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The RAD51 S181P mutation shortens lifespan of female mice

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ABSTRACT

RAD51 is critical to the homologous recombination (HR) pathway that repairs DNA double strand breaks (DSBs) and protects replication forks (RFs). Previously, we showed that the S181P (SP) mutation in *RAD51* causes defective RF maintenance but is proficient for DSB repair. Here we report that SP/SP female mice exhibit a shortened lifespan compared to +/+ females but not males. Histological analysis found that most mice in this study died from lymphoma, independent of genotype and sex. We propose that a potential cause for shortened lifespan in SP/SP females is due to the RF defect.

1. Background

Homologous recombination (HR) repairs DNA double strand breaks (DSBs) and stabilizes replication forks (RFs) [1–8]. BRCA1 and BRCA2 are important contributors to HR [9,10] and mutations in these genes lead to hereditary breast and ovarian cancer after loss of heterozygosity in humans [11]. RAD51 is the recombinase for HR and is recruited to sites of DNA damage through its interaction with a variety of proteins including BRCA2 [12,13]. BRCA2 associates with RAD51 through two separate regions: the BRC repeats [14,15] and the exon 27 encoded region (Ex27) [16,17]. The BRC repeats promote RAD51 filament formation needed to mediate strand exchange [18] by facilitating RAD51 binding to ssDNA [19]. The RAD51-Ex27 interaction stabilizes the DNA filament by binding to an interface created by two adjacent RAD51 proteins [20,21] to permit DNA replication [22]. The BRC repeats and Ex27 enable DSB repair and RF maintenance, respectively [1].

Here we report on RAD51 with serine 181 changed to proline (S181P) that selectively inhibits its interaction with BRCA2 Ex27. This mutation allows DSB repair but not RF maintenance [23]. We found that $RAD51^{S181P/S181P}$ (SP/SP) females exhibited a shortened lifespan when compared to $RAD51^{+/+}$ (+/+) females. SP/SP did not impact the lifespan of male mice. A histological analysis on these mice showed no statistically significant variance in life-threatening pathologies. Most of these mice died from lymphoma independent of genotype and sex. Based on these findings we propose a reason for this outcome: the SP mutation reduced cancer latency in females.

2. Results

SP/SP mice were generated using CRISPR/Cas9 technology in the C57Bl/6J background. These mice were analysed for lifespan. A comparison of +/+ and SP/SP mice was done for females and males. In addition, +/- mice was done for males. The reason +/- mice for males were analyzed, but not +/- females, was due to budgetary concerns. After weaning, the +/SP females were removed from this study while remaining females were consolidated into fewer cages because they do not fight unlike males who remained in their original cages. For females, the SP mutation caused a significant decrease in lifespan (Fig. 1**A** $p=0.0233$). At the point of 50 % survival, the +/+ and SP/SP females lived 925 and 802 days, respectively. For males there was no significant

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difference between the +/+, +/SP and SP genotypes (Fig. 1**B**). At the point of 50 % survival, the +/+, +/SP and SP/SP males lived 949, 921.5 and 905 days, respectively.

Necropsy and histological analyses were performed on females to determine a potential cause of death. For 28 out of 33 RAD51$^{+/+}$ females, 3 of these mice showed no signs of disease; yet 24 mice displayed lymphoma (Fig. 2**A**) and 1 mouse had adenocarcinoma. For 25 out of 30 RAD51$^{SP/SP}$ females, 3 showed no signs of disease; however, 21 mice had lymphoma (Fig. 2**B**) and 1 mouse exhibited leiomyosarcoma. Furthermore, 1 mouse exhibited angiectasis and another mouse exhibited hemangioma. Lymphoma was frequently exhibited in multiple organs, including the liver (left panel) and spleen (right panel) for both genotypes.

Necropsy and histological analyses were performed on males to determine a potential cause of death. For 28 out of 32 RAD51$^{+/+}$ males, 1 mouse showed no signs of disease, while 25 mice had lymphoma and 1 mouse exhibited fibrosarcoma. Furthermore, 1 mouse displayed suppurative dermatitis. For 22 out of 22 RAD51$^{+/SP}$ males, 1 mouse showed no signs of disease while 20 mice displayed lymphoma and 1 mouse had leukemia. For 30 out of 32 RAD51$^{SP/SP}$ males, 5 mice showed no signs of disease, while 20 mice had lymphoma, 1 mouse had fibrosarcoma and 2 exhibited hemangiosarcoma. Furthermore, 1 mouse exhibited signs for each of the following diseases: angiectasis, amyloidosis, and hepatopathy while 4 mice exhibited hemangioma. Lymphoma was frequently exhibited in multiple organs, especially the liver and spleen for both genotypes.

3. Discussion

These data show that SP had a minor effect on C57Bl/6 J mice living in a caged environment. Compared to +/+ females, SP/SP females exhibited a reduction in lifespan. Most of the +/+ and SP/SP females died from lymphoma. There was no difference in the lifespan or cause of death for +/+, +/SP and SP/SP males.

The phenotype for SP/SP is like *bcra2*$^{lex1/lex2}$ for both cells and mice. Similar to SP/SP mouse embryonic stem cells (ESCs) [23], *bcra2*$^{lex1/lex2}$ ESCs and fibroblasts exhibited hypersensitivity to crosslinking agents, chromosomal instability and replication fork maintenance defects including elevated levels of RF stalls and nascent strand degradation when compared to controls [17,24,25]. Similar to SP/SP mice, *bcra2*-$^{lex1/lex2}$ mice displayed a shortened lifespan with no significant difference in cancer formation when compared to controls [25]. One difference is that the lifespan was reduced for both sexes for *bcra2*$^{lex1/lex2}$ mice as compared to only females for the SP/SP mice. As previously demonstrated the BRC repeats are important for both DSB repair and RF maintenance while and Ex27 enables only RF maintenance [1,23]. Thus, these results suggest that RF maintenance is important for longevity assurance in mice. We hypothesize that the SP/SP mice and *bcra2*$^{lex1/lex2}$ mice died from an earlier onset of cancer that shortened their lifespan compared to control mice. These data show the consequences of defective RF maintenance in mice.

4. Methods

4.1. Generation of mice with the SP mutation

For the generation of Rad51 S181P knock-in mice, exon 7 of Rad51 was targeted: Guide sequences with specificity scores >50 were chosen using crispr.mit.edu. Rad51 S181P guide RNA sequences; 5'-AGA-TAATGTAGCATATGCGC-3'.

A complementary oligo was designed for the Rad51 S181P sgRNA construct, to clone into the T7 sgRNA construct (Transposagen) for in vitro transcription. The Rad51 S181P guide RNA oligo contained the 20-nucleotide guide sequences, proceeded by four nucleotide overhangs that were compatible with the *Bsa*I digested site of the vector. The vector was digested with *Bsa*I for 2 h at 37 °C, and then gel-purified. Paired oligos were annealed (95 °C for 5 min, 65 °C for 5 min, room temperature for 1 h), and ligated into the *Bsa*I-digested vector. The construct was sequenced to confirm guide insertion before linearization with Dra1. The construct was then transcribed in vitro using the MEGA-shortscript Kit (Ambion) and transcribed mRNA was purified using the MEGAclear Kit (Ambion), both according to manufacturer instructions. Donor oligos for injection were ordered as Ultramers from IDT and used directly.

Rad51 S181P donor oligo sequence:

5'-GGTATTCGTTTCTCACTGGACTGAGCTCTGTCTA-
CAGCCTAAAAGTCTCCTCTTTTCTAGA-
TACGGTCTCCCTGGCAGCGATGTACTAGATAATGTAGCA-
TATGCGCGAGGGTTCAACACAGACCACCA-
GACCCAGCTCCTTTACCAAGCGTCAGCCATGATGGTA-
GAATCCAGGTATG-3'.

Pronuclear injections were performed using standard procedures (Ref.: Behringer R. Manipulating the mouse embryo: a laboratory manual. Fourth edition. ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2014. xxii, 814 pages). Fertilized eggs were collected from superovulated C57BL/6 J female mice (Jackson Laboratories) approximately 9 h after mating to C57BL/6 J male mice (Jackson Laboratories). Microinjections were performed using a capillary needle with a 1–2 μm opening pulled using a Sutter P-1000 micropipette puller. The pronucleus was injected using a FemtoJet 4i (Eppendorf) with continuous flow that we estimate to result in approximately 2 picoliters of injection mix: S. pyogenes Cas9 protein 300 ng/μl (NEB), sgRNA mRNA 5 ng/μl, and Rad51 S181P doner oligo ssDNA 200 ng/μl, diluted in 10 mM Tris, 0.25 mM EDTA (pH 7.5). Following visualization of pronuclear swelling, the needle was pulled out through the cytoplasm, likely resulting in a small amount of additional RNA delivery to the cytoplasm. The injected eggs were surgically transferred to pseudo-pregnant CB6/F1 hybrid recipient females, bred at the NIH from a cross of Balb/cJ female to C57B1/6 J males. Founders were crossed to B6 mice, and the heterozygous F1 were crossed with each other to obtain homozygous F2 knock-in mouse.

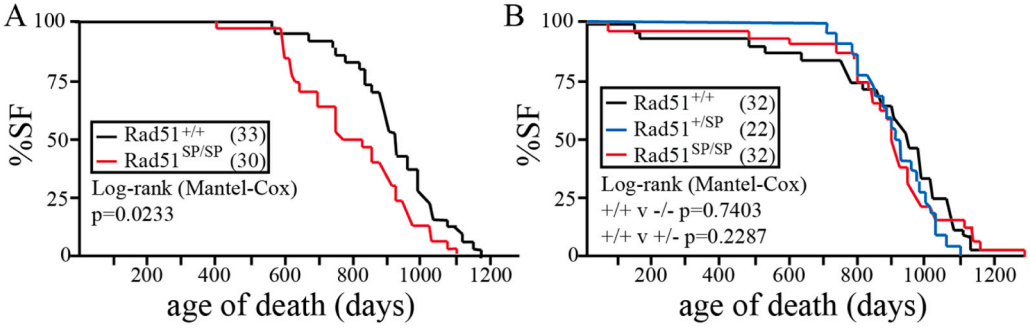

Fig. 1. Lifespan curves for (A) females and (B) males RAD51$^{+/+}$ and RAD51$^{+/SP}$ mice. Survival fraction, SF.

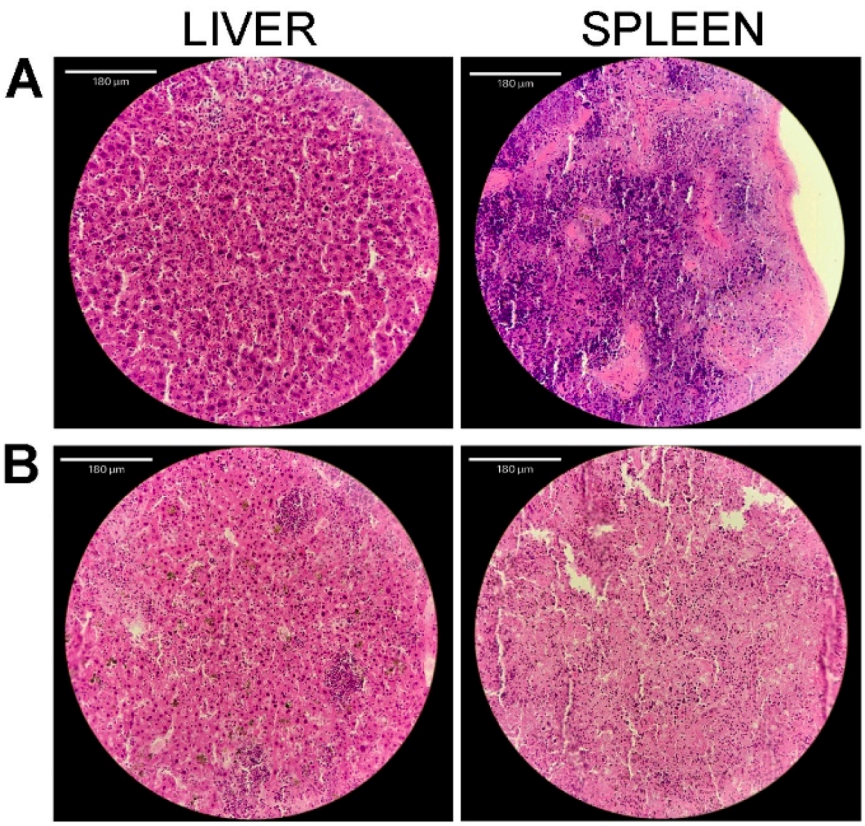

Fig. 2. H&E of lymphoma in SP/SP (**A**) and (**B**) +/+ female mice.

4.2. Genotyping

Mice were numerically identified by toe clip at 10–12 days of age. DNA was precipitated and resuspended as described [26] after Proteinase K was added.

For genotyping by endpoint PCR, 1–2 µl of DNA was used per reaction. Both the wild type (WT) and mutant (mut) reactions share the same Forward primer and both yield a 110 bp product. WT and mut reactions were performed separately using 500 nMol of the following primers:

S181P For 5'-CAC ATT TGT GGG GAC GCT GC-3'

S181P WT Rev 5'-TAG GAC ATC GCT GCC AG**A**-3'

S181P Mut Rev 5'-TAG GAC ATC GCT GCC AG**G**-3'

We performed a separate endpoint PCR reaction for sequencing to confirm no base errors were introduced during the genotyping PCR and that each genotype was correct. PCR reactions for sequencing used 500 nMol of the following primers:

S181P For 5'-CAC ATT TGT GGG GAC GCT GC-3'

S181P Rev 5'-GCC TGG TCT AAG ACA GCC AAG AC-3'

The ~200 bp PCR product was cut from the gel and extracted using a Qiagen DNA extraction kit. Sequencing reactions used 2 pmol of the S181P For primer and 50 ng of PCR product in a total of 7 µl. Sequencing was performed on an ABI 3130xl by the Nucleic Acids Core facility at University of Texas Health San Antonio.

Both the genotyping and sequencing endpoint reactions used 0.2–0.3 µl of Apex Bioresearch Products 5 U/µl Taq Polymerase (Genesee Scientific) and the following cycle conditions: Initial denaturation 95C 2 min, Cycles 1–37 (95C 30 sec, 55C 30 sec, 72C 30 sec), Final Extension 72C 5 min, 4C Hold.

To streamline the genotyping process, we designed and implemented a custom TaqMan SNP Genotyping Assay because of its ability to differentiate homozygous from heterozygous samples by using two probes with different fluorescent reporters. We first had to identify genomic interspersed repeats in the DNA so approximately 2000 bases of mouse genomic Rad51 DNA sequence including the SNP were submitted for "Repeat Masking" via Repeat Masker (Smit, AFA, Hubley, R & Green, P. *RepeatMasker Open-4.0*. 2013–2015 http://www.repeatmasker.org). Once it was determined that there were no repeats within close proximity to the SNP of interest, a sequence of ~600 bases (including the SNP) was sent to ThermoFisher/Life Technologies for analysis using their proprietary sequence preparation software. They selected ~300 bases for analysis (see below). Although there were some additional SNPs found by the software (bases in grey), all were greater than two bases away from the desired SNP (the recommended distance for successful TaqMan Genotyping probes), so we proceeded with the manufacture of the probe (Non-human TaqMan Probe 4332077, 40x, Custom ID AHHS73W). The WT base T (big font) is labeled with VIC and the mut base C (big font) is labeled with FAM. Both are quenched with MGB and use a ROX as the passive reference.

GGCTTGCCCTTACCTCTAACAGAGCAGGAATCA-
CATTTGTGGGGACGCTGCTACCTATCTTTGNNGTGATAGAAA-
TATTAGGTATTCNTTTCTCACNNGACTGAGCTCTGTCTA-
CAGCCTAAAAGTCTCCTCTTTTCTAGATANGGTCTC[**T/C**]**CTGGCAGC-GATGTCCTN**GATAATGTAGCATATGCGCGAGGGTTCAACACA-
GACCACCAGACCCAGCTCCTTTACCAAGCGTCAGCCATGATGGTA-
GAATCCAGGTATGTNCTCAGTGAGA-
CACTAAATGTGTGCCCGTGGCCCTTTCCTT

TaqMan SNP Genotyping reactions were done according to manufacturer's directions using TaqMan Genotyping Master Mix (ThermoFisher Scientific 4371353). Genomic DNA was diluted to 4 ng/ul and 9 ng was used in 5 µl reactions containing the custom TaqMan probe AHHS73W identified above. Reactions were performed on an Applied Biosystems 7900 HT Fast Real-Time PCR System (ThermoFisher Scientific). Allelic Discrimination analysis was done using the corresponding SDS software.

To confirm that our TaqMan custom probe correctly identified the SNP, we performed endpoint PCR, sequencing, and TaqMan reactions on

the first 18 mice in this study. We continued both sequencing and TaqMan SNP Genotyping Assays for an additional 27 mice. Because the genotypes were consistent regardless of method, we genotyped all remaining mice generated for this study using only the TaqMan SNP Genotyping Assay.

4.3. Mouse husbandry

We housed mice in accordance with the NIH guide for the care and use of lab animals. In our longevity studies, mice were allowed to live out their life span, *i.e.*, there was no censoring due to morbidity. Mice were euthanized only if they were either unable to eat or drink or when they were laterally recumbent and unable to right themselves. Mice were bred and enrolled in the study in a rolling fashion. Mice were separated into cages of the same sex at weaning, but genotypes were mixed. Female +/SP mice were sacrificed midway through the almost four-year study and cages were consolidated to save on cost. The +/SP males were not sacrificed because consolidation would generate a much higher risk for fighting. They were only sacrificed if they were the last remaining mouse in the cage (which is why this group only had 22 mice). Only one lab member performed the majority of the breeding, genotyping, weaning, and health monitoring of these mice so blinding was not possible, however, we feel genotype bias was minimized because mice were allowed to live until natural death as the endpoint with only some being euthanized when moribund but only if within hours of death.

4.4. Histology

Tissues were fixed in 10 % neutral buffered formalin for 24–72 h and then in 70 % ethanol until being sent to a histology core to be embedded in paraffin, cut into sections, and stained with haematoxylin and eosin by standard histological procedures. For all genotypes lymphoma was detected in the liver and/or spleen. The histology technician and pathologist were blind to our hypothesis, study design, and methods.

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CRediT authorship contribution statement

Tae Moon Kim: Writing – review & editing. **Paul Hasty:** Conceptualization, Funding acquisition, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Sherry G Dodds:** Formal analysis, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing. **Yong Jun Choi:** Methodology. **Gene Hubbard:** Investigation. **Gene Elliot:** Methodology. **Kyungjae Myung:** Funding acquisition. **Lisa Garrett:** Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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