Original Article
MicroRNA-145 induces cell cycle arrest in G1 phase by directly targeting KLF5 in colon cancer

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Abstract: Colorectal cancer (CRC) is one of the leading causes of cancer-associated mortality worldwide. There is increasing evidence that microRNAs (miRNAs) are involved in development and progression of colorectal carcinoma. miR-145 is a tumor suppressor and has been confirmed as a negative regulator of colorectal cancer. Although multiple target genes have been identified for miR-145, the molecular mechanism by which it inhibits colon cancer is still unknown. Here, we used bioinformatic analysis and a reporter assay to identify KLF5, a putative oncoprotein in colon cancer, as a potential direct target of miR-145. Ectopic expression of mir-145 robustly decreased the expression of KLF5 proteins in in CRC tissues and cell lines. Furthermore, overexpression of miR-145 induced cell cycle arrest in the G1 phase colon cancer cells. This phenomenon was consistent with siRNA-mediated down-regulation of KLF5 in colon cancer cells. Additionally, we performed a rescue experiment to demonstrate that the reexpression of KLF5 partially attenuated cell cycle arrest in the G1 phase in miR-145 transfected colon cancer cells. Real-time PCR analysis of colon cancer cell lines showed an inverse relationship between expressions of miR-145 and KLF5, providing a plausible explanation that high level KLF5 activation in colon carcinoma may be due to the reduction of miR-145. Our study demonstrated for the first time that miR-145 induces cell cycle arrest in the G1 phase by directly targeting KLF5 in colon cancer cells; further emphasizing that miR-145 may be a promising candidate in colorectal cancer therapeutics.

Keywords: KLF5, miR-145, colon cancer, cell cycle arrest

Introduction

Colorectal cancer is one of the major causes of cancer mortality and morbidity worldwide. In the past two decades, significant progress has been made in understanding the molecular mechanism responsible for the development and progression of colorectal cancer [1]. With the development of advanced genomic technology, the global genomic landscape and critical gene mutations in colorectal cancer have been identified [2-4]. Based on a several genetic models, it has been proposed that colorectal cancer develops from cumulative stepwise genetic changes in key genes with important functions such as the control of cell proliferation, apoptosis and invasion [5]. Although abnormally high activation of multiple signaling pathways such as the WNT/APC/β-catenin pathway and RAS/RAF pathway have been dem-

onstrated to be required for the development and progression of colorectal cancer, previous analyses of the genetic modifications have not clearly defined the underlying cause of this type of tumor [1, 6]. In recent years, functional studies have suggested that post-transcriptional regulation mediated by miRNAs can coordinately target multiple genes to promote the development of colorectal cancer [7]. Given the fundamental biological processes regulated by miRNAs and the knowledge that many of these processes are altered in colon cancers, it follows that we should investigate whether miRNAs play a critical role in tumorigenesis of colorectal tissues and if it can be developed as a cancer therapy [8].

MicroRNAs are endogenous small non-coding RNA molecules capable of silencing protein coding genes by binding complementary se-

quences in 3'-untranslated regions (3'-UTR) of target mRNAs to induce their degradation or translational repression [9]. An increasing body of evidence has suggested that the aberrant expression of miRNAs may lead to the initiation and progression of malignant diseases. Based on their expression patterns in cancer tissues, miRNAs can be classified as oncomirs and tumor suppressive miRNAs. The high level expression of oncomirs or the low level expression of tumor suppressive miRNAs is frequently associated with poor outcomes for clinical patients, indicating that the forced expression of tumor suppressive miRNAs or the decreased expression of endogenous oncomirs could be a promising strategy for cancer therapeutics [10]. The focus of miRNA-based cancer therapeutics is the choice of appropriate tumor suppressive miRNAs to introduce selectively into cancer cells [11]. The decreased expression of miR-145 has been detected in multiple types of cancer, including colon cancer, breast cancer, bladder cancer, prostate cancer, ovarian cancer, gastric cancer and even B-cell malignancies [12]. Given that miR-145 is located in a fragile site of the genome frequently deleted in cancer tissues, it is reasonable to define miR-145 as a tumor suppressive miRNA [13].

The transcriptional analysis of p53 directly binding to a specific DNA site on the promoter region of miR-145 further confirmed that this miRNA plays a pivotal role in p53-mediated tumor suppression [14]. Supporting evidence indicates that miR-145 inhibits proliferation, migration, and invasion and induces apoptosis of cancer cells as previously reviewed [12]. Although various putative target genes of miR-145 have been experimentally identified to explain its effect on the migration and invasion of cancer cells, the molecular mechanism of the inhibitory effect of miR-145 on the growth of cancer cells has not been elucidated clearly [15-18]. Although previous studies have shown that c-myc and IRS-1 may be the target genes of miRNAs in colon cancer cells, the specificity of the interaction was not confirmed by mutational analysis of the seed sequences in each of these studies [14, 19, 20].

In this study, by loss-of-function and gain-of-function experiments, we determined that KLF5 is a significant target of miR-145, contributing to the G1/S arrest in colon cancer cells.

KLF5, also called intestinal-enriched Krüppel-like factor IKLF, is a member of the KLF family. It functions as a transcriptional factor in a cell-type or cell-context dependent manner [21]. It is expressed predominantly in the intestinal tract and is concentrated at the base of the crypt epithelium where active cell division occurs, indicating that it may be involved in the accelerated proliferation of specific tissue-derived cells [22]. KLF5 can enhance colony formation in non-transformed cells derived from both the ileum and colon *in vitro* [23]. In recent years, it has been reported that KLF5 functions not only in small intestine and colon tissues, but also in transformed epithelium [24]. Further study showed that during the progression of intestinal cancer, KLF5 may have an oncogenic effect in non-transformed cells and even accelerate tumor-derived/Ras-transformed cells, suggesting that KLF5 plays a critical role in the development and progression of intestinal and colorectal tissues [25]. Although there is increasing evidence indicating that KLF5 has robust functions as a factor in carcinogenesis by positively regulating cellular proliferation, how KLF5 itself is regulated is still unclear. Using a high throughput screening system, the activation of MAPK and PKB signaling pathways were shown to be involved in the regulation of KLF5 at the transcriptional level [26]. However, we describe here for the first time a regulatory mechanism of KLF5 expression at the post-transcriptional level. Such in-depth analysis of the molecular mechanism of miRNA-induced cell cycle arrest may help us to develop novel strategies for cancer therapeutics.

Materials and methods

Cell culture and patient tissue samples

Colon cancer cell lines SW480, SW620, HCT-116, HT29, DLD-1, Colo205 and GES-1 immortalized gastric mucosa cells were obtained from the Classical Type Collection (Shanghai, China). Cancer cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 with RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal calf serum (FCS) with 50 U/ml penicillin and 50 µg/ml streptomycin. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma. St. Louis, MO) supplemented with 10% FCS.

Forty one pairs of CRC tissues and matched adjacent normal tissues were obtained from patients in Xijing Hospital from 2012 to 2014. The tissues were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Bioinformatic analysis of miRNA target genes

We search the following miRNA databases for the prediction and analysis of putative target genes of miR-145: http://microrna.sanger.ac.uk/; http://pictar.bio.nyu.edu/ and http://www.targetscan.org/vert_40/.

Constructs and luciferase assay

The construct pCDNA3.1-KLF5 harboring the open reading frame (ORF) of KLF5 was obtained using standard gene cloning protocols. The primers used were the following: forward 5'-ctcgagatggctacaagggtgctgag-3'; reverse 5'-ggatcctcagttctggtgcctcttcat-3'. The full DNA sequence of KLF5 ORF was confirmed by automatic DNA sequencing, and the ORF fragment was inserted into the pCDNA3.1 vector. The wild-type and mutant miR-145 binding sequences at the 3'-UTR of KLF5 and its complementary oligos were synthesized, denatured/renatured, and then cloned into the *PstI/Eco*RI sites of a luciferase gene in the pGL3 luciferase vector (Promega). The sense sequences were 5'- aattcgcaagaaaaccacaactaaaactggaaatgtatattttgtatatttgagctgca-3' (wild-type) and 5'-aattcgcaagaaaaccacaactaattccagttatgtatattttgtatatttgagctgca-3' (mutant). The antisense sequences were 5'-gctcaaatatacaaaatatacatttccagtttttagttgtggtttttcttgcg-3' (wild-type) and 5'-gctcaaatatacaaaatatacatttccagtttttagttgtggtttttcttgcg-3' (mutant). The entire 3'-UTR of KLF5 with either the wild-type or a mutant mir-145 binding site (the seed residues) was also cloned into *PstI/Eco*RI sites in the modified pGL3 vector (Promega). Dual luciferase assays were performed as previously described [27]. Briefly, all plasmids and RNA olignucleotides were transfected in HEK293T cells in 48-well plates as indicated. In each well, 10 ng of the renilla luciferase expressing phRL-TK vector (Promega, Madison, USA) was co-transfected for normalizing transfection efficiency. Reporter plasmids pGL-KLF5/UTR or pGL-KLF5/UTR-Mut

(200 ng) together with 10 nM miR-145, miR-145 inhibitor or negative control were co-transfected using Lipofectamine 2000 and Opti-MEM I reduced serum medium (Life Technologies, CA, USA). After 48 h, cells were harvested with 100 μL PLB reagent (Promega, Madison, USA), and 20 μL cell lysates were prepared in reporter lysis buffer (Promega). Firefly luciferase activity was measured for each well using the Dual Luciferase Assay Kit (Promega) with an analytical luminometer (TD-20/20, Turner Designs, Sunnyvale, USA) according to the manufacturer's instruction. Briefly, 10% volume of each cell lysate (20 μL) was added to 100 μL of Luciferase Assay Reagent II. The reaction was stopped with the addition of 100 μL Stop & Glo reagent. Normalized relative luciferase activity (RLA) was calculated using the following formula: RLA = [firefly luciferase activity]/[renilla luciferase activity].

Total RNA preparation and reverse transcription

Total RNA from cell lines were extracted using Trizol (Invitrogen, USA). Concentration and purity of total RNA were measured using a SmartSpec Plus spectrophotometer (Bio-Rad, CA, USA). The ratio of A260:A280 was used to indicate the purity of total RNA. cDNA was generated using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). According to the manufacturer's instructions, 1 μg total RNA, 1 μL miScript Reverse Transcriptase Mix, 4 μL 5 × miScript RT buffer and an appropriate volume RNase-free water were mixed well and incubated for 5 min at 95°C to inactivate the miScript Reverse Transcriptase Mix. All reverse transcriptions and no-template controls were run simultaneously.

Real-time polymerase chain reaction (PCR) analysis

A miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) was used to measure the expression of mature miR-145 in collected colon cancer cell lines after reverse transcription. Real-time PCR was performed using on an ABI 7500 QPCR System (ABI, USA), following the manufacturer's protocol. The 20 μl PCR mixture included 2 μl reverse transcription product, 10 μl 2 ×, QuantiTect SYBR Green PCR Master Mix, 2 μl 10 × miScript Universal Primer, 2 μl 10 × miScript Primer Assay (specific for miR-145,
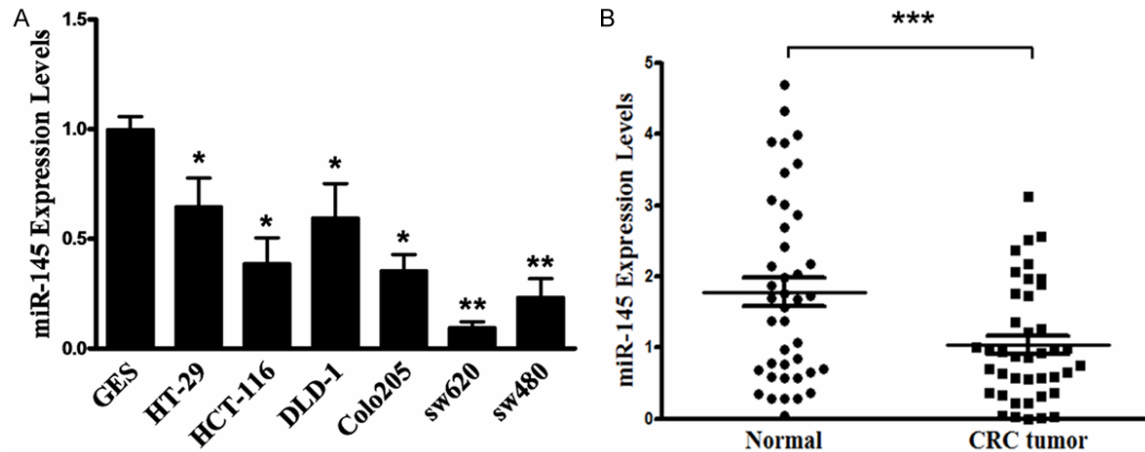
Figure 1. Downregulation of miR-145 expression in CRC tissues and cell lines. A. The expression of miR-145 in 41 pairs of CRC tissues and paired adjacent non-tumorous colorectal tissues were examined by RT-qPCR. B. The expression levels of miR-145 in immortalized gastric mucosa cells (GES) and 6 CRC cell lines. Transcription levels were normalized to U6 expression. Data are presented as means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

purchased from Qiagen, Hilden, Germany), and 4 µl RNase-free water. The reaction mixtures were incubated at 95°C for 15 min, followed by 40 amplification cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. We also quantified U6 small nuclear 2 RNA transcripts using the Hs_RNU6B_2 miScript Primer Assay (Qiagen, Hilden, Germany) for normalizing miR-145 levels.

Synthetic miR-145 mimics, inhibitor and siRNA targeting KLF5

mir-145 mimics, mir-145 inhibitor, KLF5 targeting siRNA (siKLF5-11: cacacacaccucugcuguucc; siKLF5-2: aagcucaccugaggacucaca) and negative control RNA oligonucleotides were all purchased from Genepharma Corporation (Shanghai, China). All RNA oligonucleotides are transiently transfected into cells by using Lipofectamine 2000 according to the manufacturer's procedure (Invitrogen, CA, USA) at a final concentration of 50 nM.

Cell growth assay

Cells were subjected to an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay (Sigma Aldrich) to evaluate the metabolic rate of cells as previously described [27]. Twenty-four hours after seeding (1×10^3 per well), 20 µL (5 mg/ml) of MTT was added to

each well of a 96-well plate for incubation for 4 h at 37°C. The supernatant was removed, and 150 µL of DMSO was added after 10 min of oscillation. The absorbance (OD) was determined using an ELISA detector at the wavelength of 490 nm.

Flow cytometric analysis of cell cycle distribution

One day before transfection, 5×10^5 colon cancer cells were plated into 6-well culture plates. Cells were transfected with miR-145, miR-145 inhibitor or negative control. Forty-eight hours after transfection, cells were harvested and fixed in 70% ethanol at 4°C overnight and then stained with 250 µg/mL propidium iodide (Sigma-Aldrich), 5 µg/mL RNase A (Sigma-Aldrich) and 5 mmol/L EDTA in PBS (pH 7.4) for 30 min. The cell cycle analysis was performed using a FACScan (Beckman Instruments, Fullerton, CA, USA). Each transfection was performed in triplicate.

Western blot analysis

Cells were transfected with miR-145 mimics, miR-145 inhibitor or negative control in six-well plates. After transfection, cells were cultured for 72 h, and the proteins were extracted using RIPA lysis buffer supplemented with a protease inhibitor cocktail (Sigma, USA). Protein samples (30 µg) were resolved by 12% SDS-PAGE and then transferred to nitrocellulose membranes.
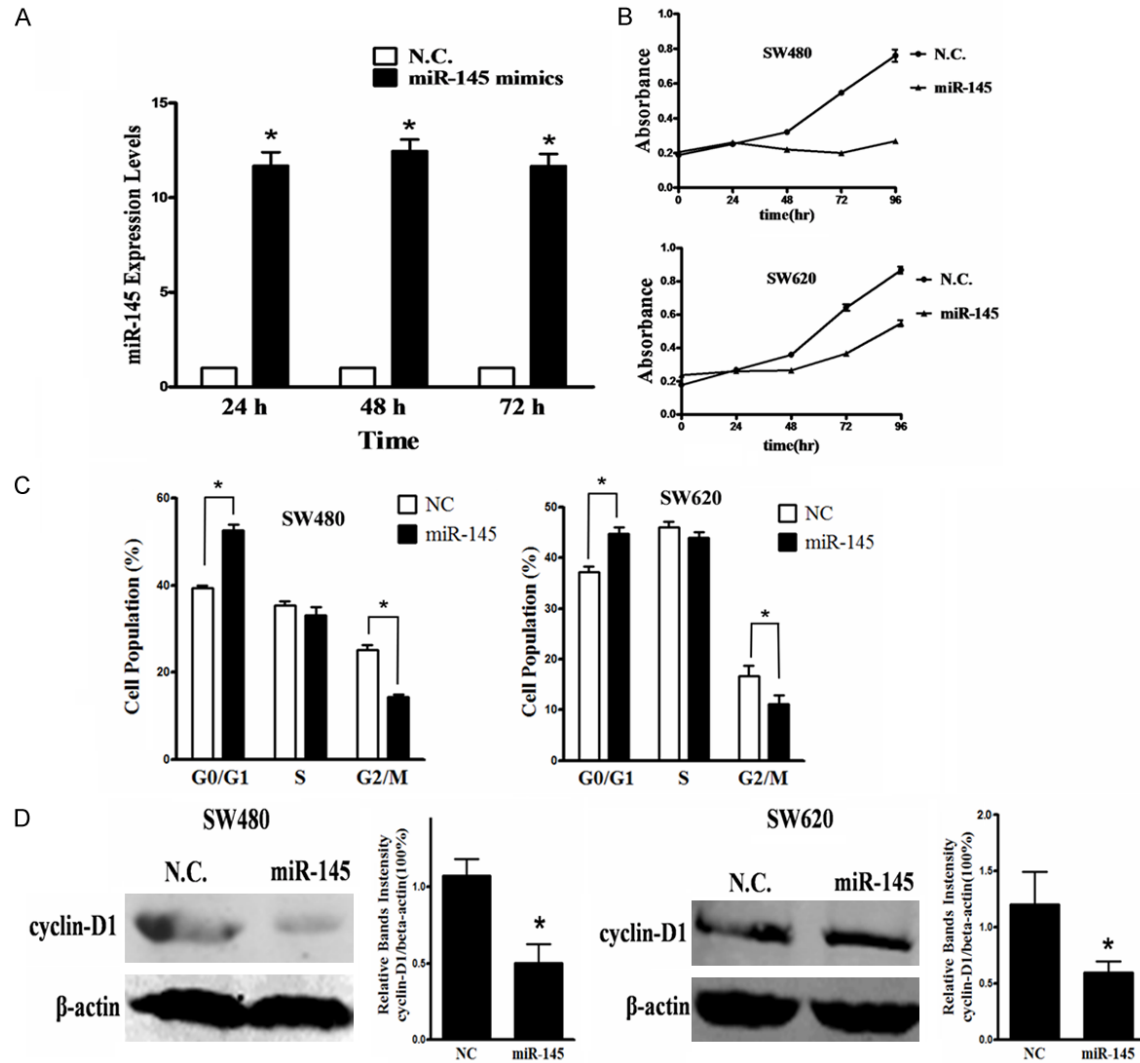
Figure 2. The effect of ectopic expression of miR-145 on the proliferation of colon cancer cells. A. qRT-PCR analysis of mature miR-145 in SW480 and SW620 colon cancer cells transfected with miR-145 mimics in the indicated time point. B. miR-145 mimics-transfected and NC mimics-transfected colon cancer cells were assessed by MTT assay with the mean data from two independent experiments, each carried out in triplicate. C. The cell cycle distribution of miR-145 mimics-transfected and NC mimics-transfected colon cancer cells was calculated by FACS assay. D. The protein expression of cyclin-D1 of miR-145 mimics-transfected and NC mimics-transfected colon cancer cells was detected by Western blot. Data are presented as means ± SD. *$P < 0.05$.

The membranes were blocked by TBST buffer (TBS plus 0.1% Tween-20) containing 5% w/v non-fat milk and hybridized with primary antibody, followed by incubation with specific HRP-conjugated secondary antibody. Protein bands were visualized by the ECL detecting system (Pierce, USA). Mouse monoclonal anti-KLF5 (Abcam, USA) and anti-cyclin-D1 (CST, USA) were used. Monoclonal anti-actin (1:5000, Sigma-Aldrich, USA) was used for the loading control.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 software (SPSS Inc., USA). Data are expressed as the mean ± standard deviation (SD) from at least three separate experiments. The differences between groups were analyzed using the Student t test to compare two groups or by one-way ANOVA for more than two groups. A value of $P < 0.05$ was considered to be statistically significant.
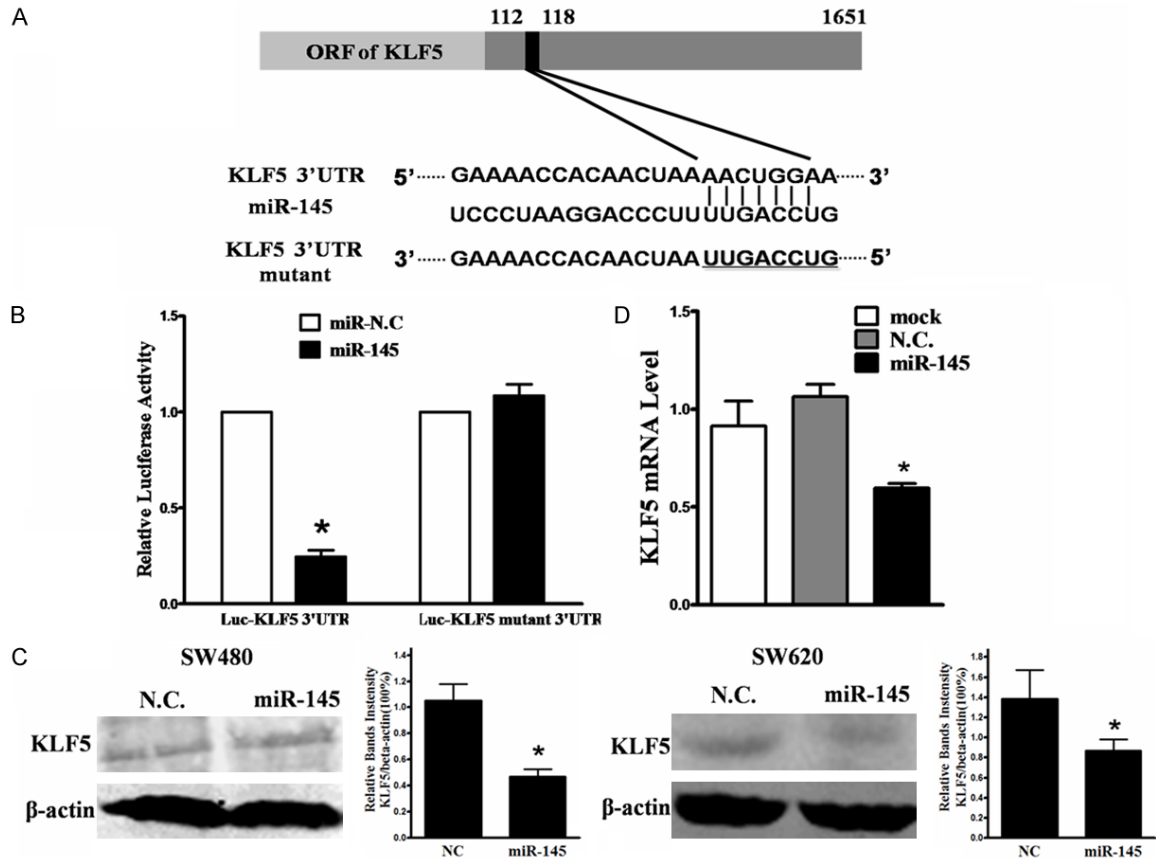
Figure 3. miR-145 targets 3'-UTR of KLF5 gene and attenuates KLF5 expression in colon cancer cells. A. Representative nucleotide sequence matches binding sites of miR-145. The panel shows predicted duplex combination between the human KLF5 3'UTR and miR-145 (upper, sequence of KLF5 3'UTR containing miR-145 binding site; middle, sequence of the miR-145; lower, sequence of KLF5 3'UTR containing mismatched miR-145 binding site as indicated in bold letters). B. The luciferase activity of a reporter plasmid containing wild-type of KLF5 3'UTR (Luc-KLF5-3'UTR) or mutated-type of KLF5-3'UTR (Luc-KLF5-mutation 3'UTR) was measured in HEK293 cells which were co-transfected with miR-145 or NC mimics. C. The KLF5 protein level was detected in miR-145 mimics-transfected and NC mimics-transfected SW480 and SW620 cells by using Western blot 48 h after transient transfection. D. The KLF5 mRNA level was detected in miR-145 mimics-transfected and NC mimics-transfected colon cancer cells by using qPCR 48 h after transient transfection. Each bar represents the mean value ± SD from three independent experiments ($^*P < 0.05$).

Results

miR-145 significantly down-regulated in CRC tissues and cell lines

To study the expression and significance of miR-145 in CRC carcinogenesis, we measured the expression of miR-145 in 41 pairs of CRC tissues and their matched adjacent normal tissues. The results showed that miR-145 expression was significantly decreased in CRC tissues compared with their matched normal tissues (**Figure 1B**). We further assessed the expression levels of miR-145 in different CRC cell lines (SW480, SW620, HCT-116, HT29, DLD-1, Colo205). Compared with most of the colon cancer cell lines examined, the intestinal epithelial-derived cell line GES had the highest level of miR-145 expression (**Figure 1A**). Taken together, these results suggest that the down-regulation of miR-145 may play important roles in CRC carcinogenesis and progression.

Ectopic expression of miR-145 inhibits proliferation of colon cancer cells by inducing cell cycle arrest at G1/S transition

There is growing evidence demonstrating that miR-145 is a *bona fide* tumor suppressive miRNA in many types of cancer, including colon cancer [28]. An *in vivo* study showed that application of miR-145 can obviously inhibit growth

of colon cancer cells in tumor-bearing nude mice [29]. However, the mechanism of tumor inhibition by miR-145 has not been properly elucidated, and whether miR-145 may induce cell cycle arrest in colon cancer cells is uncertain. In this study, we introduced exogenous miR-145 mimics into the colon cancer cell lines SW480 and SW620. After transient transfection of miRNA mimics, we examined the kinetic pattern of miR-145 expression in cancer cells by qRT-PCR. The expression level of miR-145 was upregulated by 10-fold ($P < 0.01$) in SW480 cells transfected with miR-145 mimics compared to the cells transfected with negative control (NC) mimics, and this high-level expression of miRNA was sustained for at least 72 h (**Figure 2A**), demonstrating that the effect of exogenous miR-145 in colon cancer cells may be evaluated for a sufficient amount of time over the course of our experiment. Analysis by the MTT assay showed that ectopic expression of miR-145 led to a significant reduction in cell growth compared with the control mimics in these two colon cancer cell lines (**Figure 2B**).

To further assess the effect of miR-145 on proliferation of colon cancer cells, we examined cell cycle distribution by using flow cytometry. The mir-145-transfected SW480 and SW620 cells showed higher percentages of cells in the G0/G1 phase and lower percentages of S phase or G2/M phase cells ($P < 0.05$; **Figure 2C**). It is well known that the G1-S transition is under the control of cyclinD/CDK4 complexes. As the G1/S transition is defined by accumulation of cyclin-D1, we determined its expression in colon cancer cell lines transfected with miR-145 and the control mimics by Western blot. As indicated in **Figure 2D**, the expression of cyclin-D1 was significantly decreased in miR-145-transfected SW480 and SW620 cells as compared with control miRNA-transfected cancer cells. These results suggested that the ectopic expression of miR-145 decreased cyclin-D1 expression in colon cancer cells, delineating the role of miR-145 in the inhibition of colon cancer cell proliferation.

KLF5 is a direct target of miR-145 in colon cancer cells

Given that many miRNAs have been identified as direct negative regulators of cyclin-D1, we wanted to test whether miR-145 may also be

a direct modulator of cyclin-D1 in controlling G1/S transition. Thus, we cloned the full-length 3'UTR of cyclin-D1 and constructed the cyclin-D1 3'UTR reporter plasmid to examine if miR-145 induces G1 phase arrest through the direct regulation of cyclin-D1 expression by a post-transcriptional regulatory mechanism. However, our luciferase reporter assay failed to detect an obvious effect of miR-145 mimics on the 3'UTR of cyclin-D1 (data not shown), suggesting that other yet unidentified molecule(s) may be involved in miR-145-mediated G1 phase arrest and the regulation of cyclin-D1 expression in colon cancer cells.

Using the Targetscan program and database, a serial of miR-145 target genes was found. Among them was the transcriptional factor KLF5 which contains one predicted target sequence for miR-145 from positions 123 to 134 in its 3'-UTR (**Figure 3A**). Thus, we hypothesized that KLF5 may play an important role in the miR-145-mediated control of the G1/S cell cycle transition of colon cancer cells.

To test if miR-145 directly targets the KLF5 gene, we inserted a DNA sequence containing the miR-145 binding sites (3'UTR) of the KLF5 gene into a modified luciferase reporter vector. To demonstrate the specificity of miR-145 targeting of the KLF5 gene, we also generated a mutant reporter construct containing modifications in the miR-145 binding sites within the KLF5 3'UTR (**Figure 3B**). We transfected the reporter constructs into HEK293 cells along with synthetic miR-145 or NC mimics. At 48 h after transfection, the miR-145 had suppressed the luciferase reporter activity by more than 60%, whereas mutation of the predicted miR-145 binding sites abolished the suppressive effect of miR-145 on the luciferase activity. Our result suggests that miR-145 may directly attenuate the expression of KLF5 by binding to its 3'-UTR.

Western blot analysis of the total protein extracts from SW480 and SW620 cells transfected with miR-145 showed an obvious down-regulation of KLF5 at the protein level (**Figure 3C**) compared to the control groups. Quantification of the KLF5 immuno-bands on the Western blot demonstrated that mir-145 caused a 40% or 30% reduction of KLF5 proteins in both SW480 and SW620 cells, respectively, after normalization by β-actin (**Figure 3C**).
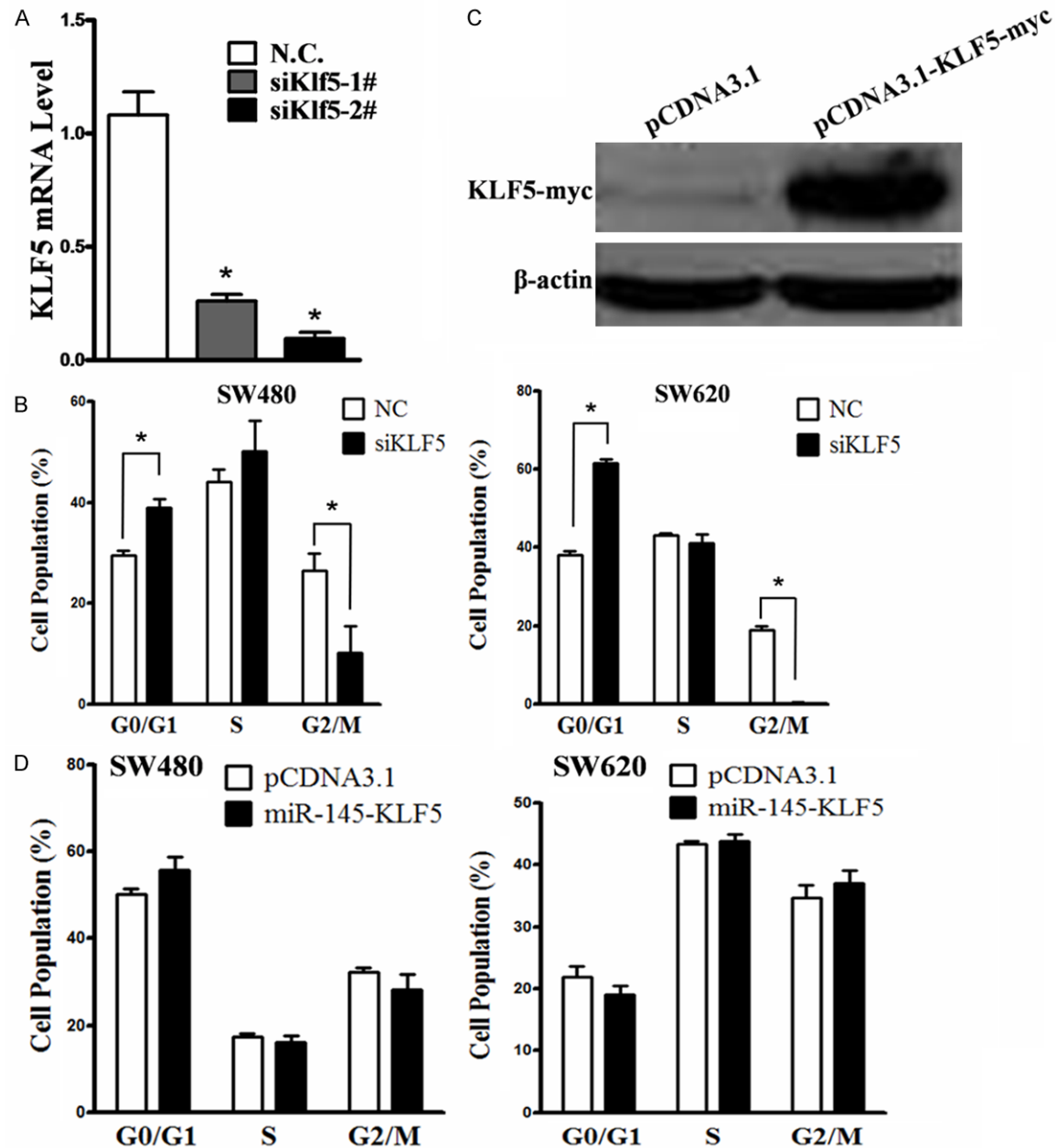
Figure 4. Targeting KLF5 is involved in tumor suppression of miR-145 in colon cancer cells. A. qPCR analysis of KLF5 expression in colon cancer cells transfected with siRNAs targeting KLF5. B. The cell cycle distribution of KLF5-siRNA-transfected and NC siRNA-transfected colon cancer cells was calculated by FACS assay. C. Western blot analysis of exogenous KLF5 expression in SW480 colon cancer cells. D. The cell cycle distribution of SW480 and SW620 cells transfected with pCDNA3.1 control as compared with the group transfected with both pCDNA3.1-KLF5-myc and miR-145 mimics. Data are presented as means ± SD. *$P < 0.05$.

These results indicated that KLF5 is a direct target of miR-145 in colon cancer cells. To further analyze the regulatory mechanism of miR-145 on KLF5 expression, we transiently transfected miR-145 mimics into colon cancer cell lines and quantatively (qRT-PCR) measured KLF5 expression at the mRNA and protein levels. The results demonstrated that miR-145

reduced accumulation of KLF5 via the degradation of KLF5 mRNA (**Figure 3D**).

miR-145 suppresses colon cancer cells by targeting KLF5

To investigate whether miR-145 regulates the cell cycle by targeting KLF5, we used siRNA to

silence KLF5 expression in SW480 and SW620 colon cancer cell lines. qRT-PCR analysis confirmed that our KLF5 siRNA targeting could obviously reduce KLF5 expression (**Figure 4A**). siRNA knockdown of KLF5 in SW480 and SW620 colon cancer cells induced significant levels of cell cycle arrest at the G1/S transition phase (**Figure 4B**). These results indicate that a reduction of KLF5 expression can mimic miR-145 to inhibit proliferation of colon cancer cells by regulating the cell cycle, suggesting that targeting KLF5 may be the mechanism of the tumor suppression by miR-145 in colon cancer cells.

Overexpression of KLF5 rescues miR-145-induced cell cycle arrest of colon cancer cells in G1/S transition

To further validate that the effect of miR-145 on the cell cycle is mediated by KLF5, we performed a "rescue" experiment to re-express KLF5 cDNA lacking the 3'-UTR prior to transfecting cells with miR-145 and testing the cell cycle profiles. The KLF5 expression level was also verified by Western blot in this experiment (**Figure 4C**). The analysis of cell cycle distribution revealed that forced KLF5 expression partially attenuated miR-145-induced cell cycle arrest in SW480 and SW620 cells (**Figure 4D**). These data suggested that the effects of miR-145 on the cell cycle were partially mediated by KLF5. Interestingly, when we transiently transfected exogenous KLF5 without the 3'UTR sequence into HT29 colon cancer cells, we did not detect an obvious change of cell cycle distribution in HT29-KLF5 cells compared with HT29-pCDNA3.1 or intact parental cells (Data not shown). This phenomenon may be explained if the proliferation of only some types of colon cancer cell lines is dependent upon KLF5.

Discussion

It has been more than a decade since miRNAs were first found to function in the development and progression of cancer [30]. Currently, miRNAs are not only used as biomarkers for the diagnosis and prognosis of malignant diseases, but they are also being developed as promising candidates in cancer therapeutics [11]. In recent years, miRNA-based therapeutic strategies have been explored to inhibit growth of cancer cells by overexpression of tumor suppressive miRNAs in several preclinical trials

[31-33]. Other anti-tumor strategies such as siRNA utilize synthetic RNA-oligonucleotides to silence only one oncogenic gene within a key malignancy pathway of cancer cells, and thus may not be practical for clinical applications given the heterogeneous nature of tumors. However, the miRNA-based therapeutic strategy is different in that it aims to restore a natural tumor-suppressive function [34]. As tumor suppressive miRNA are frequently downregulated in carcinoma cells, its restoration can suppress tumor growth by affecting several malignancy end points via downregulation of multiple oncogenes [35]. Thus, miRNA-based therapeutics may be an optimal strategy for the treatment of malignant diseases, and exploring the molecular mechanism of tumor suppression by miRNAs is indispensible for taking this approach from bench to bedside.

In our study, we focused on the molecular mechanism of miR-145-induced cell cycle arrest in the G1/S transition. Given that miR-145 is a well-known tumor suppressive miRNA, the in-depth analysis of its role in cell cycle arrest would further validate the use of miR-145-based therapeutics in colon cancer. Mir-145 is the first miRNA identified to be downregulated in colorectal carcinoma [28]. As mentioned above, the genomic region encoding miR-145 is often deleted in cancer [13]. We found that levels of miR-145 in patient colon tumor tissues were much lower than in adjacent normal tissues. Consistent with the results found in CRC tissues, miR-145 expression levels were significantly lower in all six CRC cell lines tested compared with immortalized gastric mucosa cells. Accordingly, ectopic expression of miR-145 was demonstrated to inhibit and even suppress anchorage-independent growth of many types of colon cancer cell lines, indicating that downregulation of miR-145 can promote carcinogenesis of colorectal tissue. Indeed, reintroduction of this miRNA can efficiently reverse the malignant phenotype of colorectal cancer cells [36]. Although one miRNA can simultaneously target a large number of target genes that result in detectable changes in mammalian cells [9], the previously experimentally identified direct target genes of miR-145 could not provide a reasonable explanation for miR-145-induced cell cycle arrest of colon cancer cells in G1/S transition [19, 20, 37]. Using *in silico* analysis, putative targets of

miR-145 with potential oncogenic functions have been predicted such as MYCN, FOS, YES and FLI, CCDN2, CDK3 and mitogen activated protein kinase pathway (MAPK) MAP3K3 and MAPK4K4. However, few of them have been shown to be involved in miR-145-induced cell cycle arrest [28].

Here, we predicted that KLF5, a member of the zinc finger family of proteins, may be one direct target gene negatively regulated by miR-145. To identify the *bona fide* target genes of a specific miRNA, solid experimental evidence should be obtained following several critical standard rules. First, the miRNA must be tested to determine whether it can directly bind the 3'UTR or ORF region of target genes [38]. Second, ectopic expression of the miRNA should result in obvious downregulation of the target gene at either the translational or transcriptional level [9]. Third, the phenotype of ectopically expressed miRNA should be similar to that of siRNA-induced silencing of target genes. Fourth, in a "rescue" experiment, the miRNA-mediated phenotype should be reversed by reintroduction of the target genes with no nucleotide sequences complementary to that of the miRNA. Finally, an inverse correlation between the miRNA and its target genes should be observed *in vivo*. Based on these standards, our series of experiments presented here demonstrated that KLF5 is a direct target gene of miR-145 in colon cancer cells. The results of both the reporter assay and Western blot analysis preliminarily identified the downregulation of KLF5 by miR-145 in colon cancer cells (**Figures 2** and **3**). In the functional experiment, the transient transfection of miR-145 robustly inhibited expression of KLF5 in SW480 and SW620 colon cancer cell lines. The cell cycle distribution analysis was consistent with another experiment showing that transient transfection of miR-145 mimics induced cell cycle arrest of these two colon cancer cell lines in G1/S transition. Notably, a "rescue" experiment demonstrated that restoration of KLF5 partially attenuated miR-145-induced cell cycle arrest in the SW480 cell line, indicating that KLF5 may function in miR-145-mediated cell cycle arrest in G1/S transition. We did not perform the parallel "rescue" experiment in SW620 cells since the liposome-mediated transfection of pCDNA-KLF5 was not high enough to result in an obvious change unlike with the SW480 cells.

Previous studies have demonstrated that KLF5, as a promoter of cellular proliferation, can control G1/S transition by increasing CCND1, CCNB1 and CDC2 expression at the transcriptional level [39, 40]. It was also reported that overexpression of KLF5 accelerated monolayer proliferation and anchorage-independent growth of non-transformed epithelial cells, suggesting that KLF5 may have oncogenic potential in colon cancer [23]. Accordingly, a breast cancer study also found that high level expression of KLF5 correlated with poor prognosis of clinical patients [41]. Thus, our present study together with previous reports support the notion that KLF5 plays a critical role in miR-145-induced cell cycle arrest of colon cancer cells in G1/S transition.

Although we have demonstrated that KLF5 is a *bona fide* direct target gene of miR-145 in colon cancer cells, we cannot exclude the possibility that other unidentified target genes are involved in miR-145-induced cell cycle arrest in colon cancer cells. It was reported previously that overexpression of miR-145 also has an inhibitory growth effect on HT29 colon cancer cells [28]. Our future studies will focus on discovering additional target genes involved in miR-145-mediated cell cycle regulation. Strikingly, when we transferred exogenous KLF5 into HT29 colon cancer cells, we did not detect an obvious change of cell cycle distribution (data not shown). This further emphasized that although KLF5 was demonstrated to be important in cell cycle regulation of colon cancer cells, the KLF5-dependent cancer proliferation may only occur in certain colorectal tissue-derived transformed cells.

A recent study from a triple-gene knock-in animal model has shown that KLF5 is a crucial mediator of carcinogenesis in mice harboring combined ApcMin and KRASV12 mutations [42], highlighting an important role of KLF5 in intestinal tumorigenesis. Considering that KLF5 was found to be a direct target gene of miR-145 in colon cancer cells in our present study, it can been easily concluded that the decreased miR-145 expression combined with high level activation of K-Ras and loss of APC function leads to tumorigenesis of colorectal tissue. In our future studies we will analyze the relationship between the expression pattern of these molecules and prognosis of clinical

patients. Taken together, our study for the first time demonstrated that miR-145 induces cell cycle arrest in the G1 phase, at least partially, by directly targeting KLF5 in colon cancer cells, providing further evidence that miR-145 could be a promising candidate in therapy for colorectal cancer.

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Disclosure of conflict of interest

None.

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