Received: 29 November 2021 | Revised: 14 June 2022 | Accepted: 3 July 2022

DOI: 10.1111/cas.15485

ORIGINAL ARTICLE

Cancer Science WILEY

Brca1$^{L63X/+}$ rat is a novel model of human *BRCA1* deficiency displaying susceptibility to radiation-induced mammary cancer

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Funding information
Japan Society for the Promotion of Science, Grant/Award Number: JP15H02824, JP21H03601 and JP26550036

Abstract

Women who are heterozygous for deleterious *BRCA1* germline mutations harbor a high risk of hereditary breast cancer. Previous *Brca1*-heterozygous animal models do not recapitulate the breast cancer phenotype, and thus all currently used knockout models adopt conditional, mammary-specific homozygous *Brca1* loss or addition of *Trp53* deficiency. Herein, we report the creation and characterization of a novel *Brca1* mutant rat model harboring the germline L63X mutation, which mimics a founder mutation in Japan, through CRISPR-Cas9–based genome editing. Homozygotes (*Brca1*$^{L63X/L63X}$) were embryonic lethal, whereas heterozygotes (*Brca1*$^{L63X/+}$) showed apparently normal development. Without carcinogen exposure, heterozygotes developed mammary carcinoma at a comparable incidence rate with their wild-type (WT) littermates during their lifetime. Intraperitoneal injection of 1-methyl-1-nitrosourea (25 or 50 mg/kg) at 7 weeks of age induced mammary carcinogenesis at comparable levels among the heterozygotes and their littermates. After exposure to ionizing

Abbreviations: CI, confidence interval; ER, estrogen receptor; HR, hazard ratio; MNU, 1-methyl-1-nitrosourea; qPCR, quantitative PCR; QST, National Institutes for Quantum Science and Technology; WT, wild type.

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radiation (0.1–2 Gy) at 7 weeks of age, the heterozygotes, but not WT littermates, displayed dose-dependent mammary carcinogenesis with 0.8 Gy^{-1} excess in hazard ratio during their middle age; the relative susceptibility of the heterozygotes was more prominent when rats were irradiated at 3 weeks of age. The heterozygotes had tumors with a lower estrogen receptor α immunopositivity and no evidence of somatic mutations of the WT allele. The *Brca1*$^{L63X/+}$ rats thus offer the first single-mutation, heterozygous model of *BRCA1*-associated breast cancer, especially with exposure to a DNA break-inducing carcinogen. This implies that such carcinogens are causative and a key to breast cancer prevention in individuals who carry high-risk *BRCA1* mutations.

KEYWORDS
animal model, breast cancer, genome editing, hereditary breast and ovarian cancer syndrome, radiation carcinogenesis

1 | INTRODUCTION

Breast cancer is experienced by 10% or more of women in developed countries, and pathogenic germline mutations have been estimated to cause 5%–10% of female breast cancers.[1,2] *BRCA1* and *BRCA2* are involved in homologous recombination repair of DNA double strand breaks, and one copy of pathogenic germline mutations of *BRCA* genes substantially increases the lifetime risk of breast cancer.[3,4] The risk of carcinogen exposure in people with defects in such DNA damage repair systems has hence been a matter of concern. Recent studies suggest that exposure at a young age to low-dose radiation used in diagnostic procedures or cigarette smoke increases breast cancer risk in women harboring such germline mutations, albeit with possible influence of biases and necessity for further research.[5–7] Breast cancers associated with *BRCA1* mutations are frequently negative for ovarian hormone receptors, whereas many sporadic breast cancers express those receptors.[4]

Development of preventive measure and basic understanding of key mechanisms of *BRCA*-related breast carcinogenesis are thus urgent, and good animal models will facilitate relevant research and development. More than 10 animal models of *Brca1* mutations have thus far been developed.[8–10] Notably, none of the heterozygous *Brca1* mouse mutants display increase in mammary tumorigenesis.[8–10] As mice with homozygous *Brca1* mutations do not survive, several lines of conditional knockouts have been developed in which homozygous deletion was driven by mammary-specific promoters.[8–10] Alternatively, *Brca1* heterozygous mice require a heterozygous loss of *Trp53* to achieve increased mammary tumorigenesis.[8–10] These models have contributed to establishing novel therapeutic concepts.[11] In contrast, it is difficult to assume that these models recapitulate the very early phases of breast carcinogenesis of heterozygous women carrying *BRCA1* mutations. We supposed that this issue is attributed to the low spontaneous incidence of mammary cancer in most unmodified mouse strains. Rats have higher spontaneous mammary cancer incidence, although their use has been less widespread.[12,13] A random-mutagenesis study has produced the first *Brca1* knockout rat, but this strain did not show an increased incidence of mammary cancer.[14]

BRCA1 variants have been found in ~1.4% of Japanese patients with breast cancer, and the *BRCA1*^{L63X} variant (a nonsense mutation of codon 63, which encodes leucine) is the most prevalent therein.[15–17] We thus introduced a point mutation mimicking L63X in the rat *Brca1* gene. We further characterized the normal development of the rats, as well as their mammary cancer development, with or without treatment with carcinogens including ionizing radiation (as a DNA break-inducing agent) and MNU (as a DNA alkylating agent). The present study is the first report that a single heterozygous germline mutation of *Brca1* can impose breast cancer susceptibility in a laboratory rodent when exposed to ionizing radiation, thus suggesting the use of the *Brca1*$^{L63X/+}$ rat as a new model recapitulating the features of the human disease.

2 | MATERIALS AND METHODS

2.1 | Animal experiments

All procedures below were approved by the Institutional Animal Care and Use Committee of either Kyoto University or QST and carried out essentially as described.[18–20] In brief, fertilized eggs of Jcl:SD rats (Clea Japan) were injected with guide RNA, single-stranded oligodeoxynucleotides (Figure 1A and Table 1) and Cas9 mRNA and transferred to a pseudopregnant foster mother. Genotyping was undertaken as described below. The strain (named Jcl:SD-*Brca1*^{em1kyo}) was maintained by mating mutants with purchased closed-colony Jcl:SD rats. The mutant rats and WT littermates of the second to seventh generations relative to the founder were injected intraperitoneally with MNU (Toronto Research Chemicals Inc.), whole-body irradiated with ^{137}Cs γ rays (dose rate, 0.5 Gy/min), or left untreated. The rats were palpated

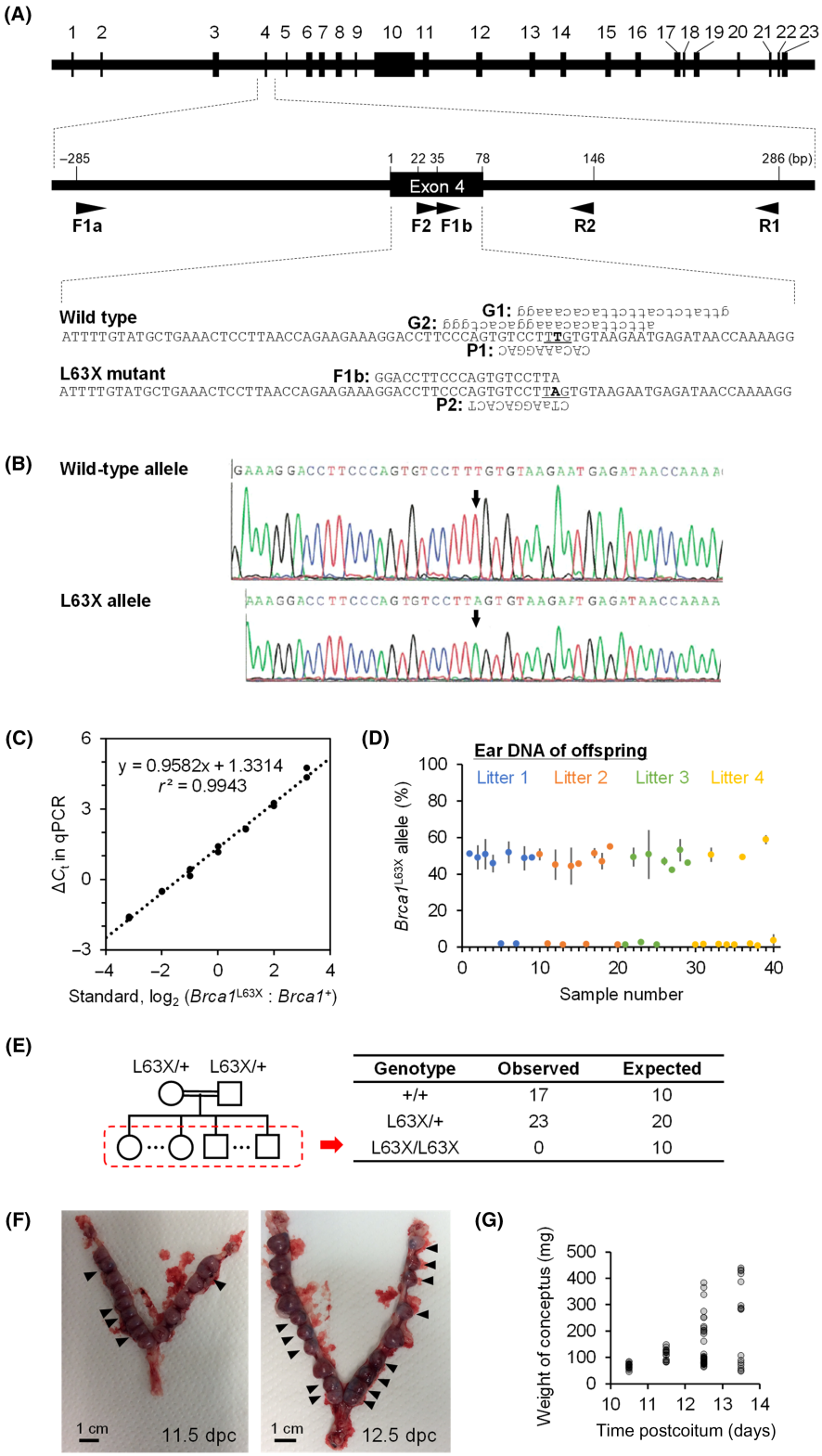


FIGURE 1 Generation and heredity of the *Brca1*^{L63X} allele. (A) Structure of rat *Brca1* and oligonucleotides used. Top, exon–intron structure. Middle, a magnified view. Bottom, nucleotide sequences. Arrowheads, primers; G1 and G2, guide RNAs; P1 and P2, probes. Uppercase, deoxyribonucleotides; lowercase, ribonucleotides; bold and underlined, location of the c.188T>A (p.L63X) mutation. (B) Sequence of cloned PCR products from the founder rat *Brca1* exon 4. Arrows, induced mutation. (C–E) Heredity of the *Brca1*^{L63X} allele. (C) Standard curve of the allele-specific quantitative PCR. ΔC_t, difference in the threshold cycles for the two molecular species. (D) Content of the *Brca1*^{L63X} allele in the genomic DNA of offspring ($n = 40$) of *Brca1*$^{L63X/+}$ heterozygotes. Mean and SD (duplicate measurements). (E) Contingency table of observed and expected numbers of offspring with each genotype. (F) Representative photographs of uteri of pregnant dams at designated days postcoitum (dpc). Arrowheads, maldeveloping conceptuses. (G) Weight of individual conceptuses

weekly, and tumors reaching ~2 cm in diameter were biopsied and diagnosed on H&E-stained sections. When rats were identified as having mammary carcinoma or showed general deterioration, they were euthanized and autopsied. A portion of each tumor was fixed in 10% phosphate-buffered formalin and histological diagnosis was confirmed.

2.2 | Genotyping

DNA was extracted from ear punches as described[21] and used for PCR (94°C for 3 min, followed by 36 cycles of 94, 58, and 72°C each for 30 s and 72°C for 5 min; primers F1a, F1b, and R1 in Table 1). The PCR products were routinely analyzed by agarose gel

electrophoresis. On occasions, PCR products were cloned by using TOPO TA Cloning Kit (Life Technologies Inc.) and sequenced by using BigDye Terminator version 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Potential off-target sequences of guide RNAs were searched by using the CRISPR Design webtool (http://crispr.mit.edu/ as of September 14, 2015) and analyzed using PCR primers F3–F11 and R3–R11 (Table 1).

2.3 | Allele-specific qPCR

Allele-specific qPCR (95°C for 10 s, followed by 45 cycles of 95°C for 5 s, 55°C for 10 s, and 72°C for 20 s; primers F2 and R2 and probes P1 and P2 in Table 1) was run by using CycleavePCR Reaction Mix (Takara Bio) on a LightCycler 96 thermal cycler (Roche Diagnostics),[22] according to the manufacturer's instructions. Mixtures of WT and mutant synthesized DNA (S1 and S2 in Table 1, respectively; 1000 copies in total; Eurofins Genomics Inc.) were used as standards.

TABLE 1 Oligonucleotides

Use	ID	Type	Sequence (5′→3′)
Genome editing	G1	Guide RNA	GTTATCTCATTCTTACACAAAGG
	G2	Guide RNA	ATTCTTACACAAAGGACACTGGG
	O1	ssODN	ACTCCTTAACCAGAAGAAAGGAC CTTCCCAGTGTCCTT[A] GTGTAAGAATGAGATAACCA AAAGGTAAATAACATG TGTAA
Genotyping	F1a	Forward primer	TGCAGGTAAGTGTAAT TTTCATAGG
	F1b	Forward primer	GGACCTTCCCAGTGTCCTT[A]
	R1	Reverse primer	CCGATGTGCATGGTACTGTC
qPCR	F2	Forward primer	TAACCAGAAGAAAGGAC
	R2	Reverse primer	CTACACATCAATTTCTACTT
	P1	Probe, FAM	CAC[a]AAGGAC
	P2	Probe, HEX	C[T]aAGGACACT
	S1	Standard	CCACACAGTGCGACCACATATT TTGCAAATTTTGTAT GCTGAAACTCCTT AACCAGAAGAAAGGA CCTTCCCAGTGTCCTT[T] GTGTAAGAATGAGAT AACCAAAAGGAGCC TACAAGGAAGTGCAAGGTTTA GTCAACTTGTTGAA GAGCTGCTGAAAAT
	S2	Standard	CCACACAGTGCGACCACATATT TTGCAAATTTTGTATGCT GAAACTCCTTAACCA GAAGAAAGGAC CTTCCCAGTGTCCTT[A] GTGTAAGAATGAGATAACC AAAAGGAGCCTACAAGGAAGT GCAAGGTTTAGTCAACTT GTTGAAGAGCTGCTGAAAAT

(Continues)

TABLE 1 (Continued)

Use	ID	Type	Sequence (5′→3′)
Sequencing	F3	Forward primer	CACTGCATAGGGAAACTGGC
	R3	Reverse primer	GGGACTGATCTAGGGGTGAC
	F4	Forward primer	AACCCTACCCAGAAAGCTCC
	R4	Reverse primer	ACCTGCAGCTGTCTTGAGAT
	F5	Forward primer	GACCCCTTCATTGTCCTCCA
	R5	Reverse primer	TCCCCTAGCTCCCTCATGAT
	F6	Forward primer	AGTCAGTCCACCATGTCAGT
	R6	Reverse primer	GCAGAGCAGACCCATCGATA
	F7	Forward primer	GCACACCACATCTCTCCTCT
	R7	Reverse primer	GCCAAACAACATGCATGACA
	F8	Forward primer	GTTTCTGGTAAGCAAGCCCC
	R8	Reverse primer	GATCAACTTCTGGGCCATGC
	F9	Forward primer	TGCCCCTTCCCATTATCACA
	R9	Reverse primer	ATCCATGGCAAAGGGAGACA
	F10	Forward primer	CCTGTAACTCCAGCTCCCAA
	R10	Reverse primer	GAAGGCAGGAGGTGAAGTCT
	F11	Forward primer	GGCAGCCTCGTTACACAATC
	R11	Reverse primer	GTGCCCCTCAGACTCTTCAT

Note: Nucleotides in bracket, location of introduced T-to-A mutation. Allele-specific quantitative PCR (qPCR) was carried out by using the cycleave PCR technique. This technique uses a chimeric DNA–RNA–DNA probe labeled with a fluorescent dye and a quencher at its ends; if the probe generates a perfect hybrid with the PCR product, it is digested with RNaseH at the RNA–DNA heteroduplex, leading to separation of the quencher and dye and increased fluorescence; any mismatch at or near the heteroduplex does not lead to RNaseH digestion. Lowercase letter, ribonucleotide; uppercase letter, deoxyribonucleotide. FAM, 6-carboxyfluorescein-labeled; HEX, hexachlorofluorescein-labeled; ssODN, single-stranded oligodeoxynucleotide.

2.4 | Whole-mount tissue preparation

Mammary tissue was extended on glass slides, fixed in 10% phosphate-buffered formalin for 1–3 days, and stored in 70% ethanol at 4°C. Tissue was subsequently defatted in acetone for 3 days and stained with hematoxylin overnight at room temperature. Staining was adjusted by decolorization in 1% hydrochloric acid/70% ethanol (up to 1 h). The tissue was then dehydrated/defatted in acetone for 2–3 h, cleared in *d*-limonene for 2 days and stored in mineral oil in heat-sealed plastic bags.

2.5 | Immunohistochemistry

Formalin-fixed paraffin-embedded sections were immunostained with an antibody against ERα (clone 6F11; Leica Biosystems), photographed, and analyzed as described.[19] The percentage of antigen-positive epithelial tumor cells was determined by automated counting using Patholoscope software (Mitani Corporation).

2.6 | Tumor genome analyses

Genomic DNA for the loss of heterozygosity (LOH) analysis was obtained from cryosections (1–3 mm^2) of tumors and normal kidney tissues of *Brca1*$^{L63X/+}$ rats with a laser microdissection system (MMI CellCut; Molecular Machines & Industries) and QIAamp DNA Micro Kits (Qiagen) and used for allele-specific qPCR as mentioned above. Genomic DNA for target sequencing was extracted from mammary carcinomas developed in the *Brca1*$^{L63X/+}$ rats in the untreated ($n = 10$), irradiated (3 weeks of age, $n = 11$), and MNU groups (dose 25 mg/kg, $n = 3$; 50 mg/kg, $n = 8$) or from normal mammary glands using Qiagen AllPrep DNA/RNA Micro Kits (Qiagen). Extracted DNA was then treated with ribonuclease A and purified using QIAmp DNA Micro Kits (Qiagen). DNA was quantified using a Qubit fluorometer (Life Technologies) and submitted to Azenta Japan for target sequencing with the DNBSEQ platform (MGI Tech). Capture probes were designed to target the rat *Brca1* gene (chr10:86,418,468–86,477,304 on the mRatBN7.2/rn7 assembly; Twist Biosciences). Somatic single-nucleotide variants and insertion/deletions in tumors were called with the VarScan 2 software (version 2.4.4).[23] A false-positive filter was then applied to remove sequencing- and alignment-related artifacts. Variants were annotated and the effect on coding sequences predicted using the SnpEff software (version 4.3).[24]

2.7 | Statistical analysis

All tests were two-sided, $p < 0.05$ considered significant, and the open-source free software R used.[25] Fisher's exact probability tests, *F* tests, Student's and Welch's *t*-tests (selected based upon the result of *F* tests), and two-way ANOVA were run on R. Kruskal–Wallis tests and Wilcoxon rank sum tests were undertaken by using the "coin" package in R.[26] Log–rank tests and simple Cox regression analyses were carried out by using the "survival" package in R.[27] Cox regression fitting to dose–response models was carried out by using the "epifit" package in R.[28] Therein, the dose–response of HR was modeled as $1+\beta_0 I_L + (\beta_L I_L+\beta_W I_W)D$, where β_0 is the excess HR for untreated *Brca1*$^{L63X/+}$ rats, I_L and I_W are dummy variables for *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats, β_L and β_W are the excess HR per Gy for *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats, and *D* is radiation dose in Gy. All R codes are provided in Document S1.

3 | RESULTS

3.1 | *Brca1*$^{L63X/+}$ rats are generated by gene editing

We injected guide RNA, single-stranded oligodeoxynucleotides (Figure 1A, Table 1), and Cas9 mRNA into fertilized eggs of Jcl:SD rats (a Sprague–Dawley strain) and transferred these eggs to a pseudopregnant foster mother to produce knockout rats. One male rat having a c.188T>A (p.L63X) mutation of *Brca1* (designated herein as the *Brca1*^{L63X} allele) was identified and used as a founder to establish the strain (named Jcl:SD-*Brca1*^{em1kyo}). The genotype of the founder was confirmed by sequencing the cloned PCR products (primers F1a and R1; Figure 1A, Table 1) from the *Brca1* alleles, indicating successful introduction of c.188T>A (Figure 1B). We compared 10 potential off-target sequences of the guide RNAs between the founder and three purchased Jcl:SD rats and confirmed their intactness (Table 2).

3.2 | *Brca1*^{L63X} homozygotes are embryonic lethal and heterozygotes are apparently normal

Using the strain established from this founder, we examined the heredity of the *Brca1*^{L63X} allele in offspring of four *Brca1*$^{L63X/+}$ female–male pairs. A well-characterized allele-specific qPCR analysis of genomic DNA (Figure 1C) indicated the absence of *Brca1*$^{L63X/L63X}$ homozygotes, a departure from Mendelian inheritance (Figure 1D,E; $p = 0.001$ by Fisher's exact probability test). Examination of uteri during pregnancy indicated many small conceptuses by 11.5 days postcoitum (Figure 1F,G). Evidence thus suggested that *Brca1*$^{L63X/L63X}$ homozygotes were embryonic lethal. The *Brca1*$^{L63X/+}$ heterozygotes showed normal postnatal growth as seen in their body weight, the weights of several organs, and the whole-mount tissue preparations of mammary gland (Figure 2).

3.3 | *Brca1*$^{L63X/+}$ rats showed apparently unchanged susceptibility to spontaneous and MNU-induced mammary carcinogenesis

We next set up untreated and carcinogen-treated groups of *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats to study characteristics of both

TABLE 2 Potential off-target sequences in the founder *Brca1*$^{L63X/+}$ rat

Guide RNA[a]	Location[b]	Reference sequence (5′→3′)[c]	Primers[a]	Result
G1	Target (*Brca1*)	**GTTATCTCATTCTTACACAAAGG**	N/A	N/A
	Chr 6: +73,719,173	GGAATCTTATTCTTACACAATGG	F3/R3	Intact
	Chr 13: −20,706,840	CTGAACTCATTCTTACACAAAAG	F4/R4	Intact
	Chr 14: +11,065,816	CTTCTCCCATTCTTACACAAAAG	F5/R5	Intact
	Chr 14: +11,066,038	CTTCTCCCATTCTTACACAAAAG	F5/R5	Absent[d]
	Chr 15: −77,797,047	TTTATGTTATTCTTACACAAAGG	F6/R6	Intact
	Chr 2: −229,084,162	TTTATCTAATTATTACACAATAG	F7/R7	Intact
	Chr 1: −280,828,323	AGTGACTCATTCTTACACAAAAG	F8/R8	Intact
G2	Target (*Brca1*)	**ATTCTTACACAAAGGACACTGGG**	N/A	N/A
	Chr 7: +111,332,798	TTCCTTACCCAAAGGACACTTAG	F9/R9	Intact[e]
	Chr 1: −157,626,260	TGTTTTAAACAAAGGACACTAAG	F10/R10	Intact
	Chr 20: +10,695,088	GTTTATACAGAAAGGACACTAAG	F11/R11	Intact

[a]Guide RNA and primer sequences are shown in Table 1.

[b]Location of the 5′-end in the Rnor 6.0 assembly. Plus/minus signs indicate sense/antisense strands.

[c]Sequence for the BN strain. Underlined bases indicate mismatches relative to the guide RNA sequence.

[d]A 222 bp sequence (chromosome [Chr] 14: 11,065,964–11,066,185), present in the reference sequence of the BN strain, was absent in the founder as well as in three untreated Jcl:SD rats.

[e]Single nucleotide polymorphism g.111332800C>T was present in the founder as well as in three untreated Jcl:SD rats. F, forward; N/A, not applicable; R, reverse.

The Significance of Bold values indicates guide RNA sequences.

spontaneous and induced mammary carcinogenesis. We chose ionizing radiation and MNU as homologous recombination-relevant and -irrelevant carcinogens, respectively, as radiation induces DNA double-strand breaks whereas MNU induces methylation of DNA bases. The treatment regimens were chosen with reference to past experiments using WT Jcl:SD rats.[19,20]

The crude analysis is summarized in Table 3. Ovarian cancers of epithelial origin, as described in humans,[4] were not observed. Comparison of untreated *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats illustrated no clear difference in mammary cancer incidence (Table 3, Figure 3A). Treatment with MNU increased the incidence rate of mammary cancer in a dose-dependent manner (Table 3, Figure 3B). The age at autopsy was significantly younger, and the number of rats with carcinoma and the number of carcinomas developing per unit time were slightly higher, in *Brca1*$^{L63X/+}$ than *Brca1*$^{+/+}$ rats treated with 25 mg/kg MNU (Table 3); however, the slight increase in the mammary cancer incidence did not reach statistical significance (Figure 3B).

3.4 | *Brca1*$^{L63X/+}$ rats are more susceptible to mammary carcinogenesis induced by radiation than WT littermates

The incidence of mammary carcinoma displayed a tendency of increase related to radiation dose over the whole observation period (Figure 4A,B), and the slopes of the dose response of HR (with unirradiated *Brca1*$^{+/+}$ as reference) were positive in both *Brca1*$^{L63X/+}$ ($p = 0.015$) and *Brca1*$^{+/+}$ ($p = 0.07$) rats (Figure 4C). The HR of early-onset mammary cancer (i.e., <40 weeks of age) showed a tendency toward dose dependence independently of the *Brca1* genotype (Figure 4D), whereas HR during middle age (40–80 weeks) was significantly dose dependent only in *Brca1*$^{L63X/+}$ rats ($p = 0.045$; in *Brca1*$^{+/+}$, $p = 0.82$; Figure 4E); no clear dose response was observed later (Figure 4F). These results suggest that the early-onset mammary cancer before 40 weeks is intrinsic to Jcl:SD rats irradiated at 7 weeks and independent of the *Brca1* genotype, whereas mammary carcinogenesis during middle age was prominent in irradiated *Brca1*$^{L63X/+}$ rats.

We previously showed that early-onset mammary carcinogenesis is suppressed, whereas that during middle age is moderately increased, in Jcl:SD rats irradiated at 3 weeks of age.[19] In *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats irradiated at this age, as expected, the early-onset component of carcinogenesis was diminished, and a clear and significant difference in mammary cancer incidence was indicated between the two genotypes (Figure 4G). Thus, the results hitherto indicate that the *Brca1*$^{L63X/+}$ rats are more susceptible to mammary carcinogenesis initiated by radiation, but not MNU, than their WT littermates.

3.5 | Mammary carcinomas of *Brca1*$^{L63X/+}$ rats show low ERα positivity

Histology of mammary cancer showed no clear difference between *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats (Figure 5A,B). We evaluated the ERα-positive rate of induced mammary carcinomas. Herein, all palpable carcinomas that developed for the first time in individual animals were immunohistochemically analyzed (Figure 5C,D). Of

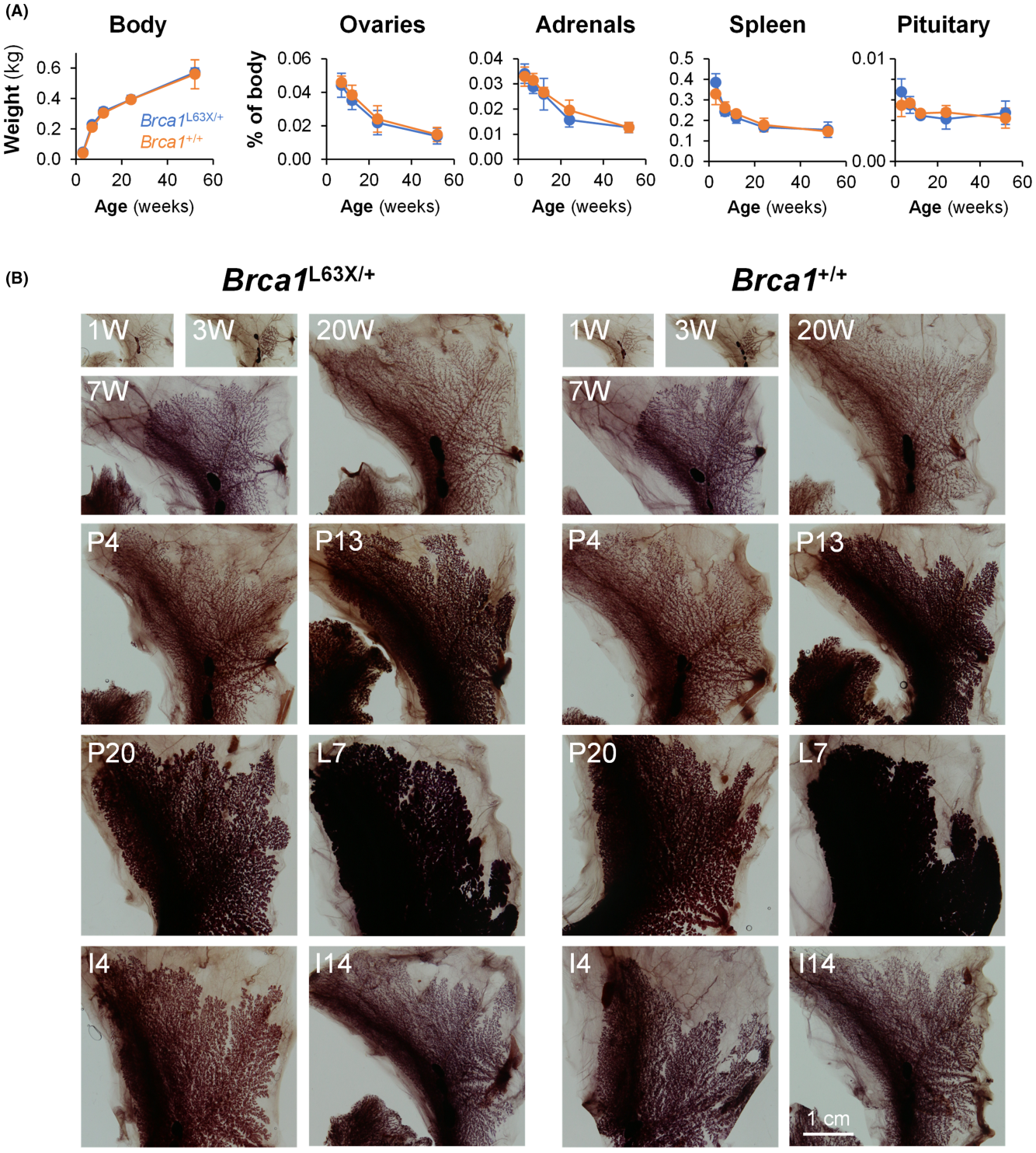


FIGURE 2 Normal development of *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats. (A) Body and organ weights of *Brca1*$^{L63X/+}$ rats (L63X/+, blue) and WT littermates (+/+, orange). Data are shown as mean ± SD ($n = 4–6$). No significant difference suggested between genotypes or interaction between genotypes and age, whereas age-related differences were significant for all measures ($p < 0.001$), by two-way ANOVA. (B) Mammary gland development. Whole mounts of inguinal mammary glands are shown. I, day of postlactational involution; L, day of lactation; P, day of pregnancy; W, weeks of age. For pregnancy and lactation, rats were mated at 11 or 12 weeks of age. Scale bar in the bottom right panel applies to all

note, carcinomas that developed spontaneously in *Brca1*$^{L63X/+}$ rats had a significantly smaller fraction of ERα-positive cells than those in *Brca1*$^{+/+}$ rats (Figure 5E). Overall, carcinomas developing in *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats irradiated at 7 weeks of age displayed similar rates of ERα positivity (Figure 5E). However, if the analysis was confined to carcinomas that developed between 40 and 80 weeks

TABLE 3 Mammary tumor incidence in *Brca1*$^{L63X/+}$ rats and their littermate *Brca1*$^{+/+}$ rats

Genotype	Treatment	Age at treatment (weeks)	Dose	*n*[a]	Age at autopsy (weeks)[b]	Carcinoma		Benign tumors	
						Rats (%)	Tumors (10^{-2}/week)[c]	Rats (%)	Tumors (10^{-2}/week)[c]
Brca1$^{L63X/+}$	None	–	–	30	82.5 ±26.0	11 (37)	0.53 ±0.15	15 (50)	1.12 ±0.28
	MNU	7	25 mg/kg	12	52.8 ±18.3***††	7 (58)	1.66 ±0.51*	4 (33)	1.42 ±0.68
	MNU	7	50 mg/kg	12	34.3 ±10.4***	10 (83)*	6.38 ±1.33***	3 (25)	0.58 ±0.30
	γ-rays	7	0.1 Gy	30	78.6 ±26.3	12 (40)	0.73 ±0.19	14 (47)	0.98 ±0.26
	γ-rays	7	0.5 Gy	30	77.8 ±22.7	9 (30)	0.50 ±0.16	15 (50)	1.29 ±0.29
	γ-rays	7	1 Gy	30	63.8 ±20.5**	13 (43)	1.16 ±0.31	21 (70)	1.72 ±0.29
	γ-rays	7	2 Gy	30	60.3 ±18.6***	17 (57)	1.46 ±0.30*	22 (73)	2.52 ±0.40**
	γ-rays	3	2 Gy	25	66.9 ±21.2*	11 (44)†	0.93 ±0.25†	20 (80)*	2.78 ±0.49**
Brca1$^{+/+}$	None	–	–	30	76.6 ±24.1	11 (37)	0.81 ±0.23	10 (33)	0.69 ±0.21
	MNU	7	25 mg/kg	12	67.8 ±13.2	6 (50)	1.34 ±0.54	7 (58)	2.46 ±0.75*
	MNU	7	50 mg/kg	9	33.1 ±6.2***	9 (100)**	8.24 ±0.88***	0 (0)	0.00 ±0.00
	γ-rays	7	0.1 Gy	30	88.1 ±22.8*	11 (37)	0.81 ±0.24	19 (63)*	1.73 ±0.34*
	γ-rays	7	0.5 Gy	30	74.9 ±22.7	12 (40)	0.66 ±0.16	18 (60)	1.45 ±0.26*
	γ-rays	7	1 Gy	30	65.5 ±24.8	13 (43)	0.92 ±0.23	21 (70)**	1.94 ±0.31**
	γ-rays	7	2 Gy	30	56.3 ±17.1**	13 (43)	1.25 ±0.33	19 (63)*	1.72 ±0.27**
	γ-rays	3	2 Gy	26	71.2 ±19.5	4 (15)	0.26 ±0.13	22 (85)***	2.36 ±0.37***

[a]Number of rats.

[b]Mean ± SD.

[c]Mean ± SEM of the number of tumors, divided by the number of weeks observed, in each rat.

*$p < 0.05$.; **$p < 0.01$.; ***$p < 0.001$ vs. the corresponding untreated group.

†$p < 0.05$.

††$p < 0.01$ vs. the corresponding *Brca1*$^{+/+}$ group.

The Kruskal–Wallis test was used to compare ages and tumor numbers among groups, followed by pairwise Wilcoxon rank sum tests to compare with the untreated group or between genotypes. Fisher's exact probability test was used to compare the number of animals with tumors. MNU, 1-methyl-1-nitrosourea.

(i.e., the period when a significant dose dependence was observed, as mentioned above), we noted a significantly reduced ERα-positive rate in *Brca1*$^{L63X/+}$ rats compared with *Brca1*$^{+/+}$ rats (Figure 5F). We previously showed that irradiation with 2 Gy at 3 weeks of age leads to premature ovarian failure and suppresses development of mammary carcinoma with high ERα positivity.[29] Indeed, carcinomas that developed after irradiation at 3 weeks showed low ERα-positive rates regardless of the genotype (Figure 5E), concordant with the idea that this irradiation regimen revealed the distinct susceptibility of the *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats to estrogen-independent mammary carcinogenesis through suppression of estrogen-dependent mechanisms.

3.6 | Mammary carcinoma of *Brca1*$^{L63X/+}$ rats retains a genetically intact *Brca1*$^{+}$ allele

To obtain insights into the mechanism of tumor development in the *Brca1*$^{L63X/+}$rats, we quantified the ratio of *Brca1*^{L63X} and *Brca1*$^{+}$ alleles in the tumors using allele-specific qPCR (Figure 1C). This analysis revealed that the *Brca1*$^{+}$ allele occupied nearly 50% (Figure 6A), indicating that LOH of the *Brca1* locus is not involved. Thus, we then searched for de novo somatic mutations of 32 tumors in the *Brca1*$^{+}$ allele by targeted sequencing. This analysis identified two exonic mutations that were either in the upstream sequence or synonymous (Figure 6B), with no evidence of loss of the *Brca1*$^{+}$ allele. These results suggest that the tumors developed through nonmutational mechanisms in the *Brca1*$^{L63X/+}$rats.

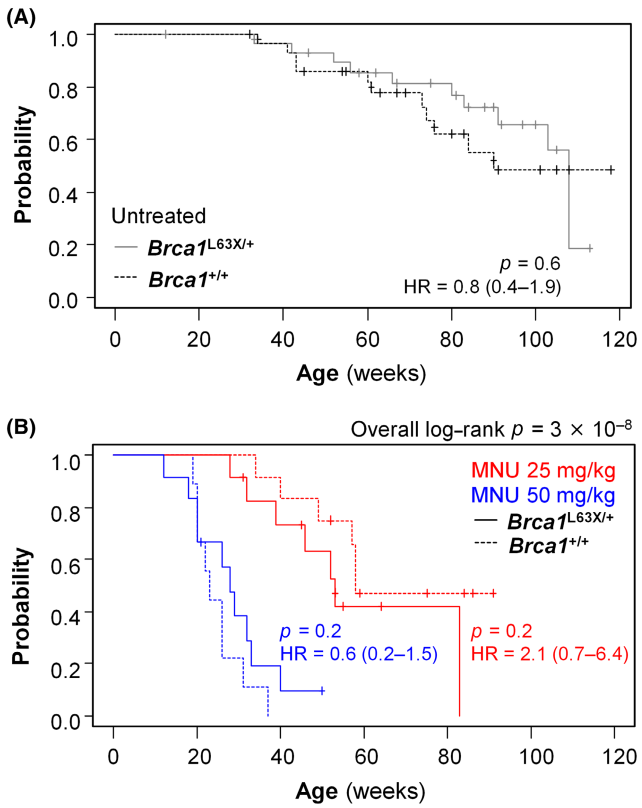


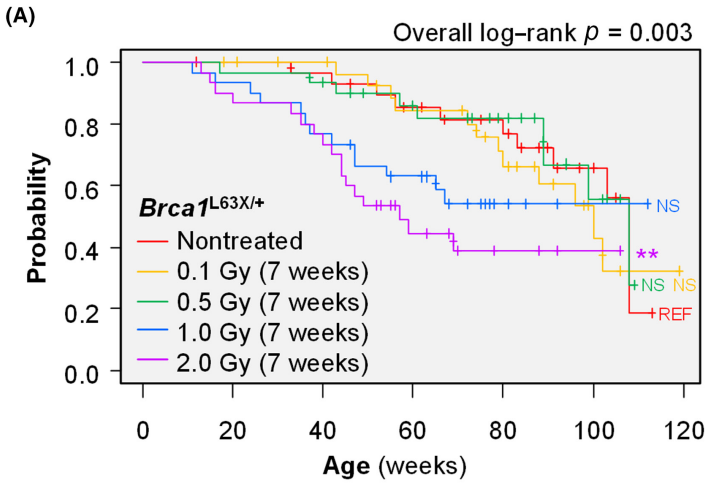
FIGURE 3 Spontaneous and 1-methyl-1-nitrosourea (MNU)-induced mammary carcinogenesis in *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats. (A) Kaplan–Meier plots depicting the incidence of palpable mammary carcinoma in the untreated groups. (B) Kaplan–Meier plots depicting the incidence of palpable mammary carcinoma in MNU-treated *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats. *p* values, log–rank test. HR, hazard ratio (95% confidence interval)

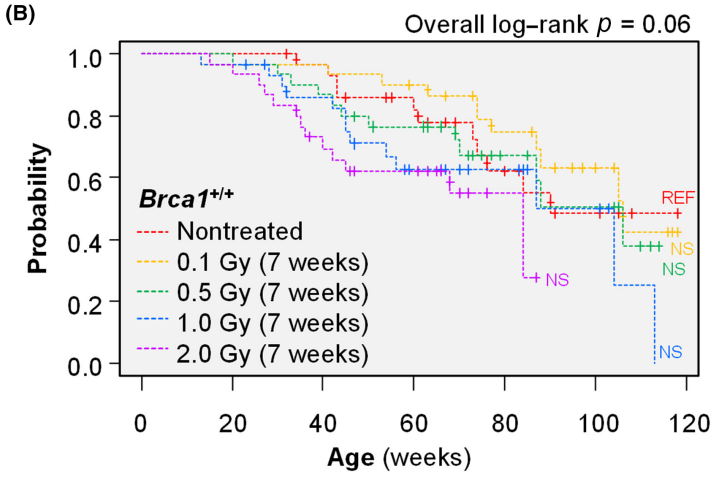
4 | DISCUSSION

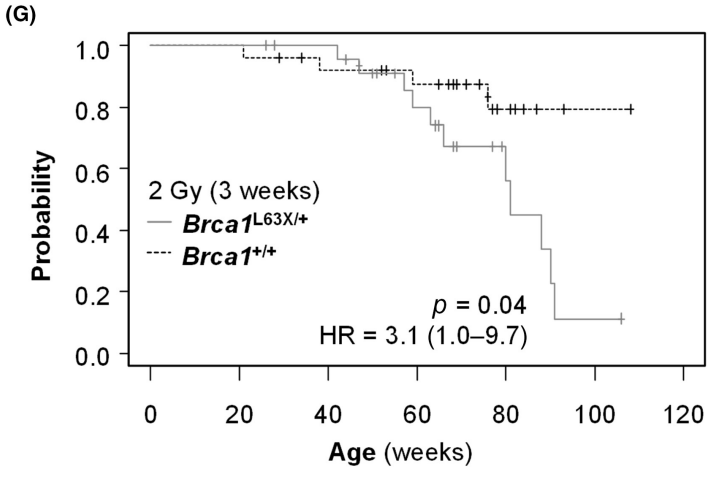
The present study reports phenotypes of a novel rat model harboring the *Brca1*^{L63X} allele. Whereas homozygotes were embryonic lethal, heterozygotes showed normal development, except that they developed mammary carcinomas with lower ERα positivity relative to WT littermates. Heterozygotes also displayed elevated susceptibility to radiation-induced, but not MNU-induced, mammary carcinogenesis.

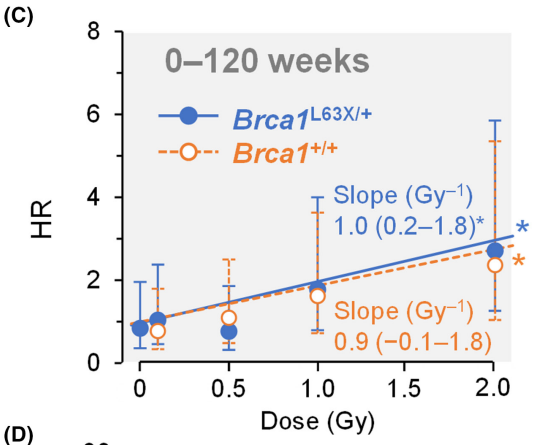
Below, we compare the above phenotypes with those of previous *Brca1* knockouts and humans to delineate the features of the present model. First, lethality of homozygotes is observed in humans,[30] as well as *Brca1*-deficient mice between embryonic days 7.5 and 13.5,[8] consistent with the present observation. Second, many mouse strains with mammary-specific homozygous *Brca1* deficiency, as in humans, develop ERα-low mammary cancer,[10] which is recapitulated by the present heterozygous rat model. Third, *Brca1*$^{L63X/+}$ rats did not display increased incidence of mammary cancer in the absence of carcinogenic exposure, a feature common to all previously reported *Brca1* heterozygous mouse/rat models.[10,14] Fourth, susceptibility to radiation-induced mammary carcinogenesis was elevated in the present model, a feature not reported on any other simple heterozygous *Brca1* models[10]; only those with double heterozygous deficiency (*Brca1* and *Trp53*) and some homozygous models show susceptibility to radiation (but not MNU) compared with WT mice.[31–33] The radiation-susceptible phenotype of the present model implies that the high spontaneous incidence of breast cancer in human carriers results from elevated susceptibility to continuous exposures to exogenous and/or endogenous carcinogens; the shorter life of rats might have hindered the susceptibility without external carcinogens. Thus, the present finding suggests that reduction of exposure to such carcinogens is key to prevention in human carriers. Finally, ovarian cancers of epithelial origin were not observed herein, unlike in human carriers. This is an expected result because epithelial ovarian cancers are rare in spontaneous and carcinogen-induced animal models.[34] Thus, the *Brca1*$^{L63X/+}$ rat model, with a single-gene, heterozygous mutation and an associated susceptibility to radiation-induced mammary cancer, recapitulates important aspects of human *BRCA1* deficiency that was not observed in previous models.

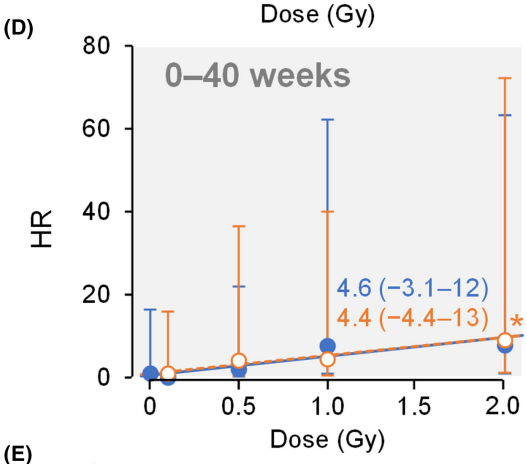
The susceptibility of carriers of *BRCA* mutations to radiation-induced carcinogenesis is a matter of concern. The use of radiation for the treatment of primary breast cancer is not associated with a risk of second cancer in the contralateral breast in carriers of *BRCA* mutations, whereas increased breast cancer risk has been suggested in carriers receiving low-dose diagnostic radiation at younger ages, albeit with a possible strong influence of biases.[35]

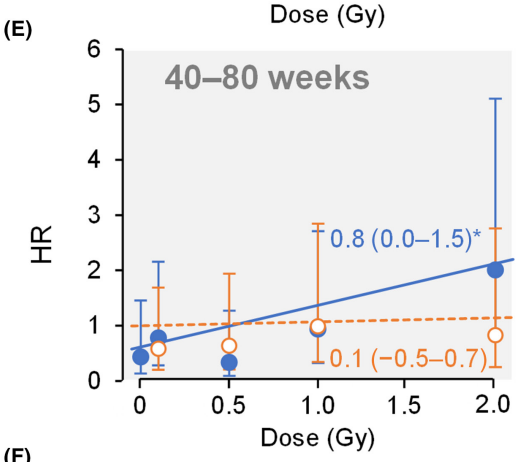

(A)
Overall log-rank p = 0.003
Probability
Brca1L63X/+
Nontreated
0.1 Gy (7 weeks)
0.5 Gy (7 weeks)
1.0 Gy (7 weeks)
2.0 Gy (7 weeks)
NS
**
NS
NS
REF
Age (weeks)


(B)
Overall log-rank p = 0.06
Probability
Brca1+/+
Nontreated
0.1 Gy (7 weeks)
0.5 Gy (7 weeks)
1.0 Gy (7 weeks)
2.0 Gy (7 weeks)
REF
NS
NS
NS
NS
Age (weeks)


(G)
Probability
2 Gy (3 weeks)
Brca1L63X/+
Brca1+/+
p = 0.04
HR = 3.1 (1.0–9.7)
Age (weeks)


(C)
0–120 weeks
Brca1L63X/+
Brca1+/+
Slope (Gy−1)
1.0 (0.2–1.8)*
Slope (Gy−1)
0.9 (−0.1–1.8)
HR
Dose (Gy)


(D)
0–40 weeks
4.6 (−3.1–12)
4.4 (−4.4–13)
HR
Dose (Gy)


(E)
40–80 weeks
0.8 (0.0–1.5)*
0.1 (−0.5–0.7)
HR
Dose (Gy)

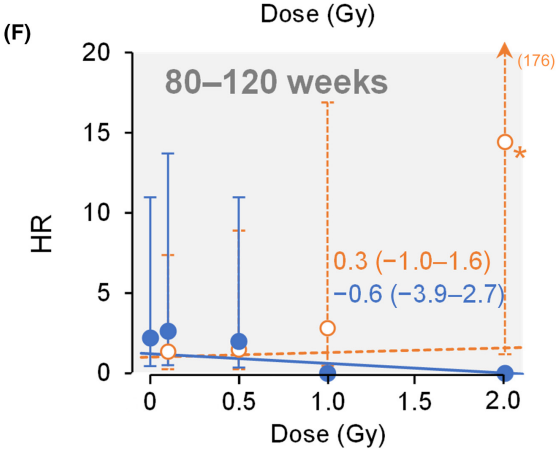

(F)
80–120 weeks
(176)
0.3 (−1.0–1.6)
−0.6 (−3.9–2.7)
HR
Dose (Gy)

FIGURE 4 Radiation-induced mammary carcinogenesis in *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats. (A,B) Kaplan–Meier plots depicting the incidence of palpable mammary carcinoma in (A) *Brca1*$^{L63X/+}$ and (B) *Brca1*$^{+/+}$ rats irradiated with 0.1–2 Gy at 7 weeks of age. Data for untreated groups (REF) are reproduced from Figure 3A. $^{**}p < 0.01$ (compared with untreated, log–rank test). NS, not significant. (C–F) Cox regression–estimated hazard ratios (HR; dots) with nonirradiated *Brca1*$^{+/+}$ rats as the reference, their 95% confidence interval (CI; error bars), and the fitted linear dose–response models (lines) during designated time periods. Numbers are linear coefficients with (95% CIs); number in parentheses in (F) indicates the upper limit of the 95% CI; $^{*}p < 0.05$ (Cox regression). (G) Kaplan–Meier plot depicting the incidence of palpable mammary carcinoma in rats irradiated with 2 Gy at 3 weeks of age

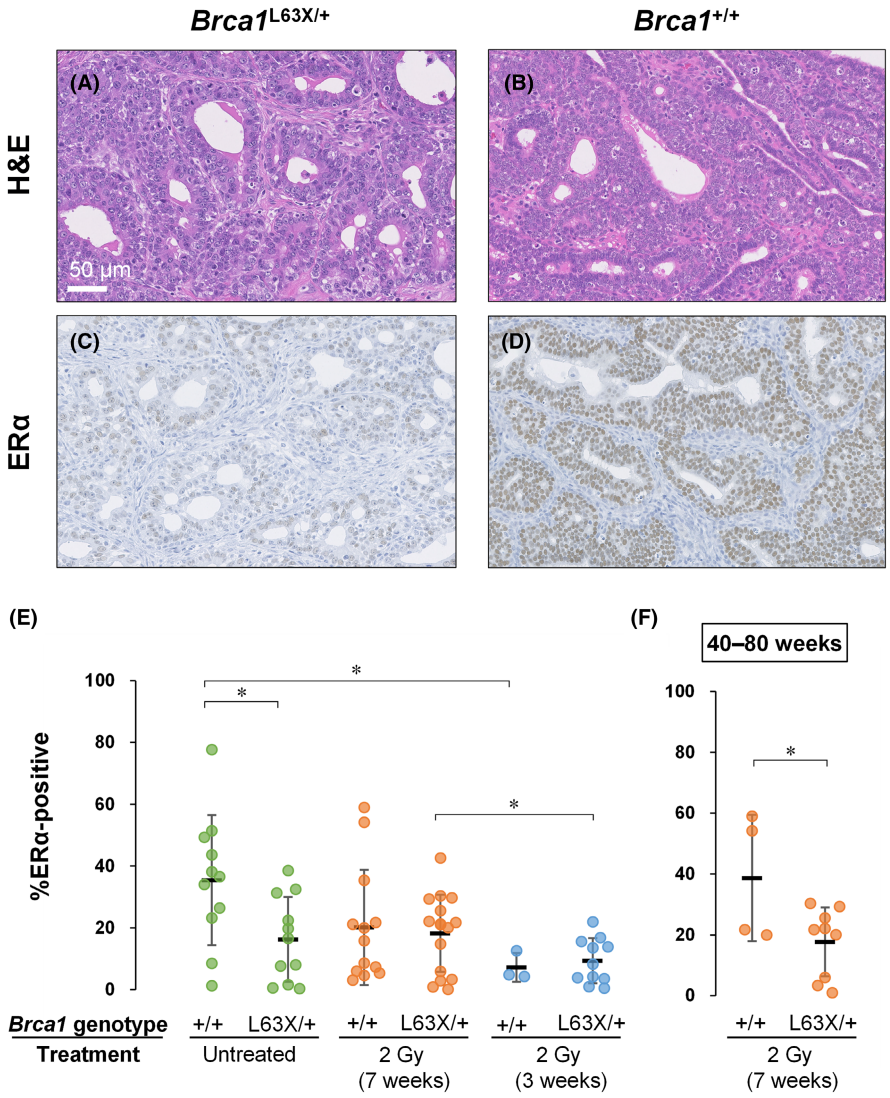
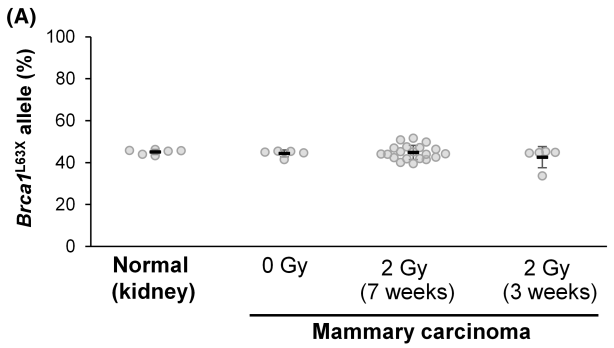


FIGURE 5 Estrogen receptor α (ERα)-positive rate of mammary carcinomas in *Brca1*$^{L63X/+}$ and *Brca1*$^{+/+}$ rats. (A, B) H&E stained tissue sections. (C, D) Immunostained sections for ERα. Brown staining indicates immunoreactivity. Carcinomas that developed in untreated *Brca1*$^{L63X/+}$ (A,C) and *Brca1*$^{+/+}$ (B,D) rats. Scale bar in (A) applies to (A–D). (E,F) ERα-positive rate of individual mammary carcinomas. Dots, individual data points; black horizontal line, mean; error bars, SD. $^{*}p < 0.05$, *t*-test

The variability between the radiotherapy and diagnostic radiation studies could reflect difference in ages at exposure, as a recent Japanese atomic bomb survivor study indicates that the susceptibility of breast tissue to radiation-induced carcinogenesis is particularly high during the peripubertal period.[36] If so, is the possible radiation-related risk in humans quantitatively consistent with our observations in the rat? Pijpe et al.[6] reported that exposure to diagnostic radiation at an average level of ~0.01 Gy is associated with a breast cancer HR of 1.62 (95% CI, 1.02–2.58) in carriers exposed before 20 years of age. The rat age of 7 weeks is a postpubertal stage and can be compared to 15–20 years of age in humans.[37] The present observation did not suggest an irregularly high risk of radiation-induced cancer at low doses, at least down to 0.1 Gy. Assuming a simple linear dose response, the observed radiation-related excess HR of 0.8 Gy^{-1} (95% CI, 0.0–1.5) in *Brca1*$^{L63X/+}$ rats predicts a very low HR of ~1.008 at 0.01 Gy (i.e., 0.008 in excess), advocating a need for careful interpretation of the studies of diagnostic radiation. The increase in the HR was manifested during middle age (40–80 weeks) of *Brca1*$^{L63X/+}$ rats irradiated at 7 weeks of age. Mammary cancer incidence before age 40 weeks is characteristic of rats irradiated after, but not before, puberty,[19] implying a specific puberty-related mechanism of tumor development before 40 weeks[29]; the present result suggests that this mechanism is independent of the *Brca1* genotype.



(B)

Group	*N*	Mutation	Note
No treatment	10	0	
Radiation (2 Gy, 3 weeks)	11	1	Upstream sequence alteration (c.−113T>A)
MNU (25 or 50 mg/kg, 7 weeks)	11	1	Synonymous alteration (c.3576C>T)

FIGURE 6 Analysis of somatic mutations of the *Brca1* locus in the mammary carcinoma of the *Brca1*$^{L63X/+}$ rats. (A) Retention of heterozygosity as revealed by allele-specific quantitative PCR. The occupancy of the *Brca1*^{L63X} allele was ~50% in normal kidney tissues and mammary carcinomas from rats that were unexposed (0 Gy) and irradiated (2 Gy) at 3 or 7 weeks of age. (B) Summarized results of the target sequencing of the *Brca1* locus. MNU, 1-methyl-1-nitrosourea; *N*, number of tumors analyzed. See Table S1 for detail

The BRCA1 protein suppresses the effects of estrogen and progesterone by binding to their receptors; hormone responsiveness is thus enhanced in cells with homozygous *Brca1* deficiency and knockdown.[38,39] Estrogen and progesterone induce the secretion of cytokines by ERα-expressing mature luminal cells, and the secreted cytokines in turn stimulate proliferation of luminal progenitor cells, which do not express ERα.[40] Thus, *Brca1* deficiency can support proliferation of ERα-negative luminal progenitors and lead to low ERα expression. Whether this mechanism holds in the situation of *Brca1* haploinsufficiency remains an open question and could be answered by use of the present model.

We did not find evidence of LOH or other somatic mutations of the *Brca1* locus in the tumors of *Brca1*$^{L63X/+}$ rats, which is surprising given that LOH is a major mechanism of biallelic inactivation of *BRCA1*.[41] Importantly, breast cancer with monoallelic *BRCA1* inactivation is more frequently observed among carriers in the Japanese population than the TCGA cohort (http://cancergenome.nih.gov).[42] It is not well understood how tumors retaining the WT allele develop. A recent systematic review indicates that hypermethylation of the promoter region of *BRCA1* is rare (i.e., ~4% of cases), although this might be an underestimation given that most studies have assessed limited methylation sites.[43] Noncoding variants are another possible explanation because functional assays provide partial evidence indicating that mutations of the promoter, upstream, intron, and 3′-untranscribed sequences can affect the expression levels of *BRCA1*.[44] Thus, the *Brca1*$^{L63X/+}$ rat model can provide a tool to identify the nonmutational mechanisms of inactivating the WT *Brca1* allele and to explore therapy strategies for *BRCA1*-related tumors without LOH or other somatic mutations. A recent publication on renal carcinogenesis also supports this idea.[45]

Taken together, our findings characterize the *Brca1*$^{L63X/+}$ rat mammary cancer model, the first single-gene, heterozygous model mimicking aspects of *BRCA1* deficiency in humans. This model is expected to provide a useful platform for studying the early phases of breast carcinogenesis and thereby preventive measures.

ACKNOWLEDGMENTS

We thank Masami Ootawara, Mari Ogawa, Ken-ichi Kudo, Hitomi Seo, Takahiro Hamoya, Misuzu Fujita, Hitomi Moriyama, staff at the Department of Radiation Effects Research and the Laboratory Animal and Genome Sciences Section, and the J-SHARE project[46] of QST, for technical support. The study was funded in part by the Japan Society for the Promotion of Science, Grant Numbers JP26550036, JP15H02824, and JP21H03601 to TI.

DISCLOSURE

Dr. Mitsue Saito supervises a researcher who receives a scholarship from Eisai Co. Ltd. The other authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data were generated by the authors and are provided with the article (Table S1).

APPROVAL OF THE RESEARCH PROTOCOL BY AN INSTITUTIONAL REVIEW BOARD

N/A.

INFORMED CONSENT

N/A.

REGISTRY AND REGISTRATION NO. OF THE STUDY/TRIAL

N/A.

ANIMAL STUDIES

Protocols were approved by the Institutional Animal Care and Use Committee.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Nakamura Y, Kubota J, Nishimura Y, et al. *Brca1*$^{L63X/+}$ rat is a novel model of human *BRCA1* deficiency displaying susceptibility to radiation-induced mammary cancer. *Cancer Sci*. 2022;113:3362-3375. doi: 10.1111/cas.15485