Deuterium-depleted water inhibits the malignant progression of colorectal cancer cells by modulating oxidative stress

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Abstract. Colorectal cancer (CRC) is one of the most common types of cancer worldwide. Alternative therapy has been widely used in CRC treatment, with deuterium-depleted water (DDW) demonstrating promising anticancer effects in a number of cancer types. The aim of the present study was to assess the anticancer effects of DDW in CRC cells and the possible mechanism involved. HT-29 and DLD-1 cells were cultured in conditioned medium prepared with DDW. Cell malignant behaviors were assessed using EdU, colony formation, tumor-sphere formation, wound-healing and Transwell assays. Stemness-related proteins, Nanog and octamer-binding transcription factor-4, were assessed using western blotting. Intracellular reactive oxygen species (ROS) levels were determined using 2',7'-dichlorodihydrofluorescein diacetate fluorescent probes. Reverse transcription-quantitative PCR and western blotting were used to assess the expression of forkhead box protein M1 (FoxM1), cyclin D1 (CCND1) and matrix metalloproteinase 9 (MMP9). The results indicated that treatment with DDW significantly inhibited the proliferation, tumor-sphere formation, migration and invasion of HT-29 and DLD-1 cells, as well as the expression of stemness-related proteins. In the mechanistic analysis, DDW treatment was revealed to decrease ROS production and downregulate the expression of FoxM1. As the downstream targets of FoxM1, the expression levels of CCND1 and MMP9 were also shown to be decreased. Moreover, H_2O_2-induced oxidative stress rescued FoxM1 expression in the presence of DDW treatment, and overexpression of FoxM1 was demonstrated to abolish the DDW-mediated tumor suppressive effects. The findings from the present study indicate that the anticancer effects of DDW in CRC cells occur by inactivating the ROS/FoxM1 signaling pathway. Moreover, the results provide a possible agent for CRC treatment.

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related mortality worldwide (1). Surgery, chemotherapy and radiotherapy are three primary modalities in CRC treatment; however, several alternative agents, including vitexin, nitric oxide and sodium selenite, have been reported to inhibit tumor progression and increase sensitivity to chemoradiotherapy in CRC (2-4). This indicates that alternative therapies could be employed to augment the efficacy of primary treatments and even achieve tumor suppressive purposes independently.

Hydrogen is the most abundant element in the universe. Deuterium and protium are two stable isotopes of hydrogen, which exhibit differences not only in physical and chemical properties but also in biological functions (5). The concentration of deuterium in natural water is ~0.015% [150 parts per million (ppm)] (6). Deuterium-depleted water (DDW) is defined as water with a deuterium concentration of <150 ppm, and it was reported to have an inhibitory effect on xenotransplanted tumor growth in mice for the first time in the 1990s (7). Subsequently, studies reported the effects of DDW in several areas, including in cancer suppression (8), anti-senescence (9), insulin sensitization (10) and neuroprotection (11). However, the functions of DDW in CRC treatment remain unclear.

Oxidative stress has been reported to serve a major role in CRC progression (12). Furthermore, the inhibition of reactive oxygen species (ROS) production is considered a critical signaling mechanism of the DDW anticancer function (13,14). Forkhead box (Fox)M1, a member of the FOX transcription factor family, is recognized as a master regulator of cancer growth, stemness and metastasis in CRC (15). Previous studies have reported crosstalk between ROS production and FoxM1 expression (16,17); however, whether the FoxM1 signaling pathway can be blocked by DDW, and the role of ROS in this process, remain unknown.

Therefore, the objective of this study was to systematically investigate the impact of DDW on malignant biological behaviors and potential molecular mechanisms in CRC cells cultured *in vitro*, specifically focusing on its effects on cell proliferation, stemness, migration, invasion and regulation of

the ROS/FoxM1 signaling pathway. The results of the present study may facilitate the clinical application of DDW for CRC adjuvant therapy in the future.

Materials and methods

Cell culture and experimental groups. The DDW (deuterium %=25 ppm) used in the present study was provided by HYD LLC. Regular ddH_2O or DDW was used to dissolve RPMI-1640 medium powder (Gibco; Thermo Fisher Scientific, Inc.). A total of two CRC cell lines, HT-29 (cat. no. STCC10801G) and DLD-1 (cat. no. STCC10810G), were purchased from Wuhan Servicebio Technology Co., Ltd. and authenticated by STR profiling. Cells were cultured in conditioned RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution (Sigma-Aldrich; Merck KGaA) at 37˚C in a humidified atmosphere containing 5% CO_2. The control group contained cells that were cultured in medium prepared with regular ddH_2O, whereas the cells in the DDW group were cultured in medium prepared with DDW.

EdU incorporation assay. The EdU assay was performed using a commercial kit (cat. no. C10310-1; Guangzhou RiboBio Co., Ltd.). HT-29 and DLD-1 cells were seeded into 6-well plates at a density of $1x10^5$ cells per well. After culturing in serum-free medium for 72 h, cells were incubated with 0.1% EdU solution for 3 h at 37˚C, then fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. Cells were then incubated with appollo staining complex for 30 min at room temperature to react with EdU, followed by incubation with Hoechst 33342 staining reagent for 30 min at room temperature to label the cell nuclei. Fluorescent staining was recorded using the Operetta CLS high-content analysis system (PerkinElmer, Inc.). A total of five visual fields were randomly selected and the number of EdU positive cells was assessed using ImageJ software (version 1.53 K; National Institutes of Health). The following formula was used to determine the percentage of EdU positive cells: EdU positive cells (%)=(EdU positive cells/total cells) x100%.

Colony formation assay. HT-29 and DLD-1 cells were seeded into 6-well plates at a density of 1x103 cells per well and cultured with conditioned medium. The medium was changed every 3 days. After 10 days, the cells were fixed with 4% PFA for 30 min at room temperature and then stained with 0.1% crystal violet for 20 min at room temperature. The colonies consisting of at least 40 cells were counted under an inverted light microscope (ECLIPSE Ts2; Nikon Corporation) using ImageJ software.

Tumor-sphere formation assay. DMEM powder and F-12 medium powder (both from Gibco; Thermo Fisher Scientific, Inc.) were dissolved with regular ddH2O or DDW. Both media were mixed at a ratio of 1:1 to prepare the DMEM/F-12 medium. HT-29 and DLD-1 cells were seeded into ultra-low attachment 24-well plates (Corning, Inc.) at a density of $1x10^3$ cells per well and cultured in DMEM/F12 medium supplemented with 1% B27, 20 ng/ml human FGF and 20 ng/ml human EGF (Invitrogen™; Thermo Fisher Scientific, Inc). Fresh medium was added every 3 days. After 2 weeks, cell spheres were counted under an inverted light microscope (ECLIPSE Ts2; Nikon Corporation), and the sphere formation efficiency (SFE) was calculated using the following formula: SFE (%)=number of spheres/1,000 x100%.

Wound healing assay. HT-29 and DLD-1 cells were cultured in 6-well plates until they formed confluent monolayers. A gap was created across the middle of the well using a 200 μl sterile tip. The cells were then cultured in serum-free medium for an additional 48 h. An inverted light microscope (ECLIPSE Ts2; Nikon Corporation) was used to capture images both before and after the 48-h incubation period to evaluate the migration rate. Experiments were repeated in quintuplicate. A visual field was randomly selected, and the same field was imaged for every time point. The area of blank space was measured using ImageJ software. The following formula was used to assess the wound healed ratio: Wound healed ratio (%)=[1-($area^{48\,h}$/$area^{0\,h}$)] x100%.

Transwell assay. HT-29 and DLD-1 cells were suspended in serum-free medium. The inner membranes of Transwell inserts (cat. no. 3464; Corning, Inc.) were coated with 10% Matrigel (cat. no. 354234; Corning, Inc.), followed by the seeding of $2x10^4$ cells with 200 μl serum-free DMEM into each upper chamber. A total of 750 μl DMEM supplemented with 10% FBS was added to each lower chamber of the 24-well plate. After incubating for 24 h at 37˚C, the cells were fixed with 4% PFA for 30 min at room temperature and then stained with 0.1% crystal violet for 20 min at room temperature. Cells that successfully invaded the bottom side of membrane were visualized using an inverted light microscope (ECLIPSE Ts2; Nikon Corporation).

Intracellular ROS detection. Intracellular ROS levels were assessed using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) method. HT-29 and DLD-1 cells were incubated with the DCFH-DA probes (Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C and then treated with H2O2 (50 μM) for 30 min at 37˚C. Cells were then fixed with 4% PFA for 30 min at room temperature. The fluorescence intensity of DCF was recorded using the Operetta CLS High-Content Analysis System (PerkinElmer, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. RT-qPCR was performed using the SuperScript™ III Platinum™ One-Step qRT-PCR Kit (cat. no. 11732020; Invitrogen; Thermo Fisher Scientific, Inc.). The sequences of the primers used for qPCR were as follows: *FoxM1* forward, 5'-ACGTCCCCAAGCCAGGCTC-3' and reverse, 5'-CTACTGTAGCTCAGGAATAA-3'; cyclin D1 (*CCND1*) forward, 5'-CGCTTCCTGTCGCTGGAGCC-3' and reverse, 5'-CTTCTCGGCCGTCAGGGGGA-3'; matrix metalloproteinase 9 (*MMP9*) forward, 5'-TTCTGCCCGGACCAAGGATA-3' and reverse, 5'-ACATAGGGTACATGAGCGCC-3'; and β-actin (reference gene) forward, 5'-CATGTACGTTGCTATCCAGGC-3' and reverse, 5'-CTCCTTAATGTCACGCACGAT-3'. The thermocycling conditions were as follows: 50˚C for 15 min

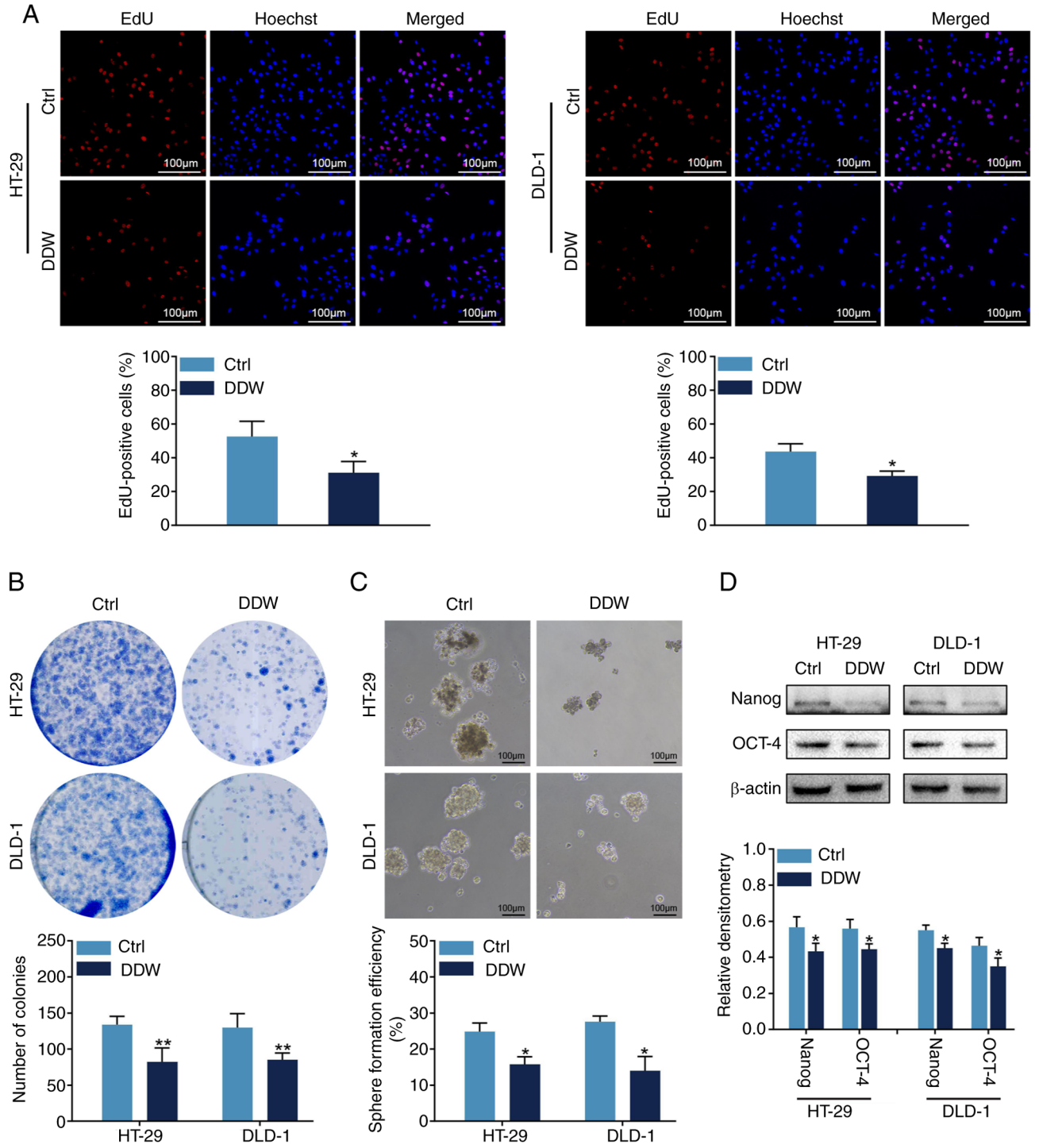

Figure 1. DDW inhibits the proliferation and stemness of colorectal cancer cells. (A) EdU incorporation and (B) colony formation assays were performed using HT-29 and DLD-1 cells to evaluate the effects of DDW on cell proliferation. (C) Tumor-sphere formation assays were used to assess the cancer stem cell-like property of HT-29 and DLD-1 cells following treatment with DDW. (D) Western blotting demonstrated the protein expression levels of Nanog and OCT-4 in HT-29 and DLD-1 cells treated with DDW. *P<0.05, **P<0.01. DDW, deuterium-depleted water; OCT-4, octamer-binding transcription factor-4; Ctrl, control.

and 95˚C for 2 min, and then 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec. The relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (18).

Lentiviral transduction. FoxM1-overexpressing (OE-FoxM1) and negative control (OE-NC) lentiviral particles ($1x10^8$ TU/ml) were purchased from Shanghai GeneChem Co., Ltd., both originating from the same plasmid backbone (Ubi-MCS-3FLAG-SV40-puromycin). HT-29 and DLD-1 cells were seeded into 6-well plates at a density of $1x10^5$ cells per well and incubated overnight. HT-29 and DLD-1 cells were transduced with the specific lentivirus at a multiplicity of infection of 10. After a 24-h incubation, fresh medium was changed to terminate the transduction. Stable infected cells were selected by puromycin (5 μg/ml) incubation for 48 h, and the surviving cells were continuously maintained in

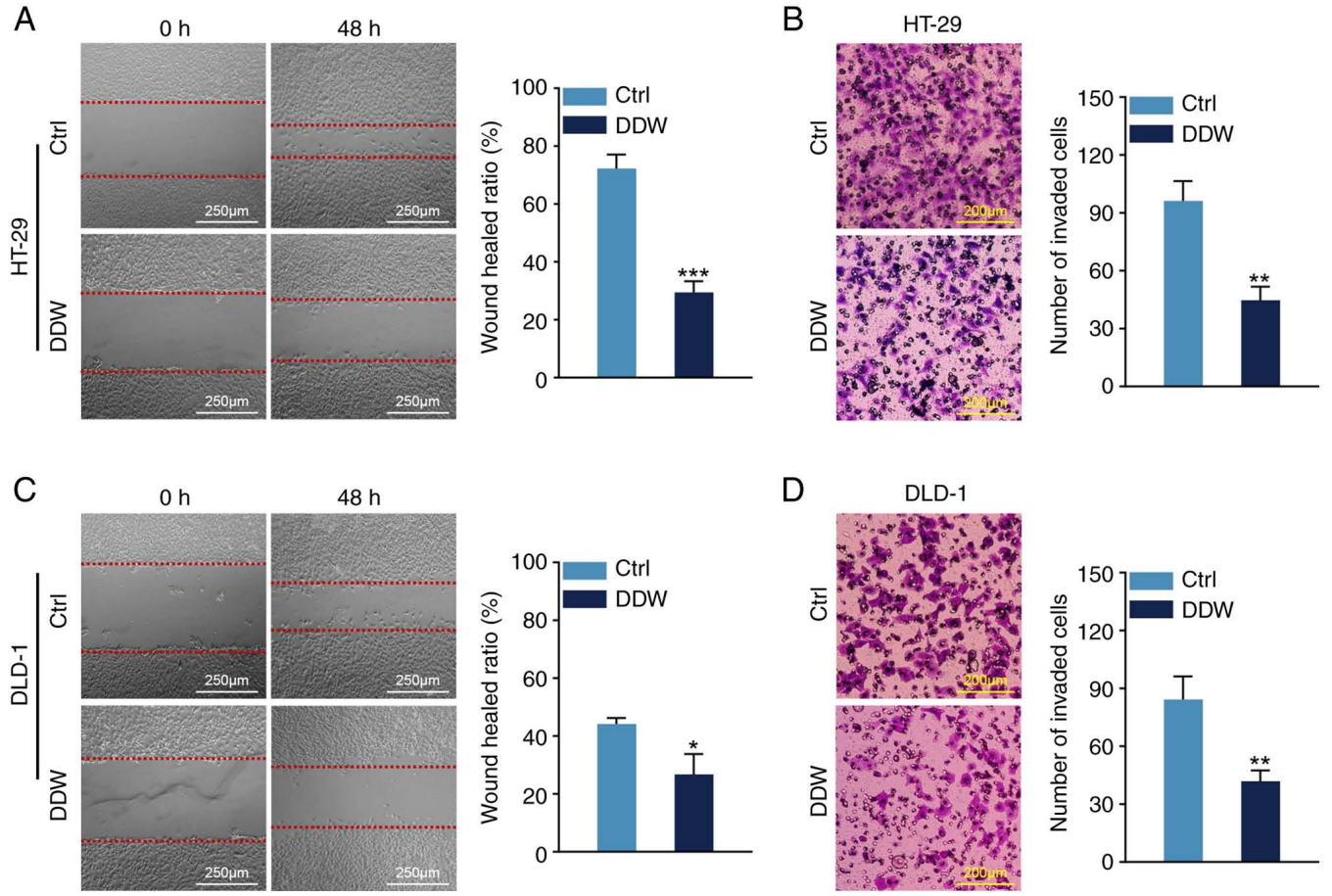

Figure 2. DDW restrains the migration and invasion of colorectal cancer cells. The effects of DDW on HT-29 cell (A) migration and (B) invasion were assessed using wound healing and Transwell assays, respectively. The effects of DDW on DLD-1 cell (C) migration and (D) invasion were assessed using wound healing and Transwell assays, respectively. *P<0.05, **P<0.01, ***P<0.001. DDW, deuterium-depleted water; Ctrl, control.

fresh DMEM with 10% FBS and 1.25 μg/ml puromycin for passaging. FoxM1 expression in the recombinant cells was validated by RT-qPCR and western blotting before subsequent experiments.

Western blotting. Total proteins from HT-29 and DLD-1 cells were extracted using RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) and quantified using the BCA Protein Assay Kit (cat. no. P0012; Beyotime Institute of Biotechnology). Proteins (40 μg per lane) were separated by 4-20% SDS-PAGE (cat. no. P0468S; Beyotime Institute of Biotechnology) and subsequently transferred onto PVDF membranes (MilliporeSigma). The membranes were blocked with 5% fat-free milk (Bio-Rad Laboratories, Inc.) for 1 h at room temperature and incubated with primary antibodies (1:1,000 dilution by PBS) against β-actin (cat. no. 66009-1-Ig), octamer-binding transcription factor-4 (OCT-4; cat. no. 11263-1-AP), Nanog (cat. no. 14295-1-AP), MMP-9 (cat. no. 10375-2-AP), CCND1 (cat. no. 26939-1-AP) and FoxM1 (cat. no. 13147-1-AP) (all Proteintech Group, Inc.) at 4˚C overnight. Protein signals were labeled with HRP-conjugated secondary antibodies (1:5,000 dilution by PBS; cat. nos. SA00001-1 or SA00001-2; Proteintech Group, Inc.) for 1 h at room temperature and visualized using an ECL kit (cat. no. WBKLS0500; MilliporeSigma). The densitometry was measured using ImageJ software.

Statistical analysis. Continuous data are expressed as mean ± standard deviation. Differences between two groups were analyzed using the unpaired Student's t-test or Mann-Whitney U test as appropriate. One-way ANOVA was used to compare the mean differences among three or more groups, and the least significant difference test was used as a post-hoc test. The data analysis for this paper was generated using GraphPad Prism 10 software (Dotmatics). P<0.05 was considered to indicate a statistically significant difference.

Results

DDW inhibits the proliferation and stemness of CRC cells. To assess the biological functions of DDW in CRC malignancy, HT-29 and DLD-1 cells were cultured in conditioned medium prepared with regular ddH_2O or DDW. It was demonstrated that, compared with the control (regular ddH_2O) treatment, DDW treatment reduced the proportion of EdU-positive cells (Fig. 1A) and the number of colonies formed (Fig. 1B). These results indicated that DDW could inhibit the proliferation of CRC cells *in vitro*. Notably, the results also revealed that DDW treatment impaired the sphere formation efficiency of HT-29 and DLD-1 cells (Fig. 1C). This finding implied that DDW might interfere with the stem cell-like phenotype of CRC cells. Therefore, the expression levels of two typical stemness markers were analyzed using western blotting. The findings demonstrated that, compared

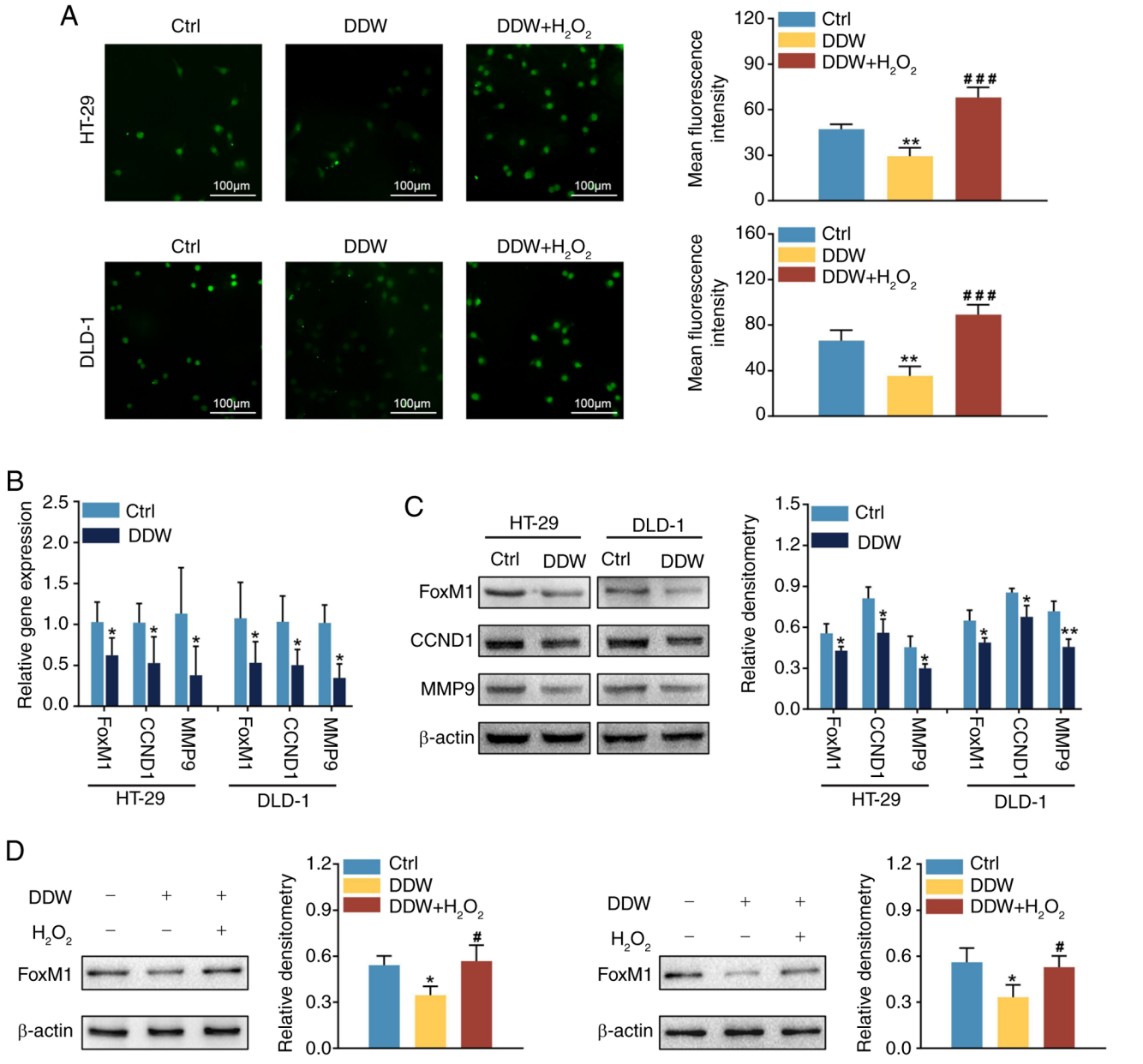

Figure 3. DDW downregulates FoxM1 signaling through suppressing ROS production in colorectal cancer cells. (A) Levels of ROS in HT-29 and DLD-1 cells treated with DDW or DDW + H_2O_2 were assessed using 2',7'-dichlorodihydrofluorescein diacetate fluorescent analysis. The mRNA and protein levels of FoxM1, CCND1 and MMP9 in HT-29 and DLD-1 cells treated with DDW were analyzed using (B) reverse transcription-quantitative PCR and (C) western blotting. (D) Protein levels of FoxM1 in HT-29 and DLD-1 cells treated with DDW or DDW + H_2O_2 were analyzed using western blotting. *P<0.05 vs. Ctrl group, **P<0.01 vs. Ctrl group, $^{\#}$P<0.05 vs. DDW group, $^{\#\#\#}$P<0.001 vs. DDW group. DDW, deuterium-depleted water; FoxM1, forkhead box protein M1; ROS, reactive oxygen species; CCND1, cyclin D1; MMP9, matrix metalloproteinase 9; Ctrl, control.

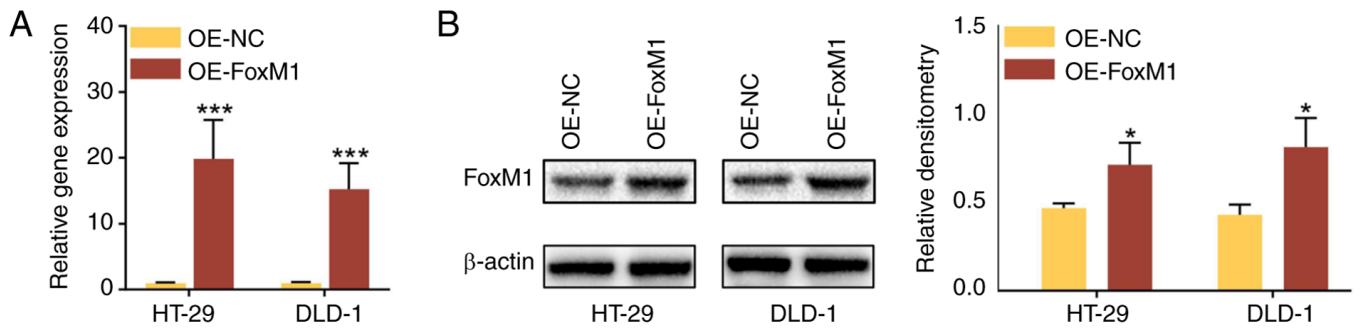

Figure 4. Overexpression of FoxM1 in colorectal cancer cells. FoxM1 was successfully overexpressed in both HT-29 and DLD-1 cells, and the transduction efficiency was assessed using (A) reverse transcription-quantitative PCR and (B) western blotting. *P<0.05, ***P<0.001. FoxM1, forkhead box protein M1; OE, overexpression; NC, negative control.

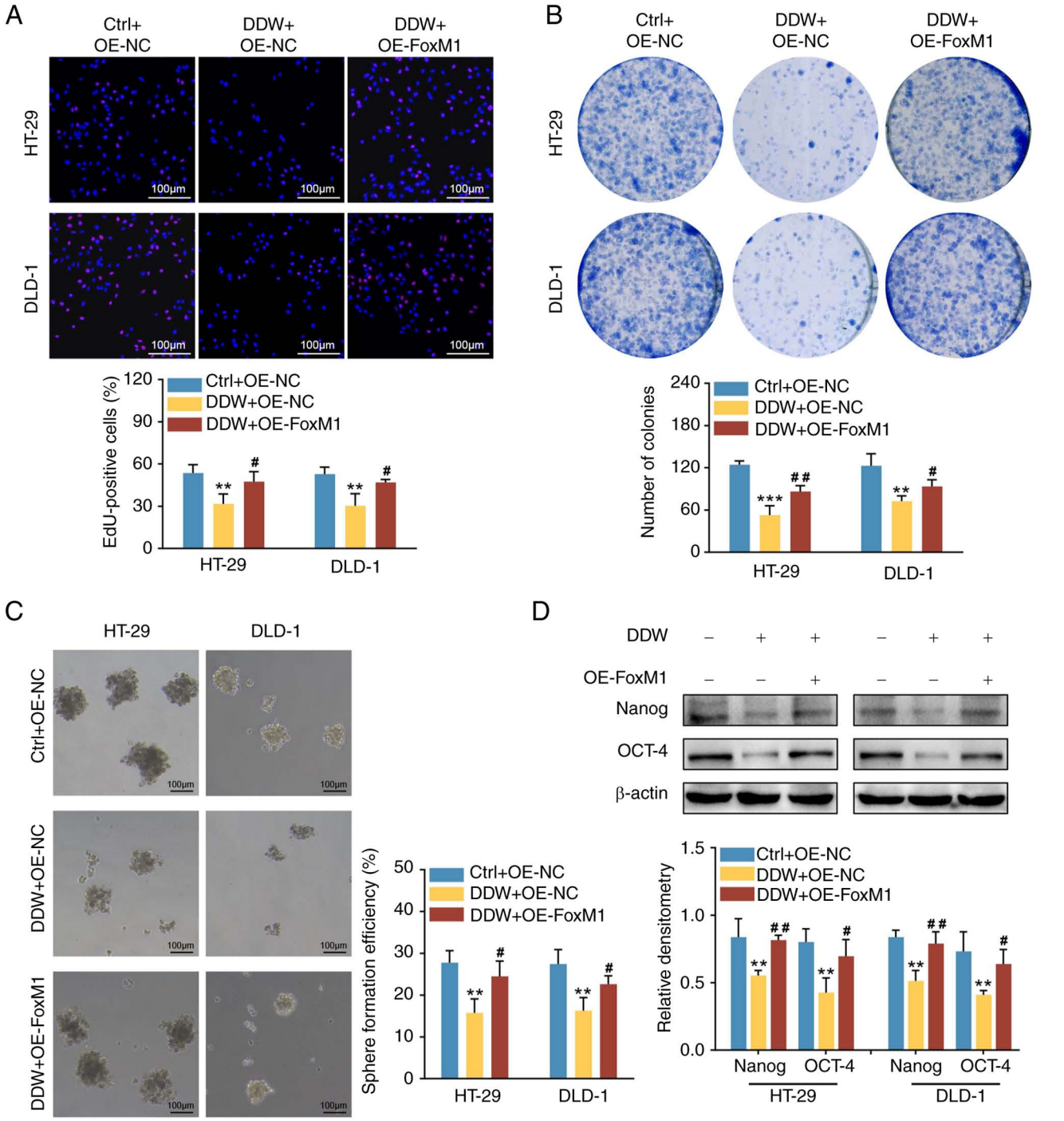

Figure 5. Overexpression of FoxM1 abolishes the inhibitory effects of DDW on proliferation and stemness in colorectal cancer cells. (A) EdU incorporation and (B) colony formation assays were performed to assess the proliferation of HT-29 and DLD-1 cells with FoxM1 overexpression in the presence of DDW treatment. (C) The cancer stem cell-like property of HT-29 and DLD-1 cells was evaluated using the tumor-sphere formation assay. (D) Protein levels of Nanog and OCT-4 in HT-29 and DLD-1 cells were assessed using western blotting. **P<0.01 vs. Ctrl + OE-NC group, ***P<0.001 vs. Ctrl + OE-NC group, $^{\#}$P<0.05 vs. DDW + OE-NC group, $^{\#\#}$P<0.01 vs. DDW + OE-NC group. FoxM1, forkhead box protein M1; DDW, deuterium-depleted water; OCT-4, octamer-binding transcription factor-4; OE, overexpression; Ctrl, control; NC, negative control.

with the control group, the protein levels of Nanog and OCT-4 were significantly downregulated in the DDW treatment group (Fig. 1D). These results indicated that DDW treatment impaired the self-renewal ability of HT-29 and DLD-1 cells.

DDW restrains the migration and invasion of CRC cells. Subsequently, wound healing and Transwell assays were performed to evaluate the effect of DDW on cancer cell mobility. Compared with the control treatment, the migrated distances of HT-29 and DLD-1 cells were significantly reduced when treated with DDW (Fig. 2A and C). Additionally, there was a notable reduction in the number of invaded cells for both the HT-29 and DLD-1 cells treated by DDW in the Transwell assays (Fig. 2B and D). These findings suggested that DDW treatment is associated with a significant reduction in CRC cell migration and invasion.

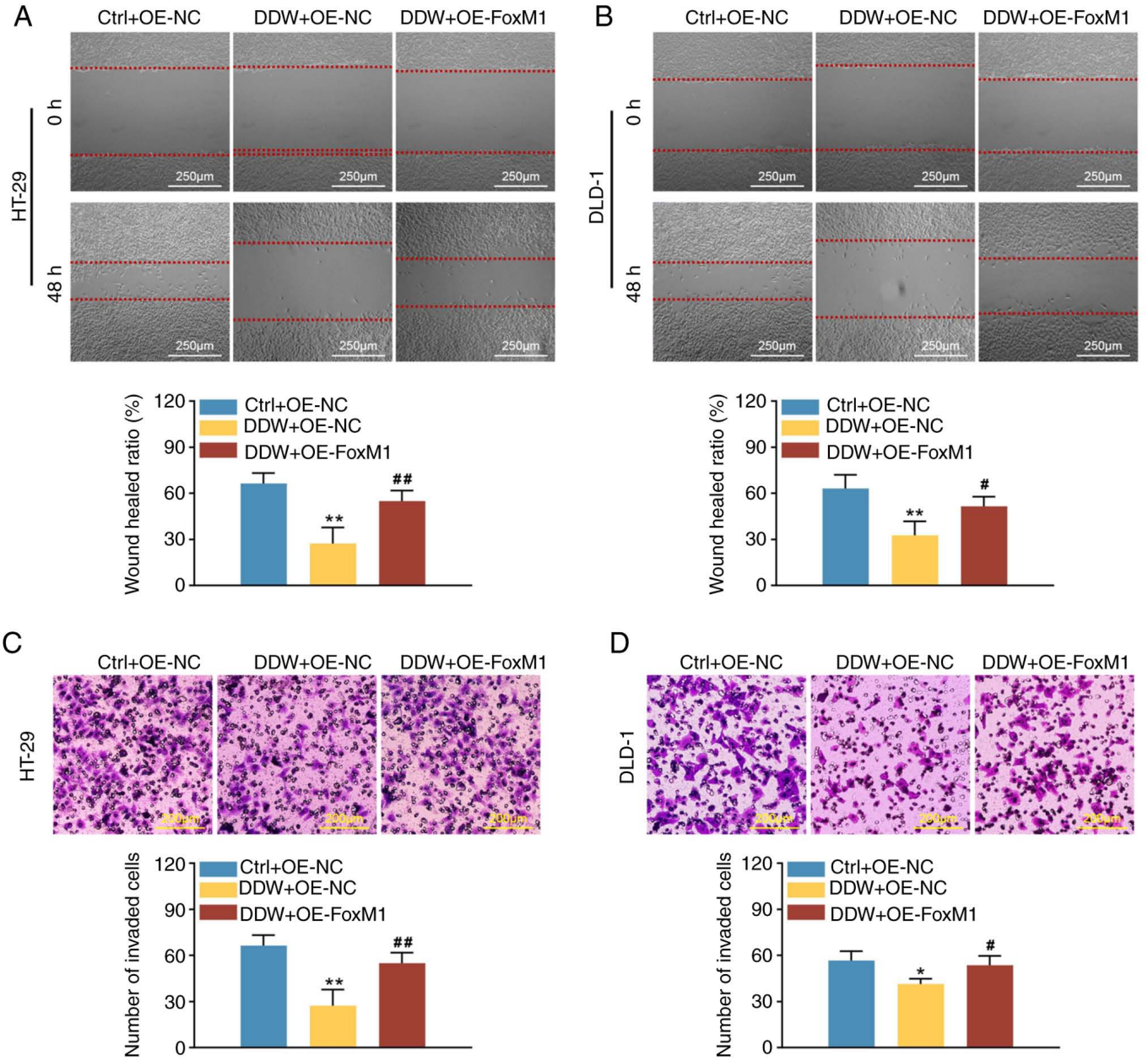

Figure 6. Overexpression of FoxM1 abrogates the effects of DDW on the migration and invasion of colorectal cancer cells. Migration was assessed using wound healing assays in (A) HT-29 and (B) DLD-1 cells with FoxM1 overexpression in the presence of DDW treatment. The invasion of (C) HT-29 and (D) DLD-1 cells was evaluated using Transwell assays. *P<0.05 vs. Ctrl + OE-NC group, **P<0.01 vs. Ctrl + OE-NC group, $^{\#}$P<0.05 vs. DDW + OE-NC group, $^{\#\#}$P<0.01 vs. DDW + OE-NC group. FoxM1, forkhead box protein M1; DDW, deuterium-depleted water; OE, overexpression; Ctrl, control; NC, negative control.

DDW downregulates FoxM1 signaling through suppressing ROS production in CRC cells. To determine whether the ROS/FoxM1 axis was involved in the aforementioned findings, ROS production in response to DDW treatment was evaluated in the CRC cells. The findings revealed that, compared with the control treatment, DDW treatment significantly reduced the ROS levels in the HT-29 and DLD-1 cell lines (Fig. 3A). Subsequently, RT-qPCR and western blotting were used to evaluate FoxM1 expression. Compared with the control treatment, DDW treatment significantly reduced the mRNA and protein levels of FoxM1 (Fig. 3B and C). Moreover, the expression levels of CCND1 and MMP9, two typical downstream targets of FoxM1 (19,20), were lower in HT-29 and DLD-1 cells treated with DDW, compared with the control treatment (Fig. 3B and C). Furthermore, H_2O_2 was utilized to induce intracellular ROS production. Compared with the cells treated by DDW alone, additional treatment with H_2O_2 not only blocked the antioxidant effect of DDW (Fig. 3A) but also rescued the expression of FoxM1 (Fig. 3D). These results indicated that DDW treatment downregulated FoxM1 expression possibly through inhibiting ROS production.

Overexpression of FoxM1 abolishes the anticancer effects of DDW in CRC cells. To assess whether the anticancer effects of DDW were dependent on the inhibition of FoxM1, FoxM1 was overexpressed in HT-29 and DLD-1 cells using lentiviral transduction. The mRNA and protein expression levels were significantly upregulated in OE-FoxM1 cells compared

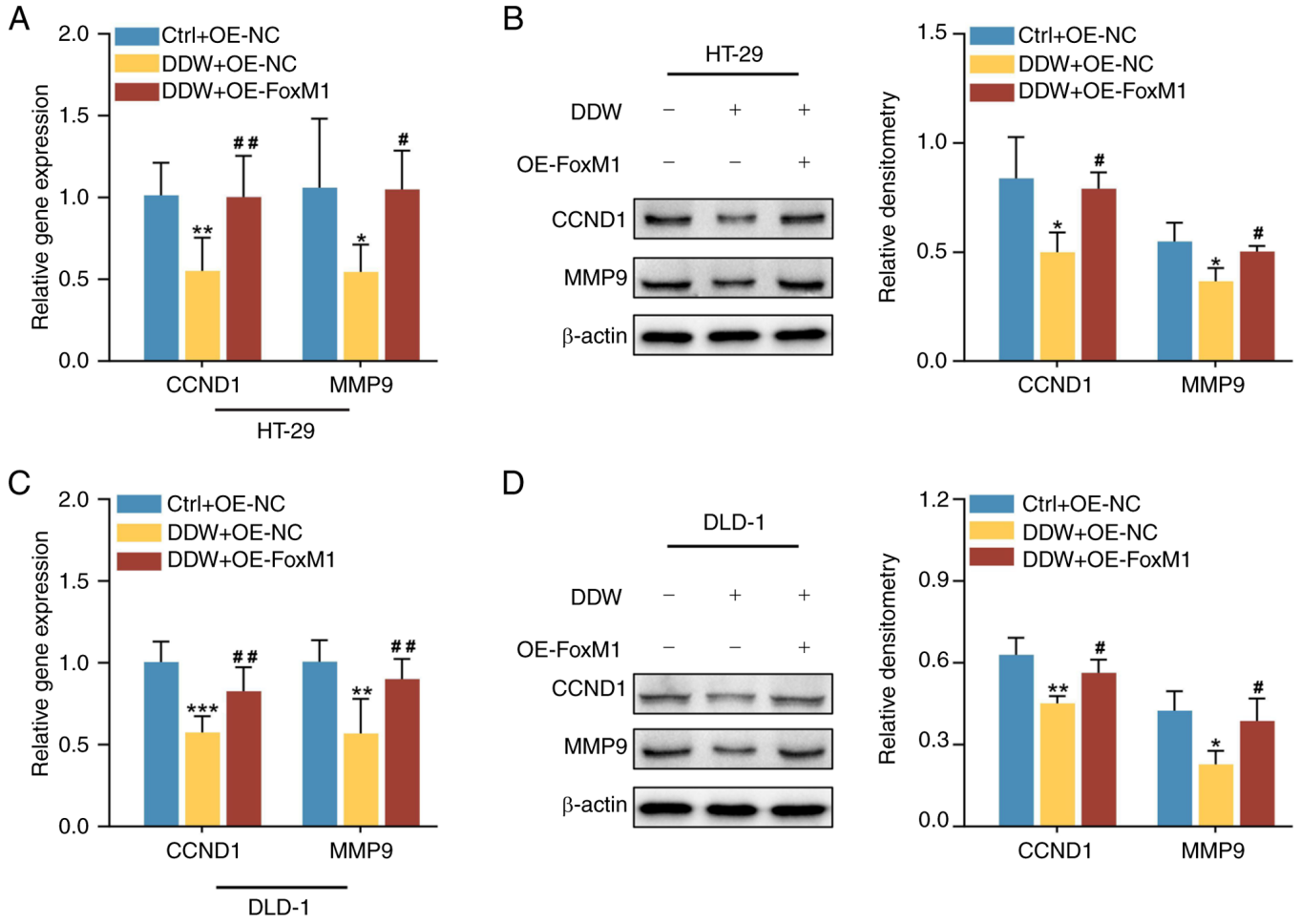

Figure 7. Overexpression of FoxM1 rescues the DDW-mediated inhibition of CCND1 and MMP9 in colorectal cancer cells. The (A) mRNA and (B) protein expression levels of CCND1 and MMP9 in HT-29 cells were assessed using RT-qPCR and western blotting, respectively. The (C) mRNA and (D) protein expression levels of CCND1 and MMP9 in DLD-1 cells were assessed using RT-qPCR and western blotting, respectively. *P<0.05 vs. Ctrl + OE-NC group, **P<0.01 vs. Ctrl + OE-NC group, ***P<0.001 vs. Ctrl + OE-NC group, $^{\#}$P<0.05 vs. DDW + OE-NC group, $^{\#\#}$P<0.01 vs. DDW + OE-NC group. FoxM1, forkhead box protein M1; DDW, deuterium-depleted water; CCND1, cyclin D1; MMP9, matrix metalloproteinase 9; OE, overexpression; Ctrl, control; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.

with the OE-NC cells (Fig. 4). Furthermore, compared with the OE-NC lentiviral transduction, the inhibitory effects of DDW on the proliferation (Fig. 5A and B), stemness (Fig. 5C and D), migration (Fig. 6A) and invasion (Fig. 6B) of HT-29 and DLD-1 cells were significantly abrogated by OE-FoxM1 lentiviral transduction. In addition, overexpression of FoxM1 also rescued the expression levels of CCND1 and MMP-9 in CRC cells treated with DDW, as demonstrated using RT-qPCR (Fig. 7A and C) and western blotting (Fig. 7B and D). These results validated that DDW may exert its tumor suppressive effects through inhibiting FoxM1 expression.

Discussion

Water is the most common compound on Earth, but its isotopic composition is heterogeneous due to kinetic isotope fractionation (21). Given that the variation of the levels of deuterium in different terrestrial water sources is minimal, it is difficult to observe significant differences in biological effects among populations consuming natural water with varying deuterium concentrations. Artificial DDW can be prepared by desalination, distillation and catalytic exchange (22). The deuterium-content of DDW used in translational and clinical studies ranges from 25 to 125 ppm; however, Yavari *et al* (23) reported that the synergistic anticancer effects of DDW combined with 5-fluorouracil gradually faded with the increase of deuterium content from 30 to 150 ppm. This indicated that a dose-dependent inhibition of cancer progression may exist in DDW-treated cells. Therefore, the present study used 25 ppm DDW to treat HT-29 and DLD-1 cells.

To the best of our knowledge, the present study is the first to demonstrate that DDW exerts anticancer effects through inhibiting the ROS/FoxM1 signaling pathway in CRC cells. This finding was demonstrated by the following results: i) DDW treatment inhibited cell proliferation, stemness, migration and invasion *in vitro*; ii) DDW treatment decreased ROS production as well as the expression of FoxM1 and its downstream targets, CCND1 and MMP-9; iii) the induction of ROS partly rescued FoxM1 expression in DDW-treated CRC cells; and iv) overexpression of FoxM1 abrogated DDW-mediated tumor suppressive effects.

DDW has been shown to suppress the proliferation of breast cancer cells by arresting them at the G0 or G1 phases (23). Notably, the results of the present study revealed that DDW

treatment decreased the expression of CCND1 in CRC cells. As a critical mediator of cell cycle progression, CCND1 is responsible for transiting cells from the G1 to S phase (24). The results of the present study also demonstrated that DDW inhibited cell migration, cell invasion and MMP-9 expression, which is in accordance with the results in nasopharyngeal carcinoma cells reported by Wang *et al* (25). Moreover, a growing body of research has reported promising outcomes regarding the clinical application of DDW. Concomitant administration of DDW with conventional therapies has been demonstrated to achieve improved overall survival rates compared with the use of chemoradiotherapy alone in patients with advanced-stage glioblastoma multiforme (26) and lung cancer (27). Furthermore, ~30% of patients with CRC who receive initial curative treatment typically develop tumor relapse (28). However, a retrospective analysis reported that consumption of DDW markedly reduced this rate to 18.5% (8), supporting the strong tumor-prevention effect of DDW.

Although overproduction of ROS induced by chemoradiotherapy leads to the death of cancer cells, slightly elevated ROS levels resulting from intracellular hypermetabolism facilitate tumor progression by activating pro-survival transcription factors including hypoxia-inducible factor 1α (HIF-1α), activator protein-1 and NF-κB (29). The present study demonstrated that manipulation of the degree of ROS production could disrupt the inhibitory efficacy of DDW toward the expression of FoxM1, and the overexpression of FoxM1 counteracted the tumor-inhibiting effects of DDW. These results indicate that ROS-induced FoxM1 could be the target of DDW in CRC treatment. However, the molecular mechanisms by which ROS upregulates FOXM1 are complex. Park *et al* (30) reported that endogenous ROS were constantly generated when cancer cells were cultured in favorable conditions with sufficient energy metabolism and oxygen supply, causing the upregulation of FoxM1 to enhance the rates of proliferation and metastasis. This viewpoint was corroborated by the finding that ROS-induced upregulation of FoxM1 promotes aerobic glycolysis in glioblastoma (31). Moreover, ROS have also been demonstrated to activate the FoxM1 promoter and induce its expression in a HIF-1α-dependent manner (32).

However, some limitations of the present study should be noted. First, the detailed mechanisms by which DDW inhibits ROS production remain unclear. Future research will focus on mitochondrial-related biological changes to explore whether DDW inhibits the production of ROS by disrupting mitochondrial function. Second, the *in vivo* anticancer effects of DDW were not evaluated in the present study. Animal experiments and population-based randomized controlled clinical trials will be introduced to further demonstrate the therapeutic effects of DDW in colorectal cancer.

In summary, the findings of the present study suggest that DDW has multiple anticancer effects in CRC cells, and DDW-mediated ROS/FoxM1 signaling inactivation could be a possible pathway in these effects. Future studies should focus on the clinical application of DDW as a promising alternative therapeutic agent in CRC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CL and YJ conceptualized the present study. CL wrote the original draft of the manuscript. CL and XC contributed to the experiments. CL, XC and YJ analyzed the data. All authors read and approved the final version of the manuscript. CL and YJ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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