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p53 suppresses hyper-recombination by modulating BRCA1 function

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Abstract

Both p53 and BRCA1 are tumor suppressors and are involved in a number of cellular processes including cell cycle arrest, apoptosis, transcriptional regulation, and DNA damage repair. Some studies have suggested that the association of BRCA1 and p53 is required for transcriptional regulation of genes involved in cell replication and DNA repair pathways. However, the relationship between the two proteins in molecular mechanisms of DNA repair is still not clear. Therefore, we sought to determine whether there is a functional link between p53 and BRCA1 in DNA repair. Firstly, using a plasmid recombination substrate, pDR-GFP, integrated into the genome of breast cancer cell line MCF7, we have demonstrated that p53 suppressed Rad51-mediated hyper-recombinational repair by two independent cell models of HPV-E6 induced p53 inactivation and p53 knockdown assay. Our study further indicated that p53 mediated homologous recombination (HR) through inhibiting BRCA1 over-function via mechanism of transcription regulation in response to DNA repair. Since it was found p53 and BRCA1 existed in a protein complex, indicating both proteins may be associated at post-transcriptional level. Moreover, defective p53-induced hyper-recombination was associated with cell radioresistance and chromosomal stability, strongly supporting the involvement of p53 in the inhibition of hyper-recombination, which led to genetic stability and cellular function in response to DNA damage. In addition, it was found that p53 loss rescued BRCA1 deficiency via recovering HR and chromosomal stability, suggesting that p53 is also involved in the HR-inhibition independently of BRCA1. Thus, our data indicated that p53 was involved in inhibiting recombination by both BRCA1-dependent and -independent mechanisms, and there is a functional link between p53-suppression and BRCA1-promotion in regulation of HR activity at transcription level and possible post-transcription level.

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Keywords

p53; BRCA1; Hyper-recombination; Chromosomal stability; Homologous recombination

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1. Introduction

Homologous recombination (HR) is an important repair mechanism for DNA double strand breaks (DSBs) that are generated by exogenous or endogenous sources, such as ionizing radiation (IR), chemotherapeutic drugs, or DNA replication fork stalling or collapse [1]. The typical molecular HR mechanism for repairing DSBs includes the following steps [2]: end resection of DNA breaks to generate single-stranded DNA tails, then nuclear filament formation of recombinase Rad51 on single-stranded DNA tails for finding identical undamaged sister-chromatid, DNA synthesis using the identical sister-chromatid as a template to fix broken DNA, and holiday junction resolution. Of these, Rad51-nuclear filament formation is the most important stage for HR mechanism. It was indicated that inappropriate activity of HR function for repairing broken DNA can result in mutation, deletion, translocation or loss of heterozygosity (LOH) that further develop genetic instability and tumorigenesis [3,4].

The tumor suppressor p53 gene, which has frequently been found to be mutated in malignancies, is involved in multiple cellular processes ranging from apoptosis, cell cycle, transcription, genomic stability, to DNA damage repair. There is emerging evidence suggesting the involvement of p53 in the regulation of DNA DSBs repair through HR repair mechanisms [5]. Loss of p53 in mice increased the frequency of HR [6], and expression of p53 mutants in human Leukemia cell line led to a noticeable increase in HR whereas wild type p53 (wtp53) decreased HR frequency [7], indicating p53 is required for HR-inhibition. It was indicated that p53's involvement in the HR process through the interaction with DNA repair proteins Rad51 and Rad54 [5,8,9], and functions at both of early and late stage during this repair process. However, p53-mediated regulation of HR is independent of its transactivation activity [10,11].

As a critical mediator, the breast cancer susceptibility gene 1 (BRCA1) plays an important role in multiple processes including DNA repair, transcription regulation, apoptosis and chromosome remodeling. Mutated BRCA1 in a mouse model can lead to highly deficient HR, genetic instability and sensitivity to different damaging agents [12]. Loss of BRCA1 in breast cancer cells significantly decreased HR, but the complementation with wild-type BRCA1 (wtBRCA1) expression will restore HR activity [12]. Many studies have demonstrated that BRCA1 colocalizes with Rad51 and BRCA2 in DNA damaged areas [13,14], and dysfunction of BRCA1 causes the decrease of damage-induced Rad51 foci formation [15], suggesting BRCA1's involvement in HR-promotion is Rad51 dependent. The BRCT domain of BRCA1 contributes the major role in regulation of HR whereas its RING finger domain only plays a partial function in this process [1,16–18].

It has been clearly shown that p53 and BRCA1 are involved in the regulation of DNA repair mechanism by opposing effects: p53 inhibits HR, but BRCA1 promotes it, this raises the question of whether there is a functional link between the two proteins, since some studies

have stated there are genetic interactions between BRCA1 and p53 in cellular processes such as apoptosis, cell cycle and tumorigenesis [19,20]. Here, our results demonstrate that the loss of p53 greatly increases the frequency of HR via enhancing BRCA1 protein level in two different isogenic cell systems. In response to DNA damage, loss of p53 recruits more Rad51 and BRCA1 to DNA broken areas. The data further suggests that hyper-recombination caused by p53 dysfunction is associated with increasing radioresistance and diminishing chromosomal aberrations. In addition, it was found that p53 loss rescued BRCA1 deficiency by recovering HR and chromosomal stability, suggesting that inhibiting HR by p53 is also BRCA1-independent. Together, our data indicate that p53 is involved in HR through suppressing BRCA1 over-function at transcriptional level, and that the functional link between two proteins plays an important role in regulating the balance between promotion and repression of HR molecular mechanisms.

2. Materials and methods

2.1. Plasmids, Cell culture, transfection, and retrovirus infection

MCF7 and H1299 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 mM Hepes, 100 µg/ml streptomycin and 100 units/ml penicillin (all purchased from Sigma). A pair of isogenic cell lines, MCF7/E6 and MCF7/LXSN cells, were established by retrovirus infection as previously described [19]. Briefly, MCF7 cells were infected by the stock solution of virus particles with E6 or LXSN, followed by selection with G418 (Mediatech) at 500 µg/ml [19], and the expression of p53 proteins in the cells were confirmed by western blotting.

The H1299 cell line was transiently transfected with the constructs of HA-wild type p53 (wtp53) or pcDNA3 as vector control using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). All cell lines were maintained in a humidified atmosphere of 5% CO₂ at 37 °C.

2.2. shRNA knock-down experiment, and restoring wtp53 assay

BRCA1 short hairpin RNAs (shRNA) was used for BRCA1 knockdown in MCF7 cells, detailed information was described previously [15]. p53 shRNAs targeting 3' untranslated regions (p53sh 3'UTR) or coding regions (p53sh CDS) for knockdown p53 in MCF7 cells were obtained from Qin Yang at Department of Radiation Oncology at Washington University School of Medicine. After 48h-infection of p53sh 3'UTR, the cells were subsequently transfected with constructs of wtp53 to restore p53 expression for the relative assay.

2.3. Clonogenic survival assay

Cells were seeded onto 100 mm cell culture dishes (Corning, NY) for 16 h. IR was applied to cells by using a Siemens Stabilipan 2 X-ray generator at 250 kVp 12 mA, at a dose rate of 2.08 Gy/min. Visible colonies containing at least 50 cells were counted after a 3 week-incubation and were identified with methanol fixation and 0.35% methylene blue staining.

Survival fractions were calculated as the plating efficiency of treated cells relative to the plating efficiency of untreated control cells.

2.4. Homologous recombination assay

HR analysis was done in MCF7 cells expressing recombination substrate pDR-GFP according to previous publications [21,22]. Briefly, MCF7 cells were transfected with pDR-GFP (a gift from Maria Jasin, Developmental Biology Program, Memorial Sloan-Kettering Cancer Center, New York) and selected with puromycin at 1.0 µg/ml for stable transfection. At least two pDR-GFP-containing clones were verified through determining the frequency of I-SceI-inducible HR, which produced green fluorescent protein (GFP)-positive cells. The one clone with inducible GFP expression was further transfected with the plasmids of pcDNA3/E6 (virus stocks were obtained from Carl Q. Maki at Harvard University, School of Public Health) or pcDNA3 vector control, and selected with G418 at 750 µg/ml for stable transfection to obtain cell population containing E6 or pcDNA3. To maintain selection, cells were cultured in complete DMEM containing 1.0 µg/ml puromycin and 500 µg/ml G418.

2.5. Cell fractionation and western blotting analysis

Whole cell extracts were used for western blot analyses. The preparation of cellular extracts was described in our previous publications [23]. Briefly, total protein concentration in each extraction was measured by BCA protein assay kit (Pierce, Rockford, USA). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were incubated with mouse anti-p53 monoclonal antibody (Ab-6, Oncogene Science (diluted 1:1000 in PBS, 3% milk); mouse anti-BRCA1 antibody (Ab-1, Oncogene Science (diluted 1:1000 in PBS, 3% milk); mouse anti-p21 (Ab-1, Oncogene Science (diluted 1:100 in PBS, 3% milk)); β-actin (Ab-1, Sigma (diluted 1:5000 in PBS, 3% milk)); respectively. Secondary antibody was goat-anti-mouse IgG-HRP conjugated (diluted 1:5000 in PBS, 3% milk). Bands were detected using the ECL chemiluminescence detection method (Amersham Biosciences) and exposed to X-ray film.

2.6. Co-immunoprecipitation

Cells cultured in 10 cm dishes were harvested and lysed in 200 µl lysis buffer (Beyotime, Shanghai, China). Subsequently, 1 mg protein lysates were incubated with normal mouse IgG, or anti-p53 (DO-1) or anti-BRCA1 (D-9) antibodies (all purchased from Santa Cruz Biotechnology) and 70 µl Protein G Plus/Protein A-Agarose beads (Calbiochem, USA) at 4°C overnight with gentle agitation. The beads were then pelleted and washed five times with lysis buffer. Immunoprecipitated proteins were eluted in 40 µl 2 × SDS-PAGE sample loading buffer and incubated at 95°C for 5 min. The supernatants were subjected to SDS-PAGE and analyzed by immunoblotting.

2.7. RT-PCR and quantitative realtime PCR analysis

Total RNA was extracted with RNeasy Mini Kit (Qiagen) and then reverse transcribed using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Quantitative realtime PCR was performed with Thermo Scientific Maxima SYBR Green qPCR Master Mix (Thermo Fisher

Scientific, USA) and specific primer pairs. The primer pairs used for amplification of GAPDH and p53 were: 5'-GAACGGGAAGCTTGTCATCA-3'(F) and 5'-AGAGGCAGGGATGTTCT-3'(R), 5'-CAGCACATGACGGAGGTTGT-3'(F) and 5'-TCATCCAAATACTCCACACGC-3'(R), respectively. The three BRCA1 primers were following: 5'-GAAACCGTGCCAAAAGA-CTTC-3'(F) and 5'-CCAAGGTTAGAGAGTTGGACAC-3'(R), 5'-ACCTTGGAAGTGTGAGAACTCT-3'(F) and 5'-TCTTGATCTCCCACACTGCAATA-3'(R), 5'-TTGTTACAAATCACCCCTCAAGG-3'(F) and 5'-CCCTGATACTTTTCTGGATGCC-3'(R). In addition, the averaged expression level of GAPDH was used as the internal reference control for RT-qPCR on all samples simultaneously. Experiments were carried out in triplicate for each data point.

The mRNA threshold cycles (C_t) of all samples was analyzed using LightCycler 480II RT-PCR System (Roche Applied Science). The relative mRNA expression levels were normalized against GAPDH and calculated by the 2^{-C_t} method, where $C_t = C_{\text{mRNA}} - C_{\text{GAPDH}}$ and $C_t = C_{\text{experimental group}} - C_{\text{control group}}$ [24].

2.8. Immunofluorescence microscopy

Cells were seeded into four-well chamber cell culture slides (Fisher Scientific) for detecting protein foci formation by immunofluorescence, the detailed procedure was described previously [25]. The following antibodies were used: rabbit anti-Rad51 (Ab-1, Oncogene Science) at a 1:200 dilution in 1% BSA-0.1% triton X-100-PBS buffer, BRCA1 (D9) at a 1:100 dilution in the buffer above, secondary antibodies of AlexaFluor 594-labeled goat anti-mouse IgG and AlexaFluor 488-labeled chicken anti-rabbit (Molecular Probe) at a 1:300 dilution. The images were collected by an Olympus microscope (BX51) and processed using Adobe PhotoShop software.

2.9. Cell cycle analysis

Treated cells were collected and fixed with 70% ethanol, and incubated for 30 min with the solution containing RNase A (Sigma, 250 µg/ml), propidium iodide (Sigma, 25 µg/ml) and 0.1% triton X-100 to detect DNA content by flow cytometry (BD Biosciences). Raw data were analyzed to determine the percentage of cells in G1, S and G2 phases.

2.10. Florescence in situ hybridization (FISH) for chromosome aberration analysis

FISH was performed using a pan-telomeric peptide nucleic acid (PNA) probe. Telomere (C₃TA₂)₃-specific probe directly labeled with Cy3 fluorescent dye was obtained from Applied Biosystems (Foster City, CA, USA). FISH analysis was performed on metaphase spreads prepared 24 h after 2 Gy irradiation. The detailed information was described in previous publications [21,26].

3. Results

3.1. p53 suppressed hyper-recombination in response to DNA damage

In this study, we first examined the role of p53 in the HR repair mechanism. To test this function, we stably expressed the recombination substrate pDR-GFP for HR assay in MCF7

cells, and confirmed I-SceI-induced DSB repair by flow cytometry (Fig. 1 A). The cells were further infected by retrovirus containing either HPV-E6 (MCF7/E6) or control vector (MCF7/LXSN) to establish the isogenic cell lines with different conditions of p53, since HPV-E6 expression can cause wtp53 ubiquitination and degradation in MCF7 cells, which is able to obtain p53-defective cell line [13,27–29]. As shown in Fig. 1B and C, after infecting retrovirus of HPV-E6, DNA damage-mediated p53 expression, p53-dependent p21 induction and p53-dependent G1 arrest were significantly disrupted after IR, suggesting that p53 function was inactivated in MCF7/E6 cells that are consistent with our previous report [19]. Disruption of p53, the frequency of spontaneous recombination (SR) (Fig. 1D left) and nuclease I-SceI-induced DSB repair (Fig. 1D right) was increased two- or seven-fold, respectively ($p<0.01$), as compared with wtp53, implying that p53 is required for the suppression of hyper-recombination.

It was reported that HPV-E6 still has other substrates besides p53 [30,31], so we used shRNA knockdown strategy in MCF7 cells to confirm the effect of p53 on HR in order to exclude some unknown side effects induced by E6 other target proteins. As shown in Fig. 1E, two shRNAs (p53sh 3'UTR and CDS) can successfully inhibit p53 expression in MCF7 cells at mRNA (Fig. 1E left) and protein level (Fig. 1E right) by RT-qPCR and western blotting assay. After knockdown of p53 by its shRNA, we also found I-SceI-induced HR frequency significantly increased as compared with consh group (Fig. 1F left, $p<0.01$), and the data further indicated that I-SceI-induced HR frequency can obviously go down again after restoring wtp53 expression in p53-defective cells (Fig. 1E and F right, $p<0.01$), these results are consistent with the data from HPV-E6 working model. Also, the cell cycle profiling was not influenced in MCF7 cells with inactivation of p53 induced by its shRNA and recovery of wtp53 (Fig. 1E right).

Thus, by two experimental strategies, our data support p53 plays a role in the regulation of HR, and further imply that p53 is required for the inhibition of hyper-recombination in response to DNA damage.

3.2. p53 inhibited the recruitment of more Rad51 to DNA breaks

Rad51 plays the central role in the HR repair process via regulating DNA strand exchange and pairing of homologous sequences. To examine whether p53-inhibited hyper-recombination is Rad51 dependent, Rad51 foci formation was tested after loss of p53 function by immunostaining. In non-irradiated cells, comparing with p53-proficient cells, the percentage of cells containing Rad51 foci formation in p53-deficient cells was significantly elevated to 21.9% (Fig. 2A and B, $p<0.05$) from 5.33%. In response to DNA damage, the percentage of cells carrying IR-induced Rad51 foci formation in p53-defective cells was significantly increased to 71% compared with p53-proficient cells (45.7%) (Fig. 2A and B, $p<0.05$), suggesting that loss of p53 led to more Rad51 proteins to DNA damage areas. We further analyzed above data in another way, which divided the cells-containing Rad51 foci into three patterns according to foci number in each cell as “<10”, “10–30”, and “>30” (Fig. 2C), it was found that cell pattern was only “<10” (5.33%) in non-irradiated cells with proficient p53, after disruption of p53, the proportion of both patterns with “<10” and “10–30” significantly increased; and after irradiation, the proportion of patterns with

“10–30” and “>30” in defective p53 cells notably increased as comparing with proficient p53 cells (Fig. 2C, $p<0.05$), further confirming that more Rad51 was recruited to broken DNA area after loss of p53. In Fig. 2D, another working system, p53sh knockdown strategy in MCF7 cells was used to determine the dynamic change of Rad51 foci formation at various time intervals over a 8 h period after exposure of cells to 10 Gy irradiation, and the data were indicated that the induction of Rad51 foci formation in p53-defective cells was significantly increased as compared with p53-proficient cells at both of early (1 h) and late (8 h) time points after IR treatment. For example, the induction of Rad51 foci was increased by 1.4 fold at 1 h after IR treatment ($p<0.05$). Thus, our data obtained by above two working systems indicated that p53-mediated HR repair pathway is through inhibiting the involvement of more Rad51 in DNA damage response against hyper-recombination.

3.3. p53 mediated HR through the regulation of BRCA1 activity

Several previous reports have indicated a functional link between p53 and BRCA1 in response to DNA damage [13,27–29]. Since both proteins are required for repairing damaged DNA, we used two independent approaches to test whether p53-inhibited HR is associated with BRCA1 function. First, MCF7 cells expressing wtp53 and wtBRCA1 were employed for testing this hypothesis. BRCA1shRNA successfully knocked down BRCA1 expression in MCF7 cells, and cell cycle changes were not observed under these conditions (Fig. 3 A), suggesting that inactivation of BRCA1 is specific to its BRCA1shRNA. Silencing BRCA1 did not affect p53 expression, while inactivation of p53 by infection of HPV-E6 virus caused a significant increase in BRCA1 protein level as compared to p53-proficient cells (Fig. 3B), indicating that p53 is able to suppress BRCA1 over-expression. In second experimental approach, H1299 cell line with null-p53 and wtBRCA1 background was used for confirming the relationship between p53 and BRCA1 protein expression, it was also found that a significant reduction of BRCA1 expression was exhibited after the complementation of wtp53 in the cells as shown in Fig. 3C, and cell cycle profiling is not affected after recovery of wtp53 in this system, further confirming wtp53 is able to regulate BRCA1 protein expression. Also, as shown in Fig. 3D, when p53sh 3'UTR successfully knocked down p53 expression in MCF7 cells and subsequently restored wtp53 expression, the patterns of BRCA1 expression further support above results. Thus, all of our data here indicated that p53 can inhibit BRCA1 over-expression.

Since BRCA1 can be recruited to DNA breaks area for repairing damaged DNA [13,27–29], we tested whether IR-induced BRCA1 foci formation is affected by p53. The data were shown in Fig. 3E. In untreated cells, the percentage of cells containing BRCA1 foci was significantly increased to 50.8% ($p<0.05$) after p53 loss as compared to p53-proficient cells (31.4%) (Fig. 3E, upper). In response to irradiation, p53 loss further increased the percentage of cells with BRCA1 foci to 78.6% ($p<0.05$) as compared with p53-proficient cells (51.4%) (Fig. 3E, upper), indicating that p53 can suppress the recruitment of more BRCA1 to damaged DNA sites. Again, the number of BRCA1 foci in each cell was also analyzed here as described in the Rad51 experiment (Fig. 2C). In non-irradiated cells, loss of p53 significantly increased the percentage of cell pattern with “10–30” as compared with p53-proficient cells (Fig. 3E, lower left). And in irradiated cells, disruption of p53 significantly enhanced both patterns of “10–30” and “>30” compared with p53-proficient

cells (Fig. 3E, lower left), further confirming that p53 was required for inhibition of increased BRCA1 function. In addition, we determined the dynamics of BRCA1 foci formation in MCF7 cells with and without p53 expression under the same condition as described in Rad51 foci formation experiment (Fig. 2D). It was found that the percent-age of the cells with BRCA1 foci formation in p53-defective cells was notably increased at each time point after 10 Gy irradiation as compared with p53-proficient cells, which was consistent with HPV-E6 induced-p53 inactivation system (Fig. 3E, lower right). For example, IR-induced BRCA1 foci formation was obviously increased by 1.3 fold at 1 h after 10 Gy irradiation ($p<0.05$). These data gained by two different working systems demonstrated that p53 is essential for inhibition of increased BRCA1 function.

Both BRCA1 and p53 can interact in vivo and in vitro and function in the same pathway following DNA damage [12,32]. Here we used the H1299 cell line complemented with wtp53 to test potential interaction between BRCA1 and p53 by immunoprecipitation. It was found that BRCA1 was present in p53-precipitated protein complex (Fig 3F), suggesting that p53 is sufficient for the interaction with BRCA1. These results indicated that the association between p53 and BRCA1 may support p53-mediated HR via inhibiting BRCA1 over-function.

It was reported that p53 can involve in DNA damage process through transcription regulation, so we have explored the mechanism of the relationship both of p53 and BRCA1 during DNA repair process. Three different primers of BRCA1 were employed for testing mRNA level of BRCA1 by RT-qPCR. It was found the mRNA level of BRCA1 tested by all primers was significantly increased after p53 inactivation (Fig. 3G, $p<0.01$), and BRCA1 mRNA level can be inhibited after restoring wtp53 expression in p53-deficient cells (Fig. 3G, $p<0.01$). Our results implied that the association between p53 and BRCA1 may be at transcription level in response to DNA repair.

3.4. p53 loss revert HR in BRCA1-deficient cells

Our above data indicated that p53-mediated Rad51-dependent HR was required for the inhibition of BRCA1 over-function. Thus, we further determined how p53 regulates HR in BRCA1-deficient background. To address this issue, we depleted p53 in BRCA1-deficient and -proficient cells and measured HR frequency using the recombination substrate pDR-GFP mentioned in Fig. 1. In BRCA1-proficient cells, inactivation of p53 increased the frequencies of I-SceI-induced homology-mediated repair or SR by five or two folds (Fig. 4A and B, $p<0.01$), which is consistent with the data described in Fig. 1. However, it is interesting to see that p53 loss in BRCA1-defective cells can restore the frequencies of I-SceI-induced HR or SR (Fig. 4A and B), suggesting that the disruption of p53 can revert HR in BRCA1-deficient cells, and p53 also involved in the regulation of general HR independent of BRCA1 function.

3.5. The dysfunction of p53 increased cell resistance to IR treatment

We next wanted to determine whether p53-inhibited hyper-recombination is associated with sensitivity to radiation-induced cytotoxicity. Cell cytotoxicity induced by irradiation was measured by clonogenic survival assay, the results showed that loss of p53 in BRCA1-

proficient cells significantly elevated radioresistance at doses of 2, 4 and 6 Gy (Fig. 5, $p < 0.01$). Inactivation of p53 in BRCA1-deficient cells caused a significant increase in radioresistance at the dose of 2 Gy and 4 Gy, however, the cell sensitivity had no difference once the dose increased to 6 Gy (Fig. 5, $p > 0.01$). Our data support the hypothesis that hyper-recombination caused by p53 loss is associated with the increase of cell radioresistance, which may be BRCA1-dependent. However, disruption of p53 in BRCA1-deficient cells may affect cell radioresistance to some extent.

3.6. The disruption of p53 enhanced chromosomal stability following IR treatment

Since both p53 and BRCA1 play key roles in maintaining genetic stability as tumor suppressors, chromosomal aberrations were investigated by FISH in the cells. Loss of p53 in BRCA1-proficient cells, spontaneous chromatid, chromosomal and radial structure aberrations had no difference and present with zero in above situations (Fig. 6A, B and C, $p > 0.01$); and p53 deletion obviously rescued chromosome and radial structure aberrations caused by BRCA1 disruption (Fig. 6A–C). In response to DNA damage, IR-induced chromosomal and radial structure aberrations were reduced after p53 disruption in both BRCA1-proficient and –deficient cells. However, double-defective BRCA1 and p53 cells showed that spontaneous and radiation-induced chromatid breaks were alleviated (Fig. 6A–C). These results further showed that p53 was involved in repairing irradiation-induced DSBs by inhibiting hyper-recombination regardless of BRCA1 status.

4. Discussion

Accumulated evidence has suggested that p53 is involved in the early and late stages of HR through direct physical binding with Rad51 and Rad54, which are the key components of recombination machinery [9,33]. During this event, the core domain and C-terminus in p53 protein may play more important roles [5,34,35]. ATM/ATR phosphoserine-15 was found to colocalize with Rad51 at DNA breaks sites in response to DNA DSBs, which suggested that serine 15 is required for p53-mediated HR inhibition [9,36]. On the other hand, serine 315 phosphorylation site in p53 is essential for promoting topoisomerase-dependent HR, indicating that different phosphorylation sites of p53 possibly have opposing effects in regulating HR [33]. However, these results do not completely explain why only defective or mutated p53 can lead to a significant increase in HR frequencies.

Based on these and other studies, we hypothesized that one main function of p53 is negative regulation of HR. In this study, we have demonstrated that p53 is able to inhibit hyper-recombination through suppressing BRCA1 over-function via transcription regulation for repairing DNA DSBs, indicating there is a functional link between p53 and BRCA1 in this repair pathway at transcriptional level. Therefore, we further proposed the following model (Fig. 7). In HR repair process there are two opposing molecular mechanisms that are p53-mediated repression and BRCA1-mediated stimulation, the balance between these two mechanisms may be key in properly maintaining repair activities. Once the balance is disrupted, it would cause defective-HR or a hyper-recombination phenotype. Our and other reports have demonstrated that defective HR caused by loss of BRCA1 is connected with genome instability by increasing chromosomal aberrations (see Fig. 6A–C) and cell

sensitivity to IR (Fig. 5) and other DNA damaging agents [15,29,37,38]. In contrast to defective-HR, it was established that the hyper-recombination phenotype can result in elevated chromosomal aberrations containing LOH, the development of aneuploidy, increased mutation and marked centrosome amplification [3,39], and LOH is correlated with human malignancies and tumor suppressor loci [4]. Even our present data have shown that loss of p53 would alleviate chromosomal aberrations including chromatid and chromosome breaks, and radial structure formation (Fig. 6A–C) which are the markers for reflecting HR deficiencies, this is not contradictory to the above data due to limitations of FISH assay in this study. However, alleviated the chromosomal aberrations in our study would explain and support why defective p53 can cause hyper-recombination. Moreover, the results demonstrated that hyper-recombination is an important mechanism in cellular radioresistance, which is in agreement with other studies [40,41]. Thus, both deficient HR and hyper-recombination are harmful for repair process, and keeping the proper balance between HR-repression and -stimulation would be important for common cellular activity to avoid potentially mutagenic DNA rearrangements and genome instability.

The functional link between p53 and BRCA1 has been reported by a number of studies. Immunoprecipitation experiments and protein binding assays indicated that there are two physical interaction regions between p53 and BRCA1 protein to form a stable complex both in vivo and in vitro, which is required for their growth inhibitory pathway [12,32]. Deng's group has shown that p53 controlled BRCA1 transcription activity both in vivo and in vitro, and disruption of *Tip53* in *BRCA1*^{11/11} embryos can remedy embryonic lethality and restore normal mammary growth [32]. Our results additionally demonstrated that the interaction of both proteins functions in controlling HR activity, which was consistent with the reports of Deng's group. Therefore, the genetic association between BRCA1 and p53 maybe essential for multiple cellular processes such as tumorigenesis, apoptosis, cell cycle and DNA damage repair.

It remains to be answered how p53 can suppress BRCA1 over-expression. Our data indicated that p53 is able to inhibit over-expression of BRCA1 mRNA level, which was consistent with the report of Lee's group that the transcriptional repression of BRCA1 expression was induced by p53 via run-on experiments and luciferase reporter assays [20]. It was clearly demonstrated that the functional link between p53-suppression and BRCA1-promotion in regulation of HR activity is through transcription regulation. However, since the core domain and C-terminus of p53 play key roles in HR repair process, and both domains can separately interact with BRCA1, it would be reasonable to suppose that the possibility that two physical interaction domains could be influenced. Since it was reported by Xia's group that direct interaction between p53 and BRCA1 may impact BRCA1 protein level and BRCA1 is a p53-dependent nuclear-cytoplasmic shuttling protein [19,40], suggesting that p53 is essential to bind BRCA1 for its translocation from nuclei to cytoplasm, in which BRCA1 protein degradation is possibly triggered. In addition, another potential possibility that p53 may have an ability to regulate BRCA1 ubiquitination needs to be explored, however there is no report to indicate p53 has E3 ubiquitin ligase activity. In summary, we have identified there may be a functional link between tumor suppressor p53 and BRCA1 for keeping a physiological balance of HR-suppression and -stimulation in DSBs repair process at transcriptional and possible post-transcription level.

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Fig. 1.

p53 inhibits homologous recombination. (A) The establishment of MCF7 cells expressing the recombination substrate pDR-GFP. MCF7 cells were transfected with construct of pDR-GFP and further selected by puromycin to obtain a stable single clone. Flow cytometry was used for screening the positive clones expressing pDR-GFP. The pictures showed the positive cell clone expressing green fluorescent protein (GFP) induced by I-SceI falling above the diagonal. (B) The establishment of cell lines with or without HPV-E6 expression. MCF7/pDR-GFP cells were infected with the virus of E6 or LXSN to obtain stable clones expressing E6 (defective in p53) and LXSN (proficient in p53). At 8 h after exposure of the stable clones to 10 Gy of irradiation, p53 and p21 were detected by western blotting. β-actin was used as loading control. (C) At 24 h after exposure of MCF7/pDR-GFP cells expressing E6 or LXSN to 10 Gy of irradiation, the cells were detected to analyze cell cycle profile using flow cytometry. (D) MCF7/pDR-GFP cells expressing E6 or LXSN were used to analyze relative spontaneous recombination (SR) (left) and I-SceI-induced homologous recombination (HR) (right). (E) MCF7 cells were infected by p53 shRNA targeting 3' untranslated regions of p53 (p53sh 3'UTR) or targeting coding region of p53 (p53sh CDS) to knockdown p53, at 48 h after infection relative mRNA of p53 expression was analyzed by RT-qPCR normalized against GAPDH (left). After restoring exogenous active p53 in the cells infected with p53sh 3'UTR, p53 and p21 protein expression in the cells were detected

by western blotting and cell cycle profiling was analyzed by flow cytometry, β -actin was used as loading control (right). (F) MCF7/pDR-GFP cells were treated as in E left or E right, then the relative I-SceI-induced HR was analyzed by flow cytometry (left and right). Each data point in E and F was from three independent experiments (mean $\pm$ SD). *P*-values were calculated by Student's *t*-test (*P<0.01).

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Fig. 2.

p53 inhibits over-recruitment of Rad51 to DNA breaks. MCF7/E6 and MCF7/LXSN cells were fixed at 8 h after 10 Gy irradiation and stained with Rad51 antibody (A, B, C). DAPI was used for nuclear staining (A). (A) Representative example of a cell containing Rad51 foci formation. (B) The percentage of cells with Rad51 foci formation. (C) The percentage of cells with different patterns divided by the number of foci. (D) The dynamics of Rad51 foci formation in MCF7 cells with and without p53sh 3'UTR treatment over an 8 h period after 10 Gy irradiation. The percentage of the cells with more than 5 nuclear foci was calculated, at least 300 cells were counted per data point, each data point was from three independent experiments (mean $\pm$ SD). *P*-values were calculated by Student's *t*-test (**P < 0.05).

Fig. 3. p53 inhibits BRCA1 over-function at protein and transcription level. (A) The knockdown of BRCA1: MCF7 cells were infected by BRCA1sh, and at 48 h after infection the cells were used for western blotting and cell cycle profiling analysis. β -actin was used as loading control. (B) The effect of p53 on BRCA1 protein levels. MCF7/E6 and MCF7/LXSN cell lines were infected with BRCA1sh or consh, at 48 h after infection, then BRCA1 and p53 protein expression in the cells were detected by western blotting. (C) H1299 cells were transfected with constructs of wtp53 or pcDNA3 as vector control, after 48 h-transfection, p53, p21 and BRCA1 were detected by western blotting. Under the same conditions, the cell cycle profiles were analyzed by flow cytometry. MCF7 cells were used as positive control for p53 expression. (D) MCF7 cells were treated as in Fig. 1E right, then BRCA1 and p53 protein expression in the cells were detected by western blotting. (E) p53 inhibits over-foci formation of BRCA1 in response to DNA damage. MCF7/E6 and MCF7/LXSN cells were treated as in Fig. 2 (A, B, C), and then cells were stained for BRCA1 (upper left and right, lower left). DAPI was used for nuclear staining (upper left). Upper left: the pictures displayed the cells containing BRCA1 foci formation. Upper right: the percentage of cells with BRCA1 foci formation. Lower left: the percentage of the cells with different patterns divided by foci number. Lower right: the dynamics of BRCA1 foci formation in MCF7 cells with and without p53sh 3'UTR treatment over an 8 h period after 10 Gy irradiation. The

percentage of cells with more than 5 nuclear foci was calculated. In each experiment, at least 300 nuclei were counted per data point, each data point was from three independent experiments (mean $\pm$ SD). *P*-values were calculated by Student's *t*-test (**P<0.05). (F) H1299 cells were transfected with the constructs of wtp53 or vector control, after 48 h-transfection, protein lysates were used for co-immunoprecipitation experiment. p53 antibody was employed to pull down the protein complex, then co-precipitated BRCA1 expression was detected in the complex. (G) MCF7 cells were treated as in Fig. 1E right. Relative mRNA expression of BRCA1 and p53 in MCF7 cells was analyzed by RT-qPCR normalized against GAPDH. Each data point was from three independent experiments (mean $\pm$ SD). *P*-values were calculated by Student's *t*-test (*P<0.01).

Fig. 4. Disruption of p53 rescues HR in BRCA1-defective cells. MCF7/pDR-GFP cells expressing E6 or LXSIN were infected with BRCA1sh, at 48 h after infection, the cells were analyzed for detecting relative recombination. (A) Relative SR in indicated cells. (B) Relative I-sceI-induced HR in the cells. Each data point was from three independent experiments (mean $\pm$ SD). *P*-values were calculated by Student's *t*-test (*P<0.01).

Fig. 5.

Effect of p53 on IR sensitivity. Cytotoxicity was assayed following different doses of IR: 0, 2, 4, 6 Gy. MCF7/E6 and MCF7/LXSN cells were treated with BRCA1sh as in Fig. 4, then the cells were further irradiated by different doses for clonogenic assay. Each data point was from three independent experiments with standard deviations (mean $\pm$ SD). *P*-values were calculated by Student's *t*-test (*P < 0.01, ***P > 0.05).

Fig. 6.

The effect of p53 on chromosomal stability. MCF7/E6 and MCF7/LXSN cells were irradiated by 2 Gy, and the frequencies of spontaneous and IR-induced chromosomal aberrations were analyzed at 24 h after IR treatment. Fluorescence in situ hybridization using telomeric probe is indicated in red. Fifty metaphases for each sample were scored. Chromatid-type (A), chromosome (B) or radial structure (C) aberrations, respectively, were represented. FISH using telomeric probe reveals the pink color. Chromosomes were stained with DAPI (blue). Each data point was from three independent experiments (mean $\pm$ SD). *P* values were calculated by Student's *t*-test (*P<0.01, ***P>0.05).

Fig. 7.
Proposed model for the role of association between p53 and BRCA1 in the regulation of HR activity.