

RNAi-mediated *HOXD3* knockdown inhibits growth in human RKO cells

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Received January 25, 2016; Accepted March 2, 2016

DOI: 10.3892/or.2016.4993

Abstract. Numerous studies have shown that the multifunctional Homeobox-containing (*HOX*) D3 gene is involved in various physiological and pathological processes. To elucidate the role and mechanism of *HOXD3* in colorectal cancer (CRC), we measured its expression in five CRC cell lines. After determining that *HOXD3* was highly expressed in the human RKO cancer cell line, we used lentiviral-mediated small interfering RNAs (siRNAs) to knock down *HOXD3* expression and assessed proliferation, cell cycle distribution, apoptosis and colony formation using cell proliferation, flow cytometric, and colony formation assays. The expression of *HOXD3* was strongly suppressed in the RKO cells infected with the lentiviruse expressing an *HOXD3* short hairpin RNA (shRNA). The downregulation of *HOXD3* expression in RKO cells significantly decreased proliferation and colony formation, and increased apoptosis *in vitro*, compared to the cells infected with the mock control ($p < 0.01$). Moreover, specific downregulation of *HOXD3* led to the accumulation of cells at the G₂ phase of the cell cycle. Our findings revealed that the *HOXD3* gene promotes CRC cell growth and plays a pivotal role in the development and survival of malignant human colorectal cancer cells.

Introduction

Worldwide, colorectal carcinoma (CRC) is one of the most common malignant cancers and is the second most frequent cause of cancer-related deaths (1-3). Despite marked advances in diagnostic and therapeutic approaches, the prognosis for CRC patients remains poor (4). Consequently, there is an urgent need to develop new CRC treatment methods.

Since homeobox-containing (*HOX*) genes encode DNA-binding proteins, they are master transcriptional

regulators of cell differentiation, morphogenesis, and organogenesis during development (5,6). In humans and other mammals, 39 *HOX* genes are clustered in four complexes called *HOXA*, *B*, *C*, and *D*. These are further subdivided into 13 paralogous groups based on their sequence similarities and relative positions along the clusters (7-11). Mutational analyses demonstrated that *HOXD* genes play a pivotal role in determining the regional specificity of cells (12,13). For example, the *HOXD13* gene is involved in the normal morphogenesis of limbs (12) and of the anal sphincter (13). *HOXD3* is a member of the third paralogous group of the *HOXD* gene family and is involved in embryonic development (14,15).

Studies have shown that the human *HOXD3* gene plays a multifunctional role in cancer. For example, *HOXD3* overexpression was found to regulate cell adhesion in human erythroleukemia HEL cells (16), induce coordinate metastasis-related gene expression, and enhance the motility and invasiveness of human lung cancer A549 (17-19) and melanoma cells (20). Additionally, studies have shown that *HOXD3* induces angiogenesis by increasing pro-angiogenic molecules (21-25). While these studies clearly demonstrated that *HOXD3* is involved in the development and growth of various types of cancers, the functional role of *HOXD3* in human CRC has not yet been determined.

In the present study, we demonstrated that *HOXD3* is highly expressed in the human CRC RKO cell line. Consequently, we used a lentiviral vector to deliver small interfering RNA (siRNA) to knock down *HOXD3* expression in the RKO cells. Finally, we assessed the effects of *HOXD3* knockdown on human CRC cell growth and survival *in vitro*.

Materials and methods

Cell lines. DLD-1, HCT-116, SW620, HT-29, and RKO human colon carcinoma cell lines were purchased from the Shanghai Cell Bank of the Chinese Academy of Science (Shanghai, China). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Corning, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Ausbian, Australia) at 37°C in a 5% CO₂ incubator.

Quantitative RT-PCR. Total RNA was extracted from the cells in each group (DLD-1, HCT-116, SW620, HT-29, and RKO)

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Key words: colorectal cancer, *HOXD3*, RKO cells, proliferation, colony formation, apoptosis

using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Two micrograms of total RNA from each sample was reverse transcribed to single-stranded cDNA. One microgram of cDNA was used as a template for the quantitative real-time PCR. The primers used were as follows: *HOXD3* forward, 5'-CGG CAA CTT CGT CGA GTC C-3' and reverse, 5'-ATG AGG GTC GCA AGG TCC A-3'; and *GAPDH* forward, 5'-TGA CTT CAA CAG CGA CAC CCA-3' and reverse, 5'-CAC CCT GTT GCT GTA GCC AAA-3'. Cycling conditions for quantitative RT-PCR were as follows: 95°C for 30 sec, then 45 cycles of 95°C for 5 sec and 60°C for 30 sec. The PCR products of *HOXD3* and *GAPDH* were 145 and 121 bp, respectively. The data were quantified using the $2^{-\Delta\Delta C_t}$ method. All analyses were performed in triplicate.

Recombinant lentiviral vector production and cell infection. To create the RNAi target site, the complementary DNA sequence (CCA AAT CAC AGC CCA ATA T) of *HOXD3* was designed by Shanghai GeneChem Co., Ltd. (Shanghai, China) using the full-length human sequence (GenBank no. NM_006898). The *HOXD3* hairpin oligonucleotides were synthesized and inserted into the pGV115-GFP (GeneChem Co. Ltd.) lentiviral vector. Lentivirus particles were prepared as previously described (26).

For lentiviral infection, RKO cells were cultured in 6-well plates. The *HOXD3*-siRNA lentivirus (sh*HOXD3*) or negative control lentivirus (shCtrl) was added according to a multiplicity of infection (MOI 1:5). At 3 days post-infection, the cells were observed for presence of the GFP marker with a fluorescence microscope (MicroPublisher 3.3RTV; Olympus, Tokyo, Japan). At 5 days post-infection, the cells were harvested and knockdown efficiency was analyzed by quantitative RT-PCR and western blot analysis.

Western blot analysis. While on ice, cell lysates were incubated for 10-15 min in ice-cold lysis buffer (100 mM Tris, pH 6.8, 2% β -mercaptoethanol, 20% glycerol, 4% SDS). The lysates were centrifuged at 12,000 x g for 15 min at 4°C, and the supernatants were collected. Protein concentration was determined using a BCA protein assay kit (Beyotime, Beijing, China). An equal amount of total protein from each sample was partially separated in a 10% SDS-PAGE gel and blotted onto PVDF membranes. Membranes were incubated with GFP or GAPDH primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) secondary antibody at room temperature. Enhanced chemiluminescence (ECL) reagent (ECL-Plus/kit; Amersham, Piscataway, NJ, USA) was used for detection. The amount of GAPDH detected was used as the protein loading internal control.

Cell proliferation assay. After being infected with the shCtrl lentivirus or sh*HOXD3* lentivirus, RKO cells were seeded in 96-well plates at a concentration of 2,000 cells/well and incubated for 5 days at 37°C with 5% CO₂. The cells were counted each day using the Cellomics ArrayScan™ VT1 HCS automated reader (Cellomics Inc., Pittsburgh, PA, USA). At least 800 cells/well were analyzed. At 1, 2, 3, 4, or 5 days

post-infection, the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml; Promega, Shanghai, China) at a final concentration of 0.5 mg/ml for 4 h. After discarding the supernatants, 150 μ l of dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., LLC, St. Louis, MO, USA) was added to each well. The plates were read at 490 nm using an ELISA reader (Tecan Infinite, Männedorf, Switzerland). All experiments were performed in triplicate.

Cell cycle distribution analysis. Flow cytometry (FCM) was used to determine cell cycle distribution as previously described (27). Briefly, RKO cells were infected with shCtrl or sh*HOXD3* vector and incubated at 37°C for 1, 2, 3, 4, or 5 days. At the indicated time-point, the cells were collected and washed with ice-cold phosphate-buffered saline (PBS). Cells were centrifuged at 1,000 x g for 10 min and then fixed in ice-cold 70% ethanol for 30 min at 4°C. The cells were washed with PBS and then resuspended and incubated in PBS containing 50 μ g/ml propidium iodide (PI; Sigma-Aldrich) and 100 μ g/ml RNase A (Fermentas, Shanghai, China) in the dark at 4°C for 30 min. Cell cycle phase was analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). All studies were performed in triplicate.

Analysis of apoptosis. FCM was used to measure apoptosis and was performed as previously described (28). At 48 h prior to transfection, the RKO cells were seeded in 6-well plates. At 72 h post-transfection, the cells were collected and washed twice with ice-cold PBS. Cell densities were adjusted to 1x10⁶/ml using 1X staining buffer. Cell suspension (100 μ l) and 5 μ l Annexin V-APC (eBioscience, San Diego, CA, USA) were thoroughly mixed and incubated in darkness at room temperature for 15 min. All rates of apoptosis were measured by FCM within 1 h. Each experiment was performed in triplicate.

Cell colony formation assay. At 72 h post-transfection, the RKO cells were reseeded at 600 cells/well in 6-well plates and cultured at 37°C for 10 days. The cells were collected and washed with ice-cold PBS. When a majority of single colonies contained more than 50 cells, the samples were fixed using 1 ml/well of 4% paraformaldehyde (Sinopharm, Shanghai, China) for 30 min at 37°C. According to instructions, the samples were stained with 500 μ l of Giemsa stain (Dingguo Bio, Shanghai, China) at room temperature for 10 min and images were acquired. All experiments were performed in triplicate.

Statistical analyses. Data for each group are presented as the mean \pm SD. Statistical analyses were performed using SPSS for Windows, version 20.0 (IBM SPSS, Chicago, IL, USA). Values of p<0.05 were deemed statistically significant.

Results

***HOXD3* mRNA levels in colorectal cancer cell lines.** *HOXD3* mRNA expression was measured in DLD-1, HCT-116, SW-620, HT-29, and RKO CRC cell lines by RT-PCR. The results showed that *HOXD3* mRNA was highly expressed in the RKO cell line (Fig. 1).

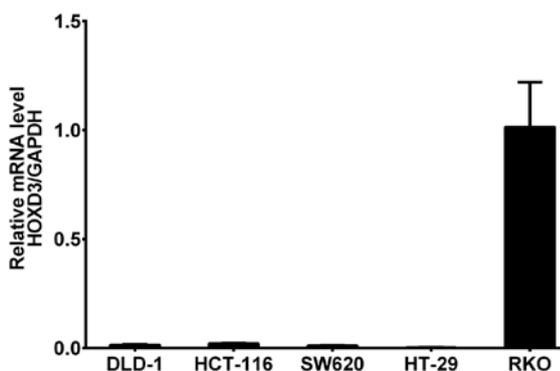


Figure 1. *HOXD3* mRNA levels in five colorectal cell lines. Expression of *HOXD3* mRNA was measured by RT-PCR and normalized to GAPDH in the indicated cell lines.

Lentiviral-mediated knockdown of *HOXD3* in RKO cells. To explore the role of *HOXD3* in CRC, RKO cells were infected with the shCtrl lentivirus or sh*HOXD3* lentivirus. As shown in Fig. 2A, by 3 days post-infection, the proportion of infected RKO cells was greater than 80% in both the sh*HOXD3* and shCtrl groups. At 5 days post-infection, *HOXD3* mRNA levels were measured by real-time PCR. sh*HOXD3* infected cultures had significantly lower levels of *HOXD3* mRNA when compared to the shCtrl-infected cultures (Fig. 2B). Fig. 2C shows *HOXD3* protein expression as detected by western blot analysis. *HOXD3* levels were greatly reduced in the sh*HOXD3* group, indicating effective knockdown of the target sequence.

***HOXD3* knockdown suppresses RKO cell proliferation.** To examine the effect of *HOXD3* knockdown on cell growth, shCtrl and sh*HOXD3* infected RKO cells were reseeded in 96-well plates and analyzed at 1, 2, 3, 4, and 5 days post-infection. As illustrated in Fig. 3A and B, shCtrl cells exhibited extensive proliferation at 5 days post-infection, while the number of sh*HOXD3* cells increased slightly. Cell growth rate was defined as: Cell count on day n/cell count on day 1, where n=2, 3, 4, or 5 (Fig. 3B). These results revealed that *HOXD3* knockdown significantly inhibited the proliferation of RKO cells.

The effect of *HOXD3* protein reduction on RKO cell proliferation was also determined by MTT assay. Although shCtrl and sh*HOXD3* cells had similar *in vitro* growth on days 1, 2, and 3, the sh*HOXD3* cells had significantly reduced *in vitro* growth on days 4 (shCtrl: 5.41 ± 0.03 vs. sh*HOXD3*: 2.90 ± 0.04 , $p < 0.01$) and 5 (shCtrl: 7.88 ± 0.12 vs. sh*HOXD3*: 3.56 ± 0.12 , $p < 0.01$) (Fig. 3C). Based on these data, *in vitro* RKO cell growth was dependent on *HOXD3* expression.

***HOXD3* knockdown leads to cell cycle arrest in the RKO cells.** To determine whether *HOXD3* is necessary for cell cycle progression in RKO cells, we measured cell cycle phases by FCM (Fig. 4A). The shCtrl group had the following distribution: G₁ phase: $48.28 \pm 0.16\%$, S phase: $40.46 \pm 1.46\%$, G₂ phase: $11.26 \pm 1.48\%$. The sh*HOXD3* group, however, had this distribution: G₁ phase: $44.83 \pm 0.31\%$, S phase: $36.56 \pm 0.77\%$, G₂ phase: $19.07 \pm 0.79\%$. As shown in Fig. 4B, sh*HOXD3* cells had significant decreases in the percentage of cells in the G₁ ($p < 0.01$) and S phases ($p = 0.015$), compared to the shCtrl cells.

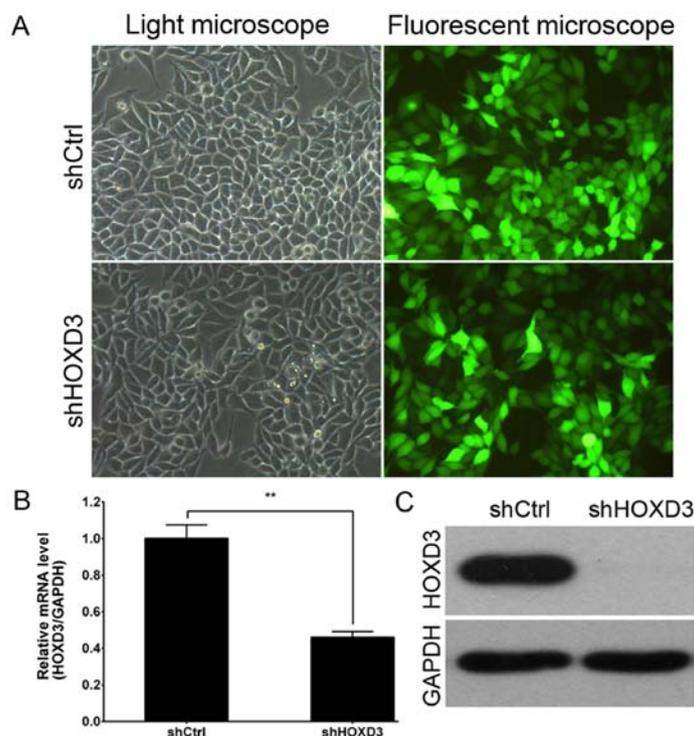


Figure 2. *HOXD3* knockdown in RKO cells infected with sh*HOXD3* or shCtrl lentiviral vectors. RKO cells were infected with the sh*HOXD3* or shCtrl lentivirus. (A) Infection efficiency as determined by light and fluorescence microscopy at 3 days post-infection. Original magnification x200. Representative images of the cultures are shown. (B) At 5 days post-infection, *HOXD3* mRNA levels were measured by qPCR and normalized to GAPDH. ** $p < 0.01$. (C) *HOXD3* protein expression was analyzed by western blot analysis in the shCtrl and sh*HOXD3* groups. GAPDH was used as an internal control.

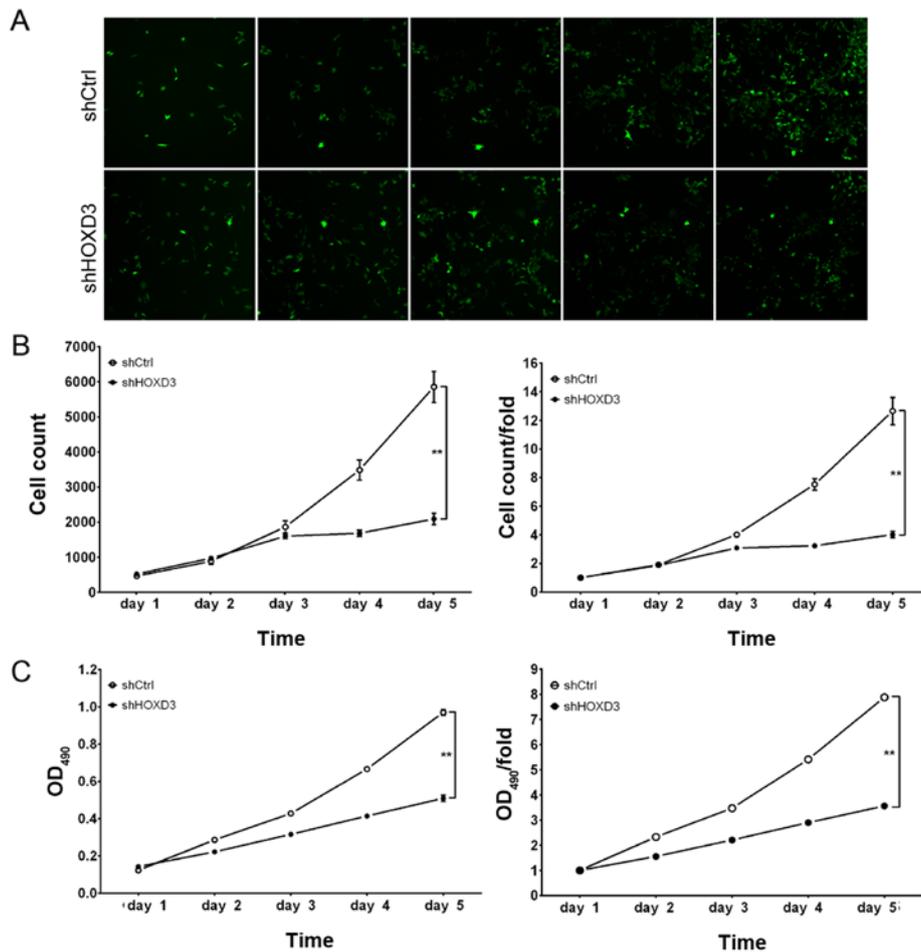


Figure 3. Effect of *HOXD3* knockdown on RKO cell growth. (A) Representative fluorescence microscopy images of cell growth taken daily after lentiviral infection. (B) Post-infection daily cell counts as measured by automated reader (shCtrl vs. shHOXD3 at days 4 and 5, $p < 0.01$). (C) Post-infection cell proliferation as measured by MTT assay. Each data point represents the mean \pm SD of absorbance values (OD_{490}) ($^{**}p < 0.01$, shCtrl vs. shHOXD3 at days 4 and 5).

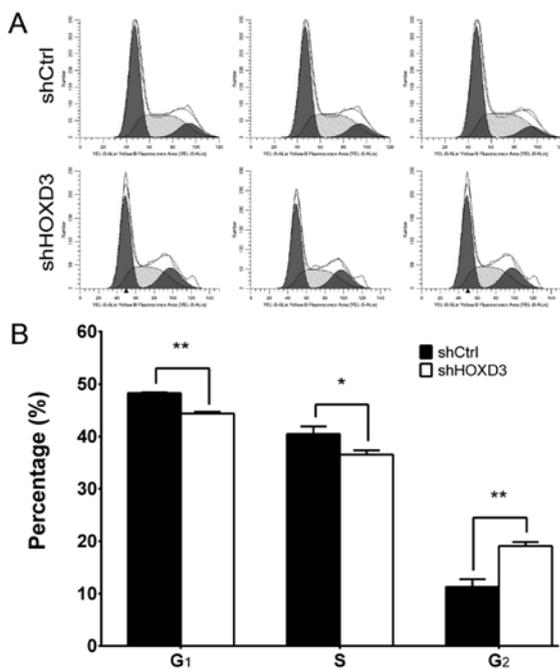


Figure 4. *HOXD3* knockdown leads to cell cycle arrest. (A) Cell cycle distribution of the RKO cells was analyzed by FCM. Graphs in triplicate for each treatment group are shown. (B) Cell cycle phase distribution is expressed as a percentage of total cells. $^{**}p < 0.01$; $^{*}p = 0.015$.

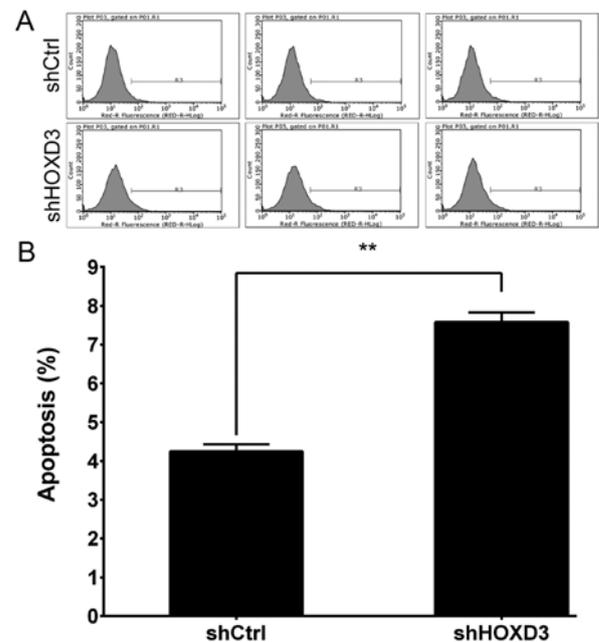


Figure 5. *HOXD3* knockdown increases RKO cell apoptosis. (A) Apoptosis as determined by Annexin V staining and FCM. Graphs in triplicate for each treatment group are shown. (B) Percentage of apoptosis quantified from the results shown in (A). $^{**}p < 0.01$.

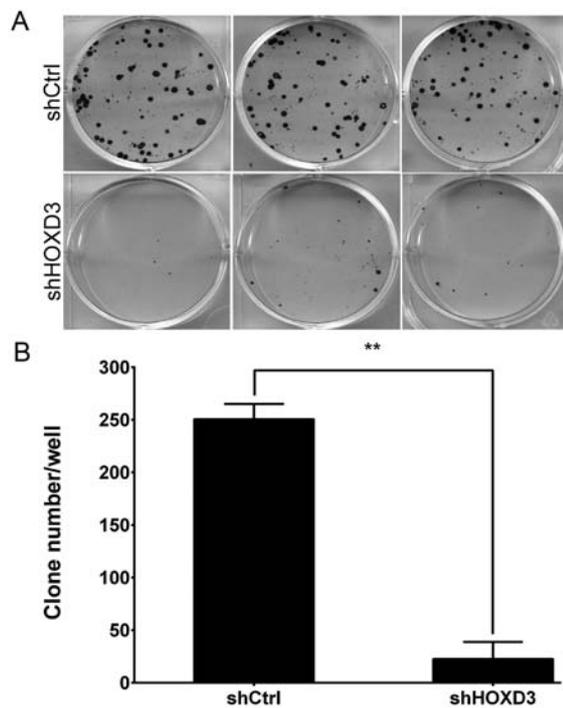


Figure 6. *HOXD3* knockdown inhibits RKO cell colony formation. (A) Photomicrographs of Giemsa-stained RKO colonies in 6-well plates 10 days post-infection. (B) An average of the number of cells in each cell colony was counted per well. ** $p < 0.01$.

Conversely, when compared to the shCtrl cells, the percentage of shHOXD3 cells in the G₂ phase ($p < 0.01$) was increased. Taken together, these data suggest that *HOXD3* regulates cell growth and can block cell cycle progression in the G₂ phase.

HOXD3 knockdown increases RKO cell apoptosis. To test whether *HOXD3* expression affects RKO cell death, we knocked down *HOXD3* and measured apoptosis. Annexin V staining followed by FCM was used to determine cell apoptosis (Fig. 5A). As shown in Fig. 5B, apoptosis was significantly increased in the shHOXD3 group compared to the shCtrl group (shCtrl: $4.24 \pm 0.19\%$ vs. shHOXD3: $7.58 \pm 0.25\%$, $p < 0.01$). These results indicate that *HOXD3* expression is an important determinant of apoptosis in RKO cells.

HOXD3 knockdown inhibits colony formation in RKO cells. Finally, we used Giemsa staining to measure the effects of *HOXD3* knockdown on colony formation in RKO cells (Fig. 6A). As shown in Fig. 6B, the cell number in a single colony was significantly reduced in the shHOXD3 group compared to the shCtrl group (shCtrl: 251 ± 14 vs. shHOXD3: 23 ± 16 , $p < 0.01$). This results indicate that reduction of endogenous *HOXD3* expression levels significantly inhibits colorectal carcinoma growth.

Discussion

Colorectal cancer is the third most common global cancer, and half of CRC patients die within 5 years of diagnosis (1). Importantly, gene therapy is being investigated as a potential cancer treatment method (29). Despite recent advances,

however, the prognosis of patients with CRC remains poor. In order to develop new therapeutic treatments, it is particularly important to unravel the underlying mechanisms of CRC development and progression.

To our knowledge, *HOXD3* expression in CRC has been largely unstudied. The present study is the first to measure *HOXD3* expression in five CRC cell lines and find high levels of expression in RKO cells. Related to our findings, previous studies have shown that abnormal *HOXD3* expression is associated with oncogenesis and tumor suppression (14,15,17). One study showed that samples from patients with invasive breast cancer had high *HOXD3* expression levels, and those patients had poor 5-year survival rates (30). Additionally, *HOXD3* overexpression in lung cancer A549 cells led to increased expression of the adhesion molecule E-cadherin (17). Another study determined that *HOXD3* overexpression altered the adhesive properties of erythroleukemia HEL cells (16).

In order to assess *HOXD3* function in CRC cell lines, we constructed the shHOXD3 lentiviral vector, which efficiently silenced *HOXD3* in the RKO cell line. Compared to shCtrl cells, the shHOXD3-infected cells had decreased proliferation and significantly decreased proportions of cells in the G₁ and S cell cycle phases. Significant increases in the G₂ phase population were also detected by FCM in the shHOXD3-infected cells. Additionally, *HOXD3* knockdown increased apoptosis and decreased colony formation in the RKO cells. Taken together, these results suggest that *HOXD3* promotes RKO cell growth. Further study is ongoing to validate the anti-apoptotic role of *HOXD3* in other CRC cell lines.

In conclusion, we demonstrated in RKO cells that downregulation of *HOXD3* expression by RNA interference inhibited cell proliferation and induced cell apoptosis. Therefore, in CRC cases where *HOXD3* is overexpressed, *HOXD3* knockdown by lentivirus-siRNA may be a valuable candidate treatment.

Acknowledgements

This study was supported by the Anhui Natural Science Research Project (no. KJ2014A147).

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