# Article

# Up-regulation of miR-1245 by c-myc targets BRCA2 and impairs DNA repair

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**BRCA2, a clinical prognostic factor, is significantly up-regulated in mRNA level, while its protein expression is often decreased in sporadic breast cancer. However, how BRCA2 protein expressions are suppressed in these tumors remains unknown. In this study, we demonstrated that miR-1245 directly suppressed BRCA2 3′-UTR and translation, impaired homologous recombination (HR)-mediated repair, reduced DNA damage-induced Rad51 nuclear foci, and rendered cells hypersensitive to γ-irradiation (IR), ultimately inducing high chromosomal abnormalities in normal breast cells and breast cancer cells. Conversely, inhibiting miR-1245 in breast cancer cells enhanced BRCA2 levels and induced resistance to IR. Furthermore, we demonstrated that c-myc up-regulated miR-1245 expression via direct binding to the miR-1245 promoter, which led to down-regulation of BRCA2 and reduction in HR efficiency. Significantly, miR-1245 levels in primary breast tumors correlated with c-myc overexpression and BRCA2 suppression. These findings uncover a BRCA2 regulatory and signaling pathway in sporadic breast cancer and support a functionally and clinically relevant epigenetic mechanism in cancer pathogenesis.**

**Keywords:** BRCA2, miR-1245, DNA damage, c-myc

## Introduction

Germline mutations or deletions in the human breast cancer suppressor gene *BRCA2* not only confer the susceptibility of carriers to early onset of breast and ovarian cancers, but also contribute to the development of pancreatic and prostate cancer as well as the D1 subtype of Fanconi anemia (Ford et al., 1998; Rahman and Stratton, 1998; Peto et al., 1999; Narod and Foulkes, 2004). Mutations of *BRCA2* commonly lead to truncations and loss of function of BRCA2 proteins, contributing to 20%–35% of total hereditary breast cancers, but *BRCA2* mutations are rarely found in sporadic (non-hereditary) breast cancer (Teng et al., 1996; Fackenthal and Olopade, 2007). While the expression level of BRCA2 mRNA was reported to be markedly up-regulated in sporadic breast tumors and may serve as a clinical prognostic factor in breast cancer patients (Bieche et al., 1999; Egawa et al., 2002), the BRCA2 protein expression was found to be frequently down-regulated in sporadic breast cancers (King, 2004; Malone et al., 2009; Li et al., 2010). On the other hand, it was reported that BRCA2 protein depletion induced by UV treatment or by silencing DSS1 could not be rescued either by proteosome inhibitors or by protease

inhibitors, and BRCA2 protein was constitutively ubiquitinated under physiologic conditions but without detectable evidence of proteasomal degradation (Wang et al., 2001; Schoenfeld et al., 2004; Li et al., 2006). These observations suggest that the suppression of BRCA2 protein in cancers might be independent of the ubiquitin/proteasome degradation pathway. However, how BRCA2 proteins are suppressed in these tumors remains unknown.

c-myc, one of the most frequently amplified oncogenes in various types of human cancers, promotes transformation through transcriptional regulation of thousands of genes, which coordinately affects various cellular processes (Meyer and Penn, 2008; Brooks and Hurley, 2009). In addition to inducing transformation, c-myc has been demonstrated to induce genomic instability (Kuttler and Mai, 2006; Prochownik and Li, 2007; Prochownik, 2008). Vafa et al. (2002) showed that deregulated c-myc could induce DNA damage and override the $G_1$ arrest induced by DNA damage. Meanwhile, Karlsson et al. (2003) reported that c-myc overexpression not only causes DNA double-strand breaks (DSBs) but also disrupts DSB repair. However, the precise mechanism of c-myc in the suppression of DSB repair remains poorly understood.

The 3′-UTR has been demonstrated to play important roles in gene expression through regulation of stability, localization,

and translation of the mRNA (Bartel and Chen, 2004; Moore, 2005; Sandberg et al., 2008). The expression of genes in cells could be modulated by expressing mRNAs with shortened 3′-UTRs that contain fewer miRNA target sites, or by expressing pseudogenes that act as decoys for the genuine genes targeted by miRNAs (Ambros, 2004; Sandberg et al., 2008; Poliseno et al., 2010). Recent studies have reported that miRNAs are associated with tumorigenesis through down-regulation of DNA repair genes, which extensively contribute to genomic instability (Crosby et al., 2009; Valeri et al., 2010; Moskwa et al., 2011). Herein, we demonstrated that up-regulation of miR-1245, activated by c-myc, suppressed the expression of BRCA2 protein via direct binding to the 3′-UTR of the BRCA2 transcript, leading to disruption of DNA repair and DNA damage, which suggest that miR-1245-mediated down-regulation of BRCA2 induces genomic instability and may represent a therapeutic target.

## Results

### The 3′-UTR of BRCA2 plays an important role in regulation of BRCA2 expression

To investigate whether the 3′-UTR of BRCA2 mRNA plays a role in the regulation of BRCA2 protein expression, we examined BRCA2 levels in the MCF-7 and MDA-MB-231 breast cancer cells transfected with GFP-BRCA2-3′UTR. Western blotting analysis revealed that ectopically expressing the GFP-BRCA2-3′UTR not only increased the BRCA2 protein level (Figure 1A), but also significantly increased the number and distribution of γ-irradiation (IR)-induced Rad51 nuclear foci (Figure 1B and Supplementary Figure S1A and B). HT1080−1885 cells were further employed to test the effect of the BRCA2 3′-UTR on homologous recombination (HR) efficiency, the major identified function of the BRCA2 protein (Xia et al., 2001; Lio et al., 2004), by using HR reporter system. Two days after BRCA2-3′UTR transfection, HT1080−1885 cells were transfected with I-SceI and then selected with puromycin. As shown in Figure 1C, the HR efficiency of cells transfected with BRCA2-3′UTR was significantly higher than that in control. These results indicate that the BRCA2 3′-UTR contributes to HR/DSB repair through up-regulation of BRCA2 protein.

To further explore the mechanism by which BRCA2 3′-UTR regulates BRCA2 expression, the effect of six fragments cloned from the 3′-UTR of BRCA2 was tested (Figure 1D, left panel). As shown in Figure 1D (middle and right panel), ectopic expression of BRCA2-3′UTR-F3, the third fragment containing nucleotide −320 to −450 of BRCA2 3′-UTR, utmostly increased the expression of BRCA2 protein, as well as the number of IR-induced Rad51 foci and HR efficiency (Supplementary Figure S1C and D), indicating that the F3 region of BRCA2 3′-UTR is pivotal for regulating the expression and function of BRCA2.

### BRCA2 is a bona fide target of miR-1245

Since miRNA regulates gene expression by binding to the 3′-UTR of the targeted mRNA (Wang et al., 2001; Ambros, 2004), we therefore examined whether the effect of BRCA2 3′-UTR on the regulation of BRCA2 expression is mediated by miRNA. Silencing Dicer, Drosha or Ago1, which is associated with formation or function of mature miRNA, was found to increase the expression of

BRCA2 protein, suggesting that miRNA is involved in the regulation of BRCA2 (Supplementary Figure S2A−C).

Since the BRCA2 3′-UTR F3 region was shown above to be critical for the regulation of BRCA2 protein, we further examined which miRNA(s) might target to this region. Analysis using TargetScan and miRanda algorithms identified the BRCA2 3′-UTR F3 region as the theoretical conserved target of miR-1245 (Figure 1E). Western blotting analysis revealed that the BRCA2 protein level was significantly decreased in cells transduced with miR-1245 and increased in those transfected with the miR-1245 inhibitor, a 2′-O-methyl modified antisense oligonucleotide designed specifically to bind to and inhibit endogenous miR-1245 (Figure 1F). However, we found that dysregulation of miR-1245 only had a slight effect on the BRCA2 mRNA level and no effect on the BRCA2 protein half-life (data not shown). Furthermore, overexpressing miR-1245 in MCF-7, MDA-MB-231, and 293FT cells could dramatically inhibit the expression of GFP which contain BRCA2-3′UTR, but did not change the expression of GFP-γ-tubulin, suggesting that miR-1245 specifically affected the BRCA2-3′UTR (Supplementary Figure S3A and B). Additionally, upon transfection with miR-1245, we found a consistent and dose-dependent reduction in luciferase activity of pGL3-BRCA2-3′UTR, which was restored with the miR-1245 inhibitor (Figure 1G, right panel). However, mutation of the miR-1245 binding site at two nucleotides in the seed region of the BRCA2 3′-UTR abrogated the suppressive ability of miR-1245 (Figure 1G, left panel). Collectively, our results demonstrate that BRCA2 is a *bona fide* target of miR-1245.

### miR-1245 induces DNA damage through down-regulation of BRCA2

Next, we evaluated the biological role of miR-1245 in DNA damage. A comet assay showed that miR-1245-transduced cells displayed significantly higher residual DNA damage relative to control cells (Figure 2A). Ectopically expressing miR-1245 significantly decreased, while suppressing miR-1245 increased, the HR efficiency and number of IR-induced Rad51 foci; notably, the inhibitory effects of miR-1245 on the HR efficiency and the formation of IR-induced Rad51 foci could be abolished upon transfection with BRCA2-3′UTR, whereas they were not affected by the BRCA2-3′UTR mutant (Figure 2B and C). Consistent with a BRCA2-deficiency phenotype, we found that up-regulation of miR-1245 resulted in rendering cells hypersensitive to IR; however, inhibition of miR-1245 induced resistance to IR treatment (Figure 2D). In addition, mitotic chromosome analysis showed that, similar to the effect of silencing BRCA2, overexpressing miR-1245 in MCF-7 cells significantly increased the number of chromatid/chromosome breaks per metaphase and complex chromosome rearrangements in the presence of mitomycin C, compared with MCF-7 control cells (Figure 2E). These results suggest that the gain of miR-1245 function leads to a phenotype similar to that with the loss of BRCA2.

Notably, overexpression of miR-1245 not only induced DSBs, but also increased the expression of γ-H2AX (Supplementary Figure S4A), indicating that miR-1245 mediated DSBs without interfering with initial recognition of DSBs. Meanwhile, we found that miR-1245 overexpression did not change the expression of multiple DNA repair-related proteins, such as BRCA1,
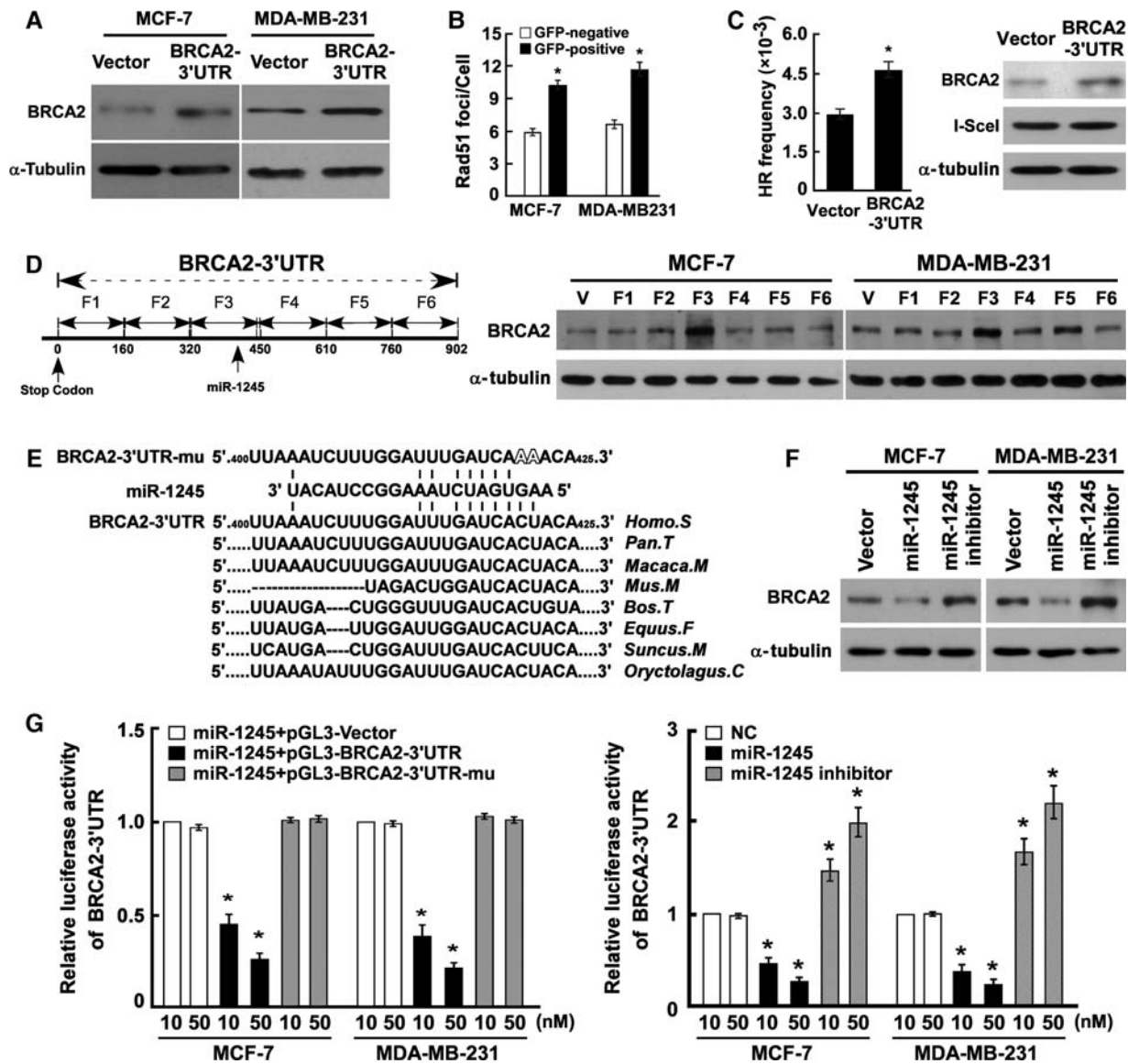
**Figure 1** miR-1245 directly suppresses the 3′-UTR of BRCA2 and its translation. (**A**) Western blot analysis of BRCA2 protein expression in MCF-7 and MDA-MB-231 breast cancer cells transfected with GFP vector or GFP-BRCA2-3′UTR. α-tubulin was used as a loading control. (**B**) Quantification of IR (5.0 Gy)-induced Rad51 foci in GFP-positive and GFP-negative cells. MCF-7 and MDA-MB-231 breast cancer cells were transfected with GFP-BRCA2-3′UTR, and the foci were scored 4.0 h after irradiation in at least 300 GFP-positive and GFP-negative cells. (**C**) DSB-induced HR assay indicated that overexpression of BRCA2-3′UTR increased the spontaneous HR frequency in HT1080−1885 cells. (**D**) Left: schematic illustration of cloned fragments of the human BRCA2-3′UTR. The 3′-UTR region was cloned as six fragments (F1−F6). Middle and right: western blot analysis of BRCA2 protein expression in MCF-7 and MDA-MB-231 breast cancer cells transfected with vector or six fragments of BRCA2-3′UTR. α-tubulin was used as a loading control. (**E**) Predicted miR-1245 target sequence in the 3′-UTR of BRCA2 (BRCA2-3′UTR) and mutant containing two mutated nucleotides in the 3′-UTR of BRCA2 (BRCA2-3′UTR-mu). (**F**) Western blot analysis of BRCA2 expression in MCF-7 and MDA-MB-231 breast cancer cells transduced with miR-1245 or vector, or transfected with miR-1245 inhibitor. α-tubulin was used as a loading control. (**G**) Luciferase assay of indicated cells transfected with pGL3-BRCA2-3′UTR or pGL3-BRCA2-3′UTR-mutant reporter with increasing amounts (10, 50 nM) of miR-1245 mimic (left panel) or miR-1245 inhibitor (right panel). Experiments (**A**−**D**, **F**, **G**) were repeated at least three times with similar results. *$P < 0.05$.

53BP1, and Rad51 (Supplementary Figure S4B), or DDIT4 and EP300 (data not shown), which were two predicated targets of miR-1245. The results of immunoprecipitation (IP) assay showing that miR-1245 had no effect on the interaction between Rad51 and BRCA2 (Supplementary Figure S4C) further support the notion that miR-1245-induced DNA damage may be through specific down-regulation of BRCA2.

*Overexpressing miR-1245 induces genomic instability in human primary normal breast epithelial cells*

To further evaluate the biological effect of miR-1245 on genomic instability, two human primary normal breast epithelial cell (NBEC) lines were established to stably express miR-1245 (Figure 3A). As expected, up-regulation of miR-1245 in both NBECs decreased BRCA2 expression, increased DSBs, reduced
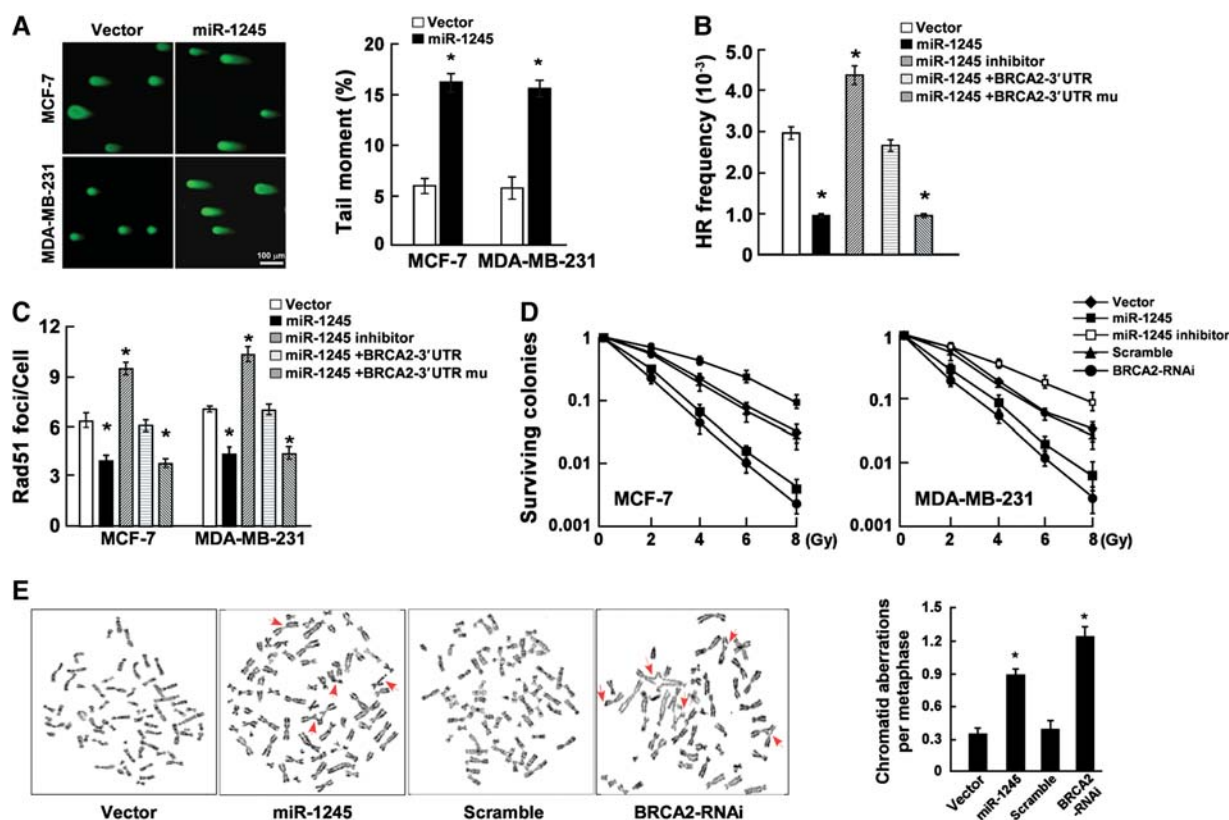
**Figure 2** miR-1245 induces DNA damage through down-regulation of BRCA2. (**A**) Representative images (left) and quantification (right) of unrepaired DSBs were analyzed by the comet assay. MCF-7 and MDA-MB-231 breast cancer cells, transduced with vector or miR-1245, were irradiated (5.0 Gy), allowed to repair for 18 h and then analyzed by the comet assay. Bar, 100 μm. (**B**) DSB-induced HR assay indicated that overexpressing miR-1245 significantly decreased the spontaneous HR frequency through down-regulation of BRCA2 in HT1080−1885 cells. (**C**) Indirect immunofluorescence (IF) staining showed that ectopic expression of miR-1245 in MCF-7 and MDA-MB-231 cells decreased the formation of IR (5.0 Gy)-induced Rad51 foci through down-regulation of BRCA2. The foci were scored 4.0 h after irradiation in at least 300 cells. (**D**) The viabilities of the indicated cells were assayed after indicated doses of γ-radiation by clonogenic cell survival assay. (**E**) Representative pictures (left) and quantification (right) of chromatid aberrations indicated cells treated by mitomycin C. Arrows in panels indicate chromatid/chromosome breaks and complex chromosome rearrangements. Experiments (**A**−**E**) were repeated at least three times with similar results. *$P < 0.05$.

the number of IR-induced Rad51 foci, and enhanced sensitivity to IR treatment (Figure 3B−E). Furthermore, a spectral karyotyping (SKY) assay showed a high frequency of chromosomal aberrations in multiple chromosomes in both miR-1245-overexpressed and BRCA2-silenced cells (Figure 4A and Supplementary Table S1). Moreover, comparative genomic hybridization (CGH) analysis not only demonstrated that up-regulation of miR-1245 caused an extremely high degree of chromosomal abnormalities, as indicated by the alterations on nearly all chromosomes and the considerably increased copy number variations (CNVs), but also showed a remarkable overlap in the patterns of chromosomal aberrations in both miR-1245-overexpressed and BRCA2-silenced cells (Figure 4B and C and Supplementary Figure S5). Collectively, our results demonstrate that miR-1245 plays an important role in inducing genomic instability in normal breast cells.

*c-myc transcriptionally up-regulates miR-1245*

Analysis of the miR-1245 promoter region (∼8.5 kb upstream of the miR-1245 stem loop) using the CONSITE program predicted nine binding sites (E-box) for c-myc, suggesting that c-myc might regulate miR-1245 (Figure 5B, left panel).

Indeed, we found that miR-1245 expression was up-regulated in c-myc-elevated cells and down-regulated in c-myc-silenced cells (Figure 5A). The luciferase reporter assay showed that overexpressing c-myc significantly increased the luciferase activity driven by the fifth E-box in the miR-1245 promoter (Figure 5B, right panel). However, neither overexpression nor knockdown of c-myc had any effect on the luciferase activities of the miR-1245 promoter that contained deleted or mutated fifth E-box (Figure 5C and D). Results of the chromatin immunoprecipitation (ChIP) assay indicated that endogenous c-myc could bind to the fifth E-box region (Figure 5E), which was further supported by the elevated binding of H3K4me3 on the same region of the miR-1245 promoter (Supplementary Figure S6). Taken together, our results demonstrate that c-myc up-regulates miR-1245 expression through directly targeting its promoter.

*c-myc down-regulates BRCA2 through up-regulation of miR-1245*

We then asked whether c-myc-mediated miR-1245 up-regulation contributes to BRCA2 protein suppression. As shown in Figure 5F and G, overexpressing c-myc decreased,
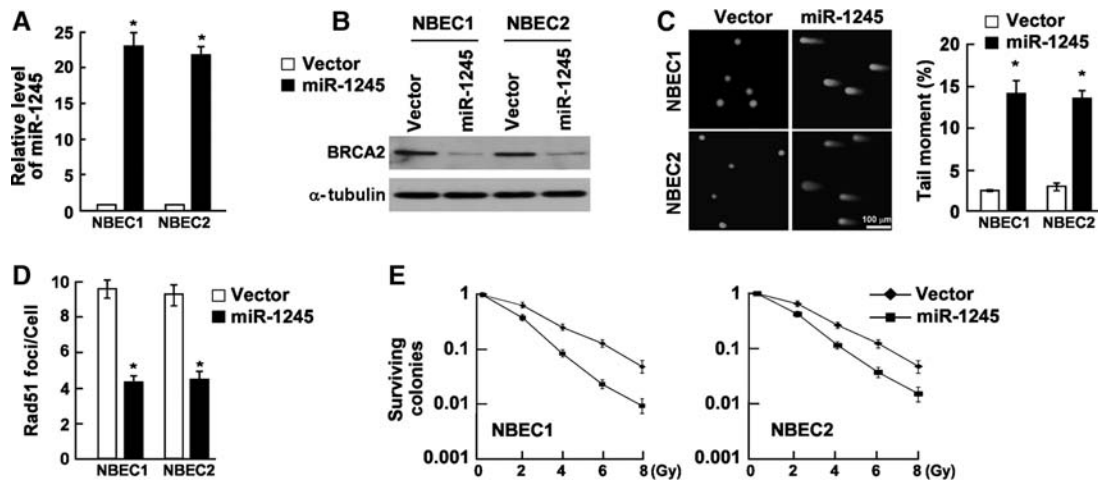
**Figure 3** Overexpressing miR-1245 induces DNA damage in human primary NBECs. (**A**) Real-time PCR analysis of miR-1245 expression in two NBECs transduced with vector and miR-1245. Transcript levels were normalized by *U6* expression. (**B**) Western blot analysis of BRCA2 expression in two NBECs transduced with vector and miR-1245. (**C**) The representative images (left) and quantification (right panel) of unrepaired DSBs in NBECs were analyzed by the comet assay. Vector or miR-1245-transduced NBECs were irradiated (5.0 Gy), allowed to repair for 18 h, and then analyzed by the comet assay. Bar, 100 μm. (**D**) Quantification of IR (5.0 Gy)-induced Rad51 foci in NBECs transduced with vector and miR-1245. The foci were scored 4.0 h after irradiation in at least 300 cells. (**E**) Viabilities of indicated NBECs after indicated doses of γ-radiation were examined by a clonogenic cell survival assay. Experiments (**A**–**E**) were repeated at least three times with similar results. *$P < 0.05$ versus control group.

while silencing c-myc increased, the expression of BRCA2 protein in the MCF-7 and MDA-MB-231 breast cancer cells. Luoto et al. (2010) recently reported that c-myc binds to the BRCA2 promoter, which prompted us to examine whether c-myc regulates BRCA2 protein at the transcriptional level. Our results showed that the expression levels of BRCA2 mRNA slightly decreased in the c-myc-overexpressing cells and increased in the c-myc knockdown cells. However, the inhibitory effect of c-myc on BRCA2 mRNA could be abrogated by transfection with the miR-1245 inhibitor (data not shown). Meanwhile, the luciferase activity of the BRCA2 3′-UTR was decreased in the c-myc-overexpressing cells but increased in the cells transfected with c-myc siRNA(s) in a dose-dependent manner (Supplementary Figure S7A). However, either overexpressing or silencing c-myc had no effect on the luciferase activity of the mutated BRCA2 3′-UTR that contained point mutations in the miR-1245 binding site (Supplementary Figure S7B). These results indicate that c-myc may mediate BRCA2 down-regulation through up-regulation of miR-1245.

It is known that c-myc can bind to the Rad51 promoter and positively regulate its expression (Luoto et al., 2010). Interestingly, we observed that c-myc overexpression resulted in decreased IR-induced Rad51 foci formation (Figure 5H). This may be caused by the reduction in BRCA2 induced by c-myc. As expected, ectopically expressing c-myc reduced the HR efficiency (Figure 5I). In addition, the inhibitory effect of c-myc on IR-induced Rad51 foci, HR efficiency, and BRCA2 expression were abolished upon transfection with the miR-1245 inhibitor or BRCA2-3′UTR (Figure 5H–J). Moreover, we found that ectopically expressing miR-1245 drastically down-regulated BRCA2 in c-myc knockdown cells (Supplementary Figure S7C). Taken together, these results suggest that miR-1245 is required for c-myc-mediated BRCA2 down-regulation.

*Clinical relevance of c-myc, miR-1245, and BRCA2 in human cancers*

Finally, we examined whether the c-myc/miR-1245/BRCA2 axis identified by our study is clinically relevant. In 63 breast cancer specimens, we found that miR-1245 expression inversely correlated with BRCA2 protein expression ($P < 0.01$), but strongly positively correlated with c-myc protein levels ($P < 0.05$) (Figure 6A). We also observed a statistically inverse correlation between the expressions of c-myc and BRCA2 ($P < 0.01$) (Supplementary Figure S8A and B). Moreover, statistical analysis of nine freshly collected breast tumors revealed that miR-1245 expression inversely correlated with BRCA2 expression ($r = -0.800$, $P < 0.05$) and strongly positively correlated with c-myc levels ($r = 0.717$, $P < 0.05$), accompanied by an inverse correlation between BRCA2 and c-myc levels ($r = -0.733$, $P < 0.05$) (Figure 6B). These data further strengthen the notion that c-myc up-regulates miR-1245 expression and down-regulates BRCA2 protein.

**Discussion**

While BRCA2 mRNA expression was reported to be significantly up-regulated in sporadic breast tumors and represented a clinically prognostic factor in breast cancer patients, BRCA2 protein expression was found to be suppressed in breast tissues (Bieche et al., 1999; Egawa et al., 2002; King, 2004; Malone et al., 2009; Li et al., 2010). However, the precise mechanism by which BRCA2 protein is suppressed in sporadic breast tumors has long been a puzzle. Herein, we demonstrated that miR-1245 directly suppressed the BRCA2 3′-UTR and its translation, providing new insights in this context. Based on our results, we propose that c-myc acts on the promoter of miR-1245 and up-regulates its expression in breast cancer. We
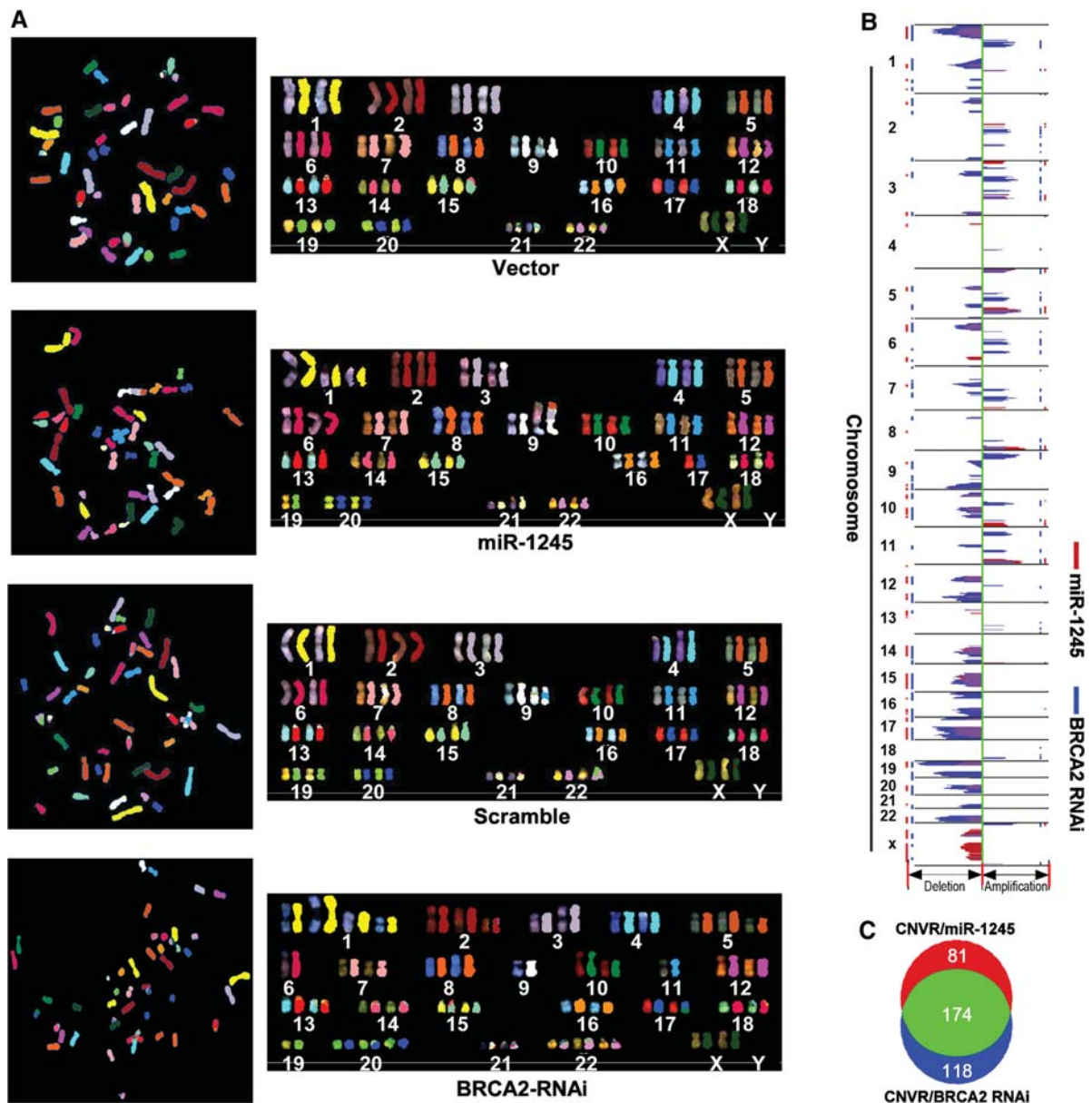
**Figure 4** Overexpressing miR-1245 induces genomic instability in human NBECs. (**A**) SKY of human primary NBECs transduced with miR-1245 or BRCA2 shRNA. SKY analysis revealed that overexpression of miR-1245 or ablation of BRCA2 induced chromosomal instability. The representative pictures of metaphase spread of primary NBEC transduced with vector, miR-1245, or Scramble, or BRCA2 shRNA. (**B**) Genomic DNA, isolated from primary NBECs either transduced with vector, miR-1245, Scramble, or BRCA2 shRNA, was analyzed by CGH assay. The lines appearing above or below the green line represent DNA amplification and deletion, respectively. (**C**) CGH analysis showed that the distribution of chromosomal aberrations obviously overlapped in miR-1245-overexpressing and BRCA2 shRNA-silenced cells. CNVs in miR-1245-overexpressing cells and BRCA2-silenced cells are shown.

observed that miR-1245 could suppress BRCA2 expression, thereby reducing HR/DSB repair and DNA damage-induced Rad51 nuclear foci, as well as rendering cells hypersensitive to IR. The ultimate effects of miR-1245 expression were found to be the significant induction of chromosomal abnormalities of normal breast cells and breast cancer cells.

The major identified function of BRCA2 protein is the direct interaction and modulation of DNA binding and recombination activities of the Rad51 recombinase that coordinates the repair of DSBs by HR, which guards the genomic integrity of cells (Xia et al., 2001). Cells with loss of wild-type BRCA2 function display

severe impairment in HR repair of DNA DSBs, hypersensitivity to DNA damaging agents, and extensive spontaneous chromosomal instability (Rahman and Stratton, 1998; Narod and Foulkes, 2004). Furthermore, conditional knockout of *BRCA2* in mammary glands results in a higher incidence of tumors after extended latency (Ludwig et al., 2001). Hence, understanding the molecular mechanism underlying the regulation of BRCA2 in breast cancer has received much attention.

Loss of heterozygosity of the BRCA2 locus has been detected in sporadic breast tumors (Gudmundsson et al., 1995; Lancaster et al., 1996), which theoretically results in suppression of the
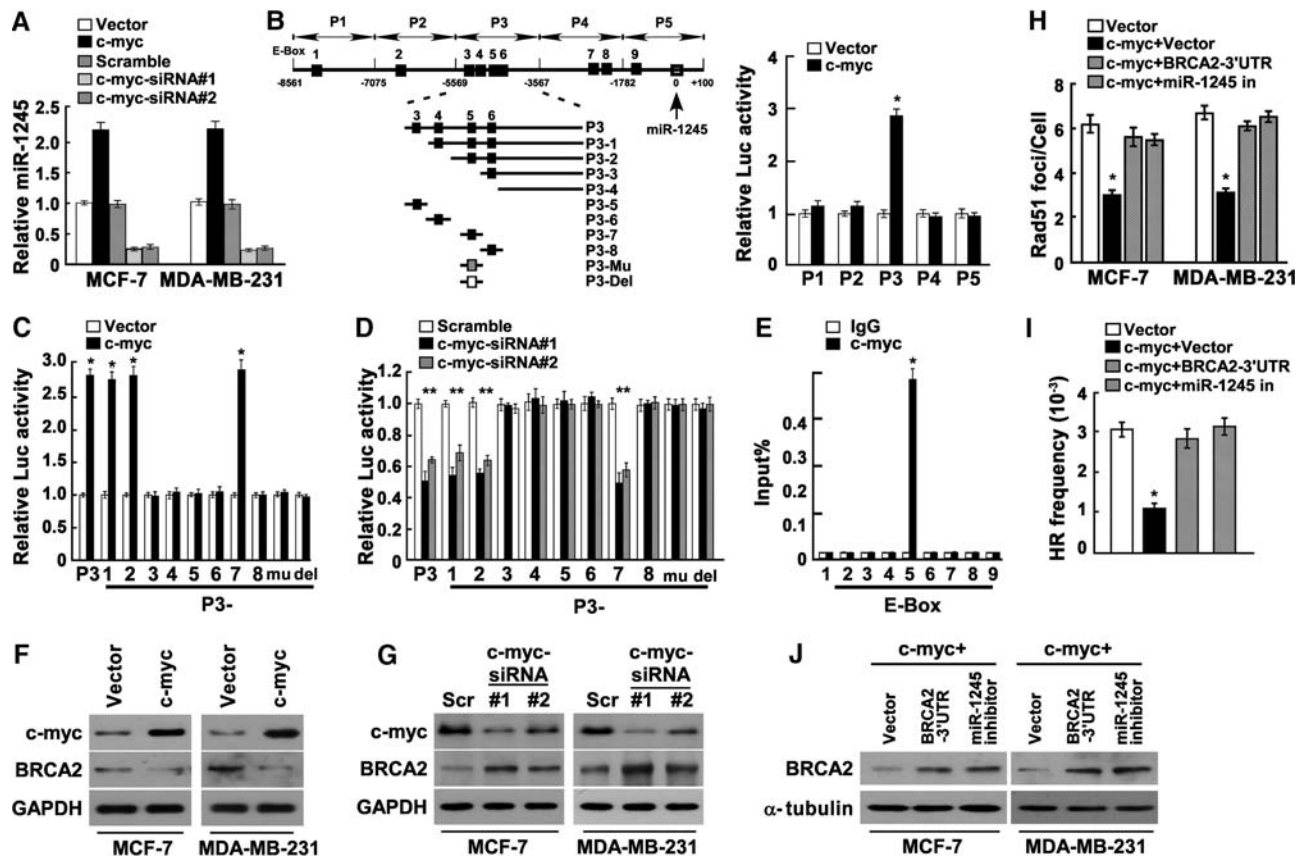
**Figure 5** c-myc down-regulates BRCA2 through up-regulation of miR-1245. (**A**) Real-time PCR analysis of miR-1245 expression in the cells transfected with c-myc or c-myc siRNA(s). Transcript levels were normalized by *U6* expression. (**B**) Left: schematic illustration of cloned fragments of the human miR-1245 promoter. The promoter region of miR-1245 was cloned as 5 fragments (P1–P5); right: transactivity of c-myc on serial miR-1245 promoter fragments as indicated in MCF-7 cells. The effect of c-myc (**C**) or c-myc siRNA(s) (**D**) on the transactivity of serial fragments of P3 region in miR-1245 promoter as indicated in MCF-7 cells. The P3 region of miR-1245 promoter was further cloned as eight fragments (P3-1–P3-8); and the fifth E-box in the P3–5 region was mutated (P3-mu) or deleted (P3-del). (**E**) Regions of miR-1245 promoter physically associated with c-myc were analyzed using the ChIP assay. Western blotting analysis of BRCA2 and c-myc expressions in the cells transfected with c-myc (**F**) or c-myc siRNA(s) (**G**). (**H**) The quantification of IR (5.0 Gy)-induced Rad51 foci was analyzed in indicated breast cancer cells. The foci were scored 4.0 h after irradiation in at least 300 cells. (**I**) The spontaneous HR frequencies were analyzed in HT1080–1885 cells co-transfected with c-myc plus vector, or plus BRCA2-3′UTR, or plus miR-1245 inhibitor, using the DSB-induced HR assay. (**J**) Western blot analysis of BRCA2 expression in the cells transfected with c-myc plus vector, or c-myc plus BRCA2-3′UTR, or c-myc plus miR-1245 inhibitor. α-tubulin was used as a loading control. Experiments (**A**–**J**) were repeated at least three times with similar results. *$P < 0.05$ versus control group; **$P < 0.01$ versus control group.
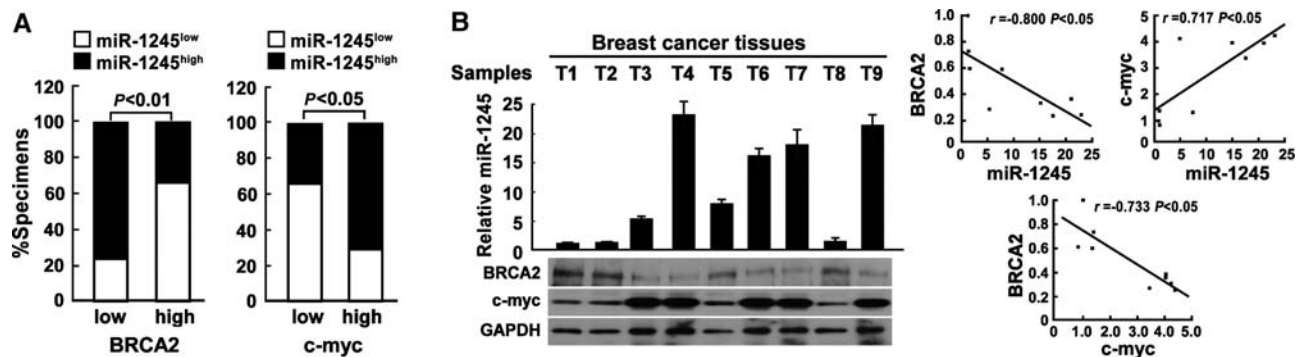


**Figure 6** Clinical relevance of c-myc, miR-1245, and BRCA2 in human cancers. (**A**) Percentages of breast cancer specimens ($n = 63$) showing low or high expression of miR-1245 relative to the expression levels of BRCA2 protein and c-myc protein. (**B**) Expression (left) and correlation (right) analyses of miR-1245, BRCA2 protein and c-myc protein in nine freshly prepared human breast cancer samples.

BRCA2 transcript. Meanwhile, the results presented by multiple previous reports have also shown that the mRNA expression of BRCA2 is significantly elevated in breast cancer tissues, but the expression of BRCA2 protein was frequently down-regulated in most sporadic breast cancers (Bieche et al., 1999; Egawa et al., 2002; King, 2004; Malone et al., 2009; Li et al., 2010), which highlights the importance of understanding the molecular mechanism of post-transcriptional regulation of BRCA2. Interestingly, degradation of BRCA2 proteins induced by UV irradiation or silencing DSS1 was reported to be mediated through a proteosome and protease-independent mechanism (Wang et al., 2001; Li et al., 2006). Schoenfeld et al. (2004) also found that constitutively ubiquitinated BRCA2 does not result in proteasomal degradation, suggesting that ubiquitination of the BRCA2 protein may be linked with activation and/or regulation of its functions instead of protein degradation. Together, these results imply that the suppression of BRCA2 protein in breast cancer may be caused by repression at the translational level. Hence, our finding that miR-1245 directly targeted the BRCA2 3′-UTR and repressed BRCA2 protein expression uncovered a new mechanism underlying BRCA2 regulation in sporadic breast cancer and may aid in identifying novel therapeutic targets.

Biological and clinical lines of evidence have established that BRCA1/BRCA2-mutant cells are extremely sensitive to inhibitors of poly(ADP-ribose) polymerase (PARP), which has been developed as therapeutic agents for patients with BRCA1/BRCA2-mutant cancer (Fong et al., 2009; Aly and Ganesan, 2011). Thus, it would be of great interest to investigate whether the expression level of miR-1245 can be used as a valuable biomarker for deciding on clinical use of PARP inhibitors. Recently, Moskwa et al. (2011) reported that up-regulation of miR-182 impairs HR efficiency and renders cells hypersensitive to IR and PARP inhibitors through down-regulation of the BRCA1 protein, suggesting that overexpressing miR-182 confers susceptibility to PARP inhibition. However, BRCA1/BRCA2-mutant cells can acquire the ability to resist PARP inhibitors through development of secondary mutations in BRCA2 that regain, at least partially, the HR-mediated repair function (Sakai et al., 2008; Wang and Figg, 2008). In this respect, determining whether overexpressing miR-1245 enhances the sensitivity to PARP inhibitors in breast cancer cells, especially in the cancer cells with BRCA2 reversion mutations, may represent a new therapeutic target for BRCA2-mutant cancer.

Overexpression of c-myc has been reported to be associated with amplification of certain genomic loci, inducing chromosomal rearrangements, tetraploidy and aneuploidy, indicating that c-myc overexpression can induce locus-specific gene amplification and whole chromosome breakage (Kuttler and Mai, 2006; Li et al., 2007; Prochownik and Li, 2007; Prochownik, 2008). Although multiple studies have documented that overexpression of c-myc resulted in DSBs through disruption of their repair (Vafa et al., 2002; Karlsson et al., 2003; Prochownik and Li, 2007), the precise mechanism of c-myc on induction of DSBs remains unclear. In the current study, we demonstrated that c-myc up-regulates miR-1245 expression via direct binding to the miR-1245 promoter, which led to down-regulation of BRCA2 and reduction in HR efficiency, suggesting that c-myc induces DSBs

and prevents their repair, at least to some extent, through down-regulation of BRCA2.

Genomic instability, which leads to widespread chromosomal damage and various genetic alterations and extensively contributes to perhaps all types of human cancers, has been identified as an evolving hallmark of almost all human cancers (Murga and Fernandez, 2007; Ricke et al., 2008; Negrini et al., 2010). Defects in any of the caretaker genes that are responsible for repairing DNA breaks and maintaining chromosomal integrity, such as *BRCA2*, would lead to genomic instability (Nowell, 1976; Loeb, 1991; Fishel et al., 1993). However, to date, the molecular mechanism regulating BRCA2 proteins in sporadic tumors has not been addressed. Our study provides key evidence supporting the notion that an epigenetic mechanism is functionally and clinically relevant to the pathogenesis of human cancer. Understanding the precise role played by miR-1245 in inducing genomic instability will not only increase our knowledge of the biological basis of the cancer, but its inhibition may also allow development of a novel therapeutic strategy and identify determinants of clinical outcomes for patients with breast cancer.

## Materials and methods
### Cells
NBECs were collected from the mammoplasty material of two women at the Department of Plastic Surgery, the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China), in accordance with rules and regulations concerning ethical issues on research use of human subjects in China, and were cultured in the keratinocyte serum-free medium (Invitrogen) supplemented with epithelial growth factor, bovine pituitary extract, and antibiotics (120 μg/ml streptomycin and 120 μg/ml penicillin). Breast cancer cell lines, including MCF-7 and MDA-MB-231, were grown in the DMEM (Invitrogen) supplemented with 10% fetal bovine serum (HyClone).

### Patient information and tissue specimens
This study was conducted on a total of 63 paraffin-embedded breast cancer samples and 9 freshly resected samples, which were histopathologically and clinically diagnosed at the Sun Yat-sen University Cancer Center from 2000 to 2010. The stages of the 63 breast cancer samples were as follows: 7 cases at stage I disease, 21 at stage II, 34 at stage III, and 1 at stage IV. The stages of the 9 freshly resected samples were as follows: 1 cases at stage I disease, 4 at stage II, and 4 at stage III. Clinical and clinicopathological classification and staging were determined according to the American Joint Committee on Cancer (AJCC) criteria (Greene et al., 2002). For the use of these clinical materials for research purposes, prior patients' consents and approval from the Institutional Research Ethics Committee were obtained.

### Rad51 focus detection
Cells were irradiated with 5.0 Gy the day after transfection, and 4.0 h later were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.2% (v/v) Triton X-100 in phosphate-buffered saline, and stained with a 1:100 dilution of rabbit anti-Rad51 polyclonal antibody (BD Pharmingen). After washing, the primary antibody was visualized with rhodamine-conjugated goat anti-rabbit IgG

and nuclei were visualized with DAPI. Foci were observed using a laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co., Ltd).

### DSB-induced HRR assay

The HT1080−1885 cell line, with a copy of installed HR substrate, was used in DSB-induced HR assay as described in previous study (Lio et al., 2004). Human HT1080 cells, 2 days after transfection with indicated plasmids or oligonucleotides, were further transfected with an equal amount of pCMV-3NLS-I-SceI plasmid that expresses NLS-tagged I-SceI enzyme or an empty vector. Forty-eight hours later, $2 \times 10^5$ cells were seeded to 10 cm dishes and selected with puromycin (1.0 μg/ml). After 7−10 days selection, the cells that underwent I-SceI-induced HR formed colonies were counted after staining. The HR frequency was measured as the number of puromycin-resistant colonies per $1 \times 10^6$ viable cells. The spontaneous HR frequency was measured in cells transfected with an empty pCMV vector. An average of three to four experiments was analyzed by Student's $t$-test to determine statistical significance.

### Single-cell gel electrophoresis (comet) assay

Comet assay was performed as per the manufacturer's instructions (Trevigen). Briefly, treated or untreated cells were collected, resuspended in ice-cold PBS at $1 \times 10^5$ cells/ml, mixed with low-melt agarose (1:10 ratio), and spread on frosted glass slides. After the agarose solidified, the slides were successively placed in lysis and alkaline solutions (Trevigen). Slides were then subjected to electrophoresis (1 V/cm of distance between electrodes) for 10 min in $1 \times$ TBE buffer, and cells were fixed with 70% ($v/v$) ethanol and stained with SYBR Green. DNA damage was quantified for 100 cells for each experimental condition by determining the tail moment, a function of both the tail length and intensity of DNA in the tail relative to the total DNA, using the software Comet Score (TriTek). Gray level images were acquired under a laser scanning microscope. Statistical analysis was done using Student's $t$-test.

### Spectral karyotyping

The slide was treated with DNase-free RNase solution (0.1 mg/ml) at 37°C for 1 h, washed in $2 \times$ SSC (sodium chloride and sodium citrate) for 10 min at room temperature, dehydrated in 70%, 85%, and 95% ethanol at room temperature for 2 min, and air-dried. Next, the slide was treated with proteinase K (0.05 g/ml) at 37°C, washed in $2 \times$ SSC at room temperature, fixed in 1% paraformaldehyde, and washed in $2 \times$ SSC at room temperature, for 10 min each, followed by dehydration in ethanol as above. The slide was placed in 70% formamide/$2 \times$ SSC at 70°C for 4 min and dehydrated in ethanol. Four microliters of SKY probe from Applied Spectral Imaging (Migdal Ha'Emek) were denatured at 80°C for 7 min. The denatured probe was incubated at 37°C for 1 h. The timing was controlled such that the incubated SKY probe was added onto the slide right after the denatured slide was dehydrated. The probed slide was incubated in a humidified chamber at 37°C for over 36 h. At least 50 metaphase spreads of good spectral hybridization quality and banding morphology were analyzed for each preparation. The procedures for detection followed the recommendations of Applied Spectral Imaging. SKY images were captured using the SkyVision Imaging System equipped with a Zeiss Axioplan 2 fluorescence microscope. Karyotyping was performed using the special software provided by Applied Spectral Imaging (SKY View 2.0).

### Immunohistochemical analysis

Immunohistochemical analysis was performed to study altered protein expression in 63 human breast cancer tissues. The degree of immunostaining of formalin-fixed, paraffin-embedded sections was reviewed and scored independently by two observers, based on both the proportion of positively stained tumor cells and the intensity of staining. The proportion of tumor cells was scored as follows: 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10%−50% positive tumor cells), and 3 (>50% positive tumor cells). The intensity of staining was graded according to the following criteria: 0 (no staining); 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). The staining index (SI) was calculated by multiplying the staining intensity score and the proportion of positive tumor cells. Using this method of assessment, we evaluated the expression of BRCA2 or c-myc in benign breast epithelium and malignant lesions by determining the SI, which scores as 0, 1, 2, 3, 4, 6, and 9. The cutoff values for BRCA2 or c-myc were chosen on the basis of a measure of heterogeneity with the log-rank test statistical analysis with respect to overall survival. An optimal cutoff value was identified: the SI score of ≥4 was used to define tumors as high expression, and ≤3 as low expression.

### Microarray data process and vitalization

Microarray hybridization, data generation, and normalization were performed in Shanghai Biochip Corporation following standard Agilent protocols on Agilent-022060 SurePrint G3 Human CGH Microarray $4 \times 180$ K. Bioinformatics analysis and vitalization of microarray data were performed on Agilent genomic workbench 6.5 in Shanghai Biochip Corporation. Genomic aberrations detected by $z$-score algorithm with a threshold of 1, vitalization with a window size of 5 Mb.

### Accession numbers

Microarray data described herein have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE26715.

Other experimental procedures are available in Supplementary Materials and methods.

### Statistical analysis

All statistical analyses were carried out using the SPSS 10.0 statistical software package. Means ± SD were calculated and two-tailed Student's $t$-test was performed using the data analysis tools provided by the software. $P < 0.05$ in all cases was considered statistically significant.

## Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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**Conflict of interest**: none declared.

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