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Permalink

https://escholarship.org/uc/item/6t40k84p

Journal

Molecular Carcinogenesis, 55(5)

ISSN

0899-1987

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Publication Date

2016-05-01

DOI

10.1002/mc.22319

Peer reviewed

miR-148a Dependent Apoptosis of Bladder Cancer Cells Is Mediated in Part by the Epigenetic Modifier DNMT1

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Urothelial cell carcinoma of the bladder (UCCB) is the most common form of bladder cancer and it is estimated that ∼15,000 people in the United States succumbed to this disease in 2013. Bladder cancer treatment options are limited and research to understand the molecular mechanisms of this disease is needed to design novel therapeutic strategies. Recent studies have shown that microRNAs play pivotal roles in the progression of cancer. miR-148a has been shown to serve as a tumor suppressor in cancers of the prostate, colon, and liver, but its role in bladder cancer has never been elucidated. Here we show that miR-148a is down-regulated in UCCB cell lines. We demonstrate that overexpression of miR-148a leads to reduced cell viability through an increase in apoptosis rather than an inhibition of proliferation. We additionally show that miR-148a exerts this effect partially by attenuating expression of DNA methyltransferase 1 (DNMT1). Finally, our studies demonstrate that treating cells with both miR-148a and either cisplatin or doxorubicin is either additive or synergistic in causing apoptosis. These data taken together suggest that miR-148a is a tumor suppressor in UCCB and could potentially serve as a novel therapeutic for this malignancy. © 2015 Wiley Periodicals, Inc.

Key words: bladder cancer; apoptosis; miR-148a; cisplatin; cell cycle; DNA methyltransferase (DNMT1); doxorubicin

INTRODUCTION

Bladder cancer in the United States is the fourth most common type of cancer in men and the ninth in women and it was estimated that ∼150 000 people died worldwide from bladder cancer in 2008 [1,2]. Approximately 90% of all bladder cancer cases are classified as urothelial cell carcinomas (UCC) which are derived from epithelial cells lining the interior of the organ [3]. Therapeutic options for UCC of the bladder (UCCB) include surgical resection and the GC (gemcitabine and cisplatin) and MVAC (methotrexate, vinblastine, doxorubicin, and cisplatin) chemotherapy regimens, but disease recurrence leading to death is common [4,5]. Little progress has been made in the development of new therapies and survival has improved little in the last 20 years [6]. Identifying alternative treatments is critical for prolonging survival.

MicroRNAs (miRNAs) are a class of small non-coding RNAs (18–24 nucleotides in length) that primarily function to negatively regulate gene expression by promoting mRNA degradation or repressing translation [7]. Recent evidence has demonstrated that miRNAs play a role in the etiology and progression of bladder cancer by affecting several facets of the disease including proliferation, apoptosis, and migration. They can serve as either oncogenes or tumor suppressors depending on context and targets. miR-708 was shown to be oncogenic in bladder cancer by

inhibiting apoptosis via targeting caspase-2 [8]. miR-182-5p was also shown to be oncogenic in bladder cancer by targeting RECK and Smad4 leading to increased proliferation, migration, and invasion [9]. A tumor suppressive role was discovered for the p53 target miR-34a due to its ability to sensitize bladder cancer cells to cisplatin treatment [10]. miR-409-3p was shown to inhibit migration and invasion by targeting c-Met [11].

miR-148a plays the role of tumor suppressor in several contexts. miR-148a was shown to suppress the epithelial to mesenchymal transition and reduce metastasis in non-small cell lung cancer and gastric

cancer respectively by targeting Rock1 [12,13]. miR-148a was shown to inhibit the epithelial to mesenchymal transition in hepatocellular carcinoma cells as well [14]. In hepatitis B associated hepatocellular carcinoma, miR-148a was shown to be a p53 target and its re-introduction into cultured liver tumor cells resulted in decreased proliferation [15]. In pancreatic and colorectal cancers, miR-148a has been shown to induce apoptosis by targeting Bcl-2 [16,17]. miR-148a down-regulation by hypermethylation has been associated with metastasis and reintroduction of this miRNA into cells with epigenetic inactivation reduced motility, tumor growth, and inhibited metastasis in a SIHN-011B head and neck cancer xenograft model [18]. Further studies on colon cancer that focused on expression of circulating miRNAs revealed that decreased circulating levels of miR-148a correlated with disease recurrence [19]. More recent studies reported that miR-148a was one of the miRNAs that were down regulated in triple negative breast cancer cell lines and this was associated with deregulation of the E2F pathway [20]. Studies in prostate cancer have also demonstrated that miR-148a levels were decreased in metastatic prostate cancer cell lines and this was implicated in drug resistance [21]. The role of miR-148a in bladder cancer has never been investigated.

DNA methylation is an epigenetic modification that plays an essential role in the control of gene expression and is integral to many processes including cellular differentiation, development, and tumorigenesis [22]. DNA methyltransferase 1 (DNMT1) is a major and ubiquitously expressed methyltransferase whose expression is regulated at multiple levels [23]. Deregulation of DNMT1 expression and the accompanying disruption of DNA methylation patterns are associated with many cancers, making DNMT1 an attractive therapeutic target [24]. Studies have revealed that DNMT1 is up-regulated in bladder cancer [25]. However, the role of DNMT1 in bladder cancer cell survival is controversial. One study reported that silencing of DNMT1 by shRNA in the T24 UCCB cell line led to decreased proliferation and increased apoptosis [26]. A conflicting report indicated that T24 cells depleted of DNMT1 exhibited no growth defects [27]. Recent studies have suggested that miR-148a serves as a tumor suppressor by targeting DNMT1 [28]. In gastric cancer cells, miR-148a overexpression was shown to suppress cellular proliferation and also to downregulate DNMT1 [29]. Whether the miR-148a/DNMT1 expression axis plays a role in bladder cancer proliferation or survival is unknown.

In this study, we characterize the expression of miR-148a in UCCB cell lines and assess its role in proliferation and induction of apoptosis. We found that miR-148a is down-regulated in several cell lines when compared to immortalized urothelial cells. Overexpression of miR-148a in vitro reduced cell viability by promoting apoptosis. Furthermore, miR-

148a decreased DNMT1 expression and we demonstrate that the effects observed following miR-148a overexpression are partially due to DNMT1 depletion. Lastly, we show that induction of cell death can be enhanced by combining miR-148a with chemotherapeutic agents.

MATERIALS AND METHODS

Cell Culture, Plasmids, siRNA, and Transfections

SV-HUC-1, T24, TCCSUP, J82, and UM-UC-3 cells were generous gifts from Drs. Sweeney and De Vere White (UC Davis). All cell lines were cultured in 10% FBS RPMI-1640 supplemented with glutamine and both penicillin and streptomycin at 37°C and 5% CO_2. All siRNA transfections were carried out using Lipofectamine RNAiMAX (Invitrogen, Waltham, MA) at an oligonucleotide concentration of 50 nM unless otherwise specified. The following oligonucleotides were used for transfection; miR-148a mimic and anti-miR-148a (Qiagen, Venlo, Limburg, Netherlands), control non-targeting oligonucleotide, and DNMT1 siRNA (Dharmacon, Lafayette, CO). The following drugs were used for treatments; cisplatin and doxorubicin (Fisher Scientific, Waltham, MA). DNMT1-PCDNA3.1 was created by inserting DNMT1 sequence excised from an LZRS vector (LZRS-DNMT1 was a gift from Paul Khavari [Addgene plasmid # 24952]) into the multiple cloning site of PCDNA3.1. UM-UC-3 cells were transfected using Lipofectamine 3 000 with empty vector or DNMT1 containing vector and subjected to G418 selection.

RNA Extraction and Quantitative PCR

Total RNA containing miRNAs was extracted using the mirVana miRNA Isolation Kit (Ambion, Waltham, MA) and reverse transcribed into cDNA using the miScript II RT Kit (Qiagen). qPCR was performed using the miScript PCR kit (Qiagen) on a ViiA 7 Real Time PCR System (Applied Biosystems, Grand Island, NY). Expression of miR-148a was normalized to RNU6-2_1 and was analyzed using the efficiency corrected ΔCt method. Primer assays; RNU6-2_1 and miR-148a (Qiagen).

Proliferation Assays

For miR-148a titration curves, cells were plated at 10 000–20 000 cells/well in a 24 well plate and transfected 24 h later with 0, 10, 25, or 50 nM miR-148a mimic and/or a balancing concentration of a control oligonucleotide. Proliferation was assessed 72 h later using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). All conditions were performed in triplicate. Data is displayed as the mean ± standard deviation.

For experiments assessing the growth of cells transfected with DNMT1 siRNA, cells were plated in a 24 well plate at 10 000–20 000 cells/well and transfected 24 h later with a control oligonucleotide or DNMT1 targeting siRNA. Proliferation was assessed 48 and 96 h later using CCK-8. All conditions were

performed in triplicate. Data is displayed as the mean ± standard deviation.

For quantitative studies using chemotherapeutic drugs in conjunction with either miR-148a mimic, anti-miR-148a, or DNMT1 targeting siRNA, cells were plated 24 h prior to transfection at 10 000–20 000 cells/well. Twenty-four hours post transfection, cells were treated with either cisplatin or doxorubicin at 0.5 μM or 0.05 μM respectively. Proliferation was assessed 48 h later using CCK-8. All conditions were performed in triplicate. Data are displayed as the mean ± standard deviation.

For qualitative studies assessing cell growth visually, T24, and UM-UC-3 cells were plated at 100 000 cells/ 35 mm dish and treated with miR-148a mimic or a control oligonucleotide and either cisplatin or doxorubicin in the same manner as above. Cell growth was assessed 48 h after drug treatment via staining with crystal violet. Images were taken using an Alpha Innotech MultiImage II system (San Jose, CA).

Colony Formation Assay

T24 and UM-UC-3 cells were plated in a 6 well plate at 1500 cells/well. The following day, cells were transfected with control oligonucleotide or miR-148a mimic in triplicate. T24 cells were allowed to grow for 10 days and UM-UC-3 cells were allowed to grow for 12 days post transfection. Colonies were then stained using crystal violet and photographed using an Alpha Innotech MultiImage II system. For the rescue experiments using DNMT1 overexpressing cells, we plated 2 000 cells/well and cultured for 11 days. All other conditions and analyses were as described above.

Flow Cytometry

T24 and UM-UC-3 cells were transfected with either a control oligonucleotide or miR-148a mimic or DNMT1 targeting siRNA in triplicate or quadruplicate. Seventy-two hours later, cells were harvested, and either ethanol fixed and propidium iodide (PI) stained (Sigma–Aldrich, St. Louis, MO) for cell cycle analysis, or were Annexin V-FITC and PI stained (eBioscience, San Diego, CA) for analysis of apoptosis. All flow cytometry was carried out on a FACS Calibur flow cytometer (BD, Franklin Lakes, NJ). Cell cycle data was analyzed using ModFit software and apoptosis data was analyzed
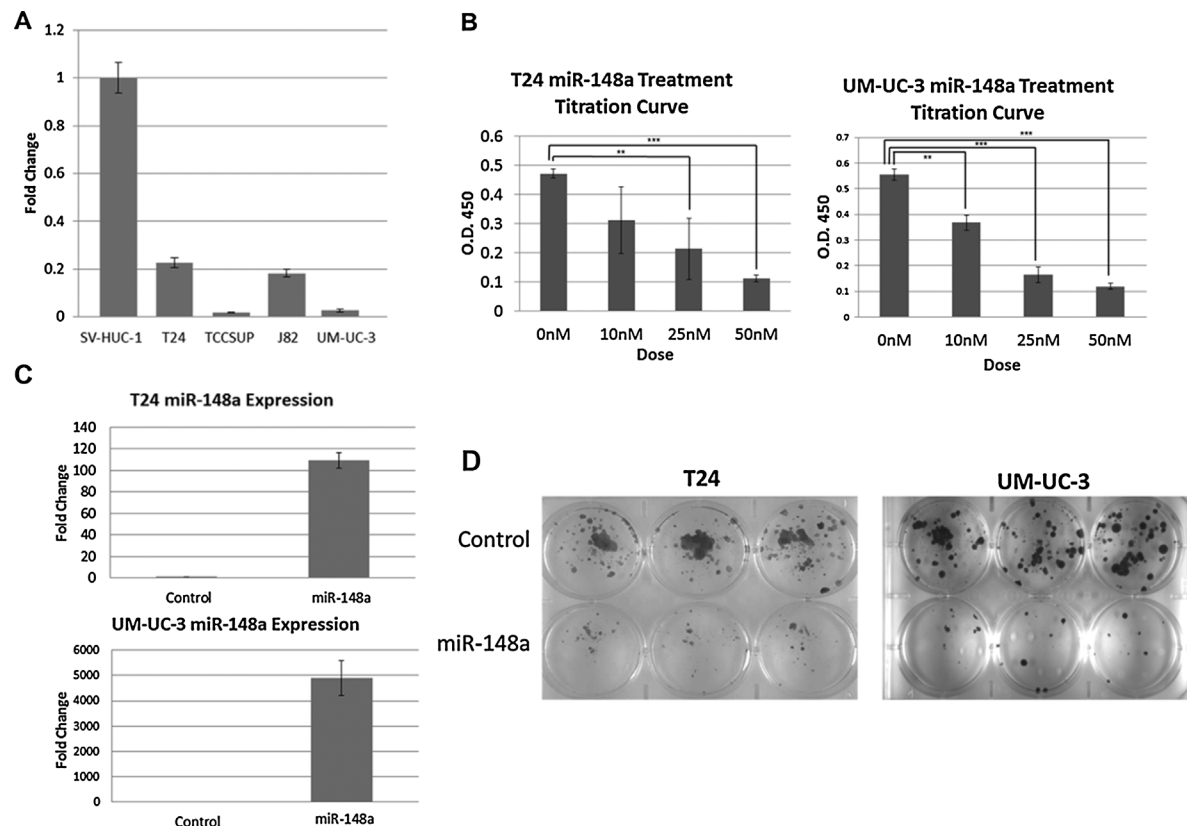

Figure 1. miR-148a expression and effects in UCCB cell lines. (A) qPCR was performed on four UCCB cell lines (T24, TCCSUP, J82, and UM-UC-3) and the immortalized urothelial cell line SV-HUC-1 to assess the relative expression of miR-148a. (B) Proliferation was assessed in T24 and UM-UC-3 cells via titration curves with treatments of 0, 10, 25, and 50 nM of either miR-148a mimic and/or a balancing amount of control oligonucleotide. (C) qPCR was used to assess expression of miR-148a in T24 and UM-UC-3 cells following 50 nM transfection with the miR-148a mimic. (D) Colony formation assays were performed on T24 and UM-UC-3 cells treated with either miR-148a mimic or a control oligonucleotide. [**]P-value ≤0.01, [***]P-value ≤0.001.

using CellQuest software. Data is displayed as the mean $\pm$ standard deviation.

Western Blot

Whole cell lysates were subjected to SDS-PAGE and transferred to 0.2 μm nitrocellulose membranes. Membranes were blocked in 5% milk in PBS-T and left to incubate with primary antibody overnight at 4°C. The following day, membranes were incubated with secondary antibody conjugated to HRP and development was carried out using the WesternBright Sirius Kit (Advansta, Menlo Park, CA) and visualized with a SRX-101A developer (Konica Minolta, Wayne, NJ) or a c-Digit digital scanner (LI-COR, Lincoln, Nebraska). Loading was assessed either by actin or tubulin. The following antibodies were used: cleaved caspase-3 and PARP/cleaved PARP (Cell Signaling, Danvers, MA), Bcl-2, DNMT1, and actin (Santa Cruz Biotechnology, Dallas, Texas), Tubulin (Thermo Scientific).

Statistics

For all assays, a two tailed two sample equal variance student's t-test was used to assess differences between samples. A $P < 0.05$ was accepted as significant.

RESULTS

miR-148a is Down-Regulated in UCCB Cell Lines

Multiple groups have reported that miR-148a functions as a tumor suppressor. In this study, we
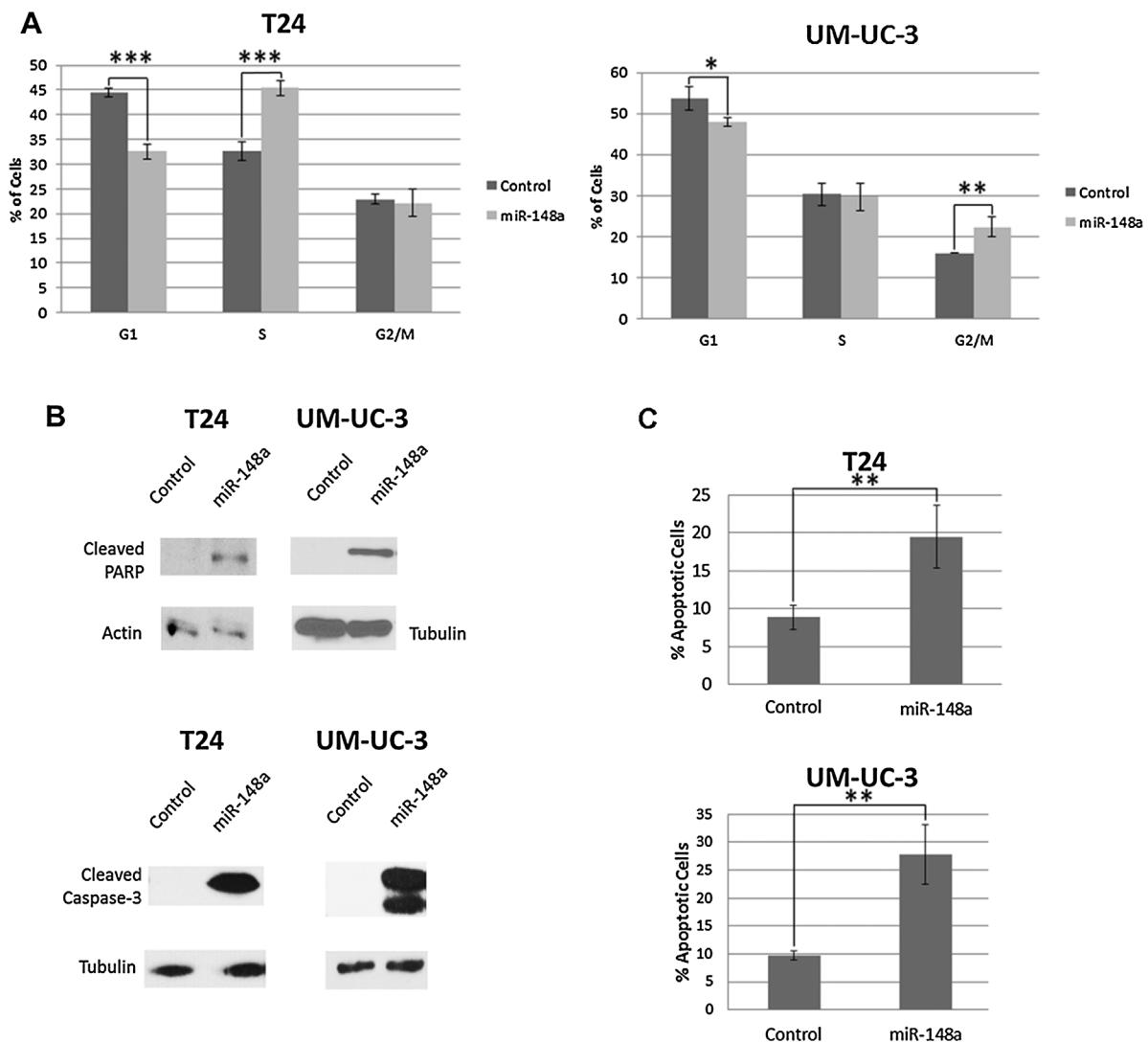

Figure 2. Effects of miR-148a mimic treatment on cell cycle and apoptosis in T24 and UM-UC-3 cells. (A) Cell cycle analysis by PI staining and flow cytometry was performed on miR-148a or control oligonucleotide treated T24 cells and UM-UC-3 cells. (B) Whole cell lysates from T24 and UM-UC-3 cells treated with either miR-148a mimic or a control oligonucleotide were used in western blot analysis of cleaved caspase-3 and cleaved PARP. Either actin or tubulin served as loading controls. (C) Annexin-V staining coupled to flow cytometry was performed to further assess induction of apoptosis in response to overexpression of miR-148a in T24 and UM-UC-3 cells. *P-value $\leq$0.05, **P-value $\leq$ 0.01, ***P-value $\leq$ 0.001.

asked if expression of miR-148a is altered in bladder tumor-derived cell lines when compared to immortalized control cells. Using qPCR, we assessed the expression of miR-148a in the UCCB cell lines T24, TCCSUP, J82, and UM-UC-3 and in the immortalized urothelial cell line SV-HUC-1 (Figure 1A). All of the tumor-derived lines have a $\geq$3-fold decrease in the levels of miR-148a in comparison to the immortalized control line, SV-HUC-1. We could further stratify the cancer lines into two classes based on relative expression; T24 and J82 have significantly more miR-148a than TCCSUP and UM-UC-3. These data demonstrate that miR-148a expression is both down-regulated and variable in UCCB. This analysis suggests that miR-148a may function as a tumor suppressor in this context.

miR-148a Overexpression Attenuates Viability in T24 and UM-UC-3 Cells

Based upon our results from the expression analysis of miR-148a in UCCB cell lines, we hypothesized that re-expression of miR-148a might lead to an inhibition of cellular growth. The T24 and UM-UC-3 cell lines were chosen for subsequent functional studies as they represent the two classes of relative expression. To determine if miR-148a overexpression attenuates cellular viability, both cell lines were transfected with 0, 10, 25, or 50 nM miR-148a mimic and dose response was assessed by proliferation assays (Figure 1B). As hypothesized, we found miR-148a

overexpression results in decreased cell growth and that this effect was dose dependent. Since the 50 nM dose was most effective, all subsequent studies used this dose. Using qPCR, we were able to confirm overexpression of miR-148a (Figure 1C). Further studies of cellular viability using colony formation assays also demonstrated a strong attenuation of cellular growth in cells treated with miR-148a mimic (Figure 1D). These results taken together demonstrate that miR-148a exerts a growth inhibitory effect in UCCB cells.

miR-148a Does Not Attenuate Cellular Viability via Cell Cycle Inhibition

The above data suggest that miR-148a acts as a tumor suppressor in the context of UCCB but does not differentiate between cell cycle inhibition and increased apoptosis. To address this question, flow cytometry was used to detect cell cycle changes of T24 and UM-UC-3 cells treated either with the miR-148a mimic or a control oligonucleotide (Figure 2A). In T24 cells, there was no statistically significant G1 or G2/M arrest that could account for the observed decrease in viability of miR-148a transfected cells. Surprisingly, there was an increase in S-phase. In UM-UC-3 cells overexpressing miR-148a, there was no change in S-phase and similar to T24, no G1 arrest. However, in UM-UC-3 cells, there was a statistically significant increase in the G2/M population of miR-148a mimic treated cells. Although this could lead to some
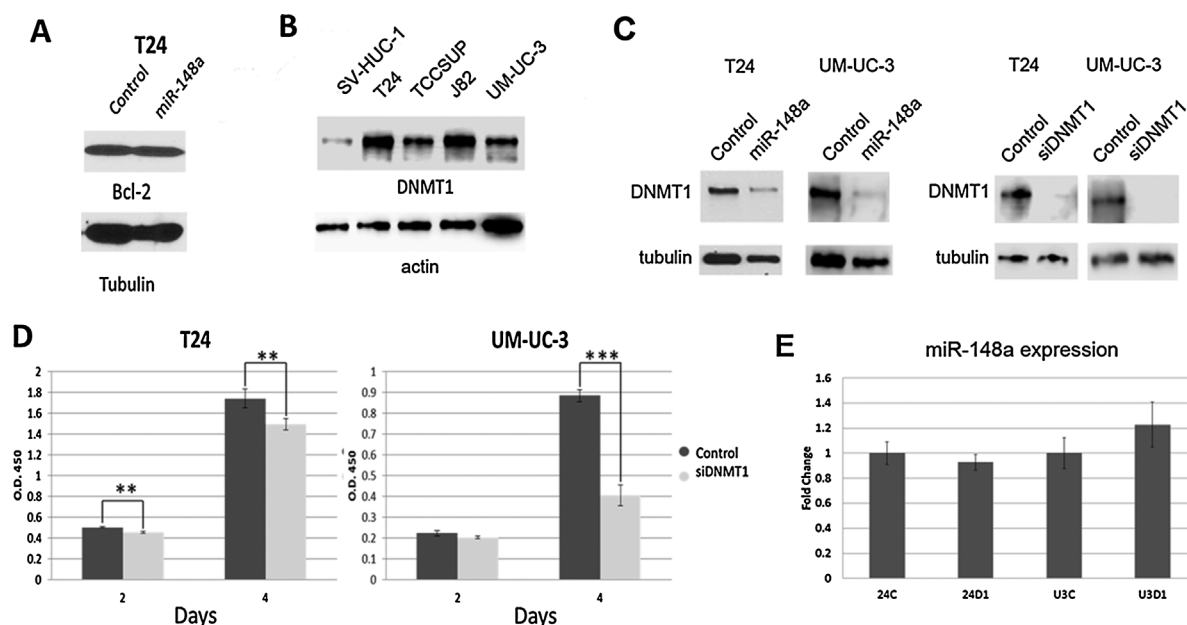

Figure 3. Assessment of DNMT1 as a downstream target of miR-148a. (A) Whole cell lysates from T24 cells treated either with miR-148a mimic or control oligonucleotide were used for western blot analysis of Bcl-2. Tubulin served as loading control. (B) Whole cell lysates from SV-HUC-1, T24, TCCSUP, J82, and UM-UC-3 cells were used for western blot analysis of DNMT1. Actin served as loading control. (C) Whole cell lysates from T24 and UM-UC-3 cells treated with either miR-148a mimic, DNMT1 targeting siRNA, or a control oligonucleotide were used for western blots for DNMT1. Tubulin or actin served as loading controls. (D) Proliferation was assessed in T24 and UM-UC-3 cells treated with either DNMT1 targeting siRNA or a control oligonucleotide. [**]P-value $\leq$ 0.01, [***]P-value $\leq$ 0.001. (E) qPCR was used to assess miR-148a expression in response to either control oligonucleotide (C) or DNMT1 targeting siRNA (D1) in both T24 (24) and UM-UC-3 (U3) cells.

proliferative inhibition, these results taken together suggest that cell cycle retardation is not the primary mechanism by which miR-148a overexpression inhibits cellular viability.

miR-148a Overexpression Induces Apoptosis in T24 and UM-UC-3 Cells

Based on the results from our cell cycle analysis, we hypothesized that overexpression of miR-148a induces apoptosis. Western blot analysis of cleaved caspase-3 and cleaved PARP demonstrated that treatment of cells with miR-148a mimic activates apoptosis (Figure 2B). Flow cytometry coupled to annexin-V staining was used to confirm the results. As anticipated, treatment of T24 and UM-UC-3 cells with miR-148a mimic induced apoptosis (Figure 2C). These data argue that the decrease in viability of bladder cancer cells observed following overexpression of miR-148a is predominately dependent on activation of apoptosis.

miR-148a Inhibits UCCB Cell Viability in Part by Targeting DNMT1

We next explored the mechanism by which overexpression of miR-148a induces apoptosis in UCCB cell lines. Previous studies in pancreatic and colorectal cancer cells reported that miR-148a induced apoptosis by targeting the anti-apoptotic oncogene Bcl-2. However, we did not observe changes in Bcl-2 levels when miR-148a was overexpressed in T24 cells (Figure 3A). It has also been shown that miR-148a can target DNMT1. Given that DNMT1 is thought to be an oncogene in bladder cancer, we first asked if DNMT1 is overexpressed in our UCCB cell line context (Figure 3B). We found that DNMT1 protein was overexpressed to varying degrees in all four lines

assayed versus SV-HUC-1. We next assessed whether the miR-148a/DNMT1 axis was mediating our observed results. Western blots of whole cell lysates from T24 and UM-UC-3 cells treated with either miR-148a mimic or a control oligonucleotide showed reduced DNMT1 levels demonstrating that in the context of UCCB, DNMT1 is a miR-148a target (Figure 3C). Using an siRNA against DNMT1, we asked if DNMT1 knockdown could phenocopy the results attained in the miR-148a studies (Figure 3C and D). Proliferation assays indicated that DNMT1 knockdown leads to a decrease in viability in both cell lines. However, the result in UM-UC-3 was far more pronounced indicating that DNMT1 is more important for survival in this cellular context than it is in T24 cells. Additionally, we asked whether there was a negative feedback loop between DNMT1 and miR-148a. Previous studies have shown that miR-148a is methylated in cancer [18]. Using qPCR, we demonstrated that miR-148a levels do not change in response to DNMT1 knockdown suggesting methylation may not be involved in miR-148a down-regulation in UCCB (Figure 3E).

We next assessed whether the effects of DNMT1 knockdown on viability were mediated by cell cycle inhibition or induction of apoptosis (Figure 4). Flow cytometry was used to analyze the cell cycle in cells treated either with DNMT1 targeting siRNA or a control oligonucleotide (Figure 4A). Similar to miR-148a overexpression, DNMT1 knockdown causes no change and a small increase in the G2/M population in T24 and UM-UC-3 cells respectively. Unlike the miR-148a overexpression experiments, we found no significant change in the G1 populations. However, there was a statistically significant decrease in S-phase in UM-UC-3 cells following DNMT1knockdown. In UM-UC-3, these results suggest that there is a modest
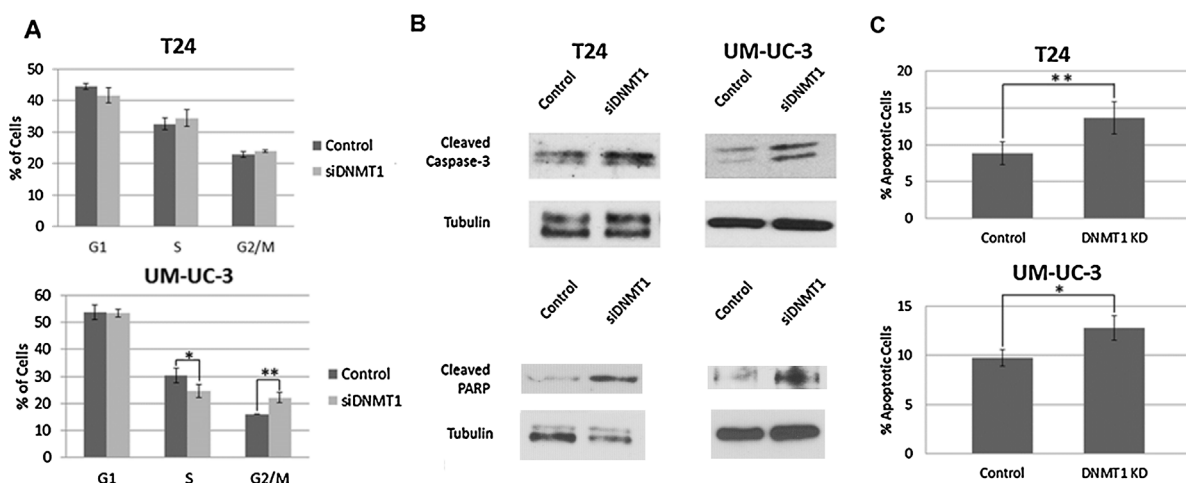

Figure 4. Effect of DNMT1 knockdown on cell cycle and apoptosis. (A) Cell cycle analysis by PI staining and flow cytometry was performed on DNMT1 siRNA treated or control oligonucleotide treated T24 cells and UM-UC-3 cells. (B) Whole cell lysates from T24 and UM-UC-3 cells treated with either siRNA targeting DNMT1 or a control oligonucleotide were used for western blots for cleaved caspase-3 and cleaved PARP. Tubulin served as a loading control. (C) Annexin-V staining coupled to flow cytometry was used to assess apoptosis on T24 and UM-UC-3 control oligonucleotide treated cells and DNMT1 targeting siRNA treated cells $^*P$-value $\leq$0.05, $^{**}P$-value $\leq$ 0.01.

retardation of the cell cycle. However, together these results suggested that decreased DNMT1 expression inhibits cellular viability predominantly through an increase in apoptosis. An analysis of apoptosis revealed that attenuation of DNMT1 leads to increased caspase-3 cleavage and cleavage of PARP (Figure 4B). To further verify the induction of apoptosis, T24 and UM-UC-3 cells treated with either siRNA targeting DNMT1 or a control oligonucleotide were subjected to annexin-V staining and flow cytometry (Figure 4C). This analysis confirmed that decreased DNMT1 expression promoted apoptosis.

We next performed a rescue experiment to deteremine if overexpression of DNMT1 abrogates the effects of miR-148a treatment. We focused this study on UM-UC-3 because DNMT1 seemed most important for survival in this cell type. We generated stable DNMT1 overexpressing UM-UC-3 cells. UM-UC-3 cells harboring the control PCDNA3.1 vector were generated to serve as a control. Western blot analysis confirmed DNMT1 overexpression (Figure 5A). Western blots for cleaved PARP demonstrated that DNMT1 overexpression completely abolishes increased PARP cleavage indicating a rescue from miR-148a- induced apoptosis (Figure 5B). We further assessed growth of these cells in response to miR-148a treatment via a colony formation assay (Figure 5C). Our data demonstrate that viability is restored in response to miR-148a overexpression when DNMT1 is also overexpressed. However, we note that the effects of miR-148a on control cell viability are not as pronounced as previously shown. We hypothesize that high levels of manipulation and stress from cloning altered the phenotype of these cells making them more resistant than their wild-type counterparts.

Taken together, these results indicate that DNMT1 is a miR-148a target in UCCB and that this pathway is partly responsible for the observed decrease in viability through an induction of apoptosis. However, because the DNMT1 data do not completely phenocopy the miR-148a data, we conclude that miR-148a has additional targets in this context.

miR-148a Used in Conjunction With Cisplatin or Doxorubicin Leads to an Additive or Synergistic Induction of Apoptosis in T24 and UM-UC-3 Cells

Based on the above results, we asked whether miR-148a overexpression could cooperate with standard of care chemotherapeutics to further decrease cellular viability. We used two types of proliferation assays (one quantitative and one qualitative) where T24 and UM-UC-3 cells were treated either with miR-148a mimic, low dose of cisplatin, or both (Figure 6). While treatment of the cells with each agent individually led to significant reductions in viability, the inhibition seen by combined treatment was additive in T24 and synergistic in UM-UC-3 cells (Figure 6A and B). We next assessed whether we could protect against cisplatin treatment using an antagomir against miR-
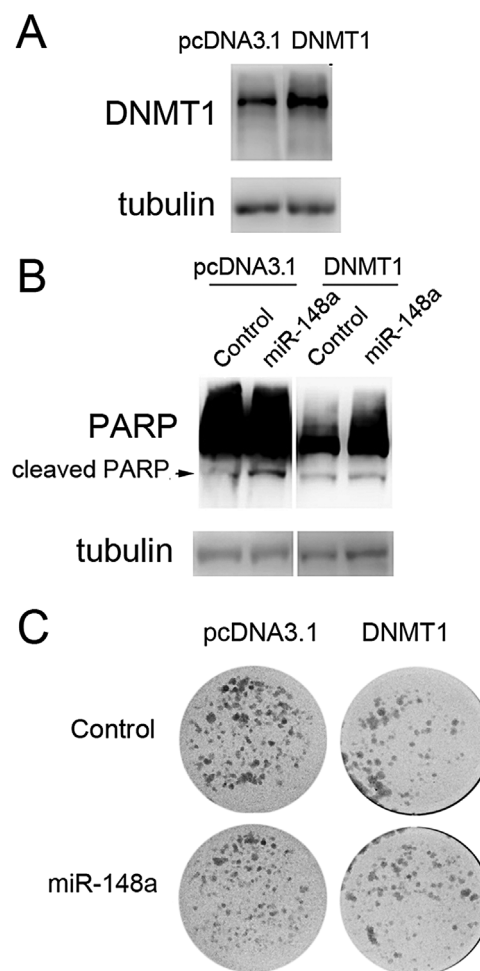

Figure 5. Overexpression of DNMT1 attenuates effect of miR-148a treatment. (A) Whole cell lysates from DNMT1 overexpressing and control empty vector UM-UC-3 cells were used for western blots to demonstrate overexpression of DNMT1. Tubulin served as a loading control. (B) Whole cell lysates from DNMT1 overexpressing and control empty vector UM-UC-3 cells treated either with control oligonucleotide or miR-148a mimic were used for western blots of PARP. Tubulin served as a loading control. (C) DNMT1 overexpressing and control empty vector UM-UC-3 cells treated either with control oligonucleotide or miR-148a mimic were used for colony formation assays to demonstrate the effect of DNMT1 overexpression on miR-148a induced reduction of viability. PCDNA3.1 = control empty vector.

148a to reduce miR-148a expression (Figure 6C). Surprisingly, our results demonstrated that anti-miR-148a has a slight anti-proliferative effect in T24 and no effect in UM-UC-3. We hypothesize that miR-148a levels are already so low in these cells that further reduction has no major effect. Accordingly, no protective effect was observed combining cisplatin with anti-miR-148a. A recent report suggested that DNMT1 expression plays a role in cisplatin sensitivity in ovarian cancer [30]. Therefore, we asked if this pathway was mediating the observed effect when combining miR-148a with cisplatin. We found that in UM-UC-3 cells, the effect of decreased DNMT1 expression, and cisplatin treatment was additive
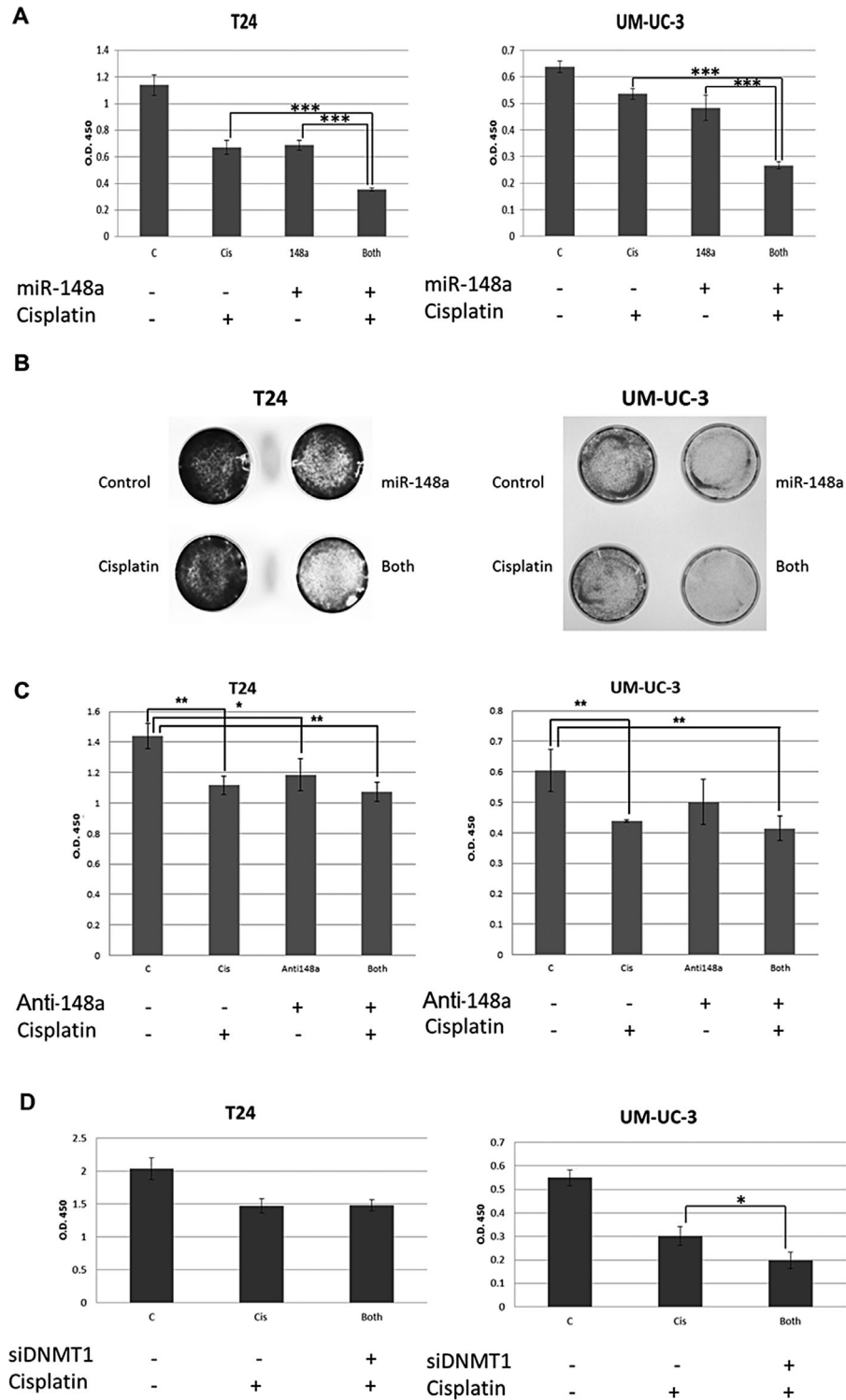
Figure 6. Effect of combining miR-148a with cisplatin in T24 and UM-UC-3 cells. (A) T24 and UM-UC-3 cells were transfected with either control oligonucleotide or miR-148a mimic and 24 h post transfection were treated with either 0.5 μM cisplatin or vehicle. Cell viability was assessed 48 h post drug treatment using the CCK-8 reagent. (B) T24 and UM-UC-3 cells were plated in 35 mm dishes and were subjected to identical treatments as described in A. Fourty-eight hours post drug treatment, cells were stained with crystal violet, and images were taken. (C) T24 and UM-UC-3 cells were transfected with either control oligonucleotide or anti-miR-148a mimic and 24 h post transfection were treated with either 0.5 μM cisplatin or vehicle. Cell viability was assessed 48 h post drug treatment using the CCK-8 reagent. (D) T24 and UM-UC-3 cells were transfected with either control oligonucleotide or DNMT1 targeting siRNA and 24 h post transfection were treated with either 0.5 μM cisplatin or vehicle. Cell viability was assessed 48 h post drug treatment using the CCK-8 reagent. * P-value ≤ 0.05, ** P-value ≤ 0.01, *** P-value ≤ 0.001.

(Figure 6D). However, the same effect was not observed for T24 cells. These data suggest again that the effects of miR-148a are context dependent and that additional miR-148a targets contribute to the pro-apoptotic effect of miR-148a.

Combination of miR-148a with doxorubicin was also assessed using the same approach described for cisplatin (Figure 7A and B). The qualitative assay argued that miR-148a could cooperate with doxorubicin in reducing cellular viability in both lines. However, the quantitative assays indicated an additive effect of the two agents for UM-UC-3 only, while the assay failed to demonstrate statistical significance in T24 cells.

DISCUSSION

Evasion of cellular death and uncontrolled cellular proliferation are two of the hallmarks of cancer and

multiple studies have focused on the role miRNAs play in regulating these processes [31]. miR-148a has been shown to be deregulated in different tumors but the downstream targets of this miRNA differ and it is unclear how miR-148a modulates cellular responses in varying cellular contexts. Many studies point to a tumor suppressor role for miR-148a. In our present study, we demonstrate that miR-148a expression is lower in UCCB cell lines versus non-transformed cells and also acts as a tumor suppressor by potently inducing apoptosis. Our results suggest that repression of miR-148a in bladder cancer cells promotes cell survival and thus miR-148a could serve as a potential therapeutic.

Moreover, our study demonstrates that miR-148a leads to induction of apoptosis in part by down-regulating DNMT1, an epigenetic modifier. Modulation of DNMT1 levels would globally modify transcription, where a decrease in DNMT1 would lead to a
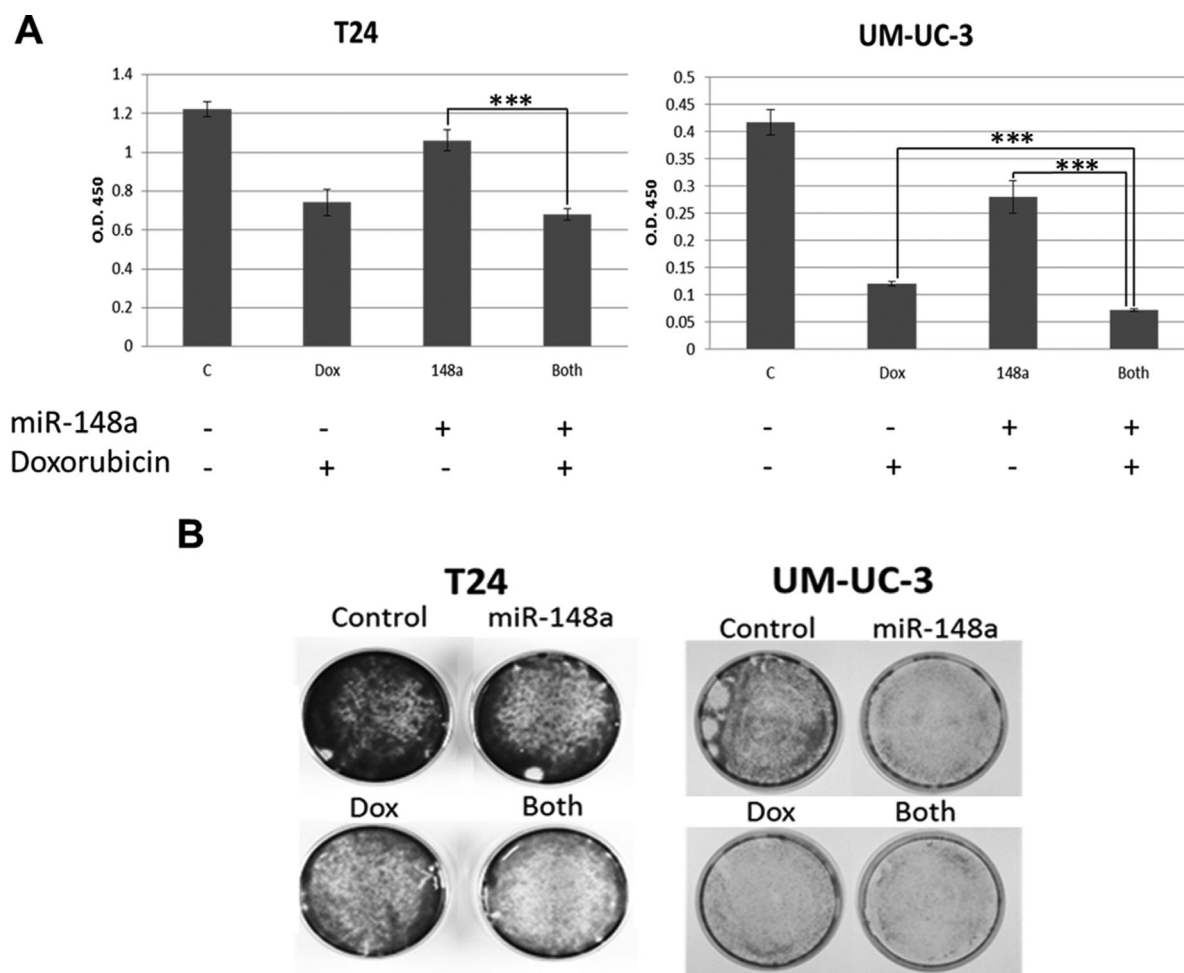

Figure 7. Effect of combining miR-148a with doxorubicin in T24 and UM-UC-3 cells. (A) T24 and UM-UC-3 cells were transfected with either control oligonucleotide or miR-148a mimic and 24 h post transfection were treated with either 0.05 μM doxorubicin or vehicle. Cell viability was assessed 48 h post drug treatment using the CCK-8 reagent. (B) T24 and UM-UC-3 cells were plated in 35 mm dishes and were subjected to identical treatments as described in A. Forty-eight hours post drug treatment, cells were stained with crystal violet, and images were taken. ***P-value ≤ 0.001.

de-repression of silenced genes. The overall effect of such wide-spread transcriptional changes would be cell context dependent. Previous studies have investigated the role of DNMT1 in bladder tumorigenesis. Wu et al. showed that DNMT1 expression was increased in bladder cancers and correlated with stage of the disease [25]. Furthermore, inhibition of DNMT1 resulted in decreased cellular viability [25]. Additional studies indicated that decreased expression of DNMT1 in T24 cells inhibited cellular proliferation and increased apoptosis [26]. Using a canine model of bladder cancer, Dhawan et al. demonstrated that targeting DNMT1 may be a viable treatment option and provided an animal model with which to translate future discoveries [32]. However, the mechanism(s) that lead to increased DNMT1 expression in bladder tumors have not been identified. Our study suggests that one mechanism which contributes to elevated DNMT1 levels in bladder tumors is a deregulation of miR-148a and further supports targeting of DNMT1 as a therapeutic strategy.

The current 5-year survival rate for muscle invasive UCCB is low and there are few treatment options [10]. Cisplatin, a potent chemotherapeutic agent, has been shown to have limited efficacy in the treatment of this malignancy. The results presented here indicate that while cisplatin and miR-148a independently induce apoptosis, the effect of combining the two agents is additive in T24 cells, and synergistic in UM-UC-3 cells resulting in significantly increased cell death. The combined treatment of cells with miR-148a and doxorubicin also enhanced apoptosis in T24 and UM-UC-3 cells compared to treatment with a single agent but the results were less impressive suggesting that the complementary effect of miR-148a was specific to the chemotherapeutic agent. Our results demonstrate that miR-148a has potential as a novel agent for treatment of UCCB and that combination therapy with specific chemotherapeutics could be used as potent therapeutic regimens.

Our work additionally points to the potential increased utility inherent in miRNAs as therapies over targeting individual oncoproteins/tumor suppressor proteins alone. We show that using siRNA to specifically target DNMT1 leads to only a partial phenocopy of the results obtained using miR-148a mimic. Thus, although DNMT1 is targeted by miR-148a in this context, it is not the only target. Use of a molecule with broader rather than restricted specificity may be a better option for promoting cell death. MiRNAs have the ability to regulate several different mRNA targets simultaneously and because of this, they are key regulators of gene networks. This provides a method to tailor de-regulated expression of several transcripts with a single molecule.

In conclusion, we have elucidated a novel pathway regulating cell death in UCCB. Our study highlights the potential utility of miR-148a as a novel therapeutic for the treatment of UCCB.

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