cancer. For example, aberrant expression of the precursors of miR-34a has been found in malignant lymphomas [14]. Chang et al have shown that miR-34a is frequently absent in pancreatic cancer cells [15]. Tazawa et al have shown that down-regulation of miR-34a may be involved in human colon cancer development [16]. Furthermore, the biological functions and targets of miR-34a have been reported. Recent studies have identified miR-34a as a miRNA component of the p53 network and shown that it can be directly transactivated by p53 [15,17]. Further evidence has revealed that miR-34a induces G1 arrest by regulation of several cell cycle genes, including cyclin E2 (CCNE2), CDK4 and hepatocyte growth factor receptor (MET) [18]. Because G1 arrest is a multi-protein regulated process and one miRNA could target multiple genes, it remains unclear whether there are other G1/S transition genes regulated by miR-34a besides CCNE2, CDK4 and MET.

Here we report that miR-34a directly regulates the expression of cyclin D1 (CCND1) and CDK6, affecting G1 arrest in A549 cells. Taken together, these results suggest that miR-34a triggers G1 arrest by regulating multiple downstream effectors including CCND1 and CDK6.

2. Materials and methods

2.1. Cell culture, reagents and antibodies

Non-small cell lung cancer A549 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 100 μg/mL penicillin/streptomycin at 37 °C in a humified atmosphere of 5% CO_2. Nocodazole was purchased from Sigma–Aldrich. Monoclonal antibodies

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Abbreviations: miRNA, microRNA ; 3′-UTR, 3′-untranslated region; mRNA, messenger RNA; CCND1, cyclin D1; CCND3, cyclin D3; CCNE2, cyclin E2; CDK4/6, cyclin-dependent kinase 4/6; MET, hepatocyte growth factor receptor

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against CCND1 (Code No. K0062-3), CDK6 (Code No. K0066-3), CCND3 (Code No. K0013-3), and CDC25A (Code No. K0072-3) were purchased from MBL Monoclonal Antibody. Polyclonal antibody against pRb (Phospho-Ser795, # 11130) was purchased from Signalway Antibody. Polyclonal antibody against Rb (10048-2-Ig) was purchased from Proteintech Group, Inc. Polyclonal antibodies against Actin (sc-1616) was purchased from Santa Cruz Biotechnology Inc.

2.2. Plasmids and transfection

Firefly luciferase reporter vectors were constructed by the following methods. Wild-type 3′-UTRs containing predicted miRNA target sites were amplified by PCR from HepG2 cell genomic DNA. Mutant 3′-UTRs were generated by overlap-extension PCR method. Both wild-type and mutant 3′-UTRs were cloned downstream of firefly luciferase coding region between the XbaI and NdeI sites of a modified pGL3-control plasmid (Promega), as described before [19]. Specific fragments including miR-34a targeting site of CCND1 and CDK6 3′-UTRs were generated by the following primers: CCND1-UTR (forward) 5′-ACG TCT AGA TGA CCT GTT TAT GAG ATG CTG-3′, CCND1-UTR (reverse) 5′-GAT CAT ATG GGG TCC ACC ATG GCT AAG TGA-3′; CDK6-UTR (forward) 5′-ACG TCT AGA TTT GGC TGT GGT ACC AAG AGA-3′, CDK6-UTR (reverse) 5′-GAT CAT ATG GAC AGT GAT ATT TCA ACA CCA-3′. And CCND1 and CDK6 mutant 3′-UTRs were generated by the following primers: MuCCND1-UTR (forward) 5′-TGT TTT ACA ATG TCA TAT ATA CCT CTG TAC TAG TTT TAG TTT TC-3′, MuCCND1-UTR (reverse) 5′-GAA AAC TAA AAC TAG TAC AGA GGT ATA TAT GAC ATT GTA AAA CA-3′; MuCDK6-UTR (forward) 5′-GAA GCA GTG TGG AAA TTA GGT GAC GGG ACA CAG TCT TAT A-3′, MuCDK6-UTR (reverse) 5′-TAT AAG ACT GTG TCC CGT CAC CTA ATT TCC ACA CTG CTT C-3′. The seed region was mutated and marked with underline. For synthetic microRNA transfection, A549 cells were transfected with miR-34a duplex or control Luc-siRNA at a final concentration of 50 nM, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

2.3. 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 produced with oligo-dT primers and RT was performed according to the protocol of Impro-II Reverse Transcriptase (Promega), qPCR was performed as described in the method of SYBR premix Ex Taq (TaKaRa) with Mx 3000p (Stratagen) supplied with analytical software. GAPDH mRNA levels were used for normalization. The oligonucleotides used as PCR primers were: CCND1 (forward) 5′-CGT GGC CTC TAA GAT GAA GG-3′, CCND1 (reverse) 5′-CTG GCA TTT TGG AGA GGA AG-3′; CDK6 (forward) 5′-TGC ACA GTG TCA CGA ACA GA-3′, CDK6 (reverse) 5′-ACC TCG GAG AAG CTG AAA CA-3′; GAPDH (forward) 5′-TCA GTG GTG GAC CTG ACC TG-3′, GAPDH (reverse) 5′-TGC TGT AGC CAA ATT CGT TG-3′.

2.4. Luciferase assays

For luciferase analysis, A549 cells were transfected with the firefly luciferase reporter vectors in 24-well plates by using Lipofectamine 2000 (Invitrogen). The transfection mixtures contained 100 ng of firefly luciferase reporter plasmid and 50 nM of synthetic miR-34a duplex. pRL-TK (Promega) as a normalization control was also transfected into A549 cells. Cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega). Cells were transfected in duplicated wells and such experiments were repeated three times.

2.5. Western blot analysis

Cells were lysed in RIPA lysis buffer (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 1% NP40, 0.5% (w/v) sodium deoxycholate, 0.1% sodium dodecylsulfate, complete mini protease inhibitors (Roche)). Lysates were sonicated and centrifuged at 12,000 r/min at 4 °C for 10 min. Per lane 40 μg of whole cell lysate was separated using 10% SDS–acrylamide gels, and transferred on Immobilon Hybond-C membranes (Amersham Biosciences). For immunodetection membranes were incubated with specific antibodies. Signals from HRP (horse-radish-peroxidase)-coupled secondary antibodies were generated by exposure to the film (Kodak).

2.6. FACS analysis

A549 cells were transiently transfected with luciferase siRNA GL-3 or miR-34a duplex at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen). Synthetic miRNA duplexes were as follows (sense/antisense): miR-34a,5′-UGG CAG UGU CUU AGC UGG UUG UU-3′/5′-CAA CCA GCU AAG ACA CUG CGA AA-3′; control-Luc-siRNAs,5′-CUU ACG CUG AGU ACU UCG ATT-3′/5′-UCG AAG UAC UCA GCG UAA GTT-3′; siRNA against Cyclin D1, 5′-GGA GAA CAA ACA GAU CAU CTT-3′/5′-GAU GAU CUG UUU GUU CUC CTC-3′; siRNA against CDK6,5′-GAU GUU GAU CAA CUA GGA ATT-3′/5′-UUC CUA GUU GAU CAA CAU CTG-3′. A549 cells were treated with nocodazole (100 ng/mL) 24 h post transfection and monitored for cell cycle distribution 16–20 h after nocodazole treatment. The cells (including the floating cells) were collected, washed with PBS, fixed in ethanol at −20 °C. The cells were washed with PBS, rehydrated and resuspended in 0.2 mL of RNase A (1 mg/mL) in PBS buffer at 37 °C for 30 min. The cells were stained with 0.3 mL propidium iodide (60 μg/mL) in a solution containing 0.1% Triton X-100, 0.1 mM EDTA. The stained cells (1×10^5) were then analyzed for DNA content with a flow cytometer (FACScaliber, Becton–Dickinson).

3. Results

3.1. miR-34a downregulates CCND1 and CDK6 proteins

Several genes involved in controlling the cell cycle have been found to be regulated by miR-34a, including CCNE2, CDK4 and MET [18]. However, a bioinformatic analysis (http://www.targetscan.org/) revealed that putative miR-34a target sites were harbored in the 3′-UTRs of many other cell cycle genes including CCND1, CCND3, CDK6 and CDC25A and others (Fig. 1). In addition, these cell cycle genes are involved in regulation of G1/S transition. To analyze whether these cell cycle genes were regulated by miR-34a we transfected A549 cells with synthetic miR-34a duplex. The results show that overexpression of miR-34a leads to a significant decrease in endogenous CCND1 and CDK6 proteins but has no effects on the expression levels of CCND3 and CDC25A (Fig. 2a). Meanwhile, we detected the mRNA levels of CCND1 and CDK6 by qRT-PCR. The results show that overexpression

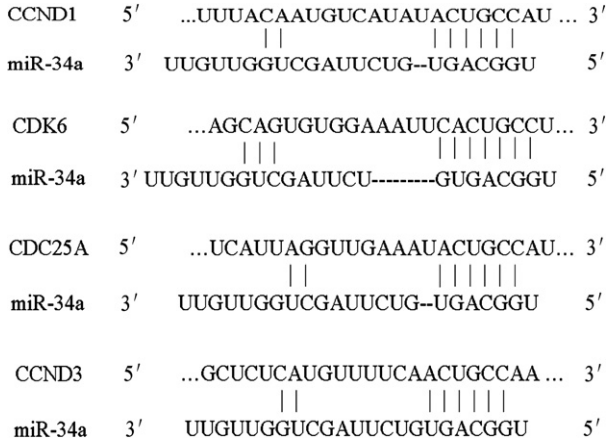

Fig. 1. miR-34a target site resides at 3′-UTR of the CCND1, CDK6, CDC25A and CCCND3. Sequence inspection by bioinformatics revealed that target site for miR-34a resides in the 3′-UTR of the CCND1, CDK6, CDC25A and CCND3 transcripts, including the theoretical miRNA:mRNA duplex pairing.

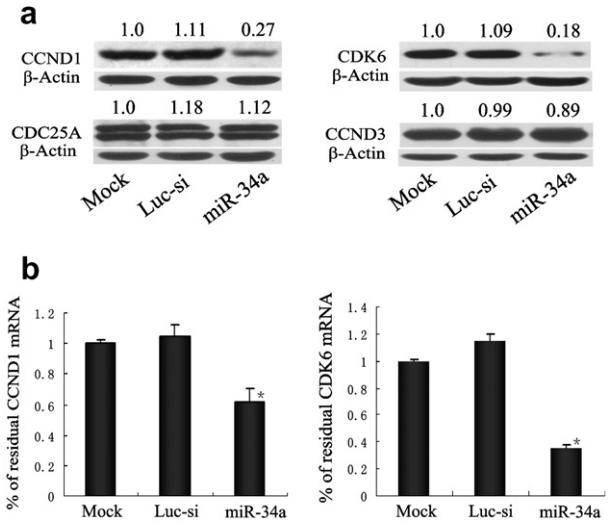

Fig. 2. miR-34a regulates CCND1 and CDK6 expression at the post-transcriptional level. (a) CCND1, CDK6, CDC25A and CCND3 proteins in A549 cells were measured by Western blot at 48 h post-transfection. A549 cells transfected with synthetic miR-34a duplex (50 nM) or luciferase siRNA as control. β-Actin was used as an internal loading control. (b) The effectiveness of miR-34a on CCND1 and CDK6 mRNA was analyzed by qRT-PCR. The mRNA levels of the *CCND1* and *CDK6* were shown and normalized against that of *GAPDH*. Data are means of three separated experiments ± S.E. $^{*}P < 0.01$.

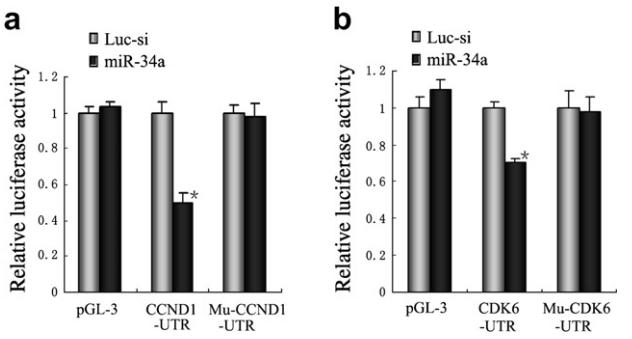

Fig. 3. 3′-UTRs of CCND1 and CDK6 are direct targets of miR-34a. (a) The effects of miR-34a on expression of CCND1 by using luciferase assays. (b) The effects of miR-34a on expression of CDK6 by using luciferase assays. Cells were cotransfected with 50 nM synthetic miR-34a duplex (black bar) or luciferase siRNA control (grey bar) with wild-type CCND1/CDK6 3′-UTR and their own different mutants of 3′-UTR, respectively. Luciferase activities were normalized by the ratio of firefly and Renilla luciferase activities. Each bar represents values from three independent experiments with duplicated samples in each experiment ($n = 6$). Values in both (a) and (b) are means of three separated experiments ± S.E. $^{*}P < 0.01$.

of miR-34a in A549 cells leads to a corresponding decrease in endogenous CCND1 and CDK6 mRNAs (Fig. 2b). These observations indicate that miR-34a downregulates the expressions of CCND1 and CDK6 at the translational level, and reduces mRNA stability simultaneously.

3.2. CCND1 and CDK6 are direct targets of miR-34a

To validate whether miR-34a directly recognized the 3′-UTRs of CCND1 and CDK6 transcripts, we cloned the fragments containing presumed target sites into the 3′-UTR of the luciferase gene. Transient transfection of A549 cells with synthetic miR-34a duplex and the pGL-3M-CCND1-3′-UTR-reporter construct, leads to a significant decrease of reporter activity as compared with the control (Fig. 3a). However, the activity of the reporter construct mutated at the specific miR-34a target site is unaffected (Fig. 3a). Similar results were obtained with cotransfection of pGL-3M-CDK6-3′UTR and miR-34a duplex (Fig. 3b). These data suggest that CCND1 and CDK6 are direct functional targets of miR-34a in cultured A549 cells.

3.3. miR-34a induces G1-arrest in A549 cell line

miR-34a as key target of p53 transcriptional factor is capable of regulating cell cycle progress and cell proliferation. Since miR-34a could downregulate expression of multiple cell-cycle related proteins, we asked whether it could change downstream biological effects of cell cycle. Ectopic miR-34a delivery decreases the level of phosphorylated retinoblastoma gene product (Fig. 4a and b), which is consistent with silencing activity of both CCND1 and CDK6. *CCND1* siRNA had stronger inhibition effectiveness on CCND1 but weaker effects on phosphorylation of Rb when compared with miR-34a. These results suggest that CCND1 is only one of the targets regulated by miR-34a in controlling cell cycle progression and some other targets of miR-34a simultaneously regulate G1/S transition. To further study the function of miR-34a in G1 arrest we transfected A549 cells with synthetic miR-34a duplex and siRNAs against CCND1 and CDK6. We examined the cell cycle distribution of the transfected cells treated with nocodazole by flow cytometry. The results show that miR-34a triggers more accumulation of cells at the G1 stage, whereas the number of cells in S-phase and G2/M-phase decrease (Fig. 4c). Silencing of these selected miR-34a targets by siRNAs leads to a substantial arrest in G1 (Fig. 4c). These results indicate miR-34a contribute to induction of G1-arrest in A549 cells, which is partially through down-regulation of CCND1 and CDK6.

4. Discussion

Recent studies have shown that miR-34a possesses anti-proliferative potential and regulates cell cycle transition from G1 to S [20,21]. In addition, emerging findings provide evidence that the gene encoding miR-34a is a direct transcriptional target of p53. Transcriptional activation of miR-34a causes dramatic reprogramming of gene expression and contributes to p53-mediated apoptosis [15,17]. Moreover, miR-34a mediates such functions through additive or synergistic effects of multiple targets, including CCNE2, CDK4 and MET [18]. In this study, our data demonstrates that CCND1 and CDK6 that are important for G1 cell cycle arrest are direct targets of miR-34a.

Summarizing the results from other laboratories and ours, miR-34a is involved in cell cycle progression and cellular proliferation in coordination with regulating multiple proteins (Fig. 5). Tumor development and progression have been shown to be dependent on cellular accumulation of various genetic and epigenetic events, including alterations in the cell-cycle machinery at G1/S checkpoint [22,23]. The G1/S phase transition is regulated primarily by D-type cyclins (D1, D2, or D3) in complex with CDK4/CDK6, and E-type cyclins

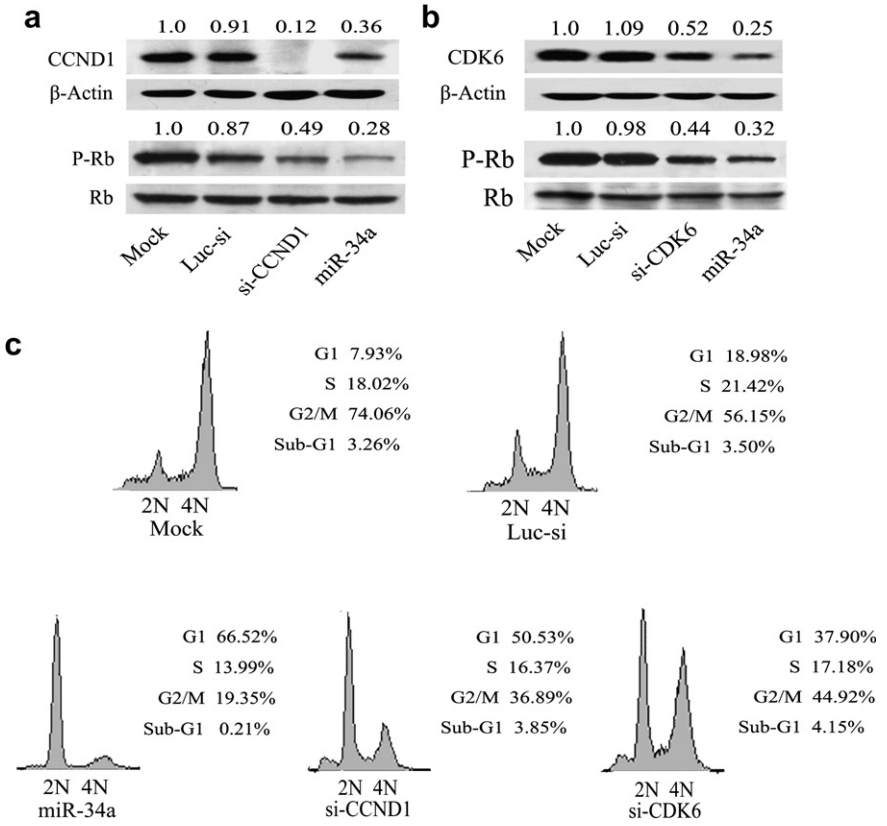

Fig. 4. Ectopic miR-34a induces G1 arrest. (a) A549 cells were transfected with *CCND1* siRNA, or miR-34a duplex at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen). Luciferase siRNA (luc-si) was used as the RNA transfection control. The levels of CCND1 and phosphorylated Rb were examined with Western blot. β-Actin was used as the loading control. (b) A549 cells were transfected with *CDK6* siRNA, or luc-si or miR-34a duplex at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen). The levels of CDK6 and phosphorylated Rb were examined with Western blot. (c) Cell cycle distribution of A549 cells transfected with miR-34a duplex or Luc-siRNA or *CCND1* siRNA or *CDK6* siRNA. 2N: cells have diploid DNA content; 4N: cells have tetraploid DNA content.

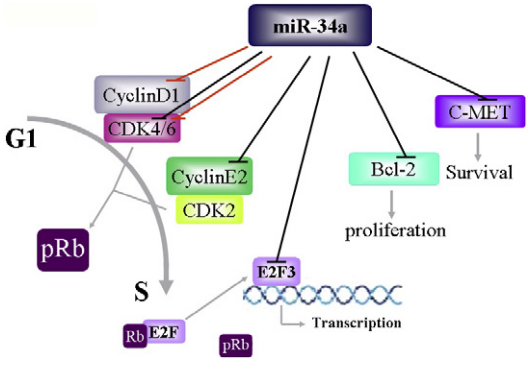

Fig. 5. A model of the miR-34a in regulating cell proliferation and cell cycle progression. miR-34a induces a G1-arrest through negative effects on cell cycle related proteins, including CCND1, CDK4, CDK6 and CCNE2. miR-34a also regulates cell proliferation by downregulating antiapoptotic factor Bcl-2, transcription factor E2F3 and hepatocyte growth factor receptor (MET).

(E1, or E2) in complex with CDK2. Cyclin D and its associated kinases are responsive to mitogenic signals and uniquely positioned to regulate cell cycle progression. Specific miRNAs regulate both cell-cycle progression and apoptosis, which reveals a new layer of complexity in the cell cycle regulation [24,25]. It is sufficiently demonstrated that miR-34a through additive or synergistic effects of multiple targets mediates negative regulation of cellular growth and proliferation. On the other hand, miR-34a maps to a region on chromosome 1p36, which is commonly deleted in neuroblastoma and other tumors [26]. In addition, ectopic miR-34a reduces the levels of E2F3 by targeting its mRNA [26]. The anti-apoptotic protein BCL2 is downregulated by miR-34a in several cell types, which is consistent with a role for miR-34a in p53-mediated apoptosis [27]. Furthermore, MET, the receptor for hepatocyte growth factor, is also regulated by miR-34a. Taken together, miR-34a might be the key effector of cell cycle and cell proliferation regulation, and its inactivation might contribute to the development of certain cancers.

In conclusion, our results reveal that miR-34a negatively regulates CCND1 as well as CDK6 expression and promotes G1 arrest. In fact, miR-34a triggers G1 arrest by regulating multiple downstream effectors including CCND1, CDK6 and other cell cycle genes. miR-34a could negatively regulate several oncogenes overexpressed in the development of a subset of human cancers, and thus has a strong rationale for cancer therapy in the future [28–30]. It is affirmative that the rationale for targeting miRNAs is superior to that for antisense mRNAs and RNAi as tools in studying gene functions and in some cases of gene therapy [31]. Monitoring miR-34a in human tumors and induction of it may thus facilitate better cancer diagnosis and cancer therapy.

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