BRCA2 Protein Deficiency Exaggerates Doxorubicin-induced Cardiomyocyte Apoptosis and Cardiac Failure

Received for publication, August 10, 2011, and in revised form, November 28, 2011. Published, JBC Papers in Press, December 8, 2011, DOI 10.1074/jbc.M111.292664

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Background: BRCA2 is widely implicated in breast and ovarian cancers, but the role of BRCA2 in the heart is unknown.

Results: Loss of BRCA2 in the heart resulted in increased doxorubicin-induced DNA damage, apoptosis, and cardiac dysfunction.

Conclusion: BRCA2 is a novel regulator of cardiomyocyte genomic integrity, survival, and function.

Significance: BRCA2 mutation carriers may be at a heightened risk of anthracycline-induced cardiac failure.

The tumor suppressor breast cancer susceptibility gene 2 (BRCA2) plays an important role in the repair of DNA damage, and loss of BRCA2 predisposes carriers to breast and ovarian cancers. Doxorubicin (DOX) remains the cornerstone of chemotherapy and evaluated their basal and post-DOX treatment phenotypes. Although CM-BRCA2−/− mice exhibited no basal cardiac phenotype, DOX treatment resulted in markedly greater cardiac dysfunction and mortality in CM-BRCA2−/− mice compared with control mice. Apoptosis in left ventricular (LV) sections from CM-BRCA2−/− mice compared with that in corresponding sections from wild-type (WT) littermate control was also significantly enhanced after DOX treatment. Microscopic examination of LV sections from DOX-treated CM-BRCA2−/− mice revealed a greater number of DNA double-stranded breaks and the absence of RAD51 focus formation, an essential marker of double-stranded break repair. The levels of p53 and the p53-related proapoptotic proteins p53-up-regulated modulator of apoptosis (PUMA) and Bax were significantly increased in samples from CM-BRCA2−/− mice. This corresponded with increased Bax to Bcl-2 ratios and elevated cytochrome c release in the LV sections of DOX-treated CM-BRCA2−/− mice. Taken together, these data suggest a critical and previously unrecognized role of BRCA2 as a gatekeeper of DOX-induced cardiomyocyte apoptosis and susceptibility to overt cardiac failure. Pharmacogenomic studies evaluating cardiac function in BRCA2 mutation carriers treated with doxorubicin are encouraged.

6 The abbreviations used are: BRCA2, breast cancer susceptibility gene 2; DOX, doxorubicin; LV, left ventricular; DSB, double-stranded break; PUMA, p53-up-regulated modulator of apoptosis; HDR, homology-directed DNA damage repair; αMHC, α-myosin heavy chain; gDNA, genomic DNA; IHC, immunohistochemistry; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; ATM, ataxia-telangiectasia mutated; p, phospho.
Chemotherapeutic agents function by directly or indirectly damaging DNA (6). Anthracyclines, such as doxorubicin (DOX), are potent and widely used antineoplastic agents in cancer therapy. However, dose-dependent cardiotoxicity, which results in refractory cardiac dysfunction, has compromised the clinical utilization of DOX (7). DOX-induced cardiomyopathy is often fatal, and no specific treatments are available for this condition. It is unknown whether a pharmacogenomic basis for DOX-induced cardiac failure exists. Several mechanisms have been suggested to link DOX treatment to cardiac failure, including cardiomyocyte apoptosis secondary to DNA-induced DNA damage, the generation of DSBs via DNA interstrand cross-linking, enhanced production of reactive oxygen species, and the activation of the tumor suppressor p53 (8–10).

BRCA2 has been previously implicated in the repair of chemotherapy-induced DSBs through homologous recombination (11). This coupled with the recognized role of BRCA2 in promoting the repair of DNA damage led us to hypothesize that BRCA2 is a guardian of the cardiomyocyte genome following DOX-induced acute DNA damage and that loss of BRCA2 may enhance susceptibility to DOX-induced cardiac failure. We used the Cre-loxP system to generate cardiomyocyte-specific BRCA2 knock-out (CM-BRCA2\(^{−/−}\)) mice. Cardiac function at base line and following DOX treatment was examined. We found that DOX-induced cardiac dysfunction was exacerbated in CM-BRCA2\(^{−/−}\) mice, and this was accompanied by greater DNA damage and reduced repair of DNA damage, leading to p53-mediated cardiomyocyte death. BRCA2 may hence serve as a pharmacogenomic clue to identify individuals at greater risk of DOX-induced cardiac failure.

### EXPERIMENTAL PROCEDURES

**Animal Studies**—All animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the St. Michael’s Hospital Animal Care Committee.

**Generation and Characterization of Cardiomyocyte-specific BRCA2 Knock-out (CM-BRCA2\(^{−/−}\)) Mice**—Cardiomyocyte-specific BRCA2 knock-out mice were generated using the Cre-loxP technology on a mixed background. Briefly, as shown in Fig. 1A, mice homozygous for the exon 11 floxed BRCA2 allele (BRCA2\(^{fl/D}\)), National Cancer Institute, Mouse Models of Human Cancers Consortium, Mouse Repository; strain, 01X9; strain name, BRCA2\(^{mibh}\) (12) were crossed with hemizygous mice expressing Cre recombinase under the control of the α-myosin heavy chain (αMHC-Cre\(^{eo}\)) promoter (13) to generate cardiomyocyte-specific BRCA2 heterozygous knock-out mice (αMHC-Cre\(^{eo};\)BRCA2\(^{fl/D}\)). αMHC-Cre\(^{eo};\)BRCA2\(^{fl/D}\) mice were subsequently crossed with BRCA2\(^{fl/+}\) mice to generate cardiomyocyte-specific BRCA2 homozygous knock-out (αMHC-Cre\(^{eo};\)BRCA2\(^{fl/+}\), denoted as CM-BRCA2\(^{−/−}\)) mice. Mice that expressed αMHC-Cre only (αMHC-Cre\(^{eo};\)BRCA2\(^{+/+}\)) displayed similar characteristics as BRCA2\(^{fl/fl}\), BRCA2\(^{fl/+}\), and BRCA2\(^{+/+}\) mice at base line and after DOX treatment. Hence, they were considered part of the WT littermate control group. Mice were genotyped using routine PCR methods as detailed in Fig. 1, B and C, with the primers described in Table 1. Total RNA and genomic DNA (gDNA) were extracted from cardiomyocytes isolated from the hearts of 10–12-week-old mice using the non-reperfusion cardiomyocyte isolation kit from Cellutron Life Technologies, Baltimore, MD. PCR performed on cDNA derived from total RNA and on gDNA using BRCA2 exon 11-specific primers (Table 1) produced the expected PCR product in samples from WT mice but not in cardiomyocytes isolated from CM-BRCA2\(^{−/−}\) mice (see Fig. 1, D and E). BRCA2 deletion was confirmed at the protein level by immunoblots performed with LV lysates and immunohistochemistry (IHC) performed on LV sections from 10–12-week-old CM-BRCA2\(^{−/−}\) mice and their WT littermate controls. Immunoblots and IHC analysis both showed reduced BRCA2 levels in the hearts of CM-BRCA2\(^{−/−}\) mice (see Fig. 1, F and G).

**DOX Treatment and Two-dimensional Echocardiography**—A single intraperitoneal dose of 10 or 20 mg/kg DOX (Sigma-Aldrich) or its diluent (water) was administered to 10–12-week-old CM-BRCA2\(^{−/−}\) mice and their WT littermate controls. Double blindered echocardiography was performed before (base line) and 7 days post-DOX (10 mg/kg) treatment under light sedation (1–1.5% isoflurane) using an HDI 5000cv echocardiographic system (Philips Ultrasound) equipped with a compact 15-MHz broadband linear transducer (CL15-7). Two-dimensional imaging was performed in the parasternal long and short axis views. An M-mode cursor was positioned perpendicular to the interventricular septum and posterior wall of the left ventricle at the level of the papillary muscles. M-mode images were obtained for measurements of chamber dimensions throughout the cardiac cycle. LV end diastolic dimension (LVEDD) and LV end systolic dimension (LVESD) were measured. During diastole, LV dimension and wall thickness

### TABLE 1

Sequences of primers used for genotyping PCRs and BRCA2 exon 11-specific PCRs

| Primer name | Sequence | Primer pair | Product size |
|-------------|----------|-------------|--------------|
| Genotyping PCR | B012 | 5’-GGGCTTCATCCAGGATGTTCT-3’ | B012/B013 | 376 bp, 5’ Lox |
| | B013 | 5’-TCCCTACAGTCTTTTACGCTG-3’ | B013/B014 | 298 bp, WT |
| | B014 | 5’-CGGTGTCAGAGTCTAGGGGA-3’ | B014/B015 | 529 bp, 3’ Lox |
| | B015 | 5’-GGCTGTCTTAGAACTTAGGCTG-3’ | 450 bp, WT |
| Exon 11-specific and p21 real time PCR | BRCA2-Ex11F | 5’-CCCATTGCTCCTTCAGCGACCTTAT-3’ | BRCA2-Ex11F/R | 296 bp, WT |
| | BRCA2-Ex11R | 5’-TGTGACCAGTTTTCCACCTG-3’ | p21-F/R | 94 bp |
| | GAPDH-F | 5’-GGGCTGCTGGTATTGGTGATTTCT-3’ | GAPDH-F/R | 177 bp |
| | GAPDH-R | 5’-GGCTGTCTTAGAACTTAGGCTG-3’ | | |
were measured from the maximum chamber cavity, and during systole, they were measured during maximum anterior motion of the posterior wall. Images were stored on the hard drive for off-line analysis. Fractional shortening was defined as (LVETD − LVESD)/LVETD. LV ejection fraction was defined as ([(LVETD − LVESD)²]/LVETD²) × 100. In all cases, the measurements were averaged from readings of three representative cardiac cycles.

Histology—Histological studies were performed on LV samples that were fixed in situ, excised, and stored in 3.7% paraformaldehyde or on hearts and livers that were frozen in optimal cutting temperature and cryosectioned (5 μm). For gross histological examination, sections (5–8 μm) were stained with hematoxylin and eosin (H&E), and the cross-sectional areas of septal cardiomyocytes were determined in ~150 transversely cut cardiomyocytes with NIH ImageJ software. Sections were examined for TUNEL staining and other proteins via routine immunohistochemical methods using antibodies from Santa Cruz Biotechnology (BRCA2, sc-28235), Cell Signaling Technology (total caspase-3, 9662; p53, 2524; cytochrome c, 4272), and Millipore (γH2A.X, 05-636; RAD51, AB3756). Semi-quantitative analysis was performed with ImageJ software to determine the extent of 3,3′-diaminobenzidine staining in each section.

RNA Extraction, cDNA Synthesis, and Real Time PCR—Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed with the Quantitect reverse transcription RNA Extraction, cDNA Synthesis, and Real Time PCR kit (both from Ontario, Canada). Real time PCR were carried out with SYBR Green Master Mix on the PRISM 7900HT system (both from Applied Biosystems) using p21-specific and GAPDH primers (Table 1).

Immunoblotting—Total protein extracted with ice-cold radioimmune precipitation assay buffer (Sigma-Aldrich) containing a protease inhibitor mixture (Roche Applied Science) was quantified with Bio-Rad Protein Assay Reagent. Protein samples (30–50 μg) were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies from Santa Cruz Biotechnology (BRCA2, sc-28235), Cell Signaling Technology (p53, 2524; p53-up-regulated mediator of apoptosis (PUMA), 4976; Bax, 2772), Millipore (Bcl-2, AB1722; GADD45α, ab3863; GAPDH, MAB374), and Abcam (ataxia-telangiectasia mutated (ATM), ab78; phospho (p) ATM, 6810; Chk2, ab47433; pChk2, ab3508; p21, ab7960). Following incubation with the appropriate horseradish peroxidase-associated secondary antibodies (Santa Cruz Biotechnology), signals were visualized with an enhanced chemiluminescence detection system (GE Healthcare) and quantified by densitometry.

Statistical Analysis—Unless otherwise stated, data are presented as mean ± S.D. Differences between multiple means were evaluated by Student’s t test or analysis of variance with post hoc Bonferroni corrections.

RESULTS

Successful Generation of Cardiomyocyte-specific BRCA2 Knock-out (CM-BRCA2−/−) Mice—To elucidate the involvement of BRCA2 in cardiac function and survival and to circumvent the embryonic lethality associated with systemic loss of BRCA2 (14), we utilized the Cre-loxP system to specifically disrupt BRCA2 in the cardiomyocytes of mice. Conditional inactivation of BRCA2 was achieved by crossing mice homozygous for the exon 11 floxed BRCA2 allele (BRCA2flfl) with hemizygous mice expressing Cre recombinase under the control of the α-myosin heavy chain (αMHC-CreGFP) promoter (Fig. 1A) (13, 15). Mice of the αMHC-CreGFP;BRCA2flfl background are denoted as cardiomyocyte-specific BRCA2 homozygous knock-out (CM-BRCA2−/−) mice. Regardless of their genotypes, were viable, fertile, and born in the expected Mendelian ratios (data not shown). We verified the successful deletion of BRCA2 in the cardiomyocytes of CM-BRCA2−/− mice at both the genomic and transcript levels. The expected PCR products for BRCA2 were obtained in samples from WT mice but not in those from the exon 11-deleted CM-BRCA2−/− mice (Fig. 1, D and E). Successful BRCA2 deletion was further confirmed at the protein level via BRCA2 immunoblotting with LV lysates and also through BRCA2 IHC examination of LV sections from 10–12-week-old CM-BRCA2−/− and WT mice (Fig. 1, F and G).

CM-BRCA2−/− Mice Show No Basal Cardiac Phenotypes—Histomorphometric assessment was performed on in situ fixed and H&E-stained hearts. Similar base-line cardiac morphologies and structures were found in CM-BRCA2−/− mice and their WT littermate controls (Fig. 2, A–C). There were no differences in the LV inner (2.4 ± 0.4 mm, WT versus CM-BRCA2−/− mice, p not significant) and outer (4.4 ± 0.8 mm, WT versus CM-BRCA2−/− mice, p not significant) diameters, septal wall thickness (1.05 ± 0.12 mm, WT versus CM-BRCA2−/− mice, p not significant), and average cardiomyocyte cross-sectional areas between the groups (Fig. 2, A–C). Echocardiographic measurements demonstrated indistinguishable cardiac function between CM-BRCA2−/− mice and their WT littermate controls (Fig. 2, D and E), which included mice with the BRCA2+/+, BRCA2flfl, and αMHC-CreGFP;BRCA2flfl genotypes. Although high levels of Cre recombinase in the heart have been associated with cardiomyopathy in aged mice, this pathology is evident only in mice that express Cre recombinase at levels 50–100 times greater than that in TG9, an αMHC-Cre transgenic strain that does not develop congestive heart failure (16). Nonetheless, to exclude the possible involvement of Cre-mediated cardiomyopathy in our model, parallel studies were conducted on littermates expressing Cre only (αMHC-CreGFP; BRCA2+/+). Because no base-line and post-Dox differences were detected between these mice and the other WT littermate controls, the former were included as part of the WT control apoptosis group as evaluation by caspase-3 and TUNEL staining of LV sections showed similar results across the groups (Fig. 2F). The extent of DSBs and DNA damage repair as measured by γH2AX and RAD51 immunostaining, respectively (Fig. 2F), along with p53 and ATM protein levels as measured by immunoblotting (Fig. 2, G and H) did not reveal any base-line differences in the hearts between the CM-BRCA2−/− and the WT littermate controls. Hence, in the absence of stress, cardiomyocyte-specific deletion of BRCA2 resulted in no grossly apparent base-line cardiac phenotype.
DOX-induced Cardiotoxicity Is Exacerbated in CM-BRCA2<sup>−/−</sup> Mice—Although DOX is a potent chemotherapeutic agent that is used for a wide variety of malignancies, its clinical utility has been limited by its dose-dependent cardiotoxic effects that result in congestive heart failure (17). DOX is a known inducer of DNA damage in cardiomyocytes and is also associated with both p53 accumulation and apoptotic cell death (8). To gather insight on the role of BRCA2 in DOX-induced cardiotoxicity, 20 mg/kg (intraperitoneal) DOX, a dose applied by other groups (18–20), was administered to 10–12-week-old CM-BRCA2<sup>−/−</sup> mice and their WT littermate controls (n = 12/group). This dose of DOX significantly increased mortality in CM-BRCA2<sup>−/−</sup> mice (seven of 12 within the first 7 days; p < 0.05 versus WT) relative to that of the WT control group (two of 12 within the first 7 days). Our subsequent investigations were performed on animals administered a single dose of 10 mg/kg (intraperitoneal) DOX. Echocardiographic examinations performed 7-days post-treatment revealed that 10 mg/kg DOX produced strikingly greater cardiac dysfunction (as per reduced ejection fraction and fractional shortening values) in CM-BRCA2<sup>−/−</sup> mice compared with their WT littermate controls (Fig. 3, A and B). DOX-induced cardiac dysfunction in CM-BRCA2<sup>−/−</sup> mice was further linked with significantly greater weight loss relative to that observed in WT littermate controls (Fig. 3C).

Loss of Cardiomyocyte-specific BRCA2 Exacerbates DOX-induced Apoptosis—DOX-induced cardiac dysfunction was exacerbated in CM-BRCA2<sup>−/−</sup> mice, and this was associated with
significantly greater percentages of TUNEL-positive nuclei in the LV sections (Fig. 3D) examined. In consideration of the possibility of DOX interference with DNA and the specificity of the TUNEL assay, microscopic assessments were conducted and indicated typical nuclear and cellular morphological features of apoptosis that correlated well with TUNEL positivity (Fig. 3D). These data were further corroborated through evaluations of caspase-3 activity via immunostaining. Caspase-3 activity in the LV sections of CM-BRCA2-/ mice was significantly increased after DOX treatment relative to that in samples from the WT littermate controls (Fig. 3E). The enhanced apoptosis observed was confined to the cardiomyocytes (see supplemental Fig. 1 indicating similar extents of DOX-induced hepatic apoptosis across the groups).

**Increased DOX-induced DNA Damage in Hearts of CM-BRCA2-/ Mice**—The increased susceptibility of CM-BRCA2-/ mice to DOX-induced cardiac dysfunction and apoptosis correlated with elevated levels of DNA damage in the
FIGURE 3. Targeted disruption of BRCA2 in cardiomyocytes exacerbates DOX-induced cardiotoxicity in CM-BRCA2−/− mice. A and B, echocardiographic determination of left ventricular ejection fraction (LVEF) (A) and fractional shortening (LVFS) (B) in CM-BRCA2−/− mice and their WT littermate controls. DOX was administered (10 mg/kg intraperitoneally) 7 days before echocardiographic examination to CM-BRCA2−/− mice and their WT littermate controls (10–12 weeks old). LV ejection fraction and LV fractional shortening values indicate significantly reduced LV performance in CM-BRCA2−/− 7 days post-DOX treatment compared with their WT littermate controls (n = 8–9/group; **, p < 0.01). C, DOX treatment induced significant weight loss in grams (gms) in CM-BRCA2−/− mice in comparison with WT littermate controls 7 days post-DOX treatment (n = 8–9; ***, p < 0.001). D and E, cardiomyocyte-specific BRCA2 loss is associated with greater DOX-induced apoptosis, DNA damage (DSBs), and diminished DNA-repair. IHC and quantification of the LV sections from CM-BRCA2−/− mice show increased DOX-induced apoptosis as evident by significantly more TUNEL-positive nuclei (blue arrowhead) (D) and caspase-3 staining (brown) (E) in comparison with the WT littermate controls (n = 6/group; *, p < 0.05; **, p < 0.01). F, immunoblot performed on total heart lysate showed higher γH2AX expression in the samples from CM-BRCA2−/− mice compared with their WT littermate controls (n = 6/group; ***, p < 0.001). G, representative micrographs and quantification of γH2AX staining of LV sections from DOX-treated CM-BRCA2−/− mice and their WT littermate controls. Significantly higher γH2AX expression (blue arrowheads) was observed in the LV sections of CM-BRCA2−/− mice (n = 6/group; ***, p < 0.001; scale bar, 10 μm). H, IHC staining and quantification for RAD51 foci on LV sections from DOX-treated CM-BRCA2−/− mice and their WT littermate controls showed significantly reduced RAD51 focus formation (blue arrowheads) in the samples from CM-BRCA2−/− mice relative to those from their WT littermate controls (n = 6/group; ***, p < 0.001; scale bar, 10 μm). For A–E and G and H, data are presented as mean ± S.D.
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LV sections of these mice relative to that in corresponding sections from the WT littermate controls (Fig. 3, F and G). Because γH2AX focus formation is the first cellular response to DNA lesions (21), the extent of DNA damage in the left ventricles of DOX-treated CM-BRCA2−/− and WT mice were evaluated by measuring γH2AX levels via immunoblotting and IHC methodologies. Immunoblots performed with LV lysates revealed increased γH2AX levels in samples from CM-BRCA2−/− mice (Fig. 3F). IHC examinations also showed more extensive γH2AX staining in the LV sections of DOX-treated CM-BRCA2−/− mice, indicating exaggerated DNA lesions compared with that seen in corresponding sections from the WT littermate control group (Fig. 3G). DNA damage causes BRCA2 to translocate RAD51 into the nucleus, and BRCA2/RAD51 focus formation is essential for prompting the repair of damaged DNA (22). Through routine IHC methods, we observed greater RAD51 focus formation in the LV sections of DOX-treated WT littermate controls relative to those from DOX-treated CM-BRCA2−/− mice. This is indicative of defective repair of DNA damage, thereby resulting in the accumulation of damaged DNA in the hearts of DOX-treated CM-BRCA2−/− mice (Fig. 3H). IHC for γH2AX and RAD51 performed on the liver sections of CM-BRCA2−/− mice and their WT littermate controls showed similar degrees of DNA lesions and DNA repair, again confirming the cardiomyocyte-specific phenotype of our model (supplemental Fig. 1).

Increased Expression of p53 and Related Apoptotic Molecules in Hearts of CM-BRCA2−/− Mice—Up-regulation of p53 itself is sufficient to induce cardiomyocyte death (23), and the embryonic lethality originally described in systemic BRCA2 knock-outs may be modulated by loss of p53 (24). It is also plausible that unrepaired chromosome breaks trigger p53 activation. Thus, we sought to determine whether the increased apoptosis and DSBs we observed in the hearts of DOX-treated CM-BRCA2−/− mice are associated with elevated p53 expression. We found greater total p53 staining in LV sections (Fig. 4A) and higher levels of total p53 in LV lysates from DOX-treated CM-BRCA2−/− mice in comparison with those from the WT littermate group (Fig. 4B). A major molecular property of p53 is that of a transcription factor that activates downstream targets like PUMA and Bax. To determine whether increases in p53 protein levels following DOX treatment result in the induction of p53 proapoptotic targets in CM-BRCA2−/− mice, we performed immunoblotting assays. These revealed elevated levels of PUMA and Bax in the left ventricles of CM-BRCA2−/− mice relative to those obtained from the WT littermate controls (Fig. 4, C and D). The critical factor in the p53-dependent survival and/or apoptotic signaling pathway is the shift in the ratio of the level of proapoptotic Bax to that of the prosurvival molecule Bcl-2 (25), both of which are recognized as fundamental regulators of cardiomyocyte apoptosis and/or survival decisions during heart failure (26, 27). The DOX-mediated increase in cardiomyocyte apoptosis in CM-BRCA2−/− mice was associated with a decrease in prosurvival Bcl-2 levels and an increase in proapoptotic Bax protein, which led to a ~5-fold increase in the Bax to Bcl-2 ratio, shifting the balance from a survival to an apoptosis mode (Fig. 4D). Release of cytochrome c from the mitochondrial intermembrane space into the cytosol is a downstream event that allows evaluation of apoptotic cell death. To assess whether the subcellular increase in p53 levels and the up-regulation of proapoptotic proteins resulted in increased cytochrome c release, we performed IHC, which uncovered significantly increased cytochrome c release in the hearts of CM-BRCA2−/− after DOX treatment (Fig. 4E).

Eukaryotic mitotic cells either induce apoptosis or activate checkpoint pathways to arrest the cell cycle and repair damaged DNA (28). ATM and checkpoint kinase Chk2, generally DSB sensors, also function as response effectors by phosphorylation of p53, which in turn promotes apoptosis or p21- and/or GADD45α-mediated cell cycle arrest (29, 30). Furthermore, DOX treatment is known to stimulate ATM autophosphorylation (Ser1981) and Chk2 (Thr68), which further activates p53 (Ser15) (31). Immunoblots for total and activated ATM, Chk2, and p53 on LV lysate from DOX-treated CM-BRCA2−/− and WT littermate controls demonstrated increased activation as well as expression of ATM and p53 and activation of Chk2 in the left ventricle of CM-BRCA2−/− mice (Fig. 4, B and F). However, the transcript (data not shown) and protein expression levels of p21 and the protein level of GADD45α in LV samples of CM-BRCA2−/− mice and WT littermate controls were unimpaired following DOX treatment (Fig. 4, G and H).

DISCUSSION

The increased risk of developing cancer as a result of mutations in BRCA1/2 has been studied extensively, and there are more than 12,000 published articles implicating BRCA1 and/or BRCA2 in cancer etiology (PubMed, October 2011). The BRCA1/2 genes are classified as “tumor suppressors” and “caretakers” on the basis of their proposed roles in maintaining genome integrity as well as the infrequency of linked sporadic tumors (2). Although both BRCA1/2 are involved in HDR, BRCA1 appears omnipresent with a wealth of biochemical data describing multiprotein interactions (32). BRCA2, on the other hand, exerts more of a direct role to promote HDR through RAD51 interactions and direct binding to single-stranded DNA (33, 34). Therefore, loss of BRCA2 leads to a defect in DNA damage repair and deranged genome integrity, and the continued failure to repair DNA damage results in the accumulation of a lethal amount of DNA damage and spontaneous chromosomal abnormalities, eventually causing cancer or apoptotic cell death in proliferating cells (5). To date, there have been no investigations delving into how loss of BRCA2 may affect DNA damage repair in terminally differentiated cells, such as cardiomyocytes. The relative contribution of homologous repair-related and non-homologous end joining pathways in the heart also remains unknown. DNA damage responses are important for cancer syndromes but may also represent a common pathological basis for diverse chronic diseases, including cardiovascular diseases (8, 35, 36). Unrepaired DNA damage is a trigger for apoptosis, a key mechanism of cell death that occurs during cardiac ischemia, and is thought to contribute to the progressive loss of cardiac function and eventual transition toward overt cardiac failure, a leading cause of death worldwide (37, 38). Recent reports show that BRCA1/2 mutation carriers have a 2 times higher risk of developing diabetes, a strong risk
for developing cardiovascular disease (39), and data from the relatives of 5,287 genotyped participants of whom 120 carried a \textit{BRCA1}/\textit{2} Ashkenazi Jewish founder mutation showed a statistically significant association between \textit{BRCA1}/\textit{2} mutations and increased rates of non-cancer mortality (40). The nature of the non-cancer mortalities was not recorded, but this association may be related to increased susceptibility to cardiovascular disease, a major contributor to non-cancer-related deaths globally.

Chemotherapeutic agents function by directly or indirectly damaging DNA through a variety of mechanisms (6). DOX is a

\textbf{FIGURE 4. Exacerbated DOX-induced cardiotoxicity in CM-BRCA2\(^{-/-}\) mice is p53-mediated.} Representative micrograph and quantification of p53 staining (brown staining) (A) and immunoblot for total and phosphorylated (serine 15) p53 (B) showed higher DOX-induced p53 expression and activation in LV sections from DOX-treated CM-BRCA2\(^{-/-}\) mice in comparison with those from their WT littermate controls \((n = 6/group;^{**}, p < 0.01;\text{ scale bar, } 50 \ \mu m).\) C and D, immunoblots for PUMA (C) and Bax and Bcl-2 (D) and corresponding quantifications showed increased PUMA expression and elevated Bax/Bcl-2 ratios in the hearts of DOX-treated CM-BRCA2\(^{-/-}\) mice in comparison with corresponding values from samples from their WT littermate controls \((n = 6/group;*, p < 0.05).\) E, increased p53, PUMA, and Bax/Bcl-2 ratios were associated with more cytochrome c release in the left ventricle of DOX-treated CM-BRCA2\(^{-/-}\) mice as evident by IHC staining (brown) and corresponding quantification \((n = 6/group;*, p < 0.05).\) Immunoblots for total ATM, phosphorylated ATM, and Chk2 (F) as well as p21 (G) and GADD45 (H) on total heart lysates from CM-BRCA2\(^{-/-}\) mice and their WT littermate controls show increased expression and activation of ATM (upper band; indicated by arrow), increased activation of Chk2, but similar p21 and GADD45 expression in the hearts of DOX-treated CM-BRCA2\(^{-/-}\) mice in comparison with the WT control littermate controls. GAPDH was used as a loading control \((n = 3–4/group;\text{ age, } 10–12 \text{ weeks). AU}, \text{ arbitrary units. For A, D, and E, data are presented as mean } \pm \text{ S.D.})
potent chemotherapeutic agent used for a wide variety of malignancies. Its clinical utility, however, has been limited by its dose-dependent cardiotoxicity, which can result in refractory cardiac dysfunction (17). DOX induces DSBs (10), and BRCA2 has been previously implicated in the repair of chemotherapy-induced DSBs through HDR (11). Thus, the role of BRCA2 in response to DOX-induced cardiotoxicity is an important subject of investigation. The BRCA2 gene is composed of 27 exons and encodes a predicted 390-kDa protein possessing no homology to other sequences in publicly available databases (41). BRCA2 harbors eight repetitive RAD51-interacting BRC motifs encoded by exon 11 (42). Because BRCA2 is a caretaker of genomic integrity, we postulated that loss of BRCA2 leads to a defect in DNA damage repair and deranged genome integrity. The continual failure to repair DNA damage can result in the accumulation of a lethal amount of DNA damage, leading to prolonged p53 activation and consequently p53-mediated activity, culminating in cardiomyocyte apoptosis, and leading to cardiac dysfunction.

An issue that is commonly encountered in the generation of animal models involving tumor suppressor genes, such as BRCA2, is the high and frequent embryonic lethality associated with homozygous mutations (14). We therefore opted to derive a tissue-specific knock-out strain. Using the Cre-loxP system, we successfully generated viable mice with cardiomyocyte-specific deletion of the BRCA2 gene. Naïve mice of this background demonstrated neither structural nor functional abnormality. Compared with the WT littermate controls, they also exhibited no apparent base-line differences in the extent of DNA damage, DNA damage repair, apoptosis, and p53 and ATM expressions. Collectively, these findings suggest that BRCA2 is dispensable in the adult heart. Genotoxic stress in the form of single dose of 20 mg/kg (intrapertitoneal) DOX, however, induced significantly higher mortality in CM-BRCA2−/− mice relative to their WT littermate controls. A single dose of 10 mg/kg DOX markedly exacerbated cardiac dysfunction and weight loss in CM-BRCA2−/− mice in comparison with their WT littermate controls (Fig. 3, A−C). Cardiomyocyte apoptosis is recognized as the underlying means of progressive ventricular remodeling and cardiac dysfunction (27, 37), and DOX-induced cardiac dysfunction was associated with a higher amount of apoptosis. Because BRCA2 is a DNA damage repair molecule, loss of cardiomyocyte BRCA2 expression would be expected to induce DNA damage and its accumulation in the cardiomyocytes of DOX-treated CM-BRCA2−/− mice. Indeed, γH2AX focus formation, the first cellular response to DNA damage (21), was significantly higher in hearts from DOX-treated CM-BRCA2−/− mice compared with those from their littermate controls, indicating greater DNA lesions (Fig. 3, F and G). Studies have shown that BRCA2 physically interacts with RAD51 to translocate RAD51 from the site of synthesis to the site of DNA damage processing. In BRCA2-deficient cells, nuclear transport of RAD51 and DNA repair are impaired (33). Although the levels of damaged DNA in the hearts of DOX-treated CM-BRCA2−/− mice were elevated, RAD51 focus formation, a hallmark of BRCA2-mediated repair (43), was absent, suggesting the presence of defective repair and thus the accumulation of unrepaired damaged DNA. Although HDR occurs preferentially in dividing cells during the late S and G2 cell cycle phases when a sister chromatid is present, HDR can also take place with the allele on the homologous chromosome acting as a template. Furthermore, HDR has been observed in G0/G1 cells albeit at levels lower than that in cells that are in the S, G2, and M phases (44). Because RAD51 accumulates indiscriminately at sites of both double- and single-stranded DNA breaks (45), our results do not allow us to distinguish between the types of DNA damage evoked by DOX. However, they do demonstrate for the first time that some form of homologous repair does transpire in terminally differentiated cardiomyocytes and may be important in the overall response to DOX. Furthermore, we provide novel evidence supporting a fundamental role for BRCA2 in mediating the repair of DOX-evoked DNA damage via RAD51.

Mouse embryos with conditional knock-out of the BRCA2 gene die via a p53-dependent mechanism, and this phenomenon can be rescued by the deletion of a p53 allele (24). Because increased p53 expression is sufficient to induce cardiomyocyte apoptosis (23) and p53 is also known to mediate DOX-induced cardiomyocyte apoptosis, we sought to investigate whether p53 is a modulator of cardiomyocyte apoptosis after DOX treatment in CM-BRCA2−/− mice. We noted significantly higher p53 protein levels along with increased expressions of the p53-regulated proapoptotic downstream targets PUMA and Bax in the hearts of DOX-treated CM-BRCA2−/− mice. The shift in the ratio between the proapoptotic Bax and prosurvival molecule Bcl-2 is critical in p53-dependent survival and/or apoptotic signaling (25). Bax and Bcl-2 are fundamental modulators of cardiomyocyte apoptosis and/or survival decisions during heart failure (26, 27). We observed that the increase in cardiomyocyte apoptosis triggered by DOX in CM-BRCA2−/− mice was associated with a ∼5-fold increase in the Bax to Bcl-2 ratio. Accordingly, this triggered a shift from a survival to an apoptotic milieu (Fig. 4D). We then identified subcellular increases in p53 levels and up-regulation of proapoptotic proteins, both of which were associated with an increase in cytochrome c release in DOX-treated CM-BRCA2−/− mice. Mitochondrial alterations have been implicated in anthracycline-associated cardiotoxicity (46). DOX-induced DNA damage has been shown to activate p53, which in turn promotes mitochondrial reactive oxygen species production and DNA damage, which result in increased cell death (46, 47). However, BRCA2 is upstream from the mitochondria, and loss of BRCA2 in cardiomyocytes culminates in accumulation of unrepaired damaged DNA after DOX treatment. Thus, it appears that in this setting unrepaired DNA damage results in prolonged activation of p53, which then either directly increases apoptosis or prompts mitochondrial reactive oxygen species production and DNA damage with apoptosis being the final outcome. DOX treatment stimulates ATM-, Chk2-, and p53-mediated events (31), which in turn result in either apoptosis or cell arrest through induction of p21 or GADD45 (29, 30). We observed up-regulation and activation of ATM and p53 as well as Chk2 activation. Conversely, p21 and GADD45α expression was unchanged, indicating that the stage of the cell cycle at which the cells were in following DOX treatment was similar between the CM-BRCA2−/− and WT littermate control groups. This is in concordance with a previous report demonstrating that
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22. Yuan, S. S., Lee, S. Y., Chen, G., Song, M., Tomlinson, G. E., and Lee, E. Y. (1999) BRCA2 is required for ionizing radiation-induced assembly of the Rad51 complex and proliferation per se. Prior to this report, there were little or no data examining the function of BRCA2 in adult tissues. In addition, cardiac tissues have a limited ability to regenerate and proliferate. Numerous studies have previously addressed this issue, and although there is evidence for a very slow turnover of adult mammalian cardiac cells, this rate is less than 1% per year basally (48). More relevant to our model, estimates suggest that following injury less than 0.1% of cardiomyocytes re-enter the cell cycle and proliferate (49). Thus, it would appear unlikely that the sole mechanism through which BRCA2 deletion exacerbates DOX-induced cardiac failure is via a block on adult cardiomyocyte proliferation because that proliferation is essentially non-existent. A final point regarding this issue is that in contrast to the adult situation there is extensive proliferation of cardiomyocytes during development. If BRCA2 was solely acting to inhibit myocyte proliferation, we would expect that MHC-Cre-mediated deletion of BRCA2 would result in some basal defect in cardiac structure or function or potentially embryonic lethality. In contrast, our results suggest no such base-line defect. As mentioned previously, the cardiac phenotype of BRCA2-deficient mice is indistinguishable from that of WT mice under naïve conditions.

The observation that BRCA2 deficiency increases susceptibility toward DOX-induced cardiac apoptosis and failure may have important translational implications. Pharmacogenomically, these data may indicate a heightened susceptibility of BRCA2 mutation carriers to anthracycline-induced cardiac failure, a cornerstone of chemotherapy for breast and ovarian cancer. Recent evidence suggests that alternative chemotherapeutic agents that elicit DNA damage, particularly nimustine and temozolomide, demonstrate increased sensitivity in BRCA2-deficient tumors (50). Although these data are derived from cancer cells as opposed to postmitotic non-dividing cardiomyocytes, they raise the possibility that DNA-damaging chemotherapy in general may be more cardiotoxic in the setting of BRCA2 deficiency. Clinical and population-based studies examining this question are encouraged. In conclusion, this study together with recently published findings lends credence to the increasing body of evidence that the BRCA family of genes may have important regulatory roles in non-cancer disorders (39, 40, 51, 52).
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