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*DNA Damage and Repair*

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# TGFβ Induces "BRCAness" and Sensitivity to PARP Inhibition in Breast Cancer by Regulating DNA-Repair Genes

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## Abstract

Transforming growth factor beta (TGFβ) proteins are multitasking cytokines, in which high levels at tumor sites generally correlate with poor prognosis in human patients with cancer. Previously, it was reported that TGFβ downregulates the expression of ataxia telangiectasia–mutated (ATM) and mutS homolog 2 (MSH2) in breast cancer cells through an miRNA-mediated mechanism. In this study, expression of a panel of DNA-repair genes was examined, identifying breast cancer 1, early onset (BRCA1) as a target downregulated by TGFβ through the miR181 family. Correlations between the expression levels of TGFβ1 and the miR181/BRCA1 axis were observed in primary breast tumor specimens. By downregulating BRCA1, ATM, and MSH2, TGFβ orchestrates DNA damage response in certain breast cancer cells to induce a "BRCAness" phenotype, including impaired DNA-repair efficiency and synthetic lethality to the inhibition of poly (ADP-ribose) polymerase (PARP). Xenograft tumors with active TGFβ signaling exhibited resistance to the DNA-damaging agent doxorubicin but increased sensitivity to the PARP inhibitor ABT-888. Combination of doxorubicin with ABT-888 significantly improved the treatment efficacy in TGFβ-active tumors. Thus, TGFβ can induce "BRCAness" in certain breast cancers carrying wild-type BRCA genes and enhance the responsiveness to PARP inhibition, and the molecular mechanism behind this is characterized.

**Implications:** These findings enable better selection of patients with sporadic breast cancer for PARP interventions, which have exhibited beneficial effects in patients carrying BRCA mutations. *Mol Cancer Res; 12(11); 1597–609. ©2014 AACR.*

## Introduction

TGFβ proteins are multitasking cytokines involved in embryonic development, cell proliferation, motility and apoptosis, extracellular matrix production, and immunomodulation (1). In solid tumors, TGFβ can be produced by cancer and niche cells and acquires a cancer-promoting function. High TGFβ levels at tumor sites correlate with high histologic grade, risk of metastasis, and poor prognosis in patients with cancer (2). Previously, we reported that a gene-expression signature induced by TGFβ activation is associated with shorter patient survival in 295 primary breast cancers and is frequently found in tumors with a basal-like molecular profile (3). Those basal-like breast cancers are mostly sporadic but often share transcriptomic characteristics with tumors carrying *BRCA1* germline mutations (4). They significantly overlap (80%) with triple-negative breast cancers (TNBC; negative for hormone receptors and HER2), exhibit high expression of DNA-repair proteins, and are associated with aggressive phenotype and poor patient outcomes (5–7). TGFβ is also implicated in resistance to chemotherapies for various cancers, including breast cancers (2). The mechanisms of TGFβ-mediated chemoresistance remain largely unknown. Those mechanisms appear to be diverse and depend on the cancer types, subtypes, stages, and the therapeutic regimens used during treatment (8–12), possibly as a result of the versatile and contextual properties of TGFβ signaling.

TGFβ can regulate gene transcription through the SMAD transcriptional factors that bind to promoters of target genes (13). More recently, TGFβ and SMADs have also been implicated in the regulation of microRNA (miRNA)

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biogenesis. miRNAs are small regulatory RNAs that base-pair with the 3′ untranslated regions (UTR) of protein-encoding mRNAs, resulting in mRNA destabilization and/or translational inhibition. Consistent with their extensive regulatory function, the biogenesis of miRNAs is tightly controlled, and dysregulation of miRNAs is linked to cancer (14, 15). Previous studies indicate that TGFβ/SMADs regulate miRNA biogenesis at both the transcriptional and posttranscriptional levels. One of the posttranscriptional regulatory mechanisms involves binding of TGFβ receptor–regulated SMADs to the stem region of primary miRNA transcripts (pri-miRNA) and to the Drosha/p68 miRNA–processing complex, possibly providing a platform to facilitate miRNA maturation (16, 17). From our previous studies, TGFβ induces levels of both miR21 and miR181 families in breast cancer cells in a SMAD4-independent pattern via the interaction of SMAD2/3 with the Drosha complex (18, 19).

We reported that *MSH2*, coding for a central component of the DNA mismatch repair (MMR) machinery, is downregulated by TGFβ in breast cancer cells through miR21 (18). An inverse correlation between *TGFB1* and *MSH2* expression is significant among primary breast cancers (18), suggesting the presence of this mechanism *in vivo*. mutS homolog 2 (MSH2) plays a key role in the recognition and repair of DNA replication errors, contributing to genomic integrity. In cancer cells, MSH2 identifies DNA adducts caused by many chemotherapeutic drugs and triggers further MMR-mediated signaling that results in cell-cycle arrest and apoptosis (20, 21). In another report, we found that TGFβ downregulates *ATM* in breast cancer cells by inducing the miR181 family, which targets the 3′UTR of *ATM* transcripts (19). Upon DNA damage, the ataxia telangiectasia–mutated (ATM) kinase phosphorylates key proteins in checkpoint control, such as P53, BRCA1, and CHEK2, resulting in cell-cycle arrest, DNA repair, or apoptosis (22). On the basis of the previous work, we focused on the effect of TGFβ on the DNA damage response and further identified *BRCA1* as a target downregulated by the TGFβ/miR181 axis. Through this mechanism, TGFβ could sensitize TNBC cells to PARP inhibitors as demonstrated by our *in vitro* and *in vivo* models.

## Materials and Methods

### Cells, plasmids, and viruses

All cell lines were obtained from the ATCC and cultured in the recommended media in a humidified 5% $CO_2$ incubator at 37°C. To generate MDA231-Alk5$^{TD}$, MDA231-Alk5$^{KR}$, and MDA231-vec, retroviruses encoding TβRI(Alk5)$^{T204D}$, Alk5$^{K232R}$ (3), or the empty pBMN-I–GFP vector were produced by transfecting Ampho-Phoenix cells and then used for transduction, followed by GFP selection. The miR181a/b and *MSH2* expression plasmids were constructed and described elsewhere (18, 19). The *BRCA1* expression construct was kindly provided by Dr. Jeffrey D. Parvin (Ohio State University, Columbus, Ohio). The *ATM* expression construct (23) was obtained from Addgene. Plasmid constructions and additional reagents are described in Supplementary Material. Cell transfection, reporter assays, production of viruses, as well as infection, and selection of transduced cells were carried out as previously described (19). Recombinant human TGFβ1 was purchased from R&D Systems. The type I/II TGFβ receptor inhibitor LY2109761 was provided by Eli Lilly and Company. ABT-888 was purchased from Chemie-Tek. 4-Amino-1,8-naphthalimide (ANI), doxorubicin, methyl methanesulfonate (MMS), and 6-thioguanine (6-TG) were purchased from Sigma.

### RNA extraction, RT-qPCR, and Western blot analysis

These procedures were performed as described previously (18, 19). Sequences of the primers can be found in Supplementary Material.

### DNA-repair reporter assays

MDA-MB-231 cells with stable expression of I-SceI/GFP–based double-strand break (DSB) repair reporters (DR-GFP and EJ5-GFP; ref. 24) were generated by transfection and puromycin selection, and subsequently pretreated with TGFβ (5 ng/mL) for 20 hours before transfected with the I-SceI expression vector or a GFP expression vector (as a control for transfection efficiency) using Lipofectamine 2000 (Life Technologies). After 3 days of culture with continuous presence or absence of TGFβ, the percentage of $GFP^+$ cells was determined by FACS analysis using a CyAn ADP analyzer (Beckman Coulter). The percentage of $GFP^+$ cells in the I-SceI–transfected group was divided by the percentage of $GFP^+$ cells in the GFP-transfected group to obtain the frequency of the repair event marked by $GFP^+$.

### Immunofluorescence and comet assay (single-cell gel electrophoresis)

Immunofluorescence was performed using a γ-H2AX antibody (EMD Millipore) and a Cyclin A antibody (Abcam) as described previously (25). For comet assay, an OxiSelect comet assay kit (Cell Biolabs) was used under a neutral condition following the manufacturer's protocol. Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss upright LSM 510 2-Photon confocal microscope. Olive tail moment was calculated using the formula tail DNA% × tail moment length.

### *HPRT* mutation frequency analysis

Selection of *HPRT* (hypoxanthine phosphorybosyltransferase) mutants was performed as described using cells that had been cleansed for preexisting $HPRT^-$ mutants (26, 27). Details can be found in Supplementary Material.

### MTT (thiazolyl blue tetrazolium bromide) cell viability assay and calculation of coefficient of drug interaction

MTT assay was performed as described previously (18). Coefficient of drug interaction (CDI) was calculated using the formula AB/(A×B), in which AB represents the ratio of the cell viability in the combination group versus that in the

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control group, whereas A and B represents the ratio of the cell viability in the single-agent group versus that in the control group. A CDI = 1 is defined as additive effect between agent A and B, CDI < 1 synergistic effect, CDI < 0.7 significantly synergistic effect, and CDI > 1 antagonistic effect.

### Xenograft tumor model

All animal experiments were approved by the Institutional Animal Care and Use Committee at City of Hope. MDA231-vec or MDA231-Alk5$^{TD}$ cells ($2 \times 10^5$) were injected into the number 4 mammary fat pad of 6-week-old female NOD/SCID/IL2Rγ-null (NSG) mice. Doxorubicin (5 mg/kg) was administered weekly through i.p. injection and ABT-888 (50 mg/kg) daily via oral gavage, both starting at day 10 after cancer cell implantation. After tumors became palpable, tumor volume ($mm^3$) was assessed by caliper measurements using the formula ($width^2 \times length$)/2. At the end of the experiment, tumors were collected and dissociated tumor cells were subjected to 6-TG selection as described above and Western blot analysis.

### *In situ* hybridization and immunohistochemistry

*In situ* hybridization (ISH) was performed using the miRCURY LNA microRNA ISH Optimization Kit (Exiqon). IHC staining was performed as previously reported (28). Details can be found in Supplementary Material.

### Statistical analysis

For breast cancer dataset inquiry, six pooled breast cancer datasets of 947 primary tumors as well as an independent dataset of 295 primary breast cancers (29, 30) were analyzed by selecting 25% highest expressers and 25% lowest expressers of *TGFB1* and comparing levels of the DNA-repair genes between the two groups. Kendall tau-b bivariate correlation analyses were used for the tissue array. Student *t* tests were used for comparison of means of quantitative data between groups. The statistical analyses were performed using SPSS 16.0 software package. Values of $P < 0.05$ were considered significant. All quantitative data are presented as mean ± SD.

## Results

### TGFβ regulates the expression of DNA-repair genes in breast cancer cells

In this study, we focused on clinically aggressive, hard-to-treat TNBCs that often exhibit active TGFβ signaling (3) and high expression of DNA-repair proteins (7). We further focused on the regulation of TGFβ of the DNA-repair pathways as our previous studies indicate that TGFβ downregulates *MSH2* and *ATM*, two important DNA-repair genes, although these studies did not address the consequent effects of TGFβ on DNA-repair function (18, 19). Treatment of MDA-MB-231 cells, a TNBC cell line, with exogenous TGFβ resulted in >50% reduction of the RNA levels of *MSH2*, *MSH6*, *MLH1*, *ATM*, and *BRCA1*. These effects were completely abolished by LY2109761, a type I/II TGFβ receptor (TβRI/II) inhibitor (Fig. 1A). Expression of a constitutively active mutant cDNA of TβRI (Alk5$^{T204D}$, abbreviated to Alk5$^{TD}$ hereafter) largely recapitulated the regulation of these genes by TGFβ (Fig. 1B). To test the role of receptor kinase activity, a kinase-dead TβRI cDNA (Alk5$^{K232R}$, abbreviated to Alk5$^{KR}$ hereafter) was expressed in MDA-MB-231. In those cells producing Alk5$^{KR}$, the expression levels of all the DNA repair or response genes were greater than the vector only cells (Fig. 1B). Similar results were observed in another TNBC line MDA-MB-468 when treated with TGFβ (Fig. 1C). At the protein level, only ATM, MSH2, and BRCA1 consistently exhibited significantly lower levels when treated with TGFβ ligand or expression of Alk5$^{TD}$ in both TNBC lines (Fig. 1D and data not shown). We therefore focused on ATM, MSH2, and BRCA1 in the subsequent studies for their potential role in mediating the effects of TGFβ on DNA repair. We also tested two luminal breast cancer lines BT474 and MCF7. Although TGFβ caused significant downregulation of BRCA1 and modest downregulation of MSH2 and ATM in BT474 cells, its effect on the DNA-repair genes was negligible in MCF7 cells treated under the same experimental conditions (Supplementary Fig. S1A and S1B).

To obtain further evidence for the regulation of TGFβ of these DNA-repair genes, we analyzed six pooled breast cancer datasets of 947 primary tumors (NKI947) as well as an independent dataset of 295 primary breast cancers (NKI295; refs. 29, 30). In the analyses of either the pooled or independent datasets, in the breast cancers that were the 25% highest *TGFB1* expressers, significantly lower levels of *BRCA1* and *MSH2* transcripts were present, compared with the breast cancers that were the 25% lowest *TGFB1* expressers (Fig. 1E and F). The association between expression of *TGFB1* and *ATM*, however, was not significant (data not shown). Nevertheless, the results showing inverse correlations of *BRCA1* and *MSH2* with *TGFB1* levels are consistent with our *in vitro* data, indicating that TGFβ1 downregulates these genes (Fig. 1A–D).

### TGFβ induces a DNA-repair deficiency in breast cancer cells

To assess the effect of TGFβ signaling on DNA repair, we first used previously described DSB reporters for homology-directed repair (HDR) and end joining (EJ): DR-GFP and EJ5-GFP, respectively (24). The results indicated that pretreatment with TGFβ significantly reduced HDR in MDA-MB-231 cells without affecting the frequency of EJ (Fig. 2A). We then examined formation of γ-H2AX foci in MDA-MB-231 cells with or without pretreatment with TGFβ. Following ionizing radiation (IR), cells with both γ-H2AX foci and expression of Cyclin A, an S/$G_2$-phase marker, were counted. TGFβ treatment significantly reduced γ-H2AX foci formation in Cyclin $A^+$ cells upon DNA damage (Fig. 2B), consistent with its ability to downregulate ATM (Fig. 1A–D). We next performed comet assays to evaluate levels of DNA damage after treatment with the genotoxic chemotherapeutic agent doxorubicin. MDA-MB-231 cells expressing Alk5$^{TD}$ constantly carried higher levels of DNA damage compared with cells expressing Alk5$^{KR}$ or the control vector,

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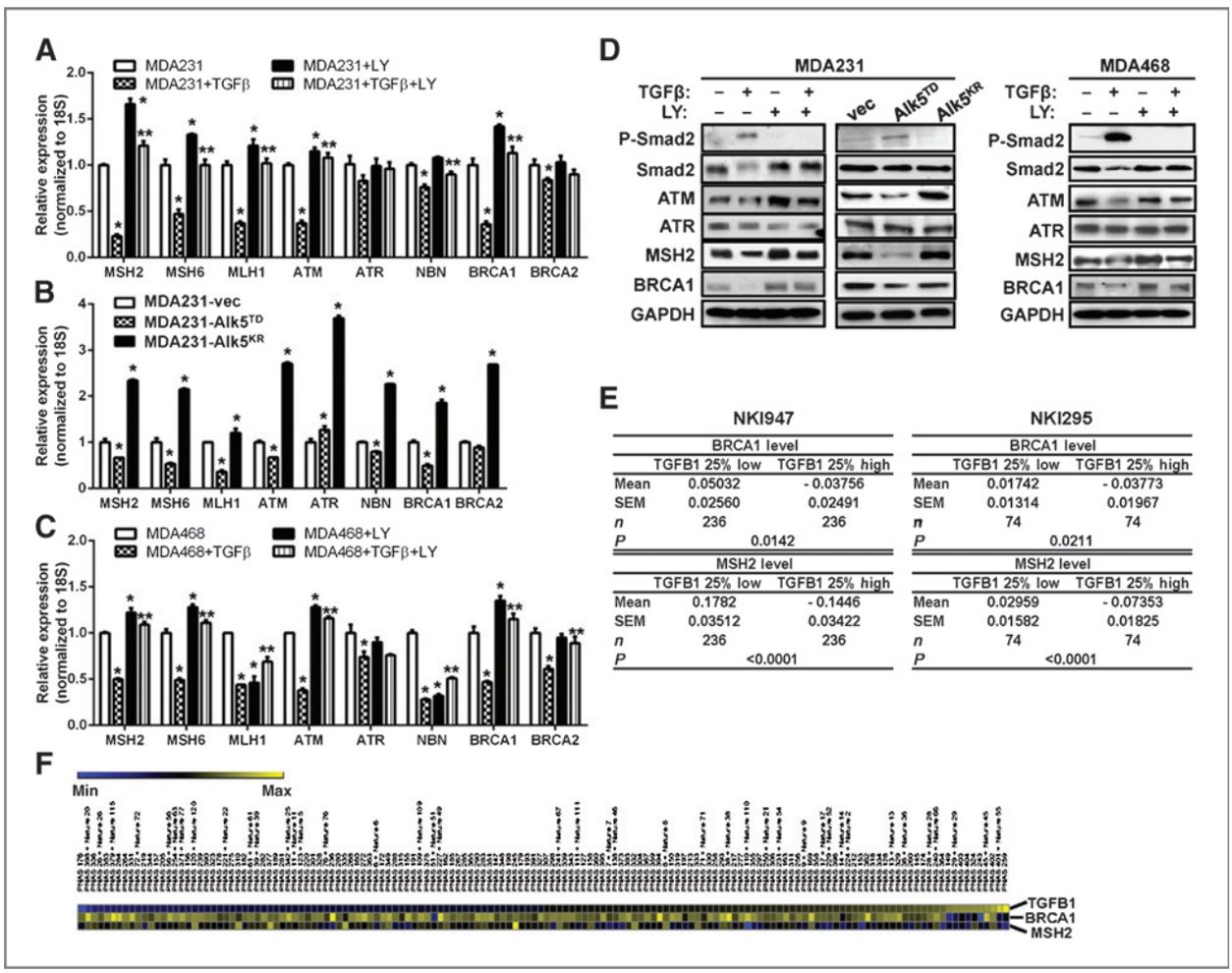


| NKI947 | BRCA1 level | | NKI295 | BRCA1 level | |
|---|---|---|---|---|---|
| | TGFB1 25% low | TGFB1 25% high | | TGFB1 25% low | TGFB1 25% high |
| Mean | 0.05032 | - 0.03756 | Mean | 0.01742 | - 0.03773 |
| SEM | 0.02560 | 0.02491 | SEM | 0.01314 | 0.01967 |
| *n* | 236 | 236 | *n* | 74 | 74 |
| *P* | 0.0142 | | *P* | 0.0211 | |
| | MSH2 level | | | MSH2 level | |
| | TGFB1 25% low | TGFB1 25% high | | TGFB1 25% low | TGFB1 25% high |
| Mean | 0.1782 | - 0.1446 | Mean | 0.02959 | - 0.07353 |
| SEM | 0.03512 | 0.03422 | SEM | 0.01582 | 0.01825 |
| *n* | 236 | 236 | *n* | 74 | 74 |
| *P* | <0.0001 | | *P* | <0.0001 | |

**Figure 1.** TGFβ regulates the expression of DNA-repair genes in breast cancer cells. A, MDA-MB-231 (abbreviated to MDA231 in figures) cells were treated with TGFβ (5 ng/mL) or/and LY2109761 (10 μmol/L), a type I/II TGFβ receptor inhibitor. At 24 hours, RNA was extracted and levels of the indicated genes were analyzed by quantitative RT-PCR; *, $P < 0.001$ compared with the control (the first treatment group); **, $P < 0.001$ compared with the TGFβ treatment group. B, MDA231 cells stably expressing a constitutively active type I TGFβ receptor construct (Alk5 with the $T^{204}D$ mutation, abbreviated to $Alk5^{TD}$), a kinase-dead type I TGFβ receptor construct (with the $K^{232}R$ mutation, abbreviated to $Alk5^{KR}$), or the empty vector, were analyzed by quantitative RT-PCR; *, $P < 0.001$ compared with the control (the first treatment group). C, MDA-MB-468 (abbreviated to MDA468 in figures) cells were treated with TGFβ or/and LY2109761 for 24 hours and analyzed by quantitative RT-PCR; *, $P < 0.001$ compared with the control (the first treatment group); **, $P < 0.001$ compared with the TGFβ treatment group. D, cells were treated as indicated for 48 hours and levels of indicated proteins were analyzed by Western blot analysis. GAPDH was used as a loading control. E, six pooled breast cancer datasets of 947 primary tumors (NKI947; ref. 30) as well as an independent dataset of 295 primary breast cancers (NKI295; ref. 29) were analyzed for the expression of *TGFB1* and indicated DNA-repair genes. The 25% highest expressers and 25% lowest expressers of *TGFB1* were compared for the levels of *BRCA1* and *MSH2*. Mean, SEM, *n*, and *P* values are shown in the tables. F, a heatmap showing levels of *TGFB1*, *BRCA1*, and *MSH2* in the 25% highest expressers and 25% lowest expressers of *TGFB1* in NKI295.

as demonstrated by an increase in olive tail moment that was observed at 6 hours after drug exposure and persisted at 24 hours (Fig. 2C). Overexpression of *ATM*, *MSH2*, or *BRCA1* cDNAs all partially reduced the DNA-damage levels, with BRCA1 exhibiting the most significant effect (Fig. 2D). These results indicate that TGFβ induces a DNA-repair deficiency in TNBC cells through downregulating DNA-repair genes.

### TGFβ induces a genomic instability through regulating DNA repair

Because DNA-repair function is tightly related to genomic stability, we further analyzed mutation frequencies at the *HPRT* gene in cells undergoing active TGFβ signaling as a means to assess the mutagenic potential of TGFβ-induced DNA-repair deficiency. MDA-MB-231 cells treated with TGFβ or expressing $Alk5^{TD}$ cDNA but not $Alk5^{KR}$ exhibited significantly higher spontaneous mutation frequency than the control cells (Fig. 3A and B). Upon treatment with DNA-damaging agents MMS and doxorubicin, the drug-induced mutation frequencies were approximately 3- to 8-fold higher when cells expressed $Alk5^{TD}$ cDNA (Fig. 3C). Again, overexpression of *ATM*, *MSH2*, or *BRCA1* cDNA partially reduced the spontaneous and doxorubicin-induced mutation frequencies, with BRCA1 exhibiting the strongest

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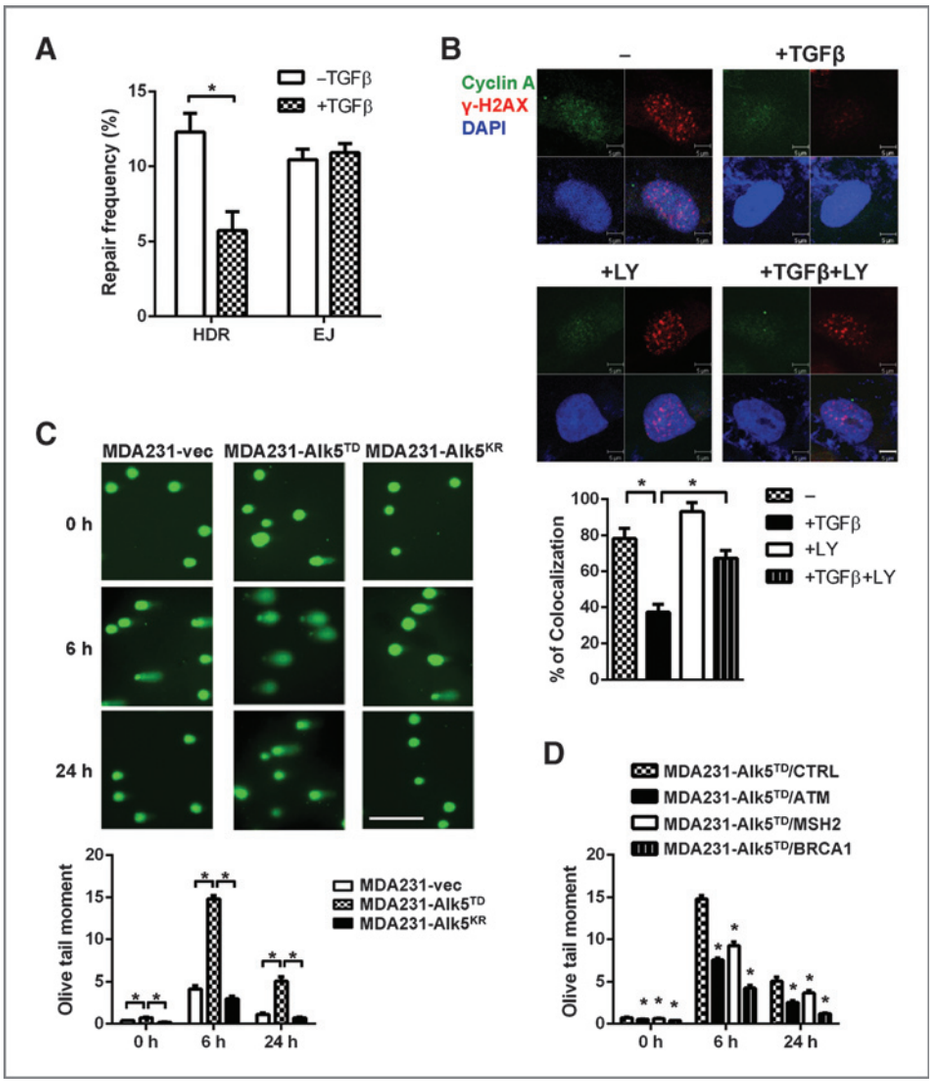


**Figure 2.** TGFβ induces a DNA-repair deficiency in breast cancer cells. A, MDA231 cells stably expressing reporters for HDR or EJ were pretreated with TGFβ (5 ng/mL) for 20 hours before transfection with the I-SceI expression vector or a GFP expression vector, and cultured for 3 days with continuous presence or absence of TGFβ. Because repair of the I-SceI–induced break by HDR or EJ in the respective reporter restores $GFP^+$, the percentage of $GFP^+$ cells was then determined by FACS analysis. To obtain the repair frequency, the GFP percentage of the I-SceI–transfected group was divided by that of the GFP-transfected group to normalize to transfected cells; *, $P < 0.001$. B, MDA231 cells were pretreated with TGFβ or/and LY2109761 for 3 days and then treated by IR at 10 Gy. After 6 hours, cells were fixed and subjected to immunofluorescent staining using a γ-H2AX antibody and a Cyclin A antibody. Nuclei were stained by DAPI. Representative images were shown; bar, 5 μm. For each treatment, 200 cells were counted and the percentage of cells with both Cyclin A expression and at least 5 γ-H2AX foci was shown; *, $P < 0.001$. C, MDA231 cells stably expressing $Alk5^{TD}$, $Alk5^{KR}$, or the empty vector and treated with doxorubicin (125 nmol/L) were subjected to comet assay. Representative images at 0, 6, and 24 hours after drug treatment were shown; bar, 50 μm. At each time point, 200 cells were counted, and the calculated olive tail moment was shown; *, $P < 0.001$. D, indicated cells expressing exogenous ATM, MSH2, BRCA1, or the empty vector (control) were analyzed by comet assay as in C after treatment with doxorubicin; *, $P < 0.001$ compared with the control (the first treatment group).

effect (Fig. 3D). Thus, the downregulation of these DNA-repair genes by TGFβ is associated with increased mutation frequency and genomic instability.

### TGFβ-mediated downregulation of *ATM*, *MSH2*, and *BRCA1* results in a synthetic lethality to PARP inhibition

Another consequence of TGFβ-mediated cosuppression of *ATM*, *MSH2*, and *BRCA1* in TNBC cells can be a dependence of cancer cells on the base excision repair pathway. PARP has roles in the base excision repair pathway, and also participates in other cellular processes. BRCA or ATM deficiency induces cancer sensitivity to PARP inhibition (31–34). As a synthetic lethal approach, PARP inhibitors have shown promising effects for *BRCA*-mutated breast cancers as well as TNBCs (31, 35). To determine whether TGFβ simulates a "BRCAness" phenotype by inducing sensitivity to PARP inhibition, we examined the BRCA-proficient MDA-MB-231 and MDA-MB-468 TNBC cells

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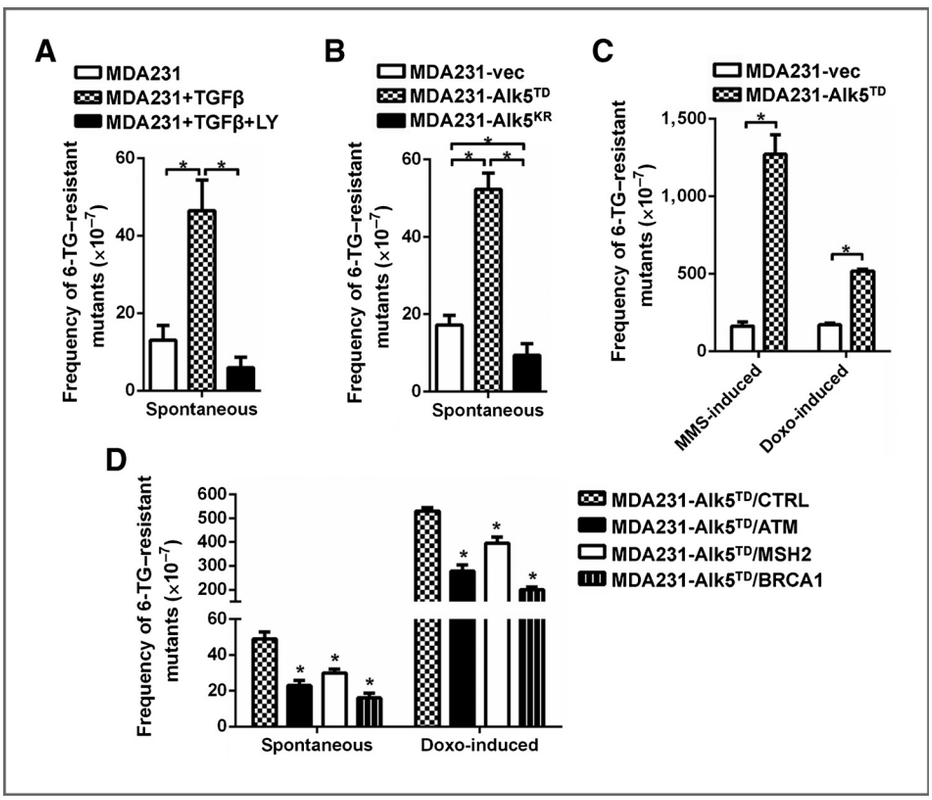


**Figure 3.** TGFβ induces a genomic instability through regulating DNA repair. A, growing MDA231 cells that were passaged every 2 days at 1:4 were treated with TGFβ in the absence or presence of LY2109761 for a total of 8 days. Cells were then plated and selected in medium containing 6-TG. Calculated frequency of the spontaneous 6-TG–resistant mutants was shown. B, indicated cells were analyzed for spontaneous frequency of 6-TG–resistant mutants. C, indicated cells were treated with MMS (20 μmol/L) for 40 minutes or doxorubicin (20 nmol/L) for 24 hours and then cultured in drug-free medium for a total of 8 days with every other day passaging at 1:4. Cells were then analyzed for drug-induced frequency of 6-TG–resistant mutants. D, indicated cells expressing exogenous ATM, MSH2, BRCA1, or the empty vector (control) were analyzed for spontaneous and doxorubicin-induced frequencies of 6-TG–resistant mutants as described above; *, $P < 0.001$.

undergoing active or suppressed TGFβ signaling. Treatment with TGFβ or expression of $Alk5^{TD}$ induced the sensitivity to PARP inhibition by ANI or ABT-888. Inhibition of TGFβ signaling by LY2109761 resulted in reduced sensitivity to PARP inhibition, and completely abolished the effect of TGFβ (Fig. 4A). To dissect the role of ATM, MSH2, and BRCA1 in mediating this effect, specific siRNAs were used to knockdown the expression of those genes either singularly or in combination (Fig. 4B). Among the single-gene knockdowns, knockdown of *BRCA1* was most effective in inducing sensitivity to PARP inhibition to a level that was comparable with that induced by *ATM* and *MSH2* double knockdown, whereas knockdown of all three genes conferred cells the highest sensitivity to ABT-888 (Fig. 4C). In contrast, overexpression of any single cDNA of *ATM*, *MSH2*, or *BRCA1* in $Alk5^{TD}$-expressing cells completely abolished the TGFβ-induced sensitivity to ABT-888 (Fig. 4D and E). Consistent with its ability to downregulate DNA-repair genes, TGFβ was able to sensitize BT474 cells, but not MCF7 cells in which it fails to regulate DNA-repair genes, to PARP inhibition by ABT-888 (Supplementary Fig. S1C and S1D).

### PARP inhibition overcomes TGFβ-mediated insensitivity to doxorubicin *in vitro* and *in vivo*

Previous results from our and other groups indicate that TGFβ induces a resistance to conventional chemotherapy drugs through various mechanisms and TGFβ inhibition enhances chemotherapy action in TNBCs (2, 8–12, 18). We therefore examined whether PARP inhibition in TNBC cells undergoing active TGFβ signaling could overcome TGFβ-mediated chemoresistance and thus might enhance the efficacy of conventional chemotherapy in these tumors. Activation of TGFβ signaling by TGFβ treatment or expression of $Alk5^{TD}$ induced a significant resistance to doxorubicin in MDA-MB-231 cells (Fig. 5A and B). Addition of ABT-888 to doxorubicin treatment overcame the resistance to the latter in $Alk5^{TD}$-expressing cells (Fig. 5C), and induced a significant synergy between the two drugs at all tested concentrations in MDA-MB-231 undergoing active TGFβ signaling (Fig. 5D).

To further examine this TGFβ effect *in vivo*, we established orthotopic xenograft tumors in NSG immunocompromised mice by injecting MDA-MB-231 cells expressing $Alk5^{TD}$ or the control vector into the mammary fat pad. ABT-888 or PBS was administered daily starting at day 10 after cancer cell implantation. The $Alk5^{TD}$-expressing tumors, but not the control tumors, responded to single-agent ABT-888 treatment, as demonstrated by significantly reduced tumor volumes (Fig. 5E). In another experiment, we compared the effect of doxorubicin single-agent treatment and the combination of doxorubicin and ABT-888 in the two types of xenograft tumors with or without TGFβ activation. The MDA-MB-231 control tumors exhibited a clear response to doxorubicin; addition of ABT-888 had no further effect on tumor growth. In contrast, the $Alk5^{TD}$-expressing tumors did not show a significant reduction in tumor volume upon doxorubicin treatment, but exhibited a significant response to the combination of doxorubicin and ABT-888 (Fig. 5F).

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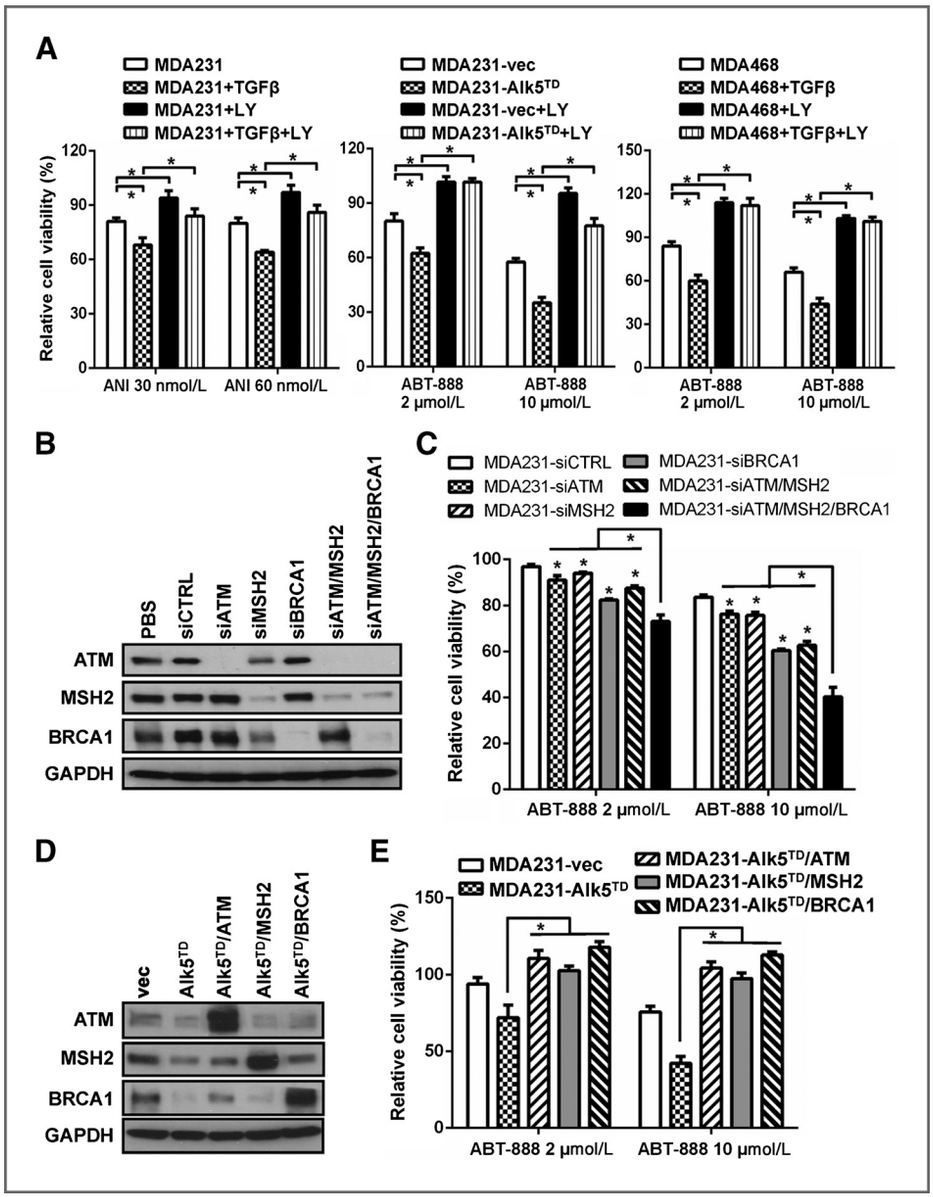


**Figure 4.** TGFβ-mediated downregulation of *ATM*, *MSH2*, and *BRCA1* results in a synthetic lethality to PARP inhibition. A, cells were pretreated with TGFβ or/and LY2109761 for 48 hours, before ANI or ABT-888 was added to the medium containing TGFβ or/and LY2109761. After 72 hours, cell viability was analyzed by MTT assay and normalized to cells that did not receive ANI or ABT-888. B, MDA231 cells transfected with indicated siRNAs were analyzed by Western blot analysis at 96 hours posttransfection. C, cells transfected as indicated were treated with ABT-888. Cell viability was determined by MTT assay and normalized to cells that did not receive ABT-888. D, MDA231-vec and MDA231-Alk5$^{TD}$ cells that stably overexpress ATM, MSH2, BRCA1, or the empty vector were analyzed by Western blot analysis. E, indicated cells were treated with ABT-888 and cell viability was determined by MTT assay; *, $P < 0.001$.

We further determined the mutation frequency in dissociated tumor cells collected from PBS- or doxorubicin-treated mice and found that the Alk5$^{TD}$-expressing tumors exhibited increased genomic instability as demonstrated by increased spontaneous and drug-induced mutation frequencies, compared with the control tumors without TGFβ activation (Fig. 5G). Levels of γ-H2AX were also lower in Alk5$^{TD}$-expressing tumors receiving PBS or doxorubicin (Fig. 5H), suggesting impaired DNA-repair function and/or reduced cell death in these tumors. Overall, the *in vitro* and *in vivo* data demonstrate that TNBC cells with active TGFβ signaling are more resistant to doxorubicin but more sensitive to PARP inhibition and suggest that single-agent treatment with ABT-888 or in combination with conventional chemotherapy would be effective against sporadic TNBCs exhibiting TGFβ activation.

### TGFβ downregulates *BRCA1* through miR181

We previously reported the miRNA-mediated mechanisms for the downregulation of *ATM* and *MSH2* by TGFβ (18, 19); however, the mechanism of TGFβ downregulation of *BRCA1*, which was the major mediator of many effects described above, remained unknown. In a search for the potential mechanisms regulating *BRCA1* expression, we scanned the 3′UTR of *BRCA1* and found a putative binding site for the miR181 family (miR181a/b/c/d sharing the same seed sequence), which we have previously reported to be unregulated by TGFβ at the posttranscriptional level in breast cancer cells (Fig. 6A; ref. 19). We then cloned the putative miR181-binding region in the *BRCA1* 3′UTR, either in the wild-type or with the miR181-recognition sequence mutated, downstream to a *Renilla* luciferase

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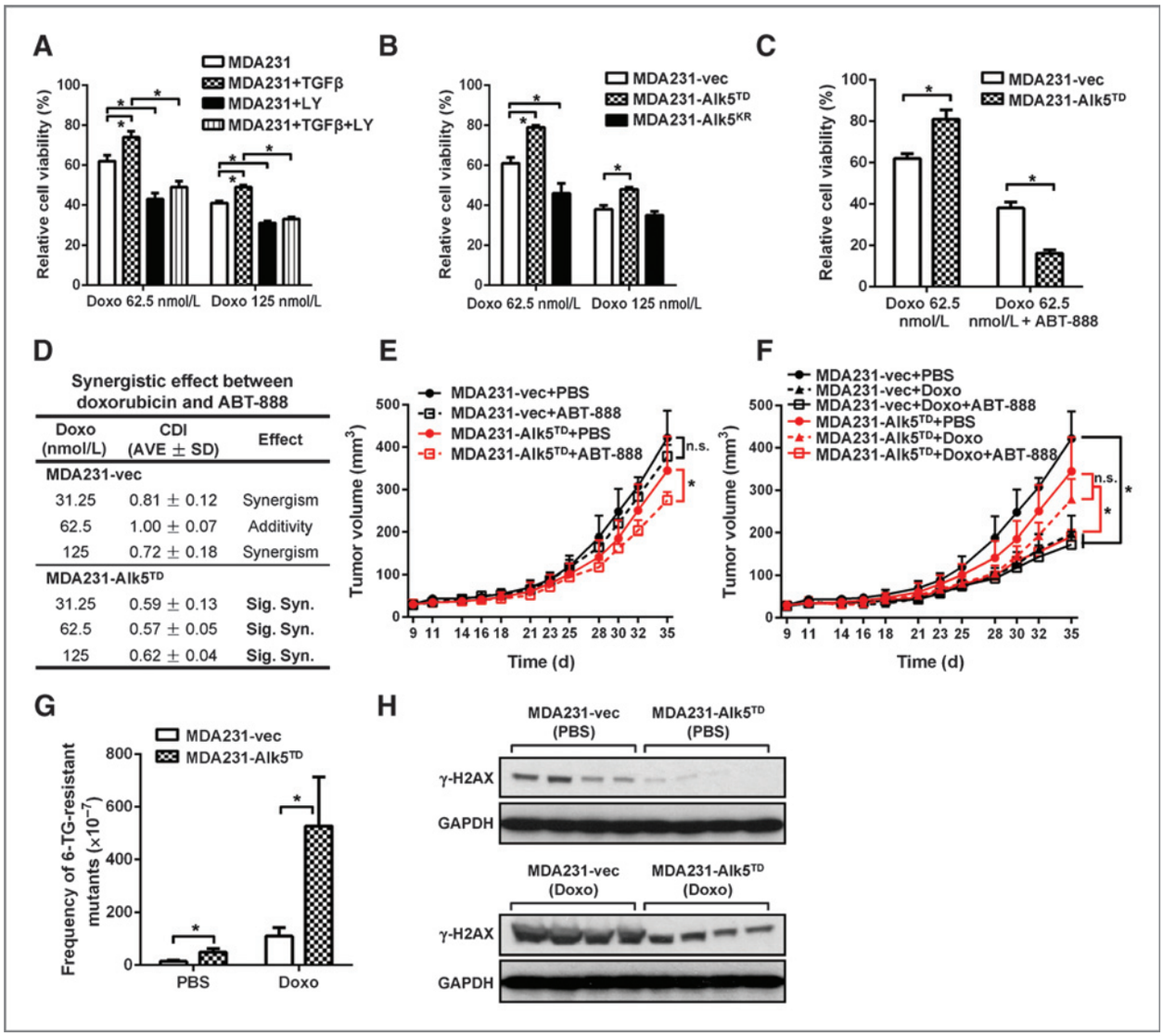


**Figure 5.** PARP inhibition overcomes TGFβ-mediated insensitivity to doxorubicin *in vitro* and *in vivo*. A, MDA231 cells were pretreated with TGFβ or/and LY2109761 for 48 hours before doxorubicin was added to the medium. After 72 hours, cell viability was analyzed by MTT assay and normalized to cells that did not receive doxorubicin. B, indicated cells were treated with doxorubicin and cell viability was determined by MTT assay. C, indicated cells were treated with doxorubicin alone or in combination with ABT-888 (10 μmol/L). Cell viability was determined by MTT assay; *, $P < 0.001$. D, MDA231-vec or MDA231-Alk5$^{TD}$ cells were treated with doxorubicin alone at the indicated concentrations or in combination with ABT-888 (10 μmol/L). Cell viability was determined by MTT assay and CDI was calculated. A CDI = 1 is defined as additive effect, CDI < 1 synergistic effect, CDI < 0.7 significantly synergistic effect, and CDI > 1 antagonistic effect. E, MDA231-vec or MDA231-Alk5$^{TD}$ cells were injected into the number 4 mammary fat pad of female NSG mice. Mice were treated with PBS or ABT-888 as described in Materials and Methods. Tumor volume was determined in each group ($n = 8$); *, $P < 0.05$; n.s., nonsignificant ($P > 0.05$). F, NSG mice that were injected with MDA231-vec or MDA231-Alk5$^{TD}$ cells into the number 4 mammary fat pad were treated with PBS, doxorubicin alone, or doxorubicin in combination with ABT-888. Tumor volume was determined in each group ($n = 6$–8); *, $P < 0.05$; n.s., nonsignificant ($P > 0.05$). G, dissociated tumor cells from indicated mouse groups were analyzed for the frequency of 6-TG–resistant mutants; *, $P < 0.001$. H, tumor lysates were analyzed by Western blot analysis for levels of γ-H2AX. GAPDH was used as a loading control.

reporter gene in the psiCHECK vector. MDA-MB-231 cells were transfected with the reporter constructs together with a miR181a/b-expressing plasmid or vector. The reporter construct carrying wild-type miR181–binding site but not the mutated site exhibited significant inhibition by miR181a/b (Fig. 6B). Consistently, overexpression of miR181a/b that also targets *ATM* (19), but not miR21 that targets *MSH2* (18, 36), resulted in downregulation of BRCA1 protein levels in both MDA-MB-231 and MDA-MB-468 TNBC cells (Fig. 6C). To further confirm that miR181 mediates the effect of TGFβ on downregulating *BRCA1* expression, MDA-MD-231 and MDA-MB-468 cells were transfected with anti-miRNAs before being treated with TGFβ. Inhibition of miR181, but not miR21, increased *BRCA1* expression and abolished the downregulation of TGFβ at the protein level (Fig. 6D and F). When cells transfected with

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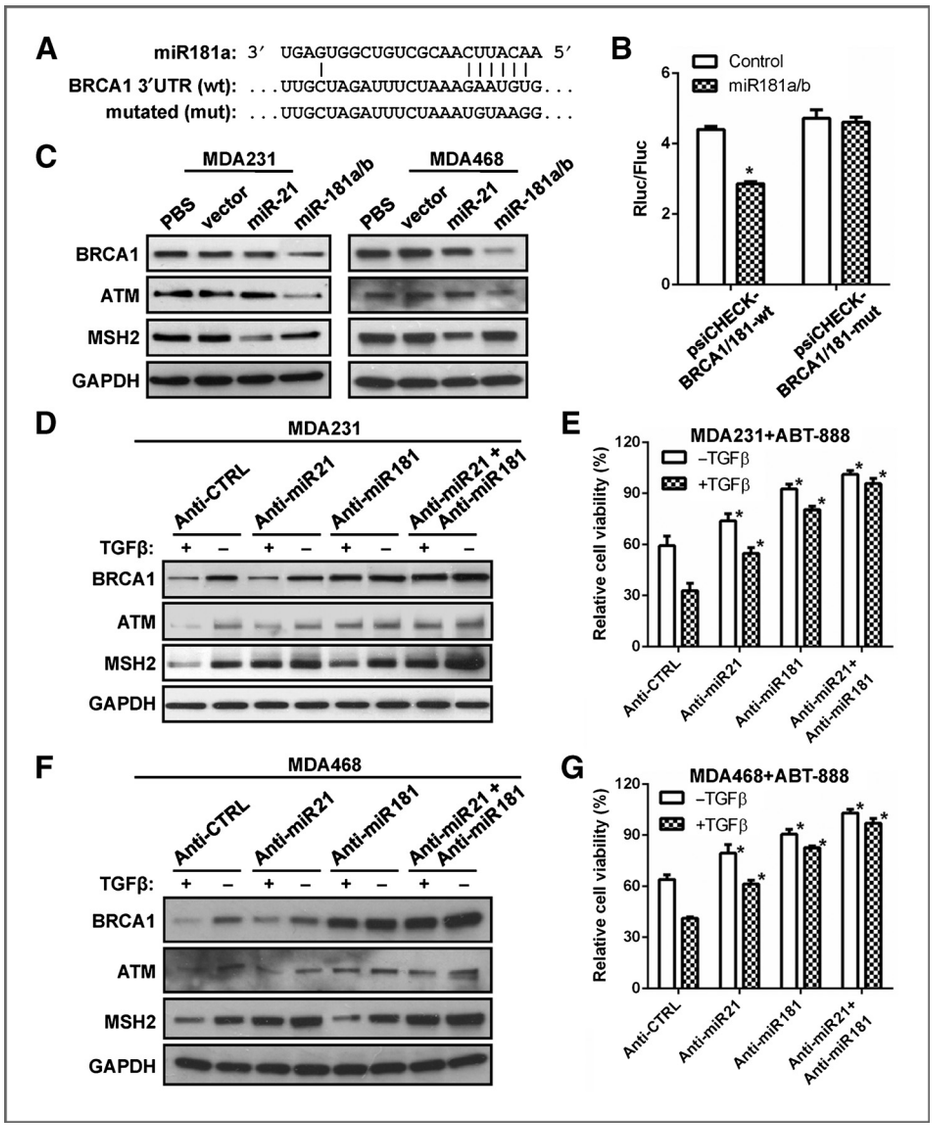


**Figure 6.** TGFβ downregulates *BRCA1* through miR181. A, the predicted miR181-targeting site in the 3′UTR of *BRCA1* mRNA. Sequences of miR181a and the mutated miR181-targeting site included in the psiCHECK–BRCA1/181-mut construct are also shown. B, the psiCHECK luciferase reporters containing the wild-type (wt) or mutated (mut) miR181-targeting site in *BRCA1* 3′UTR were used to transfect MDA231 cells together with an miR181a/b-expressing plasmid or vector (control). C, cells transfected with the expression plasmids of miR21 or miR181a/b, the empty vector, or PBS were analyzed by Western blot analysis. D, MDA231 cells transfected with indicated anti-miRNAs were treated with TGFβ or vehicle for 48 hours and analyzed by Western blot analysis. E, MDA231 cells transfected with anti-miRNAs and treated with TGFβ as indicated were treated with ABT-888 (10 μmol/L). Cell viability was analyzed by MTT assay and normalized to cells that did not receive ABT-888. F, MDA468 cells transfected with indicated anti-miRNAs were treated with TGFβ or vehicle for 48 hours before analyzed by Western blot analysis. G, MDA468 cells treated as indicated were analyzed for cell viability; *, $P < 0.001$ compared with the corresponding control group.

anti-miRNAs were examined for their responsiveness to ABT-888, anti-miR181 exhibited a greater effect on suppressing TGFβ-induced sensitivity comparing with anti-miR21, whereas coinhibition of miR181 and miR21 most effectively abolished the effect of TGFβ (Fig. 6E and G). These results are consistent with the previous observations that all three TGFβ-targeted DNA-repair genes, that is, *ATM*, *MSH2*, and *BRCA1*, individually regulated by miR181 (for *ATM* and *BRCA1*) and miR21 (for *MSH2*), contribute to TGFβ-induced sensitivity to PARP inhibition (Fig. 4B–E).

### TGFβ is associated with miR181 and BRCA1 levels as well as disease progression in primary TNBCs

To extend the herein identified mechanism to primary tumors, a tissue array, including 48 cases of TNBCs, was used to evaluate the levels of TGFβ1, miR181, and BRCA1. Significant positive correlation was detected between TGFβ1 and miR181 (Tau-b = 0.638, $P < 0.001$), whereas significant inverse correlations were detected between TGFβ1 and BRCA1 (Tau-b = −0.525, $P < 0.001$) and between miR181 and BRCA1 (Tau-b = −0.477, $P < 0.001$). In addition, higher levels of TGFβ1 and miR181 and lower levels of BRCA1 were also significantly associated with higher clinical grades and stages (Fig. 7A–D).

## Discussion

As one of the first clinical applications of synthetic lethality-based cancer therapeutics, PARP inhibition selective for BRCA1/2 deficiency has shown promising effect for the treatment of patients with tumors bearing *BRCA1/2* mutations (34, 37). As hereditary cancers with *BRCA1/2* mutations only account for about 5% to 10% of breast cancers (38) and 15% of ovarian cancers overall (39), characterizing tumors with wild-type *BRCA1/2* genes but also sensitive to PARP inhibitors is of great clinical interest. Recent studies suggest

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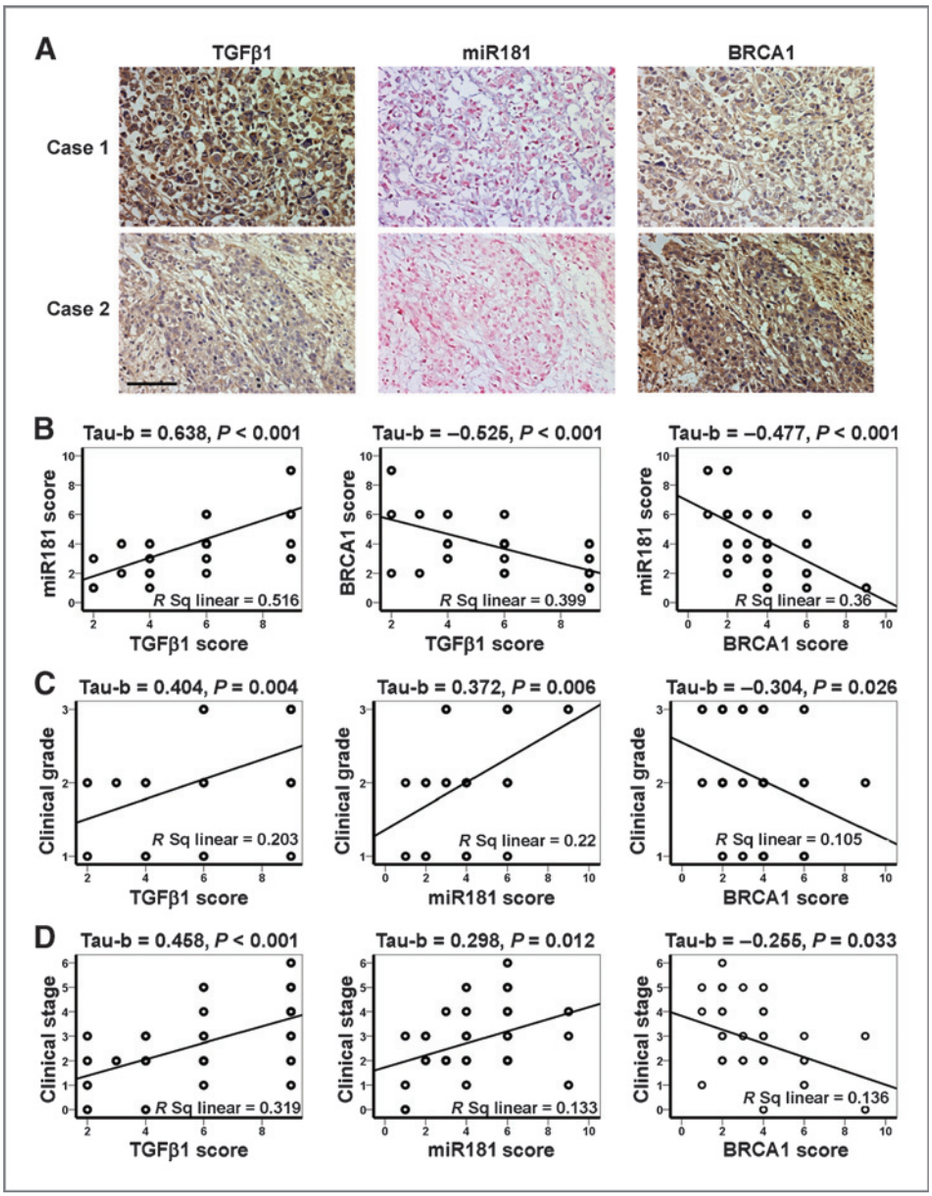


**Figure 7.** TGFβ is associated with miR181 and BRCA1 levels as well as disease progression in primary TNBCs. A, representative images of ISH and IHC staining in primary TNBCs; bar, 100 μm. B to D, levels of TGFβ1, miR181, and BRCA1 were determined by IHC or ISH in a TNBC tissue array ($n = 48$) and scored as described in Materials and Methods. Correlation analyses were carried out among their expression levels (B) and for each of them with clinical grades (C) or stages (D). Kendall Tau-b coefficient, *R* square linear, and *P* values are shown. Clinical stages are scored as: 0, stage 0; 1, stage I; 2, stage IIA; 3, stage IIB; 4, stage IIIA; 5, stage IIIB; and 6, stage IV.

that PARP inhibitors are promising agents for the treatment of TNBCs, which share similar gene-expression profiles and DNA-repair deficiencies with *BRCA1*-associated breast cancers (35, 40). Cells that manifest several recently reported epigenetic silencing mechanisms of *BRCA1/2* expression show enhanced sensitivity to PARP inhibition. These include hypermethylation of *BRCA1* CpG island (41), miRNA-mediated downregulation of *BRCA1* (42–44), and depletion of mitochondrial DNA leading to upregulation of miR1245 and the ubiquitin ligase Skp2 that, respectively, suppress BRCA2 protein translation and stability (45). Interestingly, patients with ovarian cancer carrying *BRCA1/2* mutations have better overall survival than *BRCA1/2* wild-type cases, whereas the survival for epigenetically silenced *BRCA1* cases was similar to *BRCA1/2* wild-type cases, suggesting that patient survival depends on the mechanism of *BRCA* gene inactivation (46). Genomic alterations of other genes that may affect the sensitivity of cancer cells to PARP inhibitors, including the homologous recombination genes *ATM* and *CHEK2* whose mutations have been associated with risk of breast cancers (7, 47) and *PTEN*, have been reported in breast and ovarian cancers (46, 48). In addition, inhibition of cyclin-dependent kinase 1 (CDK1), a kinase that phosphorylates BRCA1 and is, therefore, necessary for BRCA1-mediated functions, has been reported to sensitize MDA-MB-231 cells to PARP inhibition (49). Interestingly, a recent study shows that PARP-1 interacts with multiple MMR proteins and may regulate or participate in MMR (50). On the other hand, MSH2 has been shown to promote HDR (51). It is, therefore, possible that reduced expression of MSH2 results in a partial

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dependence on PARP-1 for DNA repair, which may explain the slightly enhanced sensitivity to PARP inhibition in cells with MSH2 knockdown (Fig. 4C).

Here, we show that TGFβ, a multitasking cytokine frequently elevated in tumor microenvironments, regulates DNA repair by simultaneously suppressing the expression of *ATM*, *MSH2*, and *BRCA1*. This results in a BRCAness phenotype, including impaired DNA-repair efficiency and reduced genomic stability, as well as a synthetic lethality to PARP inhibition. Our *in vitro* and *in vivo* data demonstrate that PARP inhibitors, such as ABT-888, which is under clinical trials for breast cancers, may have a more potent effect on those TNBCs with active TGFβ signaling. This may allow selection of appropriate patients with TNBC based on markers of TGFβ pathway (e.g., TGFβ and phosphorylated SMAD2/3) for PARP-targeting therapy. In addition, other factors that induce the level or activity of miR181 and/or miR21 may also affect the expression of the target genes of the miRNA, including *ATM*, *MSH2*, or *BRCA1*, and therefore may affect tumor response to PARP inhibitors. In fact, a recent study demonstrates that miR181a/b levels inversely correlate with ATM in breast cancers and determine the sensitivity of TNBC cells to PARP1 inhibition (52). Those factors regulating miR181 and miR21 may, therefore, also have values as prognostic markers for PARP-targeted therapy in sporadic breast cancers. Although our focus for this study is on clinically aggressive, hard-to-treat TNBCs that often exhibit active TGFβ signaling, the pathways identified herein may have a general application to understanding cancer and defining treatments.

TGFβ has been implicated in chemoresistance through a variety of mechanisms (2, 8–12, 18). Relevant to the study herein, downregulation of *MSH2* and *ATM*, which serve as sensors of DNA damage upon genotoxic treatment, may contribute to TGFβ-induced resistance to DNA-damaging agents such as doxorubicin (Fig. 5C and F). It is well documented that the inability of MMR-deficient cells to recognize chemotherapy-induced DNA-damage results in a damage-tolerant phenotype and drug resistance (53). In colorectal cancer cells, *MSH2* downregulation by miR21 significantly reduces 5-fluorouracil (5-FU)–induced cell-cycle arrest and apoptosis (36). ATM has a master role in triggering DNA repair upon DSBs, as evidenced by the hypersensitivity of cells from ataxia telangiectasia patients to IR (54), but there is a discrepancy of ATM deletion/suppression on cancer response to DNA-damaging therapies. A recent study revealed a mechanism for the binary effect of loss of ATM on therapeutic response. In P53-deficient tumors, suppression of ATM sensitizes cells to DNA-damaging chemotherapy, whereas in the presence of functional P53, suppression of *ATM* or *CHEK2* protects cells from genotoxic agents by blocking P53-dependent apoptosis (55).

In addition, regulation of the DNA-repair genes by TGFβ is dependent on the cellular context. In noncancerous cells, we observe an opposite inductive effect of TGFβ on MSH2 expression as a result of SMAD-mediated, P53-dependent promoter activation, which is absent due to P53 deficiency or overcome by miR21-mediated downregulation of MSH2 in cancer cells (18). TGFβ downregulates BRCA1, MSH2, and ATM and induces sensitivity to PARP inhibition in MDA-MB-231 and MDA-MB-468 TNBC cells and in BT474 luminal breast cancer cells, but not in MCF7 luminal breast cancer cells (Supplementary Fig. S1). Therefore, the ultimate effects of TGFβ on different DNA-repair pathways and, consequently, on cell response to different types of DNA damage are likely to be context-dependent. A comprehensive assessment of these contextual factors (e.g., P53 status) and the status of various DNA-repair pathways, along with assessment of TGFβ signaling, will likely provide valuable prognostic information leading to individualized treatment of breast cancers.

## Disclosure of Potential Conflicts of Interest

George Somlo is a consultant/advisory board member for Novartis, Celgene, Pfizer, and Quest. No potential conflicts of interest were disclosed by the other authors.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** L. Liu, W. Zhou, C.-T. Cheng, G. Somlo, M.Y. Fong, A.R. Chin, Y. Yu, S.T.F. O'Connor
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** C.-T. Cheng, M.Y. Fong, Y. Xu, J.M. Stark, S.E. Wang
**Writing, review, and/or revision of the manuscript:** L. Liu, C.-T. Cheng, G. Somlo, M.Y. Fong, S.T.F. O'Connor, T.R. O'Connor, J.M. Stark, S.E. Wang
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** W. Zhou, Y. Yu, Y. Xu, S.T.F. O'Connor, S.E. Wang
**Study supervision:** S.E. Wang

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Molecular Cancer Research

# TGFβ Induces "BRCAness" and Sensitivity to PARP Inhibition in Breast Cancer by Regulating DNA-Repair Genes

Liang Liu, Weiying Zhou, Chun-Ting Cheng, et al.

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