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Original article

The combination effect of Prominin1 (CD133) suppression and Oxaliplatin treatment in colorectal cancer therapy

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ABSTRACT

Colorectal cancer (CRC) is considered one of the leading types of cancer in the world. CD133, as a cancer stem cell marker, has a pivotal role in the development of drug resistance, migration, and stemness properties of CRC cells. This study was designed to check the combined effect of CD133 siRNA and Oxaliplatin on proliferation, migration, apoptosis, and stemness properties of CRC cells in the HT-29 cell line. MTT assay was performed to define the combined effect of CD133 siRNA and Oxaliplatin on the viability of HT-29 cells, and it showed that the combination of CD133 siRNA and Oxaliplatin could reduce the IC50 of this drug from 32.85 to 19.75 nmol. In order to figure out the effect of this combination therapy on CD133 expression at the gene and protein level, qRT-PCR and western blot were exploited, respectively. The results demonstrated that the silencing of CD133 could reduce the relative expression of this marker to about 0.00001 compared to the control group and reduce the protein level to 0.01. The ability of cell migration was tested by wound healing assay as well. Also, colony formation and sphere formation were conducted to assess the stemness properties in the combination group. Flow cytometry was conducted to investigate the apoptosis (15%), cell cycle (about 10% arresting in G0-G1 phase), and surface expression of CD133 in different groups (from 39.3% in the control group to 2.41 in the combination group). Finally, the expression of migration-, and stemness-associated genes were measured by qRT-PCR. We indicated that silencing of CD133 reduces the migration and stemness properties of colorectal cancerous cells. This suppression makes HT-29 cells more sensitive to Oxaliplatin and reduces the effective dose of this chemical drug. Therefore, the suppression of CD133 in combination with Oxaliplatin treatment might be a promising therapeutic approach in the treatment of colorectal cancer.

1. Introduction

Cancer is the main reason for death worldwide, and it is estimated both the cancer incidence and mortality rate will continue to rise. Colorectal cancer (CRC) is the most prevalent malignant tumor of the digestive system and the fourth most lethal cancer worldwide [1]. Although the progression of CRC treatment has been accomplished, the 5-year overall survival rate of CRC patients is still low [2]. Therefore, the detection of novel potential biomarkers for prognosis prediction and better clarification of the precise molecular mechanisms underlying CRC

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malignancy might facilitate enhanced treatments for CRC patients.

It is now well-recognized that most tumors such as colorectal tumors can self-renew and maintain their growth ability even after the treatment due to the presence of cancer stem cells (CSCs) [3]. Thus, drugs that target these abilities selectively, or strategies that increase their sensitivity to the usual therapies, suggest a better potential for cancer treatment. One such effective strategy would be to use RNA interference (RNAi) such as small interfere RNA (siRNA) to suppress genes involved in the enhancement of these properties. siRNAs are the 20–25 base pairs of RNA molecules that actively involves in the regulation of particular gene expression through monitoring the gene-silencing function [4,5].

The prominin-1 (CD133), as a CSC surface marker, is a five-transmembrane glycoprotein with 120 kDa molecular weight and is placed in membrane protrusions. CD133 has been recognized in several cancers as a potential marker for the CSCs, including brain cancer [6], ovarian cancer [6], hepatocellular carcinoma [7], prostate cancer [8,9], breast cancer [10], and pancreatic cancer [11]. It is demonstrated that CD133 plays a vital role in numerous types of cancers. CD133 has been shown to increase migration in gallbladder carcinoma [12]. CD133 upregulation contributed to the progression of hepatocellular carcinoma stimulated through STAT3 [13]. Also, CD133 + liver cancerous cells augmented angiogenesis, growth, and self-renewal of tumors. Furthermore, some studies have informed that CD133 + colorectal cancers may be more resistant to chemotherapy or chemoradiotherapy [3]. Moreover, two meta-analysis reports proposed that CD133 expression is notably associated with poor survival and may have a significant role in colorectal cancer development.

Oxaliplatin is one of the most helpful chemotherapeutic drugs used to treat CRC in several cases. As a platinum-based anti-cancer drug, Oxaliplatin shows high activity in attachment with DNA nucleobases [14]. Regarding the interaction of Oxaliplatin and DNA in CRC cells, the apoptosis pathway is excessively activated followed by DNA damage [15]. However, the cancerous cells in many CRC patients generally manifest poor sensitivity to the treatment with Oxaliplatin. CRC cells could generate some strategies to escape cell death induced by Oxaliplatin.

However, there have been few studies on the association of CD133 with the sensitivity of CRC to Oxaliplatin. Thus, the purpose of this research is to examine the function of CD133 in CRC and the effectiveness of CD133 siRNA combined with Oxaliplatin therapy for CRC treatment, which could suggest a new therapeutic strategy for CRC.

2. Materials and methods

2.1. Cell lines and culture

The human CRC cell lines, HT-29, HCT-116, and SW480 were purchased from the cell bank of Pasture Institute (Tehran, Iran). Cells were grown in RPMI-1640 medium enriched with 10% fetal bovine serum (FBS; GIBCO, Carlsbad, CA) and were incubated in a 95% humidified atmosphere with 5% CO2 at 37 °C.

2.2. Small interfering RNA transfection

Regarding the higher expression of CD133 in HT-29 cells compared to HCT-116 and SW480 cells, these cells were selected and seeded into six-well plates (2 ×105 cells per well) for transfection procedure. According to the manufacturer's protocols, jetPRIME reagent (Polyplus Co., Illkirch, France) was used to transfect 80 pmol of CD133 siRNA (Table 1), which have the better effectiveness among 20–80 pmol range of siRNA concentration and 24–72 h range of transient transfection period, into the cells. In brief, the jetPRIME reagent and CD133 siRNA were diluted in jetPRIME dilution buffer and placed at room temperature (RT) for 30 min. The mixtures of siRNA were added to the wells containing cells in the Opti-MEM medium. After incubation of plates for 5 h at 37 °C, the RPMI-1640 medium (20% FBS) was added into each well with transfected cells.

Table 1
Three different CD133 siRNA sequences.

siRNA duplexes	Sense	Antisense
CD133 siRNA (1)	5′-UUG UCA UAA UCA AUU UUG GTT-3′	5′-AAC AGU AUU AGU UAA AAC CTT-3′
CD133 siRNA (2)	5′-UGA AGU UCU GAG CAA AAU CTT-3′	5′-TTG AUU UUG CUC AGA ACU UGA-3′
CD133 siRNA (3)	5′-AGA AAG UCC UAU AAU ACU CTT-3′	5′-TTG AGU AUU AUA GGA CUU UCU-3′

2.3. Evaluation of gene expression by qRT-PCR

RNA was isolated from the cells via RiboEX reagent (GeneAll Biotechnology, South Korea, Seoul) according to the manufacturer's instruction. Next, the total RNA concentration was considered by Nanodrop (Thermo Fisher Scientific, Lenexa, KS). Then, 1 μg of total RNA was used to synthesize complementary DNA (Biofact, South Korea) via a thermal cycler system (Bio-Rad, Hercules, CA). Before the experiment, all pair primer sequences were blasted by means of the primer-blast software of the NCBI website (http://www.nchi.nlm.nih.gov). SYBR green master mix and specific primers of CD133, MMP-9, VEGF, E-cadherin, β-catenin, Sox2, Oct4, IGF-2, and MDR1 were used to perform qRT-PCR (Table 2). Also, 18 s rRNA expression was considered as a reference gene. The mRNA expression was evaluated by STEP ONE PLUS qRTPCR system (Applied Biosystems, Foster City, USA) and interpreted through the $2^{-\Delta\Delta Ct}$ method.

2.4. Proliferation assay

MTT assay was performed to examine the viability of HT-29 cells after using CD133 siRNA and Oxaliplatin, individually and in combination. In brief, 15×10^3 HT-29 cells were plated into a 96-well plate. Then, different concentrations of the CD133 siRNA were used to transfect cells, individually and in combination with Oxaliplatin. After 48 h, 50 μl of MTT (2 mg/ml) were added to the well for 2–4 h. After removal of the medium, 150 μl DMSO was added to each well and shaken for 10 min. The optical density (OD) of each well was evaluated at 570 nm with an ELISA reader (Sunrise RC, Tecan, Switzerland).

2.5. Evaluation of protein levels by western blotting

HT-29 cells were seeded into six-well plates at a density of 2×10^5 cells per well. Four groups, including control, CD133 siRNA, Oxaliplatin, and combined CD133/Oxaliplatin were considered. In order to extract the total protein, RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) was considered. The cell pellet was resuspended in lysis buffer, containing the protease inhibitor, phosphatase inhibitor, and PSMF. Cell lysates were centrifuged at 13,000 rpm for 15 min at 4 °C. Subsequently, protein samples were subjected to 12.5% SDS-PAGE electrophoresis and transferred to polyvinyl difluoride (PVDF; Roche Diagnostics) membranes. Then, these membranes were blocked with Tween 20 (0.5%) in PBS for 2 h in RT and then incubated with an anti-CD133 monoclonal antibody (Abnova, Taipei, Taiwan) (1: 1000) and β-actin (Abcam) as the reference (1:5000); (Santa Cruz Biotechnology, Santacruz, Ca). The membranes were then incubated for 1 h at RT with rabbit anti-goat secondary antibody for CD133 and rabbit anti-mouse antibody for β-actin conjugated with horseradish peroxidase (1:5000; diluted in PBS). The specific bands were identified by the Roche Diagnostics electrochemiluminescence (ECL) kit and western blot imaging system (Sabz Co., Iran). Ultimately, Image J software (National Institutes of Health, Bethesda, MD) was employed for evaluation.

Table 2
Primer sequences.

Target	Forward primer	Reverse primer
CD133	5′-GACCGACTGAGACCCAACATC − 3′	5′-GGCTAGTTTTCACGCTGGTCA − 3′
MMP-9	5′-TTGACAGCGACAAGAAGTGG-3′	5′-GCCATTCACGTCGTCCTTAT-3′
VEGF	5′-TGCAGATTATGCGGATCAAACC-3′	5′-TGCATTCACATTTGTTGTGCTGTAG-3′
E-cadherin	5′-TGCCCAGAAAATGAAAAAGG-3′	5′-GTGTATGTGGCAATGCGTTC-3′
β-catenin	5′-GCGTGGACAATGGCTACTCAAG-3′	5′-AGGTATCCACATCCTCTTCCTCAG-3′
Sox2	5′-ACATGTGAGGGCCGGACAGC-3′	5′-TTGCGTGAGTGTGGATGGGATTGG-3′
Oct4	5′-GGGCTCTTTGTCCACTTTGT-3′	5′-GGCATGCATACACACAAACAC-3′
IGF-2	5′-AACGAGTGGAGGGGATGAGG-3′	5′-AGGAGAGGGACAAAGCTGAGG-3′
MDR1	5′-GTTTGCCACCACGATAGCTGA-3′	5′-TTCTGCCCACCACTCAACTG-3′
18 s	5′-GATCAGATACCGTCGTAGTTCC -3′	5′-CTGTCAATCCTGTCCGTGTC − 3′

2.6. Flow cytometry analysis of surface expression

To evaluate the cell surface expression, 2×10^5 HT-29 cells were seeded in each well of six-well plates and were grown in a humidified incubator for 24 h. Next, four groups were described: control, CD133 siRNA, Oxaliplatin, and CD133 siRNA/Oxaliplatin. After 24 h of CD133 siRNA transfection, cells were treated with Oxaliplatin. After 24 h incubation at 37 °C, cells were centrifuged and washed twice in FACs buffer. Next, the primary antibody (100 μl) was added and cells were incubated for 30 min at RT in the dark. Then, the FITC conjugated secondary antibody (1:1000) was added to the samples, and they incubated in the dark condition for 20 min

2.7. Flow cytometry analysis of apoptosis

Apoptosis was evaluated by annexin V/propidium iodide (PI) assay. First, 2×10^5 HT-29 cells were seeded in each well of a six-well plate. After 24 h incubation, the cells were separated into four different groups, including control, CD133 siRNA, Oxaliplatin, and combined CD133 siRNA/Oxaliplatin groups. After 24 h of CD133 transfection, the cells were treated with Oxaliplatin and incubated for 24 h. Then, the cells were stained with annexin V and PI according to the manufacturer's protocols (EXBIO, Vestec, Czech Republic). For annexin V staining, 200 μl of binding buffer, annexin V (5 μl), and propidium iodide (5 μl) were added to the different groups. Next, they were incubated for 20 min at RT in the dark. Then, the groups were evaluated by flow cytometry (MiltenyBiotec™ FACS Quant 10; MiltenyBiotec, Germany). The apoptosis rate was measured by FlowJo software (Tree Star, San Carlos, CA).

2.8. Flow cytometry analysis of cell cycle arrest

First, 2×10^5 HT-29 cells were seeded into each well of 6–well plates and were grown in a humidified incubator for 24 h. Then, four groups were described: control, CD133 siRNA, Oxaliplatin, and combined CD133 siRNA/Oxaliplatin. After CD133 siRNA transfection and Oxaliplatin treatment, the cells were prepared for analysis. They were washed with PBS, and 70% ethanol was used to fix them overnight (−20 °C). Next, after washing the cells with PBS, they were resuspended in PBS containing RNase A (200 μg/ml), incubated at 37 °C for 30 min, and stained with DAPI (50 μg/ml) for analysis. The distribution of cells in each phase of the cycle was evaluated using a Flow cytometry instrument (Milteny Biotec MACSQuant 10), and the data were analyzed by the FlowJo FACS analysis software.

2.9. Scratch-wound migration assay

Migration of HT-29 cells was analyzed by the wound-healing assay. The HT-29 cells were seeded in 24-well plates (3×10^5 cells/well) and grown to 90% confluence. Then, four groups, including control, CD133 siRNA, Oxaliplatin, and combined CD133 siRNA/Oxaliplatin were considered. The cell monolayers were scratched using a 2 mm-wide tip to form a line-shaped wound. Next, the cells were transfected and treated with CD133 siRNA and Oxaliplatin, respectively. The cells were permitted to migrate, and images were collected at a different time (0, 24, and 48 h) by an inverted microscope.

2.10. Colony formation assay

Colony formation ability was evaluated by culturing the cells in a 3D cell culture medium and groups, including control, CD133 siRNA, Oxaliplatin, and combined CD133 siRNA/Oxaliplatin was investigated in HT-29 cells. So, 5×10^3 of the cells were seeded into six-well plates. The cells were transfected with CD133 siRNA and treated with Oxaliplatin. After 12 days, staining dye (including crystal violet, formaldehyde, and methanol) was used to stain the colonies for 30 min. Ultimately, the number of colonies for each group were counted using ImageJ software (NIH, MD).

2.11. Spheroid assay

Sphere formation ability of different groups, including control, CD133 siRNA, Oxaliplatin, and combined CD133 siRNA/Oxaliplatin was assessed in HT-29 cells. To this aim, these cells were seeded into six-well plates at a density of 2×10^5 cells/ well. After CD133 siRNA transfection and Oxaliplatin treatment of cells, they were incubated for 24 h at 37 °C. Then, the cells were trypsinized and transferred to 96-well plates. In a 96-well plate, a well was considered for each group, and 2x RPMI-1640 medium (Sigma, USA) containing matrigel 10%were added in wells. Next, 5×10^3 cells were seeded from each group of treated cells in each well of the 96-well plate and incubated at 37 °C. The image of spheres in different groups was captured by an OPTIKA (Italy) microscope system for 10 days.

2.12. Statistical analysis

All data analyses were performed on GraphPad Prism software version 7.0 (GraphPad Prism; San Diego, CA). Measurement data were expressed as mean ± standard deviation. Unpaired student's *t*-test and ANOVA one-way analysis of variance was used for comparing the groups with parametric data. Besides, Geisser-Greenhouse for ANOVA test and Tukey for multiple comparison test was utilized as the correction tests. $P < 0.05$ was depicted as statistical significance.

3. Results

3.1. CD133 is overexpressed in the colorectal cancer cell line, HT-29

CD133 expression was investigated in various CRC cell lines HT-29, HCT-116, and SW-480. qRT-PCR results revealed that the expression of CD133 in the HT-29 cell line is significantly higher than HCT-116 and SW-480 cell lines (Fig. 1A).

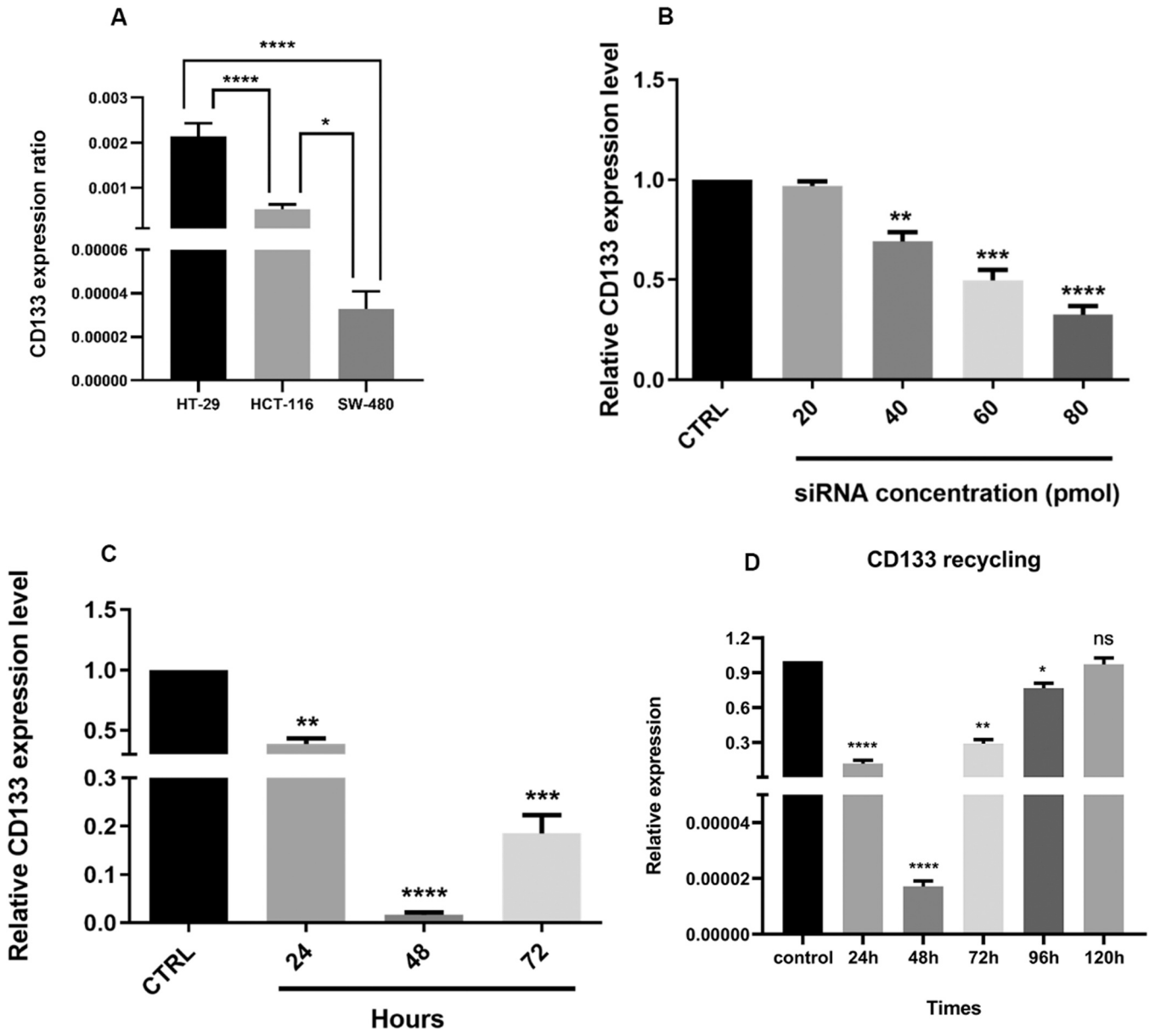

Fig. 1. A. The rational expression of CD133 in HT-29, HCT-116, and SW-480 cell lines. The expression of CD133 in the HT-29 cell line is higher than HCT-116 and SW-480 cell lines. B. the CD133 expression level in HT-29 cells, which is affected by different doses of siRNA, were decreased the most at the dose of 80 pmol. C. CD133 siRNA showed the highest rate of suppression at 48 h after transfection. D. CD133 siRNA could reduce the expression of this gene up to 48 h, and after this time, the recycling process is completed until 120 h. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). The dose of 80 pmol of CD133 siRNA was used to evaluate the recycling of CD133.

3.2. CD133 siRNA reduced the expression of CD133 in HT-29 cells

To evaluate the CD133 activity in HT-29 cells, we performed a siRNA strategy. HT-29 cells were transfected with various doses (20–80 pmol) of CD133 siRNA. After transfection of HT-29 cells with 80 pmol dose of CD133 siRNA, the cells indicated a significantly low expression of CD133 (Fig. 1B). The dose of 80 pmol was selected for all next experiments to knockdown HT-29 cells. Next, the transfection of HT-29 cells was done by CD133 siRNA for 24, 48, and 72 h. Finally, 48 h were considered as the optimum knockdown time (Fig. 1C). The qRT-PCR analysis demonstrated that transfected cells have lower expression of CD133 mRNA compared to control cells. Also, to assess the recycling process of CD133 after the transfection, the qRT-PCR was utilized for 24, 48, 72, 96, and 120 h after the transfection, and the results demonstrated that upon 120 h after the transfection, the expression level of CD133 was returned to its normal level (Fig. 1D).

3.3. CD133 knockdown has no significant effect on cell viability

MTT assay was conducted to investigate the viability and proliferation of HT-29 cells after CD133 siRNA transfection. This indicated that transfection with 20–80 pmol of CD133 siRNA after 48 h incubation has no significant effect on cell viability or proliferation compared to the control group (Fig. 2A). These results propose that CD133 is not associated with the cell viability of HT-29 cells.

3.4. Combination of CD133 siRNA and Oxaliplatin decreased cell viability in HT-29 cells

To consider the effect of CD133 siRNA and Oxaliplatin proliferation of HT-29 cells, the MTT assay was conducted to find the IC50 value of Oxaliplatin (IC50 =32.85). The results revealed that transfection of CD133 siRNA lessens the IC50 value of Oxaliplatin (IC50 =19.75). Thus,

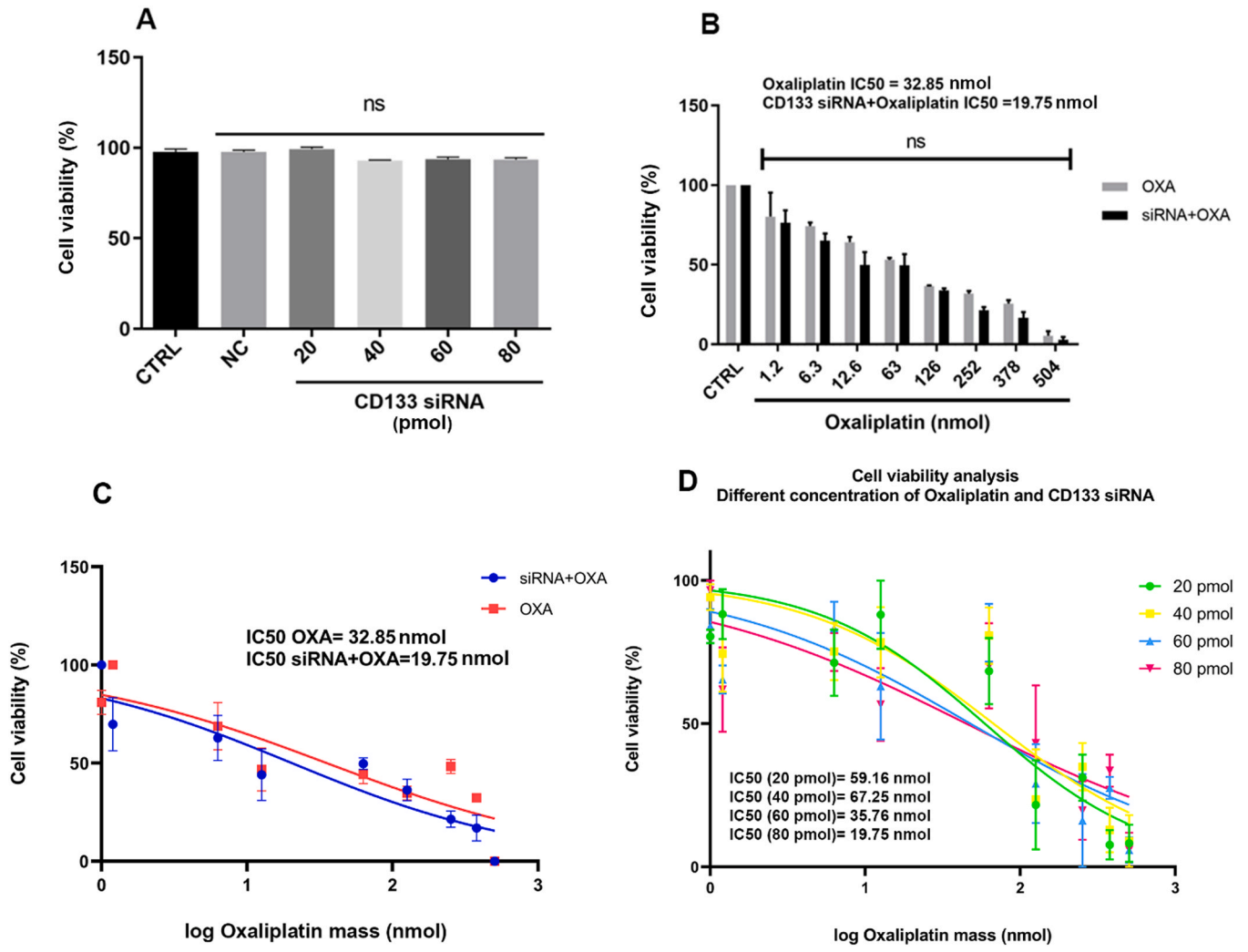

Fig. 2. The effect of CD133 siRNA on HT-29 cell survival. A. The usage of CD133 siRNA at different doses (20, 40, 60, and 80 pmol) did not have a considerable effect on the survival rate of transfected cells. B and C. Usage of CD133-siRNA (80 pmol and 48 h after the transfection) and Oxaliplatin in combination caused a significant difference in IC50 value. D. Different doses of CD133 siRNA could also have different effects on the chemosensitivity of HT-29 cells to Oxaliplatin (48 h after the transfection). The dose of 80 pmol was distinguished as the optimum dose of CD133 siRNA.

it is revealed that the combination of CD133 siRNA and Oxaliplatin makes a notable alteration in the proliferation of cells (Fig. 2B and C). These results exposed that the combined use of CD133 siRNA and Oxaliplatin simultaneously reduces the proliferation rate of HT-29 cells. Also, in order to show how Oxaliplatin reduces cell proliferation at different percentages of CD133 expression or reduction, we conducted the MTT assay and used different doses of CD133 (20, 40, 60, 80) in combination with different Oxaliplatin concentrations. Our results indicated that the dose of 80 pmol of CD133 siRNA in combination with different Oxaliplatin concentrations has the most effect on the IC50 value of Oxaliplatin (Fig. 2D).

3.5. The combined use of CD 133 siRNA and Oxaliplatin decreased the levels of CD133 mRNA and protein, as well as its surface expression in HT-29 cells

We next used CD133 siRNA in combination with Oxaliplatin in the HT-29 cell line. First, we used CD133 siRNA and Oxaliplatin separately. qRT-PCR and western blot analysis detected that the individual CD133 siRNA and Oxaliplatin results in reduced CD133 mRNA and protein expression levels. While, the combined CD133siRNA and Oxaliplatin suppressed the CD133 mRNA and protein level more than individual using (Fig. 3A, B, and C). The combined usage of CD133 siRNA and Oxaliplatin led to a decrease in CD133 surface expression. The flow-cytometric analysis revealed a decreased percentage of $CD133^{+}$ cells in individual groups of CD133 siRNA and Oxaliplatin, 15.3 and 32.4, respectively. While the combined group exhibited a lower percentage of $CD133^{+}$ cells 2.41, compared with the other groups (Fig. 3D, E). Moreover, regarding the number of cells expressing CD133 + after transfection, we checked the recycling of CD133 in HT-29 cells at different times (24, 48, 72, 96, and 120 h after transfection). The qRT-PCR results revealed that recycling of CD133 happens 120 h after transfection of CD133 siRNA (Fig. 1D).

3.6. Knockdown of CD133 in combination with Oxaliplatin could sensitize the apoptosis of CRC cells

To investigate if a single CD133 siRNA or a combination of CD133 siRNA/Oxaliplatin increases apoptosis, the annexin V/PI assay was performed. We indicated that dual usage of CD133 siRNA and Oxaliplatin induces apoptosis more than individual usage of CD133 siRNA and Oxaliplatin. The apoptosis rate for CD133 siRNA and Oxaliplatin were 2.92% and 3.85% in HT-29 cells, respectively. Interestingly, the rate of apoptosis following CD133 silencing in combination with Oxaliplatin was higher: 5.41% (Fig. 4A, B). Thus, we detected cooperative increases in apoptosis after combined usage of CD133 siRNA and

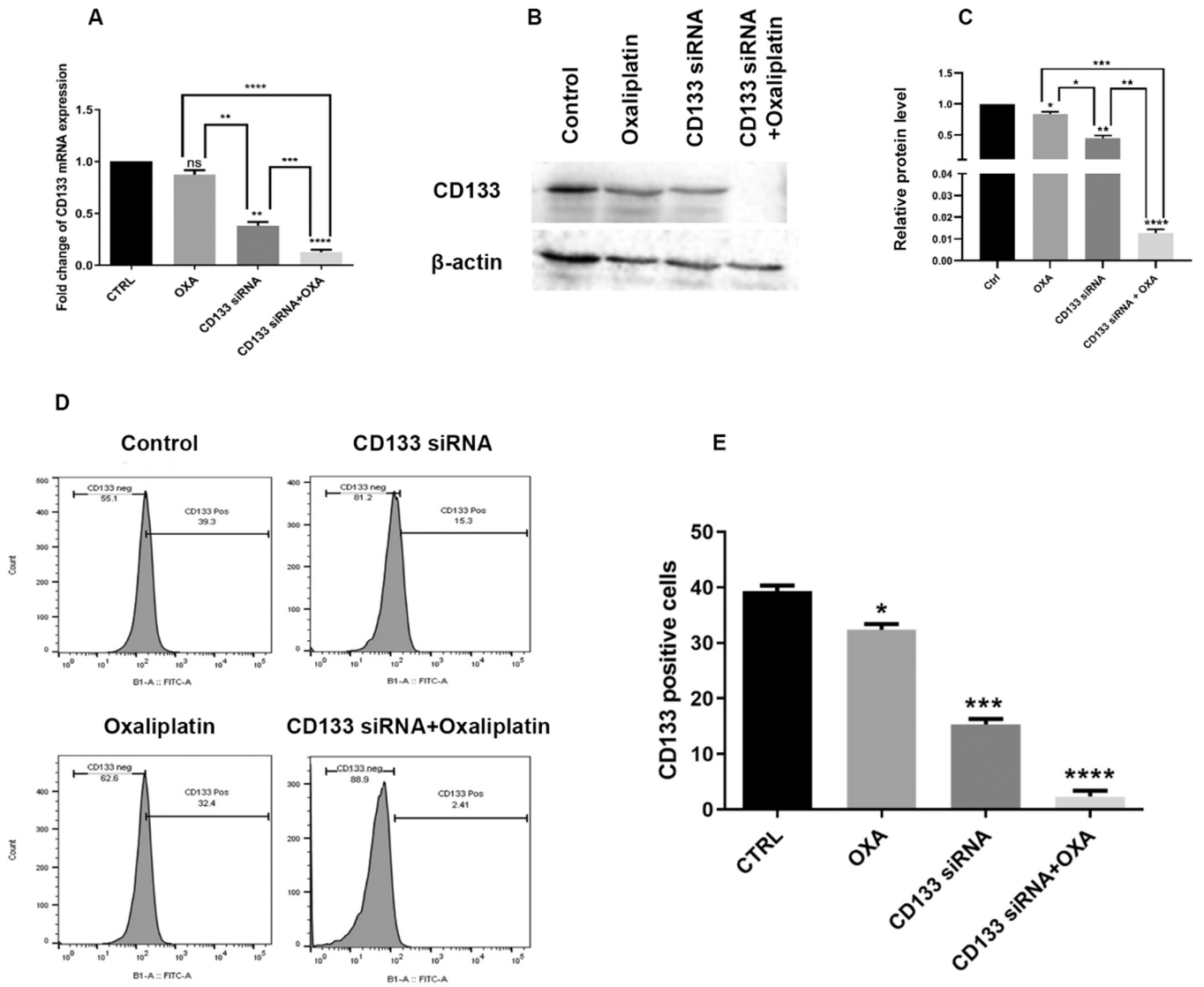

Fig. 3. The effect of CD133 siRNA and Oxaliplatin combination on the CD133 expression at mRNA and protein level as well as the surface expression. A. CD133 siRNA in combination with Oxaliplatin reduced CD133 mRNA expression. B, C. Western blot analysis showed decreased CD133 expression at protein level in the combination group. D, E. combined CD133 siRNA and Oxaliplatin significantly lessened the surface expression level of CD133 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). In all results, 80 pmol of siRNA was used and 48 h after the transfection the cells were harvested.

Oxaliplatin. These data presented that the silencing of CD133 could decrease the Oxaliplatin resistance of HT-29 cells.

3.7. Combination of CD133 siRNA/Oxaliplatin arrested HT-29 cells in the G1 phase of cell cycle

To further determine whether the combination of CD133 siRNA and Oxaliplatin affects cell proliferation, we examined the cell cycle distribution of HT-29 cells after CD133 siRNA transfection and Oxaliplatin treatment. Flow cytometry data indicated that CD133 siRNA augmented the population of G1 phase cells from 76.2% to 81.8%. Also, treatment with Oxaliplatin resulted in a rise in the population of G1 phase cells by 76.2–83.0% compared to controls. Interestingly, CD133 siRNA combined with Oxaliplatin augmented the population of cells that were in the G1 phase from 76.2% to 91.1%. It is indicated that the combination is able to enhance the percentage of HT-29 cells in the G1 phase. Actually, CD133 siRNA and Oxaliplatin dual usage could support the arrest of the cell cycle in the G1 phase (Fig. 4C, D).

3.8. Silencing of CD133 expression in combination with Oxaliplatin decreased stemness features in HT-29 cells

To recognize if individual and/or combined usage of CD133 siRNA and Oxaliplatin could dampen stemness properties, we performed the colony formation assay. In comparison to the control cells, the colony numbers were decreased after CD133 siRNA and Oxaliplatin single usage. While, the colony numbers were remarkably low in the combination group (Fig. 5A, B). Also, the result of the sphere formation assay demonstrated that using combined CD133 siRNA and Oxaliplatin leads to forming spheroids with smaller diameter compared with the individual usage of CD133 siRNA and Oxaliplatin (Fig. 5C). To support the reduction of stemness features following CD133 siRNA transfection at the molecular level, we examined the expression of stemness-related genes. qRT-PCR assays presented that Oct4, Sox2, MDR1, and IGF2 mRNA expression levels were meaningfully lessened in CD133 siRNA/Oxaliplatin combination group compared with the individual CD133 siRNA and Oxaliplatin groups (Fig. 5D, E, F, G). Therefore, both phenotypic and molecular genetic data illustrated that silencing CD133 expression in combination with Oxaliplatin decreases the stemness

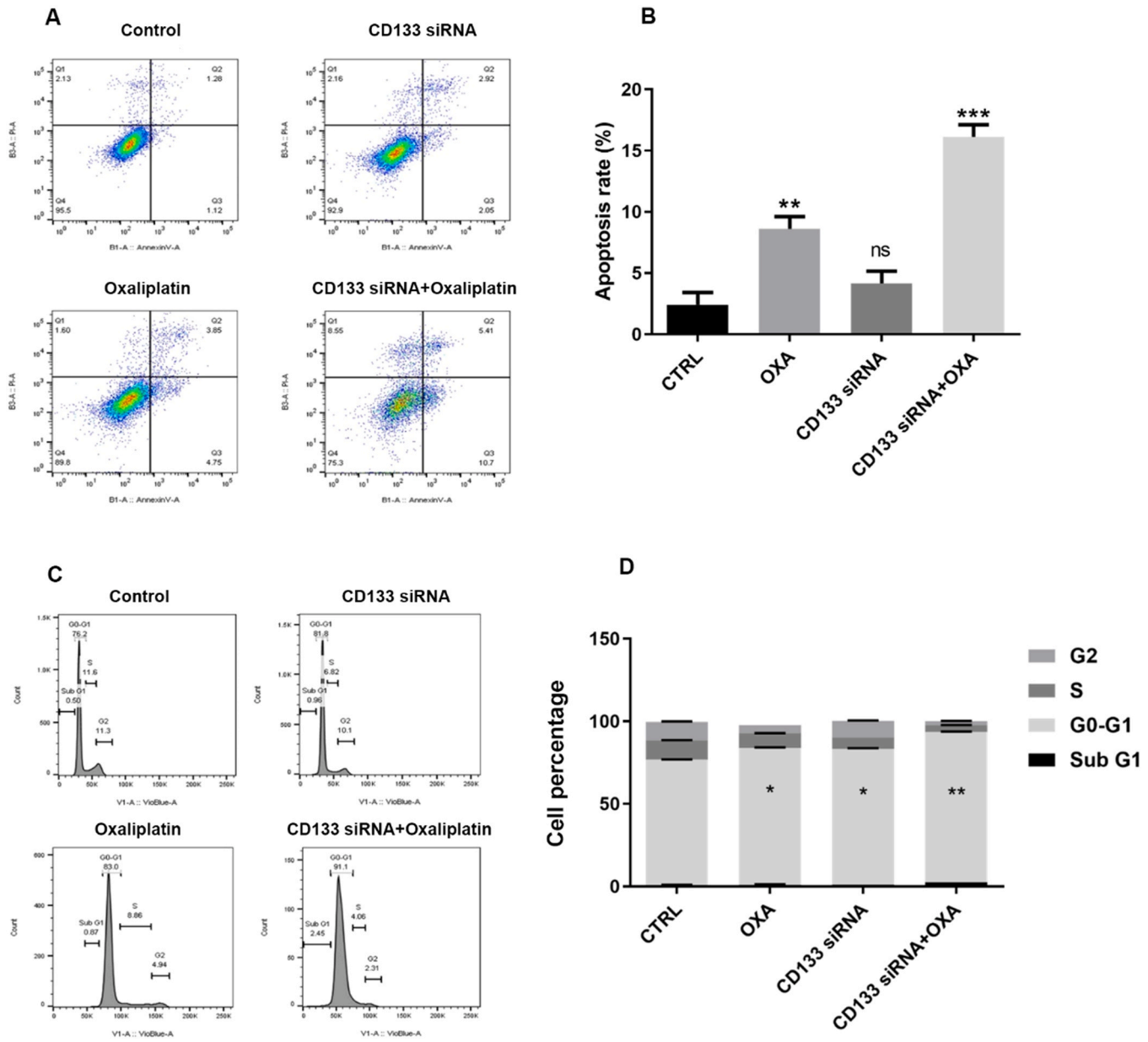

Fig. 4. The effect of CD133 siRNA in combination with Oxaliplatin on apoptosis and cell cycle. A, B. CD133 knockdown in combination with Oxaliplatin sensitized HT-29 cells to apoptosis. C, D. Combined usage of CD133 siRNA and Oxaliplatin caused cell-cycle arrest in the G1 phase (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). In all results, 80 pmol of siRNA was used and 48 h after the transfection the cells were harvested.

features of colorectal cancer cells.

3.9. CD133 siRNA and Oxaliplatin work together to inhibit migration and EMT-related genes expression in HT-29 cells

The result of the wound healing assay indicated that the combination of CD133 siRNA and Oxaliplatin reduces migration of HT-29 cells. Single usage of CD133 siRNA and Oxaliplatin decreased the migration of these cells. However, compared to the control cells, double usage of CD133 siRNA and Oxaliplatin, 48 h after transfection significantly reduced the migration rate of HT-29 cells (Fig. 6A, B). To assess if this combination alters the expression of genes associated with EMT, we investigated the expression of VEGF, MMP-9, E-cadherin, and β-catenin by qRT-PCR. This assay showed that the relative expression levels of VEGF, MMP9, and β-catenin genes were lessened in individual usage and were remarkably low after combination usage. While E-cadherin indicated increased expression in the combination group (Fig. 6C, D, E, and F). Thus, individual usage of CD133 siRNA and Oxaliplatin reduces cell migration and EMT-related gene expression and dual usage enhances these effects.

4. Discussion

Most of the cancerous cells have the ability to self-renew, differentiate into established progenies, and induce and preserve tumor development even after the treatment [16]. CD133 is one of the most well-known markers for the evaluation of CSCs [17]. It is revealed that CD133 promotes the growth and invasion of cancerous cells. According to studies, knockdown of CD133 lessens the development of hepatocellular carcinoma cells [18] and inhibits cell proliferation in colon cancer as well [19]. It is indicated that the combination of gene therapy and anti-cancer drugs can be a successful treatment strategy. In this investigation, we analyzed the combined effect of CD133 siRNA and Oxaliplatin in the HT-29 cell line of colorectal cancer.

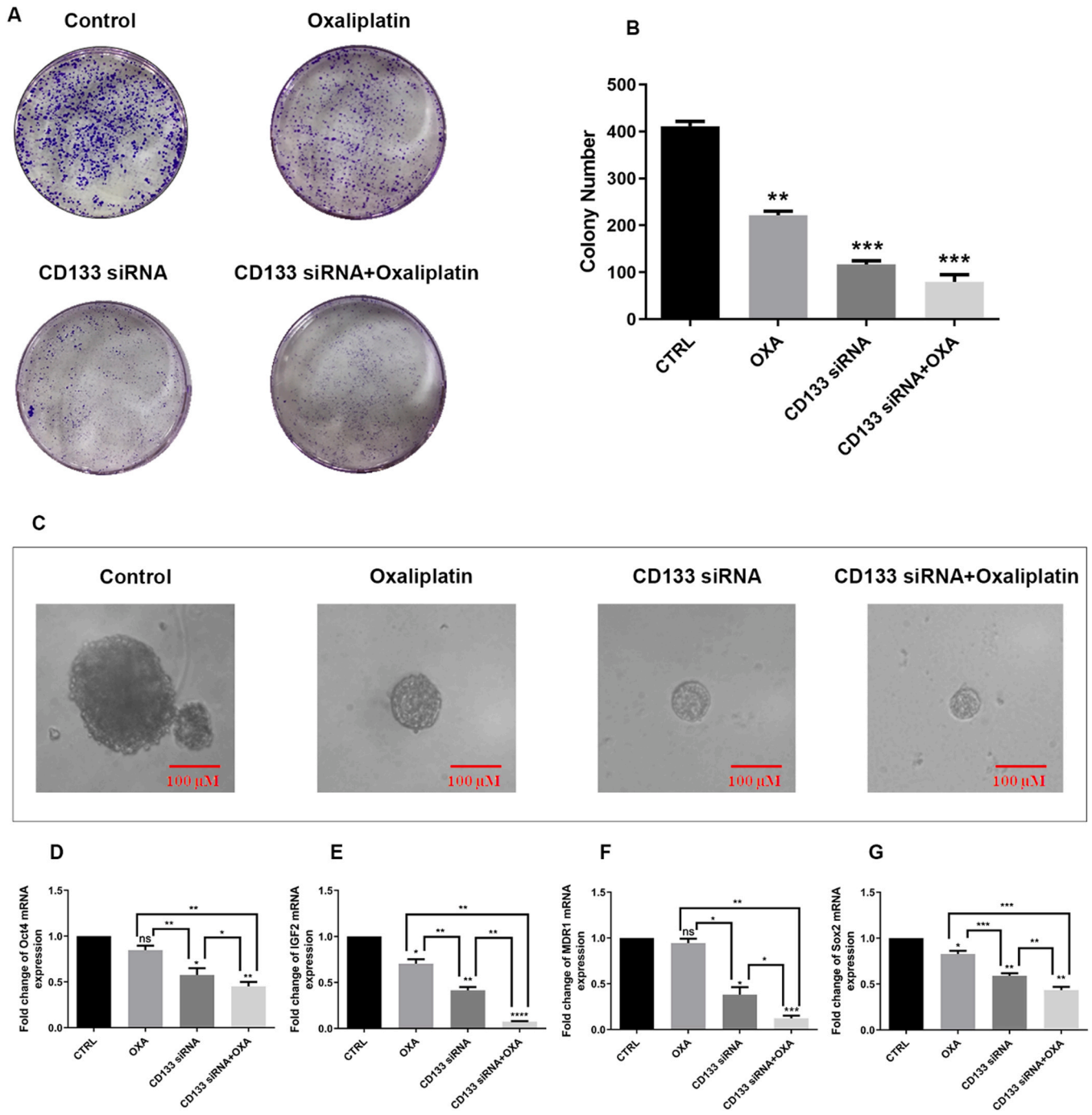

Fig. 5. The effect of CD133 siRNA in combination with Oxaliplatin on stemness features. A, B. The colony numbers were significantly decreased in the combination group. C. Also, the diameter of the spheroids was significantly decreased in the combination group. D, E, F, G. Furthermore, the expression of stemness related genes (Oct4, IGF2, MDR1 and Sox2) were decreased in dual usage group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). In all results, 80 pmol of siRNA was used and 48 h after the transfection the cells were harvested.

It is reported that CD133 reveals a notable therapeutic impact in increasing the sensitivity of chemotherapy in many cancers. In the present study, the results of the cytotoxicity assay exhibit that the effective dose of Oxaliplatin is decreased in combination with CD133 siRNA. In other words, it can be concluded that CD133 siRNA enhances the sensitivity of the HT-29 cells to Oxaliplatin. Also, it increases the stimulation of cell death by inducing a provocative effect and increasing the drug efficacy in the HT-29 cancer cell line. Our results also showed that the silencing of CD133 could be maintained up to 48 h after the transfection, and recycling of this CSC marker was completed after 120 h upon the transfection of CD133 siRNA within HT-29 cells. Several studies have been conducted to evaluate the effect of CD133 silencing on drug sensitivity. Zhang et al. demonstrated that suppressed CD133 improves the efficiency of cisplatin in laryngeal carcinoma treatment [20]. In addition, a recent study indicated that CD133 increases with the occurrence of drug-resistance phenotypes in colorectal cancer, and targeting this marker increases chemosensitivity through the AKT/NF-κB/MDR1 pathway [21].

The results of annexin-V/PI staining confirmed the MTT results and demonstrated that CD133 siRNA did not play a substantial role in the stimulation of apoptosis. However, the rate of apoptosis in the combination of CD133 siRNA and Oxaliplatin was meaningfully augmented. It

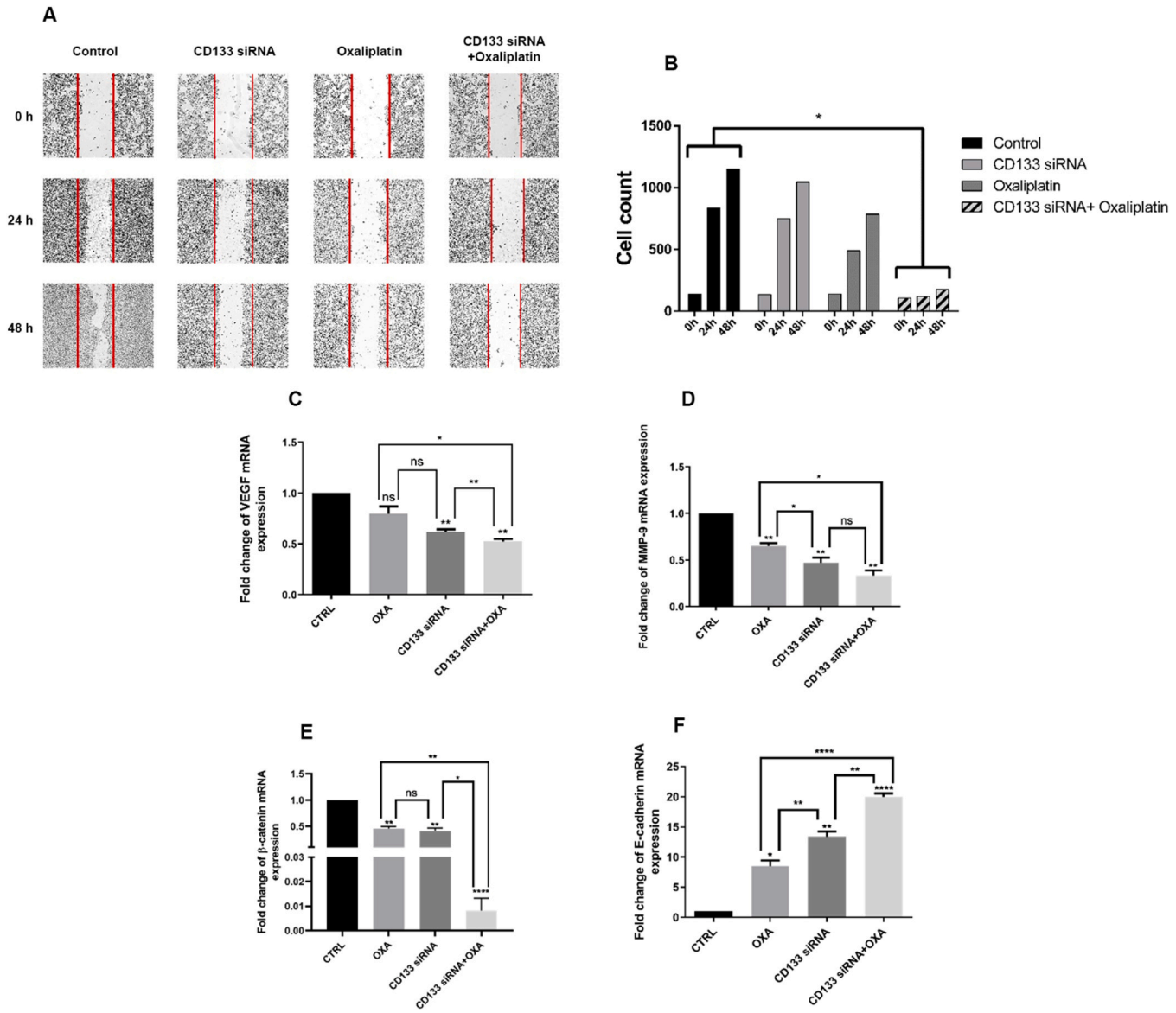

Fig. 6. The effect of CD133 siRNA in combination with Oxaliplatin on metastasis. A, B. Combination of CD133 siRNA and Oxaliplatin, 48 h after transfection meaningfully lessened the migration rate of HT-29 cells compared to the control cells. C, D, E, F. Also, the expression of metastasis-related genes, including VEGF, MMP9, and β-catenin were decreased and E-cadherin expression increased in combination group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). In all results, 80 pmol of siRNA was used and 48 h after the transfection the cells were harvested.

can be stated that CD133 siRNA induces apoptosis in CRC cells by increasing the sensitivity of these cells to the Oxaliplatin. Some researchers recognized that CD133 could hamper apoptosis and stimulate drug resistance through triggering the PI3K/AKT signaling network in gastric and hepatocellular cancer cells [22]. Cell cycle analysis indicated that the CD133-silenced cells were increased within the G0-G1 phase after treatment with Oxaliplatin. In this regard, Lan et al. revealed that the $CD133^{+}$ liver cancer cells were generally distributed in the G0/G1 phase. Following the CD133 downregulation, cells in the G0/G1 phases were reduced, which exposed that CD133 silencing induces dormant liver cancer stem cells to differentiate [23]. They reported that according to the stem cell theory, CSCs are almost exclusively in the G0/G1 phases of the cell cycle, and dormant CSCs are more resistant to chemotherapy than differentiated cancer cells. In the current study, we utilized the combination of chemotherapy drug and silencing of CD133 to determine the efficacy of this kind of therapeutic approach. To this aim, the results showed that, however, the individual silencing of CD133 could reduce the number of cells in G0/G1 phase of cell cycle (as reported in previous studies), the combination of Oxaliplatin treatment and CD133 siRNA could arrest the cancerous cells in the mentioned cell cycle phase.

One of the significant results of this research was that the inhibition of CD133 represses migration in colorectal cancer cells. Besides, the wound healing assay (scratch) results indicated that the individual use of either CD133 siRNA or Oxaliplatin lessens migration. However, the combined use of CD133 siRNA and Oxaliplatin was related to a significant reduction in migration compared to the control group, indicating the parallel effect of CD133 siRNA and Oxaliplatin. To examine the mechanism of CD133 by which the cell migration is adjusted, Liu et al. studied the $CD133^{+}$ colorectal cancer cell line SW620 and $CD133^{-}$ cell line HEK293T. Suppression of CD133 lessened cell migration and FAK phosphorylation in SW620 cells. In HEK293T cells, ectopic expression of CD133 increased cell migration and the phosphorylation level of FAK at the Tyr925 site [24]. Similarly, the downregulation of CD133 suppresses the migration and invasion of gallbladder carcinoma through decreasing Akt phosphorylation [25].

It is also indicated that CD133 has a fundamental role in the stimulation of stemness properties in various cancers. According to the results of colony formation assay, the individual use of CD133 siRNA or Oxaliplatin decreased the colony numbers. However, dual usage of CD133 siRNA and Oxaliplatin meaningfully diminished the number of colonies compared to the control group, supporting the parallel effect of CD133 siRNA and Oxaliplatin. The result of the sphere formation assay also confirmed the result of colony formation. Individual use of siRNA or Oxaliplatin decreased the size of spheroids, which was more significant in the combination group. It is revealed that CD133 overexpression supports stemness characteristics and tumorigenicity of head and neck squamous cell carcinoma. While CD133 inhibition stimulates Src activation and decreases stemness properties and tumorigenicity of this cancer both in-vitro and in-vivo [26]. In a study conducted by Lan et al., it is exhibited that $CD133^{+}$ liver cancerous cells have greater colony-forming capacity than the $CD133^{-}$ cells. CD133-expressing cells developed further and larger colonies than $CD133^{-}$ cells, suggesting that CD133 functions as a vital factor in sustaining the liver cancer cells performances [23].

In conclusion, this study revealed that the silencing of CD133, as a CSC marker, promotes the sensitivity of colorectal cancer cells to Oxaliplatin and subsequently stimulates cell death, suppresses proliferation, and migration by stimulating a provocative effect. Thus, the knockdown of CD133 could act as a possible strategy to sensitize the cancer cells to Oxaliplatin in the treatment of colorectal cancer; however, the limitations such as recycling of CD133 after the transient silencing could be improved by the identification of mechanisms, by which, the maintained silencing of CD133 would be exerted.

Conflict of interest statement

The authors declared that there is no conflict of interest related to this study.

Data availability

The data that support the findings of this study are available from Behzad Baradaran but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however, available from the authors upon reasonable request and with permission of Behzad Baradaran.

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Author Contributions

Z.A., the first author of the manuscript, performed the experiments and wrote the primary version of the manuscript. B.M., A.M., D.S., A.M., and T.K., helped in performing the experiments. N.H., A.D., O.B., improved the quality of the paper. S.S., M.A. and S.N.: contributed to the English editing of the manuscript and also helped with data categorization. B.B. and N.S.: the corresponding authors of the manuscript, contributed to the writing of the main text of the manuscript, and also supervised the manuscript. All authors have read and agreed to the published version of the manuscript.

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