Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700

Human Cancer Biology

Clinical Cancer Research

# Estrogen Receptor α Regulates ATM Expression through miRNAs in Breast Cancer

Xiaojing Guo[1,2], Chunying Yang[2], Xiaolong Qian[1], Ting Lei[1], Yaqing Li[1], Haifa Shen[3], Li Fu[1], and Bo Xu[2,4]

## Abstract

**Purpose:** Estrogen receptor α (ERα) is an essential element regulating mammary gland development and it contributes to breast cancer development and progression. Most of the ER-negative breast cancers display more aggressive clinical behaviors and are resistant to antiestrogen therapies. In addition, many ER-negative tumors show insensitivity to many chemotherapeutic drugs and radiotherapy, although mechanisms underlying this phenotype are less clear.

**Experimental Design:** We conducted immunohistochemistry on 296 cases of breast cancer tissues using a variety of antibodies. On the basis of the clinical data, we conducted siRNA knockdown to study the role of ERα on ATM expression in breast cancer cell lines. Furthermore, we used antisense oligonucleotides against micro RNAs (miRNA) or miRNA overexpression plasmids to study the role of miR-18a and -106a on ATM expression. Finally we used *in situ* hybridization to assess miR-18a and -106a expression in breast cancer tissues.

**Results:** We found that in ER-negative breast cancer tissues, expression of the ATM kinase, a critical DNA damage-response protein, is aberrantly upregulated. We also found that the locoregional recurrence rate after radiotherapy positively correlates with ATM expression. On the cellular level, we showed that ERα, but not ERβ, negatively regulates ATM expression. Furthermore, we identified that ERα activates miR-18a and -106a to downregulate ATM expression. We also showed that miR-18a and -106a were significantly underexpressed in ER-negative breast cancer tissues.

**Conclusions:** We reveal a novel mechanism involving ERα and miR-18a and -106a regulation of ATM in breast cancer. *Clin Cancer Res; 19(18); 4994–5002. ©2013 AACR.*

## Introduction

Approximately 30% of patients with breast cancer do not express estrogen receptor α (ERα; ref.1). ERα-negative tumors are a part of the triple-negative breast cancer (TNBC), a subset of the breast cancer population, which typically is associated with aggressive clinical phenotypes and poor prognosis (2). For example, ER-negative breast cancers are not responsive to antiestrogen therapies. In addition, many ER-negative patients show less sensitivity to many DNA damaging agents (3), except in some cases, such as with a Her2-positive status, patients showed variable sensitivity to chemotherapeutic drugs such as anthracycline (4, 5). At the cellular level, ERα regulates a variety of processes in mammary gland development and it also contributes to the development and progression of breast cancer (6). By binding to estradiol (E2), ERα mediates transcription by interacting directly to specific estrogen-response elements located in the promoter or enhancer region of target genes. In addition, ERα may indirectly associate with nuclear proteins such as AP1 and SP1 to stimulate transcription (7). Recent data have also shown that ERα plays a critical role in regulating (either suppress or stimulate) micro RNAs (miRNA) to control protein expression (8). Aberrant miRNA expression has been implicated in estrogen-related breast and endometrial cancers in the clinical setting (9). It has been reported that several phosphorylation events are required for a variety of cell-specific functions of ERα (10). Despite the extensive studies on ERα function in regulation of transcription, the functional mechanism driving most of ER-negative breast cancer cells resistant to many chemotherapeutic agents as well as radiotherapy is less clear.

Cellular sensitivity to DNA damaging agents such as radiotherapy and radiomimetic drugs are regulated by a cascade of DNA damage response proteins. Among them is

**Authors' Affiliations:** [1]Department of Breast Pathology and Lab, Key Laboratory of Breast Cancer Prevention and Therapy, Tianjin Medical University Cancer Institute and Hospital, Tianjin, China; Departments of [2]Radiation Oncology, and [3]Nanomedicine, The Methodist Hospital Research Institute, Houston, Texas; and [4]Department of Biochemistry and Molecular Biology, Southern Research Institute, Birmingham, Alabama

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

X. Guo and C. Yang contributed equally to this work.

**Corresponding Authors:** Bo Xu, Southern Research Institute, 2000 9th Ave. South, Birmingham, AL 35205. Phone: 205-581-2845; Fax: 205-581-2097; E-mail: xu@sri.org; and Li Fu, Tianjin Medical University Cancer Institute and Hospital, West Huanhu Road, Tianjin 300060, China. Phone: 86-22-23342606; Fax: 86-22-23340123-5813; E-mail: fuli@tmu.edu.cn

**doi:** 10.1158/1078-0432.CCR-12-3700

Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700



## Translational Relevance

Most of the estrogen receptor α (ERα)–negative breast tumors are resistant to many DNA-damaging agents, although mechanisms underlying this phenotype are less clear. In this report, we show in cellular models and clinical samples that ERα negatively regulates expression of the ATM kinase through miR-18a and -106a. These observations highlight a novel mechanism linking ERα and ATM, which explains the resistant phenotype of ER-negative cancers to chemotherapy and radiotherapy. As ER-negative breast cancers are more aggressive and unresponsive to antiestrogen therapies, other targeted therapies are urgently needed. Because ATM expression is upregulated in ER-negative cancers, ATM might represent an interesting drug target in these tumors. Using specific ATM inhibitors might achieve more clinical benefits as this might specifically increase tumor sensitivities to many of the chemotherapeutic drugs as well as radiotherapy.

the ATM kinase (11). ATM is mutated in the autosomal recessive disorder ataxia–telangiectasia, manifested by progressive neuronal degeneration, cancer predisposition, immunodeficiency, and hypersensitivity to radiotherapy (12). ATM functions in the DNA damage response by phosphorylation and activation of a series of downstream targets to regulate cell-cycle checkpoints in response to DNA double-strand breaks. ATM hyperactivation has been observed in many stages of tumor tissues (13–16). ATM activation in the early-stage of tumorigenesis is oncogene driven and represents the antitumor function of the kinase (14–16). However, hyperactivation of ATM in late stages of breast cancer contributes to breast cancer metastasis (13).

In this study, we report that ER-negative breast cancer tissues show elevated ATM expression. We show that ERα, but not ERβ, negatively regulates ATM expression in breast cancer cells. Furthermore, we have identified 2 miRNAs (miR-18a and -106a) that are targets of ERα to regulate ATM expression. Finally, we show that miR-18a and -106a expressions are aberrantly reduced in ER-negative breast cancer tissues.

## Materials and Methods

### Cell culture and materials

Breast cancer cells lines MCF-7 and MDA-MB-231, obtained from American Type Culture Collection (ATCC) were maintained in Dulbecco's modified Eagle medium containing 10% FBS (Invitrogen) and 50 mg/mL of penicillin/streptomycin (Invitrogen) in humidified 37°C chambers with 5% CO2. β-Estradiol was purchased from Sigma-Aldrich. Cell line authentication was originally done by short tandem repeat profiling at ATCC. After purchase, the cell lines were not authenticated by authors. Ionizing radiation was delivered by X-Rad 320 X-ray irradiator (Precision X-Ray Inc.). All the cellular experiments carried out in this manuscript include at least 3 independent experiments.

### Antibodies

Rabbit anti-ATM and mouse anti-phospho-ATM (S1981p) antibodies were purchased from Cell Signaling. Mouse anti-ATM, mouse anti-ERα and rabbit anti-ERβ antibodies were purchased from Abcam. All horseradish peroxidase–conjugated secondary antibodies and β-actin antibody were obtained from Santa Cruz Biotechnology.

### Plasmids, siRNAs, and transfection

miRNA overexpression plasmids (pCMV-miR18a, 106a, 101, and 421) and ATM-3′untranslated region (UTR) plasmid were purchased from Origene, ERα plasmid (pEGFP-C1-ERα) was purchased from Addgene. Plasmids were transfected into cells with Fugene HD Reagents. Control, ERα, ERβ, and ATM siRNAs were purchased from Santa Cruz Biotechnology and transfected into cells by Oligofectamine reagent (Invitrogen) according to the manufacturer's instruction. Forty-eight hours after transfection, cells were analyzed. miRNA antisense oligos against has-miR18a, 106a, 101, and 421 were synthesized from Integrated DNA Technologies. Scrambled sequences were used as negative controls. All the oligos were transfect into cells also using the oligofectamine reagent. The sequences of all the oligos are listed in the following table.

| miRNA | Antisense | Scrambled |
|---|---|---|
| miR-18a | 5′ctgcactagatgcacctta3′ | 5′gtaccacgatcatatctgc3′ |
| miR-101 | 5′ttcagttatcacagtactgta3′ | 5′agaacagacttattctttgtc3′ |
| miR-106a | 5′acctgcactgtaagcacttt3′ | 5′ctacttccgatcgaagttac3′ |
| miR-421 | 5′cgcccaattaatgtctgttga3′ | 5′ggcgctatttacaactattcg3′ |

### Luciferase reporter assay

MDA-MB-231 cells were seeded in triplicates in 48-well plates. Luciferase reporter plasmids (ATM 3′UTR or pMir-Target vector; Origene) were cotransfected with 50 nmol/L control, miR-18a, miR-106a plasmids, or both. Cells were harvested 48 hours after transfection and lysed for luciferase assays by a Dual-Glo Assay Kit (Promega) according to the manufacturer's protocol.

### Human breast cancer tissue samples

Paraffin-embedded materials from 296 invasive patients with breast cancer with operable breast cancer treated between 01/01/2003–31/05/2003 were provided by the Department of Breast Pathology and Research Laboratory, Cancer Hospital of Tianjin Medical University (Tianjin, China), with the approval of Tianjin Medical University Institutional Review Board. All cases were female with 29 to 75 years of age (the mean age is 51.8 years). All patients were treated either with modified radical mastectomy ($n = 176$) or breast-conserving therapy ($n = 120$). Locoregional radiotherapy was administered to the patients underwent with the breast-conserving therapy. The outcome of radiotherapy was evaluated by locoregional recurrence with a follow-up period from 9 to 96 (mean 74.63) months.

Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700



### Immunohistochemistry

Immunohistochemistry (IHC) using the avidin–biotin–immunoperoxidase technique was conducted for ER and ATM in the 296 cases of clinical samples. Sections of formalin-fixed tissues from all cases were conducted using a standard protocol. Briefly, 4 μm tissue sections on coated slides were heated for antigen retrieval, pretreated with a 3% solution of hydrogen peroxide for 5 to 10 minutes, rinsed and incubated with 10% normal goat serum as a blocking agent. The sections were then incubated sequentially with the primary antibody (ER in a 1:100 dilution and ATM in a 1:200 dilution), a biotinylated secondary antibody, and avidin–peroxidase conjugate. All steps were preceded by rinsing of sections with PBS (pH 7.6). The chromogen was 3,3′-diaminobenzidine. The immunoreaction for ER and ATM in the nucleus of tumor cells was evaluated independently by 2 experienced pathologists. ER was determined positive if finding of $\geq$1% of tumor cell nuclei were immunoreactive and ATM was scored as previously described (13). (−) = No positive cells, (+) = 1% to 10% of the cells stained, (++) = 11% to 50% of the cells stained, and (+++) = 51% to 100% of the cells stained.

### Western blot analysis

Cell lysates were obtained by treatment with the lysis buffer (Thermo Scientific Pierce) containing the protease inhibitor cocktail (Roche) and phosphatase I and II inhibitors (Sigma), and the protein concentration was determined using the Bradford method (Bio-Rad). Equal volumes of cell lysates were loaded into 4% to 15% Mini-protean TGX precast gels (Bio-Rad) for electrophoresis. Proteins were then transferred from gels to the nitrocellulose membrane (Bio-Rad). Following incubation with 5% of non-fat milk (LabScientific) for 30 minutes, the membrane was subsequently incubated with primary and horseradish peroxidase–conjugated secondary antibodies. Signals were detected by adding the Pierce Chemiluminescent Reagents (Thermo Scientific Pierce).

### RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted from cultured cells using TRIzol (Life Technologies). cDNA was obtained from 5 ng of total RNA using the M-MLV Reverse Transcription Kit (Promega), and the expression levels of miR-18a, miR-106a, miR-101, and miR-421 were quantified using PCR with specific primers listed in the following table.

| Primer's name | Sequences |
|---|---|
| MiR 18a-F | 5′-TGTTCTAAGGTGCATCTAGTGC-3′ |
| MiR-18a-R | 5′-TGCCAGAAGGAGCACTTAGGG-3′ |
| MiR-101-F | 5′-TGCCCTGGCTCAGTTATCAC-3′ |
| MiR-101-R | 5′-TGC CAT CCT TCA GTT ATC ACA GTA-3′ |
| MiR-106a-F | 5′-CTTGGCCATGTAAAAGTGCTTACA-3′ |
| MiR-106a-R | 5′-CCATGGTAATGTAAGAAGTGCTTCA-3′ |
| MiR-421-F | 5′-GCACATTGTAGGCCTCATTAAATG-3′ |
| MiR-421-R | 5′-GAGATCACAGAGCAGGCGCCCAA-3′ |

Real-time PCR with the SYBR green detection was conducted using an ABI Prism 7700 thermocycler with fluorescence detection (Applied Biosystems). The thermal cycling conditions were composed of 50°C for 2 minutes followed by an initial denaturation step at 95°C for 10 minutes, 45 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The experiments were carried out in duplicate for each data point. The relative quantification in gene expression was determined using the $2^{-\Delta\Delta C_t}$ method (17). Using this method, we obtained the fold changes in gene expression normalized to an internal control gene *GAPDH*.

### Detection of miRNA expression by *in situ* hybridization in human breast cancer tissues

The miRCURY LNA miRNA ISH Kit and miRNA detection has-miR18a, 106a, and control probes were purchased from Exiqon. The miRNAs *in situ* hybridization was carried out in the 296 cases of human breast cancer samples. Briefly, breast cancer tissue slides were deparaffinized and incubated with proteinase-K for 10 minutes at 37°C. After dehydration, slides were incubated with the hybridization mix and hybridized for 1 hour at 50°C to 60°C. The slides were then subsequently incubated with the blocking solution (15 minutes at room temperature), the anti-DIG reagent (60 minutes at room temperature), and with AP substrate (2 hours at 30°C). The sections were counter stained with nuclear fast red (Vector Laboratories). The stained sections were then scored for expression of has-miR18a and 106a miRNAs under the microscopy (Olympus). The sections were evaluated independently by 2 experienced pathologists according to the percentage of stained cells (18), with less than 20% of the cells stained was designated low expression of miRNA (+) and more than 20% of the cells stained was designated as high expression of miRNA (++).

### Statistical analysis

Data were analyzed by $\chi^2$ test, Student *t* test, and Pearson correlation test and *P* values $\leq$0.05 were considered significant.

## Results

### ATM expression is elevated in ER-negative breast cancer tissues

We recently reported ATM hyperactivation in breast cancer primary tissues with lymph node metastasis (13). When we further analyzed this set of the clinical data, we surprisingly found that ER-negative breast cancer tissues have a much higher expression level of ATM. In a total of 296 samples, we found that the ATM expression level was negatively correlated with ER status (Fig. 1; Table 1; $P < 0.001$, $\chi^2$ test). However, the ER status showed less correlation with expression of the active form of ATM—pS1981-ATM ($P = 0.155$, $\chi^2$ test, Table 1). As these observations were contradictory to the data reported in a previous publication by Tommiska and colleagues (19), we repeated the IHC experiment and the analysis done by 2 independent pathologists confirmed our original observation.

Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700



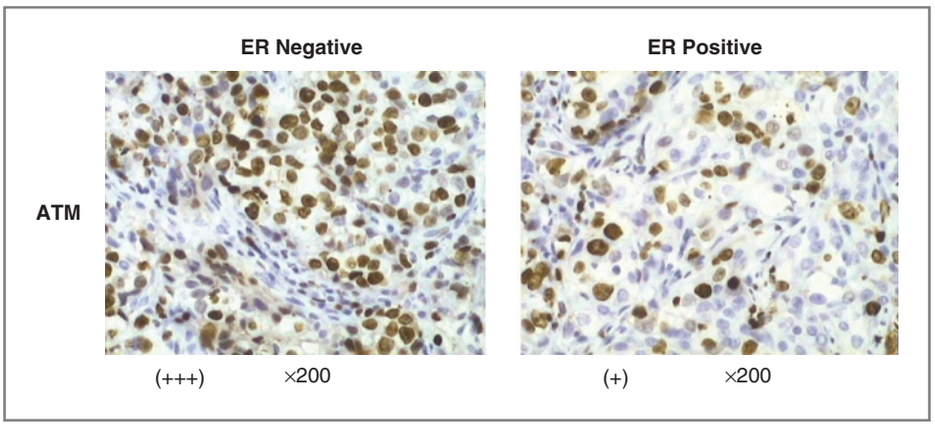


Figure 1. ATM expression is upregulated in ER-negative breast cancer tissues. IHC was conducted using the anti-ATM antibody in 296 human breast invasive ductal carcinoma tissues. Positive was defined for ER if finding of ≥1% of tumor-cell nuclei are immunoreactive. The number of positively or negatively stained cases of pan-ATM or pS1981-ATM was listed in Table 1.

A retrospective chart review of the 296 cases of breast cancer was also conducted. We focused on 120 cases who received breast-conserving surgery followed by curative radiotherapy to assess the clinical relevance of the ATM expression with patient responses to radiotherapy. Our data showed that the local recurrence rate after radiotherapy correlated with ER status (Table 2, $P = 0.012$) and ATM expression (Table 2, $P = 0.008$; Pearson correlation test). Together, these results prompted us to further study a potential connection of ER and ATM expression.

### ERα, but not ERβ, negatively regulates ATM expression in breast cancer cells

We hypothesized that ER might inhibit ATM expression. To test this notion, we first used an ER-positive breast cancer cell-line MCF-7. We transiently knocked down ERα (Fig. 2A) or ERβ (Fig. 2B) in these cells. Interestingly, we found that ATM expression level significantly increased in ERα knockdown cells (Fig. 2A). On the contrary, ERβ knockdown did not show an effect on ATM expression (Fig. 2B). We also assessed ionizing radiation-induced ATM activation using the phospho-ATM Serine 1981 antibody in cells in ER α or β knockdown cells. Unlike the effect of ERα on the basal ATM expression, ionizing radiation-induced ATM activation was unaffected by either ER α or β knockdown. This observation is consistent with the IHC data shown in Table 1.

We then overexpressed ERα in the ER-negative breast cancer cell-line MDA-MB-231 and found that ATM expression was reduced with ERα reintroduction (Fig. 2C). On the contrary, ERα expression was not affected when ATM was knocked down by siRNA in MCF-7 cells (Fig. 2D). Together, these data indicate that ERα functions as an upstream component of the signaling network that can negatively regulate ATM expression. We also measured the ATM mRNA level in MCF-7 cells when ERα was knocked-down, however, we did not observe a change in ATM mRNA (Supplementary Fig. S1), indicating that ERα regulation of ATM is not at the transcriptional level.

### ERα regulates ATM expression through miR-18a and miR-106a

We suspected that ERα regulation of ATM might be through ERα-regulated miRNAs. To test this notion, we

**Table 1.** Correlation of ER status with ATM expression in breast cancer tissues

| | ER | | |
|---|---|---|---|
| | Negative | Positive | $P^a$ |
| ATM | | | |
| − | 16 | 55 | |
| + | 23 | 82 | <0.001 |
| ++ | 26 | 51 | |
| +++ | 30 | 13 | |
| pS1981-ATM | | | |
| − | 28 | 66 | |
| + | 24 | 50 | 0.155 |
| ++ | 15 | 47 | |
| +++ | 28 | 38 | |

[a]$\chi^2$ test.

**Table 2.** Correlation of ER status and ATM expression with locoregional recurrence after breast-conserving surgery and radiotherapy

| | Local recurrence | | |
|---|---|---|---|
| | Yes | No | $P^a$ |
| ER | | | |
| Negative | 11 | 31 | 0.012 |
| Positive | 7 | 71 | |
| ATM | | | |
| − | 2 | 24 | |
| + | 3 | 35 | 0.008 |
| ++ | 5 | 26 | |
| +++ | 8 | 17 | |

[a]Pearson correlation test.

Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700



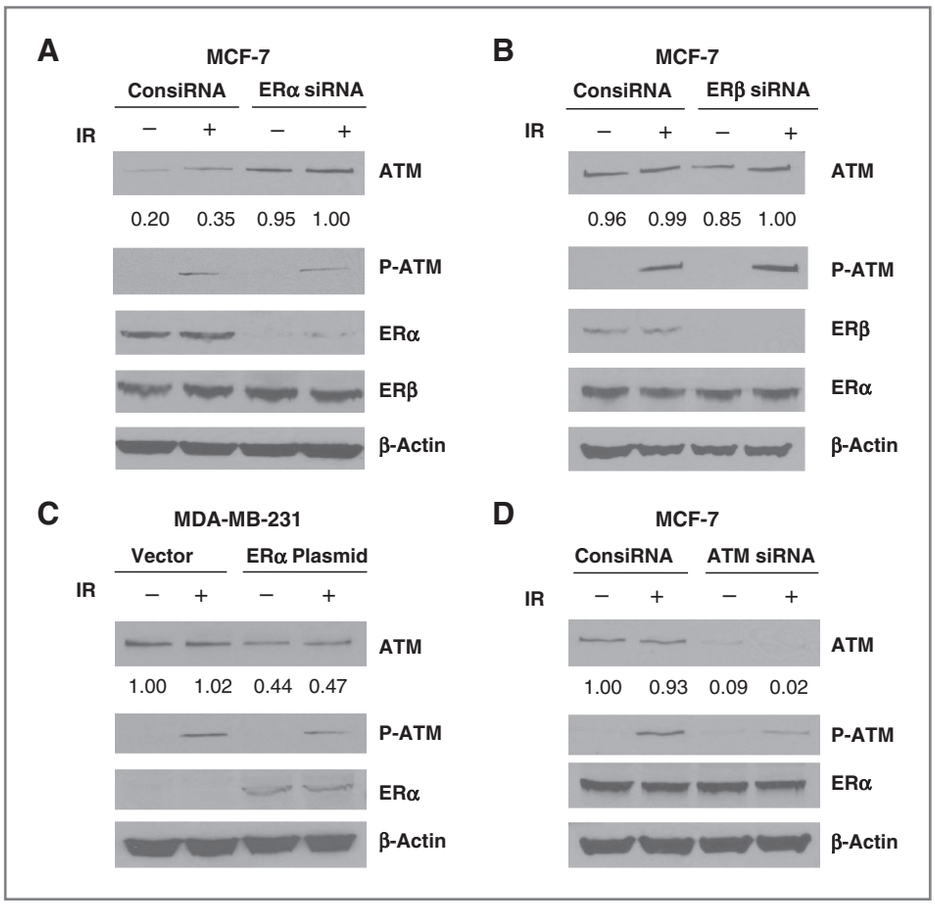


**Figure 2.** ERα negatively regulates ATM expression. A and B, MCF-7 cells were transfected with control, ERα (A) or ERβ–siRNA (B). Forty-eight hours after transfection, total cell lysates were harvested and subjected to immunoblotting using indicated antibodies. C, MDA-MB-231 cells were transiently transfected with either vector-only or ERα before they are assessed for expression of ATM, ATM S1981p, ERα, and β-actin using immunoblotting in the total cell lysates. D, MCF-7 cells were transfected with control or ATM siRNA. Forty-eight hours after transfection, total cell lysates were harvested and immunoblotting was conducted using indicated antibodies. Image J was used for quantification for ATM expression. The numbers shown represent normalized ratios of ATM and β-actin.

searched the available database containing the ERα-regulated miRNA signature. There are 19 identified miRNAs that can be transcriptionally activated by ERα (8). We explored the possibility that some of these miRNAs might regulate ATM expression. Using the TargetScan program, we searched the 3′UTR region of the *ATM* gene for miRNA binding motifs. We found that approximately 7 nucleotides at the 5′ end of 3 miRNAs (miR-18a, -101, and -106a) showed complementary sequences to the 3′UTR of the *ATM* gene (Supplementary Fig. S2). To test whether these miRNAs indeed regulate ATM expression, we designed antisense oligonucleotides against each of the 3 miRNAs. As miR-421 was previously shown to regulate ATM expression (20), we also included the antisense oligonucleotide against miR-421 as the positive control. These oligos were transfected into MCF-7 cells. As shown in Fig. 3A, we found that inhibiting miR-18a and miR-106a expression resulted in a significant increase of ATM expression, similar to the effect of inhibiting miR-421. However, knocking down miR-101 showed an opposite effect as ATM expression was reduced in antisense miR-101 expressing cells. Moreover, simultaneously knocking-down miR-18a and -106a by antisense miRNAs resulted in a more marked increase in ATM expression (Fig. 3B).

To further test ATM expression regulated by miR-18a and -106a, we used pCMV retroviral-based expression constructs containing vector or miR-18a, -106a, -101, or -421. We infected MDA-MB-231 cells with these constructs and measured the change of ATM expression. We found that overexpression of miR-18a or -106a reduced ATM expression (Fig. 4A). However, expressing miR-101 did not show any effects on ATM expression. Furthermore, we found that coexpression of miR-18a and -106a resulted in a more significant reduction of ATM expression (Fig. 4B). We also used a luciferase reporter assay following cotransfection of the luciferase reporter plasmid with miR-18a or miR-106a into MDA-MB-231 cells. Significant reductions in the luciferase activity from the reporter construct containing ATM 3′UTR were observed in the presence of miR-18a or miR-106a (Supplementary Fig. S3). Taken together, we conclude that ERα mediates ATM expression through miR-18a and -106a in breast cancer cells.

### miR-18a and -106a are underexpressed in ER-negative breast cancer tissues

To test the clinical connection of ER, miR-18a and -106a, and ATM expression, we conducted *in situ* hybridization in the 296 cases of breast cancer tissues. We found that both miRNAs showed significant reduced expression levels in ER-negative tissues ($P = 0.008$ and 0.033 for miR-18a and miR-106a, respectively; Supplementary Fig. S4; Table 3).

Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700



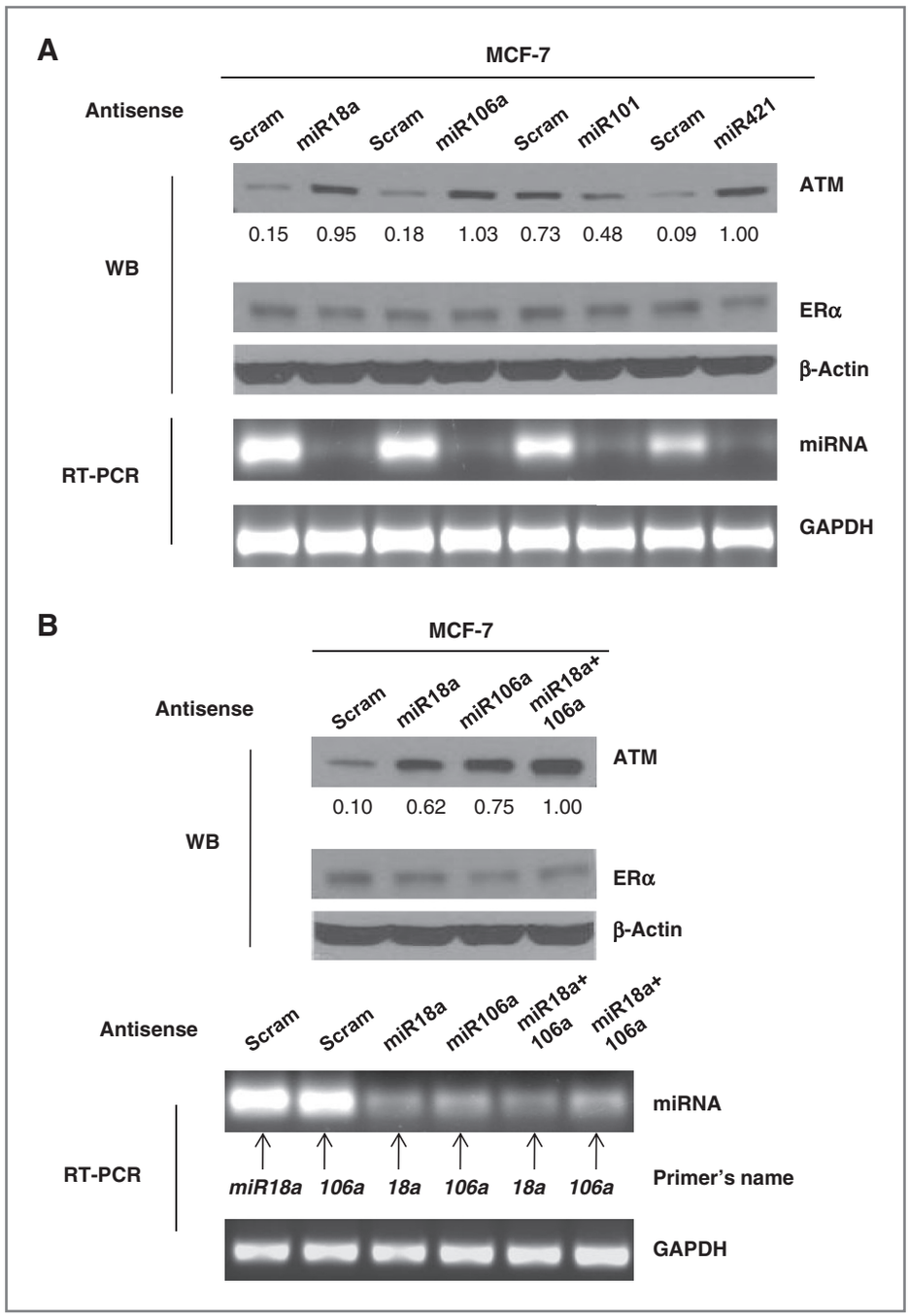


Figure 3. Knocking-down miR-18a and 106a increases ATM expression in breast cancer cells. A, MCF-7 cells were transfected with antisense oligos against miR-18a, 106a, 101, or 421. A scrambled oligo was designed as a negative control. Immunobloting was conducted using antibodies against ATM, ERα, or β-actin. Reverse transcription (RT)-PCR was conducted to measure the expression of miRNAs and GAPDH was used as a loading control. B, MCF-7 cells transfected with scrambled, antisense miR-18a, -106a, or both (miR-18a+106a) were subjected to immunoblotting using the indicated antibodies or to RT-PCR using indicated primers. ImageJ was used for quantification for ATM expression. The numbers shown represent normalized ratios of ATM and β-actin.

Furthermore, expression of miR-18a and -106a negatively correlated with ATM expression ($P < 0.001$ and $P = 0.001$ for miR-18a and miR-106a, respectively; Table 3). However, we did not observe any correlation of expression miR-18a and 106a with Serine 1981 phosphorylated form of ATM (Table 3). Combining with the data presented in Fig. 1 and Table 1 on the negative correlation of ER status and ATM expression, these observations further show the involvement of miR-18a and -106a in ERα-dependent ATM regulation.

## Discussion

In this report, we show in both cellular models and clinical samples that ERα negatively regulates expression of the ATM kinase. ATM is critical to determine the cellular survival to DNA damaging agents. In ER-negative breast cancer tissues, we observed aberrant upregulation of ATM expression. We elucidated that ERα suppressed ATM expression through regulating miR-18a and 106a. These observations highlight a novel mechanism linking ERα and ATM, which might explain the resistant

Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700



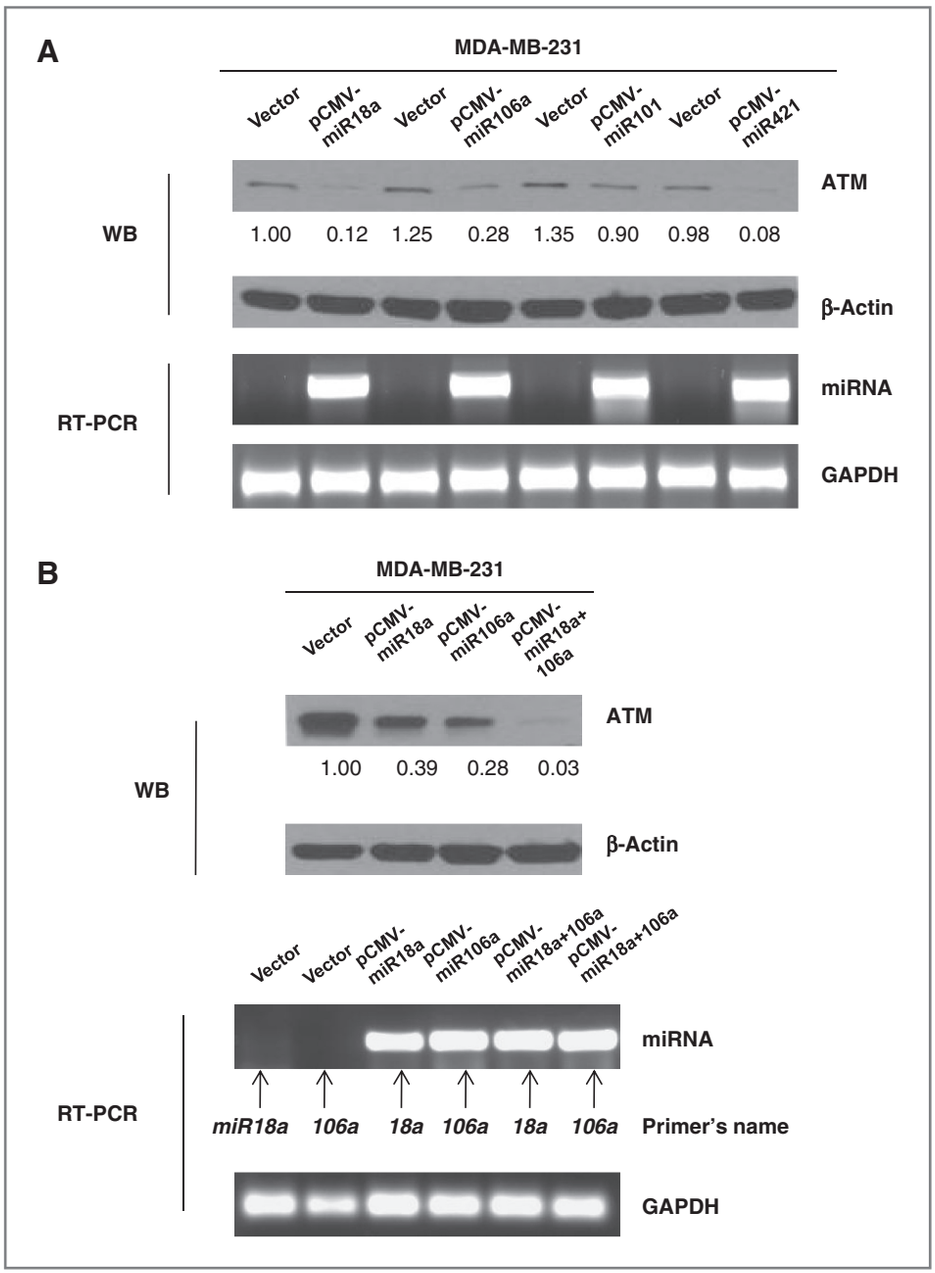


Figure 4. Overexpression of miR-18a and 106a suppresses ATM expression. A, MDA-MB-231 cells were infected with vector-only, pCMV-miR-18a, pCMV-miR-106a, pCMV-miR-101, or pCMV-miR-421 followed by immunoblotting and RT-PCR using indicated antibodies or primers. B, MDA-MB-231 cells were infected with vector, pCMV-miR-18a, pCMV-miR-106, or coinfected with pCMV-miR-18a and 106a. Forty-eight hours after infection, immunoblotting and RT-PCR were conducted. Image J was used for quantification for ATM expression. The numbers shown represent normalized ratios of ATM and β-actin.

phenotype of many ER-negative cancers to DNA damaging agents.

It is noted that there is discrepancy of our observations with a previous report that ATM expression is reduced in Brca1/2–deficient and TNBC cells (19). Although it is not clear why the results differ, one possible explanation is that the clinical samples we used were all from patients with sporadic breast cancer and in the previous article, most of the non-BRCA tumors samples were from patients with familial breast cancer.

It is interesting to observe that despite its regulation on ATM expression, ERα does not affect ATM activation in response to ionizing radiation. Although it is widely accepted that ATM Serine 1981 phosphorylation is a marker of ATM activation in response to ionizing radiation, it is much less clear to what extent ATM activation might affect the radiation response in human tissues. Furthermore, ATM Serine 1981 phosphorylation induced by 6Gy of ionizing radiation is not directly relevant to the clinical responsiveness to radiotherapy. Our data shown in Fig. 1 has suggested that ATM expression seems to be more relevant to the therapeutic outcome of breast cancer radiotherapy. It is possible that other mechanisms of ATM regulation (such as the alterations of ATM expression due

Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700



**Table 3.** Correlation of miR-18a and -106a with ER status and ATM expression in breast cancer tissues

| | miR18a | | $P^a$ | miR106a | | $P^a$ |
|---|---|---|---|---|---|---|
| | + | ++ | | + | ++ | |
| ER | | | | | | |
| Negative | 29 | 66 | 0.008 | 34 | 61 | 0.033 |
| Positive | 34 | 167 | | 48 | 153 | |
| ATM | | | | | | |
| − | 13 | 58 | <0.001 | 18 | 53 | 0.001 |
| + | 13 | 92 | | 24 | 81 | |
| ++ | 16 | 61 | | 17 | 60 | |
| +++ | 21 | 22 | | 23 | 20 | |
| pS1981-ATM | | | | | | |
| − | 23 | 71 | 0.142 | 21 | 73 | 0.376 |
| + | 11 | 63 | | 20 | 54 | |
| ++ | 18 | 44 | | 18 | 44 | |
| +++ | 11 | 55 | | 23 | 43 | |

[a] $\chi^2$ test.

to ERα and its regulated miRNAs) contribute to the outcome of locoregional recurrence. However, we do not rule out that other factors regulated directly by ERα or regulated through ERα-induced miRNAs are important for locoregional recurrence.

Unlike on ATM expression, ERα can directly regulate the DNA damage-responsive proteins. For example, a recent report showed that ERα-activated DNA-PKcs, a family member of the PI-3–like kinases (21). In addition, p53 can be a direct target of ERα (22). However, how this regulation is associated with the clinical responsiveness of ER-negative tumors to DNA damaging agents are not clear. These regulatory cascades might be related to antiestrogen resistance as hyperactivation of the PI3K pathway, the most frequently mutated pathway in breast cancer, promotes antiestrogen resistance (23).

During our study, the link of miR-18a-ATM has been reported (24). Our data provide a more comprehensive picture of miRNA regulation of ATM expression in breast cancer tissues. In addition to miR-18a, we found miR-106a also regulates the expression of ATM. More interestingly, deletion of both miRNAs results in a more significant effect on ATM expression, highlighting that multiple miRNAs regulates ATM in breast cancer tissues. As miRNAs serve as fine-tuning regulators of protein expression, we do not rule out that other mechanisms might regulate ATM expression in breast cancer tissues. For example, ATM promoter methylation shown in locally advanced breast cancer (25) might contribute low ATM expression in ER-positive tissues.

As most of the ER-negative breast cancers are more aggressive and unresponsive to antiestrogen therapies, other targeted therapies are urgently needed. Because ATM expression is upregulated in ER-negative cancers, ATM might represent a more plausible drug target in these tumors. Using specific ATM inhibitors might achieve more clinical benefits as this might specifically increase tumor sensitivities to many of the chemotherapeutic drugs as well as radiotherapy.

In summary, we identified the signaling transduction pathway involving ERα-miR18a and 106a-ATM that provides an explanation of chemo- and radioresistance in many ER-negative breast cancers.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** X. Guo, C. Yang, L. Fu, B. Xu
**Development of methodology:** X. Guo, C. Yang, L. Fu
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** X. Guo, C. Yang, T. Lei, Y. Li
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** X. Guo, C. Yang, X. Qian, B. Xu
**Writing, review, and/or revision of the manuscript:** X. Guo, C. Yang, H. Shen, L. Fu, B. Xu
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** C. Yang
**Study supervision:** C. Yang, L. Fu, B. Xu

## Acknowledgments

The authors thank all members of the Xu laboratory for help. The authors also thank Dr. Jaideep Thottassery (Southern Research Institute) and Dr. Xinmin Zhang (Temple University Hospital) for helpful comments on the manuscript.

## Grant Support

This work was supported by NIH grants R01CA133093 and R01ES016354 (to B. Xu), National Natural Science Foundation of China (Grant No. 30930038), Program for Changjiang Scholars and Innovative Research Team (Grant No. IRT0743) and the National 973 Program of China (Grant No. 2009CB521700; to L. Fu), and National Natural Science Foundation of China (Grant No. 81172531; to X. Guo).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 3, 2012; revised June 26, 2013; accepted July 3, 2013; published OnlineFirst July 15, 2013.

Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700



## References


1. Stanford JL, Szklo M, Brinton LA. Estrogen receptors and breast cancer. Epidemiol Rev 1986;8:42–59.
2. Metzger-Filho O, Tutt A, de Azambuja E, Saini KS, Viale G, Loi S, et al. Dissecting the heterogeneity of triple-negative breast cancer. J Clin Oncol 2012;30:1879–87.
3. Cleator S, Heller W, Coombes RC. Triple-negative breast cancer: therapeutic options. Lancet Oncol 2007;8:235–44.
4. Fountzilas G, Dafni U, Bobos M, Batistatou A, Kotoula V, Trihia H, et al. Differential response of immunohistochemically-defined breast cancer subtypes to anthracycline-based adjuvant chemotherapy with or without paclitaxel. PLoS ONE 2012;7:e37946.
5. Cheang MC, Voduc KD, Tu D, Jiang S, Leung S, Chia SK, et al. Responsiveness of intrinsic subtypes to adjuvant anthracycline substitution in the NCIC.CTG MA.5 randomized trial. Clin Cancer Res 2012;18:2402–12.
6. Fowler AM, Alarid ET. Amping up estrogen receptors in breast cancer. Breast Cancer Res 2007;9:305.
7. Marino M, Galluzzo P, Ascenzi P. Estrogen signaling multiple pathways to impact gene transcription. Curr Genomics 2006;7:497–508.
8. Castellano L, Giamas G, Jacob J, Coombes RC, Lucchesi W, Thiruchelvam P, et al. The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. Proc Natl Acad Sci U S A 2009;106:15732–7.
9. Klinge CM. miRNAs and estrogen action. Trends Endocrinol Metab 2012;23:223–33.
10. Maggi A. Liganded and unliganded activation of estrogen receptor and hormone replacement therapies. Biochim Biophys Acta 2011;1812:1054–60.
11. Shiloh Y. The ATM-mediated DNA-damage response: taking shape. Trends Biochem Sci 2006;31:402–10.
12. Lavin MF. Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. Nat Rev Mol Cell Biol 2008;9:759–69.
13. Sun M, Guo X, Qian X, Wang H, Yang C, Brinkman KL, et al. Activation of the ATM-Snail pathway promotes breast cancer metastasis. J Mol Cell Biol 2012;4:304–15
14. Bartek J, Lukas J, Bartkova J. DNA damage response as an anticancer barrier: damage threshold and the concept of "conditional haploinsufficiency". Cell Cycle 2007;6:2344–7.
15. Bartkova J, Bakkenist CJ, Rajpert-De Meyts E, Skakkebaek NE, Sehested M, Lukas J, et al. ATM activation in normal human tissues and testicular cancer. Cell Cycle 2005;4:838–45.
16. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 2005;434:907–13.
17. Frontini M, Kukalev A, Leo E, Ng YM, Cervantes M, Cheng CW, et al. The CDK subunit CKS2 counteracts CKS1 to control cyclin A/CDK2 activity in maintaining replicative fidelity and neurodevelopment. Dev Cell 2012;23:356–70.
18. Qian P, Zuo Z, Wu Z, Meng X, Li G, Wu Z, et al. Pivotal role of reduced let-7g expression in breast cancer invasion and metastasis. Cancer Res 2011;71:6463–74.
19. Tommiska J, Bartkova J, Heinonen M, Hautala L, Kilpivaara O, Eerola H, et al. The DNA damage signalling kinase ATM is aberrantly reduced or lost in BRCA1/BRCA2-deficient and ER/PR/ERBB2-triple-negative breast cancer. Oncogene 2008;27:2501–6.
20. Hu H, Du L, Nagabayashi G, Seeger RC, Gatti RA. ATM is down-regulated by N-Myc-regulated microRNA-421. Proc Natl Acad Sci U S A 2010;107:1506–11.
21. Medunjanin S, Weinert S, Poitz D, Schmeisser A, Strasser RH, Braun-Dullaeus RC. Transcriptional activation of DNA-dependent protein kinase catalytic subunit gene expression by oestrogen receptor-alpha. EMBO Rep 2010;11:208–13.
22. Berger CE, Qian Y, Liu G, Chen H, Chen X. p53, a target of estrogen receptor (ER) alpha, modulates DNA damage-induced growth suppression in ER-positive breast cancer cells. J Biol Chem 2012;287:30117–27.
23. Morelli C, Garofalo C, Bartucci M, Surmacz E. Estrogen receptor-alpha regulates the degradation of insulin receptor substrates 1 and 2 in breast cancer cells. Oncogene 2003;22:4007–16.
24. Song L, Lin C, Wu Z, Gong H, Zeng Y, Wu J, et al. miR-18a impairs DNA damage response through downregulation of ataxia telangiectasia mutated (ATM) kinase. PLoS ONE 2011;6:e25454.
25. Vo QN, Kim WJ, Cvitanovic L, Boudreau DA, Ginzinger DG, Brown KD. The ATM gene is a target for epigenetic silencing in locally advanced breast cancer. Oncogene 2004;23:9432–7.

Published OnlineFirst July 15, 2013; DOI: 10.1158/1078-0432.CCR-12-3700

AACR American Association for Cancer Research

Clinical Cancer Research

# Estrogen Receptor α Regulates ATM Expression through miRNAs in Breast Cancer

Xiaojing Guo, Chunying Yang, Xiaolong Qian, et al.

*Clin Cancer Res* 2013;19:4994-5002. Published OnlineFirst July 15, 2013.