von Willebrand factor rescued by miR-24 inhibition facilitates the proliferation and migration of osteosarcoma cells in vitro

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Introduction

Osteosarcoma (OS) is a malignant bone cancer with high mortality in children and young adults worldwide [1]. Although great progress has been made in both clinical diagnosis and treatment for OS during the recent decade, high distant metastasis and recurrence rate lead to the worse long-term survival and prognosis in OS patients [2]. As complicated pathologies of OS, the underlying mechanism of OS initiation and progression remains unclear so far. Therefore, a good understanding of the precise mechanism associated with OS is urgently required for developing effective therapeutic approaches.

von Willebrand factor (vWF) is a major procoagulant molecule that was shown to differentiate between metastatic and primary osteosarcoma (OS) tissues and associated with increased metastasis. However, its functional role in OS progression has been unclear yet. The expression profile of vWF and miR-24 in human OS tissues was characterized using immunofluorescence labeling and quantitative real-time PCR analysis. The interaction between miR-24 and vWF was identified by dual luciferase reporter assay. The effects of vWF and miR-24 on OS cells were assessed by cell proliferation, colony formation, and migration. The clinical significance of miR-24 in OS patients was analyzed using Kaplan–Meier analyses and Pearson’s Chi-squared test. Here, we reported that the expression of vWF was significantly increased, but miR-24 was significantly decreased in OS tissues (n=84). vWF was further validated as the target of miR-24 in MG-63 and U2OS cells. miR-24 obviously suppressed the proliferation and migration of MG-63 and U2OS cells. However, the migration-inhibiting activity of miR-24 was predominantly attenuated by vWF overexpression. Clinically, low miR-24 expression in human OS tissues was significantly associated with tumor metastasis and predicted a poor survival in OS patients. This work demonstrated that vWF, as a downstream effector of miR-24, played an important role in controlling OS cell progression. Target miR-24 or vWF, therefore, promises to be an effective biological target for OS treatment.
von Willebrand factor (vWF) is a complex multimeric plasma glycoprotein and its important role in hemostasis has been well documented [3]. Emerging roles of vWF involved in cancer cell biology, especially in tumor metastasis, were also identified in many types of human cancers [4–6]. Of note, vWF was suggested as the important mediator of platelet–tumor cells interactions, thereby promoting metastasis [6]. And this conclusion was further confirmed by the findings that vWF inhibition effectively decreased tumor metastasis in murine and reduced the adhesion of colon cancer cells to endothelial cells (ECs) [7,8]. Moreover, the high levels of plasma vWF were detected in patients with glioblastoma, acute lymphoblastic leukemia, colorectal cancer, urinary bladder cancer, and other types of cancers [9–12]. It has been reported that vWF is involved in cell death, for example, vWF would be able to control bone cancer cells apoptosis by controlling the binding of osteoprotegerin to TRAIL [13]. In addition, vWF was also shown to be highly expressed in metastatic OS tissues compared with primary OS tissues, suggesting a potential role of vWF dysregulation in OS metastasis [14]. However, the functional involvement of vWF in OS and its mechanism remain unknown.

Accumulating studies reported that miRNAs were abnormally expressed in cancer cells and played a critical role in modulating cancer cell biology, including proliferation, migration, invasion, and apoptosis [15]. As non-coding RNA transcripts, miRNAs could transcriptionally or post-transcriptionally regulate gene expression through binding to the 3'-UTR of target mRNA [16, 17]. It has been widely identified that miR-24 mediated cell proliferation and death [18]. Several miRNAs were identified to be related to malignant biological properties of OS, including miR-24. Originally, miR-24 was found to down-regulate in OS cells, and the forced miR-24 inhibited the proliferation of OS cells by targeting lysophosphatidic Acid Acyltransferase β (LPAATβ) [19]. Subsequently, increasing evidence demonstrated that miR-24 functioned as a tumor suppressor in OS progression [20]. Hence, it was reasonable to propose that miR-24 alternation has important pathological significance in OS. It experimentally revealed that miR-24 interacted with the 3'-UTR of vWF [21]. Considering their important role in OS, we therefore inferred that targeting vWF with miR-24 was involved in the progression of OS. The aim of the present study was to characterize the functional role of vWF in OS progression and to examine the existence of miR-24/vWF axis in the oncogenesis and progression of OS.

Materials and methods

Patients and specimens

The present study was approved by the Ethics Committee of the related institutions, and written informed consents were obtained from all patients enrolled in our study. A total of 84 human OS tissues and adjacent non-cancerous tissues were collected from the patients which underwent surgical resection in The First Affiliated Hospital of Soochow University, Children’s Hospital of Soochow University, The Affiliated Hospital of Xuzhou Medical University, The Friendship Hospital of Ili Kazakh Autonomous Prefecture, Jiangsu Province Hospital, The First Affiliated Hospital of Nanjing Medical University, and all patients did not receive any chemotherapy or radiation before surgery. Histological diagnosis and grading were performed by two independent pathologists. The fresh specimens were stored at −80°C for.

Immunofluorescence labeling assays

The cell glass slides and tissue cryostat sections at 5 μM of OS tissues or adjacent non-cancerous tissues were used for the immunofluorescence labeling of vWF as previously described [19]. Briefly, the sections were fixed with methyl alcohol (95%) for 5 min, then incubated with primary antibodies against vWF (Dako, Glostrup, Denmark) and PECAM (Abcam, U.K.) overnight at 4°C. The sections were washed in PBS for 3 times × 5 min and incubated with the Alexa fluor 488, 555, or 647 labeled secondary antibody (Thermo Scientific, Waltham, MA, U.S.A.) for 1 h. After DAPI staining, the images were taken under an inverted microscope (Olympus, Tokyo, Japan).

Cell culture and transfection

Human OS cell lines MG-63, U2OS, and KHOS were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). OS cell lines were cultured in RPMI-1640 medium (HyClone, Logan, UT, U.S.A.) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, U.S.A.), 100 IU/ml penicillin and 100 mg/ml streptomycin. All cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

In the present study, miR-24 mimics/inhibitor (Gangzhou RibioBio Co., Ltd., Guangzhou, China) and pcDNA-vWF (Shanghai GenePharma Co., Ltd., Shanghai, China) were used to respectively modulate the intracellular level of miR-24 and vWF. For transfection, MG-63, U2OS cells were cultured in six-well plates (1 × 10⁶ cells/well)
and then transfected with miR-24 mimics (50 nM), inhibitor (30 nM), pcDNA-vWF (2 μg), or corresponding negative control using Lipofectamine 2000 (Invitrogen, Waltham, MA, U.S.A.) according to the manufacturer’s protocol. After 48 h of the transfection, the cells were collected for further analysis.

Quantitative real-time PCR

Total RNA was extracted from the tissue specimens and cells using TRIzol reagent (Invitrogen, Waltham, MA, U.S.A.) following the manufacturer’s protocol. The purified RNA was quantitated using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, U.S.A.). For analysis of miR-24, total RNA (2.5 μg) was reverse transcribed into cDNA using miRNA First-Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, U.S.A.), and quantitative PCR was then performed with SYBR Green miRNA qRT-PCR kit (Thermo Scientific, Waltham, MA, U.S.A.) and specific primers according to the manufacturer’s protocol. For vWF mRNA measurement, reverse transcript was performed using Reverse Transcription Kit (Takara, Dalian, China). And quantitative PCR was carried out using SYBR Green qPCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.). The relative abundance of miR-24 and vWF, as determined by the 2−ΔΔC T method, was normalized to U6 and GAPDH, respectively. The gene-specific primers used in the present study were synthesized by Shanghai Sangon Biotech (Shanghai, China). The paired primer sequences were listed: miR-24, (forward) 5′-ACACTCCAGCTGGGTGCTAGTCGC-3′, (reverse) 5′-GTCAACTGGTGTCGGAGTCGCCAATTCCAG-3′; U6, (forward) 5′-CTCGTTCCGCGAAGCACCA-3′, (reverse) 5′-AACGTTCAAGATTTCGGT-3′; vWF, (forward) 5′-CGGCTTCACCCATATCCAGC-3′, (reverse) 5′-TGAGGAAGTGTATCAGCACATC-3′; GAPDH, (forward) 5′-GGCTGTGTGCATATCTCTCAGG-3′, (reverse) 5′-GGCTTTGTGTCATATCTTCC-3′.

Western blotting

Protein samples were extracted from the cultured cells using RIPA buffer (Roche, Pleasanton, CA, U.S.A.) supplemented with a protease inhibitor (cocktail) according to the manufacturer’s protocol. The concentration of total protein was determined by BCA Kit (Thermo Scientific, Waltham, MA, U.S.A.). Equal protein (25 μg) of each sample was separated by SDS/PAGE and then transferred to PVDF membrane (Millipore, Danvers, MA, U.S.A.). The PVDF membrane was blocked in 5% skim milk for 1 h and incubated with primary antibodies against vWF (dilution of 1:500, Cell Signaling Technology, Danvers, MA, U.S.A.) and β-actin (dilution of 1:1000, Cell Signaling Technology, Danvers, MA, U.S.A.) at 4°C overnight. The PVDF membrane was washed with TBS/Tween-20 and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The Immunoreactive bands were visualized by enhanced chemiluminescence substrate (Millipore, Boston, MA, U.S.A.).

Luciferase reporter assay

According to the putative binding site of miR-24 in the 3′-UTR of vWF (wild type), the site was mutated from GAGCC to ACAUU to generate the mutated vWF 3′-UTR using QuickChange Site-Directed Mutagenesis Kit (Stratagene). The wild type and mutated vWF 3′-UTR was cloned into the pmiR-GLO reporter vector (Promega Corporation, Madison, WI, U.S.A.), respectively. MG-63 cells (5 × 10^4 cells/well) were cultured in 24-well plates for 24 h, and co-transfected with luciferase reporter plasmid containing vWF 3′-UTR (wild type/mutated) and miR-24 mimic/miR-C using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 48 h of transfection, luciferase activity in MG-63 cells was detected using the Dual-Luciferase Reporter assay system (Promega, Madison, WI, U.S.A.) following the manufacturer’s instructions.

Cell proliferation and colony formation assays

Cell Counting Kit-8 (CCK-8) assay was used to assess the cell proliferation of MG-63 and U2OS cells. Cells (5 × 10^3 cells/well) were cultured in 96-well plates for 24 h, and then incubated with CCK-8 solution (10 μl/well) (Dojindo Laboratories, Kumamoto, Japan) for 2 h at 37°C. The absorbance of each well was measured at 450 nm using Microplate Reader (Bio-Rad, Foster, California, U.S.A.). In colony formation assay, Cells (1 × 10^3 cells/well) were cultured in six-well plates for 10 days. The formed colonies were washed with PBS and fixed in ethanol for 20 min. The cloning capability of cells was evaluated by means of crystal violet staining. The fixed colonies were stained with 0.1% crystal violet (Sigma–Aldrich, St. Louis, MO, U.S.A.) for 10 min. The stained colonies were counted using an inverted microscope (Olympus, Tokyo, Japan).
Cell apoptosis

OS cells apoptosis were assessed by using the fluorescein isothiocyanate annexin V Apoptosis Detection Kit (Beyotime, Shanghai, China). Briefly, cultured cells were collected, washed with cold PBS, and resuspended in 1 × binding buffer (0.01 mmol/l HEPES [pH 7.4], 0.14 mol/l NaCl, and 2.5 mmol/l CaCl₂) at a concentration of 2 × 10⁶ cells/ml. One hundred microliters of cell solution were transferred into a 5-ml culture tube, and 5 μl of annexin V was added. After gentle mixing and incubation for 15 min at room temperature in the dark, 400 μl of the 1 × binding buffer was added, and cells were analyzed by flow cytometry.

Wound-healing assays

Cells (1 × 10⁶ cells/well) were cultured in six-well plates till 90% confluence was reached. Artificial wounds were then induced by a sterile plastic tip, and the cells were washed with serum-free medium. After another 48 h of culture, the images of wound areas were recorded using an inverted microscope (Olympus, Tokyo, Japan). The migration efficiency was calculated by the following formula: migration efficiency (%) = (wound area [0 h] − wound area [48 h])/wound area (0 h) × 100%.

Statistical analysis

Data from at least three independent sample replicates were presented as the mean ± S.D. All statistical analysis was conducted using SPSS 21.0 statistical software. A value of P < 0.05 was considered as statistically significant. The statistical differences between two groups were analyzed using Student’s t test. The relative expressions of miR-24 in human OS tissues and adjacent non-cancerous tissues were compared with the Mann–Whitney U-test. The overall survivals in OS patients with or without metastasis were compared using Kaplan–Meier survival curves and log-rank tests. Pearson’s Chi-squared test was used to analyze the correlation between miR-24 expression in human OS tissues and clinicopathological parameters of OS patients.

Results

The expression of vWF was significantly increased in OS tissues and cell lines

To uncover the role of vWF in the progression of OS, the expressions of vWF were analyzed in 84 human OS tissues by immunofluorescence labeling and quantitative real-time PCR (qRT-PCR) analysis. As is shown in Figure 1A, vWF was abundant around the cancer cells and ECs, a major source of vWF, in human OS tissues (tumor). Whereas, vWF was only found around EC in the adjacent non-cancerous tissues (non-tumor). Moreover, qRT-PCR analysis revealed that the relative expression of vWF mRNA in human OS tissues (n=84) significantly higher than that of paired non-cancerous tissues (n=84) (Figure 1B). Similar results were observed in OS cell lines. MG-63 cells had higher expression of vWF than that in vWF-deficit KHOS cells (Figure 1C). Moreover, Kaplan–Meier survival analysis demonstrated that higher vWF expression showed a poor survival in OS patients (Figure 1D).

Correlation of miR-24 with overall survival and clinicopathological parameters of OS patients

Next, we analyzed the expressions of miR-24 in OS. We found that the relative expressions of miR-24 were markedly down-regulated in human OS tissues (Tumor) (n=84) (Figure 2A). To verify the functional involvement of miR-24 in OS, the correlation between miR-24 expression and clinicopathological parameters of OS patients (n=84) was analyzed. The relative expressions of miR-24 in 84 OS tissues were examined by qRT-PCR. And, the median value of all 84 OS samples was chosen as the cut-off point for separating tumors with low-level expression of miR-24 from high-level expression miR-24 tumors. Statistical analysis confirmed that low miR-24 expression in human OS tissues was significantly associated with tumor node metastasis and metastasis (Table 1). Accordingly, we noticed a lower level of miR-24 in the metastatic OS tissues (n=47) compared with primary OS tissues (n=37) (Figure 2B). And further Kaplan–Meier survival analysis demonstrated that low miR-24 expression predicted a poor survival in OS patients (Figure 2C). Together, these results revealed a potential role of miR-24 in OS progression.

vWF was a novel target gene of miR-24 in OS

Interestingly, qRT-PCR analysis showed a conspicuous negative correlation between vWF mRNA and miR-24 expression in human primary OS tissues (Table 2). And consistent with previous report [21], the bioinformatics analysis revealed that miR-24 interacted with the 3′-UTR of vWF in current study (Figure 3A). To further verify the inter-
Figure 1. Expression pattern of vWF in OS tissues and cell lines

(A) Representative immunofluorescence labeling images of vWF expression in human OS tissues (tumor) and adjacent non-cancerous tissues (non-tumor). vWF was shown in green, DAPI in blue, ECs were labeled by PECAM (red). Scale bars: 50 μm. (B) The relative expressions of vWF mRNA in human OS tissues (n=84) and adjacent non-cancerous tissues (n=84) were compared with the Mann–Whitney U-test. ***P<0.001. (C) Representative immunofluorescence labeling images of vWF expression in human OS cells MG-63 and KHOS cells. vWF was shown in green, DAPI in blue, Ki67 in red. Scale bars: 25 μm. (D) The overall survivals in OS patients with high or low vWF expression were compared using Kaplan–Meier survival curves and log-rank tests. ***P<0.001.

Figure 2. Expression of miR-24 and its correlation with overall survival in OS patients

(A) The relative expressions of miR-24 in human OS tissues (tumor) (n=84) and adjacent non-cancerous tissues (non-tumor) (n=84) were compared with the Mann–Whitney U-test. U6 was used as the internal control. ***P<0.001, compared with non-tumor. (B) Comparison of the relative expression of miR-24 between the metastatic OS tissues (n=47) and primary OS tissues (n=37). ***P<0.001, compared with OS without metastasis. (C) The overall survivals in OS patients with high or low miR-24 expression were compared using Kaplan–Meier survival curves and log-rank tests.

action between miR-24 and vWF 3′UTR, the pmirGLO luciferase vectors containing miR-24-binding site of vWF 3′UTR (vWF WT) or a mutant miR-24-binding site (vWF mutant) were constructed. Luciferase report assay showed that miR-24 overexpression markedly reduced the relative luciferase activity in the cells transfected with WT vWF, whereas miR-24 expression had no significant effect on the relative luciferase activity of cells transfected with vWF mutant, (Figure 3B). We further determined the response of vWF expression to miR-24 alternations. As shown in Figure 3C,D, miR-24 overexpression caused significant down-regulation of vWF mRNA and protein. Conversely, silencing of miR-24 resulted in a significant increase in vWF expression (Figure 3E,F). Collectively, these results supported the notion that vWF was a target gene of miR-24 in OS MG-63 cells.
Table 1 Correlation between miR-24 expression in human OS tissues and clinicopathological parameters of OS patients

| Variables              | All cases (n=84) | High expression (n=42) | Low expression (n=42) | X² value | P value |
|------------------------|------------------|------------------------|-----------------------|----------|---------|
| Age (years)            |                  |                        |                       |          |         |
| >60                    | 36               | 17 (47.22%)            | 19 (52.78%)           | 0.194    | 0.659   |
| ≤60                    | 48               | 25 (52.08%)            | 23 (47.92%)           |          |         |
| Sex                    |                  |                        |                       |          |         |
| Male                   | 66               | 34 (51.52%)            | 32 (48.48%)           | 0.426    | 0.514   |
| Female                 | 18               | 8 (44.44%)             | 10 (55.56%)           |          |         |
| Tumor size             |                  |                        |                       |          |         |
| ≤5                     | 32               | 15 (46.88%)            | 17 (53.12%)           | 0.202    | 0.653   |
| >5                     | 52               | 27 (51.92%)            | 25 (48.08%)           |          |         |
| TNM stage              |                  |                        |                       |          |         |
| I+II                   | 38               | 28 (73.68%)            | 10 (26.32%)           | 15.570   | 0.000   |
| III+IV                 | 46               | 14 (30.43%)            | 32 (69.57%)           |          |         |
| Metastasis             |                  |                        |                       |          |         |
| Yes                    | 47               | 15 (31.91%)            | 32 (68.09%)           | 13.960   | 0.000   |
| No                     | 37               | 27 (72.97%)            | 10 (27.03%)           |          |         |

Pearson’s Chi-squared test (*P<0.05). Abbreviation: TNM, tumor node metastasis.

Table 2 The expression relationship of miR-24 and vWF mRNA in human OS tissues

| Variables | miR-24 |                |                | X² value | *P value |
|-----------|--------|----------------|----------------|----------|----------|
| vWF mRNA  |        |                |                |          |          |
| High      | 42     | 12 (28.57%)    | 30 (71.43%)    | 19.091   | 0.000    |
| Low       | 42     | 32 (76.19%)    | 10 (23.81%)    |          |          |

Pearson’s correlation (*P<0.05).

Figure 3. vWF was a target of miR-24 in OS

(A) According to the putative binding site of miR-24 in the 3’-UTR of vWF (wild type), the mutated vWF 3’-UTR (mutant) was generated. (B) Dual luciferase reporter assay was performed in the OS cells MG-63 co-transfected with wild type/mutated vWF 3’-UTR reporters and miR-24 mimic (miR-24) or its control, miR-C. **P<0.01, compared with miR-C. OS cells MG-63 were transfected with miR-24 mimic or miR-C, and the level of (C) vWF mRNA and (D) protein was determined using qRT-PCR and Western blot analysis, respectively. ***P<0.001, compared with miR-C. The effect of miR-24 inhibition on (E) vWF mRNA and (F) protein expression was also evaluated in the OS cells MG-63 transfected miR-24 inhibitor (anti-miR-24) or its control, anti-miR-C. **P<0.01, compared with anti-miR-C.
miR-24 suppressed OS cell proliferation, colony formation, and migration through inhibiting vWF

Finally, we further dissected the physiological significance of the interaction between vWF and miR-24 in OS using loss- and gain-of-function approaches. Overexpression of miR-24 significantly repressed the expression of vWF in MG-63 and U2OS. Although did not significantly alter miR-24 level, pcDNA-vWF transfection increased the expression of vWF mRNA in miR-24 overexpressed MG-63 cells and U2OS cells, respectively (Figure 4A). In addition, co-overexpression of miR-24 and vWF reversed, at least in part, the miR-24-mediated decrease in the proliferation and colony formation, and increase in cell apoptosis (Figure 4B–D). Consistently, miR-24 notably inhibited the migration of MG-63 cells and U2OS cells, which could be attenuated through by co-overexpression of vWF (Figure 4E). Next, we performed the experiments to investigate the effects of miR-24 on vWF-deficit KHOS cells. We found that inhibition of miR-24 had limited effects on vWF level in KHOS cells (Figure 5A). Moreover, the inhibition of miR-24 failed to suppress vWF-deficit KHOS cell proliferation (Figure 5B), migration (Figure 5C), and colony formation (Figure 5D). These results indicate that vWF, as a downstream effector of miR-24, plays an important role in OS progression.

Discussion

vWF is a mainly expressed in ECs and megakaryocytes. vWF has important roles in hemostasis through facilitating platelet aggregation [22]. Additionally, studies have reported that plasma vWF was significantly increased in patients with colon cancer, ovarian cancer, and gastric adenocarcinoma [23, 24]. And high levels of plasma vWF were shown to be associated with cancer malignancy degree, overall survival rate, and poor prognosis [24]. Contradictorily, vWF was also reported to be involved in bone cancer cell apoptosis by inducing the binding of osteoprotegerin to TRAIL [13], implying a multiple facet of vWF in cancer progression. Recently, vWF was detected also in cancer cells of non-endothelial origin including OS [25]. Consistent with these results, vWF was found to be significantly up-regulated in metastatic OS tissues compared with primary OS tissues and associated with increased metastasis [14]. Moreover, vWF was expressed in OS cell line SAOS2 and increased the adhesion capacity of SAOS2 cells to endothelial monolayer, causing increased cancer cell extravasation and transmigration [26]. Therefore, target vWF attenuated the metastatic characteristics of OS cells, representing a potential target of OS therapy. The current study provided new evidence that vWF was abundant in human primary OS tissues.

More importantly, in human primary OS tissues, our study showed a negative correlation between the expression of vWF mRNA and miR-24, which has been demonstrated to greatly down-regulate in OS cells and to inhibit OS metastasis [21]. Accumulating studies indicated that miRNAs might serve as a potential prognostic predictor and/or therapeutic target in the tumorigenesis and progression of OS [27]. miR-24, which is abnormally expressed in various cancer types, has also been reported as one of the candidates in OS. Previous reports of dysregulated miR-24 in OS showed that miR-24 was able to inhibit the proliferation, invasion, and migration of OS cells, suggesting miR-24 as a tumor suppressor in OS progression [20, 21]. This conclusion was further strengthened by our findings that miR-24 was markedly down-regulated in the metastatic OS tissues compared with primary OS tissues and suppressed OS cell proliferation, colony formation, and migration. miR-24 was identified as an independent prognostic marker for acute leukemia, colorectal cancer, aflatoxin B1-related hepatocellular carcinoma, and human tongue squamous cell carcinoma [28-31]. In the present study, the clinical data showed that low miR-24 expression in human OS tissues was significantly associated with tumor metastasis and predicted a poor survival in OS patients, thus provided new insight into the clinical significance of miR-24 expression in OS patients.

miRNAs interacting with the 3′-UTR of mRNA constitute a transcription or post-transcription regulation mechanisms involved in various biological functions and the pathogenesis of diseases. The previous study has reported that vWF 3′-UTR as a target of miR-24 based on the bioinformatics analysis [22], the present data also evidenced that vWF was a target gene of miR-24 in OS as shown by that miR-24 mimic inhibited, whereas miR-24 inhibitor increased, both the mRNA and protein levels of vWF in OS cells. Furthermore, it was noticed that vWF overexpression predominantly attenuated the miR-24-mediated inhibition on the proliferation and migration of OS cells without altering the endogenous miR-24 level. Thus, our data indicated that vWF dysregulation was the critical mediator that contributed to tumor suppression induced by miR-24.

In summary, we demonstrated that increased vWF by miR-24 down-regulation promoted the proliferation and migration of OS cells and further identified the clinical significance of miR-24 expression in OS patients. Although additional prospective analysis is needed in other OS cell lines and in vivo, the present data confirmed the importance of miR-24/vWF signaling axis in OS progression and provided a new mechanistic insight into the functional involvement of vWF in OS in addition to its clinical observations.
Figure 4. miR-24 suppressed OS cell proliferation, migration, and colony formation through inhibiting vWF
MG-63 and U2OS cells were transfected with miR-24 mimic, miR-C, or miR-24 mimic and pcDNA-vWF (vWF), respectively. (A) The expressions of miR-24 and vWF mRNA were determined using qRT-PCR analysis. (B) The cell proliferation was assessed using CCK-8 assay. (C) Crystal violet staining was performed to detect the cell colony formation ability, and representative images were showed. (D) the cell apoptosis was assessed using Annexin V staining by flow cytometry. (E) The migration of MG-63 and U2OS cells was evaluated by wound-healing assay at 48 h after wound formation. Representative images and the cell counting results of MG-63 were showed. *P<0.05, **P<0.01, ***P<0.001.
Figure 5. **miR-24 failed to suppress vWF-deficit OS cell proliferation, migration, and colony formation**

KHOS cells were transfected with miR-C or miR-24 antagonist. (A) The expressions of miR-24 and vWF mRNA were determined using qRT-PCR analysis in cells, respectively. (B) The cell proliferation was assessed using CCK-8 assay. (C) Crystal violet staining was performed to detect the cell colony formation ability, and representative images were showed. (D) The migration of KHOS cells was evaluated by wound-healing assay at 48 h after wound formation. **P < 0.01.

**Competing interests**
The authors declare that there are no competing interests associated with the manuscript.

**Author contribution**
L.L., J.P., and H.W. designed and performed research, interpreted data. Z.M., J.Y., and F.Y. performed immunostaining experiments and analyzed data. Q.Y., L.Z., X.L., Y.Z., Z.B., and H.Y. performed cell experiments. J.L. conceptualized, analyzed data, and wrote the manuscript.

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**Abbreviations**
CCK-8, cell counting kit-8; EC, endothelial cell; OS, osteosarcoma; PVDF, polyvinylidene fluoride; vWF, von Willebrand factor.

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