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miR-128 downregulation promotes growth and metastasis of bladder cancer cells and involves VEGF-C upregulation

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Abstract. MicroRNA-128 (miR-128) serves an important role in regulating growth, invasiveness, stem cell-like traits, differentiation and apoptosis of different types of tumor cells. Vascular endothelial growth factor-C (VEGF-C) has been associated with angiogenesis, lymphangiogenesis and regional lymph node metastasis and has previously been reported to have an anti-apoptotic and proliferative role in bladder cancer (BC). To investigate the regulation of miR-128 on VEGF-C expression and their effects on proliferation and metastasis of bladder cancer, T24 and 5637 BC cells were transfected with pre-miR-128, anti-miR-128 and their respective negative control. miR-128 was downregulated in BC tissues and cell lines, while the expression levels of VEGF-C were upregulated. The present results indicated that miR-128 negatively regulated VEGF-C expression in BC T24 and 5637 BC cells. VEGF-C is a direct target of miR-128 in BC cells. Overexpression of miR-128 inhibited cell proliferation, migration and invasion. Knockdown of miR-128 promoted proliferation, migration and invasion in BC cells. Therefore, downregulation mediated malignant progression of BC may be partly attributed to increased VEGF-C expression. Consequently, the findings of the present study provide a molecular basis for the role of miR-128/VEGF-C in the progression of human BC and indicate a novel target for treatment of BC.

Introduction

Human bladder cancer (BC) is the second most frequently observed type of genitourinary cancer (1). Approximately 50% of patients diagnosed with muscle-invasive bladder cancer (MIBC) develop distant metastases in the lungs and liver, resulting in poor 5-year survival rates (2,3). Currently, the advances in suitable therapy for enhancing survival rate are limited since the underlying mechanisms that result in tumor metastasis are not well understood. Therefore, it is very important to clarify the key factors mediating bladder cancer growth and metastasis and their relative molecular mechanism for developing effective therapy.

MicroRNAs (miRNAs) are endogenous RNA molecules of about 18-25 nucleotides in length that regulate gene expression in a number of ways. In mammals, miRNAs are necessary for the regulation of numerous processes, including normal development, cell growth, differentiation, apoptosis (4). miRNAs are also known to serve significant roles in tumorigenesis. A number of types of cancer are associated with aberrantly expressed miRNAs. Both losses and gains of miRNA function have been demonstrated to contribute to cancer development and continued tumor growth (5).

miRNAs may act as both oncogenes or tumor suppressors in different situations (6). MiR-128 is an miRNA that behaves differently according to the tissue or cell background and has been studied extensively in glioma. miR-128 inhibits glioma proliferation and self-renewal by targeting Bmi-1, E2F3a and mitogenic kinases (7-9). In addition, miR-128 inhibits tumor growth and angiogenesis by targeting p70S6K1 (10), promotes cell-cell adhesion in U87 glioma cells via regulation of EphB2 (11) and modulates glioma progression by regulating the SNAI1, miR-128 and SP1 axis (12). miR-128 has been demonstrated to target polycomb repressor complexes in glioma stem cells, mediating cancer stem cell maintenance and radioresistance (13). miR-128 has also been demonstrated to serve a role in other types of cancer. In the majority of cases, miR-128 acts as a tumor inhibitor and its expression is frequently reduced in tumor cells; one of the proposed molecular mechanisms of this reduced expression is methylation of the promotor region (14). miR-128 reduces cell motility and invasiveness in neuroblastoma (15) and prostate cancer (16). It has been proposed that miR-128 mediates cell apoptosis via inhibition of NTRK3 and Bax expression and upregulation of BCL2 in neuroblastoma and embryonic kidney cells (17,18). In ovarian cancer, glioblastoma and breast tumor-initiating cells, overexpression of miR-128 may promote chemosensitivity via different signaling (19-21). By contrast, miR-128 was reported to act as an onco-miR

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in primary osteosarcoma conferring metastatic potential and unfavorable prognosis (22). However, to the best of our knowledge, there have been no previous reports about the role of miR-128 in bladder cancer.

Vascular endothelial growth factor-C (VEGF-C) has been associated with angiogenesis, lymphangiogenesis and regional lymph node metastasis and was reported to have an anti-apoptotic and proliferative role (23). It was reported that the involvement of VEGF-C expression in the promotion of lymph node metastasis could be used as a decision-making biomarker for selected patients with invasive bladder cancer who underwent bladder-preserving radical surgery and were associated with an anti-apoptotic phenotype (24-26). RNA interference-mediated VEGF-C reduction suppresses malignant progression and enhances mitomycin C sensitivity of bladder cancer T24 cells (27). It has been observed that miR-128 serves a critical role in human non-small cell lung cancer tumorigenesis, angiogenesis and lymphangiogenesis by directly targeting VEGF-C (28), but the association between miR-128 and VEGF-C in BC remains unknown.

In the present study, the expression levels of miR-128 and VEGF-C were determined in BC tissues and T24 and 5637 BC cells, using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Subsequently, the role of miR-128 expression in the migration and invasion of T24 and 5637 cells were investigated. The present study aimed to investigate VEGF-C and miR-128 as novel diagnostic or therapeutic targets for BC.

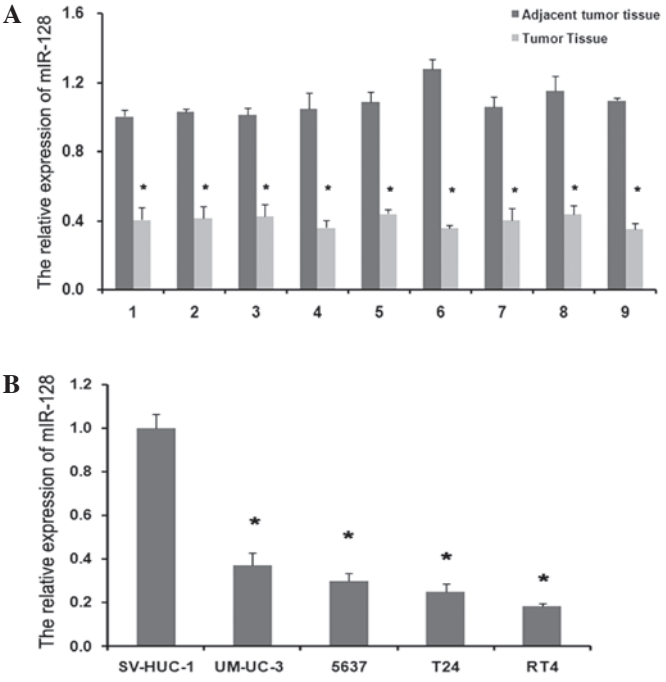

Figure 1. The mRNA level of miR-128 in bladder cancer. (A) The miR-128 mRNA level in cancer tissue relative to the adjacent normal tissue from the same patient and (B) miR-128 mRNA level in 4 bladder cancer cell lines relative to SV-HUC-1 normal cells were identified using reverse transcription-quantitative polymerase chain reaction. Data presented are the mean ± SD of a representative experiment performed in triplicate. *P<0.05 vs. normal control.

Materials and methods

Ethical statement. Prior written informed consent was obtained from all patients and the study was approved by the Protection of Human Subjects Committee of XiangYa Hospital of Central South University (Changsha, China).

Patients and tissue samples. A total of 9 BC tissue samples and adjacent non-tumorous kidney tissue counterparts were used for RT-qPCR and western blot analysis and were collected at XiangYa Hospital of Central South University. The hard and firm tumor tissues were trimmed free of normal tissue and were immediately snap frozen in liquid nitrogen. All BC cases were clinically and pathologically confirmed to be bladder carcinoma.

Cell culture and reagents. The human bladder epithelial cell line, SV-HUC-1, and the bladder tumor cell lines T24, 5637, 3-UM-UC-3 and RT4 were obtained from the Cell Bank of Central South University (Changsha, China). Cells were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% FBS (Invitrogen Life Technologies), 50 U/ml of penicillin and 50 mg/ml of streptomycin (Invitrogen Life Technologies). All cells were cultured in a sterile incubator maintained at 37˚C with 5% CO_2.

RT-qPCR analysis. Total RNA was extracted from cells with TRIzol reagent (Invitrogen Life Technologies) following the manufacturer's instructions. The relative expression level of miR-128 was determined by RT-qPCR using mirVana™ qRT-PCR microRNA detection kit (Ambion Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Specific primer sets for miR-128 (HmiRQP3056) and U6 (HmiRQP9001) were obtained from Genecopoeia, Inc. (Rockville, MD, USA). The expression levels of VEGF-C mRNA was detected by RT-qPCR using the standard SYBR Green RT-PCR Kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's instructions. The specific primer pairs are as follows: VEGF-C, sense 5'-CACGAGCTACCTCAGCAAGA-3 and antisense 5'-GCTGCCTGACACTGTGGTA-3'; and β-actin as an internal control, sense 5'-AGGGGCCGGACTCGTCATACT-3' and antisense 5'-GGCGGCACCACCATGTACCCT-3'. The relative expression levels of VEGF-C mRNA or miR-128 were quantified using GraphPad Prism software, version 4.0 (GraphPad Software, Inc., San Diego, CA, USA) and the $2^{-\Delta\Delta Ct}$ method (29).

Protein extraction and western blot analysis. Western blot analysis was performed as described previously (13). Protein levels were quantified by Bradford assay (30). A total of 30 mg protein from each sample was fractionated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (PVDF membranes; EMD Millipore, Billerica, MA, USA). The membrane was blocked in 0.1% Triton X-100 (Invitrogen Life Technologies) and 5% low fat milk powder (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline for 1 h at 4˚C and then probed with rabbit polyclonal primary anti-β-actin (1:500, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or rabbit polyclonal primary anti-VEGF-C (1:500, Immunoway, USA). After washing 3 times with Tris-buffered saline Tween-20 (Sigma-Aldrich, the membrane was incubated in peroxidase-conjugated

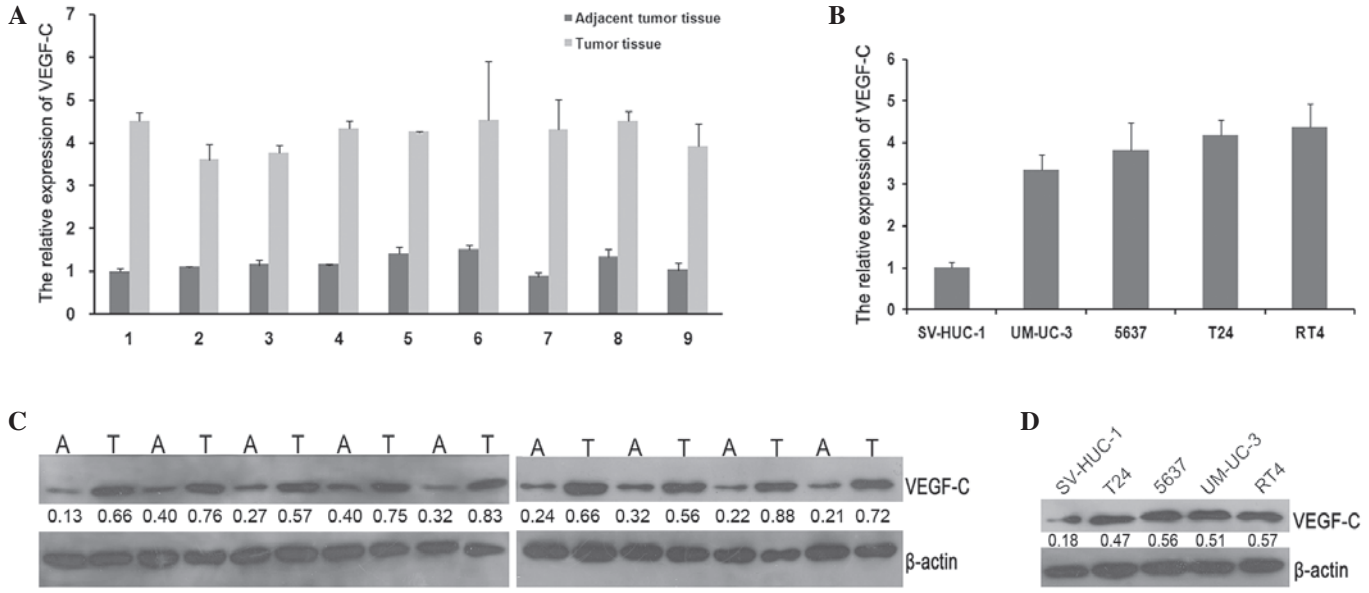

Figure 2. The mRNA and protein expression level of VEGF-C in bladder cancer. The VEGF-C (A) mRNA and (C) protein expression levels in cancer tissue relative to the adjacent normal tissue from the same patient and VEGF-C (B) mRNA and (D) protein expression levels in 4 bladder cancer cell lines relative to SV-HUC-1 cells were identified using real-time reverse transcription-quantitative polymerase chain reaction and western blot analysis. Data presented are the mean ± SD of a representative experiment performed in triplicate. A, adjacent tissue; T, tumor tissue.

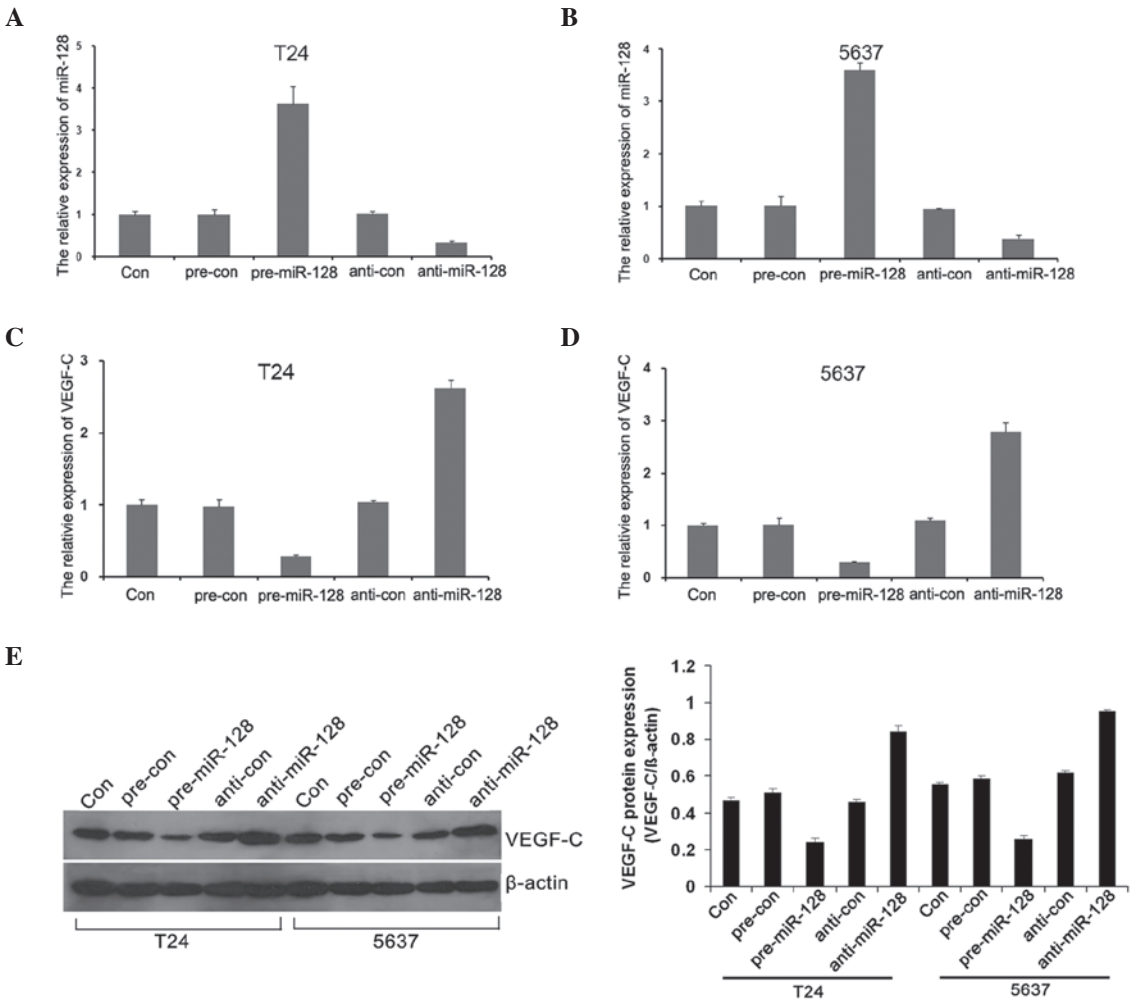

Figure 3. miR-128 and VEGF-C expression in functional cell models. The mRNA levels of miR-128 in bladder cancer (A) T24 and (B) 5637 cells transfected with pre-miR-128, anti-miR-128 or negative control (pre-con, anti-con) were detected by RT-qPCR. The mRNA levels of VEGF-C in bladder cancer (C) T24 and (D) 5637 cells and the (E) protein levels in the 2 bladder cancer cell lines transfected with pre-miR-128, anti-miR-128 or negative control (pre-con, anti-con) were detected by RT-qPCR and western blot analysis, respectively. RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

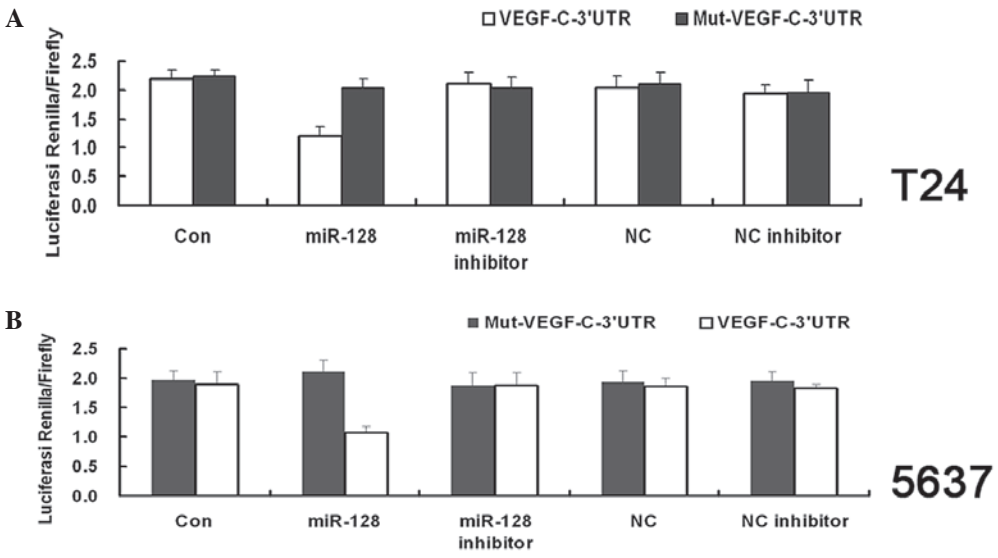

Figure 4. VEGF-C is a direct target of miR-128. Dual luciferase reporter assays were performed to test the interaction of miR-128 and its targeting sequence in the VEGF-C 3'-UTR, using constructs containing the targeting sequence (3'-UTR VEGF-C) and mutated targeting sequence (3'-UTR Mut VEGF-C) cloned into psi-CHECK2 in (A) T24 and (B) 5637 cells. Data presented are the mean ± SD of a representative experiment performed in triplicate. *P<0.05 vs. NC. NC, normal control.

goat anti-mouse/rabbit IgG antibody (1:1,000, ImmunoWay Biotechnology, Co., Newark, DE, USA). The bands were visualized by an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA, USA) using medical X-ray films (Kodak, Rochester, NY, USA) and quantified by Photoshop software (Adobe Systems, San Jose, CA, USA). The intensities of the bands of interest were expressed relative to the β-actin intensities from the same sample.

Transfection. For the miR-128 functional analysis, the pre-miR-128, pre-control [negative control (NC) of pre-miRNA], anti-miR-128 or anti-control (NC inhibitor) (Genecopoeia, Inc.) constructs were transfected into T24 and 5637 cell lines using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions.

Cell proliferation assay. Cells in exponential growth were seeded at a final concentration of $2x10^3$ cells/well in 96-well plates. The viability of the cells was evaluated by MTT assay (Invitrogen Life Technologies) after 24, 48 and 72 h of seeding. The optical density at 570 nm (OD_{570}) of each well was measured with an ELISA reader (ELX-800 type, BioTek Instruments, Inc., Winooski, VT, USA).

Colony-formation assay. For all groups, 3 ml complete medium containing 150 cells were added to each well of a 6-well plate. The plates were incubated at 37˚C, 5% CO_2 for 14 days. The cells were then gently washed and stained with Giemsa (Invitrogen Life Technologies). Colonies containing ≥50 cells (0.3-1.0 mm) were counted.

Cell invasion assay. The invasive ability of bladder cancer cells was then studied in 24-well transwell chambers coated with matrigel (EMD Millipore). For all groups, 200 µl of $1x10^6$ cells/ml cell suspension was added in triplicate wells. After a 24-h incubation, the dye on the membrane was dissolved with 10% acetic acid, dispensed into 96-well plates (150 µl/well), and the OD_{570} of each well was measured with an ELISA reader (ELX-800 type; BioTek Instruments, Inc.).

Cell migration assay. The cell migratory capability was estimated using a wound healing assay as described previously (31). In brief, cells were cultured to confluence. Wounds of approximately 1 mm width were created with a plastic scriber and cells were washed and incubated in a serum-free medium. After wounding, the cells were incubated for 24 h in a medium containing 10% fetal bovine serum. Cultures at 0 and 48 h were observed under an inverted microscope (TS100; Nikon Corporation, Tokyo, Japan).

Dual luciferase reporter assay. The 3'-UTR of VEGF-C (NM_005429.4) containing the miR-128 binding sites and its corresponding mutated sequence were cloned into the psi-CHECK2 luciferase reporter vector (Promega Corporation, Madison, WI, USA) downstream of *Renilla luciferase*, named VEGF-C-3'-UTR and VEGF-C-Mut 3'-UTR, respectively. Using Lipofectamine 2000 (Invitrogen Life Technologies), T24 and 5637 cells were co-transfected with the reporter constructs and miR-128 mimics, the miR-128 inhibitor, NC or the NC inhibitor. Luciferase activity was determined after 48 h using the Dual-Glo substrate system (Promega Corporation) and a Beckman Coulter LD400 luminometer (Beckman Coulter, Inc., Brea, CA, USA). Data are presented as the ratio of experimental (Renilla) luciferase to control (Firefly) luciferase.

Statistical analysis. Data are expressed as the mean ± standard deviation from >3 separate experiments. Statistical analysis was performed using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA). The difference between 2 groups was analyzed by the Student's *t*-test. A value of P<0.05 was considered to indicate a statistically significant difference.

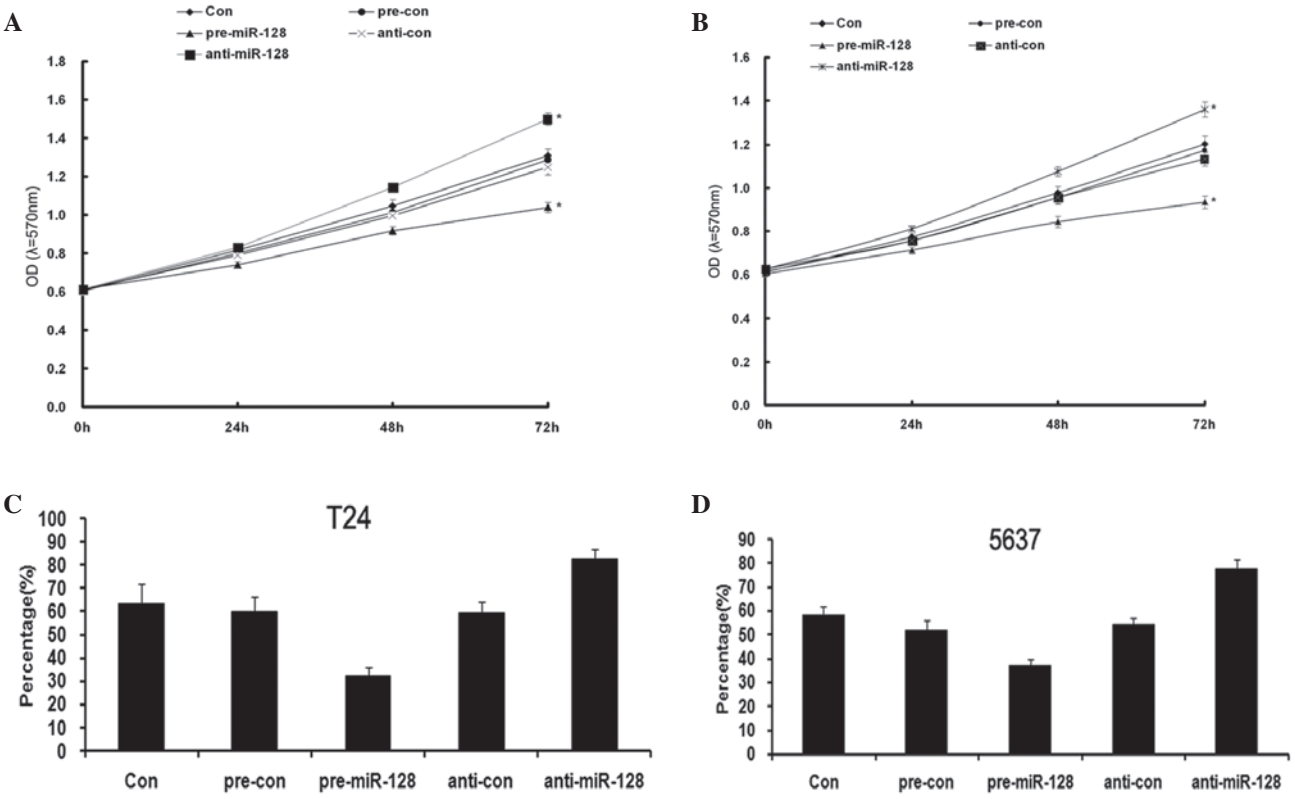

Figure 5. Effects of miR-128 on cell proliferation. The bladder cancer cells were transfected with pre-miR-128, anti-miR-128 or negative control (pre-con, anti-con). The cell viability of (A) T24 and (B) 5637 cells from 0 h to 72 h were measured by MTT assay. Cloning efficiency was calculated after cultured for 2 weeks. Representative results of colony formation assay in (C) T24 and (D) 5637 cells. Data presented are the mean ± SD of a representative experiment performed in triplicate. *P<0.05 vs. normal control.

Results

Expression of miR-128 and VEGF-C in BC tissues and cell lines. The mRNA expression levels of miR-128 and VEGF-C in clinical tissues or in BC cell lines were determined using RT-qPCR. Compared with normal adjacent tissues, the mRNA expression levels of miR-128 in tumor tissues were significantly reduced compared with in normal tissues (Fig. 1A; P=0.0017), while the mRNA expression levels of VEGF-C were reduced (Fig. 2A; P=0.013). Meanwhile, the downregulation of miR-128 and upregulation of VEGF-C were also observed in BC cell lines compared with SV-HUC-1 (Figs. 1B and 2B; P=0.004 and P=0.003, respectively). The VEGF-C protein expression was apparently increased in tumor tissues or BC cell lines compared with adjacent normal tissues or SV-HUC-1 as assessed by western blot analysis (Fig. 2C and D; P=0.0023 and P=0.015, respectively). These results indicate that miR-128 may serve an important role in malignant progression of BC. Furthermore, the coexistence of VEGF-C upregulation and miR-128 downregulation in BC cells implies a potential regulatory association between the 2 molecules.

Expression of miR-128 and VEGF-C in gain-of-function or loss-of-function model cells. To clarify the functions of miR-128 in BC cells, gain-of-function and loss-of-function cell models were constructed by transfection with pre-miR-128 and anti-miR-128. The miR-128 mRNA level was significantly upregulated (Fig. 3A and B; P=0.034 and P=0.037, respectively), while VEGF-C level was downregulated when transfected with pre-miR-128 (Fig. 3C and D; P=0.042 and P=0.039, respectively). By contrast, when transfected with anti-miR-128, the mRNA level of miR-128 was significantly downregulated (Fig. 3A and B; P=0.034 and P=0.037, respectively) and VEGF-C was significantly upregulated (Fig. 3C and D; P=0.042 and P=0.039, respectively). The results indicated that overexpression of miR-128 reduced the VEGF-C expression in T24 and 5637 cells (Fig. 3E; P=0.044).

VEGF-C is a direct target of miR-128 in T24 and 5637 cells. To assess whether VEGF-C is a direct target of miR-128, luciferase reporter assays were applied. The VEGF-C 3'-UTR fragment containing the miR-128 binding site and mutated targeting sequence were cloned into psi-CHECK2 dual luciferase reporter vectors. The miR-128 signifcantly inhibited the luciferase activity in both T24 and 5637 cells transfected with the VEGF-C-3'-UTR. However, miR-128 mimics did not suppress the luciferase activity levels in the T24 and 5637 cells transfected with Mut-VEGF-C-3'-UTR (Fig. 4A and B; P=0.024 and P=0.027, respectively). These findings indicate that VEGF-C is a direct target of miR-128.

miR-128 inhibiting proliferation of bladder cancer cells. To investigate the effects of miR-128 on proliferation of BC cells, T24 and 5637 cells were transfected with miR-128 mimics or its inhibitor. MTT and colony formation assays were used to demonstrate that overexpression of miR-128 markedly reduced the growth (Fig. 5A and B; P=0.0017 and P=0.015, respectively) and clone-formation (Fig. 5C and D; P=0.035

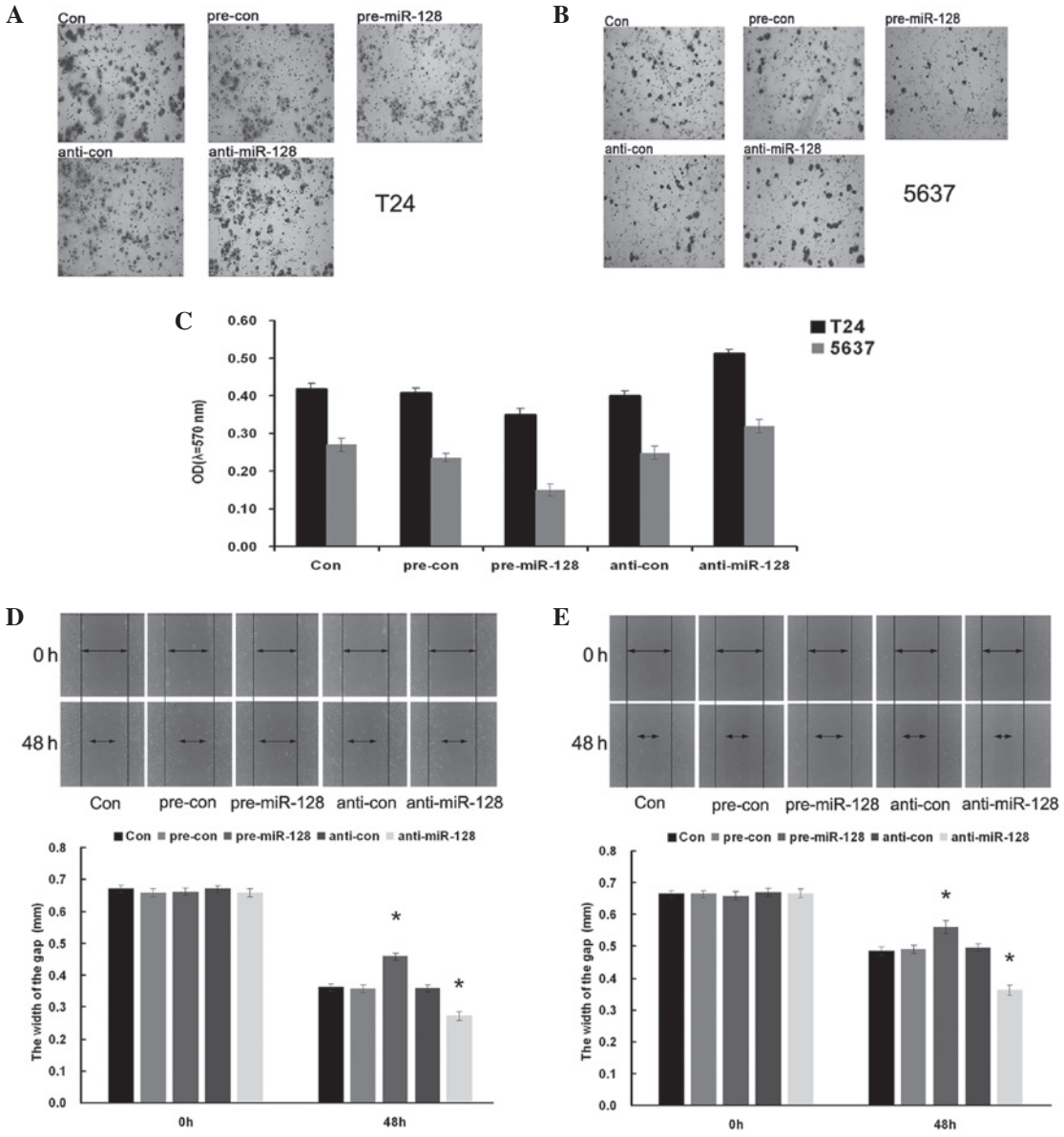

Figure 6. Effect of miR-128 on cell invasion and migration. The invasion capabilities of A) T24 and (B) 5637 cells were measured by Transwell assay. (C) Representative results of Transwell assay. The migration capabilities of (D) T24 and (E) 5637 cells were measured by scratch assay and the relative representative results are presented. Data presented are the mean ± SD of a representative experiment performed in triplicate. *P<0.05 vs. NC.

and P=0.038, respectively) rate of the 2 BC cell lines as compared with that of negative control (NC) transfected cells. However, inhibition of miR-128 increased the growth rate of the 2 BC cell lines as compared with that of NC transfected cells. These results indicated that miR-128 inhibited the proliferation of BC T24 and 5637 cells.

miR-128 suppressing migration and invasion of bladder cancer cells. To explore the functional role of miR-128 on migration and invasion in BC cells, T24 and 5637 cells were transfected with pre-miR-28 and anti-miR-128, respectively. Wound healing and transwell assays were performed to evaluate the cell metastasis capacity. The results demonstrated that cell migration and invasion capacity were significantly increased when the BC cells were transfected with pre-miR-128, while cell migration and invasion capacity were significantly reduced when cells were transfected with anti-miR-128 (Fig. 6C, P=0.037 and P=0.026, T24 and 5637 cells, respectively; Fig. 6D, P=0.031; Fig 6E, P=0.035). The results indicated that miR-128 supressed the migration and invasion of bladder cancer T24 and 5637 cells.

Discussion

miR-128 is an miR that behaves differently according to the tissue or cell background. In the majority of cases, miR-128 acts as a tumor inhibitor and its biological functions range from mediating cell proliferation to migration, apoptosis and chemoresistance. However, there is a lack of studies investigating the deregulation of miR-128 and functional role in BC is very limited.

In the present study, the mRNA level of miR-128 was investigated in 9 cases of BC and their adjacent tissues. The results demonstrated that miR-128 was reduced significantly in BC tumor tissues compared with their adjacent tissues. by

contrast, miR-128 was also downregulated in the 4 examined BC cell lines compared with normal bladder epithelial cells. The results indicate that miR-128 may be a tumor inhibitor in BC.

To investigate the biological function of miR-128 in BC, gain-of-function and loss-of-function cell models were constructed via transfection of pre-miR-128 and anti-miR-128 into T24 and 5637 BC cell lines, respectively. Using MTT, invasion, wound healing and colony-formation assays, it was demonstrated that overexpression of miR-128 inhibited growth rate, proliferation, migration and invasion capacities. These results were consistent with the results of previous studies (9,15,32,33). The involvement in cell apoptosis and chemosensitivity of miR-128 was also investigated, however no significant differences were observed between parental cells and the functional model cells.

VEGF-C has been associated with angiogenesis, lymphangiogenesis and regional lymph node metastasis and has also been reported to serve an anti-apoptotic and proliferative role (23). Bioinformatics software has been used to predict that VEGF-C is one of the target genes of miR-128 and it has been verified that miR-128 serves a critical role in human non-small cell lung cancer tumorigenesis, angiogenesis and lymphangiogenesis by directly targeting VEGF-C (28), however the association between miR-128 and VEGF-C in BC remains unknown. Using the Dual Luciferase Reporter Assay, a direct target relationship between miR-128 and VEGF-C was revealed in both T24 and 5637 cell lines. How does miR-128 exert its mediating effect on cell metastasis and proliferation? Is VEGF-C is a direct downstream factor in mediating BC cell proliferation and metastasis? To answer these questions, construction of VEGF-C knockout stable cell lines may be an effective method for future experiments. If overexpression of miR-128 in VEGF-C knockout stable cell lines results in less of an inhibitory effect on cell proliferation and metastasis than in T24 and 5637 cell lines, then one may conclude that miR-128 serves a critical role in BC proliferation, migration and invasion by directly targeting VEGF-C. This hypothesis should be investigated in future studies.

In conclusion, the present study provides evidence demonstrating that miR-128 is significantly downregulated and VEGF-C is significantly upregulated in BC tissues and cells. In addition, it was demonstrated that miR-128 directly targets VEGF-C in BC cells. The results of the present study support miR-128/VEGF-C pairs as novel diagnostic or therapeutic target for BC. Although the study provides a possible molecular mechanism for miR-128 mediated VEGF-C signaling pathway affecting BC progression, further studies are required to elucidate the details of this process.

Acknowledgements

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