

# Inhibition Effect of siRNA-Downregulated *UHRF1* on Breast Cancer Growth

Feng Yan,<sup>1</sup> Xu-Yan Tan,<sup>1</sup> Yao Geng,<sup>2</sup> Huang-Xian Ju,<sup>2</sup> Yan-Fang Gao,<sup>3</sup> and Ming-Chen Zhu<sup>1</sup>

## Abstract

The *UHRF1* gene plays important roles in both cell proliferation through its NIRF\_N domains, a PHD domain, an SRA domain, and a RING domain, and multidrug resistance in breast cancer treatment. In this work, a short-hairpin RNA (shRNA) lentiviral system was introduced in two human breast cancer cell lines (MDA-MB-231 and MCF-7) to downregulate the expression of *UHRF1* and study the specific inhibition of *UHRF1* in breast cancer growth. The effect of *UHRF1*-shRNA on breast cancer cell proliferation was examined using methylthiazole tetrazolium, bromodeoxyuridine, and colony formation assays. The proliferative potential of the *UHRF1*-shRNA-treated cells showed a remarkable decrease. Moreover, the downregulation of *UHRF1* in both breast cancer cell lines significantly inhibited the colony formation capacity. Results suggested that the inhibition of *UHRF1* via an RNA interference lentiviral system may provide an effective way for breast cancer therapy.

**Key words:** breast cancer, proliferation, RNA interference, *UHRF1*

## Introduction

THE *UHRF* FAMILY mainly includes *UHRF1* and *UHRF2* genes, which play an important role in cell proliferation through different structural domains.<sup>1</sup> The *UHRF1* gene encodes a member of a subfamily of RING-finger-type E3 ubiquitin ligases such as ICBP90 (inverted CCAAT box-binding protein of 90 kDa) in human and Np95 (nuclear protein of 95 kDa) in mouse.<sup>2,3</sup> Human *UHRF1* (ICBP90) has been identified in the one-hybrid system with an inverted CCAAT box as the DNA target sequence involved in the regulation of topoisomerase II $\alpha$  (TopoII $\alpha$ ) gene expression.<sup>4</sup> The human *UHRF1* gene spans about 35.8 kb and contains six coding exons named A to F, and exon A contains an internal splicing site. Northern blot analysis of several cancer cell lines has revealed the existence of two *UHRF1* mRNA species of 5.1 and 4.3 kb,<sup>2</sup> indicating that the expression of *UHRF1* gene may exhibit alternative splicing pattern in a cell-specific manner. In the study of the expression patterns of *UHRF1*, two peaks (in late G1 and G2/M) were observed in normal cells, whereas *UHRF1* expression was continuously expressed at a high constant level in cancer cells, suggesting that in-

creased *UHRF1* expression may be involved in carcinogenesis.<sup>1,5,6</sup> For example, *UHRF1* was found to be overexpressed in various types of lung cancer at an early pathological stage in an investigation of 56 U.S. and 322 Japanese lung cancer patients.<sup>7</sup> Significant overexpression of *UHRF1* can also be observed in bladder cancer, which is correlated with the bladder cancer stage.<sup>8</sup> This assertion is also sustained by the fact that *UHRF1* is able to overcome cell contact inhibition signaling by regulating TopoII $\alpha$  expression.<sup>9</sup>

Even though *UHRF1* has been recognized as a novel diagnostic marker in various cancers, its role in breast cancers has not been well illustrated. Chemotherapy resistance is a major problem in the management of patients with breast cancer,<sup>10,11</sup> and MDR1 remains a major cause for the failure of chemotherapy.<sup>12</sup> It has been reported that *UHRF1* plays an important role in inhibiting MDR1 promoter activity by directly binding to the MDR1 promoter.<sup>12</sup>

Consequently, to better elucidate the role of *UHRF1* in breast cancer, small interfering RNA was introduced in two breast cancer cell lines (MDA-MB-231 and MCF-7) to downregulate the expression of *UHRF1*. The present study's results show that the knockdown of *UHRF1* has an

<sup>1</sup>Department of Clinical Laboratory, Jiangsu Institute of Cancer Prevention and Cure, Nanjing, People's Republic of China.

<sup>2</sup>MOE Key Laboratory of Analytical Chemistry for Life Science, Department of Chemistry, Nanjing University, Nanjing, People's Republic of China.

<sup>3</sup>The First Clinic Medical College of Nanjing Medical University, Nanjing, People's Republic of China.

Address correspondence to: Feng Yan; Department of Clinical Laboratory, Jiangsu Institute of Cancer Prevention and Cure; No. 42 Baiziting Road, Nanjing 210009, People's Republic of China  
E-mail: yanfeng2007@sohu.com

inhibitory effect on breast cancer cell growth, suggesting that UHRF1-targeted lentivirus systems can be applied as a potentially therapeutic tool for breast cancer treatment.

## Materials and Methods

### Cell culture

Two established human breast cancer cell lines (MDA-MB-231 and MCF-7) were obtained from ATCC. Both cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Lentivirus infection (MOI of 20) was performed after 12 hours of incubation of recultured cells. The transfection efficiency determined by fluorescence microscopy was found to be 70%–90%.

### Construction of vectors and lentiviruses

A short-hairpin RNA (shRNA) sequence for human *UHRF1* gene (NM\_013282, TGTGAAATACTGGCCCGAGAA) was designed and cloned into a pLV-TM-lentiviral vector containing a GFP reporter (Shanghai Genechem Co. Ltd.). Then the vectors containing the target or nontarget sequence were transfected into MDA-MB-231 and MCF-7 cells, respectively, along with packing vectors. Lentiviruses were harvested at 3 days after purification and precipitation.

### Reverse transcription and real-time PCR

Cultured cells were infected with targeting *UHRF1* (si-*UHRF1*) and nontargeting (si-control) lentiviruses. After 72 hours, infected cells were harvested for RNA extraction by Trizol Reagent (Invitrogen). Total RNA were reverse-transcribed into cDNA with random primers, following the manufacturer's protocol (MBI Fermentas). In quantitative real-time PCR, two sets of primers were used: GAPDH, sense: 5'-CGGC ATTGTCACCAACTG-3', antisense: 5'-CGCTCGGTCAGGATCTTC-3'; UHRF1, sense: 5'-CCACATC GTCCTCACAGC-3', antisense: 5'-GGTCCACATCATCCTCATAGC-3'. All reactions were performed in triplicate according to the ABI manufacturer's protocols (Applied Biosystems, Perkin-Elmer), and relative gene expression determinations of GAPDH and UHRF1 were performed with the comparative delta-delta CT method ( $2^{-\Delta\Delta CT}$ ) as described by Livak et al.<sup>13</sup> All results were analyzed with the Light Cycler Software version 3.5 (Roche Diagnostics).

### Western blot analysis

MDA-MB-231 and MCF-7 cells were transfected with si-*UHRF1* and si-control vectors as described earlier. After infection, protein extracts of all samples were obtained with a Tris-buffered saline-based lysis buffer (Tris-buffered saline, 20 mM EDTA, and 0.1% Triton X-100). Whole cell extracts were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In western immunoblot analyses, UHRF1 and GAPDH were detected using antibodies against UHRF1 (Santa Cruz Biotechnology) and GAPDH (R&D Systems). The horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000) was purchased from Santa Cruz. Western blotting was performed using enhanced chemiluminescence (Amersham Biosciences). GAPDH antibody was used as loading control in all lanes.

### Methylthiazolotetrazolium proliferation assay

MDA-MB-231 and MCF-7 cells were trypsinized at 48 hours after lentivirus treatment, counted, and replated into 96-well plates at a concentration of 1500 cells/well. Since day 1, cells were sampled at 1-day intervals to perform the methylthiazolotetrazolium (MTT) proliferation assay. In each well, 10  $\mu$ L MTT (Sigma; 5 mg/mL in phosphate-buffered saline [PBS]) was added, and the plates were incubated at 37°C for 4 hours. After being washed with PBS, 150  $\mu$ L dimethyl sulfoxide was added to each well to dissolve the crystals. Ten (10) minutes later, at room temperature, the absorbance of each sample was recorded at 490 nm.

### Bromodeoxyuridine cell proliferation assay

MDA-MB-231 and MCF-7 cells were trypsinized at 48 hours after lentivirus treatment, counted, and replated into 96-well plates at a concentration of 1500 cells/well. The bromodeoxyuridine (BrdU) cell proliferation assay kit was used according to the manufacturer's instruction. At indicated time points, 20  $\mu$ L BrdU (1:500 in dilution) was added into each sample and incubated for 8 hours. Then the plate was fixed and washed. Anti-BrdU monoclonal (1:200 in dilution), goat anti-mouse IgG, and peroxidase conjugate antibodies were used successively for reaction. In the end, the plate was read at a dual wavelength of 450/550 nm.

### Apoptosis assay

Approximately  $5 \times 10^4$  MDA-MB-231 cells were plated in each six-well plate at 24 hours prior to infection. Twelve hours following infection with lentiviruses, the medium was removed and replaced with the original culture medium. Six (6) days after infection, the cells were treated with adriamycin (ADM, 1  $\mu$ g/mL) for 24 hours and then harvested for apoptosis assay. To investigate the apoptotic effect induced by ADM, all treated cells were collected and stained with Annexin V using an ApoScreen Annexin V Apoptosis Kit (Southern Biotech).

### In vitro colony formation assay

Cells were trypsinized at 48 hours after lentivirus treatment (si-*UHRF1* and si-control), counted, and replated in a six-well plate at a concentration of 200 cells/well. All cell samples were allowed to grow for 14 days to form natural colonies. Then the plate was washed twice with PBS solution and stained with Giemsa (MBCHEM) for 10 minutes. The stained colonies were photographed under a microscope. The total number of colonies (>50 cells/colony) and the total number of cells in each colony were counted and analyzed.

### Statistical analysis

Student's *t*-test was used to analyze the statistical significance of the differences between experimental groups. A *p*-value of < 0.05 was considered significant. All experiments were performed in triplicate.

## Results

### Inhibition to UHRF1 mRNA and protein levels by RNA interference

MDA-MB-231 and MCF-7 cells were transfected with lentiviruses containing UHRF1-targeting sequences or non-

targeting sequences as controls. Seventy-two (72) hours later, the transfection efficiency was determined by microscopy. A GFP reporter has been constructed in the vector of lentiviruses to precisely quantify the shRNA effect on *UHRF1* expression. As shown in Figure 1, in both cell lines, successful transfection was observed under fluorescence microscopy. In both MDA-MB-231 and MCF-7 cells, the transfection efficiency reached 90% with 20 MOI. The *UHRF1* expression was measured in MDA-MB-231 cells to ensure the knockdown efficiency of si-*UHRF1* lentiviruses. The mRNA expression levels of si-*UHRF1* and si-control groups were studied by real-time PCR reaction. As shown in Figure 2A, the si-*UHRF1* lentiviruses reached about 80% knockdown efficiency. Moreover, the knockdown effect of *UHRF1*-targeting lentiviruses was also corroborated by western blot analysis of cell extracts analyzed at 48 hours after treatment (Fig. 2B). Decreased *UHRF1* protein expression (nearly 80%–90%) was observed in MDA-MB-231 cells.

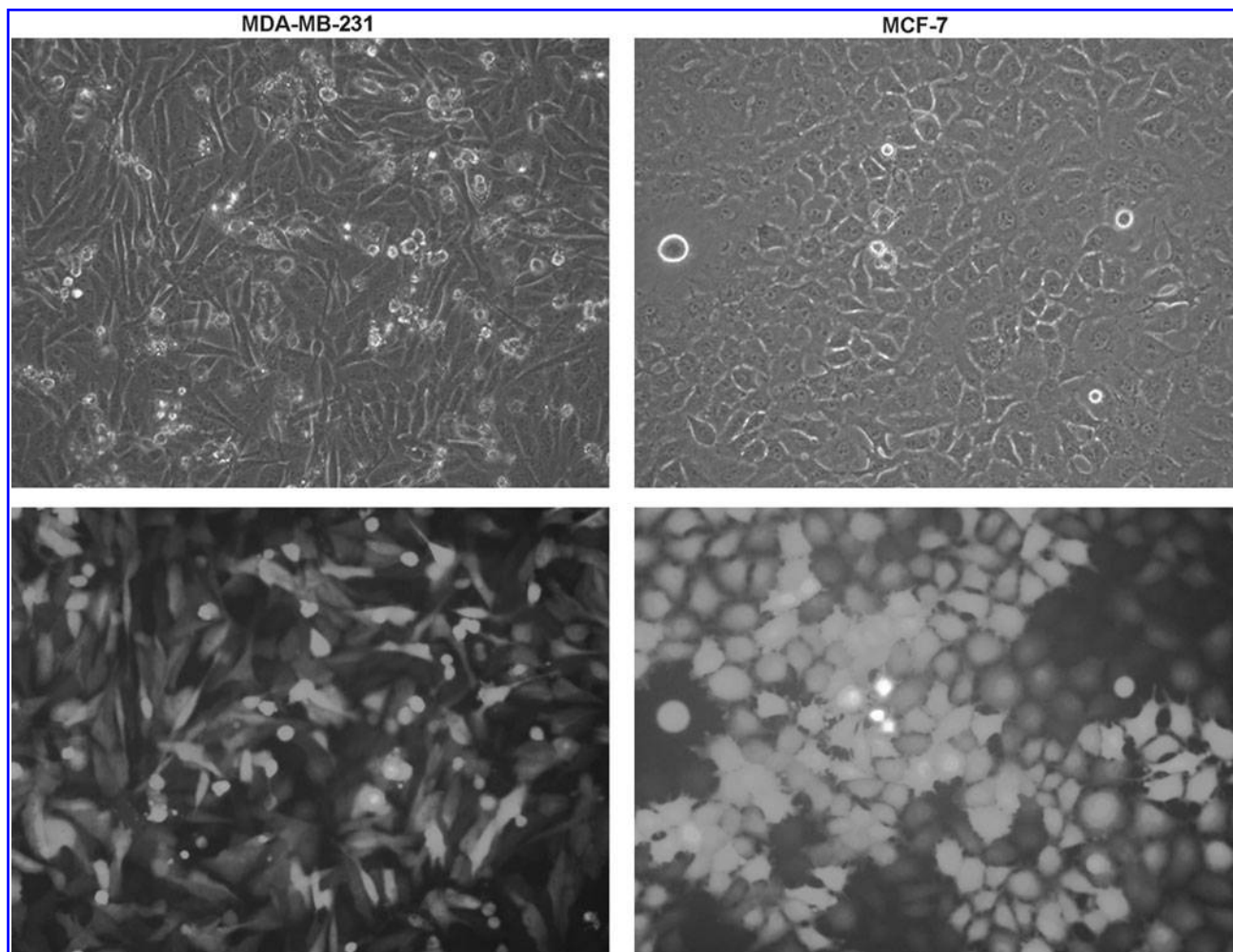
#### *si-UHRF1* inhibited proliferation of MDA-MB-231 and MCF-7 cells

To evaluate the effect of *UHRF1* knocking-down in regulating breast cancer cell proliferation, MTT and BrdU cell

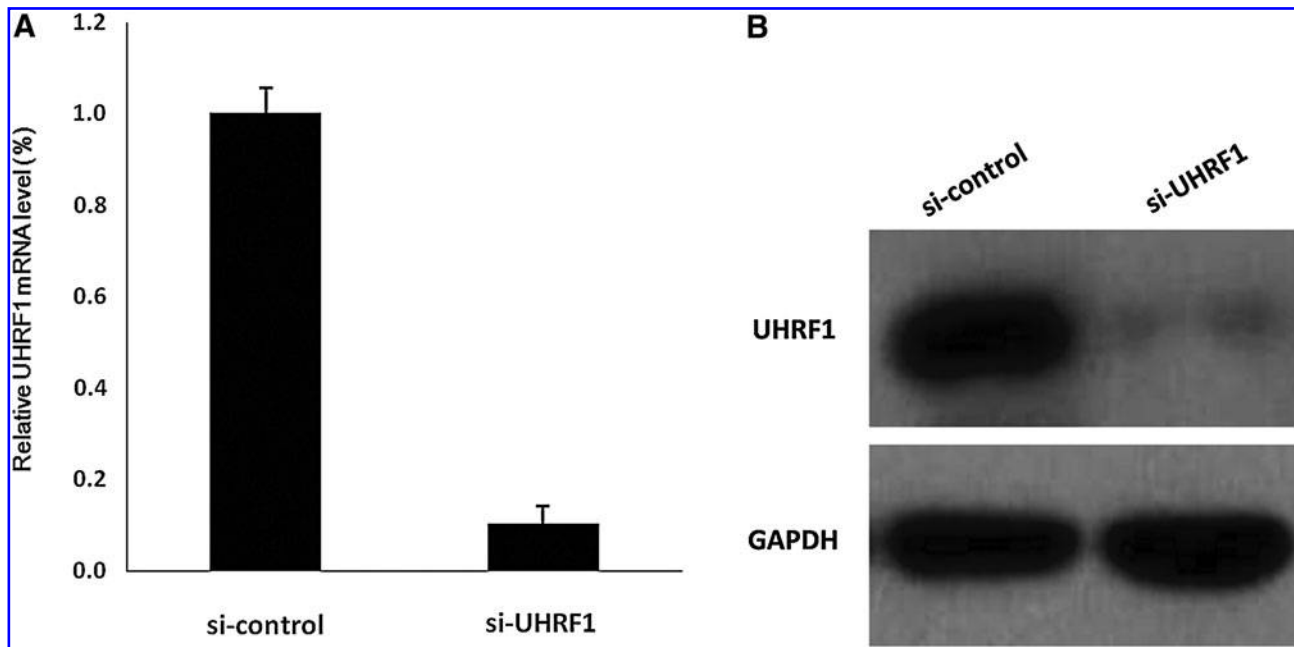
proliferation assays were used. Briefly, cells were transfected with si-*UHRF1* or si-control lentiviruses, and all treated samples were trypsinized, counted, and replated in 96-well plates (time 0 starts at this point). The MTT assays were performed with 1-day intervals, whereas BrdU assays were performed at 24 and 72 hours. In MDA-MB-231 cells, si-*UHRF1* significantly reduced cell growth in 2 days and more remarkably in 5 days ( $p < 0.05$ ), as shown in Figure 3A. The growth-inhibiting effect of si-*UHRF1* can also be supported by the results of BrdU assays at 72 hours ( $p < 0.05$ ) in Figure 3B. Additionally, the effectiveness of shRNA in inhibiting MCF-7 cell growth was assessed using MTT and BrdU assays (Fig. 3C, D), which showed similar effect in MDA-MB-231 cells.

#### *Effect of shRNA for UHRF1 on sensitivity of MDA-MB-231 cells to ADM*

To determine whether downregulation of *UHRF1* can increase the efficiency of ADM, an apoptosis assay was employed. As described in the Materials and Methods section, MDA-MB-231 cells were used as the cellular model. In Figure 4, cells infected with lentiviruses (si-*UHRF1* or si-control) were treated with ADM. Cells without ADM treatment had



**FIG. 1.** Efficient transfection with *UHRF1* short-hairpin RNA lentivirus in MDA-MB-231 and MCF-7 cells. Both cells were transfected with lentiviruses containing *UHRF1*-targeting sequences or nontargeting sequences. After 72 hours, treated cells were photographed under bright light (upper panel) and fluorescent light (lower panel) with original magnification of  $\times 200$ .



**FIG. 2.** Inhibition to UHRF1 mRNA and protein levels by RNA interference. MDA-MB-231 cells were transfected with si-UHRF1 and si-control lentiviruses, and the UHRF1 expression was measured to ensure the knockdown efficiency. **(A)** Relative expression of UHRF1 in two groups by real-time PCRs. si-UHRF1 reduced the expression of UHRF1 mRNA levels by 80% ( $p < 0.05$ ). **(B)** Whole-cell extract was obtained and subjected to western blot assay. The expression of GAPDH was used as loading control. si-UHRF1 reduced the expression of UHRF1 protein levels by 90% ( $p < 0.05$ ).

~10% apoptotic and dead cells, whereas ~40% cells were apoptotic and dead in response to ADM. However, no differences were found between the si-control (40%) and si-UHRF1 groups (42%), suggesting that UHRF1 may have no effect on drug sensitivity of breast cancer cells.

#### *shRNA for UHRF1 inhibited the colony formation capacity of MDA-MB-231 and MCF-7 cells*

Knowing that si-UHRF1 has a short-term inhibitory effect on cell proliferation, its relative long-term function on breast cancer cell growth was also examined by colony formation assays. In this experiment, MDA-MB-231 and MCF-7 cells after treatment were counted and reseeded in plates, so as to form natural colonies in the next 14 days. Before Giemsa staining, colonies (diameter: >1 mm) were observed with bright light microscopy (middle lane of Fig. 5A) and fluorescence microscopy (lower lane of Fig. 5A), showing that the size of a certain colony was reduced by si-UHRF1 treatment. Also, colonies were then stained with Giemsa and observed again to reaffirm the effect of si-UHRF1 (upper lane of Fig. 5A). The natural colonies in plates were photographed, as shown in Figure 5B. In the si-UHRF1-treated group, the number of colonies was remarkably decreased (>50%,  $p < 0.05$ ), and the number of cells in each colony was significantly reduced as well (>50%,  $p < 0.05$ ).

#### **Discussion**

Human UHRF1, also known as ICBP90, is a recently identified nuclear protein that binds to one of the inverted CCAAT boxes of the TopoII $\alpha$  gene promoter.<sup>5</sup> It has been reported that UHRF1 was dysregulated in various cancers, such as lung cancer, bladder cancer, and breast

cancer.<sup>6-8,12,14</sup> A recent study in breast cancer research has revealed its potential role in breast cancer development. In this study, the expression of *UHRF1* was investigated in low- and high-grade primary breast carcinomas compared with normal breast tissue, showing that cells expressing *UHRF1* were very low in normal breast tissue, whereas in breast carcinomas the percentage of UHRF1-positive cells appears to follow the grade.<sup>5</sup>

The present study aimed to investigate the function of *UHRF1* in breast cancer cell growth via an shRNA lentivirus system. As the constancy and efficiency of lentivirus systems have been affirmed by many studies,<sup>15,16</sup> a UHRF1-targeting shRNA sequence was constructed in vectors and lentiviruses were generated. The knockdown efficiency was then examined by real-time PCR and western blots. As revealed by MTT and BrdU assays, si-UHRF1 lentiviruses inhibited MDA-MB-231 and MCF-7 cell growth to a great extent. Additionally, si-UHRF1 decreased the formation of natural colonies in *in vitro* models in MDA-MB-231, as shown by the number of colonies and number of cells in each colony. Similarly, the proliferating potential of MCF-7 cells decreased dramatically when treated with si-UHRF1. Nevertheless, downregulation of UHRF1 did not alter the sensitivity of MDA-MB-231 cells to ADM (Fig. 4), suggesting that *UHRF1* may not be involved in the drug sensitivity of breast cancer cells. To address this question, more anticancer drugs and breast cancer cell lines should be examined in further investigations.

However, the mechanism by which *UHRF1* regulates cell growth was not investigated. Nevertheless, previous studies may provide some clues. Besides the involvement in the regulation of TopoII $\alpha$ , *UHRF1* may take part in the cell proliferation controls through other mechanisms, such as

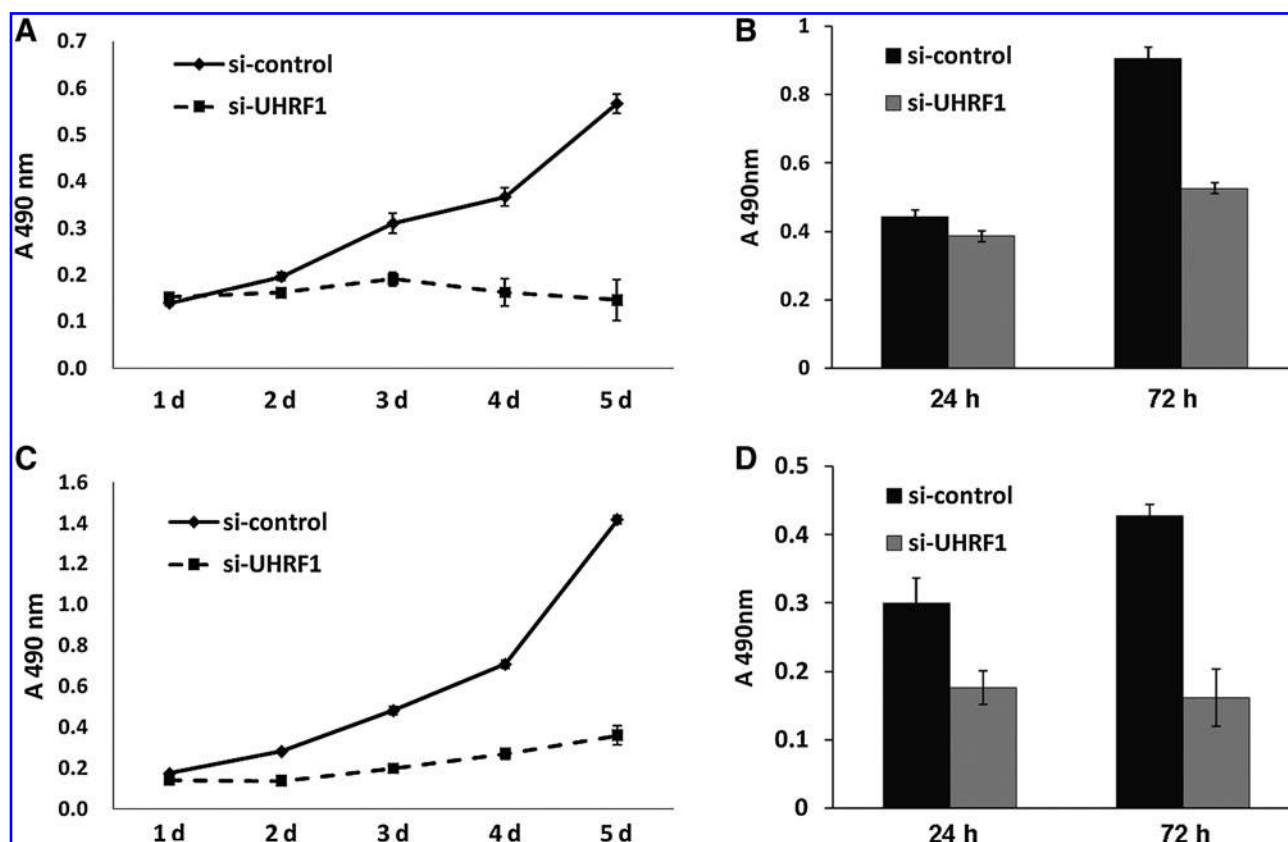


FIG. 3. Inhibition of si-UHRF1 to proliferation of MDA-MB-231 and MCF-7 cells. si-UHRF1 or si-control lentivirus-treated cells were trypsinized and replated in 96-well plates. MDA-MB-231 cells were determined by methylthiazolotetrazolium cell proliferation assays with 1-day intervals (A) and by bromodeoxyuridine assays at 24 and 72 hours (B). MCF-7 cells were determined by methylthiazolotetrazolium cell proliferation assays with 1-day intervals (C) and by bromodeoxyuridine assays at 24 and 72 hours (D).

protein kinase 2 (casein kinase 2 [CK2]), p53, p21<sup>Cip1/WAF1</sup>, and ERK1/2.<sup>4,17-20</sup> For example, CK2 is a protein serine/threonine kinase involved in cell proliferation with dysregulated expression levels. It has been reported that UHRF1 has several putative CK2 phosphorylation sites and may be a

substrate for CK2.<sup>17</sup> UHRF1 expression can be down-regulated by the p53/p21<sup>Cip1/WAF1</sup>-dependent DNA damage checkpoint signals,<sup>18</sup> and it has also been suggested that UHRF1 may serve as a focal point of transcriptional regulation mediated by G9a and other chromatin modification

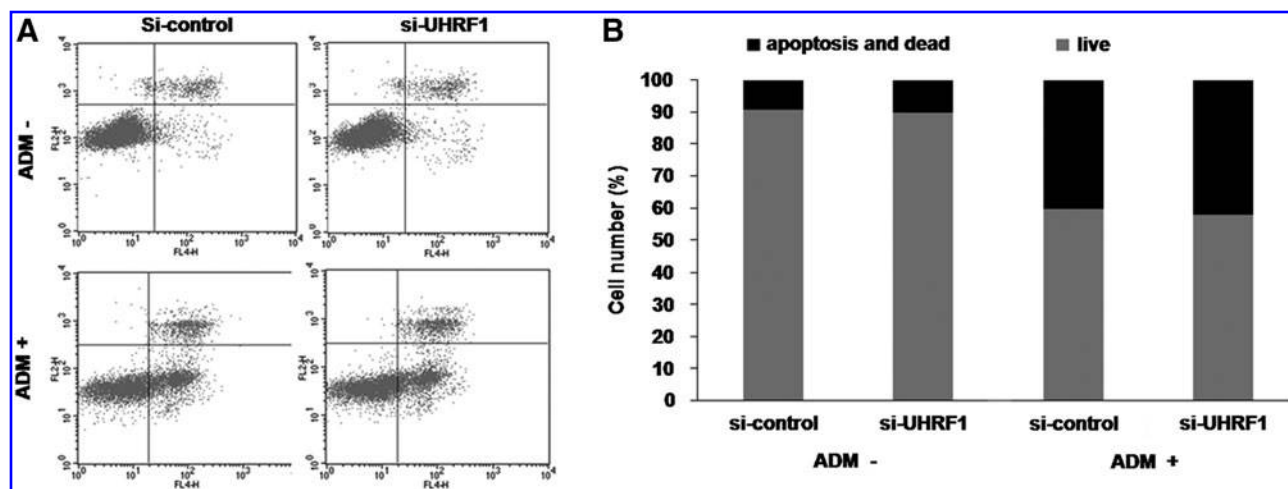
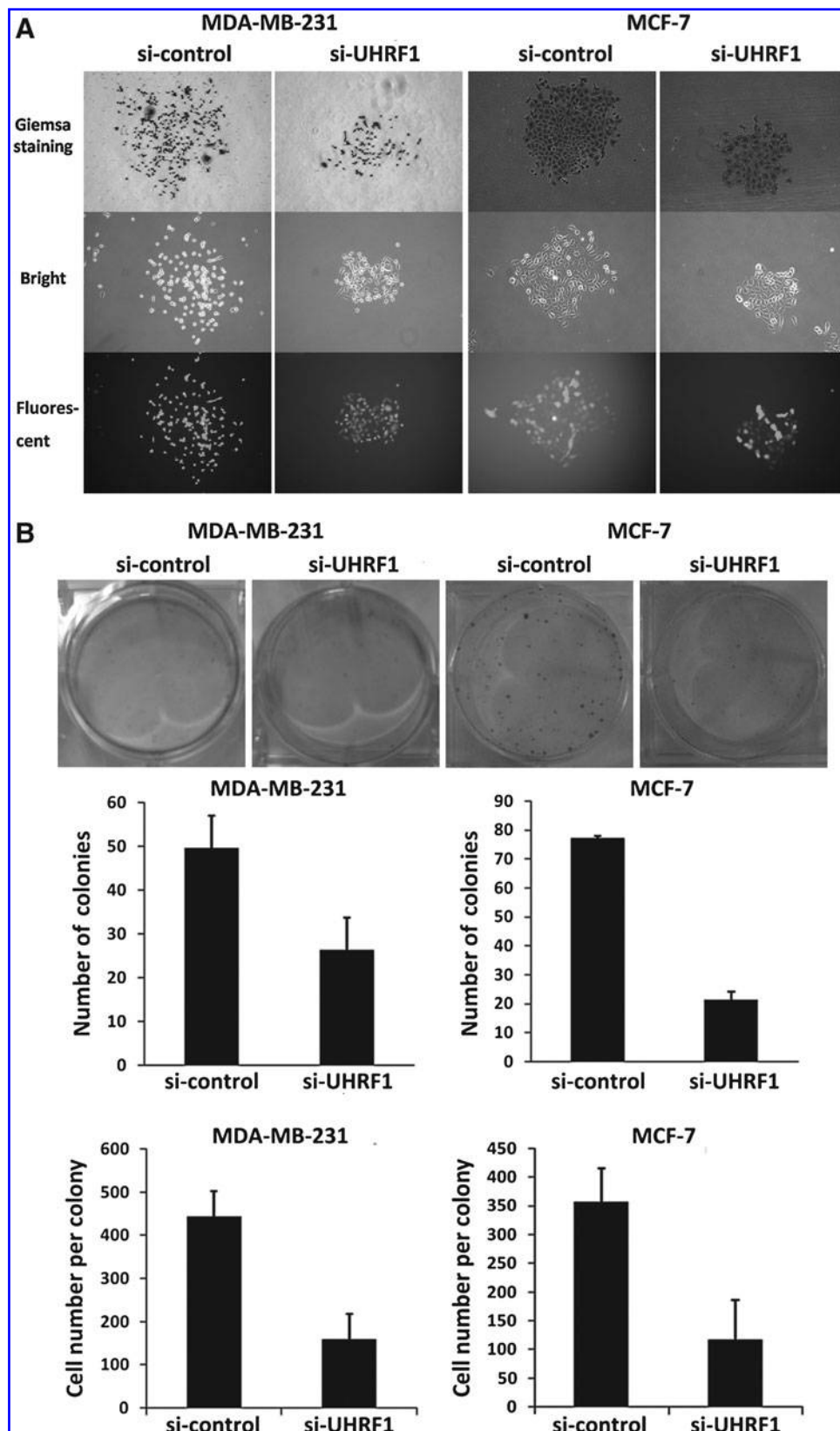


FIG. 4. Effect of si-UHRF1 on sensitivity of MDA-MB-231 cells to ADM. (A) Two-color flow cytometry analysis of untreated (ADM -, upper panel) and ADM-treated (ADM +, lower panel) MDA-MB-231 cells stained with 7AAD and Annexin V-RPE. Early apoptotic cells were located in the upper right quadrant. (B) Percentage of apoptotic and dead cells (black column) in lentivirus-infected MDA-MB-231 cells in response to ADM. ADM, adriamycin.



**FIG. 5.** Lower colony-forming efficiency of si-UHRF1-treated MDA-MB-231 and MCF-7 cells. **(A)** Colony of MDA-MB-231 and MCF-7 cells. si-UHRF1 or si-control lentivirus-treated cells were trypsinized and replated in six-well plates to form natural colonies. Fourteen days later, colonies (diameter: > 1 mm) were observed with bright light (middle lane) and fluorescent light (lower lane), and then all cells were stained with Giemsa and microphotographed (upper lane). **(B)** Naturally formed colonies of si-UHRF1- and si-control-treated cells in six-well plates were examined. The number of colonies and cell number per colony were counted and analyzed. The number of colonies in si-UHRF1-treated cells was decreased by 40% ( $p < 0.05$ ), and the cell number per colony was reduced by 50% ( $p < 0.05$ ).

enzymes.<sup>20</sup> These findings indicated that overexpression of *UHRF1* could reverse the binding status of the important transcriptional factors during breast carcinogenesis.

Moreover, *UHRF1* was also implicated in breast cancer chemotherapy resistance, because it can inhibit MDR1 gene transcription and sensitize breast cancer cells to anticancer drugs. Therefore, in conclusion, the data presented here confirm that *UHRF1* plays an important role in inhibiting breast cancer cell proliferation. And, more importantly, it supports the development of therapeutic RNAi strategies targeting *UHRF1* for breast cancer treatment.

### Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (21075055) and the Outstanding Medical Talents Program (RC2007069) from the Department of Health of Jiangsu.

### Disclosure Statement

No competing financial interests exist.

### References

- Bronner C, Achour M, Arima Y, et al. The *UHRF* family: Oncogenes that are drugable targets for cancer therapy in the near future? *Pharmacol Ther* 2007;115:419.
- Hopfner R, Mousli M, Garnier JM, et al. Genomic structure and chromosomal mapping of the gene coding for ICBP90, a protein involved in the regulation of the topoisomerase II $\alpha$  gene expression. *Gene* 2001;266:15.
- Muto M, Fujimori A, Neno M, et al. Isolation and characterization of a novel human radiosusceptibility gene, *NP95*. *Radiat Res* 2006;166:723.
- Hopfner R, Mousli M, Jeltsch JM, et al. ICBP90, a novel human CCAAT binding protein, involved in the regulation of topoisomerase II $\alpha$  expression. *Cancer Res* 2000;60:121.
- Mousli M, Hopfner R, Abbady AQ, et al. ICBP90 belongs to a new family of proteins with an expression that is down-regulated in cancer cells. *Br J Cancer* 2003;89:120.
- Jeanblanc M, Mousli M, Hopfner R, et al. The retinoblastoma gene and its product are targeted by ICBP90: A key mechanism in the G1/S transition during the cell cycle. *Oncogene* 2005;24:7337.
- Unoki M, Daigo Y, Koinuma J, et al. *UHRF1* is a novel diagnostic marker of lung cancer. *Br J Cancer* 2010;103:217.
- Unoki M, Kelly JD, Neal DE, et al. *UHRF1* is a novel molecular marker for diagnosis and the prognosis of bladder cancer. *Br J Cancer* 2009;101:98.
- Hopfner R, Mousli M, Oudet P, et al. Overexpression of ICBP90, a novel CCAAT-binding protein, overcomes cell contact inhibition by forcing topoisomerase II  $\alpha$  expression. *Anticancer Res* 2002;22:3165.
- Lehnert M. Chemotherapy resistance in breast cancer. *Anticancer Res* 1998;18:2225.
- Saeki T, Tsuruo T, Sato W, et al. Drug resistance in chemotherapy for breast cancer. *Cancer Chemother Pharm* 2005;56 Suppl 1:84.
- Jin W, Liu Y, Xu SG, et al. *UHRF1* inhibits MDR1 gene transcription and sensitizes breast cancer cells to anticancer drugs. *Breast Cancer Res Treat* 2010;124:39.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402.
- Jin W, Chen L, Chen Y, et al. *UHRF1* is associated with epigenetic silencing of BRCA1 in sporadic breast cancer. *Breast Cancer Res Treat* 2010;123:359.
- Taulli R, Accornero P, Follenzi A, et al. RNAi technology and lentiviral delivery as a powerful tool to suppress Tpr-Met-mediated tumorigenesis. *Cancer Gene Ther* 2005;12:456.
- Wang HJ, Zhu JS, Zhang Q, et al. RNAi-mediated silencing of ezrin gene reverses malignant behavior of human gastric cancer cell line SGC-7901. *J Dig Dis* 2009;10:258.
- Bronner C, Trotzier MA, Filhol O, et al. The antiapoptotic protein ICBP90 is a target for protein kinase 2. *Ann NY Acad Sci* 2004;1030:355.
- Arima Y, Hirota T, Bronner C, et al. Down-regulation of nuclear protein ICBP90 by p53/p21Cip1/WAF1-dependent DNA-damage checkpoint signals contributes to cell cycle arrest at G1/S transition. *Genes Cells* 2004;9:131.
- Fang Z, Xing F, Bronner C, et al. ICBP90 mediates the ERK1/2 signaling to regulate the proliferation of Jurkat T cells. *Cell Immunol* 2009;257:80.
- Kim JK, Esteve PO, Jacobsen SE, et al. *UHRF1* binds G9a and participates in p21 transcriptional regulation in mammalian cells. *Nucleic Acids Res* 2009;37:493.

