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ORIGINAL ARTICLE

Down-regulation of miR-9 promotes epithelial mesenchymal transition *via* regulating anoctamin-1 (ANO1) in CRC cells

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Abstract

MicroRNA-9 (miR-9) has been reported to play a suppressive or promoting role according to cancer type. In this study, we investigated the effects of anoctamin-1 (ANO1) and miR-9 on colorectal cancer (CRC) cell proliferation, migration, and invasion and determined the underlying molecular mechanisms.

Thirty-two paired CRC tissues and adjacent normal tissues were analyzed for *ANO1* expression using quantitative real-time PCR (qRT-PCR). HCT116 cells were transiently transfected with miR-9 mimic, miR-9 inhibitor, or si-ANO1. Cell proliferation was determined by MTT, and flow cytometric analysis, while cell migration and invasion were assayed by trans-well migration and invasion assay in HCT116 cells. *ANO1* was validated as a target of miR-9 using luciferase reporter assay and bioinformatics algorithms.

We found that *ANO1* expression was up-regulated in CRC tissues compared with adjacent normal tissues. *ANO1* expression was associated with advanced tumor stage and lymph node metastasis, and there was an inverse relationship between miR-9 and *ANO1* mRNA expression in CRC specimens, but no significant difference was found between miR-9 and *ANO1* expression. *ANO1* is a direct target of miR-9, and overexpression of miR-9 suppressed both mRNA and protein expression of ANO1 and inhibited cell proliferation, migration, and invasion of HCT116 cells. We also showed that overexpression of miR-9 suppressed expression of *p*-AKT, cyclin D1, and *p*-ERK in HCT116 cells.

We conclude that miR-9 inhibits CRC cell proliferation, migration, and invasion by directly targeting *ANO1*, and miR-9/ANO1 could be a potential therapeutic target for CRC.

Keywords miR-9, ANO1, Colorectal cancer, Proliferation, Migration, Invasion.

Introduction

Colorectal cancer (CRC) is one of the most common malignancy in the world, and the third leading cause of cancer-related death [1]. Metastasis is a major factor associated with the poor prognosis of CRC and is positively associated with morbidity and mortality in CRC patients [2,3]. However, little is known about the roles of miR-9 in the metastasis of

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CRC and the molecular mechanisms by which miR-9 exerts its functions.

MicroRNAs (miRNAs) are non-coding RNAs of about 22 nucleotides in length that were first described in *Caenorhabditis elegans* in 1993 [4]. miRNAs play critical regulatory roles in various cancers including gastric cancer, pancreatic cancer, and hepatocellular carcinoma by binding to the 3'-untranslated regions (3'-UTR) of target mRNAs, resulting in degradation or translational repression [5–8]. Abnormally expressed miRNAs have also been shown to contribute to the proliferation, apoptosis, and metastasis of cancer cells [9,10]. Therefore, the aberrant expression of miRNAs has been suggested to play an important role in tumorigenesis, and the expression of specific miRNAs is evaluated for cancer diagnostic, prognostic, and therapeutic purposes [11].

MicroRNA-9 (miR-9) has been identified as an oncogene in medulloblastoma, glioma, breast cancer and cystic fibrosis [12–15]. However, miR-9 has also been reported to have an anticancer effect in some cancers, including breast cancer, oral squamous cell carcinoma and gastric cancer [16–19]. Moreover, miR-9 has been shown to suppress the growth, migration, and invasion of malignant melanoma cells by targeting NRP1 and up-regulating E-cadherin [20,21]. In hepatocellular carcinoma, miR-9 suppressed expression of NF-κB *via* Yin Yang-1 (YY1) [22]. miR-9 also suppressed CRC cell proliferation and apoptosis by targeting ubiquitin-like with plant homeodomain and ring finger domains 1 (UHRF1), and functioned as a tumor suppressor in CRC [23]. In our previous study, we demonstrated that miR-9 was significantly down-regulated in CRC tissues compared with adjacent normal tissues. It also suppressed cell migration and invasion *via* transmembrane 4L six family member 1(TM4SF1) in CRC cells [24]. Based on these findings, we predicted that miR-9 might play a crucial role in CRC progression and metastasis.

ANO1, which is also known as gastrointestinal stromal tumor protein (DOG1) and transmembrane member 16A (TMEM16A), is a membrane protein [25–27]. ANO1 is a functional calcium-activated chloride channel that promotes tumor growth and progression [28,29]. Elevated expression of ANO1 has been reported in various cancers including breast cancer, prostate cancer, HNSCC, esophageal squamous cancer, and CRC [30–34]. Moreover, overexpression of ANO1 was significantly associated with distant metastasis and poor prognosis in CRC patients and inhibition of ANO1 suppressed proliferation induced apoptosis of cancer cells that originated from the epithelium [35,36]. In glioma cells, overexpression of ANO1 not only regulated the expression of nuclear factor-κB (NF-κB), but also increased the expression of cyclin D1, cyclin E, and c-myc, and matrix metalloproteinases (*MMP*)-2 and *MMP-9*, which are associated with the migration and invasion [37]. In addition, knock down of ANO1 with siRNA significantly decreased the expression of β-catenin, cyclin D1, MMP-9, snail, and N-cadherin, and increased the expression of E-cadherin in breast carcinoma cells [38]. Therefore, further study is need to clarify the exact function of ANO1 in tumor malignancy.

In this study, we investigated the role and function of anoctamin-1 (*ANO1*), another potential target gene of miR-9, in CRC. ANO1 was significantly increased in CRC specimens and cell lines, and increased expression of ANO1 was associated with advanced clinical stage and lymph node metastasis status of CRC patients. Functionally, *ANO1* is a directly target of miR-9, and overexpression of miR-9 was inhibit the CRC cell proliferation, migration, and invasion *in vitro*. Mechanically, miR-9 could suppress *p*-AKT signaling pathway and cyclin D1, *p*-ERK by inhibiting ANO1.

Our study provides evidence that miR-9 has anti-metastasis activity in CRC cells and may be an offering the effective therapeutic target for the treatment of CRC.

Table 1 Clinic pathological features in 32 patients with CRC for this study.

Clinical characteristics	Number of each group *n* (%)
Gender	
Male	15 (46.9)
Female	17 (53.1)
Age (years)	
<60[a]	13 (40.6)
≥ 60	19 (59.4)
Tumor location	
Colon	29 (90.6)
Rectum	3
Histological differentiation	(9.4)
Well	7 (21.9)
Moderate	23 (71.9)
Poor	2 (6.2)
Tumor status (T)	
T1-T2	20 (62.5)
T3-T4	12 (37.5)
Lymph node metastasis (N)	
absent	25 (78.1)
present	7(21.9)
AJCC	
I + II	23 (71.9)
III + IV	9 (28.1)

[a] Median age.

Material and methods

Patient and tissues samples

Frozen CRC tissues and clinical data were obtained from the biobank of Chonbuk National University Hospital, as shown in Table 1. This study was approved by the Institutional Review Board (IRB) of Chonbuk National University Hospital (CUH 2016-04-018-002).

Human cell lines and transfection

Human colorectal cancer cell lines (DLD-1, SW620, Caco-2, HT29 and HCT116) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were cultured as monolayer cultures in RPMI1640 or DMEM (GIBCO, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, GIBCO, Carlsbad, CA, USA). Human colon fibroblast cell line, CCD-18co, was purchased from the Korean Cell line Bank (Seoul. Korea). Cells were maintained in DMEM supplemented with at 37 °C in a humidified 5% CO_2 incubator.

Both miR-9 mimics and inhibitors were commercially synthesized by Ambion (Austin, TX, USA) and transfected into

cell lines using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. At 6 h after transfection, the medium was changed. Total RNA and proteins were extracted after 48 h and 72 h of incubation for real-time PCR and western blot analyses, respectively.

For siRNA transfection, siRNA targeting *ANO1* (si-ANO1, sc-76686) and control siRNA (si-con, sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). si-ANO1 or si-con were transiently transfected into cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cells were plated in culture plates with growth media and incubated overnight to achieve 70% confluence. The medium was removed, and serum-free Opti-MEM medium with reagent complexes containing 20 nM of si-con and si-ANO1 was added on cells.

Methylthiazol tetrazolium (MTT) assay

HCT116 cells were plated in 96-well culture plates (3×10^3 per well). After a 24 h incubation, cells were transfected with miR-9, anti-miR-9 (miR-9 inhibitor), negative control (NC), and anti-negative control (ANC) for 24, 48, and 72 h. Then 100 µL of 3-(4,5-dimethythiazol-2-yl)-2,5-diphen yl tetrazolium bromide (0.1 mg/mL; Sigma-Aldrich) was added to each well. After 3 h incubation, the MTT solution was removed and formazan crystals in the cells were solubilized using dimethyl sulfoxide (DMSO, Duksan. Pure Chemicals, Korea) with plate shaking for 30 min. Absorbance was measured on an ELISA reader at a wavelength of 450 nm. All experiments were performed in triplicate.

Luciferase reporter assay

A fragment of the *ANO1* 3'-UTR containing the miR-9 predicted seed region (WT) was amplified and cloned into the pmir GLO miR expression vector (Promega, Madision, WI, USA). EZ change™ site-directed mutagenesis kit (Enzynomics, Daejeon, South Korea) was used to create mutant type (MT). Luciferase activity was measured by the Dual Luciferase Reporter Assay system (Promega). All experiments were repeated three times independently.

RNA extraction and quantitaive real-time reverse transcription polymerase change reaction (qRT-PCR)

Total RNA, including miRNA, was extracted from CRC tissues and cultured cells using Trizol reagent (Invitrogen Life Technologies). After cDNA synthesis using M-MLV reverse transcriptase (Promega), *ANO1* mRNA levels were analyzed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems® QuantStudio™ 6 Flex Real-Time PCR system (Applied Biosystems). miR-9 expression was measured using the TaqMan MicroRNA assay kit (Applied Biosystems) according to the manufacturer's protocol. miR-9 expression was normalized to that of RNU48, and *ANO1* mRNA level was normalized to that of beta-2-microglobulin (*B2M*). Fold change was calculated using the $2^{-\triangle\triangle Ct}$ method. Primers used were as follows: *ANO1*, 5'-GATCCCATCCAGCCCAAAGTG-3' (forward), 5'-CGGGTTTTGCTGTCGAAAAAGGA-3' (reverse); *B2M*, 5'-CCTGAATTGCTATGTGTCTGGG-3' (forward), 5'- TGATGCTGCTTACATGTCTCGA-3' (reverse). All experiments were repeated three times independently.

Trans well assays

Migration and invasion of the cells were assessed by using 24 well culture dish (8 µm-pore size membranes, culture-insert 12 well, SPL, Gyeonggi-do, Korea). For the migration assays, 2.0×10^5 transfected cells were seeded in the upper chamber in serum- free media. For the invasion assay, transfected cells were plated into the insert coated with Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). Fresh medium was added to the lower chamber followed by a 48-h incubation. After incubation, the membrane was fixed and stained with methanol and crystal violet. Migratory or invasive cells were counted randomly in five fields. These assays were repeated three times for each cell type.

Western blot

Total cell lysates were extracted with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS containing protease inhibitors) (Thermo Scientific Pierce, Rockford, IL, USA) and protein amount was quantified using the Bradford protein assay (Sigma-Aldrich, St. Louis, MO, USA). Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% non-fat dry skim milk for 1 h, and treated with antibodies against ANO1 and cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), *p*-ERK and *p*-AKT (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Sigma-Aldrich) at 4 °C overnight. After three washes with TBST, blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted 1:10,000 in the blocking buffer) for 1 h. For image development, Dyne ECL reagents (Dyne, Seongnam, Korea) and a chemiluminescent image system (Fusion Solo System, Vilber Lourmat) were used. All experiments were repeated three times independently.

Apoptosis analysis

Cells transfected with anti-miR-9 or si-ANO1 were harvested at 72 h after transfection cells and stained with Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA). Apoptotic cells were assessed in triplicates and repreated three times independently by flow cytometry, BD Accuri™ C6 cytometer (BD Biosciences).

Cell cycle analysis

For the flow cytometry analysis of the cell cycle, HCT116 cells were seeded in culture plates. When the cells grew to a density of 70% confluence, they were transfected with anti-miR-9

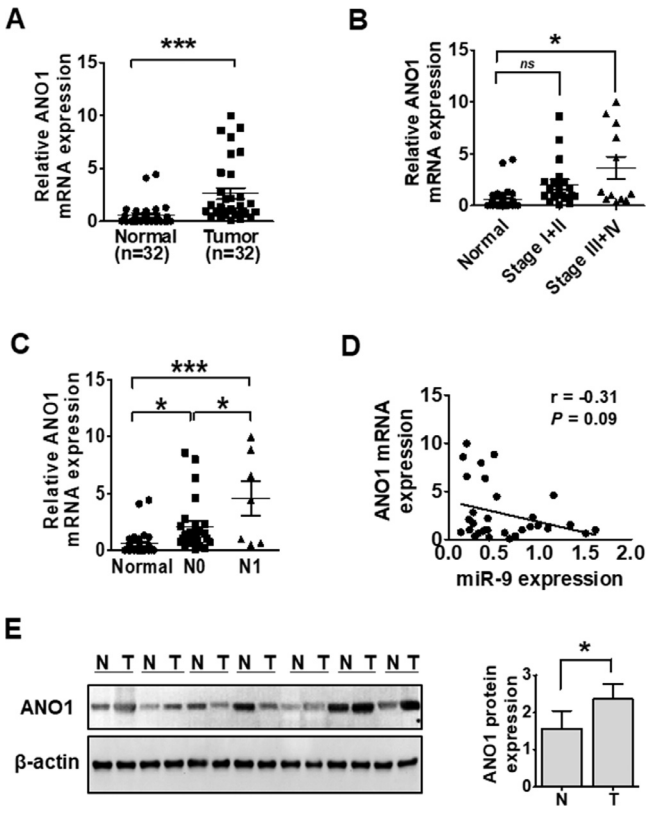

Fig. 1 ANO1 is significantly up-regulated in CRC specimens. (A) *ANO1* expression was analyzed in 32 paired CRC tissues using qRT-PCR and normalized to B2M as an endogenous control. (B) The 32 of CRC tissues were classified according to pathological stage (stage I + II and stage III + IV). (C) *ANO1* expression was frequently up-regulated in CRC with (N1) or without lymph node metastasis (N0) compared with normal tissues. (D) miR-9 expression was inversely associated with *ANO1* mRNA levels in CRC tissues. (E) Western blot results show that the protein expression level of ANO1 was higher in CRC tumor tissues (T) than paired normal tissues (N). Data shown were obtained from three independent experiments and *P*-values were calculated by *t*-test or one-way ANOVA (* $P < 0.05$, *** $P < 0.0001$).

or si-ANO1 and incubated for 72 h. After transfection, transfected cells were harvested, washed using phosphate buffer saline and fixed in 70% ethanol at 4 °C. Staining for DNA content was added 50 mg/ml propidium iodide and 1 mg/ml ribonuclease A at room temperature for 30 min. Populations in G0-G1, S, and G2-M phase were analyzed and repeated three times.

Statistical analysis

Data are presented as means ± standard errors of the means. The difference of between groups was evaluated using the Mann Whitney *t*-test when there were only two groups, and one-way ANOVA when there were more than two groups. The relationship between ANO1 and miR-9 expression was explored by a Spearman's correlation. $P < 0.05$ was considered to indicate a statistically significant difference.

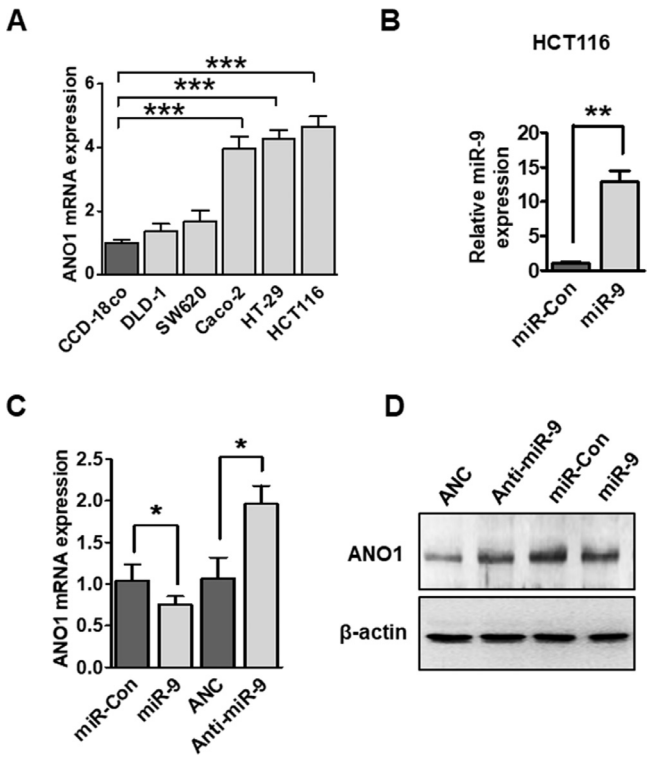

Fig. 2 Overexpression of miR-9 significantly inhibited ANO1 mRNA and protein expression in colorectal cancer cell lines. (A) *ANO1* mRNA expression in the CRC cell lines, HCT116, HT29, Caco-2, SW620, DLD-1, and CCD-18co cells. (B) HCT116 cells were transfected with either miR-9 or con-miR and analyzed using qRT-PCR. miR-9 down-regulated *ANO1* mRNA and protein levels in HCT116 cells as determined by qRT-PCR (C) and western blot (D), respectively. Data shown were obtained from three independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Results

ANO1 is up-regulated in CRC tumor tissues, advanced stage CRC, and correlated with lymph node metastasis

For this study, we determined expression levels of ANO1, nidogen 2 (NID2), and exportin 4 (XPO4), which are predicted to be miR-9 target genes by online target prediction algorithms, such as TargetScan, PicTar, and miRanda. ANO1 protein expression was suppressed by miR-9 compared with con-miR, but the expression of NID2 and XPO4 were observed similar level in HCT116 cells (data not shown).

Next, we investigated mRNA expression of *ANO1* in CRC specimens, and we founded that *ANO1* mRNA expression was significantly up-regulated in CRC specimens compared with matched normal tissues ($P < 0.0001$, for all comparisons, Fig. 1A). In addition, *ANO1* mRNA expression was positively associated with late stage (stages III + IV) compared to normal tissues ($P < 0.05$, for all comparisons, Fig. 1B). *ANO1* expression was also higher in CRC specimens with lymph node metastasis (N1) or without lymph node metastasis (N0) than matched normal tissues specimens (Fig. 1C). We also

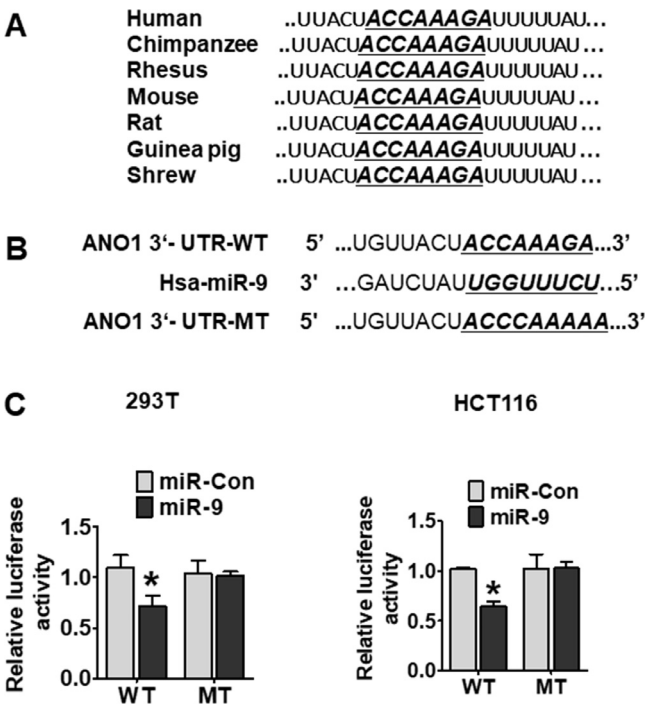

Fig. 3 ANO1 is a direct target gene of miR-9.
(A) Online target prediction algorithms were used to predict targets of miR-9, and its recognition sequence was found to be conserved among different mammalian species. (B) Human ANO1 3'-UTR containing the wild-type or mutated type miR-9 binding site was cloned into the pmir GLO miRNA expression vector. (C) 293T and HCT116 cells were co- transfected with WT or MT plasmids and miR-9 or miR-control (miR-NC), and luciferase activity was then tested.

determined that miR-9 expression was inversely correlated with *ANO1* mRNA expression in CRC specimens by analysis of Spearman's Correlation analysis, but not significant association between miR-9 and ANO1 expression (Fig. 1D). Protein expression of ANO1 was also detected in paired CRC and normal tissues by western blot analysis. ANO1 expression was up-regulated in five out of seven human colon cancer tissues compared with the corresponding normal tissues (Fig. 1E). These results suggest that *ANO1* and miR-9 might be important regulators of CRC metastasis and progression.

miR-9 inhibits ANO1 mRNA and protein expression *in vitro*

We measured endogenous expression of *ANO1* in five CRC cell lines (DLD-1, SW620, Caco-2, HT29, and HCT116) and human colon fibroblast cell, CCD-18co. As shown in Fig. 2A, *ANO1* was expressed significantly higher in human CRC cell lines than CCD-18co cells. To further evaluate the effect of miR-9 on CRC cells, we transfected HCT116 cells with miR-9 mimic or miR-control (miR-Con) for 24 h. qRT-PCR results revealed that miR-9 was significantly up-regulated in HCT116 cells (Fig. 2B). Overexpression of miR-9 significantly suppressed ANO1 expression at both the mRNA and protein levels in HCT116 cells. In contrast, inhibitor of miR-9 increased both ANO1 mRNA and protein expression in HCT116 cells (Fig. 2C and D). Based on these findings, miR-9 suppresses the expression of *ANO1* mRNA and protein in CRC cells.

ANO1 is a direct binding target of miR-9

According to bioinformatics databases, the binding site of miR-9 is highly conserved in diverse mammalian species (chimpanzees, rhesus macaques, mice, rats, guinea pigs, shrews) (Fig. 3A). To determine whether *ANO1* is a direct target of miR-9, the 3'UTR of ANO1 containing the wild-type (ANO1-WT) or a mutant (ANO1-MT) miR-9 binding site was inserted into a dual - luciferase reporter vector (Fig. 3B). Luciferase reporter activity showed that overexpression of miR-9 significantly inhibited *ANO1*-WT-driven luciferase activity, whereas it did not inhibit *ANO1*-MT-driven luciferase activity in 293T and HCT116 cells (Fig. 3C). These results indicated that ANO1 is a direct target of miR-9.

miR-9 inhibits CRC cell proliferation, migration, and invasion in HCT116 cells

Next, we performed the MTT assay to evaluate the effect of miR-9 on cell proliferation. Overexpression of miR-9 significantly inhibited the proliferation of HCT116 cells (Fig. 4A), while transfection of CRC cells with anti-miR-9 promoted proliferation (Fig. 4B). To further investigate the effects of ANO1 on growth of CRC cells, we transfected si-ANO1 into HCT116 cells and found that silencing *ANO1* inhibited proliferation *in vitro* (Fig. 4C). To confirm that migration of CRC cells is negatively regulated by miR-9, and to assess the effect of miR-9 on invasion, we performed trans-well chamber assays. Overexpression of miR-9 significantly suppressed the migration and invasion capabilities of HCT116 cells, while anti-miR-9 markedly increased both migration and invasion (Fig. 4D and E). To assess the role of ANO1 in cell migration and invasion, *ANO1* was silenced with si-ANO1 and we found that knock-down of ANO1 suppressed cell migration and invasion in HCT116 cells (Fig. 4F). These results suggest that miR-9 can suppress cell proliferation, migration, and invasion of CRC cells.

miR-9 suppresses the expression of *p*-AKT, cyclin D1, and *p*-ERK by targeting ANO1

Zhao et al. suggested that TMEM16 regulates invasion and migration of rectal carcinoma cells *via* PI3K/AKT, cyclin D1, and ERK [39]. Subsequently, to determine whether ANO1 is regulated by miR-9 in CRC cells, we analyzed the expression of *p*-AKT, cyclin D1 and *p*-ERK by western blot. As shown in Fig. 5A, overexpression of miR-9 reduced ANO1, *p*-AKT, cyclin D1, and *p*-ERK expression compared to the control. Moreover, knock-down of miR-9 increased the protein expression of ANO1, *p*-AKT, cyclin D1, and *p*-ERK *in vitro.* To validate that ANO1 regulated the expression of *p*-AKT, cyclin D1, and *p*-ERK, we silenced *ANO1* expression by transfecting in HCT116 cells with si-ANO1. Compared to the control, silencing ANO1 changed the protein expression of *p*-AKT, cyclin D1, and *p*-ERK in HCT116 cells. To further

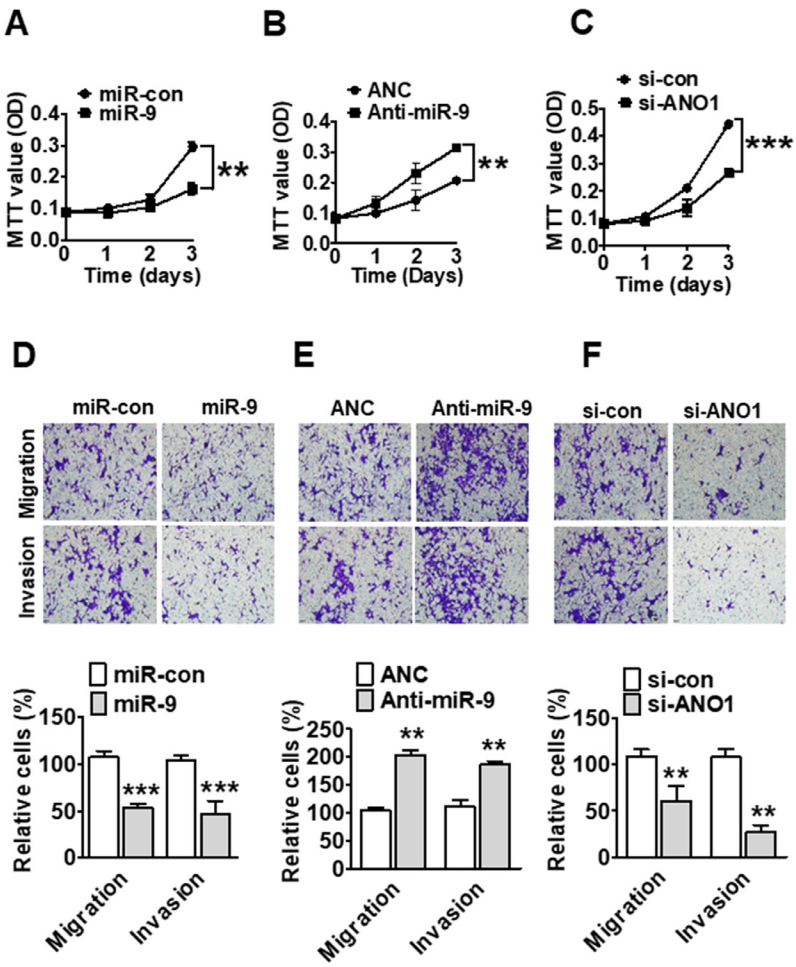

Fig. 4 Overexpression of miR-9 inhibited cell proliferation, migration, and invasion *in vitro*. The MTT assay was performed after transfecting HCT116 cells with miR-9 (A), anti-miR-9(B), and si-ANO1(C). Effect of miR-9 (D), anti-miR-9 (E), or si-ANO1 (F) on the migration and invasion of HCT116 cells was analyzed using trans-well chamber migration and invasion assays after 48 h incubation. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

confirm that *ANO1* was reduced by miR-9, we transfected with anti-miR-9 and si-ANO1 in HCT116 cells. Compared with anti-control group, overexpression of anti-miR-9 increased the expression of ANO1, *p*-AKT, cyclin D1, and *p*-ERK in HCT116 cells, while co-transfection with si-ANO1 ameliorated these increases in expression (Fig. 5B). These result suggest that ANO1 suppress the expression of *p*-AKT, cyclin D1, and *p*-ERK in HCT116 cells.

miR-9 incudes CRC cell apoptosis and cell cycle arrest *in vitro*

Next, the effect of miR-9 on cell apoptosis and cell cycle were analyzed by flow cytometry. Our data showed that the apoptotic rate was significantly suppressed in cells transfected with anti-miR-9 compared with control group, whiles co-transfection with si-ANO1 increased apoptosis rate (Fig. 6A). Moreover, cell cycle analysis showed that proportion of cells in the G0/G1 phase was inhibited in the anti-miR-9 in comparison with control group. By contrast, the transfection of HCT116 cells with si-ANO1 resulted in an accumulation of cells in the G0/G1 phase (Fig. 6B). These findings suggest that overexpression of miR-9 induced cell apoptosis and caused cell cycle arrested at G0/G1 phase in CRC cells.

Discussion

A large number of studies have identified biological processes involved in tumorigenesis and tumor progression, which include cell proliferation, apoptosis, migration, and invasion [3,5]. Here, we investigated whether miR-9 suppressed the proliferation, migration and invasion of CRC cells by negatively regulating ANO1.

miR-9 was first identified as a brain-enriched miR, that functions as an oncogene in brain cancer. Elevated expression of miR-9 is associated with poor survival in medulloblastoma patients and breast cancer patients [14,40]. However, miR-9 has also been reported to function as a tumor suppressor gene in breast, oral squamous cell carcinoma, and gastric cancers [16–19]. Deng et al. found that miR-9 suppressed cell proliferation and invasion in gastric cancer by targeting Cullin4A [41]. In our previously study,

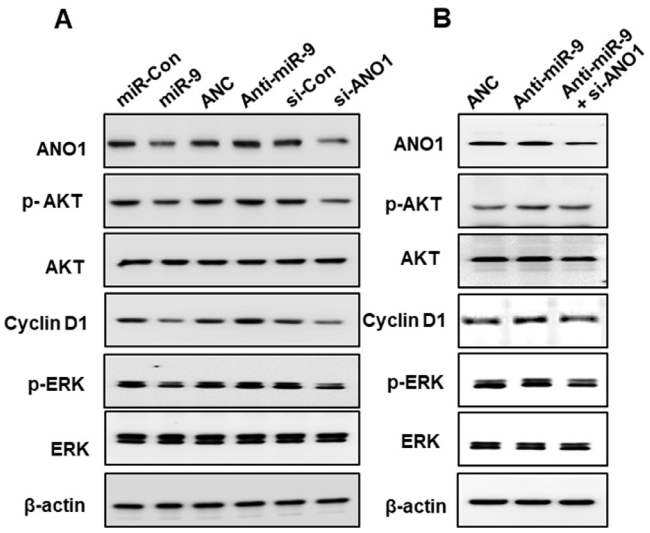

Fig. 5 miR-9 suppressed expression of *p*-AKT, cyclin D1, and *p*-ERK *via* targeting ANO1 in HCT116 cells. HCT116 cells were transiently transfected with miR-9, anti-miR-9, or si-ANO1. (A) Gain or loss of miR-9 regulated the expression level of *p*-AKT, cyclin D1 and *p*-ERK compared with control HCT116 cells. (B) Overexpression of anti-miR-9 increased the protein expression of *p*-AKT, cyclin D1, and *p*-ERK, while expression of these proteins were down-regulated by co-transfection with si-ANO1. Data were obtained from three independent experiments.

we also revealed that miR-9 expression was significantly down-regulated in CRC tissues and was negatively associated with lymph node metastasis and tumor stage in CRC patients. Moreover, overexpression of miR-9 significantly inhibited cell migration and invasion by targeting TM4SF1 in CRC cells [24]. These findings suggest that the effect of miR-9 differs according to cell type and mechanism of tumorigenesis, and miR-9 might play an important role in CRC progression and metastasis.

The ANO1 gene is localized on 11q13 chromosomal region, one of the most frequently amplified chromosomal regions in various human cancers [25]. Clinically, ANO1 overexpressed in many types of cancer including colon cancer, and promote cell proliferation, migration and invasion and which is associated with poor patient survival [30–34,42]. Knockdown of ANO1 induces cell apoptosis and inhibits cell proliferation in prostate cancer cells [36]. However, there are also reports that compared with primary tumors, TMEM16A expression decreases in metastatic lymph node of cancer with squamous cell carcinoma of the head and neck (SCCHN) and cystic fibrosis lung pathology and knockdown of TMEM16A expression suppress tumor proliferation in an orthotopic mouse model [15,43]. These results suggest that ANO1 effect might be mediated by cell type-dependent or another signaling mechanism [43]. In this study, we confirmed that the expression of *ANO1* in CRC patients. We found that *ANO1* mRNA and protein expression were up-regulated in CRC specimens compared with paired normal tissues. Moreover, *ANO1* expression was increased in CRC specimens from patients with lymph node metastasis (N1) and from patients without lymph node metastasis (N0) compared with normal tissues specimens. Additionally, we identified that miR-9 expression and *ANO1* mRNA expression inversely correlated in CRC specimens. To further investigate the function of ANO1 *in vitro*, we analyzed ANO1 mRNA expression in different CRC cell lines by qRT-PCR. When compared to normal colon cell line CCD-18co, ANO1 mRNA expression was increased in CRC cell lines such as DLD-1, SW620, Caco-2, HT-29, and HCT116 cells. Our findings suggest that miR-9 act as a tumor suppressor gene in CRC and associated with CRC metastasis and progression.

Meanwhile, AKT is a serine/threonine-specific protein kinase and is major downstream target of the phosphatidylinositol 3-kinse (PI3K)-AKT-mTOR pathway. Activation of AKT signaling promotes tumor progression by increasing metabolism, proliferation, growth and angiogenesis [44]. Phosphorylation or activation of AKT play an important regulator in various cancer type including CRC [45]. The mitogen-activated protein kinase (MAPK) is also one of the most universal transduction signaling pathway. Activation of ERK/MAPK is promote tumor cell division by the regulation of cell proliferation and differentiation. ERK1/2, as a critical member of MAPKs, mediates the motility in metastatic CRC [44]. In addition, Zhao et al. found that si-TMEM16A suppressed invasion and migration *via* the PI3K/AKT pathway [39]. TMEM16A has been shown to promote cancer progression and invasion by AKT and MAPK signaling in breast cancer and HNSCC [29,46]. Moreover, expression of cyclin D1 was positively associated with p-ERK and TMEM16A expression in human colorectal cancer cells [34]. To further explore the biological functions of ANO1 in CRC cells, *ANO1* was silenced with si-ANO1 and we found that knock down of ANO1 decreased cell proliferation, migration and invasion, and suppressed the protein expression of *p*-AKT, cyclin D1, and *p*-ERK by si-ANO1 *in vitro*.

ANO1 is also a direct target gene of miR-132, and is associated with poor prognosis in colorectal cancer [35]. Cao et al. reported that miR-381 inhibited of cell proliferation and migration *via* regulation of TMEM16A and the TGF-β signaling pathway in gastric cancer [47]. We further identified ANO1 as a downstream target gene of miR-9 using luciferase reporter assay, qRT-PCR and western blot analysis. We identified that the *ANO1* gene as a direct target of miR-9, which is regulated cell proliferation, migration, and invasion in CRC cells. Overexpression of miR-9 suppressed not only *ANO1* mRNA expression and protein expression, but also expression of *p*-AKT, cyclin D1, and *p*-ERK in CRC cells. Thus, we suggested that miR-9/ANO1 can be caused by CRC cell proliferation, migration, and invasion through regulating of p-AKT, and cyclin D1.

Although elevated *ANO1* mRNA expression has been reported to be associated with progression and lymph node metastasis in CRC samples, the prognostic significance of miR-9 could not be determined in the present study because of the small number of clinical samples evaluated. Further studies of large numbers of CRC specimens, another pathway, or *in vivo* assay are required to arrive at a definitive conclusion.

In conclusion, our study provides the evidence that *ANO1* levels were significantly up-regulated in CRC tumor specimens, and associated with lymph node metastasis and late tumor stage. ANO1 is a direct target gene of miR-9, and overexpression of miR-9 suppressed cell proliferation, migration

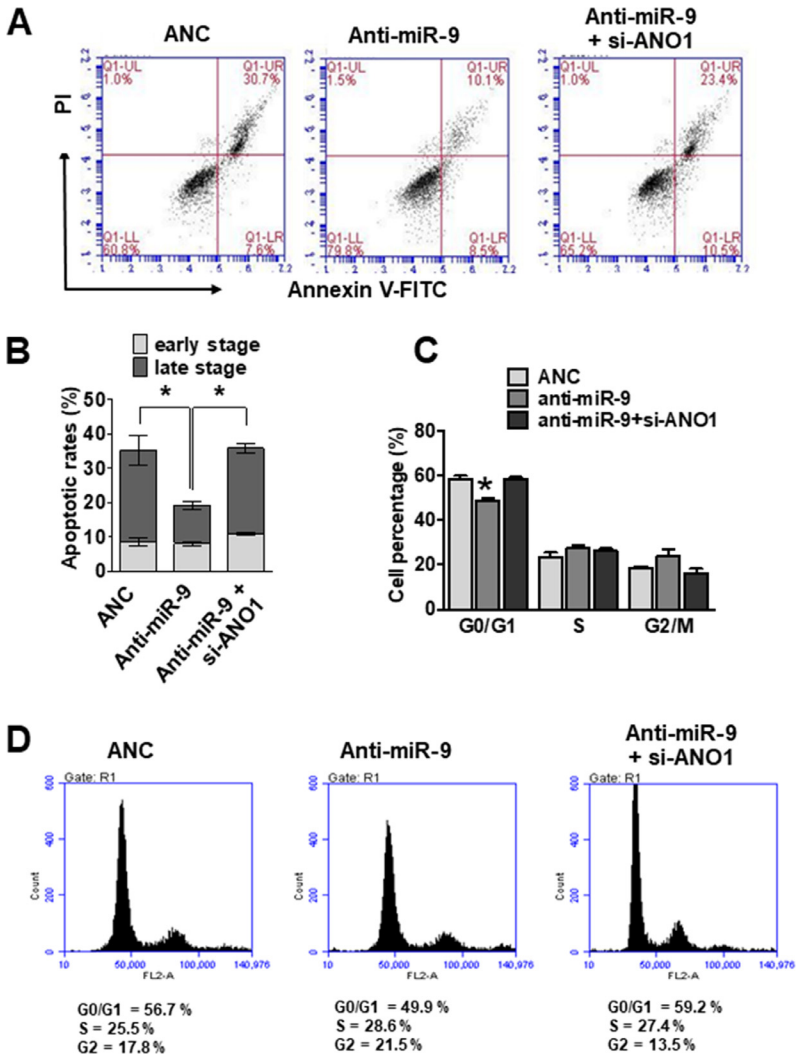

Fig. 6 miR-9 expression promoted CRC cell apoptosis and causes cell cycle arrest. After transfection with ANC, anti-miR-9, or si-ANO1, HCT116 cells were determined by Annexin-V-FITC/PI staining (A). Cell cycle distributions of HCT116 cells were measured by flow cytometry assay (B). Data were obtained from three independent experiments and the *t*-test was used to evaluate differences in luciferase expression ($P < 0.05$).

and invasion. Mechanistically, we confirmed that miR-9 reduced *p*-AKT, cyclin D1, and *p*-ERK expression by targeting *ANO1* in CRC cells. Collectively, miR-9/*ANO1* may serve as a potential diagnosis and therapeutic target for in human CRC.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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