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Research Paper

Breast cancer 1 (BRCA1)-deficient embryos develop normally but are more susceptible to ethanol-initiated DNA damage and embryopathies ☆

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ABSTRACT

The breast cancer 1 (*brca1*) gene is associated with breast and ovarian cancers, and heterozygous (+/−) *brca1* knockout progeny develop normally, suggesting a negligible developmental impact. However, our results show BRCA1 plays a broader biological role in protecting the embryo from oxidative stress. Sox2-promoted Cre-expressing hemizygous males were mated with floxed *brca1* females, and gestational day 8 +/− *brca1* conditional knockout embryos with a 28% reduction in protein expression were exposed in culture to the reactive oxygen species (ROS)-initiating drug ethanol (EtOH). Untreated +/− *brca1*-deficient embryos developed normally, but when exposed to EtOH exhibited increased levels of oxidatively damaged DNA, measured as 8-oxo-2'-deoxyguanosine, γH2AX, which is a marker of DNA double strand breaks that can result from 8-oxo-2'-deoxyguanosine, formation, and embryopathies at EtOH concentrations that did not affect their *brca1*-normal littermates. These results reveal that even modest BRCA1 deficiencies render the embryo more susceptible to drug-enhanced ROS formation, and corroborate a role for DNA oxidation in the mechanism of EtOH teratogenesis.

1. Introduction

Brca1 is a DNA repair gene commonly associated with certain familial breast and ovarian cancers [1]. Studies in knockout models have shown that mice lacking both copies of the *brca1* gene have poor genomic integrity and are not viable [2]. The morphological basis has been characterized by rapid disorganized proliferation of neuroepithelium and increased cell death [3]. However, progeny missing only one copy of the *brca1* gene appear to develop normally [3], suggesting that minor BRCA1 deficiencies have a negligible developmental impact.

A number of population-based studies have investigated the developmental consequences of *brca1* mutations on sex differences, miscarriage rates and prevalence of mutation carriers as well as double mutants. While it has been suggested in small population studies that there are differences in birth rates and sex ratios [4,5], this has not been confirmed in larger population studies [6,7]. Clinically, there have been no confirmed cases of a viable individual with two mutated copies of the *brca1* gene [8], although screening of lymphocyte DNA from patients in Scotland with breast or ovarian cancer revealed a woman who lacked the wild-type allele for *brca1*, and was the offspring of two carriers of the *brca1* mutation [9]. However, the finding that this individual carried two copies of the mutated gene was likely the result of a technical error in the screening method [10]. Taken together, population studies on *brca1* mutations and embryonic development

Abbreviations: 8-oxo-2′-deoxyguanosine, 8-oxodGuo; BER, Base excision repair; brca1, Breast cancer 1; csb, Cockayne Syndrome B; dGuo, deoxyguanosine; DSB, double strand break; ELISA, enzyme linked immunosorbent assay; EtOH, ethanol; FASD, fetal alcohol spectrum disorder; GD, gestational day; MDMA, methylenedioxymethamphetamine; METH, methamphetamine; NCC, neural crest cell; NOX, NADPH oxidases; Nrf2, nuclear factor erythroid 2-related factor 2; ogg1, oxoguanine glycosylase 1; ROS, reactive oxygen species; γH2AX, phosphorylation of histone H2AX

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suggest that double mutants are not viable, while heterozygous carriers of the mutation develop normally but are more susceptible to developing cancer later in life [7,11].

The BRCA1 protein contains a ring domain at the N-terminal primarily associated with E3 ubiquitin ligase activity and a C-terminal responsible for tumor suppression and recruitment of DNA repair enzymes [12]. When evaluating the two domains of the BRCA1 protein, it was found that the C-terminal is not essential for development. However, mice lacking the C-terminal were at significant risk of developing tumors as early as 1 month after birth in a tissue- and time-dependent manner [13]. This finding suggests that the multiple functions of BRCA1 in DNA repair are not required for normal embryonic and fetal development at least with respect to gross morphology. Evaluation of a number of other DNA repair genes in mouse models has similarly shown that progeny deficient in DNA repair exhibit normal gross morphology, but are more susceptible to developmental anomalies under conditions of chemical and radiation stress [14–16].

BRCA1 plays an important role in multiple DNA repair pathways by either interacting directly with DNA repair proteins or upregulating the expression of genes directly or indirectly involved in DNA damage repair [17]. The importance of BRCA1 in DNA repair has been demonstrated in non-homologous end joining [17], homologous recombination repair [18], nucleotide excision repair [19] and base excision repair [20]. We have found that deficiencies in several genes that are transcriptionally regulated by BRCA1, including Cockayne Syndrome B (***csb***) [21] and oxoguanine glycosylase 1 (***ogg1***) [14,16], do not affect morphological development under controlled conditions, but result in increased sensitivity to developmental anomalies caused by enhanced formation of reactive oxygen species (**ROS**) stimulated by teratogens like methamphetamine (**METH**) and ethanol (**EtOH**).

Several lines of evidence implicate ROS in the mechanism of EtOH teratogenesis. In embryo culture, ethanol induces the level of ROS-producing NADPH oxidases (**NOX**), and this induction along with ethanol-enhanced embryonic DNA oxidation and apoptosis are blocked by a NOX inhibitor [22]. CYP2E1-mediated metabolism of ethanol has also been shown to generate ROS as a byproduct [23]. ROS levels are increased in embryos and fetuses exposed *in utero* to ethanol, evidenced by DCF fluorescence and oxidatively damaged DNA [15,22,24–26]. Structural and cognitive changes caused by ethanol *in vivo* are prevented with pretreatment by the free radical scavenging agent α-phenyl-N-tert-butylnitrone (PBN) [27]. Ethanol-initiated embryopathies and DNA oxidation in whole embryo culture are respectively enhanced or reduced in genetically altered mice that either are deficient in or overexpress the antioxidative enzyme catalase, and ethanol embryopathies are blocked by pretreatment with exogenous catalase [24,28].

ROS-dependent developmental anomalies correlate with a marked increase in the formation of the oxidative DNA damage lesion 8-oxo-2′-deoxyguanosine (**8-oxodGuo**). In the case of OGG1, untreated homozygous *ogg1*-deficient progeny exhibited increased postnatal neurodevelopmental deficits, demonstrating that even physiological levels of ROS can be pathogenic in the absence of adequate DNA repair [16]. The *in utero* death of homozygous *brca1* knockout embryos may result from an overwhelming reduction in several DNA repair genes that are crucial for development. However, the absence of gross morphological anomalies in heterozygous progeny suggests that moderately reduced levels of BRCA1 have negligible developmental impact. A conditional knockout mouse line targeting the dorsal telencephalon starting on E9.5 using Emx-1 directed Cre recombinase showed increased apoptosis in homozygous but not heterozygous *brca1* knockout cells [29].

Herein, we developed a conditional *brca1* knockout model with heterozygous (+/−) *brca1* progeny that exhibit a modest 28% decrease in BRCA1 protein levels and develop normally. However, when exposed in culture to EtOH, +/− BRCA1-deficient embryos exhibited increased levels of oxidatively damaged DNA and embryopathies compared to wild-type BRCA1-normal littermates, showing that even modest deficiencies in BRCA1 increase embryonic susceptibility to the pathological effects of drug-enhanced ROS formation. These results reveal a broader biological role for BRCA1 in protecting the developing embryo from oxidative stress, and corroborate a role for DNA damage in the mechanism of ethanol embryotoxicity.

2. Materials and methods

2.1. Chemicals

EtOH was purchased from Commercial Alcohol Inc. (Brampton, ON). Saline was purchased from Baxter (Mississauga, ON). Hanks' Balanced Salt Solution, Waymouth's MB 752/1 medium, fetal bovine serum, proteinase K and calf intestinal alkaline phosphatase were purchased from Life Technologies (Burlington, ON). Nuclease P1 was purchased from Sigma Aldrich Canada (Oakville, ON).

2.2. Animals and diet

All animal studies were conducted in accordance with Canadian Council on Animal Care standards and were approved by the institutional Animal Care Committee. Mice containing LoxP sites flanking exon 11 of the *brca1* gene (*brca1*$^{LoxP/LoxP}$) were generously donated by the Frederick National Laboratory for Cancer Research (National Institute of Health, Frederick, MD). Hemizygous transgenic mice expressing Cre recombinase controlled by the Sox2 promoter (Cre$^{+/-}$) were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were housed in light- and temperature-controlled rooms (14-h light/10-h dark cycle, 20 °C, 50% humidity). Teklad 18% protein rodent chow (Harlan Laboratories, Mississauga, ON) and tap water were provided *ad libitum*. The two colonies were maintained separately and housed with up to four animals per cage, separated by sex. For overnight matings, up to 3 *brca1*$^{LoxP/LoxP}$ females were housed with one male Cre$^{+/-}$ from 17:00 to 9:00 and then separated. Females with a vaginal plug (plug designated gestational day (**GD**) 1) were placed in separate cages and were used as described below. Jackson laboratories has noted that when the Sox2-Cre females are used for cross breeding with floxed males, expression of Cre recombinase is ubiquitously present in all embryonic tissues even if the transgene is not inherited. In contrast, when male Sox2-Cre mice are used, expression of Cre recombinase is limited to offspring that carry the transgene, and is localized to cells of epiblast origin, starting on GD 6.5. Accordingly, only male Sox2-Cre mice were used in this study. All offspring had one allele with LoxP sites located in the introns flanking exon 11 of the *brca1* gene. Half of those offspring also received the Cre recombinase transgene controlled by the Sox2 promoter, which results in the deletion of exon 11, the sequence of DNA flanked by the LoxP sites, starting on GD 6.5 [30]. Deletion of exon 11 results in the truncation of the BRCA1 protein at the C-terminal [31]. Mice lacking the Cre transgene developed normally in our laboratory.

2.3. Genotyping

After weaning, mice were ear tagged and a small notch was removed from their ears for genotyping. For explanted embryos, the visceral yolk sac was used for genotyping. Crude DNA extracts were isolated using the HOTSHOT method [32]. Briefly, ear notches or visceral yolk sacs were placed in 75 μL of 25 mM NaOH

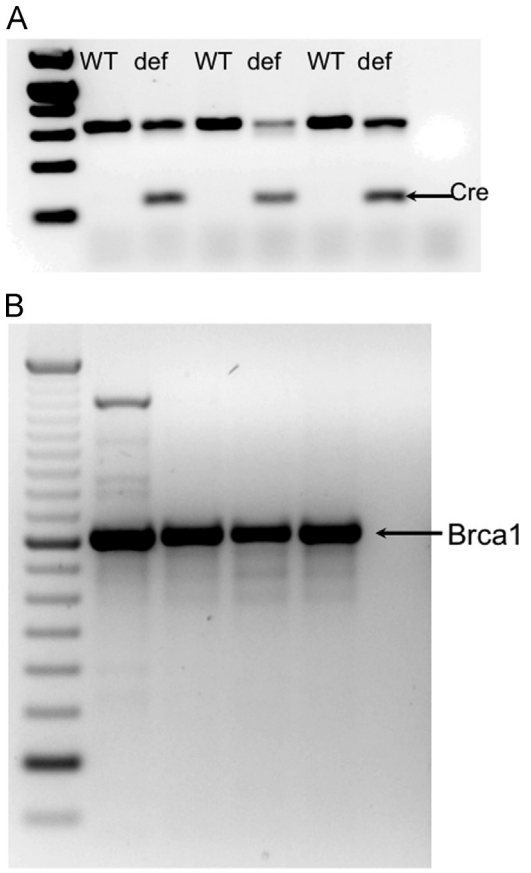

Fig. 1. Representative gels to confirm the breast cancer 1 (*brca1)* genotype of mouse strains**. (Panel A)** Agarose gel of PCR products from crude DNA extraction amplifying the Cre transgene (100 bp), with interleukin-2 as a positive control (324 bp). The presence of both bands indicates that the Cre recombinase gene is present, resulting in the heterozygous *brca1*-deficient (**def**) genotype, while lanes with only the upper band indicate an absence of Cre recombinase resulting in the wild-type (**WT**) genotype. **(Panel B)** Representative gel for the *brca1*-loxP mice showing the WT *brca1* genotype. Each lane is from a different mouse, and mice containing two copies of the floxed *brca1* allele displayed a band at 500 bp. Only mice homozygous for the floxed *brca1* allele were used in this study.

containing 0.2 mM EDTA for 1 h at 95 °C. Samples were then neutralized with 40 mM Tris (pH 5.0) and stored at 4 °C. A 2 μL aliquot of DNA was added to a 20 μL final volume reaction mix for PCR amplification.

2.3.1. Cre transgenic mice

Genotyping of the Cre transgene was performed according to a generic Cre protocol provided by Jackson Laboratories. The Cre primers were as follows: F: 5′-GCGGTCTGGCAGTAAAAACTATC-3′, R: 5′-GTGAAACAGCATTGCTGTCACTT-3′. Primers coding for interleukin 2, used as a positive control, were: F: 5′-CTAGGCCACAGAATTGAAAGATCT-3′, R: 5′-GTAGGTGGAAATTCTAGCATCATCC-3′. The PCR reaction conditions were as follows: 2.0 mM $MgCl_2$, 0.2 mM dNTP, 1 μM of each primer and 0.01 U/μL Hot Start Taq polymerase. After activation at 94 °C for 3 min, the reaction went through 35 cycles of 94 °C for 30 s, 51.7 °C for 60 s and 72 °C for 60 s, followed by 72 °C for 2 min (Fig. 1A).

2.3.2. Floxed brca1 mice

To confirm the presence of the LoxP sites, we used the following primers: F: 5′-CTGGGTAGTTTGTAAGCATGC-3′, R: 5′-CAATAAACTGCTGGTCTCAGG-3′. The reaction mixture contained 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.33 μM primers and 0.05 U/μL Hot Start Taq polymerase. The PCR cycle included activation at 94 °C for 3 min, 35 cycles of 94 °C for 1 min, 60 °C for 2 min and 72 °C for 1 min, followed by 72 °C for 3 min. The presence of a band at 500 bp indicated that the mice contained the LoxP sites (Fig. 1B).

2.4. DNA isolation for oxidative DNA damage

Pregnant dams received a single intraperitoneal (i.p.) injection of 4 g/kg EtOH or its saline vehicle (control) on GD 12 and were sacrificed 6 h later by cervical dislocation. Embryos were quickly removed from the uterus and all extraembryonic tissues were removed. Small sections of the tail were collected for genotyping, while the remaining embryonic tissues were snap frozen in liquid nitrogen and stored at −80 °C for future analysis. DNA was isolated from embryonic tissues using a modified version of the chaotropic sodium iodide method described by Ravanat et al. [33]. Approximately 50–100 mg of wet tissue was homogenized in a cold sucrose gradient lysis buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris, 0.1 mM desferoxamine, 1% (v/v) Triton X-100, pH=7.5). Nuclear cell fractions were collected by centrifugation at 1,000 g for 10 min then resuspended in an enzyme reaction solution containing 1% (*w*/*v*) SDS, 5 mM EDTA-NA_2, 0.15 mM desferoxamine and 10 mM Tris–HCl, pH=8.0. Digestion of contaminating RNA was accomplished by the addition of 624 and 312 U/mL of RNase A and T1, respectively, for 1 h at 50 °C. Protein was digested by the addition of proteinase K to a final concentration of 1.8 mg/mL for 1 h at 50 °C. DNA was precipitated out by the addition of a saturating NaI solution (7.6 M NaI, 40 mM Tris, 20 mM EDTA-NA_2, 0.3 mM desferoxamine, pH=8.0) and protein contaminants were removed by the addition of 100% isopropanol. DNA was pelleted by centrifugation at 10,000 g for 10 min before five rounds of washing in 70% (v/v) EtOH. The isolated pellet was then dissolved in sodium acetate buffer (7.6 M NaI, 40 mM Tris, 20 mM EDTA-NA_2, 0.3 mM desferoxamine, pH=8.0) and analyzed for absorbance at 260/280 nm for concentration and purity.

2.5. Digestion of DNA and analysis by enzyme linked immunosorbent assay (ELISA)

A total of 100 μg of DNA was placed in 200 μL of sodium acetate buffer and incubated for 1 h at 37 °C with 5 U of nuclease P1 to hydrolyze the DNA [33]. Nucleosidic phosphates were hydrolyzed by calf intestinal alkaline phosphatase (6 U/20 μL per reaction) for 1 h at 37 °C following the addition of 1 M Tris–HCl buffer, pH 8.5, to a final pH of 8.0. Contaminating molecules of molecular weights greater than 10 kDa were removed by filtration using Amicon YM-10 spin columns and centrifugation at 14,000 *g* for 1 h. The purified samples were placed in a competitive binding High Sensitive 8-OHdG Check ELISA Kit and analyzed according to the manufacturer's specifications (Genox Corporation, Baltimore, MD, USA). The bottoms of the ELISA plate wells were coated with a known amount of purified 8-oxodGuo. When samples and an antibody that specifically recognizes 8-oxodGuo were added, 8-oxodGuo affixed to the plate competed with free 8-oxodGuo from samples for antibody binding. Visualization was carried out by the addition of an HRP conjugated secondary antibody that oxidized tetramethylbenzidine, and the product, 3,3′,5,5′-tetramethylbenzidine diimine, was measured by absorbance at 450 nm.

2.6. Western blotting

Pregnant dams received a single i.p. injection of 4 g/kg EtOH or its saline vehicle (control) on GD 9 and were sacrificed 24 h later by cervical dislocation. Embryos were quickly removed from the uterus and all extraembryonic tissues were removed. Visceral yolk sacs were collected for genotyping while whole embryos were snap frozen in liquid nitrogen and homogenized in RIPA buffer containing complete protease inhibitor cocktail tablets (Roche

Applied Science, Laval, QC). Protein content was determined by the bicinchoninic acid (**BCA**) assay and 15 μg of total protein were loaded in each well of a 10% (v/v) polyacrylamide gel under reducing (50 mM DTT) conditions. After blocking for 1 h in blocking buffer (Tris-Buffered Saline with Tween 20 [**TBST**]+5% (*w*/*v*) milk), BRCA1 blots were cut at the 70 kDa mark. The upper region of the blot was probed against BRCA1 using a monoclonal antibody (R&D Systems Cat#MAB22101), which recognizes the C-terminal of the protein, at 1 μg/mL in blocking buffer. After washing, this upper blot was probed with a goat anti-mouse-HRP (5000 × dilution in blocking buffer) (Santa Cruz Biochemical, Santa Cruz, CA) for 1 h. The lower region of the blot was probed with mouse anti-GAPDH-HRP (Sigma Aldrich Canada, Burlington, ON) at 50,000 × dilution. Chemiluminescent detection was achieved using ECL Plus western blotting substrate (Thermo Scientific, Lafayette, CO).

For γH2AX, blots were probed with mouse anti-γH2AX, which recognizes phosphorylation of Ser^{139} on H2AX (Abcam, cat#-ab2893) at 2000 × dilution overnight at 4 °C. Both primary antibodies were probed with goat anti-mouse IgG conjugated to HRP at 5000 × dilution in 5% (*w*/*v*) milk for 1 h at room temperature. Blots were washed with TBST and visualized using ECL-plus chemiluminescent reagent (Thermo Scientific, Lafayette, CO). After probing the blots for γH2AX, blots were washed in TBST and antibodies were removed using Restore Plus Western Blot Stripping Buffer (Thermo Scientific) for 10 min at room temperature. Following another blocking step in 5% (*w*/*v*) milk, blots were treated with mouse anti-GAPDH-HRP (Sigma Aldrich Canada, Burlington, ON) at 50000 × dilution and visualized as described above.

2.7. Embryo culture

Pregnant dams were sacrificed on GD 9 by cervical dislocation and the embryos were excised. All extra-embryonic tissues, with the exception of the visceral yolk sac and ectoplacental cone, were removed. Growth medium (50 mL Waymouth's MB 752/1 medium, 500 μL of 50 U/mL penicillin and 50 mg/mL streptomycin in saline, 2.5 mM HEPES, 15 mL fetal bovine serum and 35 mL male rat serum) was pre-gassed with 5% CO_2 in air for 30 min. Embryos with 7–8 somite pairs were placed in individual wells on a 24 well plate in 2 mL of growth medium containing EtOH or saline vehicle, enclosed with an airtight seal on a platform rocker (Bellco Biotechnology, Vineland, NJ) for 24 h at 37 °C in a Sanyo model MCO-17A CO_2 incubator (Sanyo Electric Co., Ltd., Japan). Following incubation, embryos were assessed for the following parameters: anterior neuropore closure, crown-rump length, turning, head length, yolk sac diameter, number of somite pairs and heart rate. Somite development and crown-rump length were assessed only in those embryos that turned during the incubation period. Embryos were scored only if they had a heart beat. Visceral yolk sacs were removed for genotyping as described above.

2.8. Statistical analysis

Statistical analyses were performed using Prism®, Version 5 (GraphPad Software, Inc., San Diego, CA). Differences in BRCA1 expression were evaluated by Student's *t*-test. Other continuous data, expressed as mean+SD, were analyzed by one-way ANOVA with the Newman–Keuls post-hoc test for DNA oxidation and the Bonferroni post-hoc test for γH2AX blots and embryo culture. Binomial data were analyzed using the Chi-square test. The minimal level of significance used throughout was $p < 0.05$.

3. Results

3.1. Heterozygous brca1 conditional knockouts have less BRCA1 protein than wild-type littermates

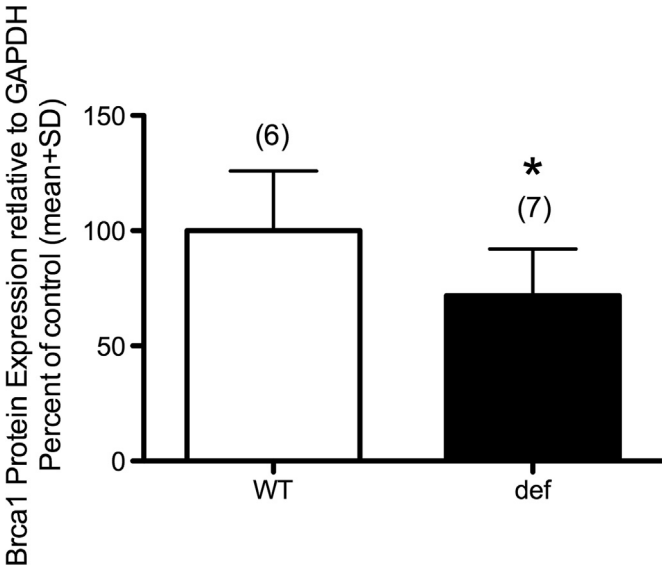

Fig. 2. BRCA1 protein expression in conditional knockouts and wild-type embryos by western blot analysis. Hemizygous Cre recombinase mice controlled by the Sox2 promoter were mated overnight (plug designated gestational day [GD] 1) with female homozygous mice containing LoxP sites flanking exon 11 of the *brca1* gene, resulting in either wild-type (**WT**) progeny with normal BRCA1 protein levels, or BRCA1-deficient (**def**) progeny with reduced protein levels. On GD 10, dams were sacrificed by cervical dislocation and whole embryos were removed and snap frozen. BRCA1 protein expression was quantified by western blot using GAPDH as a loading control. $^{*}p < 0.05$ compared to WT embryos.

Issues of embryolethality precluded the use of homozygous *brca1* knockouts for developmental studies. As a result, a Cre-Lox based approach was used for gene removal during the embryonic period, bypassing the critical period for embryolethality. To achieve a full conditional knockout, however, mice heterozygous for the *brca1*-floxed allele first had to be characterized. Frequency of genotypes appeared to follow Mendelian distribution, with an approximately 1:1 ratio of wild-type (Cre absent) to conditional *brca1*-deficient (hemizygous for Cre) progeny. No phenotypic differences were observed between the two genotypes. To determine relative BRCA1 protein levels in the two genotypes, we employed a western blot targeting the C-terminal of the BRCA1 protein. Fig. 2 shows the relative expression of BRCA1 protein in the wild-type and +/− *brca1*-deficient mice by western blot. The +/− *brca1* conditional knockout mice, carrying one floxed allele and one wild-type allele, had 28% less BRCA1 protein expression relative to their wild-type littermates ($p < 0.05$) based on semi-quantitative densitometry of western blots.

3.2. Brca1-deficient embryos exhibit more oxidatively damaged DNA than their wild-type littermates

BRCA1 is an important factor in DNA double strand break repair, but also plays an important role in the transcriptional regulation of base excision repair (**BER**) [20]. Accordingly, we evaluated levels of 8-oxodGuo as a biomarker of DNA damage and BER activity. More importantly, this DNA lesion has been shown in *ogg1* knockout mice to contribute to the mechanism of neurodegeneration caused by 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) in adults [34], and postnatal neurodevelopmental deficits caused by *in utero* exposure to methamphetamine [35] and EtOH [16]. As shown in Fig. 3, EtOH caused an increase in 8-oxodGuo levels compared to saline controls in both *brca1* wild-type (BRCA1-normal) ($p < 0.05$) and +/− *brca1* knockout (BRCA1-deficient)

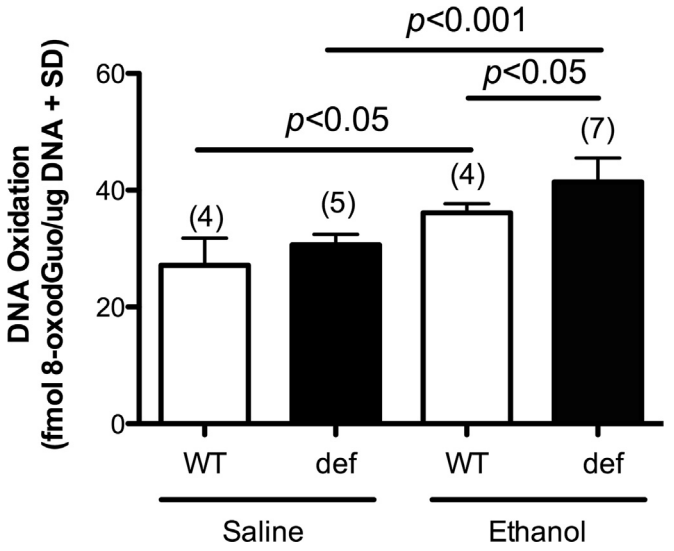

Fig. 3. *Brca1*-deficient (def) mice do not have increased levels of the oxidative DNA damage lesion 8-oxo-2′-deoxyguanosine (8-oxodGuo), but are more susceptible to ethanol (EtOH)-initiated DNA damage. Mice were bred as described in Fig. 2. On GD 12, dams received a single ip injection of 4 mg/kg EtOH or saline vehicle, and were sacrificed 6 h later by cervical dislocation. Embryos were removed and snap frozen. An aliquot of 100 μg DNA was isolated from whole embryos and analyzed by ELISA. While no differences were observed in the saline-treated groups, EtOH-exposed wild-type (WT) BRCA1-normal and heterozygous BRCA1-deficient (def) embryos had increased levels of 8-oxodGuo relative to their respective saline-treated controls, representing a drug effect. EtOH-exposed deficient embryos had elevated levels of 8-oxodGuo compared to their WT littermates, indicating a *brca1* gene effect. $^{*}p < 0.05$ compared to WT saline-exposed embryos, $^{\dagger}p < 0.001$ compared to *brca1*-def saline-exposed embryos, $^{\ddagger}p < 0.05$ compared to EtOH-exposed WT embryos.

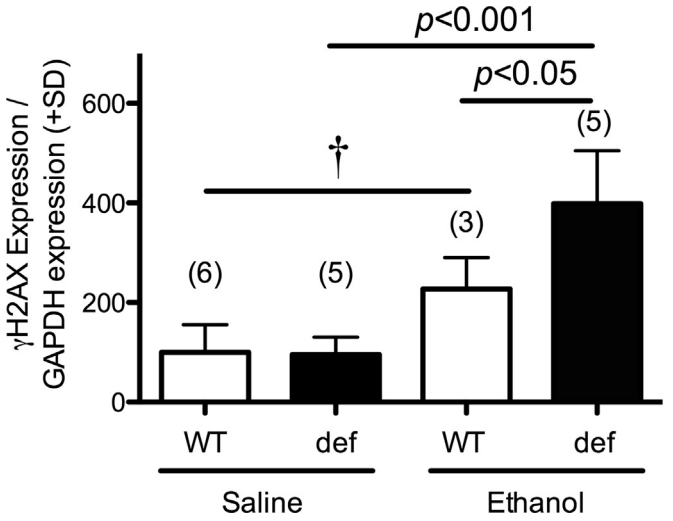

Fig. 4. *Brca1*-deficient (def) embryos are more susceptible to ethanol (EtOH)-initiated DNA double strand breaks. GD 10 embryos were explanted from dams treated as described in Fig. 3, and homogenized in RIPA buffer in the presence of protease inhibitors. DNA double strand breaks were measured by the presence of γH2AX relative to GAPDH loading control by western blot analysis. γH2AX levels were unaffected by *brca1* genotype in saline-treated control embryos. However, maternal treatment with EtOH increased phosphorylation to form γH2AX in *brca1*-deficient (def) embryos compared to both *brca1*-deficient saline-exposed progeny ($p < 0.001$), and their EtOH-exposed wild-type (WT) littermates ($p < 0.05$). † indicates a difference from saline-exposed WT littermates that was significant by Student *t*-test ($p = 0.017$), but not by ANOVA ($p = 0.082$).

($p < 0.001$) embryos, indicating a consistent drug effect regardless of *brca1* genotype. Moreover, EtOH-treated BRCA1-deficient embryos had 13% higher levels of 8-oxodGuo compared to EtOH-treated BRCA1-normal littermates ($p < 0.05$), indicating a gene-dependent increase in oxidatively damaged DNA. The *brca1* genotype had no significant effect on 8-oxodGuo levels in saline controls, although a small and non-significant trend for an increase in BRCA1-deficient embryos was observed.

3.3. EtOH-treated brca1-deficient embryos have increased expression of γH2AX relative to their wild-type littermates and vehicle controls

Following the formation of a DNA double strand break (**DSB**), H2AX is phosphorylated at Ser^{139}, forming γH2AX, to recruit other DNA repair factors to the damaged site. This makes γH2AX a robust biomarker for DNA DSBs, as well as a molecular complement to the oxidative DNA lesion 8-oxodGuo. As shown in Fig. 4, EtOH treatment increased γH2AX formation compared to saline controls by 4.2-fold in BRCA1-deficient embryos ($p < 0.001$), indicating a drug effect. A similar 2.3-fold increase in γH2AX in EtOH-treated wild-type BRCA1-normal littermates was significant by Student's *t*-test ($p = 0.017$), although not by ANOVA ($p = 0.082$). As observed for the 8-oxodGuo lesion, EtOH-treated BRCA1-deficient embryos exhibited a γH2AX level that was 75% higher than that in EtOH-treated wild-type BRCA1-normal littermates ($p < 0.05$), confirming a gene-dependent increase in DNA damage. No difference was observed between the BRCA1-deficient mice and their wild-type littermates when treated with saline vehicle.

3.4. BRCA1-dependent effect on EtOH embryopathies in mouse whole embryo culture

To determine the effect of a BRCA1-deficiency on development, embryos were cultured and assessed for a number of structural and functional parameters (Fig. 5). In saline-treated groups, no genotypic differences in any parameter were observed between wild-type and BRCA1-deficient embryos, and deficient embryos appeared to be normal. An EtOH concentration of 4 mg/mL was chosen as a threshold concentration based on previous work in *ogg1* knockout strains with a similar genetic background [15]. At this concentration, no difference in any parameter was observed in wild-type animals exposed to EtOH compared to saline controls. Upon challenge with 4 mg/mL EtOH, wild-type embryos exhibited no abnormality in any morphological parameters compared to saline controls, although a 22% increase in heart rate was observed ($p < 0.001$). In contrast, EtOH-exposed BRCA1-deficient embryos exhibited significant pathological changes in a number of parameters compared to both saline-treated littermates, indicating a drug effect, and compared to EtOH-treated wild-type BRCA1-normal littermates, indicating a gene effect. *Brca1* gene effects were evident for EtOH-initiated abnormalities in anterior neuropore closure, turning, head length and somite development in BRCA1-deficient embryos compared to wild-type littermates ($p < 0.05$).

4. Discussion

Our results show that mouse embryos with even a modest 28% reduction in BRCA1 protein levels develop normally but are at greater risk of abnormal development under conditions of relatively low oxidative stress that do not affect BRCA1-normal littermates. This suggests a broader biological role for BRCA1, not only beyond cancer, but also for embryos with only modest BRCA1 deficiencies. Such deficiencies could result from inherited +/− gene mutations or a multitude of other mechanisms, including epigenetic modifications that only modestly reduce BRCA1 levels and/or binding activity. Accordingly, the risk for BRCA1-dependent

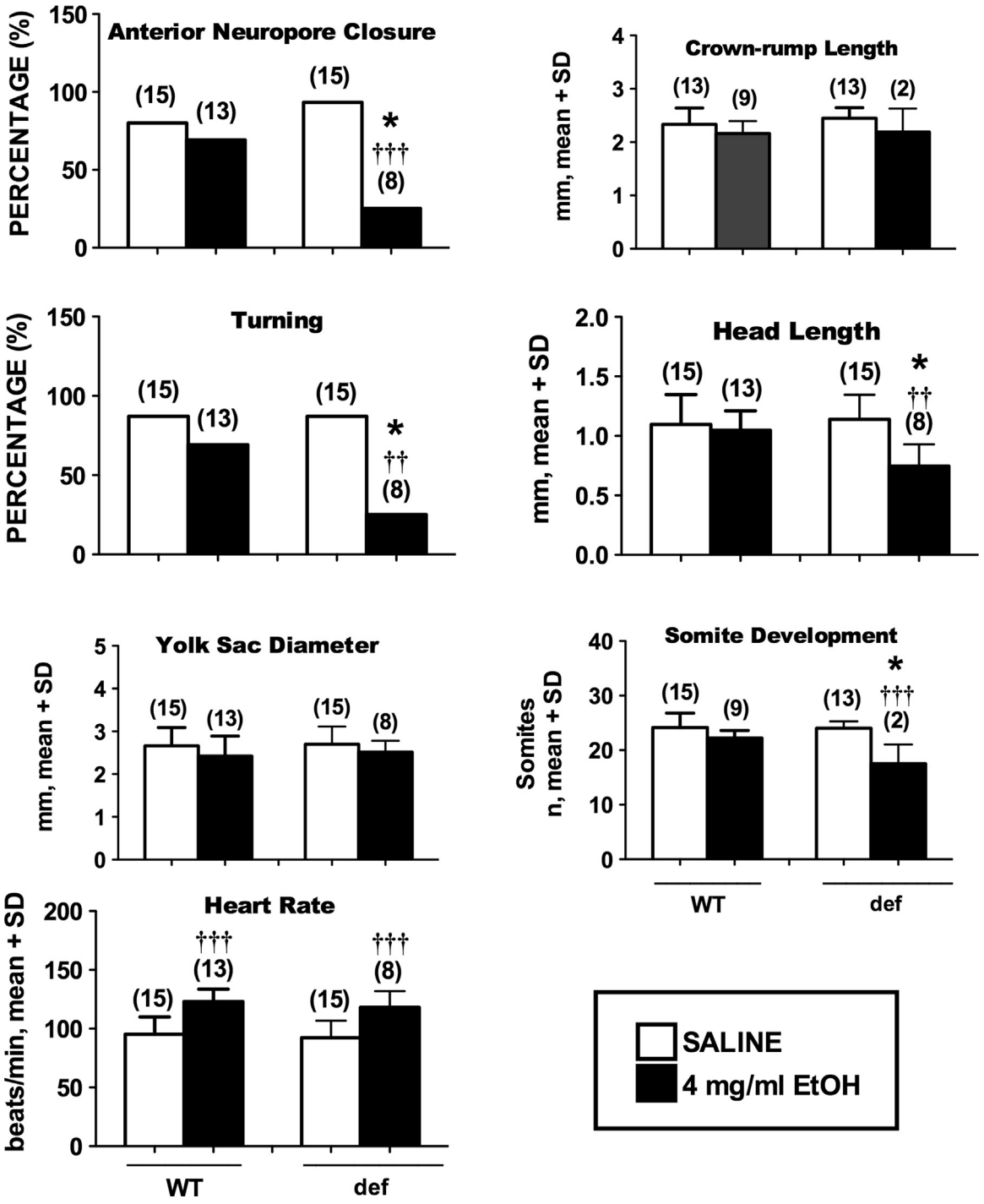

Fig. 5. *Brca1*-deficient embryos are more susceptible to ethanol (EtOH) embryopathies. Male mice hemizygous for Cre recombinase controlled by the Sox2 promoter were time mated for 2 h (plug designated GD 0) with female mice that homozygously contained LoxP sites flanking exon 11 of the *brca1* gene, resulting in wild-type (**WT**) and heterozygous (**def**) *brca1* littermates respectively exhibiting normal and deficient levels of BRCA1 protein. On GD 9, BRCA1 WT and deficient 6-8-somite embryos were explanted and incubated for 24 h with EtOH (4 mg/ml) or its saline vehicle. Embryos were evaluated under a microscope for morphological and functional (heart rate) factors. Daggers for EtOH-exposed groups indicate a difference from saline controls with the same *brca1* genotype (drug effect) ($^{\dagger\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.001$), and asterisks for EtOH-exposed BRCA1-deficient groups indicate a difference from wild-type littermates exposed to the same EtOH treatment (*brca1* genotype effect) ($p < 0.05$).

developmental abnormalities, including those due to a $+/-$ *brca1* genotype, would be expected to be substantially greater than that for breast and ovarian cancers, most of which require mutation of both *brca1* alleles resulting in a $-/-$ *brca1* genotype [36]. A corollary arising from the protective role of BRCA1 observed herein is the corroboration of a pathogenic role for the 8-oxodGuo lesion in the mechanism of EtOH embryopathies, which may ultimately prove relevant to the mechanism and risk factors for a broader range of developmental abnormalities in Fetal Alcohol Spectrum Disorders (**FASD**).

We anticipated the developmental impact of modest BRCA1 deficiencies under conditions of oxidative stress, as it has been shown that deficiencies in several genes that regulate or are directly involved in base excision repair can still result in normal developmental phenotypes [35,37,38], but are more susceptible to ROS-initiating teratogens [14–16,21]. The association of increased

levels of DNA damage with both structural and functional deficits suggests that xenobiotic-initiated oxidative stress is causing oxidatively damaged DNA, particularly the 8-oxodGuo lesion, which if not repaired, can initiate changes in gene expression resulting in a number of developmental pathologies. A pathogenic role for 8-oxodGuo is corroborated by the increase in embryopathies [15] and postnatal neurodevelopmental deficits [16] in *ogg1* knockout progeny exposed respectively in culture or *in utero* to EtOH.

Brca1-deficient mice had a 28% reduction in BRCA1 protein expression when carrying one allele that codes for the functional protein and one that is missing exon 11, resulting in a decrease in BRCA1 protein levels. Assuming complete excision of floxed *brca1* by Cre recombinase, we expected a somewhat greater suppression of protein levels. In lymphoblastoid cell lines derived from cancer patients whose tumor cells exhibit $-/-$ *brca1* mutations, BRCA1 protein expression was between 69% and 81% lower than wild-type levels [39]. A possible explanation for the discrepancy between our protein levels and those found *in vitro* may be related to the high turnover rate of BRCA1, which is controlled in part by proteolytic degradation [40,41]. *In vitro*, BRCA1 protein was found to have a half-life of 2 h, while in the presence of proteolytic inhibitors, the half-life increased to 6 h [42]. It is possible that a regulatory mechanism may attempt to compensate for the reduction by decreasing proteolysis. The relatively high turnover rate may suggest the need for tight regulation of the protein, possibly because of poor stability or overactivity. Overexpression of *brca1* in cell lines with normal levels of retinoblastoma protein has been shown to reduce cell colony formation [43]. It remains to be seen whether the protein levels observed in our study represent a population of proteins that evaded proteolysis, resulting in a population of aged BRCA1, or if the observed protein is of the same quality as that found in the wild-type controls resulting from incomplete Cre-mediated DNA digestion. The fact that this minimal level of suppression resulted in morphological and biochemical changes suggests that a very small depletion of BRCA1 is sufficient to increase susceptibility to teratogens.

Numerous forms of oxidatively damaged DNA result from the interaction of DNA with ROS, with the formation of 8-oxodGuo being the most prevalent due to the low reduction potential of deoxyguanosine (**dGuo**) [44]. EtOH has been shown to enhance the ROS-mediated formation of 8-oxodGuo [15,22,25,28,45], and elevations in 8-oxodGuo levels can lead to morphological anomalies in the developing embryo [15] and postnatal neurodevelopmental deficits [16]. In this study, we similarly observed an increase in DNA damage in both a drug- and *brca1* gene-dependent fashion. More directly, our manuscript demonstrates for the first time an increase in levels of the oxidative DNA lesion 8-oxo-dGuo in BRCA1-deficient progeny that are exposed to ethanol. Although we cannot at the current time measure oxoguanine glycosylase 1 (OGG1) activity in these mice, 8-oxo-dGuo lesion in mammals is primarily repaired by OGG1 [46], so a BRCA1-dependent decrease in OGG1 activity is a likely mechanism for the enhanced levels of 8-oxo-dGuo. This mechanism is supported by the reported role of BRCA1 in directly enhancing both OGG1 transcription and activity [47], as well as the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) [48], the latter of which we have recently shown enhances *ogg1* gene expression [49]. A decrease in OGG1 activity underlying the enhanced susceptibility of BRCA1-deficient progeny exposed to ethanol is also consistent with the enhanced susceptibility of *ogg1* knockout progeny to ethanol-initiated embryopathies in culture and fetal DNA oxidation and postnatal neurodevelopmental deficits following *in utero* ethanol exposure [15,45].

In addition to the effects on DNA repair, BRCA1 has been shown to play important roles in other pathways through transcriptional regulation. A microarray study found that cells transfected with *brca1* had increased expression of several genes involved in detoxification of reactive intermediates, such as aldehyde dehydrogenase and antioxidative enzymes [50]. Antioxidative defenses, regulated by Nrf2, are upregulated by BRCA1 [48,51]. This combination of compromised detoxification of reactive intermediates together with reduced DNA repair may collectively predispose *brca1*-deficient progeny to the pathogenic effects of ROS.

The use of mouse whole embryo culture provides a valuable tool for addressing embryonic susceptibility to chemical exposure in the absence of confounding maternal influences. In this study, we found that *brca1*-deficient embryos exposed to EtOH showed significant reductions in anterior neuropore closure, head length, turning and somite development. While these parameters do not necessarily predict structural impairments, they provide a snapshot of the state of embryos at a critical point in development. A number of parameters were affected in *brca1*-deficient embryos that represent different cellular targets: anterior neuropore closure and head length are both representative of toxicity to neural crest cells (**NCCs**), consistent with cell culture studies in which co-treatment of NCCs with EtOH and free radical scavengers resulted in increased cell viability and decreased free radical production compared to EtOH exposure alone, revealing a free radical-dependent mechanism of toxicity [52]. Somite development, which originates in paraxial mesoderm [53], is affected by EtOH treatment around gastrulation by preventing the formation of lamellipodia in cells of mesodermal origin [54]. While the latter has not been investigated for its association with ROS, the deficiencies in somite development observed may be the result of disruptions to NCC signaling, direct interaction of ROS with mesodermal tissues or involvement of a signaling cascade common to NCC and mesoderm, such as the Wnt signaling pathway. Wnt6 is essential for proper development of somites [55] and neural crest cells [56]. Embryo culture studies in which C57BL/6 mice were exposed to EtOH revealed epigenetic changes to various genes including Wnt6 that correlated with changes in expression at the RNA level [57]. This epigenetic modification may be related to the reduction of DNA repair associated with BRCA1 deficiencies.

It is interesting to note that while embryopathies in culture are commonly reported with the concentration of EtOH used herein [15,58], BRCA1 wild-type embryos were not affected. We have previously found that inbred C57BL/6 mice are susceptible to embryopathies at this concentration while outbred CD-1 mice are similarly susceptible only at a higher concentration [27]. The relative strain resistance of the wild-type *brca1* mice to EtOH embryopathies may be the result of cross breeding between the Cre transgenic mice with the floxed *brca1* strains.

While breast cancers associated with mutated *brca1* are characterized by a loss of both functional alleles [59], the embryolethality associated with carrying two non-functional alleles [8] means that mutation of only one *brca1* allele can be present throughout early embryonic development, since one wild-type functional allele is essential for survival during that period. Mutation of the second *brca1* allele could be acquired during late embryonic or fetal development, or postnatally. Our heterozygous *brca1* knockout mice accordingly represent a clinically relevant model for studying the developmental consequences of BRCA1 deficiencies. We used conditional knockout mice that express normal levels of BRCA1 until GD 6.5 when Cre recombinase expression leads to the deletion of exon 11 on one *brca1* allele [31], which is the same region deleted in the embryolethal *brca1* knockouts [3]. This approach offers the benefit of testing apparently healthy progeny in which the loss of *brca1* roughly coincides with the embryo culture period. Unlike our conditional knockout approach, conventional knockouts may have indirect pathologies associated with the absence of *brca1* from conception, which could confound interpretation. The significant developmental impact

observed with the modest 28% decrease in embryonic BRCA1 protein in our conditional heterozygous *brca1* knockout model suggests that BRCA1 plays a major role in protecting the embryo and fetus from environmentally enhanced developmental oxidative stress, which would not be discerned from adult human cancer studies limited to an analysis of *brca1* mutations. It would be useful to have more human information on the range of BRCA1 levels and binding activity to its various partners, with deficiencies potentially arising from a spectrum of mechanisms including gene mutations, altered regulation by miRNAs and post-translational changes affecting protein levels and binding activity, one or more of which may differ in the embryo and/or fetus compared to analyses in adults in cancer studies.

In the adult human population, no differences in markers of oxidative stress have been observed in individuals with *brca1* mutations [60], which is consistent with our findings in the saline-treated *brca1*-deficient embryos. Similarly, no differences in miscarriage rates have been observed between *brca1* mutation carriers and the general population [6,7]. However, it is possible that *brca1*-deficient individuals who are not exposed to ROS-enhancing environmental conditions may be masking an enhanced developmental risk in that subset of the population. As importantly, measurements of oxidative stress in adults will not reflect their own history of ROS levels *in utero*, nor will the mother's level of oxidative stress during pregnancy necessarily reflect the proximate levels of ROS in their embryo or fetus [61].

5. Conclusions

Our discovery that *brca1*-deficient mice develop normally under controlled conditions but are more susceptible to the embryopathic effects of EtOH provides the first direct evidence that moderately decreased levels of BRCA1 constitutes an important risk factor for teratogenesis initiated at least by this drug, and possibly by other ROS-enhancing environmental conditions. More information is needed concerning the human incidence and severity of BRCA1 deficiencies due not only to *brca1* mutations, but also other mechanisms leading to diminished BRCA1 protein levels and binding activities, particularly in the embryo and fetus. Our results may have implications for recommended diet and prescription drug use for pregnant women carrying offspring with a single *brca1* mutation.

Conflict of interest

The authors have no actual or potential conflicts of interest.

Authors' contributions

AMS designed the study, performed the molecular analyses and wrote the first draft of the manuscript. LMP performed the embryo culture experiments and assisted in editing and preparing the final draft of the manuscript. PGW supervised the project, provided assistance with interpretation of the findings, secured research funding, and assisted in preparing the manuscript.

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