DNA Damage Induces NF-κB-dependent MicroRNA-21 Up-regulation and Promotes Breast Cancer Cell Invasion*5

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Background: DNA damage response and miRNAs have been linked to cancer progression. Results: Genotoxic drug induces up-regulation of miR-21 in a NF-κB dependent manner. Significance: Genotoxic NF-κB signaling may serve as a drug target to reduce therapeutic resistance and metastasis in breast cancer.

This article has been withdrawn by the authors. The corresponding author contacted the editorial office to report a concern raised about some figures in this article. An investigation by the Journal determined the following. Lanes 1 and 2 of the NF-κB EMSA shown in Fig. 1E are duplicates. The TAK1 KA panel in Fig. 1G has duplicated areas in which no signal was detected. The Bay11 + Dox and KU + Dox panels in Fig. 2A have overlapping regions. The KU + Dox panel in Fig. 2C was reused in Fig. 3F as Control. Because the original phosphorimager data for Fig. 1 (E and G) could not be found, the authors state that they do not have a definitive means of verifying the data in question in these two panels. The authors state that the issues in Figs. 2A and 3F were due to errors during figure preparation, and the correct cell images for Figs. 2A and 3F were found and submitted to the Journal. Given these errors, the authors state that the responsible course of action is to withdraw the article to maintain the high standards and rigor of the scientific literature from the authors and the Journal. However, the withdrawing authors assert that these errors do not change the underlying scientific findings of the article and that the results of this article are valid.

Although significant progress has been made in early diagnosis and treatment, breast cancer still remains the second

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‡ This article contains supplemental Figs. S1–S3 and Table S1.
3 The abbreviations used are: NF-κB, nuclear factor κB; STAT3, signal transducer and activator of transcription 3; miRNA, microRNA; MSK1, mitogen- and stress-activated protein kinase 1; pCR, quantitative PCR; Dox, doxorubicin; ATM, ataxia telangiectasia mutated; IKK, IκB kinase; TNBC, triple-negative breast cancer; SASP, senescence-associated secretory phenotype; pol, polymerase; MEF, mouse embryo fibroblast.
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expression in a paracrine fashion (13, 14). Therefore, it is possible that chemotherapeutic-induced NF-κB activation may contribute to metastasis by promoting the inflammatory response.

In addition to regulating protein-coding gene transcription, accumulating evidence indicates that NF-κB also regulates microRNA expression (15–20). MicroRNAs (miRNAs) are a class of small noncoding RNAs, which primarily bind to the 3′-untranslated region (3′-UTR) of target mRNA and negatively regulate gene expression at the post-transcriptional level (21). The expression of miRNAs is frequently dysregulated in human malignancies, and miRNAs can function as oncogenes or tumor suppressors (22, 23). Although some miRNAs are down-regulated in human cancers, overexpression of miR-21 was frequently found in various solid tumors (22, 23). Moreover, miR-21 may serve as a promising therapeutic target because of its oncogenic property, and malignant cells become addicted to it during tumor progression (24, 25). As the miRNA overexpressed at the highest level in breast carcinomas compared with normal tissue (26), miR-21 overexpression has been correlated with advanced breast cancer stages, lymph node metastasis, and poor survival in patients (27, 28). However, the mechanisms underlying the regulation of miR-21 expression and the biological significance of miR-21 in breast cancer metastasis upon genotoxic treatment has not been fully elucidated.

In this report, we found that treatment with genotoxic drugs activated NF-κB in triple-negative breast cancer cell lines and contributed to elevated metastatic potential in the presence of genotoxic exposure. Genotoxic NF-κB activation leads to the expression of IL-6 and miR-21, both of which were required for enhanced cancer invasion. The signaling pathway is driven by both NF-κB/p65 and DNA damage, which may trigger NF-κB activation up-regulated by p65. miR-21 promoter also contains the NF-κB-binding sites that enhance DNA damage. Genotoxic NF-κB activation in breast cancer cell migration was significantly increased in cells regulating miR-21 via using miR-21 sponge (29). miR-21 sponge (21972) constructs obtained from Addgene have been described previously (29). Antibodies against PDCD4, PTEN, MSK1, or STAT3 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspase-8, cleaved caspase-3, phospho-MSK1 (Ser-376), p38, phospho-p38 (Thr-180/Tyr-182), and phospho-histone H3 (Ser-28) were from Cell Signaling Technology (Danvers, MA). Anti-H3S10p, anti-H3S28p, and anti-H3 were purchased from Millipore (Billerica, MA). Anti-p-STAT3 (Tyr-705) and neutralizing anti-IL6 antibody were from Calbiochem and R&D (Minneapolis, MN), respectively.

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**RESULTS**

Genotoxic Drugs Activate NF-κB in Triple-negative Breast Cancer Cells—Genotoxic anticancer drugs can induce NF-κB activation in a variety of cancers (31). We found the chemodrug camptothecin or doxorubicin (Dox) treatment induced strong NF-κB activation in MDA-MB-231 cells as determined by gel shift assays (Fig. 1A). We previously showed that NF-κB signaling induced by genotoxic agents depends on IKK, whose activation requires the upstream TAK1 and ATM kinases (9). In accordance, we found TAK1 and IKK were activated in MDA-MB-231 cells by Dox treatment with similar kinetics (Fig. 1B and supplemental Fig. S1, A and B). Depletion of TAK1 in MDA-MB-231 cells with siRNA abolished genotoxic drug-induced activation of IKK and NF-κB (Fig. 1, C and D), which was also inhibited by ATM kinase inhibitor Ku55933 (Fig. 1, E and F). Moreover, Dox-induced TAK1 activation was attenuated by

FIGURE 1. Genotoxic agents activate NF-κB in triple-negative breast cancer cells. A, MDA-MB-231 cells were treated with camptothecin (C, 10 μM, 2 h), camptothecin or doxorubicin (Dox) treatment induced strong NF-κB activation. B, MDA-MB-231 cells were transfected with control or siRNAs targeting TAK1, and treated with Dox (D) or left untreated (-). Total cell lysates were analyzed with in vitro kinase assay to assess activity of IKK or TAK1, using GST-IκBα (input) were analyzed by Western blotting with the indicated antibodies. C, siCtrl and siTAK1 were treated with Dox in the absence or presence of KU55933 (Ku, 1 μM, 2 h), and TNFα (10 ng/ml, 30 min) or left untreated (-). Total cell lysates were analyzed with in vitro kinase assay and IKK kinase assay (input) were analyzed using NF-κB or OCT1 probes (E–G), respectively. Immunoprecipitated samples and total cell extracts were treated with camptothecin (C, 10 μM, 2 h), and TNFα (10 ng/ml, 30 min) or left untreated (-). ELISA was performed in triplicate with human IL-6 ELISA kit (eBioscience, San Diego). The chemodrug camptothecin or doxorubicin (Dox) treatment induced strong NF-κB activation in a variety of cancers (31). We found the chemodrug camptothecin or doxorubicin (Dox) treatment induced strong NF-κB activation in MDA-MB-231 cells as determined by gel shift assays (Fig. 1A). We previously showed that NF-κB signaling induced by genotoxic agents depends on IKK, whose activation requires the upstream TAK1 and ATM kinases (9). In accordance, we found TAK1 and IKK were activated in MDA-MB-231 cells by Dox treatment with similar kinetics (Fig. 1B and supplemental Fig. S1, A and B). Depletion of TAK1 in MDA-MB-231 cells with siRNA abolished genotoxic drug-induced activation of IKK and NF-κB (Fig. 1, C and D), which was also inhibited by ATM kinase inhibitor Ku55933 (Fig. 1, E and F). Moreover, Dox-induced TAK1 activation was attenuated by

Wound Healing Assay—Cells were allowed to reach confluence before wounding the monolayer with a sterile pipette tip. Cellular debris was removed, and cell migration was quantified at 16–24 h after wounding. The motility was determined using the measured migration distance of cells by microscopy. Each group was measured in triplicate.

Invasion Assay—The invasion assay was performed with Matrigel-coated Transwell membrane filter inserts in 24-well culture plates. In brief, MDA-MB-231 cells were trypsinized, counted, and added to the upper chambers of 8-μm pore size Transwell inserts. A total of 1 × 10⁴ cells in a volume of 200 μl was added into each insert, and 600 μl of DMEM containing 10% FBS was added to the lower chamber. The cells in the Transwell plates were incubated at 37 °C for 24 h. Cells that remained in the inserts were removed, and cells that migrated to the underside of the inserts were fixed and stained with 2% crystal violet. The migrated cells were photographed under a microscope and counted from five randomly selected fields.

Enzyme-linked Immunosorbent Assay (ELISA)—Cells were seeded in 6-well plates, and conditioned media were collected after the indicated treatments. ELISA was performed in triplicate with human IL-6 ELISA kit (eBioscience, San Diego).

Statistical Analysis—The results were presented as means ± S.D. and analyzed with Student’s t test. p < 0.05 was denoted as statistically significant.
ATM inhibition (Fig. 1G). Because MDA-MB-231 cells were derived from basal-like triple-negative breast cancer (TNBC), we examined the genotoxic NF-κB signaling in additional TNBC cell lines HCC1937 and MDA-MB-436. Dox was also able to induce robust NF-κB activation in HCC1937 cells, which was blocked by the ATM inhibitor (Ku55933) or IKK inhibitor (Bay-11) (Fig. 1H). Consistently, genotoxic drug-induced NF-κB activation in MDA-MB-436 cells was sensitive to ATM/IKK inhibition (Fig. 1I). Taken together, this evidence suggests genotoxic agents can effectively induce NF-κB activation in TNBC cells in a manner dependent on the sequential activation of ATM, TAK1, and IKK.

Genotoxic NF-κB Activation Enhanced the Invasiveness of MDA-MB-231 Cells through Up-regulating IL-6 Expression—NF-κB is a key regulator of inflammatory response and apoptosis, which contribute to tumor metastasis. Also, acquired therapeutic resistance to radiation and chemotherapy in cancer cells highly correlates with their ability to metastasize (32, 33). Therefore, we examined whether genotoxic NF-κB activation enhances breast cancer cell invasion and migration. As shown in Fig. 2, A and B, Dox treatment significantly increased the invasiveness of MDA-MB-231 cells, which depended on genotoxic NF-κB signaling. Inhibiting either ATM or IKK activity significantly reduced MDA-MB-231 cell invasion upon Dox treatment. Moreover, migration

FIGURE 2. DNA damage-induced NF-κB activation promotes MDA-MB-231 cell migration and invasion. A and B, MDA-MB-231 cells were incubated with Ku55933 (KU) or Bay11-7085 (Bay11) along with Dox, and cell invasion was measured with a modified Boyden chamber assay. Numbers of invading cells were quantified and shown in B. Con, control. C and D, monolayer MDA-MB-231 cells were wounded and incubated with Dox alone or along with Ku55933 or Bay11-7085 for 4 h as shown, and the migrated distance was determined after 24 h and quantitation as shown in D. E, MDA-MB-231 cells were treated as indicated. Cell apoptosis was examined with flow cytometry using annexin V/propidium iodide (PI) staining. F, similar experiments were carried out as in C in the presence or absence of benzylxycarbonyl-VAD-fluoromethyl ketone (z-VAD) (20 μM), and data from three independent experiments were plotted as in D. *, p < 0.05; **, p < 0.01.
of MDA-MB-231 cells was also enhanced by genotoxic NF-κB activation in response to Dox treatment (Fig. 2, C and D). These data suggest that genotoxic NF-κB activation may play a critical role in promoting breast cancer metastasis. Because NF-κB is well known to promote cell survival via inhibiting apoptosis, we reasoned that inhibiting genotoxic NF-κB activation with ATM or IKK inhibitor may enhance MDA-MB-231 cell apoptosis upon Dox treatment, which could lead to the reduced cell migration. Indeed, treatment with ATM or IKK inhibitor significantly increased apoptosis in MDA-MB-231 cells exposed to Dox (Fig. 2E). However, incubation with a pan-caspase inhibitor, benzoxycarbonyl-VAD-fluoromethyl ketone, was not able to restore the cell migration ability hampered by ATM or IKK inhibitor (Fig. 2F), suggesting that genotoxic NF-κB activation may also promote breast cancer cell invasion via additional mechanisms.

NF-κB may promote cancer metastasis through up-regulating the expression of cytokines, such as IL-6 and TNFα, which elicit a robust inflammatory response favoring tumor invasion (34). Because extensive DNA damage may induce IL-6 expression in an NF-κB-dependent manner (11), we analyzed the IL-6 expression in Dox-treated MDA-MB-231 cells by qPCR and ELISA. Dox treatment significantly increased IL-6 transcription in MDA-MB-231 cells, suggesting that NF-κB activation may up-regulate IL-6 in breast cancer cells upon genotoxic treatment (Fig. 3A). Moreover, increased IL-6 secretion was detected in conditional media of MDA-MB-231 cells upon Dox exposure (Fig. 3B), indicating that DNA damage may function in an autocrine and/or paracrine manner to regulate both breast cancer cells and stromal cells. We also found that increased IL-6 transcription by Dox treatment was significantly suppressed when specific ATM or IKK inhibitors were used (Fig. 3C). These data strongly suggested that NF-κB activation-mediated IL-6 expression is essential for the adhesion of breast cancer cells to stromal cells exposed to DNA-damaging agents.

IL-6 was shown to promote metastasis of breast cancer, and local IL-6 expression may also serve as a cue for homing of metastatic breast cancer cells (35, 36). Accordingly, we found that the Dox treatment-induced increase in cell migration and invasion was significantly attenuated in MDA-MB-231 cells expressing an shRNA targeting IL-6 (Fig. 3F and supplemental Fig. S3D) or by treatment with neutralizing anti-IL-6 antibody (Fig. 3G), which mimicked the observation by inhibiting genotoxic NF-κB signaling (Fig. 2). These data indicated that genotoxic NF-κB activation may play a critical role in promoting breast cancer metastasis through up-regulating IL-6 expression.

**DNA Damage Up-regulates miR-21 Transcription via NF-κB in TNBC Cells**—Previous reports have indicated that NF-κB may be responsible for miR-21 transactivation in cells treated with LPS (17, 19). We have shown that interferon (IFN)-induced miR-21 expression in prostate cancer cells required NF-κB/p65 recruitment to the miR-21 promoter, which depended on STAT3 activation (16). To determine whether NF-κB can regulate miR-21 expression upon DNA damage in breast cancer cells, we transfected an miR-21-targeting luciferase reporter into MDA-MB-231 cells and measured the luciferase activity upon Dox treatment. We found that luciferase activity was significantly decreased (~50%) upon Dox treatment, compared with that in untreated cells (Fig. 4A). In contrast, inhibiting miR-21 by overexpression of an miR-21 sponge construct significantly increased miR-21 reporter activity in both untreated and Dox-treated MDA-MB-231 cells, although the Dox-induced decrease was still observed (Fig. 4A). In addition, Dox-induced up-regulation of miR-21 expression was detected by qPCR measuring either mature miR-21 (Fig. 4B) or pri-miR-21 (Fig. 4C). Furthermore, ATM or IKK inhibitors that block the genotoxic NF-κB signaling cascade significantly inhibited miR-21 up-regulation by Dox (Fig. 4, B and C). Consistently, pri-miR-21 induction by Dox was abolished in MDA-MB-231 cells expressing the 1κBα super repressor and ELKS-deficient MEFs (supplemental Fig. S2, A and B). These data strongly argued that Dox-induced increase of miR-21 expression in MDA-MB-231 cells is dependent on genotoxic NF-κB activation.

In agreement, Dox-induced increase of miR-21 expression in MDA-MB-231 cells, Dox treatment-induced up-regulation in HCC1937 cells (Fig. 2F), and Dox-induced increase of miR-21 expression in ATM-deficient MEFs (Fig. 3C) suggest that NF-κB activation-mediated miR-21 expression is conserved between species.

In addition, we identified four potential NF-κB-binding elements in the −134 to −18 region based on promoter sequence analysis. We carried out ChIP analyses to determine whether NF-κB/p65 and pol II, the primary RNA polymerase for miRNA transcription, at the remote NF-κB element (K1), which resides in the promoter region pertaining to the highest transcriptional activity (37). Collectively, these results suggest that NF-κB may serve as a direct transactivator for miR-21 transcription upon DNA damage.

**IL-6-dependent STAT3 Activation Is Required for miR-21 Expression upon Genotoxic Stress**—Previously, we showed that IFN-activated STAT3 coordinated miR-21 expression in prostate cancer cells (16). Interestingly, in MDA-MB-231 cells, genotoxic treatment enhanced IL-6 secretion, which stimulated miR-21 expression through STAT3 activation in multiple myeloma cells (38). This evidence led us to postulate that STAT3 may also be involved in transcriptional regulation of miR-21 upon genotoxic stress. To this end, we examined whether STAT3 is activated by Dox treatment in MDA-MB-231 cells. As expected, Dox treatment induced a delayed STAT3 activation (Fig. 5A), whose kinetics closely correlated with IL-6 secretion (Fig. 3B). Moreover, expression of a dominant-negative mutant of STAT3 (STAT3-Y705F) was able to significantly attenuate the miR-21 induction in Dox-treated MDA-MB-231 cells (Fig. 5B). Furthermore, in contrast to the mild induction of miR-21 in STAT3-deficient prostate cancer PC3 cells upon Dox treatment, reconstitution with STAT3-WT, but not STAT3-Y705F mutant, dramatically enhanced the
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Dox-induced miR-21 transcription in PC3 cells (Fig. 5C). Nevertheless, the deficiency or mutation of STAT3 did not affect NF-κB activation in PC3 cells induced by genotoxic treatment, although STAT3 activation was absent in PC3 cells and STAT3-Y705F-reconstituted cells (supplemental Fig. S3A). These data indicated that STAT3 activation is required for miR-21 up-regulation in response to genotoxic treatment in human cancer cells.

We showed that STAT3 was recruited to miR-21 promoter region adjacent to the remote NF-κB element in PC3 cells treated by IFN (16). In MDA-MB-231 cells, we found that STAT3 binding was also enriched at the same promoter ele-
ment in response to Dox treatment (Fig. 5D). However, in contrast to IFN treatment, Dox-induced NF-κB/p65 recruitment to miR-21 promoter is STAT3-independent (Fig. 5E and supplemental Fig. S3B), suggesting that genotoxic NF-κB signaling was activated upstream of STAT3 activation.

To explore whether genotoxic NF-κB activation-dependent IL-6 expression is essential for DNA damage-induced STAT3 activation, we examined Dox-induced STAT3 activation in the presence of the IL-6-neutralizing antibody. STAT3 activation was remarkably diminished by the IL-6-neutralizing antibody in Dox-treated MDA-MB-231 cells (Fig. 5F). Furthermore, Dox-induced miR-21 up-regulation was significantly inhibited in MDA-MB-231 cells expressing shIL-6 (Fig. 5G). Because we did not observe any overt effect of IL-6-neutralizing antibody on NF-κB activation in Dox-treated MDA-MB-231 cells (supplemental Fig. S3C), it is likely that NF-κB-dependent IL-6 induction is critical for miR-21 up-regulation upon genotoxic stress via activating STAT3.

DNA Damage-induced Histone H3 Phosphorylation Was Required for miR-21 Induction—Recent studies indicated that miRNA transcription is controlled by both upstream DNA transcription regulatory elements, such as conserved transcri-
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Consistently, we detected a significant increase of phosphorylation at H3S10 (H3S10p) and H3S28 (H3S28p) in the miR-21 promoter region around NF-κB-binding sites in Dox-treated MDA-MB-231 cells (Fig. 6A). Moreover, phosphorylation of H3S10 and H3S28 was substantially increased in MDA-MB-231 cells at 4 h after Dox treatment (Fig. 6B). Interestingly, we found phosphorylation of both H3S10 and H3S28 was inhibited by H-89 which inhibits MSK1/2 activity (Fig. 6C). This result was consistent with decreased H3S10p and H3S28p at the miR-21 promoter in the presence of H-89, as detected by ChIP (Fig. 6D). Furthermore, H-89 significantly inhibited Dox-induced transcriptional up-regulation of miR-21 in both MDA-MB-231 and HCC1937 cells, suggesting that MSK1/2 activation and subsequent phosphorylation of H3S10 and H3S28 were required for miR-21 induction in Dox-treated TNBC cells (Fig. 6, D and E).

In agreement with these data, we detected both MSK1 activation and its recruitment at the miR-21 promoter region in response to Dox treatment, which were substantially attenuated by the p38 inhibitor SB203580 (Fig. 6, F and G). MSK1 is activated under stress conditions primarily by p38-dependent phosphorylation (40). Interestingly, a recent report showed that genotoxic treatment may induce an ATM-NEMO-RIP1 com-

FIGURE 5. IL-6-dependent STAT3 activation is required for miR-21 expression upon genotoxic stress. A, MDA-MB-231 cells were treated with Dox (2 μg/ml) for the indicated times. Whole cell lysates were immunoblotted with anti-pY705 STAT3 and tubulin antibodies. B, MDA-MB-231 cells were transfected with vector or STAT3-Y705F mutant, and treated with Dox (2 μg/ml) for 8 h; miR-21 expression was determined by qPCR. C, PC3 cells stably expressing vector, STAT3-WT, or STAT3-Y705F mutant were treated with Dox as shown; miR-21 expression was analyzed by qPCR. Ctrl, control. D, STAT3 and pol II binding to miR-21 promoter region (S1) was analyzed by ChIP in MDA-MB-231 cells upon Dox treatment. Schematic representation of putative STAT3- (S) and NF-κB- (K)-binding sites within the human miR-21 promoter was shown. E, MDA-MB-231 cells were transfected with shSTAT3 and treated with Dox for 6 h; ChIP was performed to analyze STAT3 or p65 binding to miR-21 promoter region (S1/K1) in MDA-MB-231 cells upon Dox treatment. Knockdown efficiency of shSTAT3 was shown by Western blot. N.S., not significant. F, MDA-MB-231 cells were preincubated with neutralizing anti-IL-6 antibody and treated with Dox; cell lysates were immunoblotted with anti-pSTAT3 and STAT3 antibodies. G, MDA-MB-231 cells were transfected with shIL-6 and treated with Dox; miR-21 expression was determined by qPCR as in B. *p < 0.05.
plex that mediates downstream p38 activation (41). Consistently, we found Dox-induced p38 activation and subsequent MSK1 phosphorylation were remarkably reduced by ATM inhibition (Fig. 6, F and H). Taken together, these data indicate that genotoxic treatment may also induce histone H3 phosphorylation at the miR-21 promoter region, likely via ATM/p38/MSK1 signaling cascade, to facilitate miR-21 up-regulation in breast cancer cells.

miR-21 Regulates Breast Cancer Cell Migration and Apoptosis upon Persistent Dox Treatment—A high miR-21 level was associated with advanced tumor stage, lymph node metastasis, and poor survival in breast cancer patients (27, 28). Accordingly, we found antagonizing miR-21 significantly inhibited the increased migration in MDA-MB-231 cells exposed to Dox (Fig. 7A), suggesting miR-21 up-regulation may promote breast cancer metastasis during chemotherapy. To further explore the potential mechanisms involved in miR-21-promoted metastasis, we examined the expression of a validated miR-21 target gene PTEN. We found the protein level of PTEN was gradually decreased in MDA-MB-231 cells following extended Dox treatment (Fig. 7B). Moreover, the decrease of PTEN upon Dox treatment was rescued by expression of the miR-21 sponge or a dominant-negative mutant of STAT3 (Fig. 7C). These results indicated that STAT3-mediated miR-21 up-regulation upon DNA damage is responsible for the PTEN repression in MDA-MB-231 cells. Besides PTEN’s canonical function in regulating apoptosis through Akt inactivation, severe PTEN deficiency also correlates with advanced breast tumor stage, suggesting a potential role of PTEN in inhibiting cancer cell migration and/or metastasis (42). Therefore, miR-21-dependent PTEN repression may contribute to both therapeutic resistance and metastasis of breast cancer.

Genotoxic agent-induced NF-κB activation was shown to protect cancer cells from DNA damage-induced apoptosis, which may be a major mechanism of therapeutic resistance development in human malignancies (6). In addition to PTEN,
the miR-21 target gene PDCD4 may play a role in regulating apoptosis (43). We found PDCD4 expression was also decreased in MDA-MB-231 cells upon Dox treatment, and its down-regulation was reversed by expressing miR-21 sponge (Fig. 7D), indicating miR-21 may repress PDCD4 expression in Dox-treated MDA-MB-231 cells. Furthermore, overexpression of the miR-21 sponge significantly augmented MDA-MB-231 cell apoptosis induced by Dox treatment (Fig. 7E), suggesting that miR-21 may function as an anti-apoptotic effector of NF-κB activation in cancer cells treated with genotoxic agents. Down-regulation of PDCD4 by miR-21 has been shown to stimulate invasion, intravasation, and metastasis in colorectal cancer (44), and improved cell survival is likely to contribute to tumor metastatic potential. Therefore, miR-21-mediated PDCD4 repression may be also involved in increased breast cancer cell invasion upon genotoxic treatment.

**DISCUSSION**

Genotoxic treatment-induced NF-κB activation has been associated with tumor therapeutic resistance and relapse, based on the anti-apoptotic property of NF-κB (6, 7). Here, we provide additional evidence that genotoxic NF-κB activation may contribute to cancer metastasis via coordinately regulating the expression of pro-inflammatory cytokines and miRNAs (Fig. 7F). Consistent with a previous report (11), we found Dox treatment significantly increased IL-6 expression in MDA-MB-231 cells. Interestingly, IL-6 along with IL-8 were identified as two major cytokines of SASP induced by DNA damage in senescent...
cells (12). By paracrine effects of SASP, senescent cells may promote the proliferation and tumorigenesis of epithelial cells, stimulate angiogenesis, trigger an epithelial to mesenchymal transition, accelerate the invasion of malignant cells, and facilitate the growth of secondary tumors in cancer patients treated with DNA-damaging chemotherapy (13, 14). NF-κB has been proposed to be a major transcription factor regulating the expression of many SASP components (45, 46). A recent study further indicated that IL-6 can enhance NF-κB activation, thereby completing a positive feedback loop that further promotes tumor growth (47). NF-κB-dependent up-regulation of IL-6 and miR-21 may cooperatively enhance the therapeutic resistance and invasiveness in certain breast cancer subtypes, such as basal-like TNBC. It was found that chemotherapy increased the ability of breast cancer cells to metastasize (48), and TNBC is characterized by rapidly rising chemo-resistance (49). It is plausible that NF-κB activation upon genotoxic chemotherapy may contribute to both therapeutic resistance and increased metastasis in TNBC cells.

IL-6 has been shown to induce miR-21 via STAT3 activation, which promotes survival of multiple myeloma cells (38). Moreover, STAT3-driven miR-21 transactivation was found in other types of cancer, such as prostate cancer (16), glioma (50), and colon cancer (51), in response to IL-6 or IFN treatment. Our data suggested that IL-6-dependent STAT3 activation also promotes miR-21 transcription in breast cancer cells upon genotoxic stress. NF-κB was not only required for IL-6 upon DNA damage but also was recruited to the promoter, along with STAT3, which forms a complex to drive miR-21 transcription. Interestingly, we found that miR-21 transcription can be regulated by various transcription factors in different cell types and stimuli. In IFN-treated MDA-MB-231 cells, miR-21 associates with STAT3 to activate transcription of the NF-κB/p65 recruitment to miR-21 promoter, along with STAT3, which forms a complex to drive miR-21 transcription. Interestingly, we found that miR-21 transcription can be regulated by various transcription factors in different cell types and stimuli. In IFN-treated MDA-MB-231 cells, miR-21 associates with STAT3 to activate transcription of the NF-κB/p65 recruitment to miR-21 promoter, along with STAT3, which forms a complex to drive miR-21 transcription. In MMTV-PyMT-induced mouse mammary adenocarcinomas and human breast carcinomas, hypoxia-induced miR-21 required both NF-κB and cAMP-response element-binding protein (18). Besides, these transcription factors, AP-1 and androgen receptor have also been shown to regulate miR-21 induction upon PMA or androgen treatment, respectively (52, 53). Altogether this evidence suggests that miR-21 transcription can be regulated by distinct transcription factors, independently or collaboratively, in different cell types, which may also be controlled by specific signaling pathways.

DNA damage has been shown to induce epigenetic modifications such as histone phosphorylation and acetylation (54, 55). Interestingly, H3S10p and H3S28p were found to be inhibited in U2OS cells at 2 h after treated with ionizing radiation or phleomycin, and this inhibition was attributed to the decrease of M-phase due to cell cycle arrest upon DNA damage (54). We also observed the decrease of H3S10p and H3S28p at 2 h after Dox treatment in MDA-MB-231 cells. However, a significant increase of phosphorylation of H3S10 and H3S28 was detected at 4 h after Dox treatment, which is consistent with the kinetics of Dox-responsive miR-21 induction. Our data also suggested that instead of Aurora B kinase, which is responsible for H3S10p and H3S28p during mitosis (56), MSK1 was the primary kinase to phosphorylate H3S10 and H3S28 upon genotoxic stress. H3S10 and H3S28 phosphorylation have been shown to play an important role in decondensing chromatin needed for transcriptional activation of genes (56). Cytokine treatment-induced phosphorylation of H3S10 by IKKa may be critical for the activation of NF-κB-driven gene expression (57, 58). UV radiation was also shown to induce H3S28 phosphorylation by JNK (59). Our data further suggest that DNA damage-induced ATM activation may lead to H3S10 and H3S28 phosphorylation via sequential activation of p38 and MSK1, which may provide an open chromatin structure at the promoter region of miR-21 gene, resulting in effective recruitment of transcription regulators, such as NF-κB and STAT3. It is likely that both epigenetic changes and transcription regulator recruitment are required for quick and efficient transactivation of DNA damage-responsive genes.

Usually each mRNA transcript harbors numerous miRNA recognition elements in its 3’UTR region, the same miRNA recognition element can bind to a large number of mRNA transcripts. It is notable that a group of genes, including PTEN, PDCD4, PTGS2, NFIB, TIMP3, ROHB, etc., have been identified as miR-21 target genes, among which PTEN is a tumor suppressor, which is involved in inhibiting proliferation and promoting apoptosis. However, it is likely that additional genes are also involved in promoting these pathological processes, which may also be targeted by miR-21 upon genotoxic stress. These features make miR-21 an attractive drug target whose inhibition may lead to a broad impact on a group of genes involved in cancer progression. Although we only examined the miR-21 induction in cancer cells upon genotoxic drug treatment, it is plausible that the tumor stromal cells may also respond to cancer cell-released inflammatory cytokines (such as IL-6) and/or genotoxic treatment directly by increasing miR-21 expression, resulting in tumor microenvironment remodeling and cancer progression. In fact, inactivating miR-21 has been shown to result in complete tumor regression in a murine model (25). Our data indicate that genotoxic stress-induced NF-κB activation and consequent IL-6 up-regulation play a pivotal role in orchestrating miR-21 induction in cancer cells exposed to chemotherapy. Interfering with genotoxic NF-κB signaling and/or IL-6 function may serve as promising therapeutic strategies to antagonize cancer therapeutic resistance and metastasis through inactivating miR-21.

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