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MicroRNA-141 regulates the tumor suppressor DLC1 in colorectal cancer

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Our previous study has showed that DLC1 acts as a functional tumor suppressor in colorectal cancer (CRC) cell lines. The aims of this study were to determine whether DLC1 is a target of MicroRNA (miRNA) regulation and to evaluate the role of this mechanism in CRC. By bioinformatics approach and literature, miR-141 was chosen for further study. The miR-141 mimic, miR-141 inhibitor were synthesized and transfected to Lovo cells. Cell growth was determined by MTT and *in vivo* models. The flow cytometric analysis for cell cycle determination and transwell assays for evaluating the cell invasion were used. Luciferase reporter assays and Western blots showed that DLC1 was a direct target of miR-141 in CRC. The expression levels of miR-141 were obviously up-regulated in CRC tissues compared to non-cancerous tissues, while *DLC1* expression levels were down-regulated in a high proportion of clinical samples (14/18). In addition, correlation analyses revealed negative correlation between miR-141 levels and *DLC1* expression levels in CRC tissues. MiR-141 overexpression promoted cell growth *in vitro* and *in vivo*, promoted cell cycle progression and invasion in Lovo cells. Furthermore, re-introduction of DLC1 in miR-141-overexpressing Lovo cells decreased growth rate of cells, increase of the percentage in G0/G1 phase and decreased the number of migrating cells. In conclusion, we demonstrated that miR-141 is up-regulated in CRC and acts as a functional oncogene by targeting DLC1.

Key words: colorectal cancer, miR-141, DLC1

Deleted in Liver Cancer 1 (*DLC1*) was first identified as a putative tumor suppressor gene from human hepatocellular carcinoma (HCC) [1]. It was mapped to chromosome 8p21.3-22, a region recurrently deleted in HCC. As its name implied, it was suspected to be a tumor suppressor gene involved in HCC. Since the identification of *DLC1* in 1998, accumulating studies have characterized *DLC1* as a bona fide tumor suppressor in diverse human cancers [2-5]. Our previous study has showed that restoration of *DLC1* expression in colon cancer HT29 cells significantly inhibited cell proliferation and migration, induced apoptosis and cell cycle arrest [6]. In contrast, inhibition of *DLC1* gene expression by RNA interference in LoVo cell line caused adverse effects [7].

DLC1 encodes a protein comprising three major functional domains, namely, sterile alpha motif (SAM) at the N-terminus, and RhoGAP and steroidogenic acute regulatory (StAR)-related lipid transfer (START) domains at the C-terminus [8]. The linker region between the SAM and RhoGAP domains has recently been found to be a crucial region for DLC1's focal adhesion localization, interacting activities and phosphorylation [9]. Expression of the RhoGAP mutant of DLC1 fails to inhibit cell proliferation, migration and invasion, attesting to the importance of RhoGAP activity for the biological activities of DLC1 [10, 11]. Nonetheless, previous studies have shown RhoGAP-independent signaling abilities of DLC1 in various pathways within the different cellular models [12, 13].

However, the mechanisms of the DLC1 are more complex than people expected. Recent reports have provided evidence that DLC1 interacts with many proteins through its various domains to regulate its own activity [14]. Thus, post transcriptional mechanism may play an important role in the function regulation of *DLC1* gene. MicroRNAs (miRNAs) represent an important transcriptional regulation mode. These small RNA molecules of approximately 21–25 nucleotides encoded in the genomes of plants and animals are highly conserved [15]. They suppress gene expression either by repressing translation or by direct sequence-specific cleavage through the action of the RNA-induced silencing complex (RISC) following binding to the 3'-untranslated region (3'-UTR) of mRNA [16]. Previous studies have identified miR-141 and miR-200b as candidate regulators of DLC1 in HCV-infection and non-small cell lung cancer (NSCLC) [17, 18]. Thus, the aims of this study were

to determine whether DLC1 is a target of miRNA regulation and to evaluate the role of this mechanism in colorectal cancer (CRC).

Materials and methods

Cell lines and culture. Lovo cell line was purchased from the Shanghai Cell Institute Country Cell Bank. The cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China), 100 μg/mL streptomycin and 100 U/mL penicillin (Beyotime, Shanghai, China), in a 5.0% humidified CO2 atmosphere at 37°C.

Clinical specimens. Fresh cancer tissues and matched adjacent non-cancerous tissues were obtained from 18 CRC patients at Jiangsu Cancer Hospital. The samples were snap-frozen in liquid nitrogen in the operating room and stored at -80°C until total RNA extraction. Prior written informed consent and Institutional Ethics Committee approval were obtained for the use of these clinical materials for research purposes.

Quantitative reverse transcription-polymerase chain reaction (qPCR). Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen Life Technologies; Carlsbad, CA, USA). Primescript RT Reagent (Takara, Dalian, China) was used to synthesize cDNA. qRT-PCR was performed using SYBR-Green (Takara) on an ABI7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The sequences of primers were as follows: DLC1 forward, 5'-GAG CCG ATG TCG TAA TTC-3'; and reverse, 5'-TCC AAC AGG TCT ACA TCC-3; GAPDH forward, 5'-TCA CCC ACA CTG TGC CCA TCT ACGA-3'; and reverse, 5'-CAG CGG AAC CGC TCA TTG CCA ATGG-3'; hsa-miR-141 forward, 5'-CGC TAA CAC TGT CTG GTA AAG-3'; and reverse, 5'-GTG CAG GGT CCG AGGT-3'; U6 forward, 5'-ATT GGA ACG ATA CAG AGA AGA TT-3'; and reverse, 5'-GGA ACG CTT CAC GAA TTTG-3'. Samples were normalized to U6 or GAPDH. Relative expression was calculated by $2^{-\Delta\Delta Ct}$.

miRNAs transfection. The miR-141 mimic (miR-141), miR-141 inhibitor (miR-141-in) and their respective controls (NC and NC-in) were designed and synthesized by GeneP-harma (Shanghai, China). Lovo cells were seeded in 6-well plates at a density of 50%, 24 h later the cells were transfected with miRNAs using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Dual Luciferase Activity Assay. Wild-type and mutant DLC1 3'UTR reporter and control construct were purchased from GENECHEM (Shanghai, China). HEK293T cells over-expressing miR-141 and miR-NC cultured in 48-well plates were cotransfected with 1.5 μg of firefly luciferase reporter and 0.35 ng Renilla luciferase reporter with Lipofectamine 2000 reagents. 24 hours posttransfection, firefly luciferase activities were measured using the Dual Luciferase Assay (Promega, Madison, WI) and the results were normalized with Renilla luciferase according to the manufacturer's protocol.

Western blotting. Cell lysates were prepared by standard methods, then 20 μg of protein samples were separated by 5% SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membrane. After blocked with a buffer containing 5% low fat milk and 0.1% Tween-20 in Tris-buffered saline (TBST), the membrane was incubated with the primary (GAPDH, DLC1) and secondary antibody. Finally, the results were photographed with ECL substrate. RhoA-GTP Assay was performed according to previous report [19]. Antibodies were purchased from Univ-bio Inc (Shanghai, China).

Cell growth analysis. The cells were seeded onto 96-well plates at a density of 2×10^3/well in a final volume of 100 μl. After incubation for 24, 48 and 72 h, 20 μl MTT (Merck Millipore, Billerica, MA, USA) (5 mg/ml) in phosphate-buffered saline was added to each well and the cells were incubated at 37°C for a further 4 h. A total of 150 μl dimethyl sulfoxide was added to the cells in each well after the supernatants were discarded. The absorbance of each well was measured at 490 nm using a microplate reader.

Flow cytometric analysis. Cells were harvested directly or 48 h after transfection and washed with ice-cold phosphate-buffered saline (PBS). The PI/RNase staining kits (Multisciences Biotech, Hangzhou, China) were used to detect cell cycle in a FACScan instrument (Becton Dickinson).

Transwell assays. Transwell (24-well) chambers (Costar, Cambridge, MA, USA) were used to evaluate cell invasion according to NTP treatment as described previously [20]. Initially, fibronectin (2 μg/filter) was dissolved in 100 μl of MEM and poured into the upper part of the polyethylene filter (pore size, 8 μm). The wells were coated overnight in a laminar flow hood. Then, 10^5 cells (in 100 μL of growth medium) were added to the top of the filter in the upper well. The chamber was incubated for 24 h in 5% CO_2 at 37°C. Finally, attached cells in the lower section were stained with H&E, and counted using light microscopy.

In vivo models and immunohistochemistry. All animal studies were approved by the Animal Care and Use Committee of Nanjing Medical University, China. The stable miR-141-over-expression cell line Lovo-miR-141 was generated by lentivirus system expressing miR-141-GFP as described previously [21]. Lovo-miR-141 or Lovo-NC cells (1×10^6) were injected into the right back of nude mice (4 weeks of age, male, BALB/c; n= 5 mice in each group). The subcutaneous tumors were weighed and the length and width of the tumors were measured with calipers. Tumor volume was calculated using the formula: length×width^2 ×0.5. After antigen retrieval in sodium citrate buffer and quenching of endogenous peroxidase activity with 3% H_2O_2 in methanol, tissue sections (5μm thick) were incubated with primary antibody against proliferating cell nuclear antigen (PCNA, Abcam, Cambridge, UK). The Envision+ peroxidase system and DAB-chromogen were applied (Dako Japan Inc., Tokyo, Japan). Sections were counterstained with hematoxylin.

Statistical analysis. Results for continuous variables were expressed as mean ± SD. Differences were assessed using Student's t-test and value of $P<0.05$ considered to be statisti-

cally significant. All analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

miR-141 directly suppress DLC1 gene expression in CRC cell line. By employing open access softwares (TargetScan, miRBase Targets, and PicTarget) and referring to the literature, miR-141 was chosen as a preferred putative microRNA to regulate *DLC1* gene expression (Figure 1A). The relative luciferase activity of HEK293T cells transfected with miR-141 in the presence of the wild-type DLC1 3'-UTR was significantly suppressed, whereas that containing the mutant DLC1 3'-UTR was unaffected (Figure 1B), indicating that miR-141 may suppress *DLC1* gene expression through the 3'-UTR binding and silencing of *DLC1* mRNA.

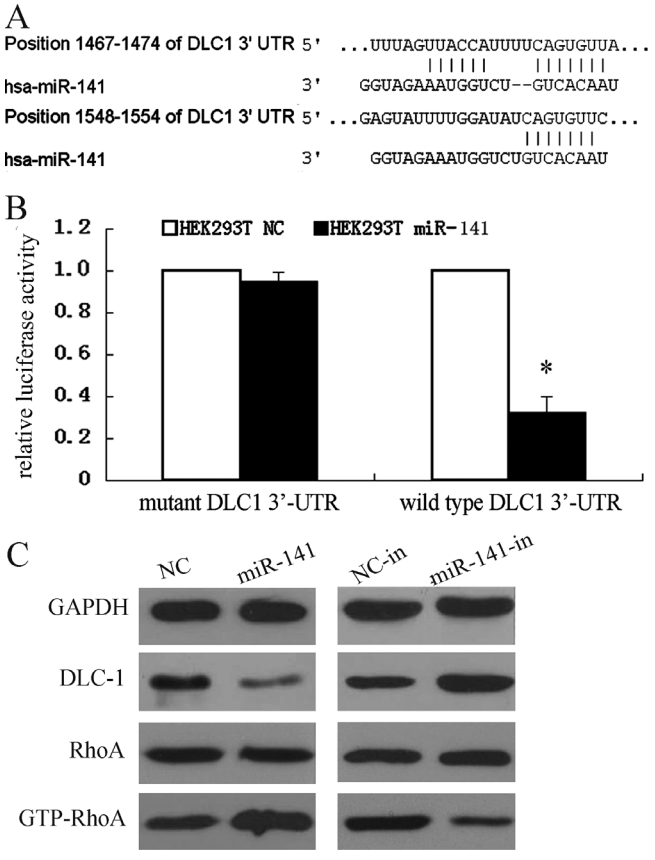

Figure 1. miR-141 directly suppresses *DLC1* gene expression in CRC cell line. (A) The complementary sites of miR-141 in DLC1 3'-UTR are shown as predicted by bioinformatics analysis. (B) Luciferase reporter assays in HEK293T cells with cotransfection of DLC1 3'-UTR and miR-141 mimic as indicated. The relative luciferase activity of DLC1 3'-UTR and miR-141 cotransfection group was significantly suppressed. (C) The inverse correlation between miR-141 expression and DLC1 protein levels in Lovo cells was verified via western blot analysis. RhoA-GTP level was determined by pull-down assay. NC, miR-141 mimic control; miR-141, miR-141 mimic; NC-in, miR-141 inhibitor control; miR-141-in, miR-141 inhibitor.

Further, the inverse correlation between miR-141 expression and DLC1 protein levels was verified via Western blot analysis (Figure 1C). The expression levels of *DLC1* were obviously decreased in Lovo cells transfected with miR-141 mimics, but were increased after inhibition of miR-141. The protein levels of GTP-RhoA were also increased in miR-141-overexpressing cells as a result of miR-141 targeted reduction of *DLC1* expression levels, but were decreased after inhibition of miR-141 (Figure 1C). These results were consistent with the functions of DLC1 to inhibit activity of RhoA.

miR-141 was up-regulated in CRC specimens and inversely correlated with DLC1 levels. We determined the

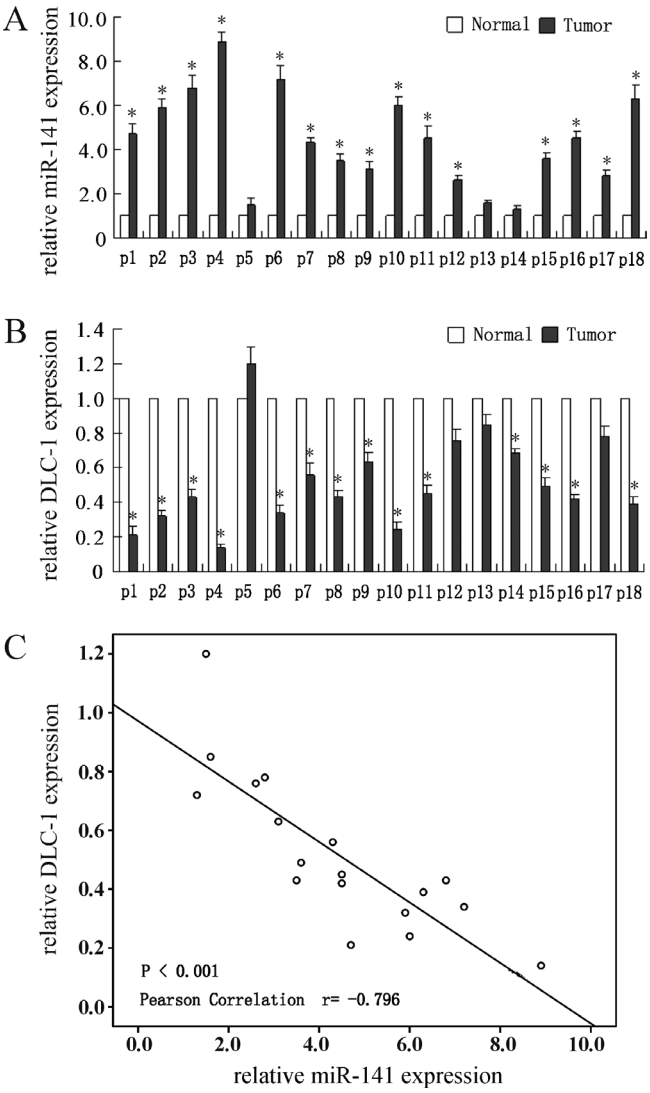

Figure 2. miR-141 is up-regulated in CRC specimens and inversely correlates with DLC1 levels. (A) the expression levels of miR-141 were obviously up-regulated in CRC tissues compared to that in non-cancerous tissues; (B) *DLC1* expression levels were down-regulated in a high proportion of clinical samples; (C) miR-141 levels were negatively correlated with *DLC1* expression levels in CRC tissues.

expression patterns of miR-141 and DLC1 by qRT-PCR in 18 pairs of CRC and matched adjacent non-cancerous tissues. As shown in Figure 2A, the expression levels of miR-141 were obviously up-regulated in CRC tissues compared to non-cancerous tissues, while *DLC1* expression levels were down-regulated in a high proportion of clinical samples (14/18). In addition, correlation analyses revealed the negative correlation between miR-141 and *DLC1* expression levels in CRC tissues (Figure 2B). These findings further demonstrate that miR-141 negatively regulates DLC1 in CRC.

miR-141 promotes proliferation and migration of CRC cells in vitro. To explore the biological function of miR-141, we transfected a CRC cell line, Lovo, with chemically synthesized miR-141 mimic (miR-141), miR-141 inhibitor (miR-141-in) or the corresponding negative controls (NC). The expression levels of miR-141 were measured by qRT-PCR (Figure 3A). MTT assays showed that overexpression of miR-141 promoted the viability of Lovo cells, while inhibition of miR-141 had the opposite effect on cell proliferation, suggesting a growth-promoting role of miR-141 in CRC (Figure 3B). Flow cytometry analysis also showed that miR-141 significantly decreased the proportion in G0/G1 phase and increased the percentage of cells in G2/M phase ($P<0.05$). In contrast, miR-141 knockdown cells displayed a significant arrest in the G0/G1-phase and a reduction in the S-phase population ($P<0.05$) (Figure 3C). These results suggest that miR-141 could promote the proliferation of CRC cells by promoting cell cycle progression. Furthermore, transwell migration assays were performed to analyze the effects of miR-141 expression on cells invasion potential. As showed in Figure 3D, the number of Lovo cells in lower section were significantly increased in the miR-141-transfected group ($P<0.05$), while reduced in the miR-141-in-transfected group ($P<0.05$), which indicated that miR-141 could promote cell invasion.

miR-141 promotes CRC tumor growth in vivo. To clarify the effect of miR-141 on CRC cell growth *in vivo*, nude mice were subcutaneously inoculated with Lovo cells stably expressing miR-141 by lentivirus infection. As shown in Figure 4A, tumors derived from miR-141-transfected Lovo cells grew more quickly compared with controls. All mice were sacrificed

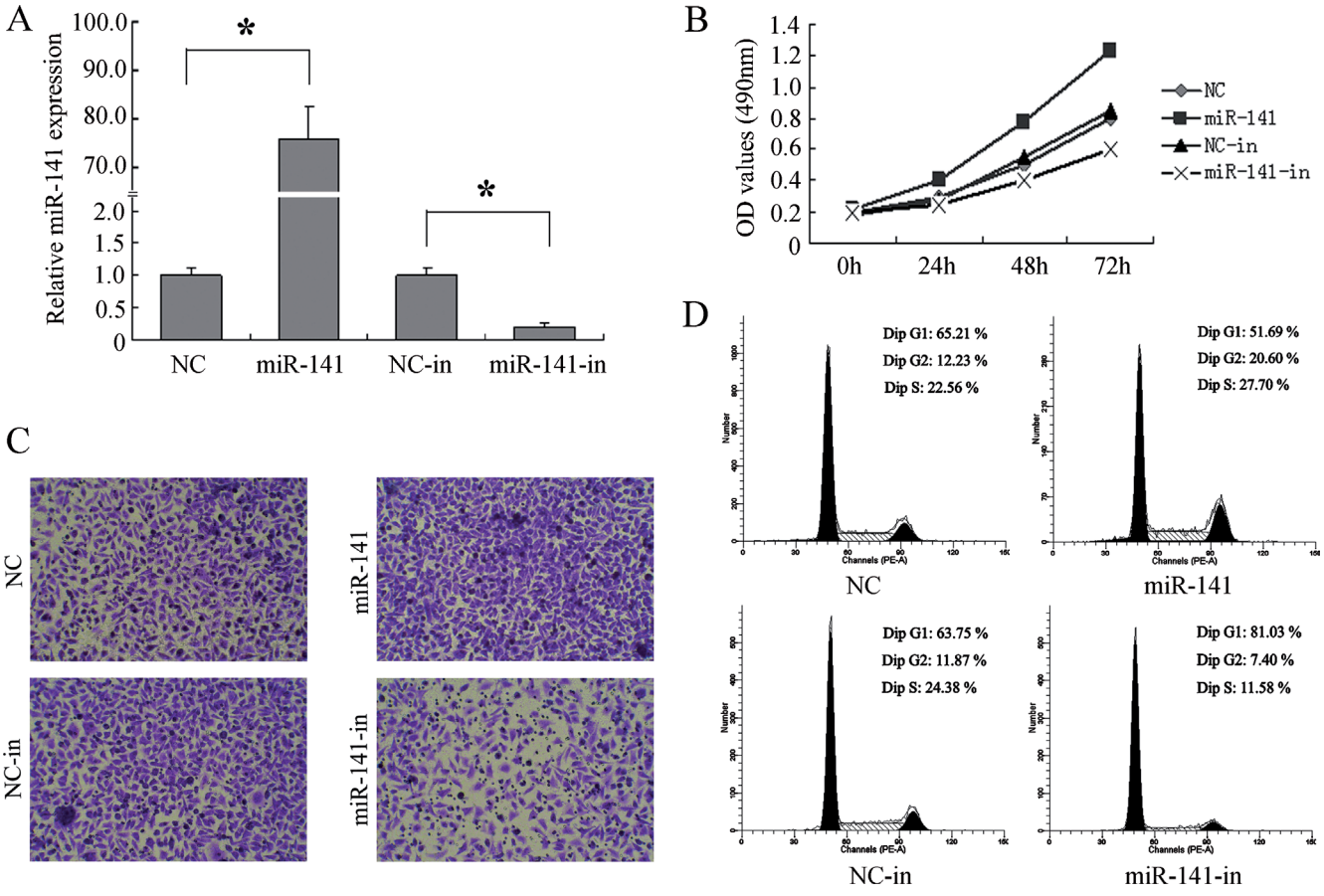

Figure 3. miR-141 promotes proliferation and migration of CRC cells in vitro. (A) the expression levels of miR-141 were measured by qRT-PCR after transfection; (B) cell growth was determined by MTT assays; (C) cell cycle was determined by flow cytometry analysis; (D) cell invasion was determined by transwell analysis. NC, miR-141 mimic control; miR-141, miR-141 mimic; NC-in, miR-141 inhibitor control; miR-141-in, miR-141 inhibitor.

2 weeks after the transplantation, and the average size of tumors derived from miR-141-transfected cells was significantly increased ($P<0.05$; Figure 4B). Moreover, the PCNA antigen in Lovo-miR-141 tumors was consistently higher than those in NC tumors, as confirmed by H&E and immunohistochemical staining (Figure 4C/D).

Repression of DLC1 plays crucial roles in miR-141-induced proliferation of CRC cells. To confirm whether the proliferation-promoting role of miR-141 in CRC cells was attributable to decreased expression of DLC1, we restored the expression of DLC1 in miR-141-overexpressing Lovo cells by transfection of DLC1 without the 3'-UTR (Figure 5A). MTT assay showed that re-introduction of DLC1 significantly decreased the growth rate of miR-141-overexpressing Lovo cells ($P<0.05$; Figure 5B). In addition, flow cytometry analysis showed that re-introduction of DLC1 into miR-141-overexpressing Lovo cells caused a significant increase of the percentage in G_0/G_1 phase ($P<0.05$) and a relative decrease of the percentage in S phase and G_2/M phase (Figure 5C). Re-introduction of DLC1 in miR-141-overexpressing Lovo cells also caused a significant decrease of the number of migrating cells ($P<0.05$; Figure 5D). These results demonstrate that repression of DLC1 plays a crucial role in the miR-141-induced proliferation of CRC cells.

Discussion

In this study, a bioinformatics approach for identifying miRNAs predicted to bind DLC1 was used at first. In total, 35 candidate regulators of DLC1 were identified from which miR-141 was chosen for further analysis according to the literature. Banaudha et al [17] have reported a direct role of miRNAs induced in HCV-infected primary human hepatocytes targeting DLC1, showing efficient HCV replication requires miR-141–mediated suppression of DLC1. Herein, we demonstrated that DLC1 was a direct target of miR-141 in CRC, via luciferase activity assay. The inverse correlation between miR-141 and DLC1 protein expression was also detected by western blotting. We concluded that up-regulation of miR-141 may be a molecular mechanism by which DLC1 exerted its functions of tumor suppressor in CRC.

MiRNA dysregulation has been observed in many human malignancies and there is evidence for miRNA involvement in tumor progression as either an oncogene or a tumor suppressor [16]. Among these miRNAs, miR-141, a member of the miR-200 family, is overexpressed in ovarian and colorectal cancers [22, 23] while down-regulated in prostate, hepatocellular, pancreatic, and renal cell carcinoma [24-27] raising a controversial issue about the role of miR-141 in cancer progression.

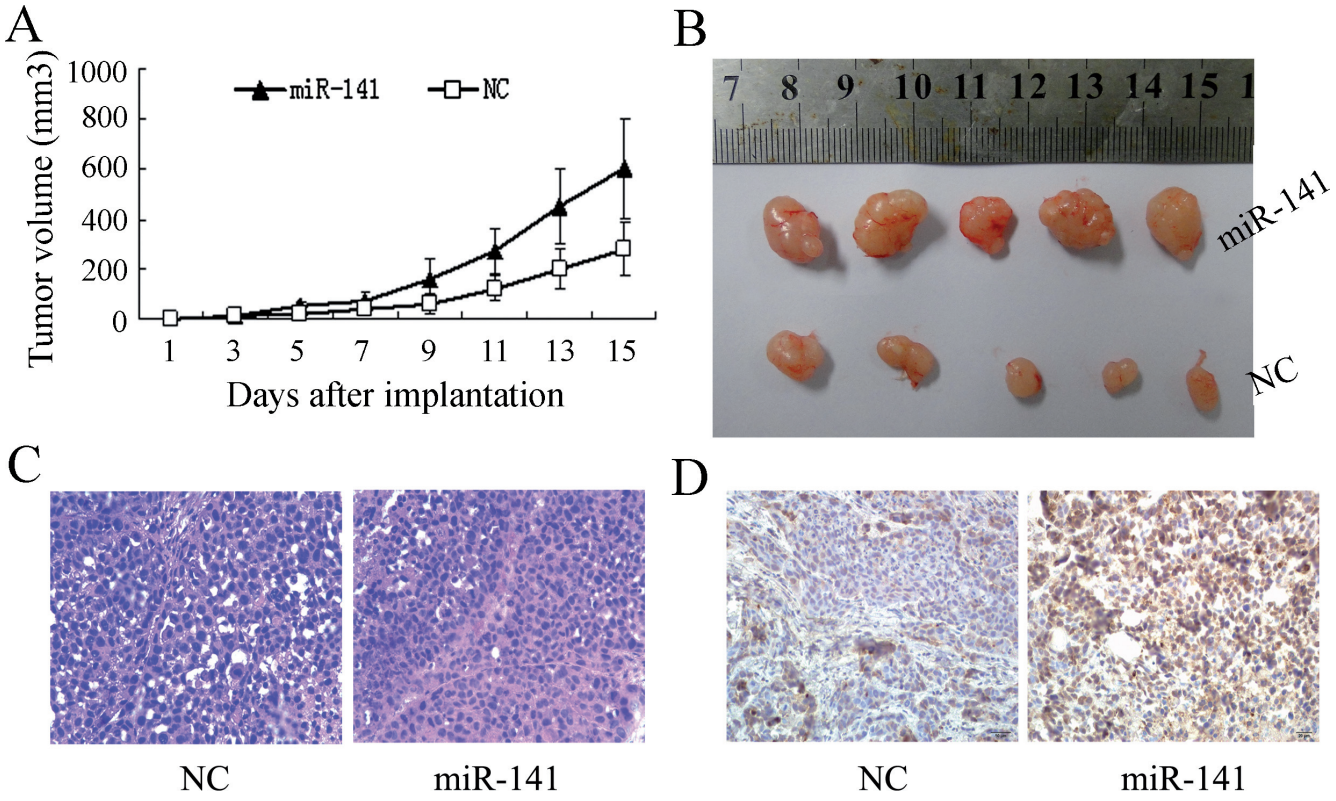

Figure 4. miR-141 promotes CRC tumor growth *in vivo*. (A) growth curve of tumors derived from miR-141-transfected Lovo cells and controls, each point represents the mean ± SD of 5 mice. (B) Representative photographs of tumors formed 2 wk after the subcutaneous transplantation. (C) Transplanted tumors with H&E staining. (D) Transplanted tumors with PCNA immunochemical staining. NC, miR-141 mimic control; miR-141, miR-141 mimic.

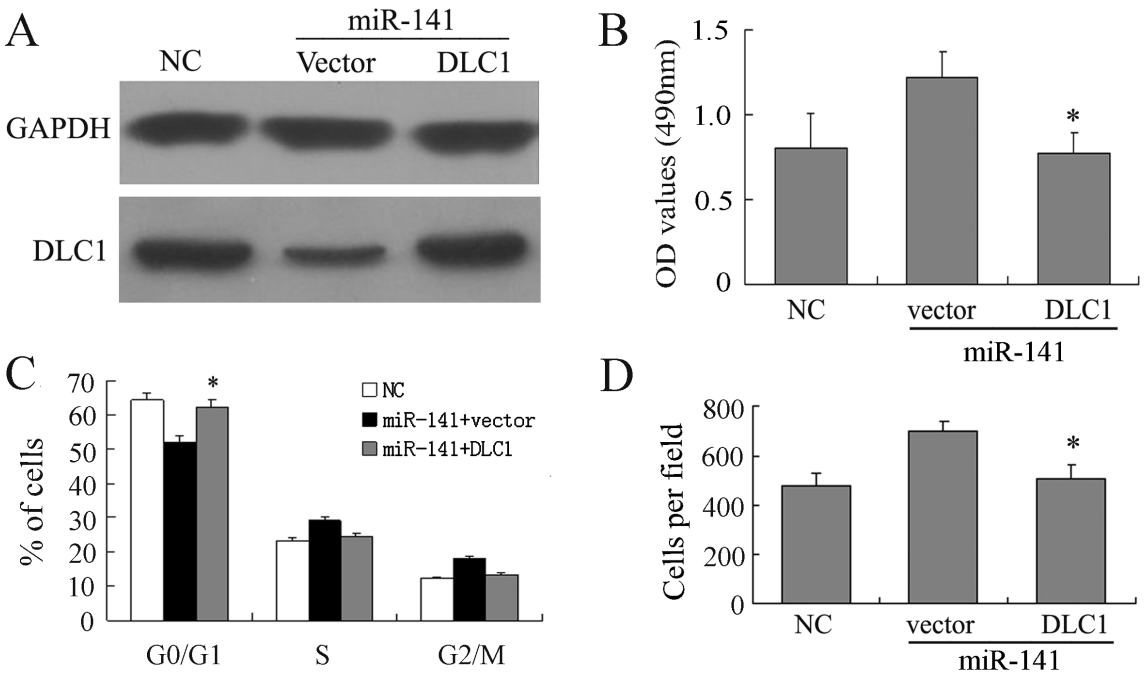

Figure 5. Repression of DLC1 reduces miR-141-induced proliferation of CRC cells. (A) Restoration of DLC1 in miR-141-overexpressing Lovo cells by transfection of DLC1 without the 3'-UTR. Re-introduction of DLC1 in miR-141-overexpressing Lovo cells (B) decreased growth rate, (C) caused a significant increase of the percentage in G0/G1 phase and a relative decrease of the percentage in S phase and G2/M phase, and (D) decreased the number of migrating cells. NC, miR-141 mimic control; miR-141, miR-141 mimic.

As a tumor suppressor gene, microRNA-141 has been shown to be downregulated in human renal cell carcinoma and regulated cell survival by targeting CDC25B [28]. Based on in situ hybridization (ISH) analysis, miR-141 was downregulated in hepatocellular carcinoma samples, and patients with low miR-141 expression had poorer overall survival rate. Moreover, functional analysis showed that miR-141 could inhibit the migration and invasion of HCC cells by targeting Tiam1 [29]. Also, Du et al [30] found significantly downregulated miR-141 in 80% (28/35) of primary gastric cancer tissues compared to pair-matched adjacent non-tumor tissues; overexpression of miR-141 with its precursors significantly inhibited the proliferation of gastric cancer cells.

However, miR-141 is up-regulated in nasopharyngeal carcinoma (NPC) specimens in comparison with normal nasopharyngeal epithelium; inhibition of miR-141 could affect cell cycle, apoptosis, cell growth, migration and invasion in NPC cells, which indicate that miR-141 exhibit oncogenic properties in NPC [31]. In ovarian tumorigenesis, miR-141 and miR-200a target p38α and modulate the oxidative stress response; enhanced expression of these microRNAs mimics p38α deficiency and increases tumor growth in mouse models [32]. miR-141 was also found to down-regulate SOX17 expression and activate the WNT signal pathway, which promotes esophageal tumorigenesis [33]. Recently, Mei et al [34] reported miR-141 promoted the proliferation of non-small cell lung cancer cells by regulating expression of PHLPP1 and PHLPP2.

The contradictory results also exist regarding CRC. Hu et al. [35] reported that microRNA-141 could regulate Smad interacting protein 1 (SIP1) and inhibit migration and invasion of colorectal cancer cells, suggesting that miR-141 acted as a tumor suppressor gene in CRC. However, two studies on >100 CRC samples founded elevated levels of circulating miR-141 were associated with liver metastasis of CRC and poor prognosis [29, 36]. Further, the combination of miR-141 levels with CEA levels improved the accuracy of CRC diagnosis [29]. These data indicated that miR-141 may promote distant metastasis of CRC. Our data revealed that up-regulated miR-141 levels in CRC tissues were negatively correlated with the decreased DLC1 expression levels. These findings further demonstrate that miR-141 may exhibit oncogenic properties in CRC by targeting DLC1.

The human *DLC1* gene is a potent tumor-suppressor gene in several cancers increasingly recognized by experts in the field as a metastasis-suppressor gene. It encodes a 1,091 amino acid protein that is highly homologous to the rat p122-RhoGAP [1]. RhoGAPs catalyze the conversion of the active GTP-bound Rho proteins to the inactive state, thereby attenuating their signal transduction activities. DLC1 suppresses proliferation, migration and invasion and induces apoptosis of human liver, breast and prostate cancer cells by acting mainly as a GAP for RhoA, RhoC and Cdc42 [10, 11]. Moreover, RhoGAP-independent mechanisms of DLC1 action mediated via its binding partners have also been linked

to tumor cell phenotype, showing that the function of DLC1 in malignancy is complex.

More than ten years of studies have established DLC1 to be downregulated in several malignancies including hematological malignancies, and lung, breast, prostate, kidney, colon, uterus, ovary, and stomach cancers [2]. Importantly, *DLC1* gene was found to be as frequently mutated as *TP53* in most aggressive cancers [4, 11]. More targeted mutations of the *DLC1* reducing the protein expression were also reported as exonic missense mutations and intronic insertions/deletions in primary ovarian and colorectal cancers [37]. *DLC1* can also be epigenetically silenced by hypermethylation of the CpG islands within the *DLC1* promoter in liver, breast, colon and prostate cancer [38]. Acetylation also modulates DLC1 activity since treatment of several cancer types with the histone deacetylase inhibitor trichostatin A reactivates DLC1 expression [39]. DLC1 is also the target of several kinases including protein kinase A (PKA), Akt/PKB, PKC and PKD [40, 41]. Phosphorylation of DLC1 on serine 567 by Akt leads to inhibition of its GAP activity [42].

Recent studies have identified microRNA as candidate regulators of DLC1 [17, 18], but the regulation of DLC1 at the post-translational level in CRC has not been well studied. A better understanding of DLC1 regulation mechanisms in human cancers will provide insights into the development of therapeutic interventions. In the present study, we found that miR-141 could promote Lovo cells growth *in vitro* and *in vivo*, promote cell cycle progression and invasion *in vivo* by using transfection with chemically synthesized miR-141 mimic and miR-141 inhibitor. This functional study firstly confirmed the oncogenic role of miR-141 in CRC development. By restoring the expression of DLC1 in miR-141-overexpressing Lovo cells, we further demonstrated a crucial role of DLC1 repression in the miR-141-induced proliferation of CRC cells, confirming that miR-141 exerted its functions by targeting *DLC1* gene.

In conclusion, we demonstrate that miR-141 is up-regulated in CRC samples and acts as a functional oncogene in CRC cell line. Targeting DLC1 may be an important mechanism involved.

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