miR-6883 Family miRNAs Target CDK4/6 to Induce G1 Phase Cell-Cycle Arrest in Colon Cancer Cells

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Abstract

CDK4/6 targeting is a promising therapeutic strategy under development for various tumor types. In this study, we used computational methods and The Cancer Genome Atlas data set analysis to identify novel miRNAs that target CDK4/6 and exhibit potential for therapeutic development in colorectal cancer. The 3'UTR of CDK4/6 mRNAs are targeted by a family of miRNAs, which includes miR-6883-5p, miR-149-5p, miR-6785-5p, and miR-4728-5p. Ectopic expression of miR-6883-5p or miR-149-5p downregulated CDK4 and CDK6 levels in human colorectal cancer cells. RNA-seq analysis revealed an inverse relationship between the expression of CDK4/6 and miR-149-5p and intronic miRNA-6883-5p encoding the clock gene PER1 in colorectal cancer patient samples. Restoring expression of miR-6883-5p and miR-149-5p blocked cell growth leading to G0–G1 phase cell-cycle arrest and apoptosis in colorectal cancer cells. CDK4/6 targeting by miR-6883-5p and miR-149-5p could only partially explain the observed antiproliferative effects. Notably, both miRNAs synergized with the frontline colorectal cancer chemotherapy drug irinotecan. Further, they resensitized mutant p53-expressing cell lines resistant to 5-fluorouracil. Taken together, our results established the foundations of a candidate miRNA-based theranostic strategy to improve colorectal cancer management.

Introduction

There were an estimated 134,490 cases of colorectal cancer and about 49,190 deaths from the disease in the United States in 2016 according to the National Cancer Institute Surveillance, Epidemiology, and End Results program (1). The 5-year survival rate for advanced (stage IV) disease still remains low, at 13.5% (1). Thus, there is ongoing need to develop treatments and biomarkers needed to address the mortality and morbidity from advanced colorectal cancer.

Dysregulation of the p16–CDK4/6–Rb–E2F pathway is a hallmark of cancer (2–4). CDK4 and 6, oncogenic kinases within this pathway, are validated anticancer targets. Different small molecule inhibitors of CDK4/6 are in development (abemaciclib) and some have been FDA approved (palbociclib and ribociclib) as cancer therapeutics (5–7). Emerging data suggest that the single-agent efficacy of these CDK4/6 inhibitors is limited and that they are best used in combination with drugs like aromatase inhibitors (8). Chemotherapeutic agents like FOLFOX, FOLFIRI, or FOLFOXIRI combinations, etoposide, taxol, or platinum are still cornerstone first-line therapy options for some solid tumors. However, these cytotoxic agents work best in actively dividing cells, and combinations of CDK4/6 inhibitors with cytotoxic agents has shown little success clinically. Hence, there is need to develop alternate therapeutics that can target the p16–CDK4/6–Rb–E2F pathway and synergize with frontline therapies.

miRNAs are 18 to 22 nucleotide small noncoding RNAs that can inhibit translation and/or affect mRNA stability by binding to the 3' untranslated region (UTR) of target genes (9, 10). Studies in different tumor models have shown that miRNAs can either be oncogenic, as in the case of miR-155, miR-21, and the miR17–92 cluster, or tumor suppressive (miR-34a, let-7 family, and miR-143; refs. 11, 12). Tumor suppressive miRNAs are generally dysregulated by multiple mechanisms including methylation of promoter sites, (13) deletion or loss of expression (12, 14). Reinforcement of tumor suppressive miRNAs known as “miRNA mimics” has become an attractive therapeutic strategy that is still being translated to the clinic. Many such miRNA mimics are currently in clinical or preclinical development for cancer and other nononcological applications (15). A disadvantage of miRNA mimics is the lack of specificity as compared with targeted therapies such as the CDK inhibitor palbociclib. However, the ability of miRNA mimics to modulate different tiers of genes within a signaling network is an advantage, especially in scenarios where the targeted therapy has shown limited success.

Contemporary CDK4/6 inhibitors work best in a subset of breast cancers (8), which must express proficient retinoblastoma (Rb) protein for therapeutic benefit. Although clinical trials are under way, CDK4/6 inhibitors have not yet shown substantial...
success in colorectal cancer. Because of this, there is an urgent need to identify alternative or complementary strategies to current CDK4/6 inhibitors. To date, no miRNA mimics have been identified, specifically targeting the CDK4/6 oncogenic network in colorectal cancer. Identification of miRNAs targeting this network independent of Rb status and understanding their regulation would provide an important complementary avenue for targeted therapy. Further, these miRNAs may serve as biomarkers of disease progression and help predict response.

In this study, we identified novel miRNAs that target CDK4/6 with potential for further therapeutic development. We discovered a novel, uncharacterized family of four miRNAs whose expression is lost in colorectal cancer. We characterized the key targets of these miRNAs as CDK4, CDK6, FOXM1, XIAP, and BCLXL, proteins with associated antiproliferative and proapoptotic effects in colorectal cancer cell lines. We also performed combination studies of both miR-6883-5p and miR-149* with irinotecan and 5-fluorouracil, used in the first-line setting to treat colorectal cancer patients in the clinic. Both miRNAs show promising synergy with either irinotecan and 5-fluorouracil. We also provide mechanistic insight and show that downregulation of both the CDK4/6-FOXM1 axis and antiapoptotic proteins BCLXL and XIAP is involved in the miRNA–drug combinations. FOXM1 is a member of the forkhead box (FOX) transcription factors, expressed exclusively in proliferating cells (16, 17). Several reports have shown that FOXM1 expression is upregulated in different tumor types, including colorectal cancer (18–22). Overexpression of FOXM1 is associated with increased cell proliferation (18), EMT (23–26), and chemoresistance (27), suggesting a protumorigenic role of the transcription factor. FOXM1 is considered a master regulator of the cell cycle, crucial for entry of cells into both the S and G2–M cell-cycle phases (28, 29). Recently, it has been shown that CDK4/6 initiate the phosphorylation and activation of FOXM1 in G1 phase of the cell cycle (30, 31). BCLXL and XIAP have been associated with poor prognosis in colorectal cancer and are important oncogenic targets in different solid tumors as well (32–35). BCLXL, in particular, has been associated with colorectal cancer tumorigenesis and progression (34).

Thus, miR-6883-5p and miR-149* mediated targeting of CDK4, CDK6, FOXM1, XIAP, and BCLXL, proteins identified in our study has therapeutic implications for colorectal cancer tumors.

Materials and Methods

Cell culture and reagents

All colorectal, pancreatic, and melanoma cell lines were obtained from ATCC and maintained in the recommended media. Cells were routinely verified as free of mycoplasma contamination. miRNA mimics for hsa-miR-6883-5p, hsa-miR-149*, and hsa-miR-206 (HMI2616, HMI0241, and HMI0364) were purchased from Sigma-Aldrich. siRNA for CDK4 and CDK6 (sc-29261 and sc-29264) were purchased from Santa Cruz Bio-technology. The CDK4-Luciferase construct was obtained from Origene Technologies.

Transfection of miRNA mimics, siRNA, and plasmid constructs

All miRNA mimic and siRNA transfections were performed by reverse transfection using Lipofectamine RNAiMAX (Life Technologies). siRNA (80 nmol/L) was used in all experiments for HT-29, RKO, and SW-480 cell lines. siRNA (40 nmol/L) was used for HCT-116 cells. miRNA mimics were transfected at concentrations of either 25 nmol/L or 50 or 100 nmol/L, as indicated in respective assays. CDK4-Luciferase vector was transfected in HCT-116 cells using Lipofectamine 3000 (Life Technologies) and stable cells were selected using G418 antibiotic (500 μg/mL).

Luciferase assays

CDK4-Luciferase containing stable HCT-116 cells was transfected with either scramble duplex or 50 nmol/L of miRNA mimics. Luciferase signal was measured at 48 hours after transfection and relative luciferase units (RLU) were calculated by normalizing luciferase signal per μg of protein per assay well. All transfections were performed in triplicate and reported as RLU units ± SEM.

Cell proliferation assays

A total of 5K–10K cells were transfected with either scramble duplex or miRNA to a net concentration of 50 nmol/L and plated in a 96-well plate. Cell viability was measured 72 hours after transfection using CellTiter-Glo Luminescent Cell Viability Assay (Promega). The percent-cell viability was calculated by normalizing the luminescence signal to scramble duplex wells. All transfections were performed in triplicates and reported as % viability ± SEM, compared with scramble.

Cell-cycle analysis

All cell lines were transfected with either scramble duplex or miRNA mimic. At 72 hours after transfection, both floating and adherent cells were collected and fixed in 70% ethanol, followed by RNase A treatment and PI staining. Cell death (sub-G1) was quantified by propidium iodide (PI) staining and flow cytometry. Flo-Jo analysis was performed to quantify the distribution of cells in G1, S, and G2–M phases of the cell cycle under different transfection conditions.

Colony formation assays

A total of 0.1 × 104 were transfected with either scramble duplex or miRNA mimics to net concentration of 50 nmol/L (HT-29 and HCT-116) or 100 nmol/L (RKO and SW-480) for 72 hours. At 72 hours, transfected cells were harvested, and 500 cells per treatment group were plated in triplicate in 6-well plates for colony formation. Colonies were stained with 0.25% crystal violet on day 14, imaged, counted and reported as number of colonies ± SEM.

Quantitative RT-PCR

Total RNA, which includes miRNA, was isolated using the Quick-RNA MiniPrep Kit (Zymo Research). Total RNA (1 μg) from each sample was subjected to cDNA synthesis using SuperScript III Reverse Transcriptase Kit (Life Technologies), for detection of CDK4, CDK6, FOXM1, FOXM1 target genes, and housekeeping genes. For detection of miRNAs, 0.5 μg of total RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). The relative expression of the reported genes and miRNAs was determined using real-time PCR performed on an Applied Biosystems 7900HT Fast Real-Time PCR system. GAPDH and RNU6B were used as the endogenous controls for mRNA and miRNA samples, respectively. Each cDNA sample was amplified using Power SYBR Green (Applied Biosystems) and miRNA components were quantified using TaqMan.
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Universal Master Mix II, no UNG (Applied Biosystems). The primers used are listed in Supplementary Table S1. TaqMan miRNA assays were purchased from Applied Biosystems and used as per the manufacturer’s instructions. ΔΔCt analysis was performed to calculate the fold change for each gene.

Western blot

Western blotting was performed as described previously. The following antibodies were used: CDK4 (Santa Cruz Biotechnology, sc-260), CDK6 [Cell Signaling Technologies (CST), D4S85], CDK1 (Santa Cruz Biotechnology, sc-54), cyclin-D1 (CST, 92G2), p-Rb (S795; CST, 9301S), Total Rb (CST, 9309L), BCL2 (CST, 27645), PARP (CST, 9542), p53-D01 (Santa Cruz Biotechnology, sc-126), p21 (CalBiochem, OP64), β-actin (Sigma, A5441), XIAP (CST, 2428), FOXM1 (CST, D12D5), p-FOXM1 (CST, 14170S), and cyclin B1 (Santa Cruz Biotechnology, sc-245).

Statistical analysis

Data are presented as the mean ± standard error of the mean from at least three replicates. The Student two-tailed t-test in GraphPad Prism was used for pairwise analysis. Statistically significant changes (*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001) are indicated.

Results

CDK4 and CDK6 can be regulated by a family of miRNAs in colorectal cancer

Overexpression of oncogenes (CDKs, cyclins, and E2Fs) or loss of tumor suppressors (p16, Rb) in the CDK4/6-Rb-E2F pathway is usually mutually exclusive and tumor-type specific. We evaluated the expression levels of CDK4 and CDK6 in colorectal cancer using RNA-seq data from The Cancer Genome Atlas (TCGA; Fig. 1A and Supplementary Fig. S1A and S1B). We found that both CDK4 and CDK6 expression is significantly higher in the tumor samples than in normal samples (P = 2.2e−16 and P = 1.594e−08, respectively). There was no significant change in the RNA levels of tumor suppressor p16 or Rb. The increased expression of CDK4 and CDK6 protein and RNA in colorectal cancer is in agreement with previous reports indicating increased protein expression as measured by immunohistochemistry (36, 37).

We wished to elucidate the molecular basis for the increased expression of CDK4 and CDK6 in colorectal cancer, focusing specifically on the role of micro-RNAs (miRNAs). Previous reports have suggested that conserved miRNAs such as miR-124-3p, and miR-15-5p are capable of targeting CDK4 and are dysregulated in melanoma and other tumor types. We used an in silico approach using TargetScan to determine if CDK4 and CDK6 mRNA have consensus miRNA binding sites in their 3’UTR. Our goal was to focus on novel and uncharacterized miRNAs that have been implicated in colorectal cancer pathogenesis. Based on a combination of TCGA data analysis and TargetScan, we identified a new family of miRNAs comprising miRs 6883-5p, 149*, 6785-5p, and 4728-5p. Sequence alignment showed identical base-pairing in the seed sequence [GGGAGGG] (binding region of miRNA–mRNA) for all four miRNAs (Supplementary Fig. S2A). Each of these miRNAs was predicted to target the 3’UTR of both CDK4 and CDK6 with 8mer and 7mer-1A binding sites, respectively (Supplementary Tables S2 and S3). We focused on miRs 6883-5p and 149* because of their novelty and relevance to colorectal cancer. As seen in Fig. 1B(left), expression of miR-149* is strikingly reduced in 11 colorectal cancer patient samples as compared with normal tissue (P = 0.0049). Loss of miR-149* is also correlated with advanced tumor stage (unpublished observations). Of note, each of the 11 colorectal cancer tumors exhibited a significant increase in CDK4 expression, with no significant alteration in other components of the CDK4/6-Rb pathway (Fig. 1B, right). While miR-6883-5p was not represented in the TCGA dataset, it should be noted that miR-6883-5p is an intronic miRNA transcribed from the gene PER1, a tumor suppressor and regulator of the cell cycle (38, 39). Importantly, PER1 expression is significantly reduced in colorectal cancer samples used in Fig. 1A (Fig. 1C and Supplementary Fig. S1C). Thus, our in silico analysis reveals that the induction of CDK4 and CDK6 in colorectal cancer is associated with the loss of two miRNAs (6883-5p and 149*) capable of binding their 3’ UTR.

miR-6883-5p and miR-149* repress expression of CDK4 and CDK6

To determine if miR-6883-5p and miR-149* regulate CDK4/6 expression, we expressed each miR in a panel of colorectal cancer cell lines: HCT116 (p53+/+), RKO (p53+/+), HT-29 (p53 R273H), and SW480 (p53 R273H/P309S). All the colorectal cancer cell lines used express Rb and p16 except SW480, which is p16-deficient (Supplementary Fig. S2B). We transfected each of the cell lines individually with miR-6883-5p or miR-149* and then examined the expression of CDK4 and CDK6 protein and RNA (Fig. 2A–D). Expression of miR-6883-5p repressed CDK4 and CDK6 at both the protein and RNA levels (Fig. 2A–D). By contrast, miR-149* did not alter CDK4 expression but potently repressed CDK6 (Fig. 2B–D). miR-6883-5p, but not miR-149*, was able to bind to the CDK6 3’UTR and repress the expression of the luciferase reporter gene in the pMirtarget-CDK4-Luciferase vector (Fig. 2E). Of note, miR-6883-5p was able to repress luciferase activity more profoundly than miR-206, which has previously been reported to target CDK4 (40). Together, these data indicate that miR-6883-5p and miR-149* regulate the expression of CDK4 and CDK6 in colorectal cancer and may play an important role in disease pathogenesis.

Expression of miR-6883-5p and miR-149* results in G0–G1 arrest and cell death

Given their ability to target CDK4/6, we investigated the functional consequences of expressing miRs-6883-5p and 149* compared with miR-206 (a known CDK4 targeting miR) in the panel of colorectal cancer cell lines. We rationalized that because the miRNAs target proteins in the cell cycle, they could affect cell proliferation, both short term and long term. All three miRNAs had comparable short-term antiproliferative effects, reducing cell viability by 30% to 75% in the panel of colorectal cancer cell lines (Fig. 3A). There was a greater than 50% to 70% inhibition of the long-term proliferation of these cells as observed in a colony formation assay in all four cell lines (Fig. 3B and C). We also checked for antiproliferative effects of miR-6883-5p and miR-149* in three melanoma cell lines. Although both miRs reduced protein levels of CDK4 and CDK6 (Supplementary Fig. S3A), there was no substantial effect on short-term proliferation of the
melanoma lines tested (Supplementary Fig. S3B). We next investigated the effects of the miRNAs on cell-cycle markers. We expected the inhibition of CDK4/6 would lead to G0–G1 arrest in all the cell lines. As seen in Fig. 3D, in all four cell lines, miR-6883-5p and miR-149 did indeed reduce levels of phosphorylated Rb (S795), indicating G0–G1 arrest and inhibition of CDK4/6 activity. We further confirmed our findings through propidium iodide staining and cell-cycle profile analysis. As seen in Fig. 3E(left), both RKO and HT-29 cells showed G0–G1 arrest following transfection with each of the miRNAs and a fraction of cells underwent cell death. However, in HCT116, expression of all miRNAs led to apoptosis, indicated by 30% to 50% cells in sub-G1 phase (Fig. 3E, right). This result was further supported with the observation of PARP cleavage by Western blot analysis indicating apoptosis (Fig. 3F). In HCT116 cells, all three miRNAs promoted increased levels of apoptosis; but in RKO and SW480 cells, 6883-5p was the most potent at inducing apoptosis, measured by PARP cleavage (Fig. 3F). No change in the p53 levels was noted, indicating that apoptosis was likely not p53-dependent in the p53+/+ cells. Interestingly, miR-6883-5p downregulated XIAP and BCLXL, which could in part explain the induction of apoptosis by miR-6883-5p. While XIAP is a predicted target of the family of miRNAs, BCLXL is not.
transfected in colorectal cancer cell lines (50 nmol/L, 72 hours, colorectal cancer cell lines transfected with 50 nmol/L of miR-149

colorectal cancer cell lines transfected with 50 nmol/L of miR-6883-5p or scrambled duplex (SCR) for 72 hours.

miR-6883-5p and miR-149

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Figure 2.

miR-6883-5p and miR-149* negatively regulate expression of CDK4 and CDK6 in colorectal cancer cell lines. A, CDK4 and CDK6 protein levels in a panel of colorectal cancer cell lines transfected with 50 nmol/L of miR-149* or scrambled duplex (SCR) for 72 hours. B, CDK4 and CDK6 protein levels in a panel of colorectal cancer cell lines transfected with 50 nmol/L of indicated miRNA mimics transfected in colorectal cancer cell lines (50 nmol/L, 72 hours, n = 3). *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. C, qRT-PCR for CDK4 in SCR or miRNA mimics transfected in colorectal cancer cell lines (50 nmol/L, 72 hours, n = 3). ***, P ≤ 0.001 relative to SCR expression. E, HCT116 cells stably transfected with CDK4-Luciferase construct were transfected with either SCR or 50 nmol/L of indicated miRNA mimics for 48 hours. Measured luciferase activities were normalized to per ug of protein for indicated samples and reported as RLU ± SEM (n = 3). *, P value relative SCR RLU. n.s., nonsignificant.

However, both BCLXL and XIAP contributed to the proapoptotic effects of the miR-6883-5p.

Silencing of CDK4 and CDK6 phenocopies the effects of miR-6883-5p and miR-149* in colorectal cancer cell lines

To determine if the phenotype associated with miR-6883-5p and miR-149* could be attributed to the direct targeting of CDK4/6 alone, we silenced the expression of CDK4/6 and measured the functional consequences. siRNA knockdown of CDK4 and CDK6 had similar effects on short-term proliferation of colorectal cancer cell lines as overexpression of the selected miRNAs (Fig. 4A). However, silencing the expression of CDK4/6 was less potent (10%–40%) in preventing long-term proliferation of colorectal cancer cell lines, especially in RKO and HT-29 cells (Fig. 4B and C). This indicates that targeting of CDK4/6 by miR-6883-5p and miR-149* can only in part explain the antiproliferative effects of these miRNAs. As expected, knockdown of CDK4 and CDK6 siRNAs arrested cells in G0–G1 phase of the cell cycle (Fig. 4D and E). However, unlike the miRNAs, knockdown of CDK4 alone led to cell death in HCT116 and SW480 cells. Because of the leakiness of the CDK4 siRNA, we can conclude that knockdown of both CDK4 and CDK6 is more potent and was needed to induce apoptosis in colorectal cancer cell lines compared with either gene alone. Thus, the dual targeting of CDK4 and CDK6 by miRs 6883-5p and 149* has potential benefits in colorectal cancer.

miR-6883-5p and miR-149* synergize with FDA-approved therapeutics for colorectal cancer

We evaluated the combinatorial potential of miR-6883-5p and miR-149* with colorectal cancer first-line chemotherapeutics irinotecan and 5-fluorouracil. As seen in Fig. 5A and B, in all four cell lines both miR-6883-5p and miR-149* synergized with irinotecan to reduce cell viability. Furthermore, the irinotecan-miRNA combination engaged the intrinsic pathway of cell death as measured by increased levels of cleaved caspase-9 and decreased levels of BCLXL and XIAP (Fig. 5C and D). Both miR-6883-5p and miR-149* increased the sensitivity of p53-mutant cell lines HT-29 and SW480 to 5-fluorouracil (Fig. 6A and B). Cells treated with miR-149* alone showed p53-independent cell-cycle arrest, as seen by induction of p21 levels (Fig. 6C and D). The combination with 5-fluorouracil led to apoptosis indicated by increased levels of cleaved PARP (Fig. 6C and D). This suggests that combinations of both miR-6883-5p and miR-149* with irinotecan/5-fluorouracil have translational potential in colorectal cancer.

miR-6883-5p and miR-149* combinations inhibit CDK4/6-FOXM1 signaling in colorectal cancer cell lines

Recently, it has been shown that CDK4/6 initiate the phosphorylation and activation of FOXM1 in G1 phase of the cell cycle (30, 31) and thereby promote downstream signaling. We
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hypothesized that targeting of CDK4/6 by miR-6883-5p and miR-149* could downregulate the CDK4/6-FOXM1 signaling pathway, particularly in combination with irinotecan and 5-fluorouracil. miR-6883-5p alone reduced levels of both p-FOX1 and FOXM1 and its transcriptional target cyclin B1 in HT-29 cells (Fig. 7A). Combination with irinotecan and 5-fluorouracil further exacerbated this signaling in HT-29 cells (Fig. 7A). Although miR-6883-5p had no effect on FOXM1 protein levels on its own in HCT116 cells, the combinations downregulated the CDK4/6-FOXM1 pathway (Fig. 7B). For HT-29 cells, we also investigated a subset of FOXM1 transcriptional targets required for progression to S or G2-M phases of the cell cycle.

Figure 3.
Restoring expression of miR-6883-5p and miR-149* results in G1 arrest and cell death in colorectal cancer cell lines. A, A panel of colorectal cancer cell lines was transfected with either 50 nmol/L SCR or 50 nmol/L of indicated miRNA mimics. Effects on cell viability were measured at 72 hours after transfection using the CellTiter-Glo assay. B, The effects of the miRNA mimics on long-term cell proliferation of colorectal cancer cell lines were assessed by colony formation assays done in 6-well plates. Cells were transfected with 100 nmol/L of SCR or indicated miRNA mimic. After 72 hours, 500 cells were seeded per well in triplicate for each condition and stained with crystal violet on day 14. Representative images of cells stained with crystal violet are shown. C, Relative colony number (n = 3) is represented graphically. All four colorectal cancer cell lines were transfected with either 100 nmol/L SCR or indicated miRNA mimics. Effects on cell-cycle markers (D) and markers of apoptosis (F) were evaluated by Western blot 72 hours after transfection. Representative Western blots are shown (n = 3). E, Cell-cycle profiles and apoptotic cells were assessed in three colorectal cancer cell lines by transfecting with either SCR or 50 nmol/L (HCT116 and HT-29) or 100 nmol/L (RKO) miRNA mimics. At 72 hours after transfection, cells were fixed, stained with PI, and analyzed by FACS. Representative results of changes G1 (RKO and HT-29) and sub-G1 (HCT-116) phases of the cell cycle are graphically represented (n = 3). *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

www.aacrjournals.org  Cancer Res; 77(24) December 15, 2017 6907

Published OnlineFirst October 23, 2017; DOI: 10.1158/0008-5472.CAN-17-1767

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cycle. qRT-PCR results indicate that miR-6883-5p downregulated expression of FOXM1 transcript by 40% and the corresponding target genes by 50% to 85% (Fig. 7C and D). Combination with both irinotecan and 5-fluorouracil further synergized with miR-6883-5p in downregulating the indicated genes (Fig. 7C and D).

miR-149 did not have significant single agent impact on any of the tested genes except CDC25B, which was reduced to 50% expression (Fig. 7C and D). However, the combination of miR-149 and irinotecan/5-fluorouracil still reduced the CDK4/6-FOXM1 signature in HT-29 cells (Fig. 7C and D).

Figure 4.
Silencing CDK4 and CDK6 phenocopies the effects on miRNA mimics. A, All four colorectal cancer cell lines were transfected with either SCR or 80 nmol/L of CDK4 or CDK6 siRNA. Effects of short-term cell proliferation were measured 72 hours after transfection using the CellTiter-Glo assay. B, Long-term effects on cell proliferation on silencing either CDK4 or CDK6 were assessed by colony formation assay done in 6-well plates. All four cell lines were transfected with 80 nmol/L CDK4 or CDK6 siRNA. At 72 hours after transfection, 500 cells were seeded per well in triplicate and stained with crystal violet on day 14. Representative images of cells stained with crystal violet are shown. C, Relative colony number (n = 3) is represented graphically. D, All four colorectal cancer cell lines were transfected with 80 nmol/L CDK4 or CDK6 siRNA. Effects on markers of the cell cycle and apoptosis were evaluated by Western blot 72 hours posttransfection. Representative Western blots are shown (n = 3). E, Cell-cycle profiles and apoptotic cells were assessed in three colorectal cancer cell lines by transfecting with either SCR or 40 nmol/L (HCT116) or 80 nmol/L (HT-29 and RKO) siRNA of CDK4 and CDK6. At 72 hours after transfection, cells were fixed, stained with PI, and analyzed by FACS. Representative results of changes G1 and sub-G1 phases of the cell cycle are graphically represented (n = 3). **, P ≤ 0.01.
In summary, our data demonstrate that miR (6883-5p and 149*) mediated targeting of cell-cycle genes CDK4/6 and anti-apoptotic genes XIAP and BCLXL has antiproliferative and proapoptotic effects in colorectal cancer cell lines (Fig. 7E). miR-6883-5p in particular is a strong candidate for combination with current colorectal cancer chemotherapy. Combinations with miR-6883-5p and miR-149* have an advantage of promoting cancer cells to apoptosis compared to growth arrest alone as observed with CDK4/6 inhibitors (Supplementary Fig. S4). We have independently reproduced the combination results observed with CDK4/6 inhibitor palbociclib and irinotecan (Supplementary Fig. S4A and S4B) or 5-florouracil (Supplementary Fig. S4C) in the recent paper by Zhang and colleagues (41). As proof of concept, we also transiently knocked down CDK4 using siRNA in HT-29 and HCT-116 cell lines, combined treatment with irinotecan and observed reduction in short-term proliferation with the combination compared siRNA or irinotecan alone (Supplementary Fig. S5A). As expected, CDK4 siRNA knockdown alone reduced levels of p-FOXM1 and its downstream target cyclin B1 (Supplementary Fig. S5B and S5C). Similar to the miR-6883-5p and miR-149* combination results, CDK4 siRNA + irinotecan induced apoptosis as assessed by PARP and cleaved caspase-3 (CC3) data (Supplementary Fig. S5C).

**Discussion**

Dysregulation of genes in the CDK4/6-Rb cell-cycle pathway is a hallmark of cancer (42). Cancer cells subvert cell-cycle checkpoints for continued growth and proliferation. Overcoming
In the present study, we used a combination of in silico prediction algorithms and TCGA dataset analysis to identify tumor suppressor miRNAs that can translate to single agent/combinatorial therapies for colorectal cancer. Previous studies described miRNAs regulating CDK4 expression in melanoma and non–small cell lung carcinoma (40, 45–47). However, there has been no report of tumor suppressor miRs identified in colorectal cancer and/or studies evaluating their use as therapeutics.

We identified a novel family of miRNAs, whose expression is lost in colorectal cancer. We have characterized the primary gene network—G1–S phase of the cell cycle—and selected apoptosis markers that are regulated by two of the four miRNAs in the family. These critical gene targets mediating the anti–proliferative and proapoptotic effects of miR-6883-5p and miR-149* include CDK4, CDK6, FOXM1, XIAP, and BCL XL. FOXM1 is a master regulator of the cell cycle and has recently been identified to be phosphorylated and activated by CDK4/6 (31). FOXM1 has also been linked to resistance to different chemotherapies like cisplatin, 5-fluorouracil (27) and is an important oncogenic target in preclinical development. Further, FOXM1 is a crucial effector of mutant p53 gain of function (GOF) toward cell-cycle progression (48). Downregulation of
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miR-6883-5p and miR-149* combinations abrogate CDK4/6-FOXM1 signaling in colorectal cancer cell lines. HT-29 and HCT116 cell lines were transfected with either 50 nmoL/L of SCR or the indicated miRNA mimics. Sixteen hours after transfection, irinotecan or 5-fluorouracil were added at the indicated doses. Expression of FOXM1 and target genes with miRNA alone or combination was assessed by Western blot and qRT-PCR.

Representative Western blots are for HT-29 and HCT116 cells shown in A and B, respectively. Representative qRT-PCR results for FOXM1 and panel of target genes in HT-29 cells combined with irinotecan (C) or 5-fluorouracil (D), respectively. E. Model depicting described role of miR-6883-5p and miR-149* in colorectal cancer cell lines.

the CDK4/6-FOXM1 axis and antiapoptotic proteins BCLXL and XIAP by our miRNA combinations thus has wide clinical implications. The investigated miRNA-drug combinations are particularly relevant in mutant p53-expressing tumors, which have varied GOFs in the cell cycle. Our study also suggests the use of miRNAs as potential adjuvant therapeutics.

miR-6883-5p is an intronic miRNA transcribed as part of the PERI gene, a tumor suppressor gene whose loss is correlated
with liver metastasis in colorectal cancer (38). PER1 also interacts with DNA-damage response proteins ATM, CHK2, and cell-cycle regulators Wee1, cyclin B1, and Cdc2 to contribute to apoptosis or cell-cycle arrest in colorectal cancer (39). By evaluating the role of miR-6883-5p, which is transcribed as part of the PER1 gene, we add the link between the circadian clock genes and their contribution to cell division and response to DNA damage.

miR-149 has previously been shown to target AKT1 and E2F1 and to contribute to apoptosis in neuroblastoma cell lines (49). Loss of miR-149 has been attributed to methylation and further linked to increased expression of its gene target PI3K (50). Our findings of miR-149 targeting CDK6 and XIAP bring to light a novel role of miR-149, along with finding a family of miRNAs with similar functions. Also, the tumor suppressor roles of miR-149 and its loss in colorectal cancer is consistent with previous findings. In melanoma, miR-149 has been described as an oncogenic miR (51). miR-149-5p has been better characterized than miR-149-3p for its target–gene network interactions. Overall studies suggest that miR-149-5p is a tumor suppressor and inhibits genes involved in the epithelial-to-mesenchymal (EMT) transition and chemoresistance networks (52–55). Thus, our study provides further evidence for the role of miR-149 as a tumor suppressive miRNA.

In summary, we have identified two novel miRNAs, miR-6883-5p, and miR-149 as direct negative regulators of CDK4 and CDK6. Restoring the expression of miR-6883-5p and miR-149 in cancer cells shows promoting antiproliferative and proapoptotic effects when used alone or in combination. Thus, this study confirms miRNA mimics as potential adjuvant therapy for colorectal cancer, providing a promising avenue of investigation to supplement the small molecule inhibitors and chemotherapeutics currently in use.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The work was presented in part at the 108th Annual Meeting of the American Association for Cancer Research (April 2017). W.S. El-Deiry is an American Cancer Society Research Professor.

Grant Support
This work was supported by grant RP-14-233-07 from the American Cancer Society to W.S. El-Deiry.

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Received June 16, 2017; revised August 22, 2017; accepted October 3, 2017; published OnlineFirst October 23, 2017.

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miR-6883 Family miRNAs Target CDK4/6 to Induce G₁ Phase Cell-Cycle Arrest in Colon Cancer Cells

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doi:10.1158/0008-5472.CAN-17-1767

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