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*Molecular and Cellular Pathobiology*

# Oncogenic Wip1 Phosphatase Is Inhibited by miR-16 in the DNA Damage Signaling Pathway

Xinna Zhang[1,2], Guohui Wan[1], Sizolwenkosi Mlotshwa[1], Vicki Vance[1], Franklin G. Berger[1,2], Hexin Chen[1,2], and Xiongbin Lu[1,2]

## Abstract

Wild-type p53-induced phosphatase 1 (Wip1) was identified as an oncogene amplified and overexpressed in several human cancers. Recent evidence suggested that Wip1 is a critical inhibitor in the ATM/ATR-p53 DNA damage signaling pathway. Wip1 dephosphorylates several key DNA damage–responsive proteins and reverses DNA damage–induced cell cycle checkpoints. Previous reports showed that Wip1 was transcriptionally induced by p53 at the early stage of the DNA damage response. To investigate the temporal and functional regulation of Wip1, we identified a microRNA, miR-16, that specifically targets the mRNA of Wip1 and thus negatively regulates the expression level of Wip1. miR-16 itself is induced immediately after DNA damage. Therefore, the increase in Wip1 protein level is significantly postponed compared with that of its mRNA level, preventing a premature inactivation of ATM/ATR signaling and allowing a functional completion of the early DNA damage response. To better understand miR-16 biological functions in the context of cancer cells, we examined its expression in mammary tumor stem cells and found it to be markedly downregulated in mammary tumor stem cells. Overexpression of miR-16 or inhibition of Wip1 suppresses the self-renewal and growth of mouse mammary tumor stem cells and sensitizes MCF-7 human breast cancer cells to the chemotherapeutic drug doxorubicin. Together, our results suggest an important role of miR-16 in the regulation of Wip1 phosphatase in the DNA damage response and mammary tumorigenesis. *Cancer Res; 70(18); 7176–86. ©2010 AACR.*

## Introduction

Eukaryotic cells have evolved a multifaceted response to counteract the potentially deleterious effects of DNA damage. On sensing DNA damage or stalled replication forks, DNA damage checkpoints are activated to arrest cell cycle progression and repair damaged DNA. Once DNA repair is completed, DNA damage signaling pathways need to be inactivated when cells return to normal. The wild-type p53-induced phosphatase 1 (Wip1 or PPM1D) is a member of the type 2C serine/threonine phosphatases. Recent evidence indicates that Wip1 is a critical regulator of DNA damage signaling pathways (1, 2).

Several Wip1 dephosphorylation targets have been identified in the ATM/ATR-p53 DNA damage signaling pathway, including ATM, Chk1, Chk2, p38 mitogen-activated protein kinase, p53, Mdm2, and MdmX (3–8). Through de-

phosphorylation of those key kinases that initiate cell cycle checkpoints, Wip1 releases cells from cell cycle arrest. Furthermore, Wip1 negatively regulates p53 levels and activities by stabilizing Mdm2 and MdmX, and thus shuts down p53-mediated apoptosis and cell cycle checkpoints. *Wip1*-null mouse embryonic fibroblasts (MEF) displayed reduced proliferation, enhanced p53 transcriptional activity, and enhanced DNA damage–induced checkpoints (9). The inhibitory roles of Wip1 on the DNA damage signaling and the tumor suppressor p53 suggested that the *WIP1* gene is an oncogene. Consistent with an oncogenic function, the *WIP1* gene is present in amplified copy numbers and is overexpressed in many human cancer types, including breast carcinomas, ovarian clear cell adenocarcinomas, neuroblastomas, pancreatic adenocarcinomas, gastric carcinomas, and medulloblastomas (10–16).

MicroRNAs (miRNA) are small (~22 nucleotides) noncoding regulatory RNA molecules that are involved in diverse biological processes and various diseases. By virtue of sequence complementarity, miRNAs bind to the mRNAs of their target genes and then block translation or accelerate their degradation (17). Emerging evidence has shown that miRNA biogenesis is regulated on DNA damage stresses. Pothof and colleagues (18) reported that UV damage triggered a cell cycle–dependent relocalization of Ago2 into stress granules and a change of miRNA expression profiling. Recent work from the Miyazono group showed that the tumor suppressor p53 promoted the posttranscriptional processing of a subgroup

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*American Association for Cancer Research*

of miRNAs. The interaction between p53 and the Drosha complex facilitates the processing of primary miRNAs to precursor miRNAs (19). miRNAs also influence DNA damage response by regulating the expression levels of their target genes. Many genes involved in the DNA damage response can be targeted by their specific miRNAs. For instance, human miR-421 was shown to target ATM (ataxia-telangiectasia mutated) transcripts and downregulate their protein expression. Consequently, overexpression of miR-421 sensitized cells to ionizing radiation (20). Human miR-15a and miR-16 cluster targets cyclin D1 (CCND1), BCL2, and WNT3A, which enhances $G_1$-S cell cycle checkpoint and inhibits tumorigenic features such as survival, proliferation, and invasion (21).

In the present study, we show that the transcripts of the *WIP1* gene are specifically targeted by miR-16. Overexpression of miR-16 abolishes the DNA damage–responsive Wip1 induction, whereas inhibition of miR-16 markedly accelerates and enhances the Wip1 induction. Deletion of the miR-16–targeted sequence in the 3′-untranslated region (UTR) of *WIP1* depleted miR-16 effects on Wip1. Previous studies reported that the 5′-UTR of the *WIP1* gene includes a conserved p53 response element, facilitating a p53-dependent induction of the *WIP1* transcripts. However, the induction of Wip1 proteins seems to have a delayed onset in contrast to an immediate induction of the *WIP1* transcripts in response to DNA damage. We presented that the level of miR-16 is rapidly induced on DNA damage stress, which postpones the accumulation of the Wip1 protein and thus allows cells to initiate functional cell cycle checkpoints in the early stage of DNA damage response. Interestingly, miR-16 is downregulated in mammospheres originated from mammary tumor stem cells. Overexpression of miR-16 in mammary tumor cells sensitizes them to doxorubicin treatment and significantly reduces the proliferation of mammary tumor stem/progenitor cells, implicating miR-16 in the regulation of the self-renewal of mammary tumor stem cells.

## Materials and Methods

### Cell lines and cell culture

U2OS (human osteosarcoma line) and MCF-7 (human breast cancer line) cell lines were obtained from the American Type Culture Collection (ATCC) in 2007 and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). Cells were cultured and stored according to the supplier's instructions and used at passages 5 to 20. Once resuscitated, cell lines obtained from ATCC are routinely authenticated (once every 6 months, cells were last tested in October 2009) through cell morphology monitoring, growth curve analysis, species verification by isoenzymology and karyotyping, identity verification using short tandem repeat profiling analysis, and contamination checks. Expression of p53 and Wip1 in these two cell lines was confirmed by immunoblotting before they were used in the experiments. Primary $Wip1^{+/+}$ and $Wip1^{-/-}$ MEFs were harvested and cultured as previously described (9).

### Plasmid constructs, cell transfection, and infection

Chemically synthesized pre-miRNAs, control miRNAs, and antagomirs were purchased from Ambion. *WIP1* short hairpin RNA (shRNA) expression vector was purchased from Open Biosystems. Using PCR primers 5′-ACTCTAGAAATG-CATCTGGGAAATGAGG-3′ and 5′-TGTCTAGAGCAGGCAT-GATGCTCAAAG-3′, the wild-type 3′-UTR of *WIP1* (1.1 kbp) was amplified from human cDNA library and cloned into the *Xba*I site of pRL vector (Promega). Mutant *WIP1* 3′-UTR was generated based on the pRL-WIP1-3′-UTR by deleting 6 nucleotides that are recognized by miR-16. Lentiviral miR-16 and miR-21 expression constructs were obtained from Genecopoeia. Lentiviral packaging vectors (Genecopoeia) and miRNA expression vector were transfected into 293T cells by Fugene HD transfection reagent (Roche) following the manufacturer's manual. Two days after transfection, cell culture supernatants were collected, filtered, and titrated using target cells. Control or pseudovirus particles expressing miRNAs were used to infect mouse or human cells at a multiplicity of infection of 3 to ensure the complete infection of target cells.

### Isolation of mouse mammary tumor cells and mammosphere culture

MMTV-ErbB2 transgenic mice [FVB/N-Tg(MMTVneu)202 Mul/J] were purchased from The Jackson Laboratory. Normal mammary tissues or mammary tumors from ErbB2 transgenic mice were mechanically dissociated and placed in a digestion medium (DMEM with 1 mmol/L glutamine, 5% FBS) supplemented with 200 units/mL collagenase (Sigma) and 100 units/mL hyaluronidase (Sigma) for 5 hours at 37°C. Cell suspensions were centrifuged at $80 \times g$, resuspended in 0.2% NaCl to lyse RBCs, and filtered through 20-μm mesh. Mammary tumor cells were plated onto ultralow attachment plates (Corning) at a density of 20,000 viable cells/mL (to obtain primary mammospheres) in a serum-free DMEM-F12 (Invitrogen) supplemented with 5 μg/mL insulin, 20 ng/mL epidermal growth factor and 20 ng/mL basic fibroblast growth factor (Sigma), and 0.4% bovine serum albumin (Sigma; ref. 22). Mammospheres were harvested after 10 days for the analysis of gene and miRNA expression. For the differentiation culture of mammospheres, collected mammospheres were treated with trypsin-EDTA (Invitrogen) and mechanically dissociated by pippetting, and then cultured in DMEM-F12 supplemented with 5% FBS (Atlanta Biologicals) without growth factors. Differentiated cells were harvested for miRNA analysis after 3 days.

### Western blot analysis, antibodies, and purified proteins

Immunoprecipitations, Western blot analysis, and immunoprecipitation–Western blot analyses were performed by standard methods described previously (4). Anti-actin, anti-p53, horseradish peroxidase (HRP) anti-goat IgG, HRP anti-rabbit IgG, and HRP anti-mouse IgG were purchased from Santa Cruz Biotechnology; anti-Wip1 was purchased from Abgent; and anti–Bcl-2 and anti–caspase-3 were purchased from Cell Signaling Technology.

## Treatments with DNA-damaging agents or Wip1 inhibitor

Cells were treated with 500 ng/mL neocarzinostatin (NCS; Sigma-Aldrich) and harvested at the indicated time points after treatment for the analyses of mRNA and proteins. In cell viability assays, MCF-7 cells were seeded at a concentration of 1,000 per well in flat-bottom 96-well microplates. After 24 hours, the cells were cultivated with doxorubicin at the indicated concentration for 72 hours. At the end of incubation, the viability of cells was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. In mammosphere culture, mouse mammary tumor cells were treated with the specific Wip1 inhibitor CCT007093 (5 μmol/L; Sigma-Aldrich) or DMSO (mock treatment) as indicated.

## Analysis of miRNAs by Northern blotting

Total RNA was extracted by Trizol reagents (Invitrogen) following the manufacturer's instructions. An equal volume of 4 mol/L LiCl and 10 mmol/L EDTA (pH 7.0) was added to the RNA solution and incubated overnight at 4°C. High–molecular weight RNA was recovered by centrifugation at 12,000 × $g$ for 15 minutes. The supernatant, containing DNA and low–molecular weight RNAs, was made 10% in polyethylene glycol 8000 and 0.5 mol/L NaCl and incubated for 30 minutes on ice to precipitate the DNA. After centrifugation to remove DNA, low–molecular weight RNA was concentrated by ethanol precipitation. RNA gel blot analysis of low–molecular weight RNA was performed exactly as described using Ambion ULTRAhyb hybridization solution at 45°C. DNA probes used were as follows: U6 RNA probe, 5′-ATATGGAACGCTTCACGAATT-3′; miR-16 probe, 5′-CGCCAATATTTACGTGCTGCTA-3′.

## Primers for quantitative reverse transcription-PCR

Primers for genes examined in the studies were as follows: human *WIP1*, 5′-TTATACCTGAACCTGACTGAC-3′ and 5′-GATCTTTTGAGGGTATGACTA-3′; mouse *Wip1*, 5′-TTGCCTCACACACCATTTGT-3′ and 5′-GGGTGTTAGCAGCACCATTT-3′; *Oct-4*, 5′-GTGGAGGAAGCCGACAACAATG-3′ and 5′-GCCTCATACTCTTCTCG-3′; *KLF4*, 5′-AGAGGAGCCCAAGCCAAAGAGG-3′ and 5′-CCACAGCCGTCCCAGTCACAGT-3′; *Keratin-14*, 5′-GCTGAGGAATGGTTCTTCAG-3′ and 5′-CAGGTTATTCTCCAGGGATG-3′; *Keratin-18*, 5′-AGATCGACAATGCCCGCCTT-3′ and 5′-CTTGCTGAGGTCCTGAGATT-3′. Primers for miRNA quantitative reverse transcription-PCR (RT-PCR) were obtained from Exiqon: U6 RNA control, miR-16, and miR-21.

## Results

### miR-16 inhibits Wip1 expression by targeting 3′-UTR of Wip1

Previous studies showed that the *WIP1* gene is transcriptionally induced by the tumor suppressor p53, but it remains unclear if posttranscriptional regulation affects the expression of Wip1 protein. To explore the possibility that miRNAs might regulate *WIP1* expression, we searched the 3′-UTR of the human WIP1 gene for miRNA-targeting motifs using the TargetScan 5.1, from which three miRNAs were predicted to bind to the 3′-UTR of *WIP1*, including miR-16, miR-217, and miR-153. Twelve nucleotides from miR-16 are complementary to the target sequence in the 3′-UTR of WIP1 (Fig. 1A). To test the *in silico* prediction, we first examined the endogenous Wip1 protein level by immunoblotting after transient transfection of a control and the precursors of the three predicted miRNAs (pre-miRNAs) into human U2OS cells. As shown in Fig. 1B, the *WIP1* protein level was significantly decreased by ectopic miR-16 expression, whereas the other two predicted miRNAs, miR-217 and miR-153, had only minor effects. miRNAs that were randomly picked as negative controls, miR-145 and miR-203, had minimal or no effect on the Wip1 level. We next examined the levels of Wip1 in cells transfected with pre-miR-16 or specific inhibitory oligonucleotide against miR-16, antigomir-16, during 0 to 4 hours after the treatment of a radiomimetic drug, NCS. Ectopic miR-16 had a pronounced suppression against the induction of Wip1 proteins, whereas antagomir-16 promoted the expression of *WIP1* (Fig. 1C).

To further validate Wip1 as a bona fide target of miR-16, we cloned the WIP1 3′-UTR portion containing the miR-16 target site into a *Renilla* luciferase reporter construct and performed a luciferase reporter assay following cotransfection of reporter constructs with precursor miR-16 into U2OS cells. A significant reduction (~75%) in the luciferase activity was observed for the reporter construct containing the WIP1 3′-UTR in the presence of pre-miR-16, but not in the presence of control or the other four pre-miRNAs (Fig. 1D, left). Transfection of antagomir-16 increased the luciferase activity by ~50% for the *WIP1* 3′-UTR reporter construct. Deletion of six nucleotides of seed sequence almost completely abolished the effects of pre-miR-16 and antagomir-16 on the luciferase transcripts with the *WIP1* 3′-UTR, suggesting that the *WIP1* gene is an authentic target of miR-16 *in vivo* (Fig. 1D, right).

### miR-16 regulates Wip1 induction in the DNA damage response

Accumulating evidence supports a role of Wip1 as a critical negative regulator of DNA damage signaling pathways. Wip1 itself is also responsive to DNA damage. The Appella group reported that Wip1 levels were transcriptionally induced in a p53-dependent manner on DNA damage (23, 24). To explore the temporal regulation of Wip1 during DNA damage response, we measured the Wip1 mRNA and protein levels in U2OS cells at 0 to 8 hours after NCS treatment. Consistent with the results from the Appella group, we observed that the Wip1 mRNA level was rapidly increased in response to NCS, reaching the peak level within 2 hours. However, the Wip1 protein level was induced at a slower pace, which reached a peak at 6 hours after treatment (Fig. 2A and B). We further explored whether there was posttranscriptional inhibition of *WIP1* expression. By Northern blotting analysis, the level of mature miR-16 increased shortly after NCS treatment and hit its peak around 2 to 4 hours after treatment when the Wip1 protein levels still remained at a relatively
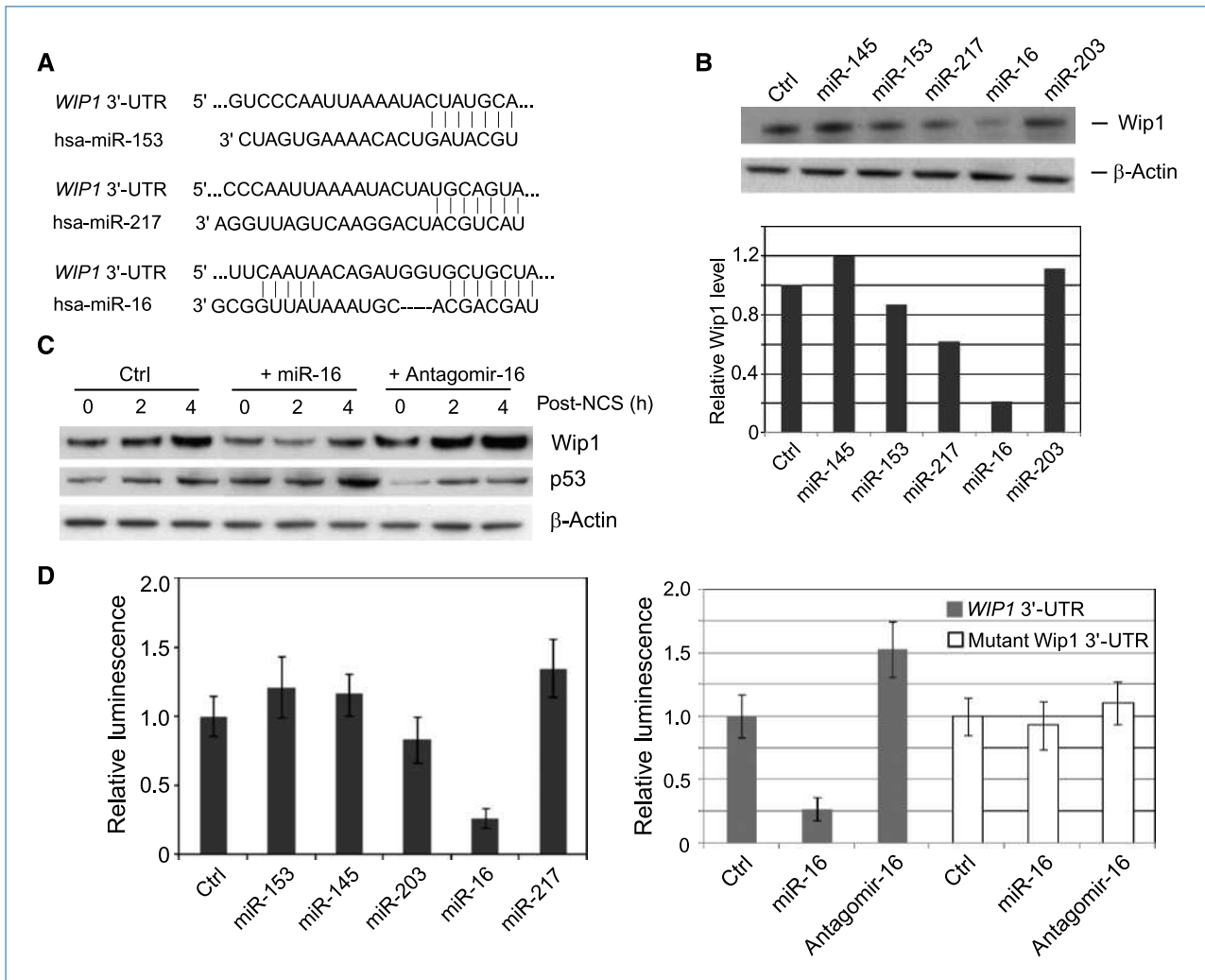
**Figure 1.** miR-16 suppresses Wip1 expression by targeting *WIP1* 3′-UTR. A, predicted miRNA sequences and their putative recognition sites within 3′-UTR of *WIP1*. B, Wip1 expression is suppressed by ectopic miR-16 transfection. U2OS cells were transfected with the indicated miRNAs. Seventy-two hours after transfection, Wip1 protein levels in each sample were detected by immunoblotting (top), quantitated according to the intensity of Wip1 bands, and normalized with the control (Ctrl) sample (bottom). C, immunoblots of endogenous Wip1 in U2OS cells transfected with control miRNA, miR-16, or antagomir-16 and treated with 500 ng/mL NCS at 72 h after transfection. D, miR-16 specifically targets the 3′-UTR of *WIP1*. A luciferase construct containing wild-type *WIP1* 3′-UTR (left) or mutant *WIP1* 3′-UTR (right) was transfected into U2OS cells with the indicated miRNAs or antagomirs. *Renilla* luciferase activities were measured 48 h after transfection and normalized to firefly luciferase. Relative luciferase activity (luminescence) was obtained after normalizing with the control number.

low level (Fig. 2C). To exclude the possibility that the effects of miR-16 are limited to cellular response to NCS, we performed the same experiments in U2OS cells treated with 10 Gy of ionizing radiation. The similar phenomena were observed on the induction of miR-16, Wip1 mRNA, and protein at the early stage of DNA damage response (Supplementary Fig. S1A and B). To further determine if altered levels of miR-16 change the temporal pattern of Wip1 induction, we examined Wip1 protein levels in the presence of ectopic pre-miR-16 or antagomir-16 over a 12-hour time course. Transfection of pre-miR-16 significantly suppressed the induction of Wip1 throughout the whole time course. Inhibiting miR-16 by antagomir boosted Wip1 expression as early as 2 hours after

NCS treatment and maintained a stable high level of Wip1 up to 6 hours (Fig. 2D). These results suggest that miR-16 is an important regulator for Wip1 induction in the early stage of DNA damage response.

## miR-16 regulates cell proliferation and sensitizes MCF-7 cells to doxorubicin

To assess whether miR-16 modulates cell proliferation, we examined the cell growth of the littermate $Wip1^{+/+}$ and $Wip1^{-/-}$ MEFs isolated from midgestation. Consistent with a previous report (9), $Wip1^{-/-}$ MEFs exhibited a much slower growth in contrast to their $Wip1^{+/+}$ counterparts. Growth curve analyses of $Wip1^{+/+}$ and $Wip1^{-/-}$ MEFs showed that

the proliferation rates of early-passage $Wip1^{-/-}$ MEFs were substantially retarded compared with $Wip1^{+/+}$ MEFs (Fig. 3A). Four days after plating, $Wip1^{-/-}$ MEFs almost stopped proliferation, whereas $Wip1^{+/+}$ MEFs were still in the doubling stage. Lentivirus particles expressing the precursor of mmu-miR-16 (mouse miR-16) were transduced into both $Wip1^{+/+}$ and $Wip1^{-/-}$ MEFs, and the growth curves of these fibroblasts were analyzed. Ectopic overexpression of

miR-16 dramatically inhibited the cell proliferation of $Wip1^{+/+}$ MEFs to a level similar to that of $Wip1^{-/-}$ MEFs. Moreover, the absence of Wip1 largely diminished the effects of miR-16 on cell proliferation. No significant effects were observed on the growth of $Wip1^{-/-}$ MEFs in the presence or absence of the lentiviral miR-16, suggesting that Wip1 may be one of the major miR-16 targets in the regulation of cell cycle checkpoints and cell proliferation.
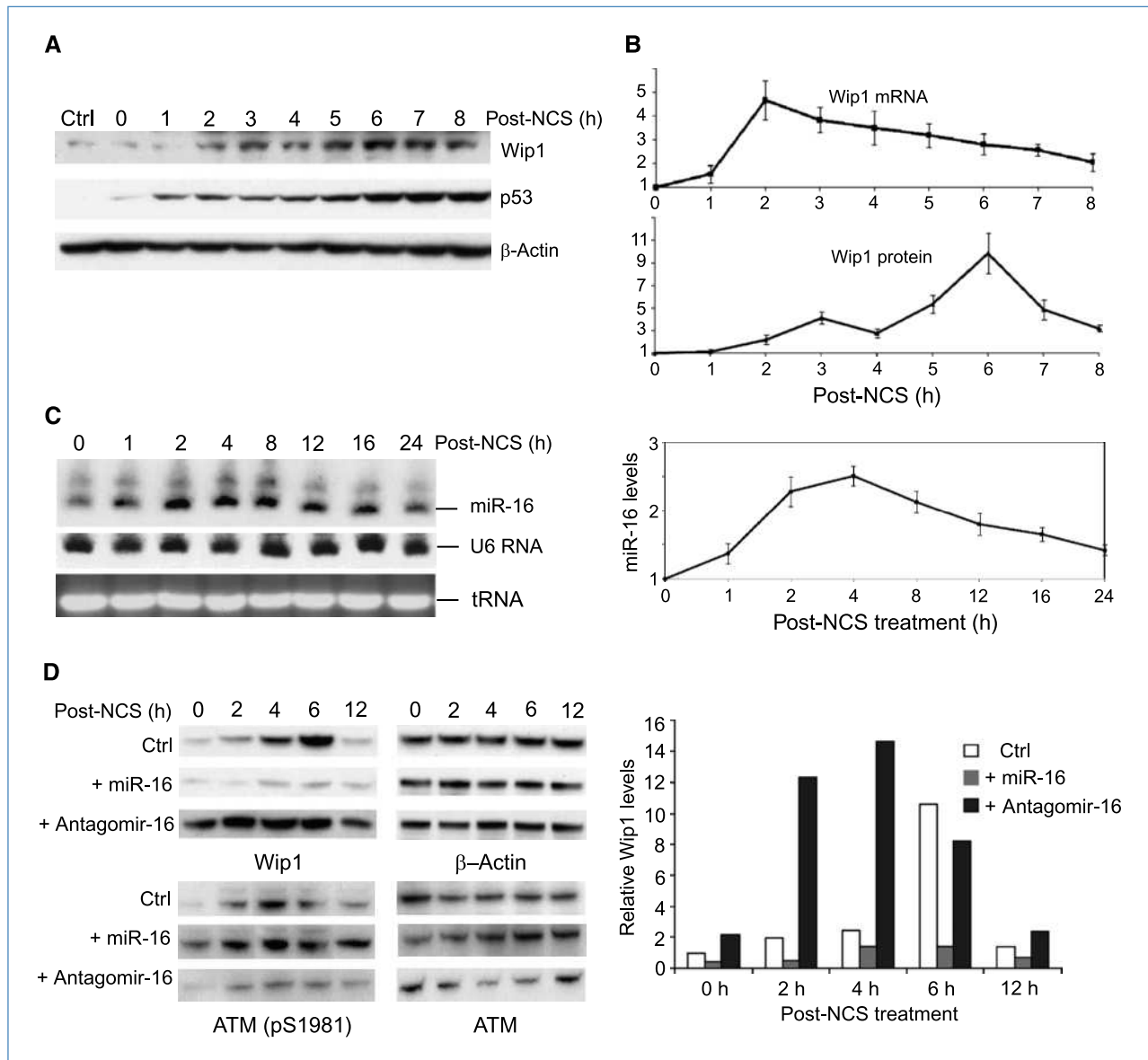


Figure 2. miR-16 inhibits the DNA damage–mediated induction of Wip1. A, Wip1 protein is induced in response to DNA damage. U2OS cells were treated with 500 ng/mL NCS, and cell lysates were harvested at indicated time points after NCS treatment. Protein levels were determined by immunoblotting. B, induction of Wip1 protein has a delayed onset compared with the induction of Wip1 mRNA. Levels of Wip1 mRNA and protein in the above NCS-treated cells were determined by quantitative RT-PCR and the intensity of Wip1 immunoblots. C, miR-16 has a rapid induction in response to DNA damage. U2OS cells were treated with 500 ng/mL NCS, and total RNAs (tRNA) were harvested at indicated time points. miR-16 levels were determined by Northern blots (left) and quantitative RT-PCR (from three sets of samples; right). D, miR-16 counteracts the induction of Wip1 in the DNA damage response. U2OS cells were transfected with control miRNA, miR-16, or antagomir-16. Left, 48 h after transfection, cells were treated with 500 ng/mL NCS and cell lysates were harvested for immunoblotting analysis. The intensity of Wip1 blots in each sample was quantified by phosphorimager and normalized with the control sample.
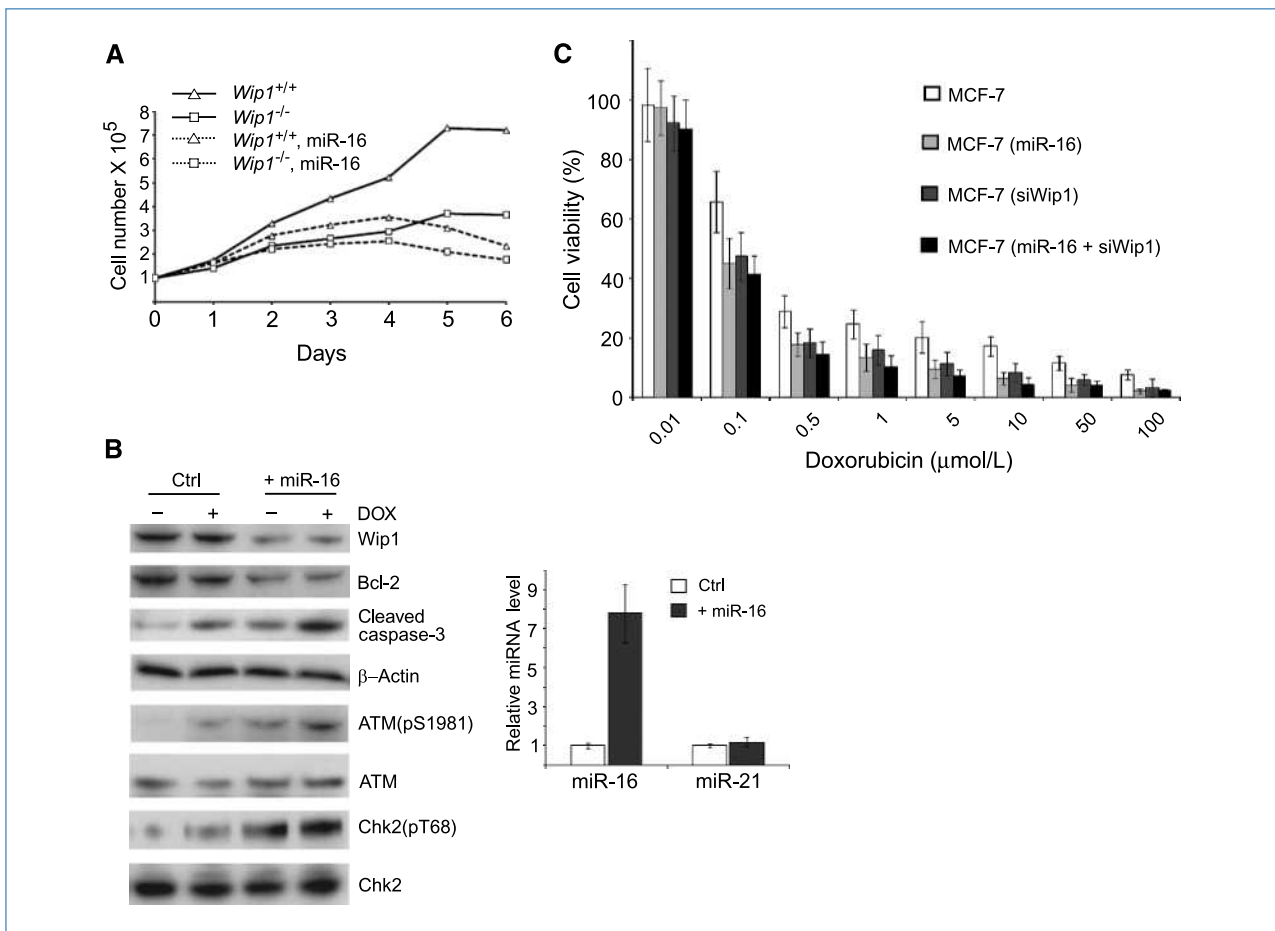
**Figure 3.** miR-16 suppresses cell proliferation and sensitizes MCF-7 cells to doxorubicin. A, growth curves of Wip1^{+/+} and Wip1^{−/−} MEFs infected with control or miR-16–expressing pseudoviruses. B, miR-16 inhibits the expression of Wip1 in MCF-7 cells. MCF-7 cells were transfected with control or miR-16 expression vector DNA. Thirty-six hours after transfection, cell lysates were harvested or total RNA was extracted for the analyses of miRNAs (by quantitative PCR; left) and proteins (by Western blotting; right). DOX, doxorubicin. C, miR-16 sensitizes MCF-7 cells to doxorubicin. MCF-7 cells were transfected with control, Wip1 shRNA, miR-16 expression vector DNA, or miR-16 and Wip1 shRNA expression vector DNA. Forty-eight hours after transfection, cells were incubated with increasing doses of doxorubicin as indicated. Cell viability was determined 3 d after incubation.

*WIP1* gene amplification occurs in many cancer types, including 11% to 18% of primary human breast cancer samples, nearly all of which express wild-type p53 (10, 25–29). This observation has led to the hypotheses that aberrantly high levels of Wip1 are a causative factor in tumorigenesis. Inhibiting Wip1 by small interfering RNA or the specific inhibitor CCT007093 reduced the cell viability selectively on cells with *WIP1* amplification. Furthermore, loss of Wip1 sensitized cells to stress- or DNA damage–induced apoptosis. To determine if miR-16 increases sensitivity of cells to chemotherapeutic drugs that cause DNA damage, we examined the viability of human breast cancer MCF-7 cells in which the *WIP1* gene is substantially amplified and overexpressed. We delivered miR-16 to MCF-7 cells by lentiviral infection. Lentiviral expression of miR-16 increased the level of total miR-16 by 7-fold in MCF-7 cells, whereas the level of the control miRNA, miR-21, was unchanged (Fig. 3B). miR-16 overexpression dramatically promoted apoptosis, indicated by a

higher level of cleaved caspase-3 in cells expressing lentiviral miR-16, compared with the control cells. Reduced Wip1 levels led to higher levels of DNA damage–induced phosphorylation of ATM (Ser[1981]) and Chk2 (Thr[68]) that are two of the identified Wip1 dephosphorylation substrates (3, 5). As expected, ectopic miR-16 knocked down the expression of Bcl-2, another known target of miR-16 (Fig. 3B; ref. 30). To examine whether miR-16 modulates the chemosensitivity of MCF-7 cells in a Wip1-dependent manner, we treated MCF-7 cells with increasing doses of doxorubicin. Cell survival was assessed by measuring the proliferation of viable cells. As shown in Fig. 3C, MCF-7 cells overexpressing miR-16 displayed a significantly higher sensitivity to doxorubicin compared with the control MCF-7 cells (IC$_{50}$: 0.037 versus 0.13 μmol/L). Knockdown of Wip1 by chemically synthesized small interfering RNA had no additional effect on the sensitivity of MCF-7 cells overexpressing miR-16 (IC$_{50}$: 0.037 versus 0.032 μmol/L; Fig. 3C). Our results indicated that miR-16

sensitizes MCF-7 cells to the treatment of doxorubicin by inhibiting Wip1.

## miR-16 is downregulated in mammary tumor stem/progenitor cells

The *WIP1* gene is frequently amplified in human breast cancers that contain wild-type p53. Deletion of *Wip1* in mice bearing mouse mammary tumor virus (MMTV) promoter-driven oncogenes Erbb2 or Hras1 impaired mammary carcinogenesis (26). However, no spontaneous mammary tumor formation was observed in *MMTV-Wip1* transgenic mice (31). This suggested that overexpression of Wip1 phosphatase alone is not sufficient to cause breast cancer in mice. Nevertheless, Wip1 can complement *in vitro* other oncogenes, such as Ras and *ErbB2*, in their ability to transform mouse embryo fibroblasts (26). We hypothesized that upregulation of Wip1 probably provides a selective advantage for the clonal expansion of cancer cells. It is now becoming clear that a subgroup of cells in mammary tumors possesses tumor-initiating cell function and contributes to tumor expansion and growth (32–35). The tumor-initiating cells, or cancer stem cells, can be isolated using cell surface markers or can be enriched in nonadherent mammospheres.

Dontu and colleagues developed a culture system in which cells derived from reduction mammoplasties were seeded in nonadherent nondifferentiating culture conditions. Cells capable of surviving and proliferating in such conditions formed discrete clusters of cells termed mammospheres (34). Such spheroids were enriched in progenitor cells capable of differentiating along multiple lineages. To determine if Wip1 plays a role in the proliferation of mammary tumor stem cells, we isolated primary tumor cells from the mammary tumors in *MMTV-Erbb2* transgenic mice. Amplification of the *Wip1* gene was not observed in any of the three studied *MMTV-Erbb2* mammary tumors. Levels of Wip1 protein and mRNA in two tumor samples were slightly increased over those in the corresponding normal tissues, whereas the level of miR-16 was 10% to 30% lower in tumor samples than in normal tissues (Fig. 4A). The whole population of primary tumor cells was cultured in regular cell culture medium or in mammosphere culture medium. As shown in Fig. 4B, ~2.5% of total cells formed mammospheres containing ~300 cells after 12 days of cultivation in nonadherent dishes. In contrast to the whole-cell population, stem cell markers (*Oct-4* and *KLF4* genes) were highly expressed exclusively in mammosphere cells, whereas differentiation markers (*Keratin-14* and *Keratin-18* expressions) were suppressed, which are typical characteristics of tumor stem cells. Quantitated by real-time PCR, the levels of miR-16 were observed to have a marked reduction of 70% to 80% in all mammospheres tested in our experiments compared with that of the whole population of tumor cells. Accordingly, the Wip1 protein levels increased by 3- to 5-fold in mammospheres (Fig. 4C). We isolated three mammospheres under the microscope and then cultured them in the regular culture medium. The progenitor cells in the mammospheres redifferentiated and proliferated. Levels of miR-16 in these recovered cancer cells from each mammospheres were similar to the original cancer cells

(Fig. 4D). The results suggested that Wip1 may be differentially expressed in normal cancer cells and cancer stem cells, and implicated that Wip1 is likely involved in the maintenance and proliferation of mammary tumor stem/progenitor cells.

To further determine the roles of Wip1 in mammary tumor stem cells, we inhibited the activity of Wip1 by its specific inhibitor CCT007093 (36), lentiviral shRNA, or ectopically overexpressed lentiviral miR-16 in mouse mammary tumor cells. In each sample, we counted the number of mammospheres that were larger than 50 µm in size. Mammosphere-forming ability of tumor cells was sharply reduced when Wip1 was inhibited or silenced (Fig. 5A). As much as 2.54% of tumor cells in the control sample formed mammospheres with an average size of 215 µm. Inhibiting Wip1 by CCT007093, shRNA, or miR-16 decreased the numbers of mammospheres to 0.17%, 0.58%, and 0.26% of total cells, respectively, and the mammospheres were significantly smaller with the size of 80 to 105 µm. As a negative control, overexpression of miR-21 had no noticeable effects on the size and number of mammospheres. Restoration of Wip1 in the miR-16 overexpressed cells remarkably reversed the inhibitory effect of miR-16 on blocking mammosphere formation in culture, suggesting that Wip1 is a primary target of miR-16 in the regulation of mammary tumor stem cells (Fig. 5B and C).

## Discussion

The *WIP1* gene was recently identified as an oncogene that is aberrantly regulated in several human cancers (10–16). In particular, the *WIP1* gene at 17q23 is amplified and overexpressed in 18% of human aggressive primary breast tumors that exclusively express wild-type p53 (29). *Wip1* removal significantly inhibited mammary tumorigenesis induced by other breast cancer oncogenes, such as *Hras1* or *Erbb2* (26). Recent studies from our laboratory and other groups have revealed the mechanisms for the oncogenicity of Wip1. Wip1 is a master inhibitor in the initiation and maintenance of DNA damage signaling and repair pathways through dephosphorylating H2AX, ATM, Chk1, Chk2, and UNG2 (4, 5, 36–39). Due to its wide-spectrum functioning in the control of DNA damage responses, Wip1 stands out from other DNA damage–responsive proteins as a very attractive target for potential breast cancer therapeutics.

On DNA damage stress, Wip1 is transcriptionally induced in p53-proficient cells but not in p53-null cells (23). Recent studies identified *WIP1* as a p53- and CREB-regulated gene. A cyclic AMP response element (CRE) and a p53 response element are located in the 5′-UTR of the *WIP1* gene. CREB binding to the CRE contributes to the regulation of basal expression of Wip1 and directs transcription initiation at upstream sites, whereas the p53 response element is required for the p53-dependent induction of transcription (24, 40). Our results showed that the level of Wip1 transcripts was immediately induced at the very early stage of the DNA damage response, but Wip1 protein levels increased at a much slower pace. The delayed onset for Wip1 protein induction allows
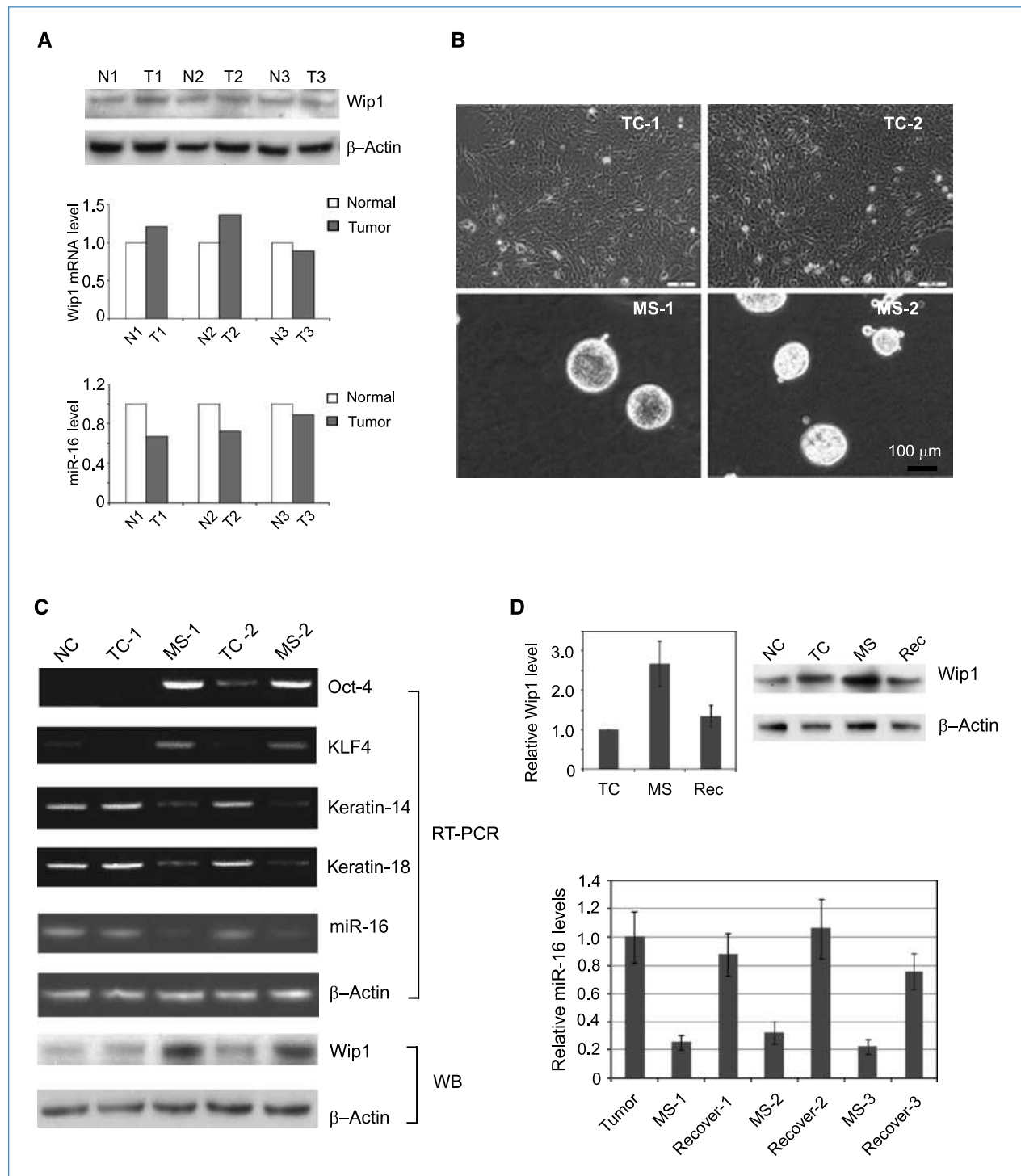
**Figure 4.** miR-16 is downregulated in mammary tumor stem cells. A, levels of Wip1 and miR-16 in mouse mammary tumors are not significantly different from those of normal mammary tissues. N1 to N3, normal mammary gland tissues; T1 to T3, mammary tumors. Levels of Wip1 mRNA and miR-16 were determined by quantitative RT-PCR. B, micrographs of mammospheres originated from mammary tumor stem cells. TC-1 and TC-2, mammary tumor cells; MS-1 and MS-2, mammospheres from the corresponding mammary tumor cells. C, expression levels of stem cell markers and differentiation markers in mammospheres and their original tumors. Stem cell markers (Oct-4 and KLF4), differentiation markers (Keratin-14 and Keratin-18), miR-16, and β-actin (as control) were analyzed by semiquantitative RT-PCR in starting tumor cells and mammospheres. NC, normal tissue cells. D, miR-16 is upregulated in cells differentiated from mammospheres. Top, Wip1 protein levels were increased in mammospheres but downregulated when mammospheres were differentiated to nonstem cells. Rec, recovered cells. In contrast, miR-16 was downregulated in each of the individual mammospheres but upregulated in redifferentiated cells. Bottom, levels of miR-16 were determined by quantitative RT-PCR.
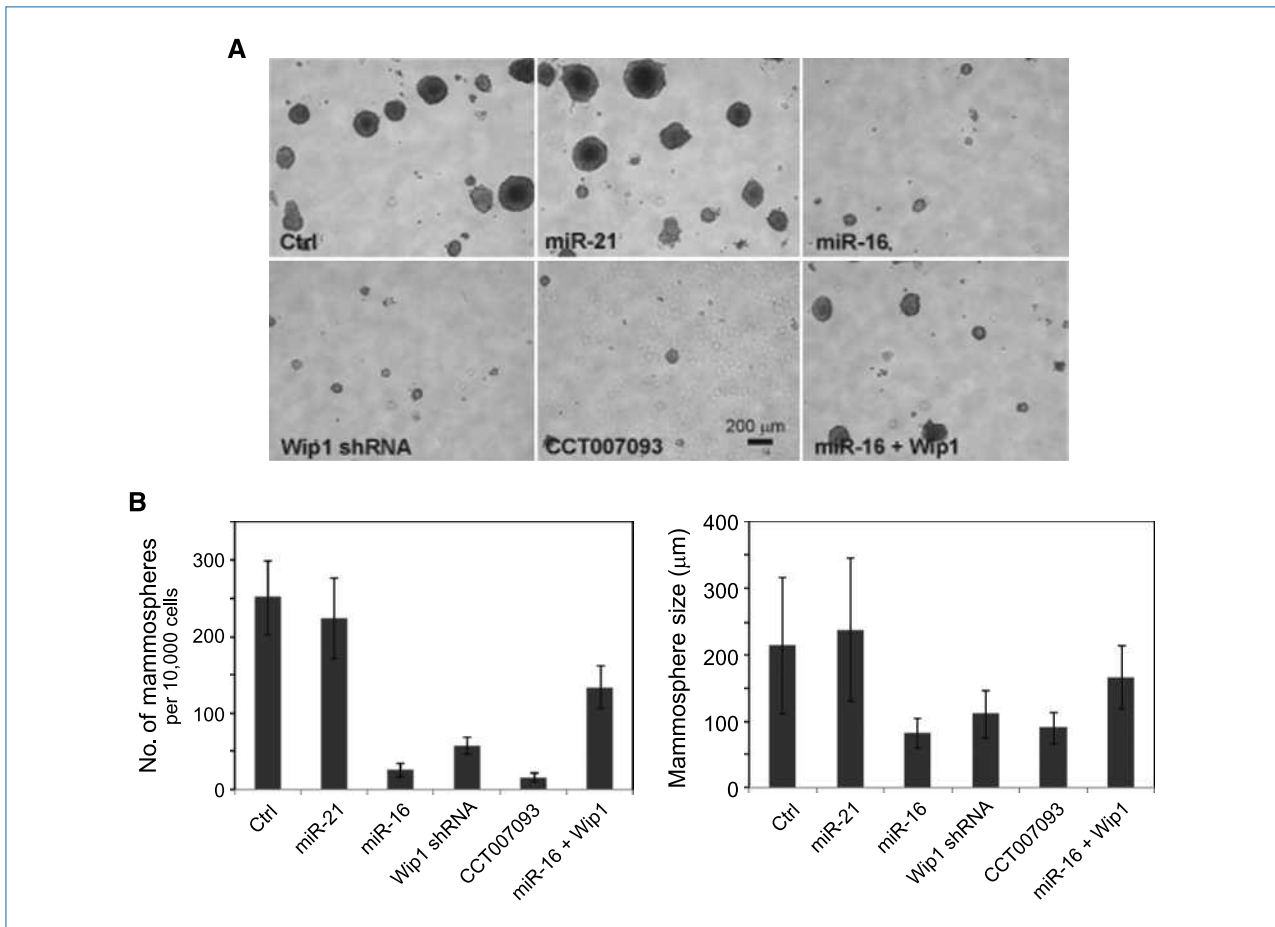
**Figure 5.** miR-16 inhibits the maintenance and proliferation of mammary tumor stem cells. A, miR-16 and the Wip1 inhibitor reduce the number of mammary tumor stem cells. Cells isolated from mammary tumors in transgenic *MMTV-Erbb2* mice were infected by control viruses or pseudoviruses expressing miR-21, miR-16, and/or mouse Wip1 and cultured in the mammosphere-forming medium with or without 5 µmol/L of the Wip1 inhibitor CCT007093. Ten days after incubation, mammospheres were observed and their sizes and numbers were determined. B, mammosphere formation is inhibited by miR-16, the Wip1 inhibitor, or Wip1 shRNA, and restoration of Wip1 reversed the effects of miR-16 on blocking mammosphere formation in culture. Mammosphere numbers (left) and sizes (right) were measured under each condition.

cells to activate a DNA damage–triggered signaling transduction cascade without early inhibition by Wip1.

miR-16 seems to be a major regulatory factor in suppressing Wip1 protein expression. The level of miR-16 rapidly increased after DNA damage and then slowly returned to normal. Altered levels of miR-16 led to dramatically different patterns of Wip1 induction. Inhibition of miR-16 resulted in greater abundance and accelerated induction of Wip1 proteins, whereas overexpression of miR-16 almost abolished the DNA damage–mediated induction of Wip1. Our studies provide strong evidence that miR-16 is a critical regulator for the induction of Wip1 phosphatase in the stress response pathway. Given the important role of Wip1 in mammary tumorigenesis, we further showed a lower abundance of miR-16 in mammospheres that initiated from mammary tumor stem/progenitor cells. Accordingly, Wip1 levels were markedly upregulated in spheroid cells. Inhibiting Wip1 or overexpressing miR-16 dramatically reduced the number and size of mammospheres, but no cumulative effects from these two treatments were achieved in our assays. This observation implicates that miR-16 may regulate the proliferation and differentiation of mammary tumor stem cells at least partly through inhibiting Wip1. Two recent publications suggest that loss of p53 permits expansion of presumptive cancer stem cells in mouse mammary tumors and in human breast cell lines. These results add restriction of cancer stem cells as a new tumor suppressor activity attributed to p53 (41, 42). Given the important roles of Wip1 in the regulation of p53 signaling, it is likely that Wip1 promotes mammary tumor stem cells through p53. The most interesting part of the miR-16–Wip1 regulatory loop is that miR-16 is responsive to DNA damage. A recent study from the Miyazono group showed that p53 enhances the posttranscriptional maturation of several miRNAs with growth-suppressive function, including miR-16. We showed here that miR-16 was induced in the DNA damage response. Putting together, miR-16 is a novel player in the ATM-p53-Wip1 autoregulatory feedback loop.

## Disclosure of Potential Conflicts of Interest

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